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Policy Forum

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Screening for and surveillance of high-risk patients with HBV-related chronic liver disease: Promoting the early detection of hepatocellular carcinoma in China

Peipei Song[1], Xiaobin Feng[2], Keming Zhang[3], Tianqiang Song[4], Kuansheng Ma[2], Norihiro Kokudo[1], Jiahong Dong[5], Linong Yao[6,*], Wei Tang[1,*]

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Summary **In China, hepatocellular carcinoma (HCC) is the second most common cancer in urban areas and first most common in rural areas. It ranks as the second leading cause of cancer-related deaths in males and the third leading cause of cancer-related deaths in females, with the total mortality rate of 26.26 per 100,000. Currently, people with hepatitis B virus (HBV) infection are a major population at risk of developing HCC in China. In fact, there are 93 million Chinese who are HBV carriers, and about 20 million of them have chronic HBV infection. Several cohort studies have shown that screening high-risk patients with HBV- or HCV-related chronic liver disease may improve the rate of early HCC detection and the rate of curative treatment. However, a government-funded national program to screen for high-risk patients with HBV-related chronic liver disease has yet to be established in China. Although several remarkable advances in HCC management have been made during the past few decades, most patients with HCC still present with advanced-stage disease, thus reducing the chance of curative treatment. Based on firsthand experience in Japan and other countries or areas, this work examined the current status, challenges, and prospects for the future of early detection of HCC in China. Findings suggested the need for a systematic guideline for the standardized management of HCC, a government-funded nationwide screening and surveillance program for high-risk patients with HBV-related chronic liver disease, and extensive use of des-γ-carboxyprothrombin (DCP) as a screening tool in China in order to facilitate the early detection of HCC in China.**

Keywords: Hepatitis B virus (HBV), hepatitis B (hepB) immunization, guideline, α-fetoprotein (AFP), des-γ-carboxyprothrombin (DCP)

1. Introduction

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 75-80% of the roughly 650,000 HCC cases reported globally each year. Of particular note is the fact that China alone accounts for 55% of HCC cases worldwide (*1*). Currently, the overall prevalence of HCC in China is 26-32 per 100,000 persons, and in some areas prevalence can be as high as 70-80 per 100,000 (*2*). HCC is now the second most common cancer in urban areas and the first most common in rural areas (*3*), and it ranks as the second leading cause of cancer-related deaths in males and the third leading cause of cancer-related deaths in females, with a total

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mortality rate of 26.26 per 100,000 in China (*4*).

The present work summarizes the current status of early detection of HCC in China. As shown in Table 1, several remarkable advances in HCC management have been made during the past few decades, such as the implementation of hepatitis B (hepB) immunization for susceptible and high-risk populations to prevent hepatitis B virus (HBV) infection and the publication of the "Expert Consensus on Treatment Standards for Hepatology Carcinoma (Chinese HCC Guideline)" (*5*) to guide clinical practice. However, most patients with HCC in China still present with advanced-stage disease (*6*). Currently, surgical resection and liver transplantation offer the best potential chances for treating HCC (*7*) but are only available to patients whose tumors are detected early. The overall 5-year survival rate for patients with HCC is about 40%, but liver resection of early HCC could result in a 5-year survival rate of 60-70% (*8*). In China, the challenge is that the majority of patients with HCC present with advanced disease, thus reducing the chance of curative treatment. Accordingly, early detection is crucial to achieving long-term disease-free survival for patients with HCC in China.

In order to determine how HCC is detected in its early stages worldwide, the literature was systematically reviewed. The reviewed literature consisted of 3,008 papers included in the PubMed database from 2001 to 2011. Also analyzed were 17 current guidelines for HCC management worldwide, including 5 guidelines from the United States of America (USA), 7 from Asia, and 5 from Europe (*9*). Several cohort studies have shown that screening high-risk patients with HBV- or HCV-related chronic liver disease may improve the rate of early HCC detection and the rate of curative treatment (*10-12*).

According to reports from the World Health Organization (WHO), approximately 2 billion people are HBV carriers, and 350 million of them have chronic HBV infection; about 1 million died due to hepatic failure, liver cirrhosis, or HCC caused by chronic HBV infection (*13*). In China, HBV is the biggest factor for developing HCC; approximately 85% of Chinese HCC cases are HBV-related, 10% of cases are HCV-related, and some cases involve HBV and HCV super-infection (*3*). Currently, people with HBV infection are a major population at risk of developing HCC in China. In fact, there are 93 million Chinese who are HBV carriers, and about 20 million of them have chronic HBV infection (*8,14*). A well-considered strategy of screening and surveillance for high-risk patients with HBV-related chronic liver disease is urgently needed in China to promote the early detection of HCC.

2. Early detection of HCC in China: Current status, challenges, and prospects for the future

2.1. *HCC guideline for the standardized management of HCC*

With the development of evidence-based medicine (EBM), the concept of "transfer of current best evidence into clinical decision-making" has garnered substantial attention worldwide. Guided by current best evidence, many clinical practice guidelines (CPGs) for HCC have been published worldwide (*15*). During the past few decades, a series of measures for standardized management of HCC have been published by the Chinese Government, and the Chinese HCC Guideline was also published in 2009 (*5*). Guidelines established by a systematic literature analysis include the guidelines established by American Association for the Study of Liver Disease (AASLD Guideline) (*16*), those of the British Society of Gastroenterology (BSG Guideline) (*17*), and the guideline established with the support of Japanese Ministry of Health, Labor, and Welfare (J-HCC Guideline) (*18*), all of which provide recommendations for the management of HCC supported by data. In contrast, the Chinese HCC Guideline was established based on a consensus of experts and not supporting

Table 1. The current status of early detection of HCC in China

Items	Current status in China
Prevalence	Overall prevalence of 26-32/100,000 (*2*).
Mortality	Total mortality rate of 26.26/100,000 (*4*).
Etiological factors	Eighty-five percent of patients with HBV infection, 10% of patients with HCV infection (*3*).
Major at-risk population	People with HBV infection; 93 million HBV carriers, 20 million people with chronic HBV infection (*13*).
Guideline	Expert Consensus on Treatment Standards for Hepatology Carcinoma (Chinese HCC Guideline) published in 2009 (*5*).
Prevention	HepB immunization for susceptible and high-risk populations.
Screening and surveillance	No government-funded nationwide screening program.
Screening tool	Ultrasonography and AFP.
Surveillance period	Six-month interval for HCC high-risk populations ages 35-40 (*5*).
Early detection	Most patients with HCC present with advanced-stage disease (*6*).

data. The Chinese HCC Guideline also covers only diagnosis and treatment, so other important aspects such as epidemiology, prevention, screening, surveillance, and follow-up are absent. This is particularly true of recommended strategies for screening and surveillance of high-risk patients with HBV-related chronic liver disease.

In Japan, there are two kinds of guidelines for HCC management. The J-HCC Guideline was established through a systematic analysis of 7,192 publications (*19*) with the support of Japanese Ministry of Health, Labor, and Welfare to guide clinical practice with recommendations supported by data. The JSH Guideline was established through a consensus of experts with the support of Japan Society of Hepatology (*20*) to provide experience-based recommendations for HCC management. The two guidelines do not contradict since they play different roles in Japan. In fact, the JSH Guideline may provide additional information based on experts' experience and up-to-date information on the management of HCC in Japan (*21*). Over the past ten years, HCC management in Japan has made remarkable progress due to the widespread acceptance and implementation of the J-HCC Guideline and JSH Guideline (*9,15,21*). More importantly, the guidelines in Japan have been systematically incorporated. The J-HCC Guideline was first published in 2005 and then revised in 2009, and the next version will be published in the near future with the incorporation of new evidence (*22*). The JSH Guideline was first published in 2007 and also be revised in 2010 (*23*).

According to the J-HCC Guideline and JSH Guideline, ultrasonography and measurement of α-fetoprotein (AFP), the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), or des-γ-carboxyprothrombin (DCP) should be performed at intervals of 3-4 months in the very-high-risk group (patients with HBV- or HCV-related liver cirrhosis) and at 6-month intervals in the high-risk group (patients with HBV- or HCV-related chronic liver disease or liver cirrhosis due to other causes) (*19,20,22,23*). Awareness of the J-HCC Guideline and its influence was studied in 2006, a survey showed that more than 70% of clinicians were aware of the guideline, and some clinicians changed their practices in line with the guideline (*24*). A survey of 200 Japanese experts was conducted in 2009 to determine the nature of HCC screening in Japan. The survey found that 72% of experts simultaneously measured the tumor markers of AFP, APF-L3, and DCP, and 44% of experts combined this measurement with ultrasonography (*25*).

The establishment and effectiveness of standardized management of HCC in Japan, and especially the periodic and simultaneous conduct of ultrasonography and measurement of AFP, AFP-L3, and DCP, may provide a good guide to HCC screening and surveillance for high-risk patients with HBV- or HCV-related chronic liver disease for other countries and areas, and especially for China.

2.2. *Nationwide screening and surveillance program for high-risk patients with HBV-related chronic liver disease*

In Asia, Japan and South Korea have implemented a nationwide screening and surveillance program for HBV and HCV infection. Similarly, Taiwan also established a screening and surveillance program to screen patients with cirrhosis every 3-6 months and patients with no cirrhosis every 6-12 months (*6,26*). However, there is no government-funded screening and surveillance program for HBV and HCV infection in Hong Kong or other parts of China. In 2002, the Japanese Ministry of Health, Labor, and Welfare started a national 5-year program to screen for HCV and HBV infection among people over 40 given the high prevalence of HCV infection in this age group (*27*). By the end of 2006, 9 million people had been screened. Of these, 112,000 were found to have HCV infection and 110,000 were found to have HBV infection (*28*). Since most high-risk patients were closely followed before developing HCC, HCC nodules was detected in the early stage in more than 60% of patients in Japan (*29*).

During the past few decades, a series of strategies have been implemented in China to control HBV infection. The "Nationwide Hepatitis B Virus Seroepidemiological Survey" was conducted in 1992 and in 2006 to ascertain epidemiological data on HBV in China (*30*). The "Chinese Chronic Hepatitis B Prevention and Cure Guideline" published in 2005 (revised version published in 2010) and the "2006-2010 National Hepatitis B Prevention and Control Plan" published in 2006 serve to guide clinical practice (*31,32*). In addition, enactment of "Blood Donation Law" and "Law for Licensing Medical Practitioners" and implementation of the "Regulations on Medical Waste Management" and "Administrative Regulations on Medical Institutions" led to further regulation of medical care. Specially, hepB immunization for infants and young children has been widely implemented in China. In 1992, hepB vaccination for infants and young children was included in the "National Hepatitis B Immunization Plan"; since 2002, the hepB vaccine for infants and young children has been subsidized by the Chinese Government; and since 2005, both the hepB vaccine and injection fee have been borne by public health insurance. Due to these efforts, the number of hepatitis B surface antigen (HBsAg) carriers among infants and young children decreased by 19 million from 1992 to 2006 and resulted in a HBsAg prevalence of 0.96% among children under 5 (*30*).

An important point to remember is that most of the implemented strategies focused on prevention, control, and curing of HBV infection in susceptible and high-risk populations. The "2006-2010 National Hepatitis B Prevention and Control Plan" seeks to establish

a national hepB conventional epidemic monitoring system, which includes revising the criteria for hepB diagnosis, to establish a national hepB laboratory testing network, and to conduct periodic evaluations of hepB diagnosis and the hepB laboratory testing network. However, a government-funded nationwide screening and surveillance program for high-risk patients with HBV-related chronic liver disease to promote early detection of HCC has yet to be established in China.

In Japan, the national 5-year program to screen people over 40 for HCV and HBV infection and the routine practice of surveillance of patients at risk of developing HCC resulted in the detection of HCC in its early stages in 60% of patients. Furthermore, the screening tools of ultrasonography, AFP, AFP-L3, and DCP are widely and routinely used to screen for HCC in Japan, and these tests are covered by Japanese national health insurance as serological biomarkers to screen for HCC in clinical settings (*14*). China needs to promptly establish a government-funded nationwide screening and surveillance program for high-risk patients with HBV-related chronic liver disease to promote early detection of HCC.

2.3. *Screening tools and surveillance period for high-risk patients with HBV-related chronic liver disease*

As mentioned before, a government-funded nationwide screening and surveillance program for high-risk patients with HBV-related chronic liver disease to promote early detection of HCC has yet to be established in China. According to the Chinese HCC guideline, AFP should be measured and ultrasound should be performed every 6 months for the HCC high-risk population ages 35-40 (*5*). In terms of cost-effectiveness, a surveillance interval of 6 months has been widely accepted worldwide. In some developed countries with advanced health insurance systems, very high-risk populations are also screened at an interval of every 3-4 months.

Imaging tools and serum tumor markers have been widely used in screening worldwide. Ultrasound is the imaging tool most often used to screen for HCC because it is simple, inexpensive, non-invasive, and allows real-time observation. However, the success of ultrasound depends on the expertise of the physician, the ultrasound equipment available, and the echo texture of the liver, so the actual sensitivity of ultrasound is difficult to assess due to the lack of a definitive standard for HCC (*33,34*). The serum tumor marker AFP is considered a useful and feasible tool for HCC screening and early diagnosis in China. The clinical usefulness of AFP in China has been confirmed by a randomized controlled trial in 2004 that involved 18,816 Chinese patients (*35*). A point to remember is that the sensitivity and specificity of AFP vary widely, and the total AFP is not always specific, especially when HCC is in its early stages (*36,37*). AFP has been found to have a sensitivity of 41-65% and specificity of 80-90% when detecting HCC given an AFP cut-off of 20 ng/mL (*38*). However, up to 50% of patients with HCC have an AFP level below 20 ng/mL (*39*), and elevated levels of AFP are also found in patients with liver diseases other than HCC, including viral hepatitis, at a rate of 10-42% (*40*). Thus, AFP cannot be used as the sole tool to screen for HCC.

Worldwide, a number of studies have looked at DCP. These studies showed that combined measurement of DCP and AFP have a sensitivity of 70-94% and specificity of 62-90%, while combined measurement of DCP and AFP-L3 have a sensitivity of 70-84% and specificity of 62-80% when detecting HCC in the early stage (*41-43*). However, DCP testing is currently approved only in Japan, South Korea, and Indonesia and has not been approved in China. In order to promote the clinical use of DCP in early detection of HCC in China, large-scale, multi-center studies of Chinese patients must be conducted to provide more data and corroborate earlier findings. Accordingly, a program involving 1,500 Chinese patients with HCC and 1,000 Chinese patients without HCC was launched by the Japan-China Joint Team for Medical Research and Cooperation on HCC in 2012 to assess the clinical usefulness of DCP in Chinese patients through a large-scale, multi-center study. Of these patients with HCC, more than 80% had HBV infection. The program found that there was no significant correlation between serum levels of DCP and AFP; DCP has a total sensitivity of 74% while the combined measurement of DCP and AFP could result in a sensitivity of 83%, which is higher than DCP or AFP alone. DCP could result in a specificity of 56% with a cut-off value of 40 mAU/mL and a specificity of 94% with a cut-off value of 100 mAU/mL (*8,14,44*). These findings provide a better perspective on the use of DCP to detect Chinese cases of HCC in their early stages. Moreover, many studies recommend that DCP be used to assess HCC progression, potentially indicating HCC recurrence after curative therapy, predicting the presence of vascular invasion and allowing the identification of recipients of liver transplants, and facilitating the development of new chemotherapeutic strategies for treating HCC (*45-48*). Thus, extensive use of DCP is expected, especially given the fact that China accounts for 55% of HCC cases worldwide.

3. Conclusion

China accounts for 55% of all HCC cases worldwide. Approximately 85% of these cases are HBV-related, and most patients with HCC present with advanced-stage disease, thus reducing the chance for curative treatment. In Japan, the establishment of standardized HCC management, implementation of a nationally

funded 5-year program to screen people over 40 for HCV and HBV infection and the routine practice of surveilling high-risk patients for HCC using ultrasound, AFP, AFP-L3, and DCP resulted in detection of HCC in its early stages in 60% of patients. In China, the established Chinese HCC Guideline lacks recommendations supported by data. This is particularly true of recommended strategies for the screening and surveillance of high-risk patients with HBV-related chronic liver disease. A government-funded national program to screen for high-risk patients with HBV-related chronic liver disease has yet to be established. In addition, AFP is the only serum biomarker that has been widely used to screen for and diagnose HCC in China. In the current work, analysis of the current status, challenges, and prospects for the future of early detection of HCC in China indicated the need for a systematic HCC guideline for the standardized management of HCC, implementation of a government-funded nationwide screening and surveillance program for high-risk patients with HBV-related chronic liver disease, and the extensive use of DCP as a screening tool in China in order to facilitate the early detection of HCC in China.

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Review

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Strategies to prevent hepatitis B virus infection in China: Immunization, screening, and standard medical practices

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Summary **China has one of the world's highest rates of hepatitis B infection. Over the past 20 years, a series of strategies have been implemented to prevent infection with the hepatitis B virus (HBV) in China. These strategies include hepatitis B (hepB) immunization for susceptible populations such as infants and young children and for high-risk populations such as health care workers and patients, premarital health care for couples of childbearing age, and standard medical practices. A series of measures implemented by the Chinese government caused the HBV infection rate in China to decrease from 9.75% in 1992 to 7.2% in 2006. However, a report on infectious diseases indicated that more than 1 million people in China were infected with hepB in 2011. There is room for improvement. The current work analyzed the current status of and challenges for strategies to prevent HBV infection in China. This work also recommends clear guidance regarding hepB immunization for parents in rural areas, more flexible premarital health care, health education for both patients and health care workers, and routine HBV screening for high-risk health care workers.**

Keywords: Hepatitis B virus (HBV), hepatitis B (hepB), prevention, high-risk population

1. Introduction

Hepatitis B (hepB) is one of the world's major health problems. Two billion people worldwide are reportedly infected with the hepatitis B virus (HBV) (*1*). HBV can cause both acute and chronic disease. HBV carriers have a substantially increased risk of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in later life (*2-4*). About 600,000 people worldwide die from hepatitis B every year (*1*). China has one of the world's highest rates of hepB infection. A nationwide HBV serosurvey conducted in 2006 showed that 7.2% of the Chinese population ages 1-59 years were hepatitis B surface antigen (HBsAg) carriers (*5*). An estimated 93 million people in China are infected with HBV.

HBV infection is irreversible, but there are some strategies to prevent HBV infection by blocking HBV's transmission. HBV can be transmitted by direct blood-to-blood contact or semen and vaginal fluid from an infected person. The common modes of transmission in developing countries are perinatal, early childhood infections, unsafe injection practices, unsafe blood transfusions, and unprotected sexual contact (*1*). Chinese strategies to prevent HBV by blocking its transmission are analyzed here. These strategies include HBV immunization of susceptible and high-risk populations, premarital health care for couples of childbearing age, and standard medical practices. Strategies have advanced over the past 20 years in China, but there is still room for improvement (see Table 1).

2. Strategies to prevent hepB in China

2.1. *Preventive strategies for infants and young children*

Infants are susceptible to HBV infection in utero, intrapartum, or postnatally through maternal transmission. Young children acquire HBV infection through close contact with their parents or other family members as part of everyday family life. Most chronic

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Table 1. Major strategies to prevent HBV in China

Target population	Strategies	Challenge	Suggestion
Infants and young children	HepB immunization, no longer paid by out-of-pocket but now government-subsidized.	Less hepB immunization in rural areas than in urban areas.	Clear guidance for parents regarding hepB immunization.
Couples of childbearing age	Premarital health care including HBV screening and health education.	Lack of awareness of the importance of premarital health care. Conflict in terms of the time spent receiving health care and at work.	1. Awareness of the importance of premarital health care. 2. More convenient services provided by maternal and child health centers.
High-risk health care workers and patients	1. HepB immunization. 2. standard medical practices.	HepB immunization for adults and especially for the elderly is less cost-effective.	1. Standard medical practices prior to immunization. 2. Health education for both health care workers and patients. 3. Routine HBV screening for high-risk health care workers with self-reported vaccination.

HBV infections are acquired in infancy and early childhood (*6,7*). People who acquire HBV infection in early life have higher levels of viral replication and severer disease than those who acquire it in later life (*8,9*).

Immunization at birth is the cornerstone of prevention, reducing mother to child transmission by > 95% and conferring long-term protection against clinical disease (*10,11*). HepB immunization was introduced in China in 1987. HepB vaccination was recommended for all infants in China pursuant to the "National Hepatitis B Immunization Plan" formulated by the Ministry of Health of the People's Republic of China in 1992. In accordance with the plan, all infants should be vaccinated with hepB vaccine three times: 24 hours after birth, 3 months later, and 6 months later. Unlike other Expanded Program on Immunization (EPI) vaccines, the hepB vaccine and injection fee are paid for out-of-pocket by parents. HepB immunization was included in the EPI in China since 2002. A hepB vaccine for children under the age of 4 was subsidized by the Chinese government starting in 2002. Parents only need pay the injection fee. From 2002 to 2007, the Global Alliance for Vaccines and Immunization (GAVI) program provided $38,678,918 for hepB vaccination of infants in poor counties in middle and western China (*12*). Reform of the health care system began in China from 2009, and the prevention of hepB received greater attention as a main public health effort. All children under age 15 who were never or incompletely immunized with the hepB vaccine had to be revaccinated from 2009 to 2011. Immunization costs were borne by public health insurance.

After those efforts, hepB immunization of infants increased from 72% in 2000 to 99% in 2010 (*13*). Children fully vaccinated with 3 doses of hepB vaccine had a significantly lower prevalence of HBsAg (1.99%) than unvaccinated children (5.56%) (*14*). HepB immunization of infants has resulted in a prevalence of HBsAg among children under 5 of less than 1% and prevented an estimated 16-20 million HBV carriers (*2*). But hepB immunization has not occurred at the same rates in urban and rural areas. HepB immunization in rural areas is 94.6% (*15*), which is lower than the national rate of 99% (*13*).

2.2. *Preventive strategies for couples of childbearing age*

There are data showing that new couples with HBV infection, and especially those in endemic areas, have 50% possibility of transmitting HBV to the next generation (*16,17*). HBV infection during pregnancy can cause HBV intrauterine transmission and damage both the mother and fetus. Gestational diabetes mellitus, antepartum hemorrhaging, and preterm deliveries are reportedly more frequent in chronic maternal HBV infection (*18-20*). A higher risk of a low birth weight and prematurity are noted with acute maternal HBV infection (*21*). During pregnancy, the immune response is depressed to prevent the rejection of the fetus. As a result, infected individuals will have a significant increase in HBV DNA and a lower level of aminotransferase (*22*). Given the high risk of HBV transmission from new couples to children and the danger to the mother and fetus, screening for HBV and interventions for couples warrant attention and investigation.

In China, the "Law on Chinese Maternal and Infant Health Care" was enacted in 1995. According to the law, both a man and woman desiring to be married must undergo a premarital medical examination that includes HBV screening and education. Utilization of premarital health care has increased yearly since 1995. The rate of premarital health care utilization reached 68% (9 million/ 13.6 million) in 2002 (*23*). From 2003 was premarital health care no longer mandatory but voluntary. Consequently, the rate of premarital health care utilization decreased to 2.7% (0.36 million/14 million) in 2004 (*24*). HBV infection is reportedly detected by premarital health care in about 3-6% of new

couples (*25-27*). Accordingly, approximately 270,000-540,000 people with HBV infection could have been detected by premarital health care in 2002, but only 10,800-21,600 people with HBV infection would have been detected by premarital health care in 2004. Since early detection of HBV infection and other diseases is missed because of the absence of premarital health care, areas such as Shanghai began to provide early detection for free starting in 2005. The rate of premarital health care utilization reached 37.1% (900,000/2,440,000) again in Shanghai in 2010 (*28*).

2.3. *Preventive strategies for health care workers and high-risk patients*

There is a potential for HBV transmission between health care workers and high-risk patients. Health care workers risk HBV infection during surgical, obstetrical, and dental procedures due to worker injuries and occupational blood exposure (*29,30*). HepB immunization of high-risk populations, and especially health care workers, is recommended (*31,32*). HepB immunization of health care workers has been funded by local governments or medical facilities in some areas of China, such as Beijing. After hepB immunization, 90% of health care workers were positive for hepB surface antibody (HBsAb) (*33,34*).

Many regulations have been implemented in China to regulate standard medical practices. These include the "Law on Infectious Disease Prevention", the "Criteria for Management of Nosocomial Infections", and the "Criteria for Management of Disinfection". Consequently, blood collection and supply are strictly regulated. Health care facilities take care to prevent nosocomial infections. As a result, transmission of HBV to patients is seldom reported in China. However, patients such as diabetics have a higher risk of HBV transmission when monitoring their blood glucose if equipment is shared or adequate hand hygiene is not used (*35*). Persons with diabetes are reported to have a 60% higher prevalence of HBV infection than are those without diabetes (*36*). HepB immunization of diabetics (type 1 and type 2 diabetes) is generally a key prevention strategy in developing countries where hepB is endemic (*37,38*). Diabetes patients who are under age 60 should be immunized with the hepB vaccine as soon after they are diagnosed as possible (*39*). In China, immunization with the hepB vaccine is now voluntary for high-risk patients such as diabetics. Thus, a government-supported strategy to promote hepB immunization of high-risk patients, such as diabetics, may be adopted in China.

3. The challenges of HBV prevention strategies in China

The HBV infection rate in China decreased from 9.75% in 1992 (*40*) to 7.2% in 2006 due to a series of measures implemented by the Chinese government (*2*). However, a report on infectious diseases indicated that more than 1 million people in China were infected with hepB in 2011 (*41*). There is room for improvement.

3.1. *HepB immunization of infants and young children*

Preventive strategies for infants and young children in China have proven successful. These strategies are estimated to have a cost-effectiveness of 1:51.01 (1:49.59 in urban areas, 1:51.91 in rural areas) (*42*). Even though there is greater cost-effectiveness in rural areas, the rate of hepB immunization is lower in rural areas than in urban areas. There may be two reasons for this difference. One is that rural families migrate more frequent than urban families. Typically, a surplus labor force moves from rural areas to urban areas to find work. Therefore, infants of rural families are more likely to fail to take all 3 hepB doses as part of immunization. The other is that parents' knowledge of HBV infection and immunization is the main factor influencing the rate of hepB immunization (*15,43*). Parents in rural areas have less knowledge of HBV than those in urban areas, so rural health care workers are responsible for informing parents of when and where to receive hepB immunization if they migrate elsewhere to find work (*44*).

3.2. *Premarital health care for couples of childbearing age*

About 30-50% of HBV infections occurred through perinatal and early childhood close contact; 90% of infants infected with HBV develop chronic infections and 30-50% of children infected with HBV develop chronic infections from the age of one to four years (*1*). Premarital health care is also hugely cost-effective because of its low cost (less than US $15) and it had the potential to block 30-50% of HBV infections, subsequently reducing the direct economic burden of hepB. The direct economic burden of hepB is estimated to be more than US $1600 per Chinese citizen annually. If hepB develops into severe hepB, the direct economic burden will be almost 95% of a family's annual income (*45*). Given the potential to decrease the economic burden of hepB, premarital health care is a cost-effective way to block HBV transmission. However, the rate of utilization of premarital health care decreased sharply after it was no longer mandatory but voluntary. Even though it is now provided for free, the rate of premarital health care utilization has only increased slightly. A survey has shown that the main reasons for the low rate of premarital health care utilization are attitudes and time spent (*46*). Some couples think that voluntary care means that the care is not necessary. Some forego care because of the conflict between work and health care. Young people need to know the

importance of premarital health care. Maternal and child health centers that are responsible for premarital health care should be government-subsidized to provide services outside the regular work week to make them more convenient.

3.3. *HepB immunization and standard medical practices for health care workers and high-risk patients*

Both hepB immunization of health care workers and high-risk patients and standard medical practices have proven effective in China (*33,34*). However, adults, and particularly the elderly have a lower response rate to hepB immunization than do young children (*47*). HepB immunization of the elderly (> 60) is not recommended given its lack of cost-effectiveness (*48*). Standard medical practices and health education are effective ways to block transmission from health care workers to patients. First, standard medical practices should be emphasized throughout an entire medical procedure and should be taught to personnel ranging from medical students to professors. Second, health care workers should receive detailed information clarifying procedures risking HBV infection and be informed of precautions. Health care workers should take care to assure the hygiene of medical instruments and take precautions. Finally, health education should be provided to patients, *e.g*. diabetics who are at risk for HBV infection should be taught how to avoid infection when monitoring their blood glucose levels. Without question, health care workers are responsible for providing health education to patients. People are accustomed to relying on health care workers for health knowledge since that knowledge is a sign of their authority. Therefore, health care workers play an important role in improving the health literacy of people (*49*). Unfortunately, health care workers reportedly have limited knowledge of the prevention and treatment of hepB in rural areas. Rural health care workers were able to correctly answer only about 70% of questions about HBV and some of their knowledge of hepB improved little (less than 80%) after health education due to low levels of literacy (*50*). Ways to improve the knowledge of hepB among health care workers should be prioritized especially in rural areas. Given the low levels of literacy, consistent techniques should be adopted until most rural health care workers have the appropriate knowledge. Increased knowledge can lead to a more positive attitude and subsequently encourage good practices by both health care workers and patients.

HBV screening of high-risk populations is recommended (*51*). Serological testing of at-risk health care workers is crucial to preventing further HBV transmission (*35*). On one hand, health care workers found to have an HBV infection benefit by being treated as early as possible. On the other hand, identifying health care workers who are infected allows them to be transferred away from patients so that they do not transmit the infection. Levels of HBV DNA and hepBe antigen should be monitored in health care workers who have an HBV infection to facilitate blocking of further transmission. HBV screening combined with self-reported vaccination may be efficient and less expensive.

4. Conclusion

China has one of the world's highest rates of HBV infection. Many strategies including hepB immunization, premarital health care, and standard medical practices have been used to control HBV infection over the past 20 years. The HBV infection rate in China decreased from 9.75% in 1992 to 7.2% in 2006 due to the implementation of these strategies. But there is still room for improvement. Clear guidance for parents regarding hepB immunization can help to promote hepB immunization especially in rural areas. Premarital health care should be adequately utilized by the population and provided flexibly by maternal and child health centers. Standard medical practices should be prioritized over hepB immunization of health care workers and high-risk patients. Health education for both health care workers and patients is needed. Serologic HBV testing of at-risk health care workers is also necessary

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Werner syndrome: A changing pattern of clinical manifestations in Japan (1917~2008)

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Summary **As ~75% of the Werner syndrome (WS) patients recognized between 1904 and 2008 all over the world are of Japanese origin, the most case reports and clinical studies on WS has been published in Japanese journals. Thus, the detailed English-written clinical review on the recent WS case reports has been warranted. Although WS has been characterized by a variety of clinical manifestations mimicking premature aging, the recent longevity and delayed age-associated manifestations observed both from Japanese WS and general population may suggest a common environmental effect on some gene(s) other than WRN and may give us a newer pathophysiological look at WS and also natural aging through the molecular dysfunction of WRN.**

Keywords: Aging, cancer-prone syndrome, helicase, inflammation, longevity, premature aging, Werner syndrome

1. Introduction

Werner syndrome (WS:MIM#27770) is an autosomal-recessively inherited disease caused by the mutation of RecQ3 helicase (WRN) located at chr8p11-12 (*1-3*). WS patients are usually paid no special attention either from the family members or doctors concerning to any developmental abnormality until the usual premature termination of the teenage growth spurt and voice changes, followed by the age-related pathophysiology mimicking advanced aging (*4*). WS has been classified as an adult form of progeria, the representative natural model for human aging, a caricature of human aging, or as a segmental progeroid syndrome (*4-7*). The clinical manifestations recognized in WS, irrespective of ethnic origin, are commonly scheduled by hierarchical deterioration of the connective tissue system, the endocrine-metabolic system, and later to lesser degree the immune system, and the central nervous system (*4,5*). Like other helicases, the intact WRN helicase functions at the time of cell proliferation/division inside the mitotic cells, while the mutated/truncated WRN helicase protein does not (*8-10*). Thus, the systems/organs mainly consisting of post-mitotic cells like central nervous system and cardiac muscles may have at the most minor changes in WS. Actually no apparent association has been reported in WS with Alzheimer disease, Parkinson disease, and cardiomyopathy that may be frequently observed in elderly general population.

Since the first description of WS by Otto Werner in 1904 (*11*), additional WS case reports have accumulated worldwide, the majority being from Japan (*4,12*). So, we would like to review the details of the up-dated information written in Japanese to the non-Japanese readers (*13*).

2. Database

In the series of the previous reports, the literature has been searched for publications on WS through a citation index in and out of Japan between 1904 and 1995 (*3,4,14,15*). We have continuously searched the literatures and the referral cases between 1996 and 2008

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in the present communication. Bibliographies of each case were examined for additional references. Care was taken to exclude multiple reports of the same patient, recognized by details of family and personal histories and demographic characteristics.

Diagnosis of WS was based on the presence of 3 of 4 criteria under age 35 as described below (*3,4,14,15*): *i*) Characteristic habitus and stature:short stature and light body weight, slender extremities with stocky trunk and beak-shaped nose. *ii*) Premature senescence:bird-like appearance, alopecia/gray hair, skin hyperpigmentation, hoarseness, diffuse arteriosclerosis, juvenile bilateral cataracts and osteoporosis. *iii*) Scleroderma-like skin changes:atrophic skin and muscle, circumscribed hyperkeratosis, telangiectasia, tight skin over bones of feet, skin ulcers and localized calcification. *iv*) Endocrine-metabolic abnormalities: diabetes mellitus (DM) and hypogonadism. Diagnosis of neoplasia was as given by the original authors.

Approximately 200 Japanese WS patients (WS0101-WS60001) diagnosed by our criteria were further confirmed by the loss of intact WRN protein and the presence of WRN mutations as previously reported (*3,16,17*). B-lymphoblastoid cells and skin fibroblasts from ~200 WS patients and their family members are deposited at RIKEN Bio-Resource Center (Tsukuba Japan) as Goto Collection of Werner Syndrome and can be used by researchers upon request (*http://biolod.org/class/cria304u12i/Goto_Collection_EBV_transformed_B_cell_lines_ and_primary_fibroblast_derived_from_Werner_syndrome_patient*).

3. Brief history of clinical characterization of Werner syndrome

The history of WS research begins with the publication of a doctoral dissertation by a German general physician (later an ophthalmologist) at the University of Kiel in 1904, Otto Werner (*11*). He described several progeric manifestations, in addition to skin sclerosis and bilateral juvenile cataracts. Werner carefully differentiated the clinical manifestations in his patients from those with similar phenotypic manifestations formerly described by Rothmund (*18*), later known as Rothmund-Thomson syndrome (RTS: MIM#26840; *18-20*). Interestingly, we found the mutation of RecQ4 (RECQL4) located at chr 8p24.3, belonging to the same RecQ helicase family as WS causes RTS (*21*).

In Japan, Ishida, an ophthalmologist in Kyoto University, reported the first probable Japanese case of WS in 1917 (*22*), followed by a continuous publication of Japanese cases up to 1,128, while a total of 359 outside of Japan at the end of 2008 (Figure 1) (*4,12,23*). The number of patients associated with malignancy (between 1907 and 2008) was shown in the parentheses within the table in Figure 1.

Thirty years later from Werner's original observation, the door to WS research was reopened by two New York internists, Oppenheimer and Kugel, in 1934 and 1941 (*24,25*). They coined the name: "Werner's syndrome" and published the first necropsy case. The first reference of the rare malignancy in WS, fibroliposarcoma, was reported by Agatson and Gartner in 1939 (*23,26*). A Boston internist, Thannhauser reviewed WS and RTS as the discrete syndromes in 1945 (*20*), and Seattle-based geneticist group, Epstein *et al*. released a landmark overview on 125 cases of WS including 8 Japanese cases in 1966 (*5*).

Since the Ishida's first case report, the first necropsy Japanese WS case was documented in 1966 by Hamada (*27*), followed by the first association case report of WS with malignancy: malignant melanoma in 1968 by

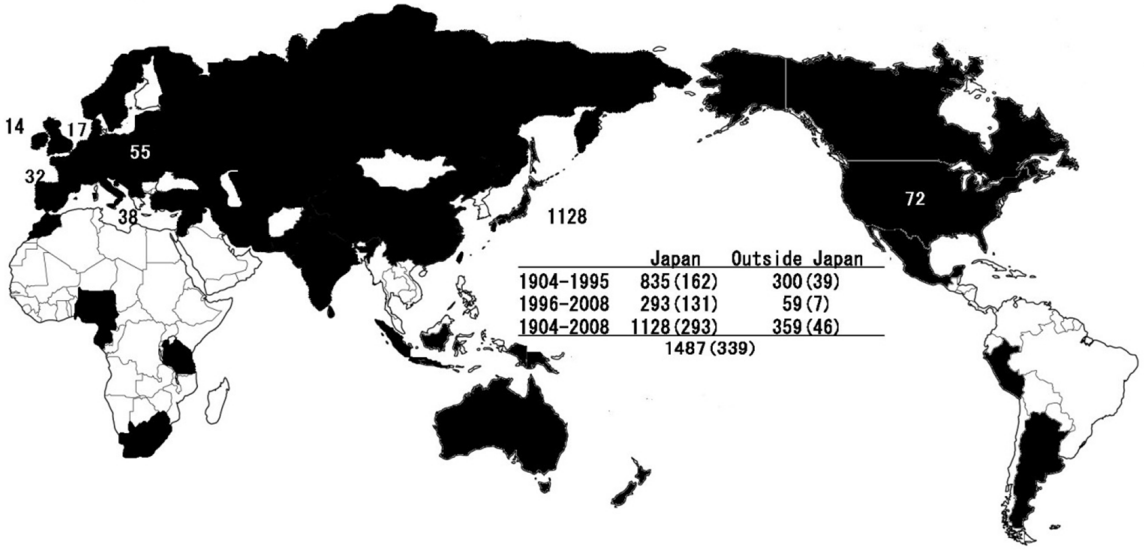

Figure 1. World distribution of Werner syndrome. The country with at least one reported patient is shaded. The number indicated for each country is the number of reported patients. The table within the figure indicates the number of reported patient for the respective term in and outside Japan. The number of malignancy associated with the patients is shown in the parentheses.

Koga (*23,28*). We proposed in 1981 for the first time the initial screening criteria for the possible WS patient as described (*3,4,14,15*).

4. Changing clinical manifestations

4.1. *Characteristic bird-like appearance and body habitus* (100% at age 35 years)

Short stature, light body weight, and stocky trunk with extremely thin extremities were noted in all the patients at age 26 years old. These characteristic appearance including bird-like faces, extremities mimicking Cushing syndrome or Klinefelter syndrome is still a hallmark of WS (*4,12,14*). Body size (height: 122-161 cm and weight: 19-52 kg) is also still small, but has been expanding in concert with the growing constitution in general Japanese population. Some patients exceeded 177 cm high and weighed over 70 kg.

4.2. *Premature senescence* (100% at age 35 years)

Gray hair/alopecia, bilateral cataracts, hoarseness, osteoporosis with osteosclerosis, and atherosclerosis are the hallmarks of WS. Interestingly, osteoporosis is usually more pronounced in postmenopausal women than men in general population, but this is not the case in WS. WS men have severer osteoporosis, either peripheral or vertebral, than women (*29,30*). Osteoarthritis, one of the commonest features in a general elderly population, has not been frequently reported in WS (*31*).

4.3. *Scleroderma-like skin changes* (100% at age 35 years)

Skin atrophy, skin sclerosis, skin ulcers, hypo/hyper-pigmentation, telangiectasia, sarcopenia, subcutaneous calcification, painful corns, and flat feet were included. Skin sclerosis such as extremely tight skin over malleoli and painful corns: the historical hallmark of WS has been rarer recently, though the reason is unknown. The skin ulcers in WS especially of lower extremities were induced by the daily mechanical stress in combination with the decreased cellular replicative potential and the subcutaneous fat defect (*14,32,35,36*). The skin ulcers in WS have been still well-known as the most incurable and occasionally leading to the amputation of the legs resulted from gangrene among dermatologists and orthopedic surgeons. Neither skin ulcers nor subcutaneous calcifications is usually associated with natural aging. Approximately 25% WS patients escape from skin ulcers (*32,35*). As described above, subcutaneous calcification, especially along the Achilles tendon is the must for diagnosing WS (*36*). So-far, all the mutation-proven WS have the Achilles tendon calcification, although relatively mild in some cases

4.4. *Endocrine-metabolic disorders* (type II DM, primary/secondary hypogonadism, thyroid hyper/hypo-dysfunction, hyperuricemia and hyper- lipidemia; 80% at age 35 years old)

The frequency of DM among Japanese WS patients has been constant in contrast to the rapid increase among general Japanese population. Of particular interest, the age at onset of DM in WS has delayed (*12*). The average age of onset of DM in WS was 33.7 years old in 1966, while 39.7 years old in 2004 and 39.3 years old in 2008. Since body mass index (BMI) was an accurate indicator of DM in the general population, the BMI in most WS patients was constantly below 22 (*12,33,37*), though all the WS patients showed an intravisceral fat accumulation revealed by MRI examination (*33,38*).

The serum adiponectin level decreased and the TNFα level increased in diabetic patients in general. In WS, adiponectin was significantly suppressed and TNFα is significantly enhanced compared with the controls. In addition, treatment of DM by pioglitazone normalized adipocytokines in WS (*38*). Recently, the WS patients associated with NASH (non-alcoholic steatohepatitis) have been reported (*39*).

Hypogonadism from both sexes was observed in 40%. Of special interest, one of the most prominent features in normal elderly male: prostate hypertrophy has never been recognized in WS. The thyroid dysfunction either of hyper-, hypo-function or malignancy that had been observed in the ~15% among WS patients has been rarer (*15*). All types of hyperuricemia were found in 10% in WS, though hyperuricemia was not usually associated with normal aging (*40*). Some patients had a history of gouty attack (*41*). Hyperlipidemia, characterized by hypertriglyceridemia was still a biochemical hallmark of WS (*4,37*).

The pathogenetical concept of atherosclerosis has been changing, though hyperlipidemia is still a risk factor for atherosclerosis. Atherosclerosis has been viewed as a sort of 'silent auto-inflammation' caused by the pro-inflammatory cytokines produced by macrophages that phagocytose modified lipoprotein particles (*42*). Interestingly, one autopsy case of 51 years old female WS patient who neither had dyslipidemia nor DM showed mild atherosclerosis similar to her age (*43*). She died of myelodysplastic syndrome (MDS).

Since the first description by Tokunaga *et al.*, the elevation of hyaluronan both in the urine and the serum has been constantly reported in WS. WS has been formerly inadvertently classified as a new group of hereditary mucopolysaccharidosis (*44-46*). Hyaluronan elevation either from urine and serum has been believed as a biomarker of normal aging and progeroid syndromes such as WS and progeria (*46-49*) and the International Registry of Werner syndrome included hyaluronuria for diagnosing WS (*http://www.pathology. washington.edu/ research/werner/registry/diagnostic.html*). However, the excessive production

of hyaluronan has been widely reported in other inflammatory conditions such as rheumatoid arthritis and liver diseases (*50-52*). The increased serum/urine hyaluronan level in aging and WS has been recognized as the result from chronic inflammation (*48,49,53,54*).

4.5. *Immune system disorders*

Immune system has been believed as the most sensitive organ to normal aging (*55-60*). At the age of 35 years, Approximately 80% of the WS patients show signs of mild immune abnormalities. A deficiency in so-far unidentified T cell subsets similar to the healthy nanogerian was detected in WS (*56*). Decreased natural killer cell activity, which recovered after the interferon stimulation, was observed in most WS patients (*57*). Most patients had low titers of several autoantibodies, including IgG anti-double-stranded DNA antibody, anti-nuclear antibody, and rheumatoid factor, as is usually observed in the healthy population over the age of 60 years (*58,59*). The autoantibody specific to autoimmune systemic sclerosis: anti-topoisomerase I (Scl-70), has never been detected in WS, although antinucleolar antibody of an undefined type was detected in some cases (*59,60*). Interestingly, a small percentage of the patients had autoimmune diseases, including Graves' disease, systemic lupus erythematosus, Sjogren's syndrome, and autoimmune hepatitis (*60*). However, WS patients were not abnormally sensitive to bacterial or viral infection at any stage of their life, even though the third major cause of death in WS is bacterial pneumonitis similar to the general Japanese population (*4*). The various types of low titer of auto-antibody production have been presumed as the result from the imbalance between inflammatory/anti-inflammatory responses against natural/modified antigens.

We have proposed the sub-normal immune system in WS may induce 'silent inflammation', 'inflammaging', or para-inflammation normally responsive to the daily infectious attacks and persistent oxidative stress, leading to the pathophysiology of DM, atherosclerosis, malignancy, auto-antibody production, and hyaluronan production (*48,50,52-54,61*).

4.6. *Nervous system disorders* (frequency: 40% at age 35)

WS has been believed to have a relatively normal central nervous system, consisting of mainly post-mitotic cells that may escape from WRN helicase dysfunction (*62*). However, with the recent advance of medical devices including computed tomography and magnetic resonance imaging (MRI), brain atrophy has been observed in 40% of WS patients, even before the age of 40 years (*4,63,64*). At least 3 WS patients had senile dementia due to subcortical arteriosclerotic encephalopathy or multiple meningioma, but not of the Alzheimer type by clinical determination and autopsy (*65-67*). Of great interest, 10% patients had schizophrenia of paranoia type at the age of ~37 years (*4*). Either bipolar or monopolar mood disorder has been rare in WS (*68*). Although ~10% WS had hearing loss as a result of otitis media infection and ~15% mental retardation before 1970, both symptoms have never been reported recently.

4.7. *Changing pattern of malignancy*

The average age of onset of malignancy in WS was 36.9 years old in 1966, while 48.8 years old in 2004 and 48.9 years old in 2008. As already reported, non-epithelial neoplasms including soft-tissue sarcoma (STS), osteosarcoma, malignant melanoma, benign meningioma, and myeloid disorders are still a hallmark of WS as listed in Table 1 (*23*). WS has been classified as a member of hereditary cancer-prone syndrome. The ratio of epithelial to non-epithelial cancers was about 1:1.5 instead of the usual 10:1. Thyroid carcinoma and malignant melanoma have been frequently associated with Japanese WS as was reported (*69*). Among epithelial neoplasms three WS were associated with pulmonary carcinoma that has never been reported before (*23*), though neither additional prostatic nor colorectal carcinoma has been reported as shown in Table 1.

Multiple primary neoplasms or myeloid disorders are also a hallmark in WS (Table 2) (*23*). Twenty five Japanese with WS had primary neoplasms, or neoplasia with MDS. Thyroid carcinoma, malignant melanoma, meningioma, MDS, and MFH (malignant fibrous histiocytoma) were the frequent counterpart in the multiple primary neoplasms. Of note, six primary neoplasms were found in a Japanese man with possible

Table 1. Neoplasms in Japanese Werner syndrome (1996~2008)

Diagnosis	No.
Non-epithelial	
Soft-tissue sarcoma	
MFH*	8
Others	12
Osteosarcoma	6
Malignant melanoma	18
Meningioma	9
Hematologic disorders	
AML**	4
MDS***	11
Others	8
Epithelial	
Thyroid	9
Liver	6
Skin	5
Lung	5
Others	30
Grand total	131

*MFH: malignant fibrous histiocytoma. **AML: acute myelogenous leukemia. ***MDS:myelodysplastic syndrome.

Table 2. Multiple primary neoplasms or myeloid disorders in Werner syndrome

Case	Mutation*	Sex	Diagnosis (age)
WS15001	6//6	M	Malignant melanoma, conjunctiva + osteosarcoma (all at 52)
WS32201	?//?	M	Malignant melanoma, acral lentigious (21) + malignant melanoma,intranasal (31)
WS31801	?//?	M	Malignant melanoma + multiple myeloma (all at 56)
WS25101	?//?	F	Malignant melanoma + leiomyosarcoma (all at 55)
WS57501	1//?	F	Pancreas carcinoma (60) + malignant melanoma (61)
WS32701	?//?	F	Thyroid, papillary + Bowen disease (all at 54)
WS33201	?//?	F	Thyroid, medullary (38) + fibrosarcoma (43)
WS10201	6//6	F	Thyroid, follicular (43) + ureter carcinoma (50) + adrenal carcinoma (50)
WS65001	?//?	M	Thyroid, type unknown(46) + meningioma (49)
WS10501	6//6	F	Thyroid, type unknown + hepatocellular carcinoma + MDS** (all at 56)
WS23801	?//?	M	Thyroid, type unknown + meningioma + MDS** (all at 62)
WS24701	?//?	M	MDS** (53) + meningioma (54)
WS35801	?//?	F	Meningioma + osteosarcoma (all at 58)
WS24101	?//?	F	Meningioma (63) + hepatocellular carcinoma (66) + cholangiocarcinoma (66)
WS32301	?//?	M	Fibrosarcoma + MFH*** (all at 51)
WS26401	?//?	M	MFH*** + malignant peripheral nerve sheath tumor + osteosarcoma (M, all at 58)
WS0402	4//4	F	SCC****, skin (53) + MFH*** (55) + bladder carcinoma (56)
WS24001	4//4	F	Breast (40) + MDS** (55)
WS36001	?//?	F	Malignant peripheral nerve sheath tumor + olfactory neuroblastoma (all at 41)
WS32601	?//?	M	Pheochromocytoma + malignant adrenal gland tumor (all at 55)
WS36101	?//?	M	"SCC****, oral soft palate (69) + left edge tongue (74) + right edge tongue (75) + hard plate (79) + esophagus (79) + ureter, tansitional cell carcinoma (82)"
WS56201	?//?	M	Bowen disease + SCC**** (all at 70)
WS52801	6//6	F	Uterine carcinoma (40) + leiomyosarcoma (52)
WS17601	?//?	M	Hepatocellular + basal cell carcinoma (all at 44)
WS14701	4//4	M	Gastric + renal carcinoma (all at 45)

*See details in Table 3; **MDS:myelodysplatic syndrome; ***MFH:malignant fibrous histiocytoma; ****SCC:squamous cell carcinoma.

WS, though the WRN mutation in the patient was not examined (*70*).

5. Genetics

5.1. *Geographical distribution and mutation type*

WS is a genetic disease transmitted by autosomally recessive inheritance (*14*). Although consanguineous marriage especially in rural areas may still contribute the relatively high incidence of WS in Japan, consanguinity (mostly first-cousin marriage) was noted in only ~45% among ~200 mutation-proven WS patients. In addition, familial occurrence has been infrequent since 1996. Most cases recently reported are sporadic. The patients have been reported from all area of Japan and outside of Japan (Figure 1), while there are still several clustering areas in Japan as have been already reported (*4,14,71*).

Since the discovery of WRN gene in 1996 (*2*), the mutation type of the responsible gene:WRN has been reported in and outside of Japan. According to the International Registry of Werner syndrome (*http://www.pathology.washington.edu/research/werner/registry/diagnostic. html*), the number of the mutation type has accumulated up to ~100 worldwide. Approximately 200 Japanese WS patients (WS0101-WS61901) diagnosed by our criteria were further confirmed by the loss of intact WRN protein and the presence of WRN mutations (*3,16,17*). Although the precise mutation in ~15% patients with defective WRN protein is not identified yet, the mutation types so-far recognized in Japan are quite different from those outside Japan as shown in Table 3. Interestingly, the majority of the Japanese patients with WS have mutation type 4, that has never been found in the non-Japanese WS.

Japan is the largest producer of WS (*4,14,71*), probably because of an extremely high incidence (1:100) of heterozygous carriers in Japan (*14,71,72*). Although few patients with WS have been reported in ethnically similar Asian countries such as Korea, the incidence of heterozygous carriers in general population in the representative 3 areas of Korea (Seoul, Pusan, and Gwangju) was less than 1:1,000 (*73*, personal communication from Drs. F. Takeuchi and A. Park).

5.2. *Changing pattern of longevity*

Like the decreased life-span of the cultivated and *in vivo* skin fibroblast, the WS patients have been believed to have a shorter life-span than normal (*34,74,75*). This notion may be generally true. However, the increase in

Table 3. Mutations in Werner syndrome in Japan

Nomination	Site (nucleotide no.)	Codon	Mutation type	Nucleotide sequence	Comments	Predicted protein (a.a)	% Mutated alleles in WS
Mut1	4,144	1,305	substitution	CGA --> TGA	nonsense	1,304	10
Mut4	1-bp upstream from 5' end of exon 26	1,047-1,048	substitution	aatagGGTAGA --> aatacggtaga	exon skip and frame shift	1,060	52.3
Mut5	4,146	1,305	1-bp deletion	CGAGCA --> CGAAGC	frame shift	1,017	1.4
Mut6	1,336	369	substitution	CGA --> TGA	nonsense	368	18.9
Mut7	3,677	1,149	1-bp deletion	GAGCGA --> GGCAGG	frame shift	1,160	0.9
Mut8	7-bp upstream from 5' end of exon 30(3690-3691)	1,153-1,154	substitution	tttgttcagATT --> ttagTTCAGATT	frame shift	1,162	0.6
					nonsense	462	0.6
Mut9	1,620	463	substitution	TAT --> TAA			
Mut10	733-734	168	2-bp deletion	AAGCTG --> GCTGAA	frame shift	176	0.6

*See details in Table 3; **MDS:myelodysplatic syndrome; ***MFH:malignant fibrous histiocytoma; ****SCC:squamous cell carcinoma.

Table 4. Association of the earliest progeroid symptoms with life-span

Onset (y.o)	Progeroid symptoms	Death (y.o)	Cause of death	ID/ Sex	Mutation*
4	Cataract	23	Cerebral bleeding	WS15701F	6//?***
7	Cataract	> 61	Still alive	WS0402F	4//4
8	Hoarseness	> 61	Still alive	WS8401M	4//4
8	Characteristic habitus	53	AMI**	WS5001F	4//4
10	Scleroderma	46	Malignancy	WS14701M	4//4
10	Characteristic habitus	51	Malignancy	WS12501M	1//4
10	Characteristic habitus	57	Malignancy	WS10001M	1//1
10	Characteristic habitus	77	AMI	WS1801M	4//4
11	Skin atrophy	39	Infection	WS53101F	1//1
11	Hyperkeratosis	53	AMI	WS2101F	4//4
12	Cataract	44	AMI	WS7001M	4//4
13	Cataract	49	Infection	WS0101M	1//4
13	Hoarseness	57	Malignancy	WS0401M	4//4
13	Characteristic habitus	70	AMI	WS61901M	4//4
14	Characteristic habitus	45	Malignancy	WS4401F	4//4
15	Cataract	39	Malignancy	WS23703M	4//6
15	Characteristic habitus	58	AMI	WS4801M	4//6
15	Characteristic habitus	59	Infection	WS0801F	4//4
15	Skin ulcer	61	Malignancy	WS1701F	6//6
16	Short stature	38	Infection	WS8601F	1//1
16	Skin ulcer	46	AMI	WS15301M	4//4
17	Cataract	59	AMI	WS6901F	6//6
20	Cataract	49	Infection	WS11201M	10//10
20	Cataract	55	Malignancy	WS11502F	6//6
20	Characteristic habitus	57	Malignancy	WS16001F	4//?
21	Cataract	47	Malignancy	WS9301M	4//4
22	Cataract	43	AMI	WS10402M	4//4
24	Diabetes mellitus	55	Malignancy	WS24002F	4//4
24	Cataract	43	Malignancy	WS24001M	4//4
25	Cataract	45	Malignancy	WS20001F	4//4
27	Gray hair	42	Malignancy	WS6201F	7//7
27	Skin ulcer	52	Malignancy	WS15001M	6//6
28	Cataract	56	Malignancy	WS55201M	4//4
29	Cataract	61	Malignancy	WS57501F	1//?
30	Cataract	38	Malignancy	WS5901M	6//6
30	Cataract	48	Malignancy	WS25402M	5//5
30	Cataract	50	Malignancy	WS10201F	6//6
30	Cataract	66	AMI	WS51002M	4//4
32	Cataract	50	Malignancy	WS25401F	5//5
32	Cataract	56	Malignancy	WS10501F	6//6
35	Cataract	43	Malignancy	WS50902F	1//6
35	Cataract	49	Infection	WS51001F	4//4
36	Cataract	55	Malignancy	WS16301F	6//6
37	Malignancy	37	Malignancy	WS8602F	1//1
37	Cataract	> 65	Still alive	WS4702F	6//6
37	Cataract	70	AMI	WS4701F	6//6
38	Gray hair	52	Malignancy	WS9501M	6//6
39	Cataract	52	AMI	WS4704M	6//6
40	Characteristic habitus	> 62	Still alive	WS6701F	4//4
41	Malignancy	46	Malignancy	WS15501F	4//4
44	AMI	63	AMI	WS9101F	4//4
52	Cataract	59	AMI	WS8801F	1//4
53	Skin ulcer	63	Infection	WS12401F	4//4
63	Malignancy	63	Malignancy	WS52301M	4//4

*See details for Table 3; **AMI: acute myocardial infarction; ***//?: mutation unidentified.

Table 5. Longevity of heterozygote members in Werner syndrome family

WS/ Sex	Age at death	Cause of death	ID	Mutation*
WS0101M	97	Malignancy	Grand father	4//w**
WS7901F	92	Infection	Mother	?***//w
WS4401M	92	Malignancy	Father	4//w
WS0101M	>90	Still alive	Father	1//w
WS0801F	90	Infection	Grand mother	4//w
WS0801F	90	Malignancy	Mother	4//w
WS61901M	90	Infection	Mother	4//w
WS5701F	90	Cerebral bleeding	Mother	4//w
WS4501M	90	Infection	Grand father	?//w

*See details for Table 3; **w: wild type; ***?: mutation unidentified.

the number of elderly WS patients (age > 63 years) in Japan paralleled the increased longevity in the general population, as described in Table 4. Death occurred on an average of 52.8 years in 2004 (82.7 years in general population) and 55.0 years in 2008 (82.7 years in general population), although the life-span of WS was ~38.2 years in 1966 (71.7 years in general population) (*12*). The major causes of death are still malignancy and myocardial infarction (*4,5,12,14,15*). Interestingly, the way of the appearance of age-related pathophysiology followed by death observed in WS was similar irrespective of the ethnic origin and mutation type (*4,5,15*).

5.3. *Does early pathophysiology determine the life-span?*

Analyses of the way how aging-related conditions may rapidly arise in WS may give us the fundamental insight into the pathophysiological mechanisms of human aging. Since the most clinical manifestations characteristic in WS usually overlap with those of natural aging process at an early stage of life, most patients, family members and even doctors do not acknowledge the presence of the disease before the age of 36.7 ± 10.1 years (*4*), even if additional family members are already recognized as affected. The parents of children with WS may in some time recognize the abnormality either by their lack of the prepubertal growth spurt, the loss of sex maturation and voice change or the early onset of cataract. Reliable records, particularly of early pathophysiological manifestations of WS, are therefore limited. As listed in Table 4, we examined in mutation-proven WS; *i*) if longer life-span (age > 63years) was linked with slower progeroid outcomes (age < 40years), and *ii*) if earlier onset of clinical symptoms (age < 13years) was associated with a shorter life-span (age < 40years). These results may suggest a possible common environmental/epigenetical link between the longevity in WS and the general population (*12*).

Several long-lived patients (age > 63 years old) were not diagnosed with WS prior to 35 years of age. In a few patients who had shorter life-spans of age < 40 years, premature aged phenotypes typical of WS before age 10 were noted. The percentage of WS patients suffering from early death at < 40 years of age was 34.9% prior to 1985, and 13.3% after 1986 for both sexes. There was no significant difference between males and females in the frequency of early death. The major causes of death in WS have been malignancy, acute myocardial infarction (AMI) and infection; similar to the general population.

Thus, although data is limited, there appears to be no clear-cut correlation between delayed onset of WS-specific progeroid symptoms and a longer life-span or *vice versa*.

5.4. *Family analysis: Does WRN heterozygote contribute longevity?*

The longevity (> 90 years old) members have been sometimes encountered among heterozygous carriers in WS families as listed in Table 5.

Although the data is still limited and we do not know if WRN heterozygosity may contribute longevity, WRN has been recently reported to modulate mitochondrial ROS (reactive oxygen species) production by the repression of HIF-1 (hypoxia inducible factor-1) activity (*76*). So, the 50% WRN function may moderately suppress ROS production leading to longevity. Obviously, this notion is highly speculative and further study may be required.

6. Conclusion

We should bear in mind that as the rapid improvement and changes in the average life-span and life-style in general population all over the world, the life-span and clinical manifestations even in the genetically-determined disease like WS may change extensively as already described (*12*). This may suggest the possible interventional treatment for the clinical changes in WS, age-related diseases in the general population and even pathophysiology of natural aging.

Although the recent delayed onset of typical progeroid phenotypes in WS caused by the loss of function of WRN may be explained by the environmental changes including life-style and medical improvement, genes normally cooperated with WRN may possibly

contribute unexpected phenomenon . While this notion is highly speculative, the *in vitro* molecular studies and the prospective cohort study by using the larger number of mutation-proven Japanese patients may allow direct testing of these concepts in the future.

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Review

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Trends in the use of preconditioning to hypoxia for early prevention of future life diseases

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Summary **Environmental factors during fetal life program the health outcomes regarding many diseases in future life. This idea has been supported by worldwide epidemiological studies, but the underlying mechanisms are still poorly understood. Three questions should be answered. (*i*) Does a common underlying cause of ordinary pathological fetal development exist? (*ii*) If such a cause exists, which mechanism might develop disease in later life? (*iii*) Is it possible to prevent this underlying cause and therefore the associated obstetric complications to primarily prevent future life diseases? The objective of this review is to attempt to answer these three questions by using PubMed (extending to October 2012) and other sources. Three data-based answers corresponding to these questions were found: (*i*) hypoxia, (*ii*) excessive stimulation of neurogenesis, and (*iii*) preconditioning/adaptation to hypoxia. The method for such preconditioning/adaptation is intermittent hypoxic training (IHT), in which air with low oxygen concentration is breathed through a mask to protect against subsequent strong adverse influences. Data are cited for IHT applications for the prevention/treatment of diseases in different fields, particularly in obstetrics. Data suggested that all common fetal origins of adult diseases are likely predetermined by changes in the fetal brain; therefore, early detection of these changes must be very important. The use of IHT may be a real means to primarily prevent obstetric complications and therefore, prevent future life diseases.**

Keywords: Fetus, pregnancy, neurogenesis, primary prevention

1. Introduction

Environmental factors during fetal life program the health outcomes in future life. This David Barker's hypothesis (*1*) has been supported by worldwide studies, including large scale epidemiological studies (*2-5*). These studies confirmed that abnormalities of early growth, including preterm birth, intrauterine growth restriction/retardation, and low weight/height at birth, are tightly associated with future life diseases: cardiovascular and cardiopulmonary diseases, diabetes and obesity, neuropsychiatric, and others. Majority of authors believe that the most important causative factor here is undernutrition. However, Morley considers that "human studies in general provide limited and unconvincing evidence that differences in maternal macronutrient intake are important. Nevertheless there is a need to understand the underlying causal pathways" (*6*). All of this shows that profound underlying causes of these abnormalities are still poorly understood.

The aim of this review paper is to attempt to clarify these causes by answering the following questions: Does a common underlying cause of ordinary pathological fetal development exist? If such a common cause exists, which mechanism might develop disease in later life? Is it possible to prevent the underlying cause and therefore the associated obstetric complications to prevent future life diseases?

A literature review was conducted using the PubMed database and other sources, with a time frame extending to October 2012. The review was conducted from the viewpoint of hypoxia, an important factor in any pathological process.

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2. Causes of abnormalities during pregnancy: Relationship to hypoxia

2.1. *Obstetric complications: Relationship to hypoxia*

The main obstetric complications are considered to be as follows: hypoxic hypoxia, asphyxia at birth, hypoxia/ischemia, hypoxic/ischemic encephalopathy, preeclampsia, infection/inflammation, and maternal psychological stress. We will not consider here the effects of undernutrition, fetal nicotine, cocaine, alcohol, and glucocorticoids exposure.

Hypoxic hypoxia results from insufficient oxygen reaching the blood, as might occur by breathing air with low oxygen content, for example, in the mountains.

Asphyxia at birth and hypoxia/ischemia (with its consequence in a form of hypoxic/ischemic encephalopathy) are related to stagnant (circulatory) hypoxia. These types of hypoxia are associated with the failure to transport sufficient oxygen because of inadequate blood flow.

Preeclampsia is a multisystem disorder affecting about 5-10% of all pregnancies. It is a major cause of maternal, fetal and neonatal mortality and morbidity. Despite intensive research, the aetiology of this disease remains unknown. Preeclampsia originates in the placenta, starting with inadequate cytotrophoblast invasion and ending with widespread maternal endothelial dysfunction. Production of placental anti-angiogenic factors has been shown to be up-regulated in preeclampsia. These factors are released into the maternal circulation where their actions disrupt the maternal endothelium and result in hypertension, proteinuria, and the other systemic manifestations of preeclampsia. The molecular basis for placental dysregulation of these pathogenic factors remains unknown, although hypoxia is likely an important regulator (*7*).

An important role of the systemic inflammatory response syndrome in preeclampsia, which is tightly connected with tissue hypoxia, is suggested in previous studies (*8,9*). Tissue (histotoxic, cytotoxic, cytopathic) hypoxia appears when tissues are unable to use oxygen despite normal oxygen delivery.

Infection/inflammation is a pathological process which is widely recognized as the inflammatory response syndrome (*8-10*) based on tissue hypoxia. The cells under 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 (*11*).

Maternal psychological stress produces fetal asphyxia (*12*), this was revealed in animal experiments. Stress experienced during pregnancy not only leads to pregnancy complications like miscarriage, preeclampsia, preterm parturition, low birth weight or major congenital malformations, stress also increases the risk of the child to develop diseases in the subsequent periods of life (*13*). Note that any obstetric complication or adverse event/process may be accompanied by maternal psychological stress, and it may be difficult to distinguish their effects.

This data show that all considered obstetric complications are tightly related to some types of hypoxia.

2.2. *Abnormalities of early growth: Relationship to hypoxia*

The role of different types of hypoxia in abnormalities of early growth (preterm birth, intrauterine growth restriction/retardation, low weight/height at birth) was clarified in many studies. Preterm birth may be caused by hypoxic hypoxia (*14*), infection/inflammation (*15,16*), preeclampsia (*17*), or maternal psychological stress (*18*). Intrauterine growth restriction/retardation may be caused by hypoxic hypoxia (*19*), preeclampsia (*20*), or maternal psychological stress (*21*). Low weight/height at birth may be caused by hypoxic hypoxia (*22*) or preeclampsia (*20*). Therefore, all considered abnormalities of early growth are tightly related to some types of hypoxia.

2.3. *Abnormalities during pregnancy: Consequences for future offspring's life*

Consequences for future offspring's life due to abnormalities during pregnancy, including obstetric complications and abnormalities of early growth, have been known for a long time. However, the list of these consequences increased sufficiently during the last 20-30 years because of the works of David Barker and his followers. Many of these works are the epidemiologic studies with great numbers of participants, so the results of the studies are reliable. It was found that abnormalities during pregnancy might give many pathologic consequences for future offspring's life, for example: cardiovascular and cardiopulmonary diseases, including high blood pressure and risk of stroke (*1-5,23-25*); behavioral, neurological and mental diseases, including cerebral palsy, depression, schizophrenia, epilepsy (*24,26-30*); metabolic diseases, including overweight, type 2 diabetes (*2-5,31-33*); bronchopulmonary diseases, including asthma (*34,35*); hearing loss (*36*). This data show that abnormalities during pregnancy, including obstetric complications and abnormalities of early growth, are associated with or caused by some types of hypoxia.

3. The role of hypoxia in the neurogenesis stimulation

The abnormalities during pregnancy are tightly connected with hypoxia, and involvement of neurogenesis should be considered.

The role of hypoxia in neural stem cells (NSC) development and functioning is discussed (*37*). The

authors noted that scant information on intermittent hypoxia effects on stem cells that was obtained generally in cell culture models, reveals that intermittent hypoxia at certain duration and intensity is a more potent trigger of transcription activation than constant hypoxia. In future, a method of intermittent hypoxia training/treatment could be effectively used for correction of physiological changes and disorders.

NSCs exist within a "physiological hypoxic" environment of 1 to 5% O_2 in both embryonic and adult brains (*38*). The studies showed that hypoxia could promote the growth of NSCs and maintain its survival *in vitro*. *In vivo* studies also showed that ischemia/hypoxia increased the number of endogenous NSCs in the subventricular zone and dentate gyrus. In addition, hypoxia could influence the differentiation of NSCs. More neurons, especially more doparminergic neurons, were produced under hypoxic condition.

Contrary to the long-held dogma, neurogenesis occurs in discrete areas of the adult brain, the hippocampus and the subventricular zone, and NSCs reside in the adult central nervous system. Proliferation of NSCs was observed in experiments involving adult rats treated in a hypobaric chamber (*39*). Researchers reported activation of protein synthesis and an increase of RNA concentration in the brain.

Recent studies have shown that neurogenesis is increased in the diseased brains, after strokes and traumatic brain injuries, and that new neuronal cells are generated at the sites of injury, where they replace some of the degenerated nerve cells. Thus, the central nervous system has the capacity to regenerate after injury (*40*). Endogenous neurogenesis in the hippocampus of developing rat after intrauterine infection was observed in the study (*41*). That is, in essence, the influence of tissue hypoxia, tightly connected with infection/inflammation.

Hypoxic hypoxia was used in animal experiments to develop pathologic neurogenesis to mimic diseases including schizophrenia (*42*) and bronchopulmonary dysplasia (*43*) in the offspring's future life.

Thereby hypoxia of any type stimulates neurogenesis, especially during gestational age.

Considering that the brain is the organ most vulnerable to hypoxic influence, excessive hypoxia produces the damage in the brain, for example, white matter damage (*44*). This programs future life diseases. David Barker (*1*) points to the importance of long-term programming in early life and parallel findings in clinical and animal research. Above cited data show that "programmer" of the future life diseases is most likely the brain, so the way to avoid future life diseases is to early detect and correct pathological brain changes (the "program") instead of treating the disease as it appears. The most difficult task here is probably to find these early changes related to nonmental diseases. Currently, the brain changes have been found in newborns with congenital heart disease (*45,46*). For other diseases, changes have been found in adult brain for type 2 diabetes (*47-50*), asthma (*51,52*), and chronic obstructive pulmonary disease (*53-55*). Improvements in diagnostic methods will make it possible to establish changes during early life. This trend is in its beginning just now, and more favourable trends will be considered in the text sections.

4. Trends in the studies and in the routine use of hypoxic hypoxia for prevention and treatment

Some preventive or treatment methods have been proposed for obstetric complications: maternal nutrition, physical activity, vaccination, the use of vitamins, magnesium sulphate; hypothermia (which improves oxygen supply by reducing oxygen demand). No method was found to be effective and safe. Particularly, for preeclampsia the only successful treatment is delivery; no definitive preventive strategies have been identified (*7*). Therefore, it may be important to examine the possibility of the use of hypoxia as a preventive or therapeutic means.

4.1. *Hypoxic hypoxia as a general protective means: Animal studies*

Many animal studies have been performed with the use of hypoxic hypoxia as a protective means. Generally, these studies describe hypoxia-induced tolerance to hypoxia, or preconditioning/adaptation to hypoxia.

The first fundamental study on the protective features of hypoxic hypoxia (*56*) contained the results of numerous animal experiments (rats, mice, and rabbits). Hypoxic hypoxia (10% O_2) was administered once for 30 min before a harmful pharmaceutical agent was injected or was administered during 10-15 days for 30 min daily before applying physical force or introducing an infection. The following data were obtained (control *vs*. experiment):

- Asphyxia: heartbeat stopped in pregnant rabbits, min: 34.5 ± 4.8 *vs*. 66.2 ± 5.4; heartbeat stopped in the rabbit fetus after the mother's asphyxia, min: 93.0 ± 8.2 *vs*. 136.0 ± 6.4.
- Acute hypoxia with hypercapnia: lifetime of the rats, min: 18.1 ± 0.36 *vs*. 25.5 ± 0.5.
- Haemorrhagic shock: breathing stopped in rats, min: 9.9 ± 0.3 *vs*. 18.5 ± 0.6; heartbeat stopped in rats, min: 18.3 ± 0.4 *vs*. 30.5 ± 0.4; breathing stopped in rabbits, min: 23.8 ± 0.3 *vs*. 41.7 ± 0.4.
- Physical load: duration of swimming of rats, min: 4.6 ± 0.3 *vs*. 8.0 ± 0.3; heartbeat stopped after submersion on the bottom: 5.8 ± 0.2 *vs*. 9.4 ± 0.4.
- Survival rate of mice after tick-borne encephalitis virus infection (%): 33.3 ± 5.1 *vs*. 51.7 ± 5.4.

Sufficient data were also presented in (*56*) on the survival rate in mice after injection of pharmacological

agents (eight types).

Useful review on hypoxic preconditioning is done by Lin (*57*).

Hypoxic preconditioning also protects against brain injury or attenuates its consequences. For example, it attenuates global cerebral ischemic injury following asphyxial cardiac arrest through the regulation of the delta opioid receptor system (*58*), protects against cerebral and cardiac ischemia (*59*), protects the right ventricle from ischemia and reperfusion (*60*), protects the brain and likely other organs of neonatal and adult rats (*61*).

Protective effects of hypoxic preconditioning on the development of depressive states in rat models were studied. Three episodes of intermittent preconditioning using hypobaric hypoxia (360 mmHg, 2 h) prevented the onset of depressive behavioral 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 (*62*). The authors consider that the data received indicate the possible use of hypoxic preconditioning for the prophylaxis of post-stress depressive episodes.

Prenatal hypoxia preconditioning improves the hypoxic ventilatory response and reduces mortality in neonatal rats (*63*).

Preventive influence of hypoxic hypoxia on cerebral circulation was studies in a model of acoustic stress in the KM line rats genetically predisposed to audiogenic seizures (*64*). The 2 h influence of an 'altitude' of 5000 m reduces the death rate and the extent of neurological changes (the frequency and severity of motion disorders and the development of intracranial haemorrhages) under conditions of acoustic stress.

After 2 weeks of adaptation to simulated altitude in adult rats (*65*), cardiac output was increased by 22% and total peripheral resistance was decreased by the same value. Angiogenesis seems to increase the stability of oxygen transport in microcirculation.

Adaptation to periodic hypoxic hypoxia effectively prevented oxidative and nitrosative stress, protecting against neurodegenerative changes, and protecting cognitive functions in experimental Alzheimer's disease (*66*).

An important role of hypoxia-inducible factor in hypoxic preconditioning is discussed in several reviews (*59,61,67*). Oxygen-independent activation of this factor is a promising therapeutic strategy for the prevention of organ injury and failure (*67*).

Mechanism of hypoxic influence has been the subject of many studies. Over the course of evolution, aerobic organisms have developed sophisticated systems for responding to alterations in oxygen concentration, as oxygen acts as a final electron acceptor in oxidative phosphorylation for energy production. Hypoxia-inducible factor (HIF) plays a central role in the adaptive regulation of energy metabolism, by triggering a switch from mitochondrial oxidative phosphorylation to anaerobic glycolysis in hypoxic conditions. HIF also reduces oxygen consumption in mitochondria by inhibiting conversion of pyruvate to acetyl coenzyme A, suppressing mitochondrial biogenesis and activating autophagy of mitochondria concomitantly with reduction in reactive oxygen species production (*68*).

Studies carried out by Sharp *et al*. (*59*) show that animals exposed to brief periods of moderate hypoxia (8% to 10% oxygen for 3 h) are protected against cerebral and cardiac ischemia between 1 and 2 days later. Hypoxia preconditioning requires new RNA and protein synthesis. The mechanism of this hypoxia-induced tolerance correlates with the induction of HIF, a transcription factor heterodimeric complex composed of inducible HIF-1α and constitutive HIF-1β proteins that bind to the hypoxia response elements in a number of HIF target genes. Studies show that HIF-1α correlates with hypoxia induced tolerance in neonatal rat brain. HIF target genes, also induced following hypoxia-induced tolerance, include vascular endothelial growth factor, erythropoietin, glucose transporters, glycolytic enzymes, and many other genes. Particularly, the role of erythropoietin was studied previously (*69*). The authors concluded that, in mice, IHT reduces bodyweight and serum glucose by increasing EPO synthesis which secondarily increases leptin and insulin production in liver.

A bioenergetic mechanism for development of urgent and long-term adaptation to hypoxia was considered also in a paper (*70*). Hypoxia induces reprogramming of respiratory chain function and switching from oxidation of NAD-related substrates to succinate oxidation. Succinate therfore is a signaling molecule, which effects are realized at three levels in hypoxia, intramitochondrial, intracellular and intercellular.

4.2. *IHT and its clinical applications*

IHT, also known as intermittent hypoxic treatment, intermittent hypoxic therapy, normobaric intermittent hypoxic therapy, normobaric hypoxytherapy, or hypoxytherapy, is a method for treatment or prevention of diseases by hypoxic preconditioning or adaptation to hypoxic hypoxia. Such an adaptation is produced by breathing air with low oxygen content, usually 10-12% through a mask, at normobaric conditions, *e.g.* in a room at sea level. This method was developed in the former USSR beginning in the 1970s, by Professor Rostislav Strelkov and colleagues, originally as a radioprotective method for military and oncological (hypoxiradiotherapy) applications. Methodical recommendations prepared by Strelkov and colleagues and issued by the Russian Health Ministry (*71*) (also see subsequent editions) recommend the use of IHT (10-

12% O_2, 5 min breathing, 5 min rest, 1 h per session, 1-4 weeks per course) for the treatment of various diseases. This drug-free method, which is almost without contraindications, has been routinely used by about 2 million patients in the last 30 years. The method is also applied to increase physical working capacity and endurance, especially in sports (*56,72*).

Much literature and practical pictures may be found on the websites *www.go2altitude.com* (mostly sport), particularly *http://www.go2altitude.com/iht.html* – some IHT centers worldwide; and *www.bionova.ru* (mostly medicine), particularly *http://www.bionova.ru/?page=4* and *http://www.bionova.ru/?page=6#pol* – the use of the IHT in the different fields of medicine in Russia.

The effects of high altitude stay on the incidence of common disorders in men were described (*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 Indo-Chinese conflict). A significantly lower number of cases of most disorders were found among the men at high altitude than among those at sea level. In particular, the difference in morbidity rates per thousand was 0.16/1.25 (diabetes mellitus), 0.22/0.96 (ischemic heart diseases), 0.37/2.15 (asthma), and 1.07/2.82 (neuroses).

Some trials were performed by means of sojourns in the high mountains, by the use of hypobaric chamber and by the use of normobaric hypoxia (*74*). The results were negligible or insufficiently strong (for schizophrenia) or moderate (for depression). One of these trials carried out in the USA in 1930s have used acute hypoxic hypoxia and gave encouraging results initially, but unfortunately was not completed.

The IHT was also used as a method to enhance nonspecific resistance in epilepsy treatment (*75,76*). The optimizing effect of hypoxic hypoxia on physiological functions of the patients with epilepsy consisted in increased level of hemoglobin and erythrocytes in the blood, less frequent systole, systolic and diastolic pressure reduction and prolongation of breath holding during Stange's test). As a result of these changes, the frequency of epileptic attacks decreased and normalization of behavioural responses was observed.

The use of IHT together with standard drug treatment in patients with migraine without aura (*77*) resulted in a decrease of the rate and severity of migraine attacks, an improvement in the state of the autonomic nervous system, and a decreased level of personal anxiety and degree of manifestation of depression to a markedly greater extent than in control patients.

Beneficial results of the application of IHT were obtained for bronchial asthma and chronic obstructive pulmonary disease (*78*). Bronchial obstruction decreased by 10-15%, exercise tolerance, general condition, ventilation, and haemodynamic and immunological parameters improved, and the frequency of bronchopulmonary infection exacerbations decreased 2-fold.

Hypoxytherapy was also applied for treatment of hypertension (*79*). It was concluded that hypoxytherapy exerted a robust, persistent therapeutic effect and can be considered as an alternative, nonpharmacological treatment for patients with stage 1 arterial hypertension. The antihypertensive action of IHC is associated with normalization of nitric oxide production.

IHT has also been used for preparation to surgery to increase patient's nonspecific resistance: general (*80*); in patients with ischemic cardiomiopathie preparing to coronary bypass with artificial circulation (*81*) (see an official Instruction of the Belarus Health Ministry (*82*)); before cesarean section (*83,84*); before and after gynecological surgery (*85*).

Combined hypoxic-hyperoxic training was used in the treatment of the metabolic syndrome (*86*). The use of hypo-hyperoxic exercise (alone or in combination with systemic hyperthermia and hardware vibratory) leads to a significant reduction in body weight. It was achieved mainly by reducing fat mass accompanied by a reduction of total cholesterol, LDL (low-density lipoprotein), FPG (fasting plasma glucose), optimization of blood pressure, increased hypoxic stability, physical endurance, improved mental status.

IHT was used to increase nonspecific systemic resistance in 107 patients with chronic salpingo-oophoritis for treatment or rehabilitation purposes (*87*). IHT promoted the recovery of compromised oxygen metabolism in all patients, resulting in activation of oxygen transport mechanisms and the normalization of tissue respiration. Recovery was recorded in 67.3% of patients, and the frequency of aggravations of the chronic condition was reduced in the rest.

4.3. *IHT as a possible method for the primary prevention of fetal origins of future life diseases*

IHT could prevent adverse hypoxic influences and is routinely used in general clinics. The use of IHT, as a drug-free method, is especially important in obstetrics, where it has also been recommended (*71,88*).

In one study (*89*) researchers reported the discovery of hypoxic cycles with a 2-fold difference in PO_2 levels of oxygen content in the uterine tissue of pregnant (3-5 days) rats as compared with non-pregnant rats. The frequency of the PO_2 pulsation was much lower in the uterine tissue of non-pregnant rats. The hypoxic cycles were assessed as a mechanism of rhythmic periodic stimulation of metabolic reactions directed towards not only the increased resistance to hypoxia, but also towards the nonspecific resistance of uterine fetal tissues and the female body in and out of pregnancy. This discovery suggests that IHT acts as a natural biorhythmic process. Impulse biorhythm change of

cyclic pO_2 in the uterus tissues and intrauterine fetus of rats, guinea pigs and dogs is regarded as evolution-fixed physiological mechanism aimed to increase nonspecific resistance of the fetus (*90*).

Research was conducted on the development of children born to mothers with preeclampsia who were treated with normobaric hypoxia (*91*). One hundred women cured by IHT and fifty 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 a mask, interrupted by 5 min of breathing atmospheric air, with a total of six 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, haemoglobin 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.

In another study, researchers examined the efficiency of preventive usage of IHT in 44 pregnant females at high risk for preeclampsia in the presence of essential hypertension, stages I-II, and neurocirculatory asthenia of the hypertensive type. The authors paid attention to a decrease in the incidence of preeclampsia, in particular its severity patterns, and perinatal mortality (*92*).

Pregnant females at high risk of preeclampsia who underwent IHT in the second and third trimester, compared with controls, showed (*93*) more successful delivery; less frequent occurrence of nephropathy, fetal hypoxia, and premature labour; and better physical condition of newborns.

In the paper (*94*) oxygen metabolism kinetics was investigated in 90 pregnant females at high risk for preeclampsia and associated vascular disorders. 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 preeclampsia, 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 preeclampsia prevention. Energy metabolism of maternal and fetal tissues during preconditioning/adaptation to intermittent experimental normobaric hypoxia was also considered in (*95*).

Experimental studies have also been conducted on increasing the nonspecific body resistance of mother, fetus and newborn to extreme factors by hypoxic training (*96*). Strelkov *et al*. (*97*) conclude "the use of

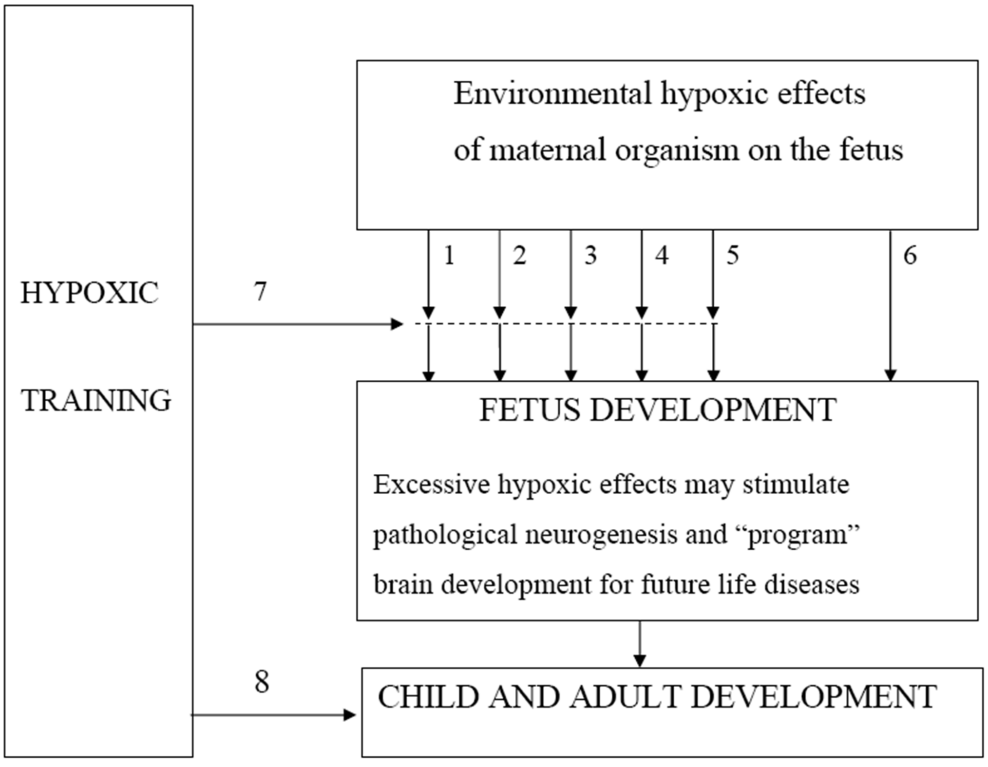

Figure 1. Simplified scheme of hypoxic influences on development. 1-6, environmental effects of maternal organism on the fetus, including harmful and useful (**1-5**) effects (**6**). 1, preeclampsia; **2**, hypoxia/ischemia; **3**, asphyxia at birth; **4**, infection/inflammation; **5**, maternal psychological stress; **6**, natural hypoxic training of the fetus by maternal organism: increased pO_2 levels of pulsation of oxygen content in the uterine tissue of pregnant rats as compared with non-pregnant rats. **7** and **8**, preventive/therapeutic effects of hypoxic training. All of those effects are tightly connected with hypoxia.

IHT with 10% O_2 is not only absolutely harmless for the fetus with no unfavourable effects on the course of the pregnancy or its outcome, but was 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%". Many other clinical data in support the harmlessness of IHT have been provided.

Data from the literature (*71,88,91-94*) related to the IHT procedure, suggest, particularly in preventive obstetrical applications, one course of IHT before pregnancy and one or two courses during pregnancy after the 16th week. All authors consider this procedure as effective and safe, but improved doubling study is needed.

The given data of this article are illustrated by the simplified scheme of hypoxic influences on development (Figure 1).

5. Conclusion

Data cited show the following trends in the studies: (*i*) hypoxia of different types plays a key role in almost all ordinary abnormalities and complications of pregnancy; (*ii*) hypoxia stimulates neurogenesis and is necessary for normal neurodevelopment, but excessive hypoxia leads to brain injuries and pathological development of different organs; and (*iii*) preconditioning/adaptation to hypoxic hypoxia primarily prevents obstetric complications and therefore future life diseases. It is a clear trend to use IHT for such adaptation, but improved doubling research is needed before wide using this method for primary prevention of obstetric complications.

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Review

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Promotion of osteoclast differentiation and activation in spite of impeded osteoblast-lineage differentiation under acidosis: Effects of acidosis on bone metabolism

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Summary **The acidosis that accompanies many diseases and pathological conditions can promote osteoclast formation and activation. Acidosis mainly acts on the last phase of osteoclast formation to generate large osteoclasts and promote bone resorption. There are several acid-sensing mechanisms, among which transient receptor potential (TRP) channels and G protein-related receptors have been focused on. TRPV4 channels appear to be, at least partly, implicated in acidosis-promoted large osteoclast formation. Other TRP channels including TRPV1 and TRPV2 might be components of the acid-sensing machinery. Several reports suggest the involvement of ovarian cancer G protein-coupled receptor 1 (OGR1), a G-protein-related acid sensor, in receptor activator of nuclear factor kappa-B ligand (RANKL) expression via cyclooxygenase-2 (COX-2). On the other hand, acidosis impairs osteoblast differentiation, which is further impeded in the presence of inflammatory cytokines.**

Keywords: Acidosis, acid sensing, osteoclast, osteoblast, bone metabolism

1. Introduction

The acid-base balance in the body has vital effects on cellular functions, because the structure and function of proteins are strictly controlled by the proton concentrations in tissue fluid, therefore, it could broadly influence the activity of enzymes in bone-related cells, the activity of transcription factors and the structure of other proteins involved in bone metabolism. We will explain the widely varying effects of acidosis on bone metabolism, mainly focusing on the mechanism behind the formation and activation of large osteoclasts. We will also describe the inhibitory effects of acidosis on osteogenic-lineage populations. There have recently been reports regarding the candidates of acid-sensing machinery, to which we will refer. We will pick up key mechanisms, which may explain acidosis effects on the metabolism of the bone system.

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2. Systemic acidosis and local acidosis

The pH of blood is of great importance to the body. A change of more than ± 0.05 from the physiologically neutral pH 7.4, results in acidosis (pH 7.35 or less) and alkalosis (pH 7.45 or more). The pH balance of extracellular fluids, blood, lymph, and interstitial fluid, are primarily attributed to the equilibrium between the acid-base balance composed of carbon dioxide (CO_2) and sodium bicarbonate ($NaHCO_3$). This balance cannot be maintained when the functions of organs deteriorate. The inability of the lungs to properly expel CO_2 can lead to respiratory acidosis, while defects in the kidney's function to excrete protons result in the consumption of bicarbonate ions (HCO_3^-) by remaining acids, metabolic acidosis (*1,2*).

Although organ failure invites acidosis systemically, numerous conditions and diseases can induce acidosis locally, local acidosis. These include tumors, inflammation, injury, infections, bone fractures, ischemia, hypoxia, and retardation of metabolic waste. These conditions necessarily affect the function of

surrounding tissues. Acidosis has been reported to drive bone metabolism toward bone resorption (*3*). On the other hand, mild alkalosis promotes osteoblast differentiation (*4*).

3. Acidosis and bone resorption

Acidosis impedes bone formation and promotes Ca^{2+} excretion. Alkalosis-inducing bicarbonate ions (HCO_3^-) in drinking water are reported to suppress Ca^{2+} excretion, whereas acidosis-inducing ammonium chloride (NH_4Cl) promotes Ca^{2+} excretion (*5-7*). Foods rich in nonvolatile acid precursors, for example, meat containing phosphorous or sulfur, lower blood pH (*2*).

Calvarial cultures under acidic conditions exhibit osteoclast activity to form pits on the surface of calvariae (*8*). This was confirmed using osteoclasts recovered from cocultures of bone marrow cells and osteoblastic cells on a collagen gel in the presence of 1,25,dihydroxy vitamin D_3 (1,25 $(OH)_2$ VD_3) and prostaglandine E_2 (PGE_2) in a regular culture medium for one week. We can control pH of the culture systems by adding different amount of $NaHCO_3$ to the cultures at 5% CO_2. When cultures consisting of osteoclasts and their nursing osteoblastic cells were transferred onto dentine slices in media with different pH to test for the ability to form pits, osteoclasts in acidic media, at pH 7.0 and pH6.8, formed pits more than in a neutral medium at pH7.4, indicating that acidosis activates osteoclast resorption (Figure 1). Although acidosis is important for osteoclast activity to resorb bone, interaction with osteoblasts appears to be vital to the activity of osteoclasts. Whereas osteoclasts derived from bone marrow cells using soluble receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) showed only weak resorptive activity, they exerted strong activity when osteoblasts activated with 1,25 $(OH)_2$ VD_3 and PGE_2 were added to the osteoclast cultures just before the pit formation assay on dentine slices (Figure 2).

In an *in vitro* cell system, intracellular Ca^{2+} elevation under acidosis activates calcineurin which activates NFATc1; NFATc1 moves to the nucleus, where it acts as a critical transcription factor for osteoclast activation. Acidosis also appears to inactivate several protein kinases

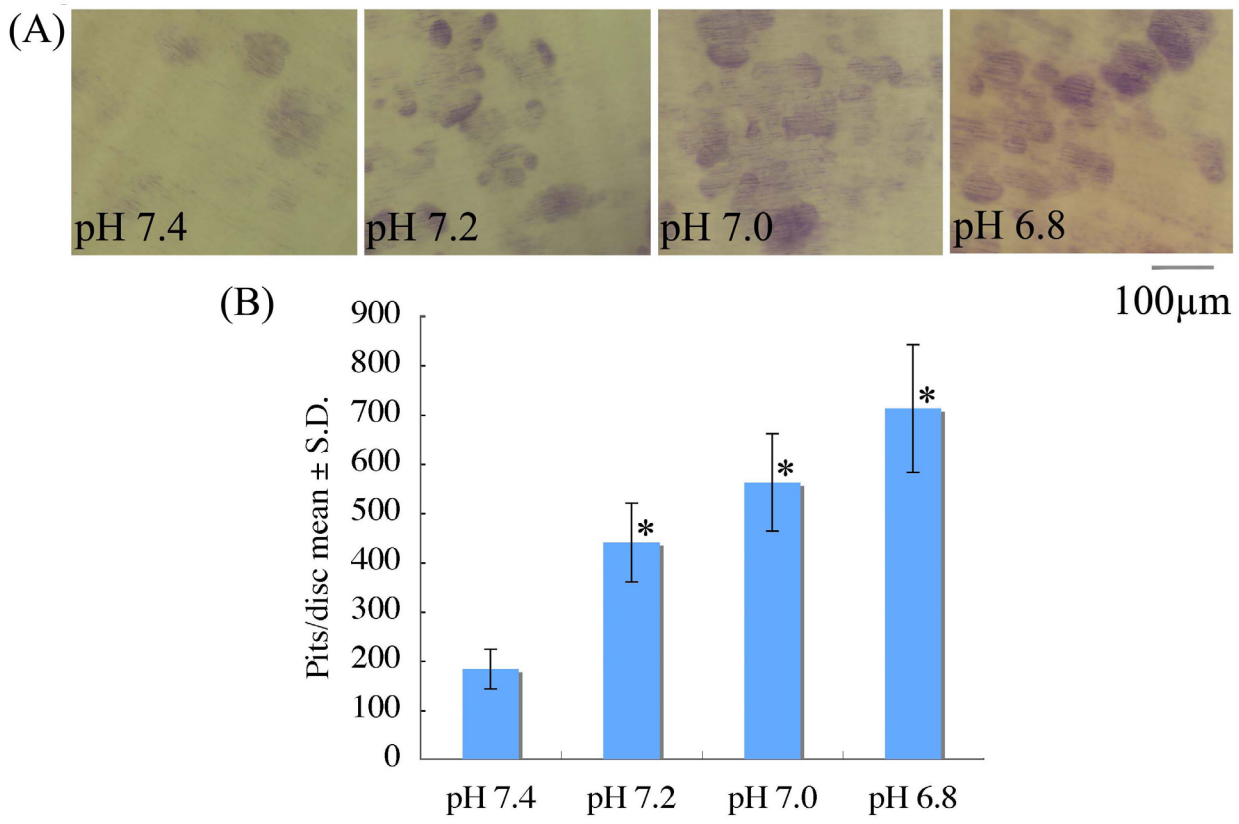

Figure 1. Acidosis promotes osteoclast activity. Mouse (male ddY) bone marrow cells were cocultured with TMS-12 cells, an osteoblastic cell line, on a type 1 collagen gel (2.4 mg/mL) in an α-MEM, 10% fetal calf serum, 10 nM 1,25 $(OH)_2VD_3$ and 1 μM PGE_2 at pH 7.3 for 7 days. After the digestion of the gel by 0.1% collagenase plus 0.2% dispase in α-MEM, cells were recovered, divided and suspended in media at pH 7.4, 7.2, 7.0, and 6.8. Cell suspensions were placed in 96-well plates with previously set discs of dentine slices at the bottom and kept in a humid atmosphere at 37ºC for 24 h. **(A)** Stains represent the pits. Bar under the photo represents 100 μm. **(B)** Pits of the resorbed trace on a disc were counted under a microscope after staining with 1% toluidine blue and scraping off the cells on the disc. Asterisks (*) represent differences that are statistically significant from control values at pH 7.4, $p < 0.05$, $n = 4$. All of the procedures for animal experiments were approved by the University Committee of Animal use.

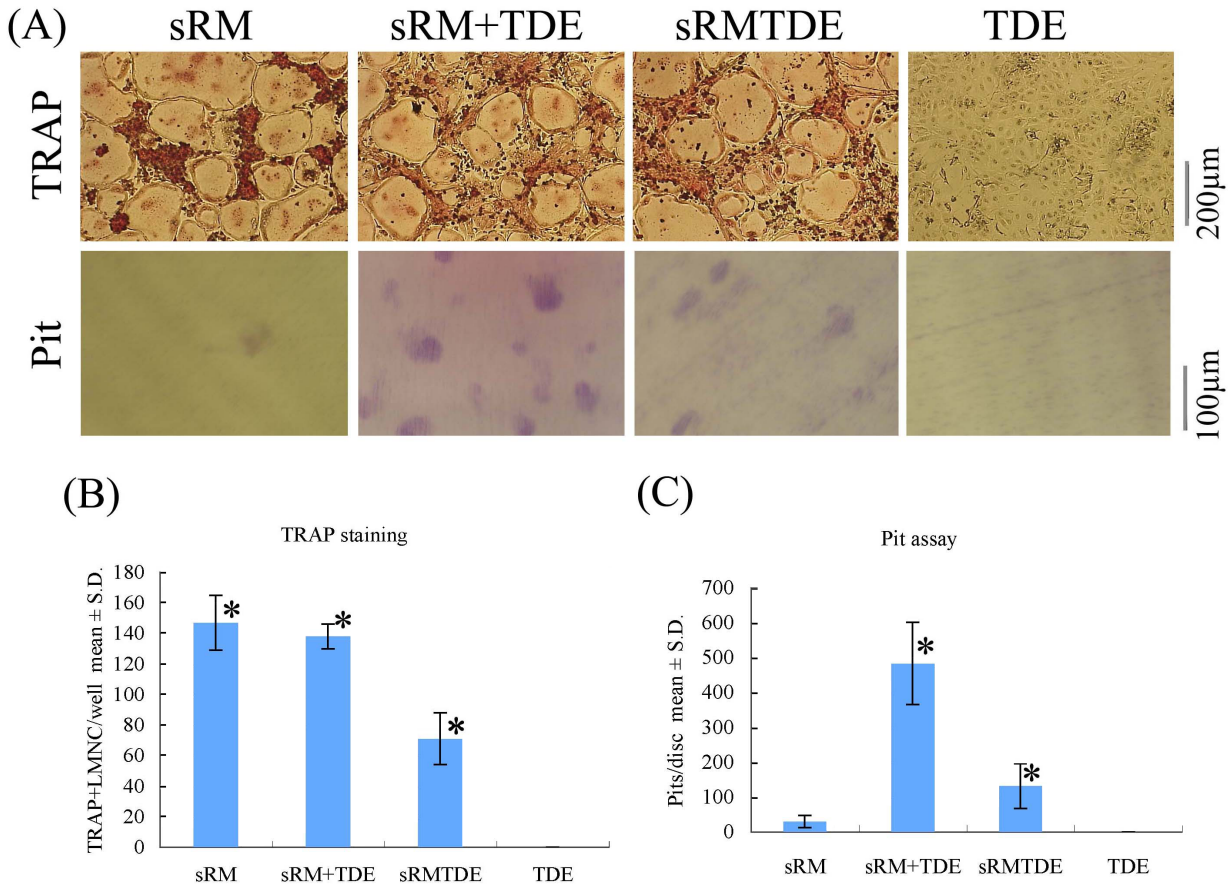

Figure 2. The cooperation between osteoclasts and osteoblasts appears to potentiate the bone resorptive activity of osteoclasts derived from bone marrow cells treated with soluble RANKL (sRANKL) and M-CSF. Mouse bone marrow cells cultured with 50 ng/mL sRANKL (sR) and 30 ng/mL M-CSF (M) in α-MEM-10% fetal calf serum at pH 7.3 for 3 days, sRM, were prepared. On the other hand, TMS-12 cells activated with 10 nM 1,25 $(OH)_2VD_3$(D) and 1 μM PGE2 (E) for 3 days, TDE. On day 3 sRM was combined with TDE, cultured in the presence of sRANKL and M-CSF at pH 6.8 for 24 h. **(A)** Tartarate-resistant acid phosphatase-positive large multinuclear cells (TRAP+LMNC: large osteoclasts) per well of 96-well plates. sRM cultures formed large osteoclasts. Although unstimulated TMS-12 cells and TMS-12 stimulated not enough suppressed large osteoclast formation from sRM, sRMTDE still stimulated pit formation. Bars under the photos of TRAP staining represents and those of pit formation assay represent 200 μm and 100 μm, respectively. **(B)** and **(C)**: graphical indications of TRAP staining and Pit formation assay, respectively. sRM cultures require activated TMS-12 to exert full resorptive activity. Asterisks (*) represent differences that are statistically significant from control values of sRM (cultures only containing activated TMS-12), $p < 0.05$, $n = 4$. TRAP + LMNC/large osteoclast: tertarate-resistant acid-phosphatase, a maker of osteoclasts, positive large multinuclear cells with 10 or more nuclei.

involved in the inactivation of NFATc1, maintaining NFATc1in the nucleus (*9*). Other than acidosis, PGE_2 appears to promote osteoclast formation in the absence of osteoblasts, implying the direct action of PGE_2 on RANKL-activated osteoclast lineage cells (*10*).

TRPV5, a member of a subfamily of the transient receptor potential (TRP) channel family, is reported to be indispensable for the differentiation of functional osteoclasts and for Ca^{2+} homeostasis in epithelial cells of the intestine and kidney. The loss of TRPV5 results in hypercalciuria, an increase in the numbers of less functional osteoclasts and loss of bone thickness (*11*). RANKL treatment induces intracellular Ca^{2+} elevation *via* TRPV5, but siRNA treatment to knockdown TRPV5 increased the bone resorptive activity of osteoclasts (*12*). Therefore, TRPV5 channels might function to monitor bone resorption. The effect of acidosis on TRPV5 channels remains to be clarified.

4. Acidosis and osteoclast formation

Acidosis also promotes the formation of osteoclasts, especially, large osteoclasts in several systems. It has been unclear where acidosis acts in the course of osteoclast formation, although there have been several reports that acidosis exerts promotive effects on osteoclastogenesis (*13,14*). We investigated this point to demonstrate that acidosis primarily acts on osteoclast differentiation in the last stage of the process, just before large-scale cell fusion (Figures 3A and 3B) (*15*). In a different coculture system, where osteoclasts were induced to differentiate with 1,25 $(OH)_2$ VD_3 and PGE_2 on a collagen gel in media with different pH, osteoclast formation was promoted at acidic pH and bone-resorbing activity of the cultures was also higher at lower pH than at a physiologically neutral pH7.4 (Figure 3C) (data not shown).

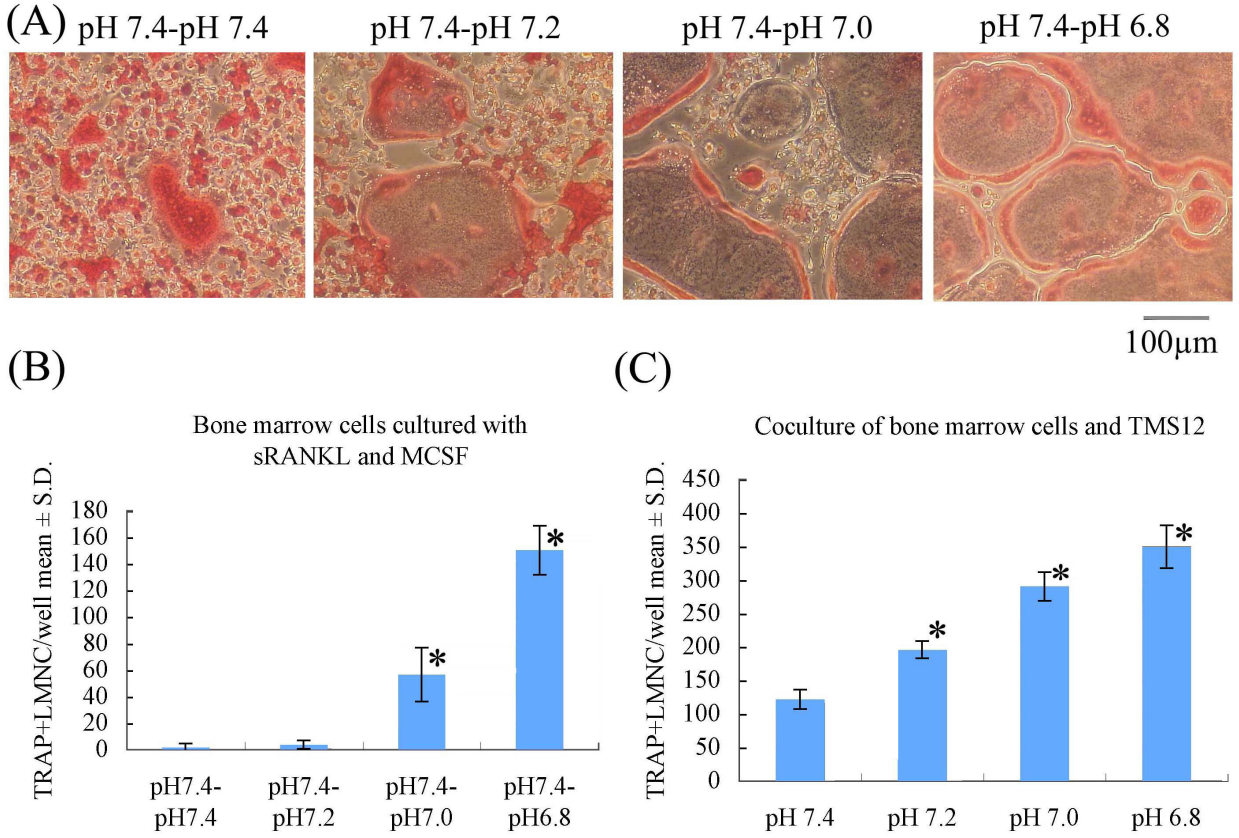

Figure 3. Acidosis acts on the last phase of large osteoclast formation in two different culture systems, monoculture and coculture on a collagen gel. (A) Mouse bone marrow cells were treated with sRANKL and M-CSF in α-MEM-10% fetal calf serum at pH 7.4 for 3 days. Cells were then cultured overnight in different media at pH 7.4, 7.2, 7.0, and 6.8. TRAP staining was performed on day 4. Photos represent TRAP + LMNC at 10 × 10 magnification. Bar under the photos represents 100 μm. **(B)** TRAP + LMNC numbers were counted after staining. Asterisks (*) represent differences that are statistically significant from control values at pH 7.4, $p < 0.05$, $n = 4$. **(C)** Mouse bone marrow cells were cocultured with TMS-12 cells, an osteoblastic cell line, on a type 1 collagen gel (2.4 mg/mL) in α-MEM, 10% fetal calf serum, 10 nM 1,25 $(OH)_2VD_3$ and 1 μM PGE_2 at pH 7.3 for 5 days. Then the cultures were divided into 4 groups; the media were replaced by a medium with pH 7.4, 7.2, 7.0, or 6.8; cultures were maintained for another 3 days, from day 5 to day 8; cells were recovered from the gel to test TRAP staining. Asterisks (*) represent differences that are statistically significant from control values at pH 7.4, $p < 0.05$, $n = 4$.

5. Acid-sensing machinery

Several types of acid-sensing systems have been reported, including members of acid-sensing G protein-related receptors, ovarian cancer G protein-coupled receptor 1 (OGR1) and T cell death-associated gene 8 (TDAG8) (*16-18*), of TRP, TRPV1 and TRPV4 (*19,20*), and of acid-sensing ion channels (ASICs) (*21*). TRPV1 and TRPV4 channels are permeable to several cations including Ca^{2+} and Na^+ and ASICs are specific for Na^+ ions. OGR1 was originally reported as a receptor for several lysophospholipids, lysophorylcholine, and sphyngosilphosphorylcholine (*16*), but later recognized as an acid-sensing receptor coupling with the Gq protein. On recognizing protons it changes molecule structure based on histidine residue-mediated transformation in an acidic environment. TDAG8 senses acid to move Gs, resulting in an elevation in the intracellular concentration of cyclic AMP (cAMP) (*18*).

The TRP family consists of cation-permeable channels with a variety of characteristics, differing in selectivity to cations including Ca^{2+} and Na^+. TRPV1, known as the receptor for capsaicin, is activated by acid. Several reports suggest the involvement of TRPV1 in osteoclastogenesis. Capsaicin, a TRPV1-specific agonist, enhanced osteoclast formation in murine bone marrow cultures treated with RANKL and M-CSF (*22*). On the other hand, capsazepine, a TRPV1-specific antagonist, suppressed osteoclast formation and the bone resorptive activity of osteoclasts (*23*). TRPV1-specific antagonists, capsazepin and 5-resiniferatoxin, also suppressed osteoblast differentiation. In our acidosis-promoted osteoclast formation system, capsaicin did not potentiate osteoclast formation, and AMG9810, a TRPV1-specific antagonist, did not inhibit osteoclast formation. The difference in systems used may cause different results.

TRPV4 was first reported as a channel sensing low osmolarity (*20*), but later found to be sensitive to mechanical stress (*24*). Vascular endothelial cells are known for their sensitivity to stretching, rotating

their orientation or spindle-shape perpendicular to the direction of stretch when scattered into elastic wells and cultured with cyclic stretch, which is attributed to TRPV4 (*25*). Several reports refer to knockout mice with depletion of TRPV4 (*26*). They are insensitive to the tail suspension test, which mimics microgravity (*27*). The mice also showed defects in osteoclast formation and activity, with large osteoclasts small in number (*28*). TRPV4 shows a weak response to acid (*29*), and is activated by src under acidotic conditions (*30*), implying that TRPV4 could be activated during acidosis.

TRPV4 channels are produced in the last phase of osteoclast differentiation, acting as Ca^{2+} channels to sustain the intracellular Ca^{2+} level for the maintenance of active NFATc1 (*28*). The precise control system for TRPV4 has recently been reported, with the calmodulin kinase system having important roles downstream of the channels (*31*).

Several membrane-derived arachidonic acid metabolites are known to activate TRPV4 (*20*). PGE_2 activates TRPV1 channels *via* PKC phospholyration in which EP1 is involved (*32*). PGE_2 potentiated osteoclast formation by soluble RANKL and M-CSF, which is sensitive to treatment with RN1734, a TRPV4-specific antagonist (*15*), showing that PGE_2 may activate TRPV4 in a similar way. Acidosis itself could release PGE_2, implying that it would also release arachidonic acid, the precursor of PGE_2 (*33*), and downstream metabolites, which could activate TRPV4 channels. Arachidonic acid and its metabolites can activate a wide variety of cation channels (*34,35*).

Ca^{2+} influx through TRP channels appears to be important to the formation of large osteoclasts, because lowering the extracellular Ca^{2+} concentration using EGTA, a Ca^{2+}-specific chelating reagent, reduced the degree of acidosis-promoted osteoclast formation. ASICs are unlikely important to acidosis-promoted osteoclast formation, because Ca^{2+} influx appears to be of primary importance in acidosis-promoted osteoclastogenesis. A Gs-related system is also unlikely to act on preosteoclasts in acidosis-promoted osteoclastogenesis, because dibutylyl-cAMP, a membrane-permeable derivative of cAMP, and forskolin, an adenylatecyclase activator, inhibit osteoclast formation (*36*).

TRPV4 channels appear to contribute to acidosis-promoted osteoclast formation because RN1734, a TRPV4-specific antagonist, partially inhibited, and 4-α PDD, a TRPV4-specific agonist, enhanced osteoclst formation under mild acidosis. Other unidentified TRP family cation channels permeable to Ca^{2+} may also contribute to acidosis-promoted osteoclast formation. In our experiment Ruthenium red, a general blocker of TRP channels, potently suppressed the osteoclastogenesis. Ruthenium red also blocks Ca^{2+}-dependent Ca^{2+} release (CICR) *via* ryanodine receptors. Another CICR blocker, dantrolen, did not show inhibitory effects on acidosis-promoted osteoclast formation, suggesting that Ruthenium red primarily acted on TRP channels in this system (*15*).

TRPV2 channels, members of the TRPV family, are sensitive to temperature and mechanical force, and necessary for osteoclast formation. These channels might also be candidates for the acidosis-sensitive machinery which drives osteoclast formation, partly because TRPV2 has structural homology with TRPV1 and TRPV4, is induced by RANKL, is responsible for Ca^{2+} oscillation during preosteoclast differentiation, and is maintained until a comparatively late phase of osteoclast formation (*37*). Studies using specific agonists and antagonists should provide clues as to whether this is the case or not. Anti-OGR1 specific to the extracellular domain of OGR1 was not able to suppress large osteoclast formation when added at the last phase of acidosis-promoted osteoclast formation.

6. Acid-sensing machinery

Osteoclasts are vulnerable to apoptosis under physiological conditions. Acidosis is reported to promote survival through NFATc1-independent and PKC-dependent pathways under the control of OGR1 (*38*). Acidosis is also reported to potentiate osteoclast survival to activate bone resorption *via* upregulation of osteopontin, promoting cell survival through integrin binding, augmentation of adhesion and spreading *via* activation of pyk-2, Cbl-b and src activation (*39*). However, Teramoto has reported that acidosis does not modulate osteoclast survival (*14)*.

7. Acidosis and *RANKL* gene expression

Several groups have addressed how acidosis works *via* OGR1. They used osteoblastic cells and calvarial organ cultures. Acidosis activated OGR1 to elevate intracellular Ca^{2+} levels *via* Gq stimulation, resulting in cyclooxygenase 2 (COX-2) expression. This led to the production of PGE_2, which is reported to activate osteoblasts to induce RANKL expression. siRNA against OGR1 blocked acidosis-induced COX-2 expression in a human osteoblastic cell line. YM-254890, a Gq antagonist, and PKC inhibitors blocked COX-2 expression at low pH (*40*). This result is in line with a report that acid-induced PGE_2 is essential for Ca^{2+} release from cultured calvariae. Pharmacological blocking of intracellular Ca^{2+} was able to suppress COX-2 expression, PGE_2 production and RANKL expression (*41*). This cascade from OGR1 to COX-2 and RANKL might be an event in the induction process by acidosis. Acidosis-promoted osteoclast formation in our experiments, where bone marrow cells were supported by soluble RANKL and M-CSF, was not blocked in the presence of Indomethacin, a general

inhibitor of cyclooxygenases, implying that PGE_2 production does not have primary role in the late phase of osteoclast formation. We have observed that the role of COX-2 in RANKL induction appears to be during a limited period in the course of osteoclast formation (unpublished data).

8. Bicarbonate ions repress osteoclast formation

As mentioned at the beginning, alkalosis suppresses bone resorption. One reason for this is that bicarbonate ions are able to activate a soluble type adenylatecyclase, which produces cAMP (*42*). cAMP-elevating reagents acting on osteoblasts, PGE_2 and PTH (parathyroid hormone), generally induce RANKL expression. On the other hand, those acting on osteoclasts suppress osteoclast formation and activation, for example, forskolin, dibutyl cAMP and calcitonin (*36*). Thus local and systemic control of HCO_3^- ions appears to be of great importance for maintaining bone mineral content.

9. Acidosis and osteoblast-lineage differentiation, survival and functions

Osteoblast differentiation is inhibited under acidosis. In a regular system, where the osteoblasts are derived from bone marrow cells in a 5% CO_2/HCO_3 system, osteoblasts differentiate better at higher than lower pH (Figure 4A). Acidosis is reported to impede the production of collagen type 1, osteocalcin and other osteoblast marker proteins (*43*). Inflammatory cytokines, tumor necrosis factor (TNF)-α and IL-1 β also deteriorate osteoblast formation with different strengths (Figure 4B). TNF-α showed potent inhibitory activity under acidotic conditions. We confirmed that acidosis and TNF-α cooperate to inhibit osteoblast differentiation from bone marrow cells (Figure 4C).

Connexins are a group of proteins, which contain short cytoplasmic N-terminal sequences, intracellular four transmembrane domains and long intracellular C-terminal sequences and assemble as the hexamer connexon on the plasma membrane (*44*). So far the

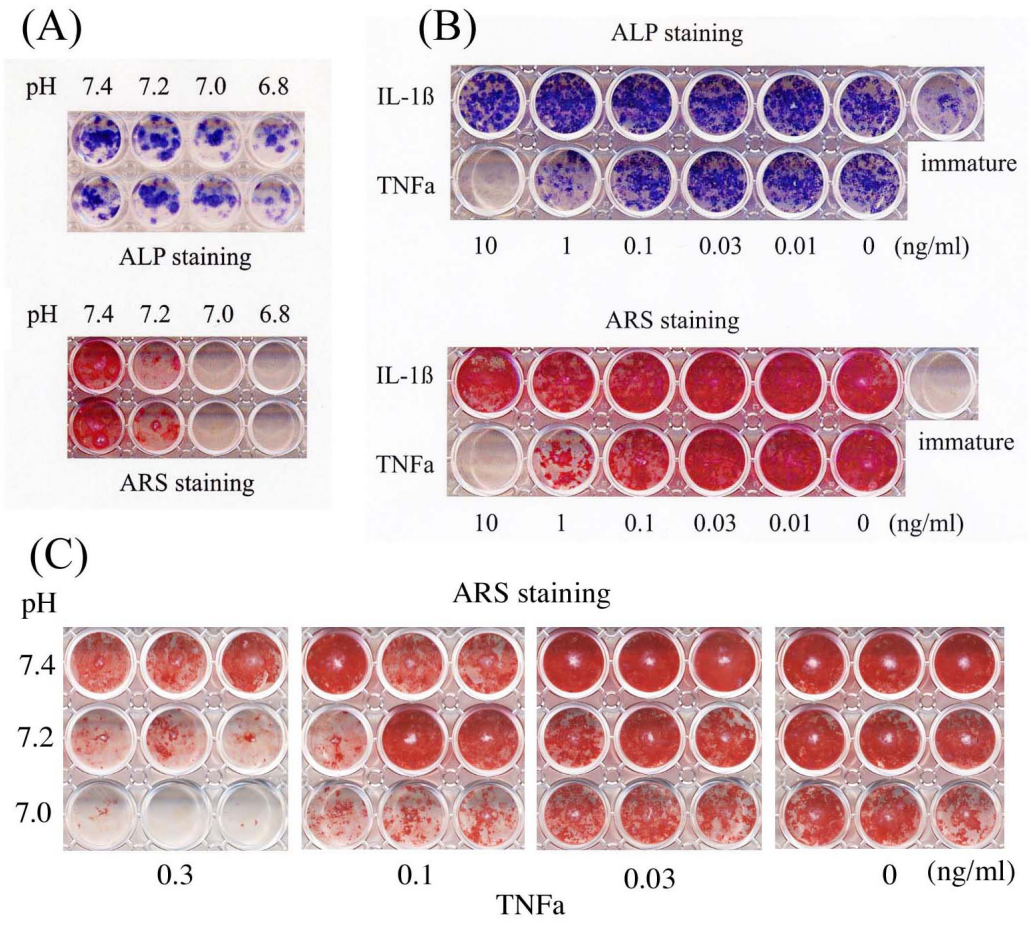

Figure 4. Acidosis impedes the differentiation of osteoblast lineage cells from bone marrow cells. Mouce bone marrow cells (1,000,000 cells/1 mL/well) were cultured in 24-well plates in α-MEM-10% fetal calf serum in the presence of 50 μg/mL ascorbic acid 2-phosphate (Ascp) and 10 mM glycerol 2-phosphate (Gp) at pH 7.4, 7.2, 7.0, and 6.8. The effects of IL-1 beta (IL-1β) and TNF-α were tested at pH 7.3. The culture medium was changed every third day in all cases. Cultures without Ascp and Gp were called immature in the figure. **(A)** Alkaline phosphatase staining (ALP) was conducted on day 10 for early differentiation of osteoblasts to test early differentiation of osteoblasts. **(B)** Alizarin red S staining (ARS) was performed on day 21 to certify nodule calcification. **(C)** TNF-α potently acts against osteoblast differentiation under acidotic conditions.

group has 21 members. Connexin 43 (Cx43) is the most dominant in bone tissue. There have been numerous investigations into the roles of Cx43 using many types of tissues and cells, including cell differentiation and cell survival (*45,46*). Cx43 is found in osteoblast-lineage cells, especially in osteocytes. Cx43 connexons are thought to act as hemichannels, platforms for the assembly of proteins through C-terminal sequences, intercellular channels to transmit or exchange Ca^{2+}, other ions, cAMP and small peptides and nucleotides with a molecular weight of less than around 1,000. There are several reports on Cx43 knockout mice specific to the osteoblast-lineage (*47*). A delay of osteoblast lineage differentiation and deterioration of function and survival are common phenotypes of Cx43 conditional knockout mice (*48,49*), although the mechanism remains to be elucidated. Astrocytes are reported to internalize Cx43 under acidosis (*50*). This is an interesting example because osteoblast differentiation is delayed when Cx43 is depleted (*51*). The survival of osteocytes is influenced by Cx43 (*48*). These examples suggest that the differentiation of osteoblast-lineage cells from mesenchymal stem cells and their survival would be impeded under acidotic environments. In addition to acidosis, hypoxic conditions cause Cx43 internalization (*52*).

10. Acidosis, hypoxia and reactive oxygen species

Hypoxic conditions impair bone formation, partly because oxygen is necessary for the production of collagen molecules, rich in hydroxylized lysine and proline residues. Biosynthesis generally requires energy for putting materials into whole molecules that hold ordered structures. When tissues are left in a hypoxic environment, aerobic metabolic cascades slow down and an anaerobic respiratory system dominates, producing more lactic acid. Degenerated biomaterials often contain nonvolatile acidic products, sulfates, phosphates and so on. Thus, acidosis is closely related with hypoxia and both are favorable for bone resorption (*4,53*). Both would work cooperatively, impeding bone formation and accelerating bone resorption.

Acidosis is an environmental factor for deteriorating bone formation, promoting osteoclast formation and activity, impeding osteoblast differentiation. Although how acidosis acts on preosteoclasts and preosteoblasts to exert inverse effects, promotion and suppression of differentiation, respectively, remains to be elucidated, several hints appear to be in the researches regarding the roles of reactive oxygen species in bone metabolism.

RANKL stimulates osteoclast differentiation by stimulating several signal pathways converging on NFATc1 activation, where reactive oxygen species induced by RANKL stimulation promote long-lasting Ca^{2+} oscillation required for osteoclast formation (*54*). Glucocorticoide and TNF-α are known to suppress osteoblast differentiation, where reactive oxygen works to eventually decrease the amount of active form β-catenin, a key transcriptional factor required for osteoblast differentiation (*55*). In cancer cells acidic environment itself leads to the generation of reactive oxygen species (*56*).

11. Conclusions

Acidosis is deeply involved in a variety of diseases and pathological conditions, promoting bone resorption and impeding bone formation. It therefore could be a candidate for intervention in the treatment of diseases.

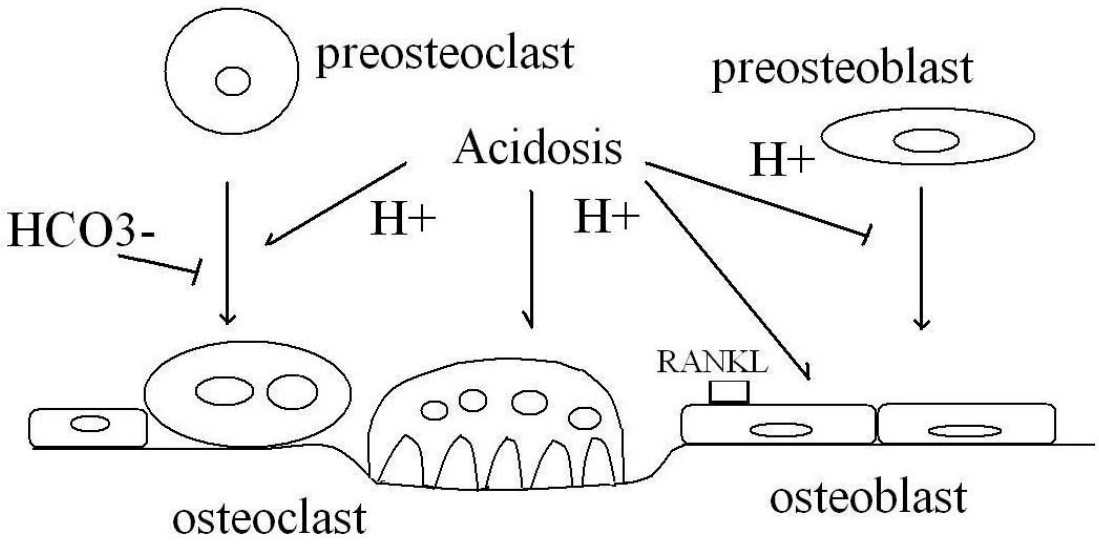

Figure 5. Acidosis promotes osteoclast differentiation and activation and inhibits osteoblast differentiation. This is a schematic indication of the contens of this review. Acidosis has promotive effects on osteoclast lineage cells. On the other hand acidosis acts against osteoblast differentiation.

The acid-base balance is a basic factor for the body. Therefore, this influences a wide variety of targets, conferring accents on more system-specific reactions. Elucidation of the relationship among acidosis, hypoxia, redox state, RANKL production, mechanical force, Wnt signaling and inflammatory cytokines would provide a more precise understanding of bone physiology and pathology of bone-related diseases. Figure 5 is a schematic drawing of the contents of the text.

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Original Article

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Prognostic significance of β-catenin expression in patients with non-small cell lung cancer: A meta-analysis

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Summary **β-Catenin has been reported to play a crucial role in the invasion and metastasis of lung cancer. However, the value of β-catenin as a prognostic factor for non-small cell lung cancer (NSCLC) remains controversial. The present study systematically reviewed the evidence of predicting significance of β-catenin expression in NSCLC patients with meta-analysis. Twelve literatures were included by searching PubMed, Cochrane library, and EMBASE databases. Separate hazard ratio estimates and a 95% confidence interval (CI) for the prognostic value of β-catenin in NSCLC were extracted and merged from the included literatures. The summary hazard ratios were 1.91 (95% CI 1.60-2.28), indicating a worse overall survival for NSCLC patients with reduced β-catenin expression. There was no significant heterogeneity among the studies (X^2 = 12.41, p = 0.413, I^2 = 3.3%). Publication bias was not statistically significant. Sensitivity analysis showed that omission of any single study had little effect on the combined risk estimates. This meta-study revealed that decreased β-catenin expression denoted a poor prognosis in NSCLC patients.**

Keywords: β-Catenin, non-small cell lung cancer, prognosis, overall survival

1. Introduction

Lung cancer is the leading cause of death in malignant neoplasm around the world (*1*), accounting for 1.1 million deaths annually world-wide (*2*). In China, lung cancer is one of the principal malignant neoplasms, with an increasing tendency in both morbidity and mortality in recent years (*3*). The prognosis of lung cancer is also dismal, with a 5-year survival of merely 15% (*4*). Non-small cell lung cancer (NSCLC), mainly including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for nearly 90% of all lung cancer cases (*5*). Though new chemotherapies have remarkably improved the outcome of NSCLC patients, the prognosis remains poor on the whole. Approximately 30% of patients with stage I NSCLC will die within 5 years after surgery (*6*) due to metastasis.

Tumor cells escaping from the primary tumor is the initial step of metastasis, which depends in part on cell adhesion molecules (CAMs) (*7*). Once the CAMs are altered, metastasis would be promoted (*8*) and a poor prognosis will be induced (*9*). β-Catenin, a multifunctional protein encoded in chromosome 3p21 (*10*), is one of the essential components of CAMs and plays a crucial role in cell-cell adhesion and tissue remodeling (*11*). It participates in cell-cell adhesion by binding to the intracellular domain of E-cadherin. The latter is a homotypic cell-cell interaction molecule which is ubiquitously expressed on epithelial cells (*8*) and has proven to be related to a poor outcome in NSCLC (*12*). β-Catenin is also important in Wnt/β-catenin signaling pathway by activating transcription of target genes and leading to cell proliferation, invasion, and metastasis (*13*). It is reported that reduced expression of β-catenin is an important determinant for the metastatic capability of certain cancer cells (*14*). Indeed, decreased β-catenin expression has been widely reported to be related to poor differentiation, lymph node spread, and metastasis in various human

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carcinomas such as breast cancer (*15*), gastric cancer (*16*), and prostate cancer (*17*).

Recently, the relationship between β-catenin expression and survival of patients with NSCLC has been intensively studied. But the prognostic significance of β-catenin expression in NSCLC remains controversial. Actually, several studies claimed that reduced β-catenin expression was associated with poor outcome of NSCLC patients, while others did not support the conclusion. Therefore, we performed this meta-analysis to assess the prognostic value of β-catenin expression for NSCLC patients.

2. Methods

2.1. *Search strategy and study selection*

We searched PubMed, Cochrane library, and EMBASE databases for relevant articles published until November 1st, 2012. Articles were identified using the following search terms: "Beta-catenin, β-catenin, or CTNNB1", "prognostic, prognosis, or survival" and "lung neoplasm, lung cancer, or lung carcinoma". No lower date or language limits were applied initially, but for full-text review and data analysis, only articles in English were included finally. References of identified articles were also searched manually. To make this study meet the high standards, the following criteria were used: (*i*) the patients were diagnosed as NSCLC by pathology; (*ii*) β-catenin expression was measured by immunohistological chemistry (IHC) method in primary lung cancer tissue; (*iii*) information on overall survival comparing patients with and without impaired expression of β-catenin were provided; (*iv*) sufficient data of the value of hazard ratio (HR) and 95% confidence interval (CI) between β-catenin expression and overall survival were given; (*v*) the patients were followed-up for at least 3 years. We excluded articles of studies on animals, reviews and studies with insufficient data. When an individual author published several articles with data obtained from the same patient population, only the newest or most informative article was selected.

In selecting literature, we first screened the title and abstract to see whether they met the including criteria. Then, based on the initial screening, we scrutinized the full manuscript of studies that needed further examination. Two reviewers (Song and Mei) independently verified study eligibility. All disagreements in judging study eligibility were resolved by consensus.

2.2. *Data extraction and quality assessment*

The following information were retrieved independently by 2 reviewers (Mei and Su) from the final set of literatures: publication year, first author, number of patients enrolled, histology and disease stage, method of HR estimation, cut off value, percentage of decreased expression, HR and 95% CI as well as the other related events.

Two reviewers (Mei and Su) read the articles independently and performed quality scoring using the mean global quality score method according to Steele's (*18*). The overall score evaluated various aspects of the methodology, and was grouped into four main categories: scientific design, description of the method used to identify abnormal β-catenin expression, the generality of the results, and the analysis method of data. A maximum of 10 points was given for each category with an inclusive maximum score of 40 points. When an item was not appropriate in a study, its value was abandoned. Final scores were expressed as percentages ranging from 0% to 100%, with higher values indicating better methodology.

2.3. *Statistical analyses*

We chose HR as the effect indicator to compare time-to-event results for its distinctive advantages: accounting for censoring, including all data and describing all of patients' experience. The individual HR estimate was combined into overall HRs with the methods published by Yusuf *et al*. (*19*). Some of the studies that provided HR and a 95% CI value were pooled directly. For studies not provided directly, we obtained the value from the available data or by reading Kaplan-Meier survival cure in the original studies (*20-22*). The way to obtain HR and a 95%CI from the Kaplan-Meier survival cure was reported by Parmar MK (*21*) and has been widely applied in meta-analysis about prognostic factors (*16,18,23,24*). Engauge Digitizer version 2.11 (free software from *http://sourceforge.net*) was used in reading the Kaplan–Meier curves. If a study provided both the results of multivariate analysis and univariate analysis, we chose the former. The available data contained the total number of events, the log-rank statistic and its *p*-value, or the O-*E* statistic (difference between numbers of observed and expected events). Heterogeneity among studies was assessed by the Chi-squared test and *Q*-test. The I^2 value was used to evaluate the heterogeneity (I^2 = 0-40%, no or moderate heterogeneity; I^2 > 40%, significant heterogeneity). Fixed-effect model was used if there was no significant heterogeneity. Otherwise, the random-effect model was used. Funnel plot and Egger's linear regression test were performed to identify the possibility of publication bias. The robustness of the combined results was confirmed by sensitivity analysis in which the data of an individual study were removed each time. The pooled HR > 1 indicated that NSCLC patients with decreased β-catenin expression had a poor survival. The impact of decreased β-catenin expression on overall survival was considered statistically significant if the 95% CI

did not overlap with 1. Nonparametric tests were used to compare the distribution of quality scores according to the value of a discrete variable. All the *p*-values were two sided, and $p < 0.05$ was considered statistically significant. All statistical analyses were conducted with STATA software version 11.0.

3. Results

3.1. *Statistical analyses*

The flow diagram of article selection is shown in Figure 1. Initially, ninety six articles were identified. After reading the title and abstract, twenty-one studies were included for further confirming. Nine studies were excluded by scrutinizing the entire paper. Of those excluded studies, five had insufficient data (*22,25-28*) and one analyzed the relationship between β-catenin mRNA level and the outcome in NSCLC (*29*). One reported pulmonary metastases from colorectal carcinoma (*30*). One evaluated the association between nuclear β-catenin expression and survival in NSCLC (*31*). The influence of decreased β-catenin expression on NSCLC survival was estimated by disease-free survival in another article (*32*). Additionally, one article (*33*) provided information about adenocarcinoma and squamous cell carcinoma, which we processed independently as two studies in the meta-analysis. Eventually, twelve literatures containing thirteen studies that met the inclusion criteria were selected (*17,33-43*).

The major characteristics of the included studies are outlined in Table 1. The total number of patients was 1,964, with sample sizes ranging from 35 to 522 patients. The reduced rate of β-catenin expression varied from 5.26% to 67.5%. HR and 95% CI were obtained from the original studies directly in six of

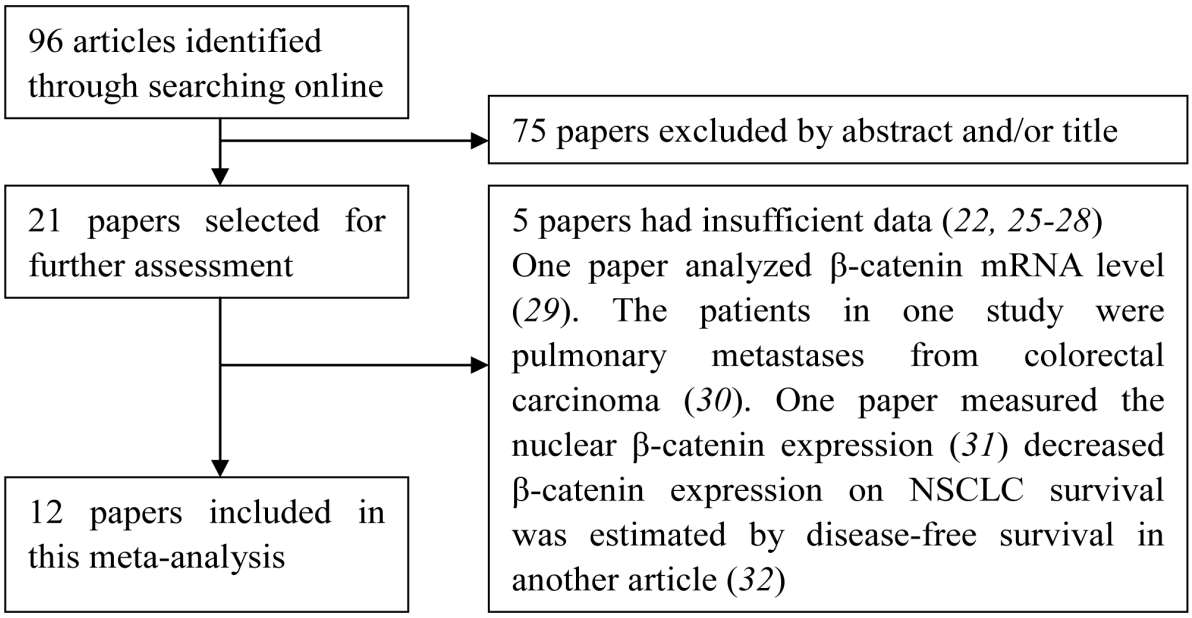

Figure 1. The flow diagram of article selection.

Table 1. Basic characteristics of the included studies in the meta-analysis

First author	Year	Histology	No.	Cut off	Reduced/ negative (%)	HR estimate	HR (95%CI)
Chiu *et al*. (*17*)	2012	NSCLC (AD:219; SQ:242; others:61)	522	5	28.0	HR (M)	3.18 (1.46-6.91)
Zhang *et al*. (*34*)	2012	NSCLC (AD:54; SQ:56)	110	10	50.0	Curve	1.79 (1.17-2.74)
Xu *et al*. (*35*)	2011	NSCLC (AD:165; SQ:97)	262	70	22.9	HR (M)	2.17 (1.09-4.29)
Yamashita *et al*. (*36*)	2010	NSCLC (SQ:31; others:86)	117	70	29.1	HR (M)	1.26 (0.65-2.43)
Yang *et al*. (*37*)	2010	NSCLC (AD:26; SQ:17)	120	50	67.5	HR (M)	2.39 (1.04-5.51)
Zhao *et al*. (*38*)	2010	NSCLC (AD:58; SQ:44)	102	10	62.7	HR (M)	1.13 (0.56-2.26)
Woenckhaus *et al*. (*33*) (*1*)	2008	AD	38	5	5.26	A (U)	2.92 (1.27-6.73)
Woenckhaus *et al*. (*33*) (*2*)	2008	SQ	38	5	13.2	A (U)	1.09 (0.36-3.33)
Nozawa *et al*. (*39*)	2006	AD	35	88	37.1	A (U)	2.41 (1.15-5.05)
Hommura *et al*. (*40*)	2002	NSCLC (AD:108; SQ:92; others:17)	148	25	23.6	A (U)	2.38 (1.03-5.52)
Lee *et al*. (*41*)	2002	NSCLC	75	50	13.3	Curve	3.02 (1.52-5.93)
Kimura *et al*. (*42*)	2000	NSCLC (AD:75; SQ:9; LCC:1; others:1)	86	80	48.8	Curve	1.40 (1.03-2.52)
Kase *et al*. (*43*)	2000	NSCLC (AD:227; SQ:104)	311	70	37.0	HR (M)	2.21 (1.36-3.60)

M: multivariate analysis; U: univariate analysis; AD: adenocarcinoma; SQ: squamous cell carcinoma; A: available data; Curve: Kaplan–Meier curve.

twelve studies (*17,35-38,43*) and calculated from available data in the other three original literatures (*33,39,40*). For the remaining three studies (*34,41,42*), HR and 95% CI were extrapolated from Kaplan-Meier curves. On statistical method, six studies (*17,34-37,43*) provided the results of multivariate analysis and the others (*33,38-42*) provided results with univariate analysis. The cut-off point ranged widely. Three studies (*35,36,43*) selected a proportion of < 70% as reduced staining. Two studies (*17,33*) used 5% as the cut-off value, two studies (*34,38*) selected cut-off points at 10%, and two other studies used 50% (*37,41*). The three remaining studies used < 25% (*40*), < 88% (*39*), and < 80% (*42*), respectively.

3.2. *Quality assessment*

Overall, the mean global quality score of the included studies was 55.3%. There was no statistical difference between the ten positive and three negative studies (55.5% *versus* 55%, $p = 0.25$). All of the results of methodological assessment are shown in Table 2.

3. 3. *Meta-analysis*

Forest plot showed that combined HR was 1.91 and 95% CI 1.60-2.28 by fixed-effect model for all studies and the heterogeneity was not statistically significant ($X^2 = 12.41$, $p = 0.413$, $I^2 = 3.3\%$, Figure 2). Of the thirteen studies, ten studies located in the right side of equivalent line supported the assumption that reduced β-catenin expression was associated with poor survival of NSCLC patients. The bars of 95% CI of the other three studies overlapped with the equivalent line, which did not support the conclusion.

When limiting the histology to adenocarcinoma, two studies were assessable. The combined HR and 95% CI by the fixed-effect model were 2.62 (1.51-4.56) and no heterogeneity was observed. Based on the stage, the results of two studies of stage I patients indicated a significant association between β-catenin expression and overall survival (HR: 1.92, 95% CI: 1.20-3.09).

We also divided the studies on statistical method and analyzed them separately. Of twelve studies, six studies used multivariate analysis while the others used univariate analysis. Both combined results of multivariate and univariate analysis indicated similar statistically significance (HR = 1.91, 95% CI: 1.43-2.50, Figure 3 and HR = 1.91, 95% CI: 1.51-2.41, Figure 4). The results supported the assumption that reduced β-catenin expression was associated with a poor survival in NSCLC patients. Similarly, no significant heterogeneity was observed in any of the subgroups ($I^2 = 18.2\%$, $p = 0.295$ and $I^2 = 4.7\%$, $p = 0.391$, respectively).

Table 2. Results of the methodology assessment

Items	N	Global score (%)	Design (/10)	Laboratory methodology (/10)	Generalizability (/10)	Results analysis (/10)
All studies	13	55.3	5.3	5.1	6.1	5.6
Negative	3	55.0	5.4	5.2	5.9	5.5
Positive	10	55.5	5.3	5.1	6.2	5.6
p-value		0.25	0.36	0.13	0.17	0.09

Score distributions are summarized by the median values; Negative: no significant prognostic factor for survival; Positive: as significant positive prognostic factor for survival.

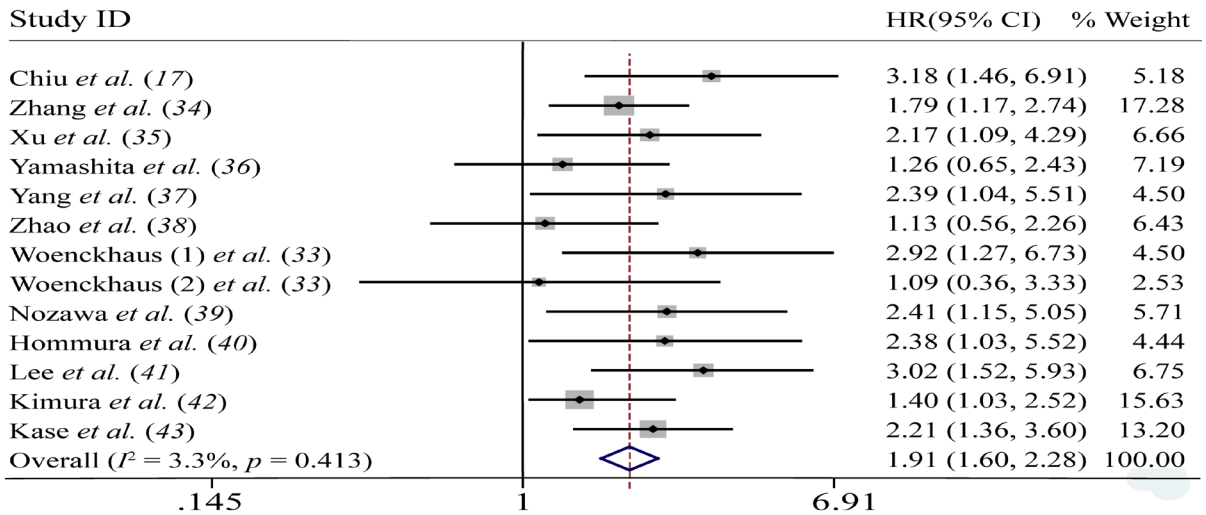

Figure 2. Meta-analysis of roles of β-catenin expression on survival in patients with NSCLC. Hazard ratio (HR) and 95% confidence interval (CI) of reduced β-catenin expression on overall survival for NSCLC patients. Results are expressed as individuals (squares) and overall HRs (diamonds) and their respective 95% CIs (horizontal bars). An HR higher than 1 indicates a poor prognosis for NSCLC patients with reduced β-catenin expression.

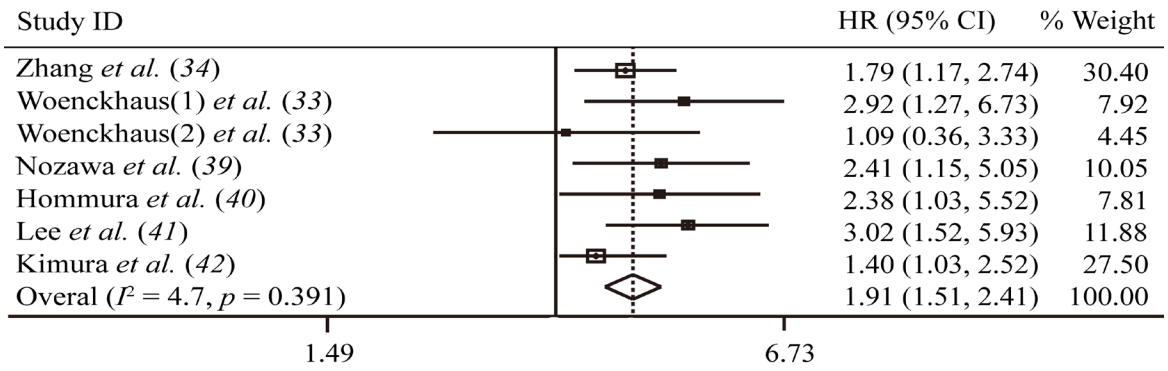

Figure 3. Forest plot of the effect of β-catenin expression on overall survival in NSCLC in studies with multivariate analysis. The bars of 95% CI of four studies do not overlap with the equivalent line compared with the two others. The summary HRs is 1.91 (95% CI: 1.14-2.50) favoring the assumption that decreased β-catenin expression is associated with poor prognosis in NSCLC patients.

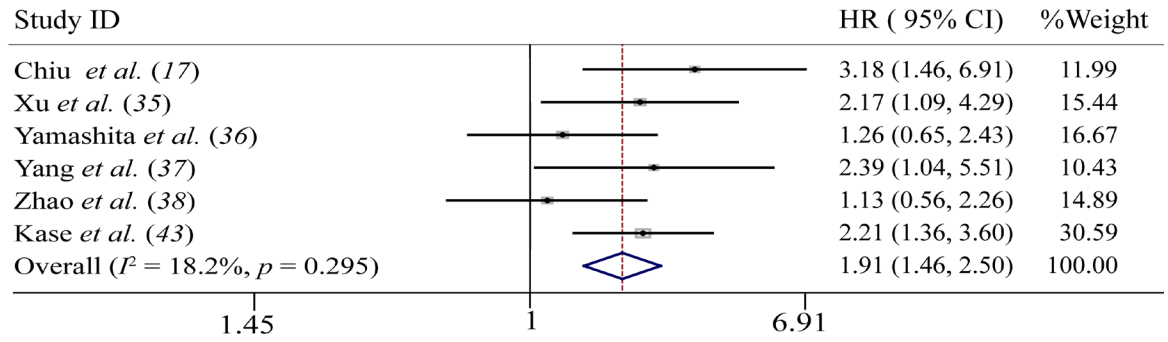

Figure 4. Forest plot of the effect of β-catenin expression on overall survival in NSCLC patients in studies with univariate analysis. The aggregated HR is 1.91 (95% CI: 1.51-2.41) which supports the assumption that reduced β-catenin expression is associated with a poor outcome of NSCLS patients.

3. 4. *Publication bias*

Funnel plot and Egger's test were both performed to evaluate the publication bias. Funnel plot did not reflect obvious asymmetry in this meta-analysis (Figure 5). Also, no indication of publication bias was found from the Egger's test (t = 0.87, p = 0.402 > 0.05).

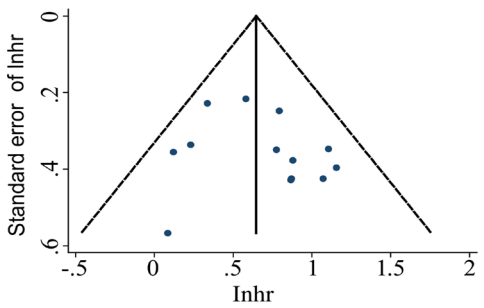

Figure 5. Begg's funnel plot for publication bias test of the studies with pseudo 95% confidence limits. The funnel graph plots hazard ratio against the standard error of the log hazard ratio. Each point represents a separate study for the indicated association. The publication bias is not significant (p = 0.402 > 0.05).

3. 5. *Sensitivity analysis*

To evaluate the robustness of the result of combined HR, sensitivity analysis was performed by removing one study each time. The results were shown in Figure 6. The pooled HRs and 95% CIs were not significantly altered when any part of the study was omitted, which indicated that any single study had little impact on the combined risk estimates and confirmed the robustness of the result of this meta-analysis.

4. Discussion

Despite remarkable advances in treatment, the prognosis of NSCLC remains gloomy at present (*4*). Metastasis and recurrence are the main causes of poor prognosis. For better management of NSCLC patients, many efforts have been made to find a predictor of prognosis. Some prognostic markers such as p16 (*44*) and Ki-67 (*45*) were evaluated. Several molecules including mmp9 (*23*), survivin (*24*), p53 (*18*), and cyclinD1 (*16*) have been suggested the prognostic factors for NSCLC. However, none of these markers could predict the outcome of NSCLC patients exactly

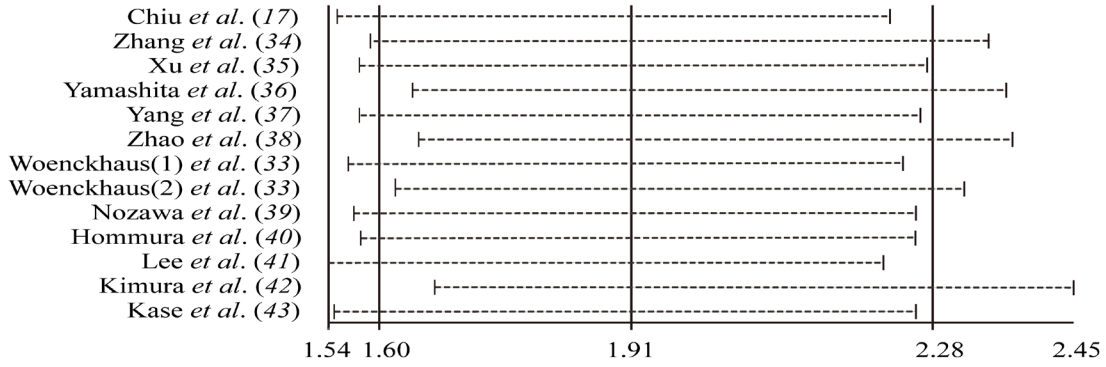

Figure 6. Sensitivity analyses of all the studies. Omission of any study did not affect the whole estimate results significantly.

and reliably and more markers are needed. Recently, one systemic review concluded that reduced E-cadherin expression was associated with a poor survival in NSCLC (*12*) which suggested that CAMs might be underlying predictive factors for NSCLC patients.

In this meta-analysis, the association between reduced β-catenin expression and overall survival in patients with NSCLC was comprehensively reviewed. The aggregation of all included studies produced statistically significant HRs: 1.91 (95% CI: 1.60-2.28), favoring the assumption that reduced β-catenin expression is associated with a poor prognosis in patients with NSCLC. Subgroup analyses on histology, stage and multivariate or univariate analysis also demonstrated similar results.

The initial step of metastasis is tumor cell escaping from the primary tumor, which is regulated by cell adhesion molecules. Decreased cell connection has proven to contribute to invasion and metastasis in tumor development (*46*). It is noted that intact complexes of β-catenin/E-cadherin are important adhesion molecules and inhibitors of cancer invasion and metastasis (*11*). β-Catenin, a component of the β-catenin/E-cadherin complex, has been reported to be involved in tumor metastasis (*7*). When β-catenin expression is decreased, the stability and function of β-catenin/E-cadherin complex will change. The prognostic value of β-catenin expression in NSCLC patients has been extensively investigated recently (*17,33-43*). Many of these articles claimed that reduced β-catenin expression was a predictor for poor outcome of NSCLC patients. The results of our present meta-analysis supports this conclusion in general.

Exploring heterogeneity is one of the important goals of meta-analysis (*47*). One of the advantages of the present meta-analysis is that no significant heterogeneity was found among the included studies ($p = 0.413$, $I^2 = 3.3\%$). Sensitivity analysis also showed that omission of any single study did not have significant impact on the combined risk estimates. Furthermore, funnel plot did not reflect obvious asymmetry, and Egger's test further indicated no considerable publication bias in this meta-analysis. This made the results of this meta-study more reliable to some extent.

Be that as it may, there remained some limitations in this meta-analysis. In the studies included, the antibodies used in detecting β-catenin expression were not the same. The definition of cut off value was also different, and varied from 5% to 88%. Furthermore, in the thirteen studies, six studies used multivariate analysis while the remaining adopted univariate analysis. Besides, other clinical factors such as age, sex and different chemotherapies in each study might lead to bias. Determining whether or not these factors influence the results of this meta-analysis would need further investigation.

We did not include non-English publications in this study. Some HR results were obtained indirectly from available data or by reading the survival curve. These approaches may have produced errors because of possible inaccurate reading. Additionally, among the nine excluded studies, five studies were excluded because of insufficient data. None of the five studies reported significant association between reduced β-catenin expression and survival in NSCLC. All of the above factors could lead to possible bias and should not be neglected.

In conclusion, the results of our meta-analysis suggest, as a whole, that reduced β-catenin expression is associated with a poor overall survival in NSCLC patients. Decreased β-catenin expression could be a prognostic predictor for NSCLC patients. Some limitations mentioned above should not be ignored. More prospective well-designed studies with standardized detecting methods, unified cut-off values and statistical methods are needed to further confirm and establish the utility of prognostic value of β-catenin expression in NSCLC patients.

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Original Article

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Downregulating immunogenicity of Schwann cells *via* inhibiting a potential target of class II transactivator (*CIITA*) gene

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Summary **Immunological rejection induced by allogeneic Schwann cells remains a problem for construction of artificial nerves. Class II transactivator (*CIITA*) gene is a chief regulator of major histocompatibility complex class II (MHC II) molecules which contributes to the immunogenicity of Schwann cells. This study aimed to downregulate MHC II expression by suppressing CIITA expression, therefore reducing the immunogenicity of Schwann cells. Recombinant siRNA expression vectors targeting the *CIITA* gene were produced and subsequently transfected into rat RSC96 Schwann cells. Interferon (IFN)-γ was used to augment immunological rejection of RSC96 cells. The mRNA levels of CIITA and MHC II were assessed by fluorescence quantitative PCR. The protein levels of MHC II were determined using flow cytometry assays. Finally, the immunogenicity of RSC96 cells was analyzed using mixed lymphocytes reactions. Results indicated the expression of MHC II molecules was at a low level in cultured RSC96 cells, while significantly elevated after treatment with IFN-γ. Concurrent treatment with the constructed CIITA siRNAs efficiently downregulated the mRNA levels of CIITA and MHC II in RSC96 cells at 48 h post-transfection. MHC II protein levels were also significantly reduced after CIITA siRNAs transfection. Correspondingly, the immunogenicity of RSC96 cells was significantly downregulated post-transfection. These studies suggest suppressing *CIITA* gene was efficient in reducing MHC II expression and thus decreasing the immunogenicity of rat Schwann cells.**

Keywords: Schwann cells, class II transactivator (*CIITA*) gene, major histocompatibility complex class II (MHC II), RNAi

1. Introduction

Reconstruction of peripheral nerve defects remains a great challenge for surgeons. Nerve autografts are considered the golden standard for clinical treatments in repairing large lesion gaps in the peripheral nervous system; the disadvantages include limited availability of donor nerves and donor site morbidity (*1-3*). Therefore, intensive research has been focused on artificial nerves. However, obtaining an adequate number of autologous Schwann cells for constructing artificial nerves requires much time, which contributes to delaying repair of the peripheral nerve injuries and has a negative impact on nerve regeneration. As for allogeneic Schwann cells, immunological rejection remains a problem when contributing to construction of artificial nerves (*4-6*).

Schwann cells contribute to immunogenicity of artificial nerve *via* expressing major histocompatibility complex (MHC) I and MHC II antigens (*7,8*). Immunosuppressive agents have been applied to prolong the survival of Schwann cells; however, it has been found that there was extensive loss of regenerated axons in the allograft when immunosuppression was withdrawn (*9*).

Mosahebi *et al.* (*4*) demonstrated that the increase of expression of MHC II at 3 weeks in the conduits containing allogeneic Schwann cells corresponded to an increase of infiltration of T-lymphocytes as well as macrophages. Furthermore, there was a corresponding reduction in X-gal staining at 3 weeks pointing to a rejection process of allogeneic Schwann cells. Therefore, downregulation of the *MHC II* gene is important for survival of Schwann cells.

Class II transactivator (CIITA) is referred to as a

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chief regulator of MHC II transcription (*10*). It is also important for both constitutive expression of MHC II in B-cells or dendritic cells as well as cytokine-induced expression of MHC-II in a variety of other cell types including fibroblasts and vascular endothelial cells. Recent research (*11*) showed that disruption of function of CIITA played a beneficial role in preventing normal allogeneic T-cell responses and thus can prolong survival of *CIITA*-deficient hearts as compared to wild-type grafts. Therefore, we believe that *CIITA* is a potential target gene to downregulate immunogenicity of Schwann cells.

The aim of the present study is to investigate the feasibility of downregulating immunogenicity of Schwann cells *via* inhibiting a potential target of the *CIITA* gene, which might contribute to suppress immunological rejection of allogeneic Schwann cells.

2. Material and Methods

2.1. *Cell lines and cell culture*

The rat RSC96 Schwann cells were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). Schwann cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA), with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90% 4.5 g/L glucose and 10% fetal bovine serum, at 37°C in a humidified incubator with 5% CO_2.

2.2. *Construction of siRNA expression vectors*

According to the recommendations of Ambion (Life Technologies) and Genscript (GenScript, Piscataway, NJ, USA) on the RNAi target sequence, ten pairs of DNA oligonucleotides were designed and synthesized for hairpin RNA expression by Sangon (Shanghai, China) (Table 1). Oligonucleotides were dissolved in sterile, nuclease-free H_2O to a concentration of 3 mg/mL, and kept at −20°C. Oligonucletides were subsequently assembled using an annealing reaction by mixing 1 μL of each oligonucleotide (sense + antisense) with 48 μL annealing buffer. The mixture was incubated at 90°C for 4 min, at 70°C for additional 10 min, and then was slowly cooled to 10°C.

To linearize 1 μL of the pSUPER vector with *Bgl* II and *Hin*d III restriction enzymes, oligonucleotides were inserted into the linearized pSUPER vectors at a molar ratio of 3:1 with the aid of T4 DNA ligase. Colonies were picked randomly. Then the recombinant plasmids were transformed into Top10F competent cells (Novegen, Darmstadt, Germany). The plasmids were extracted and purified, and digested with *Eco* RI and *Hin*d III to confirm the presence of insert.

2.3. *Vectors transfected into rat Schwann cells*

The RSC96 cells were plated at a density of 2×10^5/L per well. When the cell confluence reached 80%, a given amount of each siRNA was mixed with LipofectAMINE 2000 (Life Technologies) for 20 min at room temperature according to the manufacturer's instructions. The mixtures were applied to the cells. After incubation for 9 h at 37°C in a humidified incubator with 5% CO_2, 10^6 U/L IFN-γ was added. Seven groups were set as follows: (*i*) siRNA 1 with interferon (IFN)-γ (10^6 U/L) added, (*ii*) siRNA 2 with INF-γ (10^6 U/L) added, (*iii*) siRNA 3 with INF-γ (10^6 U/L) added, (*iv*) siRNA 4 with INF-γ (10^6 U/L) added, (*v*) nonspecific vector control

Table 1. Interference target gene sequence and oligo-DNA sequence of recombinant vectors

Target gene sequences	Name	Oligo DNA sequences
CTCAACTCAgAgACAgACA 1.Position in gene seqTence: 524 GC content: 42.9%	M1 sense	5'-gATCCCCCTCAACTCAgAgACAgACATTCAAgAgATgTCTgTCTCTgAgTTgAgTTTTTA-3'
	M1 antisense	5'-AgCTTAAAAACTCAACTCAgAgACAgACATCTCTTgAATgTCTgTCTCTgAgTTgAgggg-3'
TCAggAgAgAAgCCTCAgA 2.Position in gene seqTence: 761 GC content: 47.6%	M2 sense	5'-gATCCCCTCAggAgAgAAgCCTCAgATTCAAgAgA TCTgAggCTTCTCTCCTgATTTTTA-3'
	M2 antisense	5'-AgCTTAAAAATCAggAgAgAAgCCTCAgATCTCTTgAATCTgAggCTTCTCTCCTgA ggg-3'
GTggTCCTCTggTAgCCAT 3.Position in gene seqTence: 1291 GC content: 52.4%	M3 sense	5'-gATCCCCgTggTCCTCTggTAgCCATTTCAAgAgAATggCTACCAgAggACCACTTTTTA-3'
	M3 antisense	5'-AgCTTAAAAAgTggTCCTCTggTAgCCATTCTCTTgAAATggCTACCAgAggACCACggg-3'
CAgTCCTCCTggAgCCTTA 4.Position in gene seqTence: 2234 GC content: 52.4%	M4 sense	5'-gATCCCCCAgTCCTCCTggAgCCTTATTCAAgAgATAAggCTCCAggAggACTgTTTTTA-3'
	M4 antisense	5'-AgCTTAAAAACAgTCCTCCTggAgCCTTATCTCTTgAATAAggCTCCAggAggACTgggg-3'
CACAgAgTCCATgTCACAA Irrelevant sequence control	NTC sense	5'-gATCCCCCACAgAgTCCATgTCACAATTCAAgAgATTgTgACATggACTCTgTgTTTTTA-3'
	NTC antisense	5'-AgCTTAAAAACACAgAgTCCATgTCACAATCTCTTgAATTgTgACATggACTCTgTgggg-3'

with INF-γ (10^6 U/L) added, (*vi*) negative control with INF-γ (10^6 U/L) added, (*vii*) negative control without IFN-γ added. Cells were then cultured for an additional 48 h at 37°C before further analysis. The best siRNA sequence was chosen for *CIITA*.

2.4. *Fluorescence quantitative PCR*

Total RNA of the cells was isolated and collected with RNase-free DNase Set (Qiagen, Valencia, CA, USA). RT-PCR was performed using the RNA PCR kit (TaKaRa, Ver.3.0) and employing 0.4 mg total RNA as the template per time point. For CIITA mRNA amplification, the primers 5'-GCCTGAGATGACCC TGCTGTA-3' and 5'-CAGTTCAAGGTCCAGCATG GT-3' were used. For MHC II mRNA amplification, the primers 5'-GCATACGGCTCGTGATCAGA-3' and 5'-CCCACGTCGCTGTCGAA-3' were used. Cycling conditions were as follows: 90 sec at 95°C; followed by 40 cycles of 5 sec at 95°C, 30 sec at 58°C, and 1 min at 95°C, 1 min at 58°C; a touchdown (0.5°C/cycle) annealing for 10 sec, with the last cycle concluding with a reaction for 7 min at 72°C. The obtained PCR products were separated using 1.5% agarose gel electrophoresis, analyzed by AlphaImager 2000 (Alpha Innotech Corporation, San Leandro, CA, USA), and quantitated by a digitalized software (Kodak Digital Science™ ID Image Analysis Software; Eastman Kodak Co., Rochester, NY, USA).

2.5. *Flow cytometry*

RSC96 Schwann cells were collected and washed in phosphate buffered saline (PBS); cell concentration was adjusted to $5 \times 10^6 - 1 \times 10^7$/mL. Cells were stained with specific MHC II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then washed with PBS. After washing, the cells were stained with goat anti-rat fluorescent antibody, and fixed in 10 mL of ice-cold 75% ethanol. After 24 h of incubation at 20°C, cells were washed twice in PBS and resuspended in 3 mL of PBS for 5 min. Three-color flow cytometry was employed using an Enzymatic Amplification Staining Kit (Flow-Amp Systems, Tebu-bio, Le Perray en Yvelines, France) (*12*).

2.6. *Mixed lymphocytes reaction*

Normal peripheral blood mononuclear cells (PBMCs) were isolated from heparinized, vacutainer-collected peripheral blood using Ficoll-Hypaque density gradient centrifugation at 2,000 rpm for 10 min. Stimulative cells were subgrouped as (*i*) Schwann cells without IFN-γ treatment; (*ii*) IFN-γ-treated Schwann cells (negative control); (*iii*) IFN-γ-treated Schwann cells transfected with siRNA for 24 h; (*iv*) IFN-γ-treated Schwann cells transfected with siRNA for 48 h. Cell concentration was 10^6 cells/100 μL. PBMCs and stimulative cells were mixed and cultured for 3 days. Then mononuclear cell proliferation was detected by MTT assay. Briefly, 20 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg/mL was added into each well and incubated for 4 h in a 37°C, 5% CO_2 and 90% humidity incubator. The medium was then removed and 150 μL DMSO (Fisher Scientific, Loughborough, UK) was added to each well to extract and solubilize the formazan crystal by incubating for 10 min. Finally, the plate was read at 570 nm using a microplate photometer (Multiskan Ascent, Thermo Fisher Scientific, Waltham, MA, USA).

2.7. *Statistical analysis*

Data are displayed as mean ± SD, SPSS 11.5 (SPSS Inc., Chicago, IL, USA) and one-way analysis of variance (ANOVA) tests were used for statistical analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *CIITA and MHC II mRNA expression after siRNA transfection*

The mRNA level of CIITA and MHC II were measured by fluorescence quantitative PCR. IFN-γ treatment of Schwann cells elevated the expression of *CIITA* and *MHC II* genes. Forty eight hours after transfection, the mRNA level of CIITA and MHC II were significantly decreased in all siRNA groups, compared to the control group (Table 2). However, the difference between non-specific vector controls and the negative control group was not significant ($p > 0.05$). Among the four siRNA groups, the siRNA group 2 was the most efficient, mRNA levels of CIITA and MHCII decreased by an average of 88.32 ± 0.93% and 86.54 ± 0.69%, respectively. Thus we chose siRNA 2 as interference vector in the subsequent experiments.

3.2. *MHC II protein expression after siRNA transfection*

The effect of transfection on MHC II expression on Schwann cells was assessed by flow cytometry. Results are shown in Table 3. The expression of MHC II of Schwann cells was at a low level without IFN-γ treatment, however, the expression of MHC II increased significantly after exposure to IFN-γ. Forty eight hours after transfection, the protein expression of MHC II was decreased significantly compared to the control group ($p < 0.01$).

3.3. *Mixed lymphocyte reaction*

Schwann cells without IFN-γ treatment stimulated a low level of PBMCs proliferation, while IFN-γ-treated Schwann cells stimulated a higher level of PBMCs proliferation. After transfection with CIITA siRNA for

Table 2. Levels of expression of CIITA mRNA and MHCII mRNA in Schwann cells after transfection with different siRNA sequences

Group	CIITA copies (copies/10^6 GAPDH)	CIITA mRNA *vs.* negative control (%)	MHC II copies (copies/10^6 GAPDH)	MHC II mRNA *vs.* negative control (%)
1. Negative control with INF-γ (10^6 U/L) added	$5.63 \pm 0.31 \times 10^4$	100	$2.66 \pm 0.31 \times 10^4$	100
2. Nonspecific vector control with INF-γ (10^6 U/L) added	$5.47 \pm 0.26 \times 10^4$	98.3 ± 1.0	$2.47 \pm 0.66 \times 10^4$	93.2 ± 5.6
3. siRNA 1 with IFN-γ (10^6 U/L) added	$1.56 \pm 1.35 \times 10^{4\,*}$	28.4 ± 3.4	$8.52 \pm 0.73 \times 10^{3\,*}$	32.7 ± 1.1
4. siRNA 2 with INF-γ (10^6 U/L) added	$6.25 \pm 0.51 \times 10^{3\,**}$	12.3 ± 0.6	$3.19 \pm 0.12 \times 10^{3\,**}$	12.5 ± 1.8
5. siRNA 3 with INF-γ (10^6 U/L) added	$8.77 \pm 0.38 \times 10^{3\,**}$	16.4 ± 0.8	$4.67 \pm 0.38 \times 10^{3\,**}$	18.5 ± 2.8
6. siRNA 4 with INF-γ (10^6 U/L) added	$1.51 \pm 0.86 \times 10^{4\,*}$	27.5 ± 2.4	$7.51 \pm 0.64 \times 10^{3\,*}$	27.7 ± 2.8
7. Negative control without IFN-γ added	$7.46 \pm 0.11 \times 10^2$	1.4 ± 0.4	$8.35 \pm 0.23 \times 10^2$	3.6 ± 0.6

Mean ± SD, $n = 3$. * $p < 0.05$, ** $p < 0.01$.

Table 3. Flow cytometry analysis of MHC II molecules expression by Schwann cells transfected with CIITA siRNA

Group	MHC II molecules on Schwann cells	Inhibitory rate (%)
1. Schwann cells without IFN-γ treatment	135 ± 25	---
2. IFN-γ-treated Schwann cells (negative control)	847 ± 84	---
3. IFN-γ-treated Schwann Cells were transfected with siRNA for 24 h	646 ± 106	23.6 ± 11.2
4. IFN-γ-treated Schwann Cells were transfected with siRNA for 48 h	152 ± 32	81.6 ± 3.1*

Mean ± SD, $n = 3$. * $p < 0.05$.

Table 4. Inhibitory rate of PBMCs proliferation after siRNA in mixed lymphocytes reactions assay

Group	OD_{570}	Inhibitory rate (%)
1. Schwann cells without IFN-γ treatment	0.32 ± 0.07	---
2. IFN-γ-treated Schwann cells (negative control)	1.43 ± 0.65	---
3. IFN-γ-treated Schwann cells were transfected with siRNA for 24 h	0.76 ± 0.25	43.2 ± 22.9
4. IFN-γ-treated Schwann cells were transfected with siRNA for 48 h	0.47 ± 0.17	75.9 ± 20.8*

Mean ± SD, $n = 3$. * $p < 0.05$.

24 h, the proliferation of PBMCs was slightly inhibited compared to the control group (inhibitory rate 43.2 ± 22.9%, $p > 0.05$). After RNAi for 48 h, the proliferation was decreased significantly (inhibitory rate 75.9 ± 20.8%, $p < 0.05$). Results are presented in Table 4 and Figure 1.

4. Discussion

Schwann cells are critical for nerve regeneration, and are the major cells contributing to the immunological rejection of nerve allografts because of MHC expression (*13*). Schwann cells can act as nonprofessional antigen presenting cells (APC) under certain conditions, and can activate T cells *in vitro* in an antigen-specific and MHC-restricted manner (*14,15*). Allogeneic Schwann cells seem to induce the upregulation of inflammatory cytokines such as IFN-γ, which are known to participate in immunological rejection. The authors have observed low level expression of MHC II molecules on cultured rat Schwann cells, and the present study confirmed that rat Schwann cells can be induced by IFN-γ to express a high level of MHC II molecules.

The expression of classical and nonclassical *MHC II* genes is regulated primarily by CIITA (*10*), which is achieved by three independent CIITA promoters (pI, pIII, and pIV), promoter pIV can be activated by IFN-γ. CIITA is the chief regulator of *MHC II* gene transcription and MHC II-restricted antigen presentation. The present study confirmed that there was a clear correlation between *MHC II* genes and the *CIITA* gene, the expression of MHC II molecules and the *CIITA* gene on Schwann cells increased simultaneously followed by IFN-γ induction. The *CIITA* gene is an ideal target for inhibiting the expression of *MHC II* genes.

RNA interference can inhibit the expression of the *CIITA* gene. In the present study, the pSUPER plasmid was used as the vector. The plasmid contained the RNA polymerase III H1 promoter, and could transcribe the hairpin RNA with a 9-nt stem-loop structure which can produce the target sequence. After transfection with pSUPER recombinant vectors (*16*), the induced MHC II expression on cell surface was significantly inhibited, and the CIITA mRNA level was also decreased. The expression of CIITA and MHC II were significantly inhibited after transfection for 48 h, indicating that 48 h was the optimal time for RNA interference. We also found that the inhibitory degree of *MHC II* gene expression was lower than that of the *CIITA* gene; therefore, we speculated that the *MHC II* gene may be also regulated by an additional regulation approach

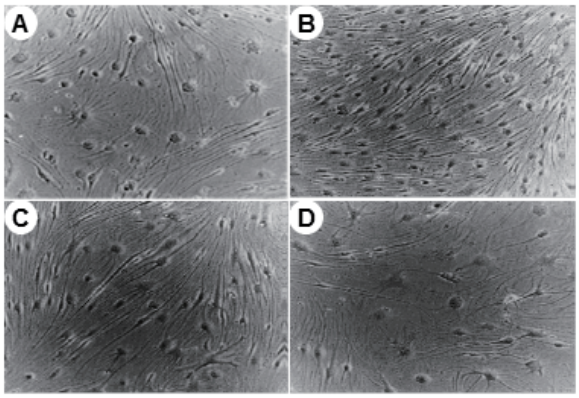

Figure 1. Mixed lymphocytes reaction. Untreated Schwann cell stimulated low level PBMCs proliferation **(A)**; IFN-γ-treated Schwann cell stimulated high level of PBMCs proliferation **(B)**; Proliferation of PBMCs was slightly decreased after siRNA for 24 h **(C)**; Proliferation of PBMCs was significantly decreased after siRNA for 48 h **(D)**.

except for *CIITA*.

Mixed lymphocyte reaction is an assay to detect response of alloreactive T cells to foreign MHC molecules and is used as a predictive test for T cell-mediated graft rejection (*17-20*). In the present study, a one-way mixed lymphocyte reaction was applied. PBMCs were used as reaction cells, and Schwann cells were used as stimulative cells. Alloantigen recognition of mixed lymphocyte reaction *in vitro* occurs *via* a direct recognition pathway, which plays a dominant role in acute rejection. The results of mixed lymphocyte assays demonstrated that the immunogenicity of rat Schwann cells increased after exposure to IFN-γ, and there was a decline in the immunogenicity after 48 h of transfection, which confirmed that the immunogenicity of Schwann cells depended on the expression of MHC II molecules. In direct recognition, alloreactive T cells are stimulated by donor APCs which express the allogeneic MHC, however, in the present study, PBMCs were stimulated successfully without the existence of any professional donor APCs. Therefore, we believethat Schwann cells may be able to take up antigens and act as non-professional APCs with IFN-γ treatment.

In conclusion, the pSUPER recombinant vectors targeting the *CIITA* gene can specifically suppress gene expression of CIITA and MHC II and thus significantly downregulate the immunogenicity of rat Schwann cells *in vitro*. Further experimentation will be needed to determine whether the decline in the immunogenicity of Schwann cells can be sustained.

Acknowledgments:

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Original Article

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Chronic stress promoted the growth of ovarian carcinoma *via* increasing serum levels of norepinephrine and interleukin-10 and altering nm23 and NDRG1 expression in tumor tissues in nude mice

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Summary **The current study aimed to examine the effects and underlying mechanisms of chronic psychological stress on the growth of ovarian carcinoma. Human ovarian carcinoma cells SKOV-3 were subcutaneously inoculated into nude mice to establish an ectopic mouse model. The animals were experimentally stressed 6 h daily for a total of 42 days with a physical restraint system. We examined the effects of stress on the growth of tumor cells that were inoculated 7 days after the initiation of stress. The growth of SKOV-3 xenografts in the stress group showed a more rapid trend than that in the control. The mean weight of tumors that were removed at the end of the experiment increased by 71.7% in the stress group as compared to the control. In order to explore the underlying mechanisms, we first determined the serum levels of norepinephrine (NE) and interleukin 10 (IL-10) in the mice using an enzyme-linked immunoabsorbent assay (ELISA) and then analyzed protein expression profiles of SKOV-3 xenografts using a proteomics-based approach combining two-dimensional electrophoresis with ultra performance liquid chromatography-electrospray tandem mass spectrometry (nanoUPLC-ESI-MS/MS). Results demonstrated that serum levels of NE and IL-10 were obviously increased in the mice receiving 6 h of immobilization daily for 42 days. In xenografts exposed to stress, a tumor promoting protein nm23 was significantly upregulated while a tumor suppressing protein NDRG1 was obviously downregulated, which were confirmed by subsequent Western blot analysis. Results obtained in the current study suggested that chronic stress promoted the growth of ovarian carcinoma in nude mice through increasing serum levels of NE and IL-10 and altering nm23 and NDRG1 expression in tumor tissues.**

Keywords: Chronic stress, ovarian carcinoma, norepinephrine, interleukin 10, nm23, NDRG1

1. Introduction

Ovarian carcinoma is the second most common gynecologic cancer, with the incidence and mortality of 224,747 and 140,163 cases worldwide in 2008 according to the statistics published by World Health Organization (*1*). Due to non-specific symptoms in the early stage, approximately two thirds of patients are at the advanced stage of this disease upon diagnosis (*2*). Thus far, ovarian carcinoma is still the leading cause of death among gynecological cancers, with overall five-year survival rates of 19-39% (*3*). Although studies indicated that the initiation and progression of ovarian carcinoma involves alternations or dysregulation of multiple genes and signal transduction pathways (*4*), factors that drive tumor growth and the underlying mechanisms are not well understood.

Substantial evidence indicated that the onset and progression of cancer is influenced by psychological factors such as stress, and depression as well as social isolation, and adequate psychotherapies are beneficial to cancer patients (*5-7*). The mechanisms underlying the effects of psychological stress on cancer cells were revealed to be related to the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis, which further act on the immunological

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system and consequently influence tumor development and prognosis (*8*). In a previous study, Sood and colleagues demonstrated that chronic stress promoted tumor growth and angiogenesis in a mouse model of ovarian carcinoma *via* activation of the tumor cell cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway (*9*). These results suggested psychosocial factors are implicated in the pathologies of ovarian carcinoma and provided clues for possible therapeutic interventions in managing this disease.

In the current study we used a mouse model in which human ovarian carcinoma cells SKOV-3 were subcutaneously inoculated to further illustrate the mechanisms behind the effects of chronic stress on the progression of ovarian carcinoma. From neurochemical and immunological perspectives, we examined alternations of serum levels of norepinephrine (NE) and interleukin-10 (IL-10) in mice exposed to chronic stress. In addition, differential expression of proteins in ovarian cancer tissues were analyzed from the view point of proteomics.

2. Materials and Methods

2.1. *Cell lines and cell culture*

The human ovarian cancer cell line, SKOV-3, was obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences, China. Cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific) and penicillin-streptomycin (50 U/mL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were harvested after brief incubation in trypsin.

2.2. *Chronic restraint stress model*

Female BALB/c-nu mice (4-6 weeks old) were purchased from the Laboratory Animal Research Center of Hubei Province (Wuhan, Hubei, China), and housed in laminar air flow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule and fed autoclaved standard chow and water. The research protocol was in accordance with the institutional guidelines of the Animal Care and Use Committee.

Mice were randomly divided into stress and control groups after a week in the new environment (six mice per group). Mice in the control group were allowed freedom of action throughout the experiment. For the chronic restraint stress group, each mouse was subjected to an established chronic physical restraint protocol (*10,11*), in which the mouse was restrained for 6 h daily (from 11 a.m. to 5 p.m.) in a 50 mL conical centrifuge tube filled with multiple punctures to allow ventilation. The mice were neither physically compressed nor experiencing pain. For both groups, SKOV-3 cells (2×10^7) were suspended in 100 μL of Matrigel (Collaborative Biomedical, Bedford, USA) and were injected subcutaneously into the right lateral chest wall in close proximity to the axilla on day 8 after starting the experiment. All the mice were kept according to the protocol for 35 consecutive days after tumor cells implantation. Tumors were measured using callipers and volumes were approximated by the formula, volume = $1/6\pi ab^2$, where a and b represent two perpendicular tumor diameters (*12*). Blood was drawn from the postorbital venous plexus of the mice for hematological analysis as described below. Mice in each group were sacrificed by cervical dislocation at the end of the experiment. Tumors were removed, weighed, and then stored at −80°C for further analysis. Tumor growth inhibition rates were defined as a percentage of the control tumor weight.

2.3. *Serum NE and IL-10 assay*

Blood samples were centrifuged at 3,000 rpm for 10 min immediately after collection, and plasma was frozen until analysis. The serum NE and IL-10 levels were determined using Mouse NE (Wuhan Youersheng Technology Co., Ltd., Wuhan, Hubei, China) and IL-10 (Wuhan Boster Biological Engineering Co., Ltd., Wuhan, Hubei, China) ELISA kits, respectively, according to the manual instructions.

2.4. *Protein extraction*

Frozen xenograft tissue samples were homogenized on ice using a glass tissue grinder. For every 100 mg tissue, 1 mL lysis buffer (consisting of 40 mmol/L tris buffer (pH 7.5), 7 mol/L urea, 2 mol/L thiourea, 1% dithiothreitol, 4% 3-[3-(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 1 mmol/L ethylenediam inetetraacetic acid (EDTA)) and 10 μL protease inhibitor cocktail were added. The homogenates were sonicated on ice using a sonifier (Sonoiprep150, SANYO Electric Co., Ltd., Moriguchi, Osaka, Japan). After sonication, 5 μL (10 μg/μL) DNase and 5 μL (10 μg/μL) RNase were added. Subsequently, the sample was incubated for 20 min on ice. Cellular debris was removed by centrifugation at 14,000 rpm/min for 20 min at 4°C, and the supernatants were collected. The protein concentrations were quantified by the Bradford protein assay. The obtained protein samples were sub-packaged, labeled, and stored at −80°C.

2.5. *Two-dimensional electrophoresis (2-DE)*

Protein samples (100 μg) were mixed with rehydration solution (8 mol/L urea, 2% CHAPS, 0.5% IPG buffer, 18 mmol/L DTT and a trace of bromophenol blue) to a volume of 350 μL. This sample was loaded into strip holders together with 18 cm Immobiline DryStrips (linear pH gradient from pH 3 to 10), and the loaded strips were

covered with Drystrip Cover Fluid. The rehydration holders were placed in the IPGphor isoelectric focusing electrophoresis (IEF) system for passive rehydration for 12 h at 30 V at 20°C. Subsequently, isoelectric focusing of the first dimension was carried out. The proteins were focused at 20°C for 1 h at 500 V, then 1 h at 1,000 V, and finally 6 h at 8,000 V. After completion of the IEF program, the strips were equilibrated at room temperature in two steps: 15 min in an IPG equilibration buffer (50 mmol/L Tris-HCl solution (pH 8.8), 6 mol/L urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), and a trace of bromophenol blue) plus 1% DTT, followed by 15 min in IPG equilibration buffer plus 2.5% iodoacetamide (IAA). For the second dimension, SDS-PAGE with a 13% polyacrylamide gel was used. The IPG strips were placed on the top of the gel and the proteins were then separated according to their molecular weights. Electrophoresis was carried out at 20°C, 15 mA/gel for 15 min, followed by a 6 h run at 30 mA/gel until the bromophenol blue indicator front reached the bottom of the gels. Three 2-DE gels were performed for each group.

2.6. *Gel scanning and image analysis*

After silver staining, the two-dimensional gels were imaged on an image scanner in a transmission mode, and quantitative analyses of the digitized images were performed using the Image Master 2D PlatinumTM software (Amersham Pharmacia Biotech, Amersham, UK) according to the protocols provided by the manufacturer, which included background subtraction, spot detection, defining landmark annotations, and matching. The intensity of each spot was normalized to the total valid spot intensity. Gels from the control and stress group were analyzed simultaneously. Each sample was analyzed based on triplicate gels produced in identical conditions to demonstrate reproducibility and diminish experimental errors. An average gel would be selected as a standard gel in each group by matching analysis, with more homogeneous spots, fewer impurities and better representation of the spot distribution. Using image analysis software, target spots were compared to the average gel to detect increases/decreases in expression (to more than double/half that of control, respectively), and to catalogue significant inter-group variations.

2.7. *Protein identification by ultra performance liquid chromatography-electrospray tandem mass spectrometry (nanoUPLC-ESI-MS/MS)*

Considering its compatibility to nanoUPLC-ESI-MS/MS analysis, we chose Coomassie Brilliant Blue G-250 staining in this study. We selected the preparative gels to perform Coomassie Brilliant Blue G-250 staining. Differentially expressed protein spots were selected, which were chosen according to the spots of silver staining gels. The spots were carefully excised from gels using a biopsy scalpel, and spot pieces were digested with trypsin in a 1.5 mL siliconized Eppendorf tube. Spot pieces were washed twice with Milli-Q water, destained in 50% acetonitrile containing 100 mmol/L ammonium bicarbonate for 20 min at room temperature, dehydrated and dried using a vacuum centrifuge. The dried gel pieces were incubated in 50 mmol/L ammonium bicarbonate containing 0.1 μg/μL modified trypsin for digestion at 37°C overnight (12-16 h). The resulting harvested peptide mixture was prepared as a sample solution as described previously (*13*). Sample solution (5 μL) was injected into a nano-Acquity system and subjected to nanoUPLC-ESI-MS/MS analysis. The UPLC-ESI-MS/MS system consists of a nano-ACQUITY UPLC system and a Synapt high definition mass spectrometer using an electrospray ionization source Z-spray. The ACQUITY UPLC analytical column uses a 75 μm × 250 mm BEH C18 column packed with 1.7 μm particles, and the enrichment column is a 180 μm × 20 mm symmetry C18 packed with 5 μm particles. The column temperature was maintained at 35°C. Optimum separation was achieved with a gradient mobile phase (which flowed at a rate of 200 nL/min) and two mobile phases consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The gradient conditions were 80 min 1-40% B, 10 min 40-80% B, 10 min 80% B, and 20 min 100% B, then a return to initial conditions. For ESI-MS/MS, ionization was achieved by using nano-electrospray ionization positive ions with a capillary voltage of 2.5 kV and a cone voltage of 35 V, the source temperature and desolvation temperature were set at 90 and 300°C, respectively. Nitrogen was used as the cone gas and for desolvation, with a flow rate of 50 and 500 L/h, respectively. Argon was used as the collision gas set at 2.5×10^{-3} mbar. Data were acquired in data dependent acquisition (DDA) mode, and the two highest intensity ions were selected from each scan, which was carried out using tandem mass spectrometry. MS/MS spectra were processed using total data acquisition software (PLGS, v2.3), and then analyzed with the Mascot search engine (*www.matrixscience.com*) against the NCBInr database including two variable modifications: Carbamidomethyl (C) and Oxidation (M). One missed cleavage site was allowed for trypsin digestion, all mass values were considered monoisotopic, and the MS/MS tolerance was set at ±0.2 Da. Individual ion scores of > 38 indicate identity or extensive homology ($p < 0.05$).

2.8. *Western blot analysis*

For Western blotting, the method was essentially as previously described (*14-16*). Briefly, protein extracts were separated on SDS-PAGE and then blotted to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After incubating for 1 h with PBS containing 0.1% Tween 20 and 5% non-fat dried milk, primary antibodies were added and the membrane was incubated at room temperature

overnight. Primary antibodies in this study were rabbit anti-NDRG1 (Millipore, USA), and mouse anti-nm23 (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). After washing, goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondantibody were added, depending on the species of the primary antibody. After incubation for 1 h, membranes were decolorized with phosphate buffered saline (PBS) at room temperature, subjected to enhanced chemiluminescence, and exposed to film. The exposed films were examined visually and photographed or scanned.

2.9. *Statistical analysis*

Data was described as the mean ± SD, and analyzed by Student's two-tailed *t*-test. The limit of statistical significance was $p < 0.05$. Statistical analysis was done with SPSS/Win17.0 software (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. *Chronic restraint stress enhanced tumor growth*

Mice in the stress group generally manifested as hyperactive with biting of the restraint tube for the first several days, indicating that they were in a state of dysphoria and anxiety. These manifestations were moderately improved in the subsequent days until the end of the experiment. Tumor nodules that were palpable appeared at about the fourth day after subcutaneous inoculation of SKOV-3 cells and started to grow quickly approximately at the ninth day in both groups. However, the growth of xenografts in the stress group showed a more rapid trend than that in the control group (Figure 1). Tumor volumes measured in the stress group were obviously larger than those in the control group at the time points of 24, 27, 30, 33, and 35 days ($p < 0.05$ at each indicated time point). The SKOV-3 xenografts were removed after the experiment and weighed 1.717 ± 0.571 g and 1.083 ± 0.286 g for the stress group and control group, respectively, indicating that the chronic restraint stress significantly enhanced tumor growth ($p < 0.05$).

3.2. *Chronic restraint stress increased serum levels of NE and IL-10*

Blood samples of mice in both the control and stress groups were obtained and centrifuged. The supernatant serum was subjected to ELISA assays for determination of NE and IL-10. NE concentrations of the stress and control group were measured at 315.95 ± 55.87 and 199.18 ± 27.96 ng/mL, respectively. There is a significant difference in NE concentration between the two groups ($p < 0.01$). The serum level of IL-10 in the stress group was determined at 240.03 ± 22.25 pg/mL, which is also obviously higher than that (201.08 ± 21.30 pg/mL) in the control group ($p < 0.05$). These results indicated chronic restraint stress increased the serum levels of both NE and IL-10 in mice (Figure 2).

3.3. *2-DE map of SKOV-3 xenografts after exposure to chronic restraint stress*

Proteins extracted from SKOV-3 xenografts were separated using 2-DE. Similar patterns of protein expression were detected in the tumor tissues from control and chronic restraint stress treated mice. On average, 1,400 protein spots were detected per gel in the stress and control group. Nineteen protein spots showed a significant difference in expression levels between the stress group and control ($p < 0.05$), including 14 spots that were upregulated, 4 spots that were downregulated, and 1 spot that was only detected in the stress group. The spots were all distributed between pI of 4-10 and molecular weight (MW) of 14-60 kDa. Among these differential staining spots, the expression levels in two spots (designated names S7121 and S5543) positioned

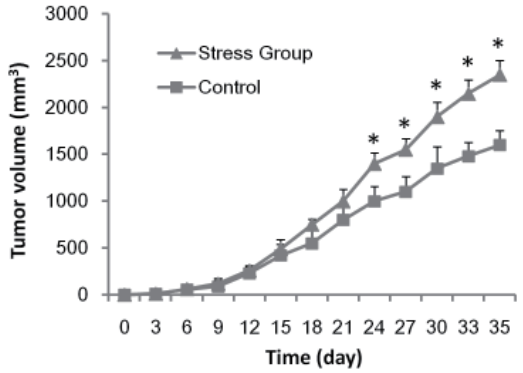

Figure 1. Tumor volumes measured in the stress group and control. SKOV-3 cells were inoculated into the mice 7 days after initiation of stress and were allowed to grow in nude mice for 35 days. Tumor volumes measured at a 3 day interval until the end of the experiment. The growth of SKOV-3 xenografts in stress group demonstrated a more rapid trend compared to control. * $p < 0.05$, stress group *vs.* control.

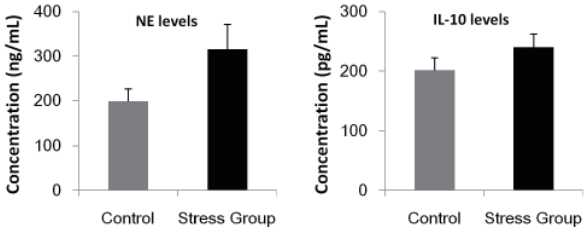

Figure 2. NE and IL-10 levels determined in the stress group and control. The mice in the stress group were immobilized 6 h daily for a total of 42 days with a restraint system. Blood samples were obtained at the end of the experiment and serum concentrations of NE and IL-10 were determined using ELISA. Chronic stress significantly increased the serum levels of NE and IL-10 compared to control. * $p < 0.05$, ** $p < 0.01$, stress group *vs.* control.

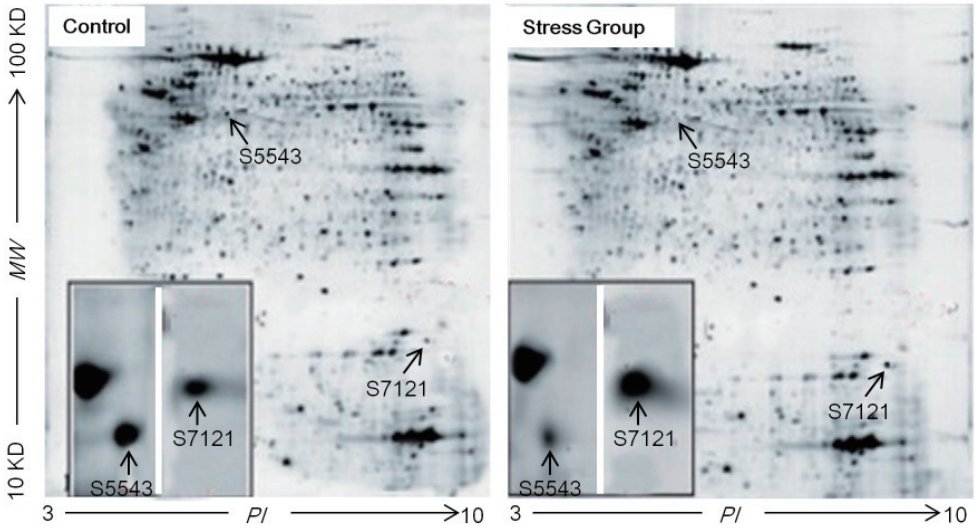

Figure 3. 2-DE maps of SKOV-3 xenografts in the stress group and control. Tumor tissues were removed at the end of experiment and subjected to 2-DE. After silver staining, the two-dimensional gels were imaged on an image scanner in a transmission mode, and quantitative analyses of the digitized images were performed. Protein S7121 (pI 7.1, MW 21 kDa) was significantly upregulated while protein S5543 (pI 5.5, MW 43 kDa) was obviously downregulated in tumor tissues of stress group.

Table 1. NanoUPLC-ESI-MS/MS analyses of nm23 and NDRG1 in SKOV-3 xenografts

Protein name	Nominal mass (Mr)/ calculated PI value	Score[a]	Sequence coverage (%)	Change in expression[b]	Change-fold (mean ± SD)	*p*-value[c]
nm23	20398/7.1	349	33	Up	2.28 ± 0.16	< 0.05
NDRG1	42808/5.5	736	36	Down	−(2.42 ± 0.17)	< 0.05

[a] Individual ions scores > 38 indicate identity or extensive homology. [b] Up- or down-regulated in the stress group *vs*. the control group. [c] Data were analyzed with an independent Student's *t*-test with SPSS version 17.0 software. Differences was considered significant if $p < 0.05$.

at pI 7.1, MW 21 kDa and pI 5.5, MW 43 kDa were profoundly different between the two groups (Figure 3). Expression level of protein S7121 in the stress group was up-regulated and was shown to be 2.2-fold over that in the control. On the contrary, expression level of protein S5543 in the stress group was down-regulated and was about 0.4-fold over that in the control. These results suggested chronic restraint stress altered protein expression profiles in SKOV-3 xenografts.

3.4. *Identification of differentially expressed proteins in SKOV-3 xenografts*

To identify differentially expressed proteins in the xenografts after exposure of chronic restraint stress, protein spots S7121 and S5543 were excised from 2-DE gels, and then subjected to nanoUPLC-ESI-MS/MS analysis. Proteins S7121 and S5543 were identified as nm23 and NDRG1 by searching the NCBInr database. An overview of these two proteins is presented in Table 1.

In order to verify the results of proteomic analyses, tumor tissues from both groups were grinded and total proteins were extracted and subjected to Western blotting analysis. As shown in Figure 4, the average band intensity of nm23 in the stress group was increased compared to control, whereas the signal of NDRG1 was decreased in this group. These results indicated that expression of nm23 was enhanced and expression of NDRG1 was reduced after exposure to chronic restraint stress, which were in agreement with the results of proteomic analyses.

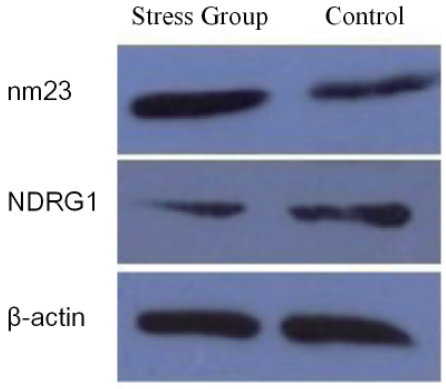

Figure 4. Western blot analyses of nm23 and NDRG1 expression in SKOV-3 xenografts. Compared to control, nm23 expression is increased while NDRG1 expression is downregulated in the stress group.

4. Discussion

The current study used an established ectopic mouse model in which human ovarian carcinoma cells SKOV-3 were subcutaneously inoculated into the right lateral chest wall in close proximity to the axilla of nude mice. We experimentally stressed animals 6 h daily for a total of 42 days with a physical restraint system, in which periodic immobilization is supposed to induce high levels of SNS and HPA activity characteristic of chronic stress. We examined the effects of stress on the growth of tumor cells which were inoculated 7 days after the initiation of stress. In mice exposed to stress, mean tumor weight increased by 71.7% compared to control. We found that serum levels of NE and IL-10 were obviously increased in the mice receiving stress. Furthermore, we demonstrated that two proteins nm23 and NDRG1 were differentially expressed in tumor tissues in the stress group. These results suggested that alteration of serum levels of NE and IL-10 and tissue expressions of nm23 and NDRG1 may be involved in the effects of chronic stress in promoting the growth of ovarian carcinoma.

Previous studies suggested that changes in stress-related neuroendocrine transmitters such as NE during psychological stress lead to a modulation of immune cells and tumor microenvironment (*8*). Mechanisms underlying modulation of the immune function by NE were demonstrated that adrenergic receptors in immune cells bind NE to activate the cAMP response element-binding protein (CREB), which in turn induces the transcription of genes encoding for a variety of cytokines such as IL-10 (*17-20*). IL-10 is an immunosuppressive cytokine that has a variety of inhibition effects on the anti-tumor immune response such as reducing macrophage inflammatory response (*21*). Besides those indirect antitumor effects by NE through suppressing the immune function of organisms, research has recognized that NE could exhibit direct influence on tumor progression and this effect was mainly through NE and the β-adrenergic receptor (β-AR) signal pathway in cancer cells (*7,9,22-23*). β-AR signals can activate several common intracellular pro-proliferative and pro-migratory signaling pathways, such as the cAMP/PKA, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) and phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathways (*24,25*). Thus, the total effects of psychological stress converging on ovarian cancer cells observed in our study are probably mediated by indirect immune suppression and direct tumor promotion processes.

We further explored the changes in protein expression in ovarian carcinoma after receiving chronic psychological stress and found that the stress altered the protein expression profiles of tumor tissues. Among a series of differentially expressed proteins, levels of two proteins which were identified as nm23 and NDRG1 were profoundly different between the stress group and control. We found that nm23 was significantly upregulated while NDRG1 was obviously downregulated in tumor tissues when the mice were exposed to stress. The *nm23* gene is a putative metastasis-suppressor gene that was originally identified by screening of cDNA libraries from murine melanoma cell sublines of varying metastatic potential (*26*). However, subsequent studies showed that this protein plays controversial or tissue-specific roles in cancer progression (*27,32*). In ovarian carcinoma, evidencein favor of its role in promoting tumor progression were reported (*33,34*). These studies suggested that nm23 has a biological function that leads to poor clinical outcomes in ovarian carcinoma. In light of those findings, upregulation of nm23 may contribute to the effects of chronic stress in stimulating the growth of ovarian carcinoma. Another protein NDRG1 that was found significantly downregulated in stress-imposed tumor tissue is encoded by the gene belonging to the N-myc downregulated gene family. NDRG1 is a cytoplasmic protein involved in stress responses, hormone responses, cell growth, and differentiation (*35,36*). Studies demonstrated that NDRG1 expression was decreased in cancer primary and metastatic cells when compared to normal cells and was thought to function as a metastasis suppressor in cancer types including ovarian, colon, and prostate cancers (*37-39*). NDRG1 has also been reported to be necessary for p53-mediated apoptosis and it plays a role in suppressing malignant cell growth (*40*). Thus, the stimulatory effects of chronic stress on the growth of ovarian carcinoma may also be partially ascribed to decreased expression of NDRG1 in tumor tissues.

In conclusion, the current study confirmed that chronic psychological stress exhibited an adverse effect on the progression of ovarian carcinoma in a mouse model. Mechanisms underlying this effect were revealed to be related to increasing the serum levels of NE and IL-10 in the mice as well as upregulating and downregulating the expression of a tumor promoting protein nm23 and a tumor suppressing protein NDRG1, respectively, in ovarian carcinoma tissues. Evidence provided in this study should help further understanding of the molecular mechanisms of the adverse effects of psychological stress on the progression of ovarian carcinoma and designing corresponding strategies to cope with this disease.

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