ISSN 1881-7815 Online ISSN 1881-7823

BST BioScience Trends

Volume 7, Number 4
August, 2013

IRCA BSSA

IACMHR

www.biosciencetrends.com

BST
BioScience Trends

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

BioScience Trends is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA and Shandong University China-Japan Cooperation Center for Drug Discovery & Screening (SDU-DDSC).

BioScience Trends devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

BioScience Trends publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, News, and Letters on all aspects of the field of life science. All contributions should seek to promote international collaboration.

Editorial Board

Editorial Office

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan
Tel: +81-3-5840-8764
Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com

BioScience Trends

Editorial and Head Office

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

Tel: +81-3-5840-8764, Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

Editorial Board Members

CONTENTS

Volume 7, Number 4, 2013

Policy Forum

Review

Original Articles

Policy Forum

DOI: 10.5582/bst.2013.v7.4.159

Prevention of human papillomavirus (HPV) infection and cervical cancer in China: How does HPV vaccination bring about benefits to Chinese women?

Xiaoyan Liu[1,2], Aihua Feng[3], Yimeng Cui[1], Ruoyan Gai Tobe[1,*]

[1] *School of Public Health, Shandong University, Ji'nan, Shandong, China;*
[2] *Qilu Hospital of Shandong University, Ji'nan, Shandong, China;*
[3] *The Fourth People's Hospital of Jinan, Ji'nan, Shandong, China.*

Summary **Cervical cancer is the second leading cause of cancer deaths among women in the world and more than 85% of cervical cancer cases occur in women living in developing countries. Human papillomavirus (HPV) infection is the major cause of cervical cancer. Since 2006, two prophylactic vaccines against the high-risk strains of HPV have been developed and approved in more than 100 countries around the world. However, in China, HPV vaccines are still under clinical trials for government approval. In this paper feasibility and justification of HPV vaccine introduction into China is examined by reviewing experiences in both developed and developing countries where the vaccination program has been implemented. The vaccination program has showed significant cost-effectiveness and great health and economic impacts on cervical cancer prevention and control in both high-income and middle- and low-income countries. On the other hand, based on the lessons from both developed and developing countries, secondary prevention alone cannot fully play a role to reduce the incidence and the disease burden, and neither does the vaccination program. The epidemiological characteristics in China suggest an urgent need to introduce the vaccines and the geographically diversified prevalence of oncogenic HPV types as well as socioeconomic status also highlight the importance of region-driven approaches for cervical cancer prevention and control by integration of a screening and vaccination program.**

Keywords: Human papillomavirus (HPV), cervical cancer, vaccination, screening, China

1. Introduction

Cervical cancer is the second leading cause of cancer death among women in the world and more than 85% of cervical cancer cases occur in women living in developing countries, where approximately 529,000 new cases and 275,000 deaths occur every year (*1*). Human papillomavirus (HPV) infection acquired from sexual activities is the most common viral infection of the reproductive tract and the causal relationship between HPV infection and the cervix and cervical cancer was built by zur Hausen, the Nobelist in Physiology and Medicine in 2008 for his epoch-making findings. The infection can be detected in more than 95% of carcinoma issues (*2*). So far, more than 100 different HPV genotypes have been detected, and among them type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are a high-risk for cervical carcinoma. The most common genotypes among infected women are 16, 18, 31, 58, and 52, accounting for approximately half of HPV infections (*3*).

Recently, there has been a milestone which prevents HPV infection and development of cervical cancer: two prophylactic vaccines against the high-risk strains of HPV, a quadrivalent vaccine *Gardasil* developed by *Merck* and a bivalent vaccine *Cervarix* by *GlaxoSmithKline* have been developed and approved in more than 100 countries around the world. The prophylactic vaccines mainly target HPV-16 and -18 types, which are the most prevalent genotypes globally

**Address correspondence to:*
Dr. Ruoyan Gai Tobe, School of Public Health, Shandong University, No.44 Wen-hua-xi Road, Ji'nan 250012, Shandong, China.
E-mail: ruoyangtobe@sdu.edu.cn

and responsible for about 70% of cervical cancer worldwide (*1*). To fully play a role for the prevention of cervical cancer and diseases related to HPV infection, they need to be widely implemented in the appropriate target population, as both are only effective for those not infected with HPV, preferably prior to sexual debut. When girls begin having sex, the risk of HPV infection increases dramatically, weakening the effectiveness of the vaccination strategy. Table 1 summarizes basic information of the two vaccines (*4,5*). Both vaccines have shown excellent efficacy with minimal toxicity; on the other hand, numerous questions remain, such as delivery strategies, accessibility to vaccination for underserved populations, social acceptance, monitoring of safety and effectiveness post-licensure, and integration of current existing HPV screening in both developed and developing countries. Monitoring and evaluation of the long-term health and socioeconomic impacts including side effects is necessary not only for each country, but also for the global society.

Having characterized the geographical diversity, epidemiological characteristics of HPV infection in China is quite different by region (*6*). The disease burden of cervical cancer is high, particularly in the rural area. It is estimated that among Chinese women aged 30 to 50 years, the prevalence of infection with high-risk HPV is 15.0~20.8%, and the mortality of cervical cancer increases 4.1% per year (*7*). On the other hand, the two prophylactic vaccines are still under clinical trials among the Chinese population for government approval. Although time is needed for the introduction of the HPV vaccines in China, it is expected to bring benefits to Chinese women with more effective prevention of HPV infection and cervical cancer in the long-term and the feasibility and justification should be examined by reviewing experiences in developed and developing countries where the vaccination program has been launched.

2. Strategies and impacts of HPV vaccination on the prevention of HPV infection and cervical cancer

The World Health Organization (WHO) strongly recommended an introduction and scaling-up of the HPV vaccination program (*1*). So far, there are more than 160 countries which have approved the prophylactic vaccines and have gradually introduced the vaccines into the national routine immunization program. Generally, the program in most countries targets pre-adolescent and adolescent girls whose age ranges from 9 to 13 years old by school-based or healthcare facility-based or mixed approaches with a catch-up group aged up to 26 years old. It provides 3 injections during a 6 month period, as the protective effect by the vaccines has been shown prior to exposure of the risk to HPV infection, *i.e.*, the sexual debut. In

Table 1. Basic information of two types of HPV vaccine

Items	*Gardasil* (*quadrivalent*)[a]	*Cervarix* (*bivalent*)[b]
Pharmaceutical company	Merck, Whitehouse Station, NJ, USA	GlaxoSmithKline, Rixensart, Belgium
HPV types	6, 11, 16, 18	16, 18
Prevention of diseases	Girls and women • Cervical, vulvar, vaginal and anal cancer caused by HPV types 16 and 18 • Genital warts (condyloma acuminata) caused by HPV types 6 and 11 • Precancerous or dysplastic lesions caused by HPV types 6, 11, 16, and 18 Boys and men • Anal cancer caused by HPV types 16 and 18 • Genital warts (condyloma acuminata) caused by HPV types 6 and 11 • Precancerous or dysplastic lesions caused by HPV types 6, 11, 16, and 18	Girls and women Following diseases caused by HPV types 16 and 18 • cervical cancer • cervical intraepithelial neoplasia (CIN) grade 2 or worse and adenocarcinoma *in situ*, and cervical intraepithelial neoplasia (CIN) grade 1
Target population	Females and males aged 9 to 26 years	Females aged 9 to 25 years
Administration	Intramuscular shoulder injection	Intramuscular shoulder injection
Efficacy against precancerous lesions	For females without infection of types 16 and 18: 98% (95% CI: 86-100%) For females with infection of types 16 and 18: 44% (95% CI: 26-58%)	For females without infection of types 16 and 18: 93% (95% CI 80-98%) For females with infection of types 16 and 18: less efficient
Cross-protection effects	Protection against types 31 was 46% (95% CI 15-66) for persistent infection and 57% (29-75) for any CIN or adenocarcinoma in situ	Protection against CIN2 or worse associated with types 31 and 33 in lesions with no co-infection with the vaccine types, and to a lesser extent against types 45 and 51
Safety	0.8% of individuals who received the vaccine and 1.0% of individuals who received the placebo reported a serious systemic adverse reaction	5.3% of individuals who received the vaccine and 5.9% of individuals who received the placebo reported at least one serious adverse event, without regard to causality

[a], Merck & Co. Gardasil (Human papillomavirus quadrivalent (types 6, 11, 16, and 18) vaccine, recombinant; [b], GlaxoSmithKline. Cervarix (Human papillomavirus bivalent (types 16 and 18) vaccine, recombinant).

developed countries, during the past several years, the coverage of the vaccines has increased and caused a significant impact on reduction of the incidence of cervical and other HPV-associated cancers, including prevalence of HPV infection, genital warts and cervical lesions (*8*). With evidence that showed high efficacy for prevention of genital warts and anal precancerous lesions in males, *Gardasil* is also licensed and recommended for use in boys in some countries such as the United States since last year (*9*). In middle- and low-income countries, although several challenges such as affordability, infrastructure for delivery, coverage, and communication strategies to boost public acceptability still remain in the national cervical cancer prevention and control strategies (*10*), the vaccination program has been adopted by public financing, out-of-pocket payment or donations. The GAVI Alliance has subsidized low-income countries to increase financial feasibility for the introduction.

As summarized in Table 2 (*11-42*), the vaccination program has showed significant cost-effectiveness and great health and economic impacts on cervical cancer prevention and control in both high-income and middle- and low-income countries, even if the protocol of vaccination and coverage is diversified depending on the specific situation of each country. In middle- and low-income countries, the combination of vaccination and screening showed better cost-effectiveness than that of vaccination or screening alone. On the other hand, uncertainties for the cost-effectiveness of the different options of the prevention and control programs included coverage, price of vaccines, oncogenic and epidemiological characteristics of HPV infection, suggesting the issues should be carefully examined when introducing the vaccination program. Moreover, the vaccination program has also brought significant cross-protection impacts on prevention of HPV-related diseases, such as cervical intraepithelial neoplasia (CIN), adding further value to the preventive potential and clinical benefits of the vaccines (*43*).

3. Cervical cancer control in China: current status and challenges

3.1. *Strategies and limitations of early detection of cervical cancer by the screening program*

Recently in China, without official approval of the vaccines, the most essential strategy for cervical cancer prevention and control is the routine screening program targeting women at reproductive age for early detection and treatment of HPV infection, CIN (from which developing cervical cancer generally needs several years) and cervical cancer at an early stage. Based on previous large-scale studies, a systematic routine screening program can reduce the incidence of cervical cancer by at least 60% and has been recommended by WHO, particularly in developing countries (*44*). The major methodologies of screening include: *i*) Pap smear cytology test; *ii*) liquid-based cytology test; *iii*) HPV DNA test (HC-II); *iv*) visual inspection with acetic acid (VIA); *v*) colposcopy. Table 3 briefly lists advantages and limitations of each methodology (*45*).

The Ministry of Health of China has launched the Guideline for Screening and Early Detection and Treatment of Cervical Cancer. According to the guideline, the target population is women older than 21 years old or with sexual intercourse experience for more than three years. The guideline also defined high-risk women as those with several sexual partners, too early sexual debut, HPV infection, low immunity, poor health knowledge and accessibility to healthcare. Depending on diversified geographical socioeconomic status and levels of exposure to the risks of the population, the guideline has recommended three protocols with a different combination of methodologies based on feasibility and a cost-effectiveness evaluation. The three protocols are: *i*) primary screening by liquid-based cytology test + HPV DNA test, an optimal one with best sensitivity and specificity but relevantly high cost, suitable for a screening program in developed regions and/or women with good economic status; *ii*) primary screening by Pap smear cytology test + HPV DNA test, suitable in moderately developed regions; *iii*) primary screening by VIA, a basic one as an alternative in low-resource settings. High-risk women are prioritized in the guideline, with a recommended more intensive frequency of screening and follow-up. Ideally, with such a strengthened HPV screening program, early detection and treatment of cervical cancer can be realized and incidence as well as disease burden can be reduced.

For women living in rural areas, among whom the incidence and the disease burden of cervical cancer is much higher, since 2009 the nationwide pilot campaign of free screening for cervical cancer and breast cancer has been implemented with a strong political commitment from the annual government report and the 12th Five-Year Plan for Healthcare System Reform. On the other hand, due to huge geographical diversity in socioeconomic development, the current screening program has limitations accomplishing its function. Although the national guideline recognized the huge gap in technical capacity and accessibility in different regions, the current recommended region-driven protocols for screening need to be re-examined, because incidence and disease burden in less developed regions tends to be higher and the difference of the protocol implementation may enlarge the gap. In reality, a relevant amount of high-risk cases from patients living in low resource regions may be missed due to low sensitivity of the screening protocols and bad performance of healthcare facilities at the primary level, in which the technical capacity tends to be poor and human resources are lacking. This remains an

Table 2. Strategy of HPV vaccination in some countries

Country/Region (Year introduced)	Protocol of vaccination	Estimated coverage	Impacts, effectiveness and economic evaluation	References
High-income				
The United States (2006)	3 doses targeting females aged 11-12 and catch-up ages 13-26, delivered at facilities	32%	Vaccination for HPV in combination with screening can be a cost-effective health intervention, but it depends on maintaining effectiveness during the ages of peak oncogenic HPV incidence.	Kulasingam *et al*, 2003; Markowitz *et al*, 2013
Canada (2007)	3 doses targeting females aged 12 and catch-up ages different by regions, delivered at schools	75% (estimated in the simulation model)	The bivalent vaccine had lower ICER compared to quadrivalent vaccine. It is associated with more reduced cervical cancer morbidity and mortality. Differences in these outcomes depend on the extent of cervical disease prevented by cross-protection and the burden of GW caused by HPV-6/11.	Kohli *et al*, 2012; Anonychuk *et al*, 2009
The United Kingdom (2008)	3 doses targeting females and males aged 12-13 and catch-up ages 13-17, delivered at schools	84%	The quadrivalent HPV vaccination programme that includes a catch-up strategy can reduce the incidence of cervical cancer, CIN and genital warts at a cost per QALY ratio within the range typically regarded as cost-effective. Vaccination with screening, compared to screening alone, was associated with an incremental cost-effectiveness ratio of 21,059 pound per QALY.	Dasbash *et al*, 2008; Kulashingam *et al*, 2008
France (2007)	3 doses targeting females aged 14 and catch-up ages 15-23, delivered at schools or facilities	80% (estimated in the simulation model)	The incremental cost per QALY for the introduction of HPV vaccination alongside the French cervical cancer screening program was €8,408, suggesting that a quadrivalent HPV vaccine in the current screening program in France is cost-effective.	Bergeron *et al,* 2008
Germany (2007)	3 doses targeting females aged 12-17, delivered at schools or facilities	-	The quadrivalent HPV vaccination programme of females ages 12 to 17 in Germany is cost-effective with an ICER of 5,525€/QALY. The current vaccination and cervical cancer screening programmes in Germany will substantially reduce the incidence of cervical cancer, CIN and genital warts.	Damm *et al*, 2009; Schobert *et al*, 2012
Italy (2008)	3 doses targeting females aged 11 and catch-up ages different by regions, delivered at schools or facilities	56%	The bivalent vaccine would prevent an additional reduction of 7976 abnormal pap smears, 601 CIN1, 1826 CIN2/3 and 295 CC cases compared to the quadrivalent vaccine while 25,848 genital wart cases would be prevented by the quadrivalent vaccine. The additional cost averted with the bivalent vaccine was estimated at €2,385,354 per year.	Capri *et al,* 2011; Mennini *et al*, 2009
Netherlands (2010)	3 doses targeting females aged 12 with catch-up ages 13-16, delivered at schools or facilities	50% (estimated in the simulation model)	The bivalent or quadrivalent vaccine reduces the cervical cancer incidence by 221 and 207 /100,000, corresponding to ICERs of €17,600 /QALY and €18,900 /QALY, respectively. The quadrivalent vaccine additionally prevents 4390 cases of genital warts, reducing the ICER to €16,300 /QALY.	Westra *et al*, 2013
Belgium (2007)	3 doses targeting females aged 12-18 with catch-up ages 13-18, delivered at schools or facilities	82%	The vaccine reduces the lifetime risk of cervical cancer from 0.94% to 0.34%, preventing 362 cases of cervical cancer and 131 related deaths in a cohort of 60,000 girls aged 12 years. The vaccination with current screening is at €10,546 /QALY.	Annemans *et al,* 2009
Irelands (2010)	3 doses targeting females aged 12-13, delivered at schools or facilities	-	ICER for quadrivalent vaccine would be 25,349 euros/QALY and 30,460 euros/QALY for the bivalent vaccine. At current prices, the bivalent vaccine would need to be 22% cheaper than the quadrivalent vaccine in order to have equivalent cost effectiveness.	Dee *et al*, 2010
Norway (2009)	3 doses targeting females aged 11-12 with catch-up ages 12-24, delivered at schools	63%	Implementation of a quadrivalent HPV vaccine national program in Norway could reduce the incidence of cervical cancer, cervical intraepithelial neoplasia and genital warts at a cost-effectiveness ratio.	Dasbash *et al*, 2008; Sander *et al*, 2012
Switzerland (2008)	3 doses targeting females aged 10-14 with catch-up ages 14-19, delivered at schools or facilities	80% (estimated in the simulation model)	Compared to screening only, adding a quadrivalent vaccine could prevent over lifetime 62% of cervical cancers and related deaths, 19% of Cervical Intraepithelial Neoplasia (CIN 1), 43% of CIN 2, 45% of CIN 3 and 66% of genital warts per cohort. ICER were estimated to be CHF45,008 /life-year and CHF26,005 / QALY.	Szucs *et al*, 2008
Australia (2007)	3 doses targeting females aged 12-13 with catch-up ages 13-26, delivered at schools	71%	Vaccination with screening compared with screening alone was associated with ICER of $51,103 /life-year and $18,735 /QALY.	Kulasingam *et al*, 2007
Japan (2009)	3 doses targeting females aged 12-16, delivered at schools or facilities	-	Vaccinating a 12-year-old cohort can reduce CC incidence and deaths from CC by 73%, associated with ICER of yen1.8 million per QALY gained.The vaccination program is more cost-effective to increase the coverage of the screening along with the universal administration of HPV vaccine.	Yamamoto *et al*, 2012; Konno *et al*, 2010

(continued on next page)

(Table 2. continued)

Country/Region (Year introduced)	Protocol of vaccination	Estimated coverage	Impacts, effectiveness and economic evaluation	References
Singapore (2010)	3 doses targeting females aged 9-26, delivered at facilities	-	Comparing the bivalent to the quadrivalent vaccine, the ICER was $12,488 per life-year saved. The quadrivalent vaccine dominates to the bivalent vaccine due to the additional QALY effect from reduction in genital warts. The overall outcomes were most sensitive to vaccine cost and coverage.	Lee *et al*, 2011
Taiwan (2006)	3 doses targeting females aged 9-26, delivered at facilities	-	An additional 768 QALY and 11.6 million new Taiwan dollars costs saved for the bivalent vaccine versus the quadrivalent vaccine after discounting.	Demarteau *et al*, 2012
Middle- and low- income				
Thailand (2010)	3 doses targeting females aged 9-12, delivered at schools or facilities	80% (estimated in the simulation model)	Pre-adolescent HPV vaccination alone was projected to reduce the lifetime risk of cervical cancer by 55%, which was greater than any strategy of screening alone. Pre-adolescent vaccination and HPV DNA testing five times per lifetime, starting at age 35 years, reduced the lifetime cervical cancer risk by 70%, and had a cost-effectiveness ratio less than Thailand's GDP per capita.	Sharma *et al*, 2012
Malaysia (2010)	3 doses targeting females before age 13 with catch-up ages 13-18, delivered at schools	-	Vaccination increase life expectancy with better QOL of women when cancer can be avoided. Cost effective strategies will include increasing the screening coverage to 70% or higher. Since feasibility and long term screening adherence is doubtful among Malaysian women, vaccination is a more cost effective strategy against cervical cancers.	Ezat *et al*, 2010
India (2011)	3 doses targeting females aged 11-12 with catch-up ages 13-26, delivered at facilities	70% (estimated in the simulation model)	If high coverage of pre-adolescent girls with a low-cost HPV vaccine that provides long-term protection is achievable, vaccination followed by screening three times per lifetime is expected to reduce cancer deaths by half, and be cost-effective.	Diaz *et al*, 2008
Hungary (2010)	3 doses targeting females aged 12 with catch-up ages 12-24, delivered at facilities	-	The ICER of adding bivalent vaccine to the current national cancer screening program was estimated to be 27 588 $/QALY. By quadrivalent vaccine, the ICER of the routine vaccination targeting females aged 12 and the routine vaccination plus the catch-up group were €9,577 and €10,646 per QALY.	Voko *et al*, 2011; Dasbash *et al*, 2010
Mexico (2008)	3 doses targeting females aged 9-12 with catch-up ages 12-24, delivered at schools or facilities	67%	The quadrivalent vaccine could reduce the probability of persistent HPV-16/18 infection by at least 60%, resulting in a near-proportional reduction in HPV-16/18-associated invasive cervical cancer and CIN 3. The most effective strategy therein was vaccination of 12-year-olds, plus a temporary 12-24-year-old catch-up program covering both sexes; whereby HPV 6/11/16/18-related cervical cancer, high-grade cervical pre-cancer and genital wart incidence was reduced by 84-98%.	Reynales-Shigematsu *et al*, 2009; Insinga *et al*, 2007; CDC, 2011
Peru (2011)	3 doses targeting females aged 10-13, delivered at schools	82%	Enhanced screening in adult women combined with preadolescent vaccination had incremental cost-effectiveness ratios lower than Peru's 2005 per capita GDP and considered to be cost-effective.	Goldie *et al*, 2012
Brazil (2006)	3 doses targeting females aged 9-12, delivered at schools or facilities	50% (estimated in the simulation model)	Vaccination in addition to the current screening programme is likely to save years of life and, depending on the cost of vaccination, may even save resources.	Vanni *et al*, 2012

obstacle for implementation and effective coverage of the regular screening program. Accessibility to regular screening is limited for quite a large number of high-risk women due to lack of knowledge and awareness as well, leading to a loss of opportunity for prevention of cancer development. Therefore, the technical capacity at the primary level and in less developed regions urgently needs to be strengthened; moreover, in the long-term, the introduction of the prophylactic vaccines is crucial to supplement the screening program, especially targeting high-risk women.

3.2. *Factors potentially affecting the introduction of HPV vaccination*

Although it showed great health and socioeconomic impacts in numerous countries, the worldwide introduction of the HPV vaccination program still has a short history with various questions remaining. In China, the government has showed a conservative attitude for approval of the current vaccines and has strictly required clinical trials targeting the Chinese population, even though it met with opposition from some scholars and the mass media. Because political commitment plays a core role in the introduction, solid evidence is necessary to persuade the policy makers. Table 4 summarizes factors potentially affecting the introduction based on results of studies having been implemented in China so far, including epidemiological characteristics, efficacy

Table 3. Major advantages and limitations of the screening methodologies for cervical cancer

Methodology	Advantages	Limitations
Pap smear cytology test	Regular screening tool for more than 50 years in settings where the cytology screening system has been established, with relevantly high sensitivity (50-80%) and specificity (85-90%)	The operation requires well trained personnel, maturelaboratory technique, high financial costs, and three or more diagnostic follow-ups and treatment for the positive cases.
Liquid-based cytology test	Similar to Pap smear cytology test, while the operation improved collection efficacy accuracy of samples, with higher sensitivity (85%) and specificity (90%). In settings where the laboratory technical capacity for the cytology screening is weak, samples can be restored and sent to outside.	High financial costs (prolonged screening interval may reduce the costs)
HPV DNA test (HC-II)	It can explore the level of the risks and determine the screening interval with higher sensitivity than liquid-based cytology test for detecting significant precancerous lesions. Moreover, the processing of results can be automated, making the test more objective and requiring less training of personnel. It is especially suitable for large-scale screening in high-risk populations.	High financial costs (prolonged screening interval may reduce the costs)
Visual inspection with acetic acid (VIA)	Simple, less personnel and laboratory requirement, low financial costs, quick result and easy to operate, particularly suitable for low-resource setting with limited technical capacity of the cytology screening	Relatively low sensitivity (50-70%) and specificity (85%)
Colposcopy	As an essential supplementary tool for the early detection of cervical cancer and precancerous lesions, it is conducted for suspicious cases and positive results from the screening test. The combination of colposcopy with HPV DNA test and the cytology tests can further improve sensitivity and specificity.	Facility-based, intensive personnel and technical requirement and not suitable for a large-scale screening

Table 4. Literature review for factors affecting implementation of HPV vaccination program in China

Literature review
Epidemiological characteristics of HPV infection
● In rural Guangdong Province: HPV types 16 and 18 accounted for 28.52% of total infection while types 52 and 58 presented 48.24%. (Chen *et al*, 2012)
● HPV 16 (76.7%) and HPV 18 (7.8%) were the most common, together accounting for 84.5% of squamous cell carcinoma (SCC), followed by HPV 31 (3.2%), HPV 52 (2.2%), and HPV 58 (2.2%). Positive HPV in SCC did not differ notably by region. The potential impact of vaccines against oncogenic HPV types 16 and 18 is estimated to be high (84.5%) against total SCC. (Chen *et al*, 2009)
● In Western China: HPV-16 and -58 were the most prevalent types, with prevalence of 37.8% and 21.8%, respectively; HPV-18 and -45 were uncommon types. (Li *et al*, 2012)
● In Wufeng County: HPV 16, 52, and 58 are common genotypes. (Zhang *et al*, 2012)
● The most prevalent HPV are types 52 and 58 with positive rate of 42.5% in cervical cancer patients, greater than types 16 and 18 in Shanghai, in southern China the HPV 52- and 58-positive rate is greater than that in northern China. (Lo *et al*, 2002)
● The most prevalent types found were HPV16 (2.9 %), HPV52 (1.7 %), HPV58 (1.5 %), HPV33 (1 %), and HPV18 (0.8 %). Patterns of HPV prevalence differed by age, geographic region, and cytology findings. (Wu *et al*, 2013)
Efficacy, safety and immunogenicity
● A double-blinded RCT in China showed the quadrivalent vaccine was generally well tolerated, with no vaccine-related serious adverse events. High antibody levels were observed for each of the four HPV types and sero-conversion was > 96%. (Li *et al*, 2010)
Cost-effectiveness of cervical cancer prevention and control strategies
● Per-dose HPV vaccine cost of approximately < $9-14 would be required for strategies involving vaccination to be cost-effective. Combined screening and vaccination approaches are required to maximize outcomes in rural China. (Canfell *et al*, 2011)
● Assuming a cost per vaccinated girl of I$25, the cost per DALY averted is I$1,360 in China, reflecting the greater number of girls that need to be vaccinated to prevent a death from cervical cancer in China. Vaccine price has an even greater effect on predicted affordability. (Goldie *et al*, 2008)
● Making an HPV16, 18 vaccine accessible to 70% of young adolescent girls in 72 countries including China could prevent the future deaths of more than four million women vaccinated over the next decade. Provided the cost per vaccinated girl is less than $10-$25, adolescent HPV16,18 vaccination would be cost-effective even in relatively poor countries. (Goldie *et al*, 2008)
Knowledge and attitude
● Only 15.0% of women have ever heard of HPV, and this knowledge differs by rural (9.3%) and metropolitan areas (21.6%) and also by education. Most (84.6%) participants were willing to be vaccinated if HPV vaccine became available to them. (Li *et al*, 2009)
● Knowledge of HPV among the general female population was low; only 24% had heard of HPV. Less than 20% of healthcare providers recognized sexually naive women as the most appropriate population for HPV vaccination. There was high acceptance of the HPV vaccine for all categories of respondents. Only 6% of women were willing to pay more than US $300 for the vaccine. (Zhao *et al*, 2012)

and safety of the vaccines, cost-effectiveness of cervical cancer prevention and control strategies Sexual behavior of the population, and knowledge and attitudes towards the vaccines and HPV-related diseases are also important (*46-57*).

In China, the prevalence of HPV 16 and 18, which are prevented by the current vaccines, is generally high, while there is a huge geographic diversity of the prevalence of oncogenic HPV types. Other prevalent genotypes include HPV 52 and 58, as well. Such epidemiological trends suggest introduction of the prophylactic vaccines and development of vaccines targeting other prevalent genotypes have potential clinical and social benefits. The clinical trials for the development are ongoing. Based on the current data, the quadrivalent vaccine showed great efficacy in the Chinese population and no vaccine-related serious adverse events were reported so far. Based on a mathematical simulation model, introduction of the vaccines and integrated vaccination and screening program will potentially be very cost-effective in China. Providing the program universally covers the high-risk and high-burden population and the price is low enough to ensure accessibility for the underserved. Like other developing countries, there is a concern about financial costs and affordability, highlighting the need for lowering vaccine prices, cost-efficient mechanisms for delivery of vaccinations to high-risk and high-burden populations, and creative sources of financing. A strong political commitment by the government is essential, because universal coverage is expected to be achieved by injection of public subsidies and adaptation of medical insurance, rather than out-of-pocket payment, particularly for the poor living in rural areas. An interesting finding shown in the previous studies is that although the overall related knowledge is lacking, people have a good willingness to receive the vaccines when they are available in China. Strengthening of health education on the prevention and control of cervical cancer and HPV-related disease is another important issue to improve potential coverage.

4. Prospects for the future

Although the disease burden of cervical cancer is relatively high in China, the two current prophylactic vaccines are currently not available due to ongoing clinical trials among Chinese women and multivalent vaccines that encompass additional oncogenic HPV strains are under development as well. The major strategy for cervical cancer prevention and control is screening of women at reproductive ages and secondary prevention. Time is still needed to introduce the prophylactic vaccines, currently, early detection and treatment by the universal coverage screening program is the core of a comprehensive strategy with region-driven approaches for cervical cancer prevention and control.

So far, based on lessons from both developed and developing countries, secondary prevention alone cannot fully play a role to reduce the incidence and the disease burden, and neither does the vaccination program. As a preventive tool, both these vaccines prefer girls and women not yet exposed to the risk of infection, *e.g.*, prior to first sexual intercourse, and are not effective for those already infected. Moreover, because immunity to HPV is primarily type specific, protection by the current generation of vaccines with a limited number of HPV types cannot provide complete protection against all oncogenic HPV types. Therefore, the functional screening program is still necessary. Previous economic evaluations indicated that an integrated vaccination and screening program is the most effective tool for great cost-effectiveness and health impact. According to WHO, a HPV vaccination program combined with regular screening in women over age 30 for precancerous lesions followed by adequate treatment are key tools to prevent the 530,000 new cervical cancer cases diagnosed every year (*1*). Therefore, like other countries in the Asia Pacific area and the world, the short-term goal for cervical cancer control is to identify feasible and effective screening measures, and to find the most effective way to combine vaccination with sustainable screening programs (*58,59*).

The high prevalence of HPV 16 and 18 in the overall population in China suggests an urgent need to introduce the current vaccines (*60*). The geographically diversified prevalence of oncogenic HPV types as well as socioeconomic status also highlights the importance of region-driven approaches for cervical cancer prevention and control. With the profound tendency for cervical cancer epidemics and the tremendous task of control, in the long-term the introduction of the vaccines and the development of the new generation vaccines for additional oncogenic HPV types are crucial. Besides the current genotypes protected by the vaccines, cross-reactivity suggests that even better clinical benefits may be achieved by its wide application. Challenges for ensuring the benefits for Chinese women by the HPV vaccines include political commitment of the government, provision of solid evidence for policy making, monitoring of safety and side effects, health education, affordable prices and possible public subsidies for the poor and the vulnerable, and strengthened screening programs particularly for the high-risk population and for primary level healthcare facilities with poor technical capacity.

Acknowledgements

This work is granted by the National Natural Science Foundation of China (H2606-81202225) and Independent Innovation Foundation of Shandong University (2012TB017).

References

1. The World Health Organization. Comprehensive cervical cancer prevention and control: A healthier future for girls and women. The World Health Organization, Geneva, Swizerland, 2013.
2. Lowy DR, Solomon D, Hildesheim A, Schiller JT, Schiffman M. Human papillomavirus infection and the primary and secondary prevention of cervical cancer. Cancer. 2008; 113 (Suppl 7):1980-1993.
3. de Sanjosé S, Diaz M, Castellsagué X, Clifford G, Bruni L, Muñoz N, Bosch FX. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: A meta-analysis. Lancet Infect Dis. 2007; 7:453-459.
4. Merck & Co. Gardasil [Human papillomavirus quadrivalent (types 6, 11, 16, and 18) vaccine, recombinant]. *http://www.merck.com/product/usa/pi_circulars/g/gardasil/gardasil_pi.pdf* (accessed June 02, 2013).
5. GlaxoSmithKline. Cervarix [Human papillomavirus bivalent (types 16 and 18) vaccine, recombinant]. *http://us.gsk.com/products/assets/us_cervarix.pdf* (accessed June 02, 2013).
6. Zhao FH, Lewkowitz AK, Hu SY, *et al*. Prevalence of human papillomavirus and cervical intraepithelial neoplasia in China: A pooled analysis of 17 population-based studies. Int J Cancer. 2012; 131:2929-2938.
7. Li J, Kang LN, Qiao YL. Review of the cervical cancer disease burden in mainland China. Asian Pac J Cancer Prev. 2011; 12:1149-1153.
8. Markowitz LE, Tsu V, Deeks SL, Cubie H, Wang SA, Vicari AS, Brotherton JM. Human papillomavirus vaccine introduction – The first five years. Vaccine. 2012; 30(Suppl.):F139-148.
9. Palefsky JM, Giuliano AR, Goldstone S, *et al*. HPV vaccine against anal HPV infection and anal intraepithelial neoplasia. N Eng J Med. 2011; 365:1576-1585.
10. Kane MA, Serrano B, Sanjose S, Witter S. Implementation of human papillomavirus immunization in the developing world. Vaccine. 2012; 30(Suppl.):F192-200.
11. Westra TA, Stirbu-Wagner I, Dorsman S, Tutuhatunewa ED, de Vrij EL, Nijman HW, Daemen T, Wilschut JC, Postma MJ. Inclusion of the benefits of enhanced cross-protection against cervical cancer and prevention of genital warts in the cost-effectiveness analysis of human papillomavirus vaccination in the Netherlands. BMC Infect Dis. 2013; 13:75.
12. Annemans L, Remy V, Oyee J, Largeron N. Cost-effectiveness evaluation of a quadrivalent human papillomavirus vaccine in Belgium. Pharmacoeconomics. 2009; 27:231-245.
13. Kulasingam S, Connelly L, Conway E, Hocking JS, Myers E, Regan DG, Roder D, Ross J, Wain G. A cost-effectiveness analysis of adding a human papillomavirus vaccine to the Australian National Cervical Cancer Screening Program. Sex Health. 2007; 4:165-175.
14. Szucs TD, Largeron N, Dedes KJ, Rafia R, Benard S. Cost-effectiveness analysis of adding a quadrivalent HPV vaccine to the cervical cancer screening program in Switzerland. Curr Med Res Opin. 2008; 24:1473-1483.
15. Lee VJ, Tay SK, Teoh YL, Yok MY. Cost-effectiveness of different human papillomavirus vaccines in Singapore. BMC Public Health. 2011; 11:203.
16. Dee A, Howell F. A cost-utility analysis of adding a bivalent or quadrivalent HPV vaccine to the Irish cervical screening program. Eur J Public Health. 2010; 20:213-219.
17. Kohli M, Lawrence D, Haig, J, Anonychuk A, Demarteau N. Modeling the impact of the difference in cross-protection data between a human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine and human papillomavirus (HPV)-6/11/16/18 vaccine in Canada. BMC Public Health. 2012; 12:872.
18. Anonychuk AM, Bauch CT, Merid MF, Van Kriekinge G, Demarteau. A cost-utility analysis of cervical cancer vaccination in preadolescent Canadian females. BMC Public Health. 2009; 9:401.
19. Demarteau N, Tang CH, Chen HC, Chen CJ, Van Kriekinge G. Cost-effectiveness analysis of the bivalent compared with the quadrivalent human papillomavirus vaccines in Taiwan. Value Health. 2012; 15:622-631.
20. Dasbach EJ, Insinga RP, Elbasha EH. The epidemiological and economic impact of a quadrivalent human papillomavirus vaccine (6/11/16/18) in the UK. BJOG. 2008; 115:947-956.
21. Kulasingam SL, Benard S, Barnabas RV, Largeron N, Myers ER. Adding a quadrivalent human papillomavirus vaccine to the UK cervical cancer screening program: A cost-effectiveness analysis. Cost Eff Resour Alloc. 2008; 6:4.
22. Capri S, Gasparini R, Panatto D, Demarteau N. Cost-consequences evaluation between bivalent and quadrivalent HPV vaccines in Italy: The potential impact of different cross-protection profiles. Gynecol Oncol. 2011; 121:514-521.
23. Mennini FS, Giorgi Rossi P, Palazzo F, Largeron N. Health and economic impact associated with a quadrivalent HPV vaccine in Italy. Gynecol Oncol 2009; 112:370-376.
24. Damm O, Nocon M, Roll S, Vauth C, Willich S, Greiner W. Human papillomavirus (HPV) vaccination for the prevention of HPV 16/18 induced cervical cancer and its precursors. GMS Health Technol Assess. 2009; 5:Doc04.
25. Schobert D, Remy V, Schoeffski O. Cost-effectiveness of vaccination with a quadrivalent HPV vaccine in Germany using a dynamic transmission model. Health Econ Rev. 2012; 2:19.
26. Bergeron C, Largeron N, McAllister R, Mathevet P, Remy V. Cost-effectiveness analysis of the introduction of a quadrivalent human papillomavirus vaccine in France. Int J Technol Assess Health Care. 2008; 24:10-19.
27. Kulasingam SL, Myers ER. Potential health and economic impact of adding a human papillomavirus vaccine to screening programs. JAMA. 2003; 290:781-789.
28. Markowitz LE, Hariri S, Lin C, Dunne EF, Steinau M, McQuillan G, Unger ER. Reduction in human papillomavirus prevalence among young women following HPV vaccine introduction in the United States, National Health and Nutrition Examination Surveys, 2003-2010. J Infect Dis 2013; 208:385-393.
29. Dasbash EJ (b), Largeron N, Elbasha EH. Assessment of the cost-effectiveness of a quadrivalent HPV vaccine in Norway using a dynamic transmission model. Expert Rev Pharmacoecon Outcomes Res. 2008; 8:491-500.
30. Sander BB, Rebolj M, Valentiner-Branth P, Lynge E. Introduction of human papillomavirus vaccination in Nordic countries. Vaccine 2012; 30:1425-1433.
31. Yamamoto N, Mori R, Jacklin P, Osuga Y, Kawana K, Shibuya K, Taketani Y. Introducing HPV vaccine and scaling up screening procedures to prevent deaths from cervical cancer in Japan: A cost-effectiveness analysis.

BJOG. 2012; 119:177-186.
32. Konno R, Sasagawa T, Fukuda T, Van Kriekinge G, Demarteau N. Cost-effectiveness analysis of prophylactic cervical cancer vaccination in Japanese women. Int J Gynecol Cancer. 2010; 20:385-392.
33. Voko Z, Nagyjanosi L, Kalo Z. Cost-effectiveness of adding vaccination with AS04-adjuvanted human papillomavirus 16/18 vaccine to cervical cancer screening in Hungary. BMC Public Health. 2011; 12:924.
34. Dasbach EJ, Nagy L, Brandtmüller A, Elbasha EH. The cost effectiveness of a quadrivalent human papillomavirus vaccine (6/11/16/18) in Hungary. J Med Econ. 2010; 13:110-118.
35. Reynales-Shigematru LM, Rodrigues ER, Lazcano-Ponce E. Cost-effectiveness analysis of a quadrivalent human papilloma virus vaccine in Mexico. Arch Med Res. 2009; 40:503-513.
36. Inshiga RP, Dasbash EJ, Elbasha EH, Piug A, Reynales-Shigematsu LM. Cost-effectiveness of quadrivalent human papillomavirus (HPV) vaccination in Mexico: A transmission dynamic model-based evaluation. Vaccine. 2007; 26:128-139.
37. Centers for Disease Control and Prevention (CDC). Progress toward implementation of human papillomavirus vaccination – the Americas, 2006-2010. MMWR Morb Mortal Wkly Rep. 2011; 60:1382-1384.
38. Vanni T, Mendes Luz P, Foss A, Mesa-Frias M, Legood R. Economic modeling assessment of the HPV quadrivalent vaccine in Brazil: A dynamic individual-based approach. Vaccine. 2012; 30:4866-4871.
39. Goldie SJ, Levin C, Mosqueira-Lovón NR, Ortendahl J, Kim J, O'Shea M, Diaz Sanchez M, Mendoza Araujo MA. Health and economic impact of human papillomavirus 16 and 18 vaccination of preadolescent girls and cervical cancer screening of adult women in Peru. Rev Panam Salud Publica. 2012; 32:426-434.
40. Diaz M, Kim JJ, Albero G, de Sanjosé S, Clifford G, Bosch FX, Goldie SJ. Health and economic impact of HPV 16 and 18 vaccination and cervical cancer screening in India. Br J Cancer. 2008; 99:230-238.
41. Sharma M, Ortendahl J, van der Ham E, Sy S, Kim JJ. Cost-effectiveness of human papillomavirus vaccination and cervical cancer screening in Thailand. BJOG. 2012; 119:166-176.
42. Ezat WP, Aljunid S. Cost-effectiveness of HPV vaccination in the prevention of cervical cancer in Malaysia. Asian Pac J Cancer Prev. 2010; 11:79-90.
43. De Vincenzo R, Ricci C, Conte C, Scambia G. HPV vaccine cross-protection: Highlights on additional clinical benefit. Gynecol Oncol. 2013. (doi: 10.1016/j.ygyno.2013.05.033)
44. The World Health Organization. Cervical cancer screening in developing countries: Report of a WHO consultation. The World Health Organization, Geneva, 2002.
45. Dong Z. The Guideline for Screening and Early Detection and Treatment of Cancer in China. Peking University Medical Press, Beijing, China, 2005.
46. Chen Q, Xie LX, Qing ZR, *et al*. Epidemiologic characterization of human papillomavirus infection in rural Chaozhou, Eastern Guandong Province of China. PLoS One. 2012; 7:e32149.
47. Chen W, Zhang X, Molijn A, *et al*. Human papillomavirus type-distribution in cervical cancer in China: The importance of HPV 16 and 18. Cancer Causes Control. 2009; 20:1705-1713.
48. Li J, Mei J, Wang X, Hu L, Lin Y, Yang P. Human papillomavirus type-specific prevalence in women with cervical intraepithelial neoplasm in Western China. J Clin Microbiol. 2012; 50:1079-1081.
49. Zhang L, Wang Y, Peng M, She Q, Xiang Q, Chen Q, Liu Z, Zhang W, Tao N, Qiu L, Wu X. Prevalence and type distribution of high-risk human papillomavirus infections among women in Wufeng County, China. Arch Gynecol Obstet. 2012; 286: 695-699.
50. Lo KW, Wong YF, Chan MK, *et al*. Prevalence of human papillomavirus in cervical cancer: A multicenter study in China. Int J Cancer. 2002; 100:327-331.
51. Wu EQ, Liu B, Cui JF, *et al*. Prevalence of type-specific human papillomavirus and pap results in Chinese women: A multi-center, population-based cross-sectional study. Cancer Causes Control. 2013; 24:795-803.
52. Li R, Li Y, Radley D, Liu Y, Huang T, Sings HL, Zhang L, Wang W, Zhong X, Saah AJ. Safety and immunogenicity of a vaccine targeting human papillomavirus types 6, 11, 16 and 18: A randomized, double-blinded placebo-controlled trials in Chinese males and females. Vaccine. 2010; 30:4284-4291.
53. Canfell K, Shi JF, Lew JB, Walker R, Zhao FH, Simonella L, Chen JF, Legood R, Smith MA, Nickson C, Qiao YL. Prevention of cervical cancer in rural China: Evaluation of HPV vaccination and primary HPV screening strategies. Vaccine. 2011; 29:2487-2494.
54. Goldie SJ, Diaz M, Kim SY, Levin CE, Van Minh H, Kim JJ. Mathematical models of cervical cancer prevention in the Asia Pacific Region. Vaccine. 2008; 26(Suppl 12):M17-M29.
55. Goldie SJ, O'Shea M, Diaz M, Kim SY. Benefits, cost requirements and cost-effectiveness of the HPV 16, 18 vaccines for cervical cancer prevention in developing countries: Policy implications. Reprod Health Matters. 2008; 16:86-96.
56. Li J, Li LK, Ma JF, Wei LH, Niyazi M, Li CQ, Xu AD, Wang JB, Liang H, Belinson J, Qiao YL. Knowledge and attitudes about human papillomavirus and HPV vaccines among women living in metropolitan and rural regions of China. Vaccine. 2009; 27:1210-1215.
57. Zhao FH, Tiggelaar SM, Hu SY, *et al*. A multi-center survey of HPV knowledge and attitudes toward HPV vaccination among women, government officials and medical personnel in China. Asian Pac J Cancer Prev. 2012; 13:2369-2378.
58. Garland SM, Cuzick J, Domingo EJ, Goldie SJ, Kim YT, Konno R, Parkin DM, Qiao YL, Sankaranarayanan R, Stern PL, Tay SK, Bosch FX. Recommendations for cervical cancer prevention in Asia Pacific. Vaccine. 2008; 26(Suppl 12):M89-M98.
59. Hakim AA, Dinh TA. Worldwide impact of the human papillomavirus vaccine. Curr Treat Options Oncol. 2009; 10:44-53.
60. Shi JF, Qiao YL, Smith JS, Dondog B, Bao YP, Dai M, Clifford GM, Franceschi S. Epidemiology and prevention of human papillomavirus and cervical cancer in China and Mongolia. Vaccine. 2008; 26(Suppl 12):M53-M59.

(Received July 27, 2013; Revised August 16, 2013; Accepted August 18, 2013)

Policy Forum

DOI: 10.5582/bst.2013.v7.4.168

Measures to combat H7N9 virus infection in China: Live poultry purchasing habits, poultry handling, and living conditions increase the risk of exposure to contaminated environments

Peipei Song[1,2], Jufeng Xia[3], Jianjun Gao[3], Lingzhong Xu[2], Yong Huang[4], Linong Yao[5], Wei Tang[2,3,*]

[1] *Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *School of Public Health, Shandong University, Ji'nan, Shandong, China;*
[3] *Department of Surgery, The University of Tokyo Hospital, Tokyo, Japan;*
[4] *Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[5] *Zhejiang Provincial Center of Disease Control and Prevention, Hangzhou, Zhejiang, China.*

Summary **From March 31 to May 31, 2013, 132 cases of humans were infected with the H7N9 avian influenza virus, 39 of which resulted in death in China, which sparked global concerns about public health. Fortunately, no new case was reported in China since May 8, which seems like to make it step into a stable stage, and the emergency response to the event launched by Shanghai, Jiangsu, Zhejiang, Anhui, Jiangxi, Shandong, and Hu'nan of China have been terminated currently. However, on July 20 and August 10, two new cases were reported from two provinces - Hebei and Guangdong - where no case was reported during the period of spring of 2013. The emerged two new cases rung an alarm bell, thus, the continued public health response cannot let down its guard. Based on our before studies, we found that live poultry purchasing habits, poultry handling, and living conditions increase the risk of exposure to H7N9 virus contaminated environments in China. Due to the difficulty in changing live poultry purchasing habits and in thoroughly removing or closing live poultry markets in China, we suggest that enhanced regulation of poultry markets would be a more feasible and effective strategy to fight against H7N9 virus infection in China. Moreover, in view of the fact that frequent and inevitable contact between rural residents and poultry where rural residents lived also exists due to poultry handling and living conditions, the enhanced regulations on environmental health are also needed for free-range poultry, especially in rural areas.**

Keywords: H7N9 virus, avian influenza, environmental exposure, live poultry market

1. Introduction

On July 20, 2013, a 61 years of age female living in Hebei was laboratory-confirmed with H7N9 virus infection and dead on August 11 (*1*), besides, on August 10, another 51 years of age female infected with H7N9 virus was also laboratory confirmed in Guangdong and now remains in hospital in a critical condition (*2*). Both of the two new cases in China had a history of exposure to live animals in live poultry markets and were from the provinces where no case was reported during the period of spring of 2013, thus causing concerns in public that whether the virus will return in a more dangerous form and may spread to even more provinces in the near future?

From March 31 to May 31, 2013, 132 cases of human were infected with the H7N9 avian influenza virus in China, 39 of which were resulted in death (*3,4*), which sparked global concerns about public health. Fortunately, no new case was reported with H7N9 virus infection in China since May 8, and the emergency response to the event launched by Shanghai, Jiangsu, Zhejiang, Anhui, Jiangxi, Shandong, and Hu'nan of

**Address correspondence to:*
Dr. Wei Tang, Department of Surgery, The University of Tokyo Hospital, Tokyo, Japan, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: tang-sur@h.u-tokyo.ac.jp

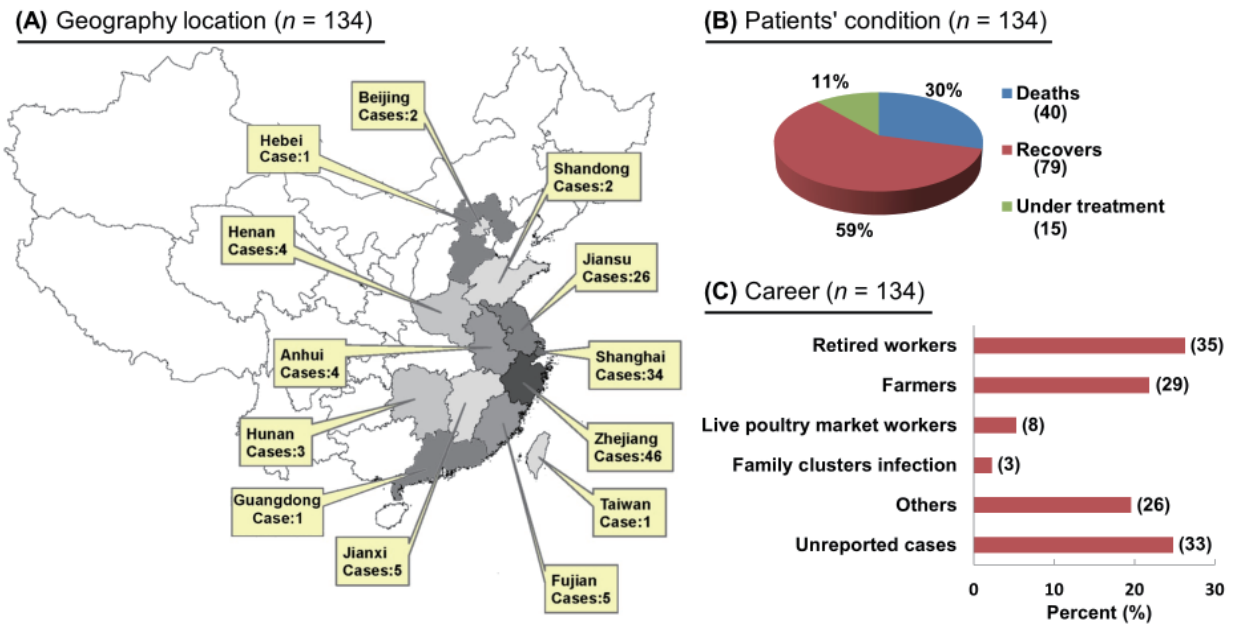

Figure 1. Epidemiologic characteristics of 134 confirmed cases of human infection with H7N9 virus in China. Data collection and analysis based on the information from weekly report on number of confirmed human cases of avian influenza A(H7N9) reported to WHO, the prevention and control work of human infection with avian influenza A(H7N9) by the National Health and Family Planning Commission (NHFPC) of China, and the epidemic reports form 13 provinces with infected patients in China during the period from March 31 to August 10, 2013.

China have been terminated, which seems like to make it step into a stable stage. However, the two new laboratory-confirmed cases emerged on July 20 and August 10 rung an alarm bell, thus, the continued public health response cannot let down its guard.

2. The public health response to combat H7N9 virus infection in China

Until now, 134 cases of H7N9 infection were reported in 43 cities of 13 provinces in China; most were in Zhejiang (46 cases, 7 deaths), Shanghai (34 cases, 13 deaths), and Jiangsu (26 cases, 8 deaths) (Figure 1A and 1B). Based on experience fighting SARS, H5N1, and H1N1 outbreaks over the past ten years, China's public health response to H7N9 virus infection is faster and more effective in terms of transparency in reporting, surveillance, screening, and stockpiling of antimicrobials (*5,6*). Moreover, important information such as viral gene sequence data and patient information were shared in a timely manner within China and with the international community *via* the WHO under International Health Regulations (*7*).

After the outbreak of infection with the H7N9 virus, China promptly initiated a survey of the virus' prevalence. In total, 84,444 specimens were collected from 473 live poultry markets, 32 poultry slaughterhouses, 896 poultry farms, 79 wild bird habitats, 36 pig slaughterhouses, and 137 environmental sampling sites. Results were announced by the Ministry of Agriculture of China on April 17 (*8*). This results and the update information involving a total of 899,758 specimens announced on May 23 showed that Avian influenza (H7N9) viruses were only isolated in samples from "wet" poultry markets and wild pigeons but not from poultry farms, wild bird habitats, or slaughterhouses (*8,9*). From April 18-24, a joint team of international and Chinese influenza experts convened by the WHO visited poultry markets and neighborhoods in Shanghai where infections were reported to assess the prevalence of H7N9 in China (*10*). Results indicated that the risk of H7N9 infection stemmed from live poultry markets and that people could be infected through contact with virus-carrying birds or exposure to contaminated environments.

On April 29, a joint report on the environmental exposure risk at poultry farms from the Ministry of Agriculture and World Organization for Animal Health indicated that there were previously no cases of human or animal H7N9 infections. And virological data have indicated that the new H7N9 virus is a recombination of H7N3, H9N2, and the original H7N9 (*11,12*). Genetic recombination and key gene mutations have presumably occurred in poultry markets and in transit but not at poultry farms. Thus, there may be no pressing need for large-scale culling at henneries and sampling and inspection of poultry farms may prove adequate.

In this condition, focus on live poultry markets, active public health responses have been implemented in China, including closure of affected markets, halting the sale of fowl, thorough disinfection, poultry culling, and prohibiting the entry of exotic live poultry that

could benefit in preventing more people infected with H7N9 virus. Currently, closing live poultry markets is regarded as a major prevention measure in China.

3. Administration of live poultry markets in management of H7N9 virus infection: thorough closure or enhanced regulation?

It is expected to avoid H7N9 virus infection at large if the live poultry markets could be closed thoroughly in the nationwide. However, it should be noted that nationwide thoroughly closing live poultry markets faces huge challenge in China. Currently, the measures of closing live poultry markets usually start to enforce after finding H7N9 virus positive samples in the affected market, just like the measures that have been implemented in affected areas such as Shanghai, and the measure are implementing in Hebei and Guangdong since the new cases were reported. Actually, as early as 2006, the Chinese Government have issued a regulation to direct live poultry markets to move out of densely populated areas in large and medium-sized cities and encouraging large cites to take the lead in prohibiting the sale and slaughter of live poultry in live poultry markets (*13*). But until now, a large amount of live poultry are still sold and slaughtered in markets, even in Shanghai, Jiangsu, and Zhejiang where most of the cases of H7N9 infection were reported.

Several studies have indicated that the greater risk factor for infection in urban residents is contact with virus-carrying birds or exposure to contaminated environments in live poultry markets. This risk is closely related to the habit of purchasing freshly slaughtered poultry. Changing live poultry purchasing habits will be difficult and take a long time. Correspondingly, thoroughly removing or closing live poultry markets face challenge and need a long time to solve it. So, due to the difficulty in changing live poultry purchasing habits and in thoroughly removing or closing live poultry markets in China, enhanced regulation of poultry markets will be a more feasible and effective strategy expected to be implemented. Such regulation would be expected to establish quarantine areas at production sites, establish fixed locations for slaughtering and quarantine, implement regular spot checks, implement bio-safety level disinfection, and establish better traceability of frozen poultry.

4. Other environments involving close contact with poultry: poultry handling, and living conditions in rural areas

Beside the environmental exposure risk at live poultry markets and poultry farms, the environments involving close contact with poultry also exist in rural areas. According to available information on 134 laboratory-confirmed cases of H7N9 virus infection, 35 cases involved urban retirees and 29 involved farmers (Figure 1C); most affected individuals had a history of exposure to live animals, and especially chickens.

In order to ascertain the relationship between poultry handling and avian influenza in rural areas, we conducted a cross-sectional study involving 1,379 participants in Shandong Province, Anhui Province, and the Inner Mongolia Autonomous Region from September 2007 to January 2008 (*14*). Results showed that the risk of environmental exposure to avian influenza is closely related to Chinese living conditions that influence poultry handling: of 1,379 participants, half had contact with wild birds ("often" and "occasionally"); nearly 50% have a semi-closed or open yard that poultry could pass through, 51% kept poultry in their homes. Some respondents had special chicken coops while others did not. Respondents who did not have special chicken coops instead had poultry that were raised in a common room, an extra room, or a restroom. Poultry feces were often found in the yard, living room, restroom, or kitchen of most homes with poultry and even in those with special coops. Thus, enhanced regulations on environmental health are needed for free-range poultry, especially in rural areas.

5. Conclusion

The emerged two new laboratory-confirmed cases infected with H7N9 virus on July 20 and August 10 rung an alarm bell, thus, the continued public health response cannot let down its guard. Based on our before studies, we found that live poultry purchasing habits increase the risk of exposure to contaminated environments. Due to the difficulty in changing live poultry purchasing habits and in thoroughly removing or closing live poultry markets in China, we suggest that enhanced regulation of poultry markets would be a more feasible and effective strategy to fight against H7N9 virus infection in China. Such regulation would be expected to establish quarantine areas at production sites, establish fixed locations for slaughtering and quarantine, implement regular spot checks, implement bio-safety level disinfection, and establish better traceability of frozen poultry. Moreover, in view of the fact that frequent and inevitable contact between rural residents and poultry where rural residents lived also exists due to poultry handling and living conditions, the enhanced regulations on environmental health are also needed for free-range poultry, especially in rural areas.

References

1 Health Department of Hebei Province. One new case of human infected with H7N9 virus. *http://www.hebwst.gov.cn/index.do?id=50348&templet=con_news&cid=2* (accessed July 20, 2013).
2 Health Department of Guangdong Province. One

new case of human infected with H7N9 virus was laboratory confirmed. *http://www.gdwst.gov.cn/a/zwxw/2013081010827.html* (accessed August 10, 2013).
3 The National Health and Family Planning Commission of China. The prevention and control work of human infection with avian influenza A (H7N9). *http://www.moh.gov.cn/mohwsyjbgs/h7n9/list.shtml* (accessed June 15, 2013).
4 WHO. Number of confirmed human cases of avian influenza A (H7N9) reported to WHO. *http://www.who.int/influenza/human_animal_interface/influenza_h7n9/Data_Reports/en/index.html* (accessed June 17, 2013).
5 Mei L, Song P, Tang Q, Shan K, Tobe RG, Selotlegeng L, Ali AH, Cheng Y, Xu L. Changes in and shortcomings of control strategies, drug stockpiles, and vaccine development during outbreaks of avian influenza A H5N1, H1N1, and H7N9 among humans. Biosci Trends. 2012; 7:64-76.
6 Mei L, Tang Q, Cui YM, Tobe RG, Selotlegeng L, Ali AH, Xu LZ. Changes in and shortcomings of drug stockpiling, vaccine development and related policies during outbreaks of avian influenza A H5N1, H1N1, and H7N9 among humans. Drug Discov Ther. 2013; 7:95-100.
7 WHO. Sixty-sixth World Health Assembly. *http://www.who.int/mediacentre/events/2013/wha66/en/index.html* (accessed June 20, 2013).
8 The Ministry of Agriculture. Avian influenza (H7N9) viruses were not isolated from poultry farms. *http://www.moa.gov.cn/zwllm/zwdt/201304/t20130417_3436622.htm* (accessed May 31, 2013).
9 The Ministry of Agriculture. The national surveillance for animal H7N9 Avian influenza has been finished: Avian influenza (H7N9) viruses were not isolated from poultry farms. *http://www.moa.gov.cn/zwllm/zwdt/201305/t20130523_3471497.htm* (accessed May 31, 2013).
10 WHO. China – WHO Joint Mission on Human Infection with Avian Influenza A (H7N9) Virus. *http://www.who.int/influenza/human_animal_interface/influenza_h7n9/ChinaH7N9JointMissionReport2013u.pdf* (accessed June 6, 2013).
11 Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, *et al*. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013; 368:1888-1897.
12 Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, *et al.* Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: Clinical analysis and characterisation of viral genome. Lancet. 2013; 381:1916-1925.
13 The General Office of the State Council. The opinions on rectifying and regulating live poultry market to strengthen prevention and control work for highly pathogenic avian influenza. *http://www.gov.cn/zwgk/2006-11/24/content_452941.htm* (accessed June 27, 2013).
14 Gai R, Wang X, Zhang Y, Xu L. Knowledge and practice of poultry handling and living environments of rural residents in China. Biosci Trends. 2008; 2:61-63.

(Received July 28, 2013; Revised August 12, 2013; Accepted August 15, 2013)

Mini-Review

DOI: 10.5582/bst.2013.v7.4.172

Advances in the study of biomarkers of idiopathic pulmonary fibrosis in Japan

Haibo Huang[1,2,*], Xiaonu Peng[1], Jun Nakajima[2]

[1] *Department of Thoracic Surgery, Yantai Yuhuangding Hospital, Medical College of Qingdao University, Yantai, Shandong, China;*
[2] *Department of Thoracic Surgery, The University of Tokyo Hospital, Tokyo, Japan.*

Summary **Idiopathic pulmonary fibrosis is an intractable disease with a median survival time of 2 to 3 years. Serum levels of Krebs von den Lungen-6 (KL-6), surfactant protein A (SP-A), and surfactant protein D (SP-D) are useful biomarkers for idiopathic pulmonary fibrosis and they are widely used in Japan. Based on clinical use in Japan, a combination of KL-6, SP-A, and SP-D is useful at diagnosing interstitial lung diseases and predicting the prognoses for patients with these diseases. However, the differential diagnosis of idiopathic pulmonary fibrosis from other interstitial lung diseases is still challenging. Several other biomarkers have been identified and are being studied in Japan.**

Keywords: Interstitial lung disease, biomarker, KL-6, SP-A, SP-D

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive, fibrosing interstitial pneumonia of unknown etiology (*1*). Although its exact epidemiology is still unknown, IPF is reported to have no distinct geographical distribution, a greater prevalence in males, and mostly affect individuals who smoke (*2*). IPF has the characteristic appearance of usual interstitial pneumonia (UIP), which is usually limited to the lungs. It is the most common and severe form of idiopathic interstitial pneumonia (IIP). It is progressive, irreversible, and associated with an extremely poor prognosis. No effective pharmacological therapies have been identified to date. The median survival time for patients with IPF is 2 to 3 years from the time of diagnosis (*3*).

The prevalence of IPF in Japan is reported to be 11.8 cases per 100,000 population. IPF has been designated as an intractable disease by the Ministry of Health, Labor, and Welfare of Japan, and patients diagnosed with the condition receive full insurance coverage (*4*). In 1989, Kohno first reported Krebs von den Lungen-6 (KL-6) as a new serum indicator for IPF (*5*). Since then, several types of biomarkers were identified and have been studied extensively mostly in Japanese. Several types of biomarkers for IPF have been used clinically in Japan. The current article briefly reviews advances in the study of biomarkers for IPF in Japan.

2. Search strategy

A search of the Medline database was done using a combination of the keywords "idiopathic pulmonary fibrosis" and "biomarker" or a combination of the keywords "idiopathic pulmonary fibrosis" and "marker". All articles related to Japan were reviewed. Searches of the databases of the Japan Science and Technology Agency (J-GLOBAL) and Japan Medical Abstracts Society (JMAS) were also done using a combination of the keyword "idiopathic pulmonary fibrosis" or "idiopathic interstitial pneumonia" and "biomarker." All abstracts were reviewed and the full text of relevant articles was reviewed.

3. Biomarkers used in clinic in Japan

The diagnosis of IPF was once a major challenge to clinicians. According to the American Thoracic Society's (ATS) 2000 guidelines on diagnosis and treatment of IPF (*6*), the golden standard for diagnosing IPF is by pathological findings. However, a lung biopsy

**Address correspondence to:*
Dr. Haibo Huang, Department of Thoracic Surgery, Yantai Yuhuangding Hospital, No. 20 Yuhuangding Donglu, Yantai 264000, Shandong, China.
E-mail: doctorhuanghaibo@hotmail.com

is not feasible for many patients suspected of having IPF, so the identification of diagnostic biomarkers for IPF would help both clinicians and patients, particularly in cases where a lung biopsy cannot be obtained. With the development of high-resolution computed tomography (HRCT), the ATS had revised its criteria so that patients with UIP patterns according to HRCT can be diagnosed as having IPF without the need for a lung biopsy (*1*). This sounds as though the value of biomarkers for IPF has decreased. Because of the potential side effects of many types of therapies for IPF, however, IPF should be carefully be diagnosed, and biomarkers may help in this regard. Biomarkers also have important value in predicting the progression and activity of IPF as well as patient response and prognosis.

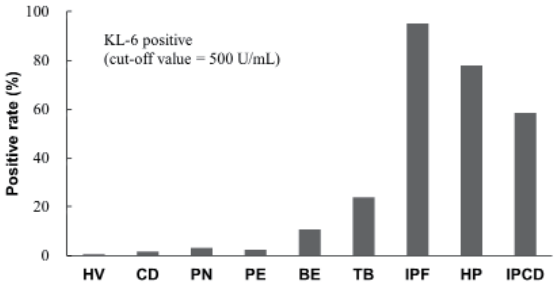

Figure 1. Different rates at which KL-6 tests positive in different lung diseases (*Ref. 13*)**.** Abbreviations: HV, health volunteer; CD, collagen disease; PN, ordinary pneumonia; PE, pulmonary emphysema; BE, bronchiectasis; TB, pulmonary tuberculosis; HP, hypersensitivity pneumonia; IPCD, interstitial pneumonia with collagen disease.

3.1. *KL-6*

KL-6 is the earliest and most intensively studied biomarker of interstitial lung diseases (ILDs) in Japan. KL-6 is a high-molecular-weight glycoprotein classified as a polymorphic epithelial mucin. It is expressed on the surface of various epithelial cells and highly expressed by regenerating type II pneumocytes. It was first investigated as a serum tumor biomarker, but it resulted in a very high rate of false positives in patients with pulmonary fibrosis (*7*). A later study (*5*) identified it as a biomarker for ILDs.

KL-6's value in diagnosing patients with ILDs compared to healthy controls has been demonstrated by several studies (*8,9*). The clinical cut-off value for distinguishing patients with ILDs from healthy subjects and patients with lung diseases other than ILDs had been set at the level of 500 U/mL (*10*). That said, serum levels of KL-6 were also significantly higher in patients with active pulmonary tuberculosis, with a false positive rate of 28% (*11*). A study (*12*) found that serum levels of KL-6 were elevated in 70-100% of patients with ILDs and therefore cannot be used to differentiate IPF from other interstitial pneumonias. Data from a study (*13*) with a large Japanese sample were used to create Figure 1 to show the different rates at which KL-6 tests positive based on a cut-off value of 500 U/mL; data are from 225 patients with various lung diseases and 200 healthy individuals.

Serum levels of KL-6 are also related to therapeutic efficacy. A study treated 14 Japanese patients with rapidly progressive IPF (*14*) with high-dose corticosteroid pulse therapy and it followed all of the patients for at least 3 weeks. Patients whose serum levels of KL-6 significantly decreased had a better response and better long-time survival. Several studies (*15,16*) found that serum levels of KL-6 were related to the extent and activity of IPF.

Serum levels of KL-6 are also related to the prognosis for a patient with IPF. Elevated serum levels of KL-6, and especially levels over 1,000 U/mL, upon initial measurement are reported to be related to increased mortality (*17*). The progression of IPF is significantly faster in patients with KL-6 levels of 1,000 U/mL or higher upon initial measurement than in patients with KL-6 levels below 1,000 U/mL (*18*).

3.2. *Surfactant proteins*

Surfactant protein A (SP-A) and surfactant protein D (SP-D) are both water-soluble lung-specific proteins. They are also collectins, a subgroup of the C-type lectin family. They are mainly produced by alveolar epithelial type II pneumocytes and Clara cells within the lungs. Elevated serum levels of SP-A in patients with IPF were first noted in 1993 (*19*). Serum levels of SP-A in patients with IPF and pulmonary alveolar proteinosis (PAP) were found to be significantly higher than those in healthy volunteers and patients with other non-interstitial lung diseases. A study found that serum levels of SP-A indicate the activity of IPF (*20*). Another study (*12*) found that SP-A was better at distinguishing IPF from other ILDs. That study found that serum levels of SP-A in patients with UIP were significantly higher than in patients with non-specific interstitial pneumonia (NSIP).

Elevated serum levels of SP-D were first found to be a possible marker for IPF in 1995 (*21*). In that study, serum levels of SP-D were significantly higher in patients with IPF, IPCD, and PAP. Serum levels of SP-D were significantly related to the activity of IPF and IPCD. They also closely indicated the severity of PAP.

Serum levels of SP-A and SP-D were also reported to indicate the prognosis for patients with IPF (*22*). In that study, serum levels of SP-A and SP-D were significantly correlated with the extent of alveolitis. Serum levels of SP-D were also related to the extent of parenchymal collapse on CT and deterioration in pulmonary function. Patients with higher levels of SP-A

Table 1. Rates at which SP-A and SP-D test positive in different lung diseases (*Ref. 23*)

Rate of testing positive (%)	SP-A	SP-D
70-100	IPF	IPF, IPCD, PAP
50-70	PAP	
30-50	IPCD	
10-30	SAR, PN, DPB, TB, PC	SAR, PN, DPB, TB, PE
0-10	BA, PE	BA

Abbreviations: IPF, idiopathic pulmonary fibrosis; IPCD, interstitial pneumonia with collagen disease; PAP, pulmonary alveolar proteinosis; SAR, pulmonary sarcoidosis; PN, ordinary pneumonia; DPB, diffuse panbronchiolitis; TB, pulmonary tuberculosis; PE, pulmonary emphysema; BA, bronchial asthma.

and SP-D had a worse 3-year survival rate.

A study (*23*) of serum levels of SP-A and SP-D in a large Japanese sample set the cut-off value of serum SP-A and SP-D at 48.3 ng/mL and 109 ng/mL, respectively. The study described serum levels of SP-A and SP-D in 87 patients with IPF, 303 patients with HV, and 215 patients with other lung diseases (Table 1).

Many studies have investigated the levels of KL-6, SP-A, and SP-D in bronchoalveolar lavage fluid (BALF). Levels of KL-6 in BALF were reported to be consistent with those in serum (*5,8,9*), whereas the levels of SP-A and SP-D in BALF were reported to be inversely correlated with levels in serum (*19-21*). Although the levels of KL-6, SP-A and SP-D in BALF are potential biomarkers for IPF, the inconvenience of obtaining BALF has resulted in their limited use clinically.

KL-6, SP-A, and SP-D have been recognized by Japan's National Health Insurance System as diagnostic markers for ILDs in 1999. KL-6 has been used in clinical practice for more than 10 years in Japan, and KL-6 levels are examined in more than 2,000,000 samples per year in Japan (*24*). Kits to measure KL-6, SP-A, and SP-D have been successfully developed by reagent manufacturers (Picolumi KL-6: Sanko Junyaku Co., Tokyo, Japan; SP-A test-F: Kokusai Shiyaku Co., Hyogo, Japan; SP-D kit Yamasa; YamasaShoyu Co., Tokyo, Japan). KL-6, SP-A, and SP-D have been included a fourth edition of clinical diagnostic criteria for IPF from a Study Group on IPF of the Ministry of Health, Labor, and Welfare, and all three markers are widely used in many hospitals in Japan.

3.3. *The comparative diagnostic value of KL-6, SP-A, and SP-D*

A study (*25*) compared the usefulness of KL-6, SP-A, and SP-D in clinically diagnosing ILDs (Figure 2). KL-6 had the highest specificity and sensitivity for ILDs, but SP-A had the best ability to differentiate IPF from other ILDs (*12*). To date, KL-6, SP-A, and SP-D have proven value at diagnosing ILDs, but the differential diagnosis of ILDs is still difficult. Thus, a combination of KL-6, SP-A, and SP-D is needed to diagnose IPF.

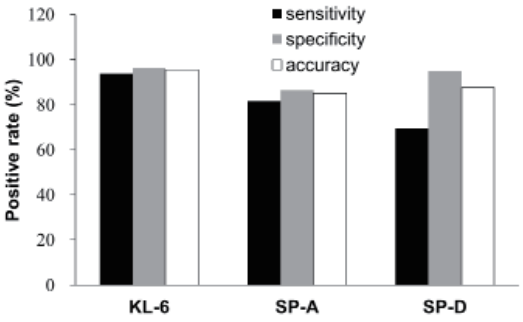

Figure 2. Different diagnostic values of KL-6, SP-A, and SP-D for ILDs (*Ref. 25*)**.**

4. Potential biomarkers being studied in Japan

4.1. *Vascular endothelial growth factor (VEGF)*

Since many interstitial lung diseases are associated with aberrant angiogenesis, the key angiogenesis regulator VEGF has been investigated as a potential biomarker for IPF. A study in the UK (*26*) found that serum levels of VEGF were not related to IPF. A study in Japan (*27*) grouped patients depending on the alveolar-arterial difference in oxygen ($AaDO_2$) and the study found that there were significant differences in the serum levels of VEGF in different groups with a certain $AaDO_2$ and healthy volunteers. IPF patients with serum levels of VEGF above the median tended to have a worse survival than those with serum levels of VEGF below the median.

4.2. *YKL-40*

YKL-40, also known as human cartilage glycoprotein 39, is a chitinase-like protein. Its roles in tissue remodeling and fibrosis have been investigated. A study in Japan (*28*) found that YKL-40 levels in serum and BALF were significantly higher in patients with IPF than in healthy controls. Serum levels of YKL-40 are significantly related to serum levels of KL-6. This means that YKL-40 may be a potential biomarker for IPF.

4.3. *Osteopontin*

Osteopontin is a type of glycoprotein involved in immune response and tissue repair. Its role in promoting the migration, adhesion, and proliferation of fibroblasts has been demonstrated in a mouse model of bleomycin-induced pulmonary fibrosis (*29*). A study in 2005 (*30*) found that levels of osteopontin in plasma were significantly higher in patients with ILDs than in those

with sarcoidosis or in healthy controls. No significant differences in the plasma concentration of osteopontin were noted in patients with IPF or other ILDs.

4.4. *Periostin*

Periostin is a type of protein known for its roles in the maintenance and development of bones, teeth, and the heart. A recent study (*31*) found that serum levels of periostin were significantly higher in patients with IPF than in healthy volunteers and patients with cryptogenic organizing pneumonia (COP). Furthermore, periostin levels were inversely correlated with patients' pulmonary function.

4.5. *Napsin A*

Napsin A, which is expressed in type II pneumocytes and alveolar macrophages, is a type of aspartic proteinase. A recent study (*32*) found that serum levels of napsin A were significantly higher in patients with IPF than in healthy controls. Serum levels of napsin A are also correlated with the severity of disease.

4.6. *Connective tissue growth factor (CCN2)*

CCN2 (also known as CTGF) is a type of secreted peptide that mainly serves to produce extracellular matrix and perform other profibrotic activities. Plasma levels of CCN2 were reported to be significantly higher in patients with IPF than in patients with non-IPF IIPs and healthy controls (*33*). That study also reported a correlation between plasma CCN2 and changes in the 6-month forced vital capacity (FVC).

4.7. *S100A9*

S100A9 is a type of calcium-binding protein also known as calgranulin B. It is reported to be a useful marker for non-infectious inflammatory diseases. A study of BALF (*34*) found that S100A9 levels were significantly higher in patients IPF than in patients with other IPDs and healthy volunteers.

4.8. *Heat shock protein 47 (HSP47)*

HSP47 is a type of collagen-specific molecular chaperone that mainly functions in biosynthesis and the secretion of collagen. Serum levels of HSP47 in cases of acute exacerbation of IPF (AE-IPF) are reported to be significantly higher than those in cases of stable IPF (*35*).

Details are shown in Table 2.

Table 2. Details on other biomarkers being studied in Japan

Biomarker	Location	Difference between ILDs and HV	Difference between IPF and other ILDs	Correlation with disease activity or severity	Correlation with KL-6	Correlation with SP-A or SP-D	Number of HV	Number of IPF	Other IIPs	*Ref.*
VEGF	serum	Yes*	--	HRCT	--	--	43	41	--	(*27*)
YKL-40	serum	Yes	--	DLco and PaO_2	Yes	--	41	63		(*28*)
	BALF	Yes	--	--	--	No	16	18		(*28*)
Osteopontin	plasma	Yes	No	PaO_2	Yes	--	20	8	8	(*29,30*)
Periostin	serum	Yes	No	Pulmonary function	--		--	51	41	(*31*)
Napsin A	serum	Yes	--	Forced vital capacity		--	20	20	--	(*32*)
CCN2	plasma	Yes	Yes	Forced vital capacity	--	--	101	33	14	(*33*)
S100A9	BALF	Yes	Yes	--	--	--	23	28	70	(*34*)
	serum	No	No	--	--		23	28	70	(*34*)
HSP47	serum	--	--	Exacerbation *vs.* stable			--	53	--	(*35*)

Abbreviations: DLco, diffusing capacity for carbon monoxide; PaO2, partial pressure of oxygen. *Differences were significant for the subgroup with a certain alveolar-arterial difference in oxygen.

5. Conclusion

This review briefly presented advances in the study of biomarkers for IPF in Japan. Although no specific

biomarkers for IPF had been identified to date, KL-6, SP-A, and SP-D have shown good specificity and sensitivity at diagnosing ILDs. Of the three, KL-6 has the best specificity and sensitivity at diagnosing ILDs while SP-A is specifically able to differentiate IPF from other ILDs. Based on clinical use in Japan, the combination of KL-6, SP-A, and SP-D provides certain value at diagnosing IPF and predicting the prognosis for patients with the condition.

That said, the differentiation of IPF from other ILDS is still challenging. Biomarkers should help to overcome this challenge. Several other biomarkers have also been identified, but further research and evaluation are still needed.

References

1. Raghu G, Collard HR, Egan JJ, *et al*. An official ATS/ERS/JRS/ALAT statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med. 2011; 183:788-824.
2. Iwai K, Mori T, Yamada N, Yamaguchi M, Hosoda Y. Idiopathic pulmonary fibrosis: Epidemiologic approaches to occupational exposure. Am J Respir Crit Care Med. 1994; 150:670-675.
3. King TE Jr, Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron JA Jr, Flint A, Thurlbeck W, Cherniack RM. Idiopathic pulmonary fibrosis: Relationship between histopathologic features and mortality. Am J Respir Crit Care Med. 2001; 164:1025-1032.
4. Song PP, Kokudo N. Revision of measures to combat intractable diseases in Japan: Three pillars will play an even greater role in the future. Intractable Rare Dis Res. 2013; 2:33-34.
5. Kohno N, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, Akiyama M. New serum indicator of interstitial pneumonitis activity. Sialylated carbohydrate antigen KL-6. Chest. 1989; 96:68-73.
6. No authors listed. Idiopathic Pulmonary Fibrosis: Diagnosis and Treatment, International Consensus Statement. Am J Respir Crit Care Med. 2000; 161:646-664.
7. Kohno N, Akiyama M, Kyoizumi S, Hakoda M, Kobuke K, Yamakido M. Detection of soluble tumor-associated antigens in sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. Jpn J Clin Oncol. 1988; 18:203-216. (in Japanese)
8. Ohnishi H, Yokoyama A, Kondo K, Hamada H, Abe M, Nishimura K, Hiwada K, Kohno N. Comparative study of KL-6, surfactant protein-A, surfactant protein-D, and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. Am J Respir Crit Care Med. 2002; 165:378-381.
9. Kohno N, Awaya Y, Oyama T, Yamakido M, Akiyama M, Inoue Y, Yokoyama A, Hamada H, Fujioka S, Hiwada K. KL-6, a mucin-like glycoprotein, in bronchoalveolar lavage fluid from patients with interstitial lung disease. Am Rev Respir Dis. 1993; 148:637-642.
10. Kitamura S, Hiwada K, Kobayashi J, *et al*. Use of the ED046 kit to analyze serum KL-6 in patients with pneumonitis. Nihon Kyobu Shikkan Gakkai Zasshi. 1996; 34:639-645. (in Japanese)
11. Inoue Y, Nishimura K, Shiode M, Akutsu H, Hamada H, Fujioka S, Fujino S, Yokoyama A, Kohno N, Hiwada K. Evaluation of serum KL-6 levels in patients with pulmonary tuberculosis. Tuber Lung Dis. 1995; 76:230-233.
12. Ishii H, Mukae H, Kadota J, Kaida H, Nagata T, Abe K, Matsukura S, Kohno S. High serum concentrations of surfactant protein A in usual interstitial pneumonia compared with non-specific interstitial pneumonia. Thorax. 2003; 58:52-54.
13. Kohno N. Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. J Med Invest. 1999; 46:151-158.
14. Yokoyama A, Kohno N, Hamada H, Sakatani M, Ueda E, Kondo K, Hirasawa Y, Hiwada K.Circulating KL-6 predicts the outcome of rapidly progressive idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1998; 158:1680-1684.
15. Sakamoto K, Taniguchi H, Kondoh Y, Johkoh T, Sumikawa H, Kimura T, Nishiyama O, Kato K, Kataoka K, Ono K, Kitaichi M, Hasegawa Y.Serum KL-6 in fibrotic NSIP: Correlations with physiologic and radiologic parameters. Respir Med. 2010; 104:127-133.
16. Ichiyasu H, Ichikado K, Yamashita A, Iyonaga K, Sakamoto O, Suga M, Kohrogi H. Pneumocyte biomarkers KL-6 and surfactant protein D reflect the distinct findings of high-resolution computed tomography in non-specific interstitial pneumonia. Respiration. 2012; 83:190-197.
17. Yokoyama A, Kondo K, Nakajima M, Matsushima T, Takahashi T, Nishimura M, Bando M, Sugiyama Y, Totani Y, Ishizaki T, Ichiyasu H, Suga M, Hamada H, Kohno N. Prognostic value of circulating KL-6 in idiopathic pulmonary fibrosis. Respirology. 2006; 11:164-168.
18. Satoh H, Kurishima K, Ishikawa H, Ohtsuka M. Increased levels ofKL-6 and subsequent mortality in patients with interstitial lung diseases. J Intern Med. 2006; 260:429-434.
19. Kuroki Y, Tsutahara S, Shijubo N, Takahashi H, Shiratori M, Hattori A, Honda Y, Abe S, Akino T. Elevated levels of lung surfactant protein A in sera from patients with idiopathic pulmonary fibrosis and pulmonary alveolar proteinosis. Am Rev Respir Dis. 1993; 147:723-729.
20. Honda Y, Kuroki Y, Shijubo N, Fujishima T, Takahashi H, Hosoda K, Akino T, Abe S. Aberrant appearance of lung surfactant protein A in sera of patients with idiopathic pulmonary fibrosis and its clinical significance. Respiration. 1995; 62:64-69.
21. Honda Y, Kuroki Y, Matsuura E, Nagae H, Takahashi H, Akino T, Abe S. Pulmonary surfactant protein D in sera and bronchoalveolar lavage fluids. Am J Respir Crit Care Med. 1995; 152:1860-1866.
22. Takahashi H, Fujishima T, Koba H, *et al*. Serum surfactant proteins A and D as prognostic factors in idiopathic pulmonary fibrosis and their relationship to disease extent. Am J Respir Crit Care Med. 2000; 162:1109-1114.
23. Yasuhito H. Clinical significance of serum surfactant proteins A and D in idiopathic interstitial pneumonia. Jpn J Thoracic Diseases. 1996; 34:181-185. (in Japanese)
24. Ishikawa N, Hattori N, Yokoyama A, Kohno N. Utility of KL-6/MUC1 in the clinical management of interstitial

lung diseases. Respir Investig. 2012; 50:3-13.
25. Ohnishi H, Yokoyama A, Kondo K, Hamada H, Abe M, Nishimura K, Hiwada K, Kohno N. Comparative study of KL-6, surfactant protein-A, surfactant protein-D, and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. Am J Respir Crit Care Med. 2002; 165:378-381.
26. Simler NR, Brenchley PE, Horrocks AW, Greaves SM, Hasleton PS, Egan JJ. Angiogenic cytokines in patients with idiopathic interstitial pneumonia. Thorax. 2004; 59:581-585.
27. Ando M, Miyazaki E, Ito T, Hiroshige S, Nureki SI, Ueno T, Takenaka R, Fukami T, Kumamoto T. Significance of serum vascular endothelial growth factor level in patients with idiopathic pulmonary fibrosis. Lung. 2010; 188:247-252.
28. Furuhashi K, Suda T, Nakamura Y, Inui N, Hashimoto D, Miwa S, Hayakawa H, Kusagaya H, Nakano Y, Nakamura H, Chida K. Increased expression of YKL-40, a chitinase-like protein, in serum and lung of patients with idiopathic pulmonary fibrosis. Respir Med. 2010; 104:1204-1210.
29. Takahashi F, Takahashi K, Okazaki T, Maeda K, Ienaga H, Maeda M, Kon S, Uede T, Fukuchi Y. Role of osteopontin in the pathogenesis of bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol. 2001; 24:264-271.
30. Kadota J, Mizunoe S, Mito K, Mukae H, Yoshioka S, Kawakami K, Koguchi Y, Fukushima K, Kon S, Kohno S, Saito A, Uede T, Nasu M. High plasma concentrations of osteopontin in patients with interstitial pneumonia. Respir Med.2005; 99:111-117.
31. Okamoto M, Hoshino T, Kitasato Y, Sakazaki Y, Kawayama T, Fujimoto K, Ohshima K, Shiraishi H, Uchida M, Ono J, Ohta S, Kato S, Izuhara K, Aizawa H. Periostin, a matrix protein, is a novel biomarker for idiopathic interstitial pneumonias. Eur Respir J. 2011; 37:1119-1127.
32. Samukawa T, Hamada T, Uto H, Yanagi M, Tsukuya G, Nosaki T, Maeda M, Hirano T, Tsubouchi H, Inoue H. The elevation of serum napsin A in idiopathic pulmonary fibrosis, compared with KL-6, surfactant protein-A and surfactant protein-D. BMC Pulm Med. 2012;12:55-62.
33. Kono M, Nakamura Y, Suda T, *et al*. PlasmaCCN2 (connective tissue growth factor; CTGF) is a potential biomarker in idiopathic pulmonary fibrosis (IPF). Clin Chim Acta. 2011; 412:2211-2215.
34. Hara A, Sakamoto N, Ishimatsu Y, Kakugawa T, Nakashima S, Hara S, Adachi M, Fujita H, Mukae H, Kohno S. S100A9 in BALF is a candidate biomarker of idiopathic pulmonary fibrosis. Respir Med. 2012; 106:571-580
35. Kakugawa T, Yokota SI, Ishimatsu Y, Hayashi T, Nakashima S, Hara S, Sakamoto N, Kubota H, Mine M, Matsuoka Y, Mukae H, Nagata K, Kohno S. Serumheat shock protein 47 levels are elevated in acute exacerbation of idiopathic pulmonary fibrosis. Cell Stress Chaperones. 2013; 18:581-590.

(Received May 3, 2013; Revised June 25, 2013; Accepted July 12, 2013)

Original Article

DOI: 10.5582/bst.2013.v7.4.178

Factors affecting childhood immunization in Lao People's Democratic Republic: A cross-sectional study from nationwide, population-based, multistage cluster sampling

Tomomi Kitamura[1,*], Kenichi Komada[1], Anonh Xeuatvongsa[2], Masahiko Hachiya[1]

[1] *Bureau of International Medical Cooperation, National Center for Global Health and Medicine, Shinjuku, Tokyo, Japan;*
[2] *Ministry of Health, Vientian, Lao People's Democratic Republic.*

Summary **Vaccines are one of the most important achievements in public health, and a major contributor to this success is the Expanded Programme on Immunization (EPI). The effective vaccination series of the EPI should be used by its target population. Various factors influence the utilization of the EPI vaccination series. In Lao People's Democratic Republic (Lao PDR), immunization coverage was lower than the regional average. This study evaluates risk factors affecting immunization underutilization among children five to nine years of age. It is a cross-sectional study from nationwide, population-based, multistage cluster sampling. The children who have received 'standard six' antigens and those who have been partially immunized are compared. In a bivariate analysis, household occupation, maternal age, means of transportation, time to the nearest health facilities, the child's birthplace, birth attended by medical staff, and notification of vaccination date by medical staff, village authority, or megaphone were associated with vaccination status. The final multivariate logistic regression model revealed that maternal age and notification of vaccination date by the village authority increased the odds of full vaccination, while notification of vaccination date by megaphone had decreased those odds. Further detailed qualitative research may be needed to discover how maternal sociodemographic factors influence the utilization of these services. Future research needs to target younger children and must include health care provider factors related to vaccination services.**

Keywords: Expanded programme on immunization (EPI), full vaccination, childhood vaccination

1. Introduction

Vaccines have substantially reduced the global burden of infectious diseases. They are considered one of the most important achievements in public health and one of the most cost-effective preventive services for children (*1-7*). The major contributor to this success is the Expanded Programme on Immunization (EPI) of the World Health Organization (WHO), United Nations Children's Fund (UNICEF) and Global Alliance Vaccine Initiative (GAVI) (*6*). The EPI was launched in 1974 as a worldwide alliance of collaborating nations whose goal was to expand immunization services and coverage (*6*).

The success of EPI does not only depend on effective vaccination series, but also on achieving optimal use by its target population and high immunization coverage (*4,8*). Pinpointing non-vaccination factors is important for achieving the EPI targets (*9,10*). The utilization of vaccination services depends on numerous factors such as provision of EPI services including outreach services, accessibility of these services, number of health workers, availability of safe needles, syringes, and cold chain, as well as health education and knowledge and attitude of mothers (*5,11,12*). Once a child enters the vaccination system, completion of the series is determined by the mother's educational level, employment status, experience with vaccination services, adequate schedule information, immigration status, and overall socioeconomic status (*13-15*). Various factors

**Address correspondence to:*
Dr. Tomomi Kitamura, Bureau of International Medical Cooperation, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku, Tokyo 162-8655, Japan.
E-mail: tkitamura.imcj@gmail.com

are important for the initiation and completion of the vaccination series.

The EPI was initiated in Lao People's Democratic Republic (Lao PDR) in 1979 (*16*). This programme greatly contributed to the progress of basic immunization coverage through the support of international partners and the government of Japan (*17*). However, immunization coverage in Laos PDR became stagnant after the WHO Western Pacific Region achieved regional polio eradication in 2000 (*17*). Immunization coverage was lower than regional average: measles immunization coverage among 1-year-olds was 64%, diphtheria tetanus toxoid and pertussis (DTP3) immunization coverage among 1-year-olds was 74%, hepatitis B (HepB) immunization coverage among 1-year-olds was 74%, tuberculosis (Bacille Calmette-Guérin vaccine: BCG) immunization coverage among 1-year-olds was 72% and polio (OPV3) immunization coverage among 1-year-olds was 76% (*18,22*). The risk factors for non-vaccination need to be studied to achieve optimal use of the vaccination services in Lao PDR. Therefore, the aim of this study is to evaluate risk factors affecting underutilization of childhood immunization among children five to nine years of age.

2. Material and Methods

2.1. *Study location and population*

Lao PDR is located in Southeast Asia and bordered by five countries: Burma, China, Vietnam, Cambodia, and Thailand. In 2010, its population was approximately 6.2 million and its under-five mortality rate was 54 out of 1,000 live births (*19*).

2.2. *Sampling and sampling frame*

This analysis has been done in the sub-population of the nation-wide survey for Hepatitis B sero-prevalence. All 143 districts in Lao PDR were stratified into two strata according to their immunization coverage. Twelve districts were randomly selected from each stratum, and two villages were selected from each district *via* probability of population proportional to size sampling. After randomly selecting 21 children (five to nine years old) and their mothers (15 to 45 years old) from the selected villages, questionnaires were administered.

One thousand and eight pairs of mothers and children were recruited and assessed for eligibility for this study. Forty-three pairs were excluded either because mothers were younger than 15 years of age or older than 45 years of age, or children were younger than five years of age or older than nine years of age. Four hundred and sixty-five pairs were excluded because they did not have vaccination certifications such as yellow cards or mother and child handbooks. Three pairs were excluded because possession of the vaccination certification was unknown. Among the 497 pairs with the vaccination certification, 284 pairs were excluded because they could not show their certification on the day of the survey. From this, 213 pairs were included in this study.

In Lao PDR, the yellow cards or mother and child handbooks are provided to children who received any vaccination regulated by the Laos PDR Ministry of Health. Vaccination dates were transcribed from those cards, and any vaccination documentation was considered sufficient evidence (*20*). Each child's immunization record was checked against the EPI immunization schedule recommended by the WHO (*21,22*). The following categories were used: fully immunized, if the "standard six" antigens – BCG, DTP3 (3 doses), OPV3 (3 doses), and measles vaccines – have been received on the day of interview; and partially immunized, if at least one recommended vaccine dose was not given (*23,24)*. This study compared factors between children who completed their standard vaccinations (full vaccination) and children who had not completed their vaccinations at the time of the survey (partial vaccination).

2.3. *Collection of data*

The survey, which used a face-to-face, interview-based questionnaire, was conducted from 25 January 2012 to 4 February 2012 by a survey team. A pilot study was conducted prior to the survey to check for clarity and consistency of the questionnaire. The survey team collected demographic information, vaccination status, and other relevant information. Each survey team had two surveyors. These surveyors received two days of training, which consisted of an overview of research methods, interview strategies, and ethical considerations. Before each interview, written consent was obtained from the child's mothers and vaccination dates were transcribed from yellow cards or mother and child handbooks.

2.4. *Statistical analysis*

The proportions of children who received all the recommended routine vaccination according to the policy of the Expanded Programme of Immunization (EPI) in Lao PDR and its 95% confidence interval (CI) were calculated. Bivariate analysis was performed to assess the relationship between vaccination status and its risk factors. A chi-square test was used for categorical variables and Student's *t* test was used for continuous variables. Crude odds ratios (ORs) and 95% CIs were calculated. Based on the results of the bivariate analysis), all significant factors and ethnicity were entered into the multivariate logistic regression analysis. Ethnicity was added to the final multivariate model because its diversity presented a major challenge in health service delivery due to cultural and language barriers. However, the variable "birthplace of the children" was excluded

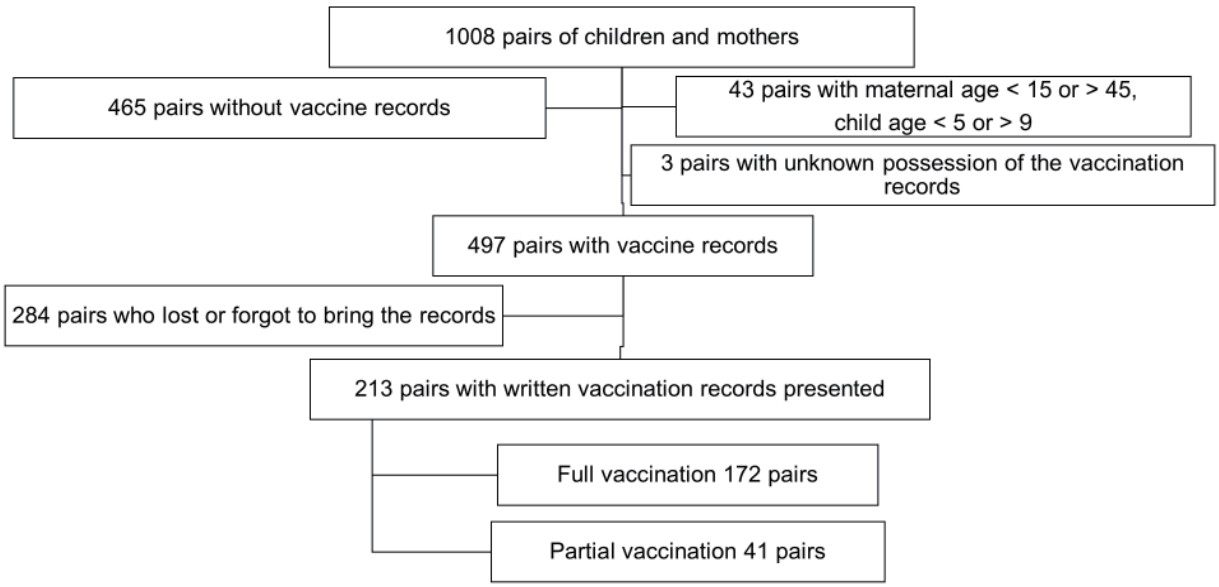

Figure 1. Study profile.

from the final multivariate logistic regression analysis since it showed the highest multicollinearity among the variables in the model. Multicollinearity was detected by using the variance inflation factor (VIF). Stata version 11.0 was used to perform all statistical analyses.

2.5. *Ethical considerations*

The survey was reviewed and approved by the Ethical Committee of the Ministry of Health, Lao PDR, and the institutional review board of the National Center for Global Health and Medicine, Japan (NCGM-G-001130-00). Access to selected households was granted by the Ministry of Health and the provincial and district government authorities.

3. Results

3.1. *Study profile of all subjects*

Figure 1 shows the profile of research subjects. 213 pairs were eligible. The case (full vaccination) group had 172 children and the control (partial vaccination) group had 41 children. The proportion of children, aged five to nine, who hold certificates of vaccination (either a yellow card, a mother and child handbook, or both) was 49.3% of the sample, while the proportion of children with an eligible record of vaccination was 21.1% of the sample (data not shown).

3.2. *Proportion of children vaccinated by antigen*

The statistics of children vaccinated with each antigen are presented in Table 1. All vaccination rates were over 80% except for the HepB birth dose, which was 19.2%. Among those 19.2%, only five children received the birth dose on the day of birth or the next day. The dates for the HepB birth dose for the rest of the children (80.8%) were not accurate. This low rate and record confusion may be due to the fact that the HepB vaccination for newborns was integrated into the EPI in 2004, which gradually expanded from central hospitals to rural areas. Most (80.8%) of the children were fully immunized between the ages of five and nine, according to the recommended EPI schedule.

Table 1. Proportion of children having received the various EPI antigens according to vaccination cards

EPI antigen	Total (%), (n = 213)	95%CI
HepB0	19.2	13.9-24.6
BCG	97.2	94.9-99.4
OPV1	97.7	95.6-99.7
OPV2	94.8	91.8-97.8
OPV3	93.4	90.1-96.8
DPT1	98.6	97.0-100
DPT2	95.8	93.1-98.5
DPT3	93.4	90.1-96.8
MV1	83.6	78.6-88.6
Full vaccination***	80.8	75.4-86.1

***Full vaccination – BCG, diphtheria-tetanus perttusis (DTP) (3 doses), polio (3 doses), and measles vaccines

3.3. *Risk factors of childhood immunization*

Among the sociodemographic factors of the family, household occupation, maternal age, means of transportation, and time to the nearest health facilities were associated with full vaccination of the children (Table 2). Birthplace of the children and birth attended by medical staff were associated with vaccination status (Table 3). Notification of vaccination date by

Table 2. Risk factors of childhood immunization (sociodemographic factors)

Items	Full vaccination $n = 172$, (%)	Partial vaccination $n = 41$, (%)	Crude OR (95%CI)	p-value
Ethnicity				
Lowland Lao	102 (59.3)	25 (61.0)	Reference	
Midland Lao	61 (35.5)	14 (34.2)	1.07 (0.52-2.21)	0.86
Highland Lao	9 (5.2)	32 (4.9)	1.10 (0.22-5.43)	0.90
Household occupation			Reference	
farmer	126 (73.3)	35 (89.7)	0.31 (0.10-0.94)	0.03
not farmers	46 (26.7)	4 (10.3)		
Mean maternal age (years)	32.7 (CI; 31.8-33.7)	29.3 (CI; 27.5-31.2)		0.00
Maternal education				
no education	68 (39.5)	16 (39.0)	Reference	0.32
primary school	55 (32.0)	19 (46.3)	0.68 (0.32-1.45)	0.25
junior high school	34 (19.8)	4 (9.8)	2.00 (0.62-6.45)	0.43
high school	10 (5.8)	1 (2.4)	2.35 (0.28-19.73)	0.89
university	5 (2.9)	1 (2.4)	1.18 (0.13-10.78)	
Sex of children				
boy	74 (43.0)	21 (51.2)	0.69 (0.34-1.38)	0.29
girl	97 (56.4)	41 (46.3)	Reference	
unknown	1 (0.6)	1 (2.4)		
Mean number of children	3.4 (CI; 3.1-3.7)	3.2 (CI; 2.6-3.9)		0.71
Transportation				
walk	65 (37.8)	13 (31.7)	Reference	
bicycle	3 (1.7)	0 (0)		
bike	44 (25.6)	18 (43.9)	0.49 (0.22-1.10)	0.08
car	27 (15.7)	0 (0)		
tractor	33 (19.2)	5 (12.2)	1.32 (0.43-4.02)	0.63
boat	0 (0)	3 (7.3)		
N/A	0 (0)	2 (4.9)		
Mean time to the nearest health facilities (minutes)	29.5 (CI; 25.0-33.9), $n = 152$	48.1 (CI; 20.5-75.7), $n = 37$		0.02
Decision-maker for vaccination				
Father	36 (20.9)	9 (22.0)	Reference	
Mother	129 (75.0)	29 (70.7)	1.11 (0.48-2.56)	0.80
Grandparents	4 (2.3)	3 (7.3)	0.33 (0.06-1.76)	0.20
Village leader	2 (1.2)	0 (0)		
Others	1 (0.6)	0 (0)		

N/A; Non applicable, CI; 95% confidence interval.

Table 3. Risk factors of childhood immunization (delivery history)

Items	Full vaccination $n = 172$, (%)	Partial vaccination $n = 41$, (%)	Crude OR (95%CI)	p-value
Birth place of children				
provincial hospital	38 (22.1)	3 (7.3)	Reference	
district hospital	24 (14.0)	9 (16.9)	0.21 (0.05-0.86)	0.03
health centre	4 (2.3)	1 (2.4)	0.32 (0.03-3.80)	0.36
private clinic	2 (1.2)	1 (2.4)	0.16 (0.01-2.29)	018
home	72 (41.9)	26 (63.4)	0.22 (0.06-0.77)	0.02
bush near house	31 (18.0)	0 (0)		
N/A	1 (0.6)	1 (2.4)		
Birth attended by the medical staff				
yes	94 (54.7)	14 (34.2)	2.27 (1.09-4.69)	
no	77 (44.8)	26 (63.4)	Reference	0.02
N/A	1 (0.6)	1 (2.4)		
Birth attended by the village health volunteer				
yes	32 (18.6)	3 (7.3)	2.88 (0.83-10.10)	0.08
no	137 (79.7)	37 (90.2)	Reference	
N/A	3 (1.7)	1 (2.4)		
Birth attended by the traditional birth attendant				
yes	33 (19.2)	9 (22.0)	0.81 (0.35-1.87)	0.62
no	136 (79.1)	30 (73.2)	Reference	
N/A	3 (1.7)	2 (4.9)		
Birth attended by the family member				
yes	75 (43.6)	19 (46.3)	0.79 (0.39-1.60)	0.51
no	95 (55.2)	19 (46.3)	Reference	
N/A	2 (1.2)	3 (7.3)		
Birth attended by nobody				
yes	4 (2.3)	2 (4.8)	0.46 (0.08-2.60)	0.36
no	167 (97.1)	38 (92.7)	Reference	
N/A	1 (0.6)	1 (2.4)		

N/A; Non applicable, CI; 95% confidence interval.

Table 4. Risk factors of childhood immunization (source of information of vaccination)

Items	Full vaccination $n = 172$, (%)	Partial vaccination $n = 41$, (%)	Crude OR (95%CI)	p-value
Source of information of vaccination (medical staff)				
yes	129 (75.0)	32 (78.1)	0.66 (0.27-1.60)	0.35
no	43 (25.0)	7 (17.1)	Reference	
N/A	0 (0)	2 (4.9)		
Source of information of vaccination (information on the vaccination cards)				
yes	51 (24.8)	9 (22.0)	1.45 (0.64-3.29)	0.37
no	117 (68.0)	30 (73.2)	Reference	
N/A	4 (1.3)	2 (4.9)		
Source of information of vaccination (family member or friends)				
yes	40 (23.3)	14 (34.2)	0.58 (0.27-1.21)	0.14
no	129 (75.0)	26 (63.4)	Reference	
N/A	3 (1.7)	1 (2.4)		
Source of information of vaccination (TV)				
yes	67 (39.0)	13 (31.7)	1.36 (0.66-2.84)	0.40
no	102 (59.3)	27 (65.9)	Reference	
N/A	3 (1.7)	1 (2.4)		
Source of information of vaccination (local authority)				
yes	97 (56.4)	28 (68.3)	0.58 (0.27-1.22)	0.14
no	72 (41.9)	12 (29.3)	Reference	
N/A	3 (1.7)	1 (2.4)		
Source of information of vaccination date (medical staff)				
1. yes	33 (19.2)	18 (43.9)	0.31 (0.14- 0.64)	0.00
2. no	138 (80.2)	23 (56.1)	Reference	
N/A	1 (0.6)	0		
Source of information of vaccination date (village health volunteer)				
yes	62 (36.1)	24 (58.5)	1.16 (0.57-2.36)	0.68
no	108 (62.8)	16 (39.0)	Reference	
N/A	2 (1.2)	1 (2.4)		
Source of information of vaccination date (local authority)				
yes	170 (98.8)	38 (92.7)	6.71 (1.05-42.7)	0.02
no	2 (1.2)	3 (7.3)	Reference	
Source of information of vaccination date (woman's union)				
yes	78 (45.4)	19 (46.3)	0.96 (0.48-1.90)	0.91
no	94 (54.7)	22 (53.7)	Reference	
Source of information of vaccination date (official letter from district governor)				
yes	27 (15.7)	6 (14.6)	1.09 (0.42-2.86)	0.85
no	144 (83.7)	35 (85.4)	Reference	
N/A	1 (0.6)	0		
Source of information of vaccination date (megaphone)				
yes	40 (23.3)	20 (48.8)	0.32 (0.15-0.66)	0.00
no	132 (76.7)	21 (51.2)	Reference	
Source of information of vaccination date (poster)				
yes	27 (15.7)	5 (12.2)	0.57 (0.48-3.74)	0.57
no	145 (84.3)	36 (87.8)	Reference	

N/A; Non applicable, CI; 95% confidence interval.

medical staff, village authority, or megaphone was also associated with full vaccination (Table 4).

Based on the bivariate analysis all variables significantly associated with full vaccination status and ethnicity were included in the multivariate logistic regression model. The results revealed that maternal age and obtaining information of the vaccination date by the village authority increased the odds of full vaccination. However, obtaining information on the vaccination date by megaphone had decreased the odds of full vaccination (Table 5).

4. Discussion

This study highlights the factors associated with the vaccination status of children aged five to nine in Lao PDR. Multivariate analysis of the risk factors for childhood immunization showed that maternal age and notification of the vaccination date by the village authority were positively associated with full

Table 5. Factors associated with full vaccination of children (logistic regression); occupation of household head-grouped

Factors	Adjusted odds ratio	95%CI	*p*-value
Sociodemographic factors			
Ethnicity	1.321	0.575-3.036	0.512
Household occupation	0.269	0.067-1.085	0.065
Maternal age	1.087	1.008-1.172	0.031
Transportation to the health facility	1.174	0.852-1.618	0.327
Time to the nearest health facility	0.989	0.977-1.001	0.076
Delivery history			
Birth attended by medical staff	2.617	0.936-7.317	0.067
Source of EPI information			
Source of information of vaccination date by medical staff	0.422	0.150-1.185	0.102
Source of information of vaccination date by local authority	17.430	1.827-166.280	0.013
Source of information of vaccination date by megaphone	0.204	0.065-0.637	0.006

immunization. Notification of the vaccination date by megaphone was negatively associated with full immunization.

4.1. *Sociodemographic factors*

According to the bivariate analysis, maternal age was associated with vaccination status. Furthermore, there was a linear trend that showed that full vaccination increases with maternal age. However, the relationship between childhood immunization and maternal age varies in the literature. There are some studies that show that maternal age was not associated with childhood immunization (*6*). One study from Africa showed the influence of younger maternal age on the utilisation of medical care (*8*).

This study did not show that maternal education was associated with vaccination status, which may be due to the contextual effects of maternal education on children's immunization in Lao PDR (*25*). Many studies have shown that maternal education or literacy is positively associated with the vaccination status of the children (*1,6,8,9,13,25*). In addition, some studies showed that the mother's knowledge of specific immunizations was associated with full vaccination (*3,5,17*). However, in Mali, Koumaré *et al*. reported no difference associated with parental knowledge about EPI diseases and full vaccination (*3*). In India, Parashar showed that literate women in a village may influence other women's capacity to seek and take advantage of state-provided healthcare, and even children of uneducated mothers may have better health knowledge due to residential or employment proximity to literate women through social influence (*25*). Further detailed research is needed to determine why maternal education was not associated with childhood immunization in this context.

Maekawa *et al*. showed that distance to health facilities in a rural region of Lao PDR was associated with vaccination status (*17*). This study focused on time rather than distance to health facilities since access depends on not only the distance but also the accessibility of the road and the availability of transportation. Therefore, mean time to the nearest health facilities was associated with full vaccination status in the bivariate analysis; however, it was not significant in the logistic regression model, which may be due to the established nationwide outreach service of EPI (*26*). Further research is needed to explore the association of vaccination status and the actual accessibility to the vaccination sites, combining precise information on distance, time to the vaccination sites, and availability of transportation.

4.2. *Delivery history*

Birth attended by medical staff was associated with full vaccination of the children; however, it was not significant in the multivariate analysis. According to Maekawa e*t al.*, bivariate analysis showed that immunization status was associated with whether mothers obtained information on immunisation before delivery; however, this factor was not significant in the multivariate analysis (*17*). Maekawa *et al.* also revealed that household visits and receipt of information before delivery influenced the number of fully immunized children . Further analysis revealed that household visits contributed to higher full-vaccination rates, especially among illiterate mothers. Therefore, it may not be sufficient to give information only once before or after the delivery; it might be better to provide information over several household visits.

4.3. *Source of EPI information*

The notification of the vaccination date by village authority had higher odds of full vaccination. On the other hand, notification of the vaccination date by megaphone was negatively associated with vaccination status. These results show that comprehensive and appropriate information dissemination may be key for vaccinating children against the EPI diseases (*3,6*).

There are several strengths in this study. This information has been collected through a nationwide survey using multistage cluster sampling. Geographically, the survey dissemination covered a wide portion of

Lao PDR and the ethnic minorities. A sampling frame allowed sampling bias to be a minimum. Only written records were used to avoid inaccuracies in vaccination history. Yellow cards and mother and child handbooks were the sole source of immunization information in this study. Parent's recall was not included in the interview. Rodewald *et al*. stated that the gold standard for measuring vaccination status, other than serological testing to detect immunity, is a parent-linked and provider-validated immunization status measure (*2*). Parent-linked means that parents name all immunization providers and provider-validated means obtaining the immunization records for each child and combining those data into a single record. Multiple studies have documented the inaccuracy of parents as the sole source of immunization status. The single-provider record check method, used in this study, lies between the parental recall and the parent-linked, provider-verified measure. The validity of this method depends on the frequency and reliability with which immunizations are included in the medical records (*2*). However, this study has several limitations. Firstly, this analysis has been done in the sub-population of the nation-wide survey for Hepatitis B sero-prevalence and only included the children and mothers who possessed any certifications of vaccination; therefore, the number of children in full and partial vaccination is skewed and no immunization or risk factor information was included from the children without the vaccination certifications. Second, the target of this study was children five to nine years old, which is older than the normal target population for a risk factor-based study. Thirdly, this study did not focus on health care provider factors, which may influence the vaccination status. Utilization of vaccination services is dependent on sociodemographic factors of the target population, as well as the number of health workers and availability of safe needles and syringes (*5*). Two studies show that children missed immunizations due to the provider's reluctance to vaccinate children while sick or when visiting health care centres for other purposes (*9,28*). This study did not focus on these provider risk factors. Lastly, this is a cross-sectional study; therefore, it is difficult to generate cause-and-effect relationships of childhood immunization and risk factors due to the study's design.

In conclusion, this study reinforces the importance of appropriate means of notification or provision of information on vaccination services in order to ensure full immunization (*13*). In the policy and programme level in Lao PDR, it may be necessary to implement appropriate information system on vaccination services at the community level such as mobilizing the village authorities. Older mothers showed higher odds of having fully immunized children, however, other sociodemographic factors such as maternal education was not associated with vaccination status. Further detailed qualitative research may be needed to discover how this factor influences vaccination services, along with other maternal sociodemographic factors such as education. Future research needs to target younger children to accurately collect information on vaccination records and other sociodemographic factors. Future studies also have to include health care provider factors that affect vaccination services.

Acknowledgements

This research was supported by Grants of the National Center for Global Health and Medicine from the Ministry of Health, Labour, and Welfare, Japan (25-8).

References

1. Muhsen K, Abed El-Hai R, Amit-Aharon A, Nehama H, Gondia M, Davidovitch N, Goren S, Cohen D. Risk factors of underutilization of childhood immunizations in ultraorthodox Jewish communities in Israel despite high access to health care services. Vaccine. 2012; 30:2109-2115.
2. Rodewald L, Maes E, Stevenson J, Lyons B, Stokley S, Szilagyi P. Immunization performance measurement in a changing immunization environment. Pediatrics. 1999; 103(4 Pt 2):889-897.
3. Koumaré AK, Traore D, Haidara F, Sissoko F, Traoré I, Dramé S, Sangaré K, Diakité K, Coulibaly B, Togola B, Maïga A. Evaluation of immunization coverage within the Expanded Program on Immunization in Kita Circle, Mali: A cross-sectional survey. BMC Int Health Hum Rights. 2009; 9(Suppl 1):S13.
4. Linkins RW, Salmon DA, Omer SB, Pan WK, Stokley S, Halsey NA. Support for immunization registries among parents of vaccinated and unvaccinated school-aged children: A case control study. BMC Public Health. 2006; 6:236.
5. Odusanya OO, Alufohai EF, Meurice FP, Ahonkhai VI. Determinants of vaccination coverage in rural Nigeria. BMC Public Health. 2008; 8:381.
6. Jamil K, Bhuiya A, Streatfield K, Chakrabarty N. The immunization programme in Bangladesh: Impressive gains in coverage, but gaps remain. Health Policy Plan. 1999; 14:49-58.
7. Xie J, Dow WH. Longitudinal study of child immunization determinants in China. Soc Sci Med. 2005; 61:601-611.
8. Fosu GB. Childhood morbidity and health services utilization: Cross-national comparisons of user-related factors from DHS data. Soc Sci Med. 1994; 38:1209-1220.
9. Torun SD, Bakirci N. Vaccination coverage and reasons for non-vaccination in a district of Istanbul. BMC Public Health. 2006; 6:125.
10. Salmon DA, Moulton LH, Omer SB, DeHart MP, Stokley S, Halsey NA. Factors associated with refusal of childhood vaccines among parents of school-aged children: A case-control study. Arch Pediatr Adolesc Med. 2005; 159:470-476.
11. Balraj V, Mukundan S, Samuel R, John TJ. Factors affecting immunization coverage levels in a district of India. Int J Epidemiol. 1993; 22:1146-1153.

12. Perks C, Toole MJ, Phouthonsy K. District health programmes and health-sector reform: Case study in the Lao People's Democratic Republic. Bull World Health Organ. 2006; 84:132-138.
13. Topuzoglu A, Ozaydin GA, Cali S, Cebeci D, Kalaca S, Harmanci H. Assessment of sociodemographic factors and socio-economic status affecting the coverage of compulsory and private immunization services in Istanbul, Turkey. Public Health. 2005; 119:862-869.
14. Bishai D, Suzuki E, McQuestion M, Chakraborty J, Koenig M. The role of public health programmes in reducing socioeconomic inequities in childhood immunization coverage. Health Policy Plan. 2002; 17:412-419.
15. Streatfield K, Singarimbun M, Diamond I. Maternal education and child immunization. Demography. 1990; 27:447-455.
16. World Health Organization. International Review of the Expanded Programme on Immunization in the Lao People's Democratic Republic, May 2012. *http://www.wpro.who.int/immunization/documents/Intl_Review_of_EPI_in_Lao.pdf* (accessed July 14, 2013).
17. Maekawa M, Douangmala S, Sakisaka K, Takahashi K, Phathammavong O, Xeuatvongsa A, Kuroiwa C. Factors affecting routine immunization coverage among children aged 12-59 months in Lao PDR after regional polio eradication in western Pacific region. Biosci Trends. 2007; 1:43-51.
18. World Health Organization. Global Health Observatory Data Repository 2011. *http://apps.who.int/gho/data/view.main* (accessed March 27, 2012).
19. United Nations Children's Fund. At a glance: Lao People's Democratic Republic. *http://www.unicef.org/infobycountry/laopdr_statistics.html* (accessed April 14, 2013).
20. Rainey JJ, Lacapère F, Danovaro-Holliday MC, Mung K, Magloire R, Kananda G, Cadet JR, Lee CE, Chamouillet H, Luman ET. Vaccination coverage in Haiti: Results from the 2009 national survey. Vaccine. 2012; 30:1746-1751.
21. World Health Organization. Vaccine schedule selection form 2011. *http://apps.who.int/immunization_monitoring/en/globalsummary/ScheduleResult.cfm.* (accessed August 20, 2012).
22. United Nations Children's Fund. Immunization Summary 2006. *http://www.unicef.org/lac/Immunization_Summary_2006(1).pdf* (accessed August 4, 2012).
23. World Health Organization. Immunization coverage cluster survey 2005. *http://www.who.int/vaccines-documents/DocsPDF05/www767.pdf* (accessed August 4, 2012).
24. Senessie C, Gage GN, von Elm E. Delays in childhood immunization in a conflict area: A study from Sierra Leone during civil war. Confl Health. 2007; 1:14.
25. Parashar S. Moving beyond the mother-child dyad: Women's education, child immunization, and the importance of context in rural India. Soc Sci Med. 2005; 61:989-1000.
26. Brugha RF, Kevany JP, Swan AV. An investigation of the role of fathers in immunization uptake. Int J Epidemiol. 1996; 25:840-845.
27. Brugha RF, Kevany JP. Maximizing immunization coverage through home visits: A controlled trial in an urban area of Ghana. Bull World Health Organ. 1996; 74:517-524.
28. Robison SG, Kurosky SK, Young CM, Gallia CA, Arbor SA. Immunization milestones: A more comprehensive picture of age-appropriate vaccination. J Biomed Biotechnol. 2010; 2010:916525.

(Received July 21, 2013; Revised August 6, 2013; Accepted August 7, 2013)

Original Article

DOI: 10.5582/bst.2013.v7.4.186

An iTRAQ approach to quantitative proteome analysis of cerebrospinal fluid from patients with tuberculous meningitis

Qiang Ou[1,2], Xiaohui Liu[3], Xunjia Cheng[1,3,*]

[1] *Department of Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai, China;*
[2] *Department of Infectious Diseases, Shanghai Tongji Hospital, Shanghai, China;*
[3] *Institute of Biomedical Science, Fudan University, Shanghai, China.*

Summary **To study the cerebrospinal fluid (CSF) protein profiles of tuberculous meningitis (TBM) and discover potential biomarkers for TBM, differential expression of proteins in the CSF of patients with TBM, patients with cryptococcal meningitis, and a control group were compared using isobaric tags for relative and absolute quantitation labelling (iTRAQ) coupled with 2-dimensional liquid chromatography-tandem mass spectrometry (LC-MS). As a result, a total of 208 unique proteins with a molecular weight ranging from 10 KD to 135 KD were identified and quantified in CSF samples from patients with TBM. Of the proteins, 9 were expressed at levels differing 2.0 fold, 6 were up-regulated, and 3 were down-regulated. These proteins appear to be involved in calcium ion binding, lipoprotein metabolism, immune response, and signal conduction. Two differentially expressed proteins were identified using ELISA. The present study represents the successful use of iTRAQ to examine CSF from patients with TBM. The differentially expressed proteins identified may be potential diagnostic biomarkers and provide valuable insight into the underlying mechanisms of TBM. This study also demonstrated that the differential protein profiles of diseases can be quickly determined using iTRAQ-LC-MS, a potential method for quantitative comparative proteomics.**

Keywords: Tuberculous meningitis (TBM), iTRAQ, LC-MS, biomarker

1. Introduction

Tuberculous meningitis (TBM) is an infection of the meninges caused by *Mycobacterium tuberculosis* (MTB) and is associated with high mortality rates and severe neurological sequelae. Its prognosis largely depends on early diagnosis as well as timely treatment (*1,2*). Currently, however, the diagnosis of TBM remains a major challenge due to inadequate diagnostic methods and poor sensitivity and/or specificity of existing markers. Thus, the search for more potential biomarkers to facilitate diagnosis and predict prognosis is of great importance.

Central nervous system (CNS) diseases can cause changes in protein expression in cerebrospinal fluid (CSF) (*3*). At present, differential proteomics provides a powerful approach to screen for variations in protein levels and posttranslational modifications associated with disease, culminating in the identification of many potential therapeutic targets and disease-related biomarkers. Numerous researchers have discovered potential biomarkers of disease using proteomics (*4-6*). Kumar *et al*. performed quantitative protein expression profiling of the brains of patients with TBM and normal individuals using isobaric tags for relative and absolute quantitation labelling (iTRAQ) labelling and LC-MS/MS, and they found several potential diagnostic biomarkers for tuberculous (*7*). However, the global proteomic profiling of CSF from individuals with TBM has proceeded very slowly, and changes in the CSF proteomes of patients with TBM are rarely reported.

Although 2-dimensional gel electrophoresis is commonly used in conventional proteomics, its drawbacks have been noted; these include low sensitivity,

**Address correspondence to:*
Dr. Xunjia Cheng, Department of Medical Microbiology and Parasitology, Shanghai Medical College of Fudan University, Shanghai 200032, China.
E-mail: xjcheng@shmu.edu.cn

taking an excessive amount of time, and failure to detect low-abundance proteins (*8*). A new labelling method that uses isobaric tags for relative and absolute quantitation labelling (iTRAQ) avoids these drawbacks and offers several advantages (*9*). iTRAQ reagents have been developed for the relative and/or absolute quantification of complex protein mixtures by labelling tryptic fragments of protein mixtures with reporter tags at the N-terminus and lysine residues (*10*). Quantification is achieved by comparing reporter ion abundance (*i.e.*, m/z 114, 115, 116, and 117) in the MS/MS spectra. Isotope-coded affinity tag (ICAT) reagents react with cysteine residues, but iTRAQ labels the unblocked N-termini of tryptic peptides and lysines, thus improving protein coverage.

In the present study, iTRAQ and liquid chromatography-tandem mass spectrometry (LC-MS) were used to identify differential proteins in patients with TBM. Nine proteins that were differentially expressed in CSF from patients with TBM were identified. These proteins may be potential diagnostic protein markers for tuberculous. Two of these potential biomarkers were identified using ELISA.

2. Material and Methods

2.1. *Subjects*

Subjects were patients with TBM and patients with cryptococcal meningitis seen by the Department of Infectious Diseases of Shanghai Public Health Clinical Center from January 2009 to December 2010 and healthy volunteers. *i*) Patients with TBM: twenty patients definitively diagnosed with TBM (having the disease no longer than 2 weeks) consisting of 13 males and 7 females. Their ages ranged from 18 to 52 years, with an average age of 32.6 years. All patients had typical clinical manifestations of TBM and CSF that tested positive for *M. tuberculosis* DNA according to acid-fast bacillus smears, cultures, or PCR results. The patients were yet to receive treatment. *ii*) Patients with cryptococcal meningitis: twenty patients consisting of 12 males and 8 females. Their ages ranged from 18 to 65 years, with an average age of 35.8 years. These patients had typical clinical manifestations, positive smear or culture results, and were yet to receive treatment. *iii*) Normal control group: twenty healthy volunteers consisting of 13 males and 7 females. Their ages ranged from 16 to 55 years, with an average age of 38.4 years. Individuals in this group were free of CNS infections and had normal CSF biochemical and routine test results.

There were no statistically significant differences between the patients and healthy individuals in terms of age and gender ($p > 0.05$). The present study was approved by the Ethics Committee of Shanghai Public Health Clinical Center. All subjects provided written informed consent for the collection of samples and subsequent analysis.

2.2. *Preparation of CSF samples*

CSF was extracted *via* a lumbar puncture. Pooled TBM, cryptococcal meningitis, and control samples were created by combining 800 μL of each individual CSF sample. Proteins were precipitated by mixing 10 volumes of cold acetone containing 10% trichloroacetic acid (TCA) with the CSF samples. The mixtures were then incubated overnight at –20°C, followed by centrifugation at 8,000 rpm/min for 15 min at 4°C. The concentrations of pooled samples were measured using the Bradford method (*11*).

2.3. *iTRAQ labelling and strong cation exchange (SCX) chromatography*

About 100 μg of each of three pooled samples was labelled. iTRAQ labelling was performed in accordance with a kit protocol (Applied Biosystem Inc, Foster City, CA, USA) and a previous report (*12*). After trypsin digestion, iTRAQ reagents (114, 115, and 117) in 70 μL ethanol were added separately to each tube and incubated at room temperature for 1 h (114 for patients with TBM, 115 for patients with cryptococcal meningitis, and 117 for the control group). All labelled peptide solutions were then pooled in a new vial and dried using a rotary vacuum concentrator (Christ RVC 2-25, Christ, Germany). Pooled iTRAQ-labelled peptide samples were desalted prior to SCX chromatographic fractionation and LC-MS/MS analysis.

iTRAQ-labelled mixed peptides were fractionated using SCX chromatography in a 20 AD HPLC system (Shimadzu, Kyoto, Japan) using a polysulfoethyl column (2.1 mm × 100 mm, 5 μm, 200 Å, The Nest Group, Southborough, MA, USA). The peptide mixture was reconstituted in Buffer A [10 mM KH2PO4 in 25% ACN (Fisher Scientific, Fair Lawn, New Jersey), pH 2.6] and loaded onto the column. The peptides were separated at a flow rate of 200 μL/min for 60 min with a gradient of 0% to 80% Buffer B (Buffer A containing 350 mM KCl) in Buffer A. Fractions were collected in 3-min increments and vacuum-dried.

2.4. *Protein identification by MS*

Fractions were vacuum-dried and resuspended in 50 μL HPLC Buffer A (5% ACN, 0.1% formic acid (TEDIA, Fairfield, USA)), loaded onto the RPLC column (ZORBAX 300 SB-C18 column, 5 μm, 300 Å, 0.1 mm × 15 mm, Microm, Auburn, CA, USA) and analyzed on a QSTAR XL System (Applied Biosystem, Inc.) and a 20AD HPLC system (Shimadzu). MS data were acquired automatically using Analyst QS 1.1 Service Pack 8 software (ABI/MDS SCIEX, Concord, Canada).

Survey scans were acquired from m/z 400 to 1,800, with up to 6 precursors selected for MS/MS from m/z 100 to m/z 2,000.

Ratios of 114.1, 115.1, and 117.1 amu signature mass tags were generated from MS/MS fragmentation of iTRAQ-labelled tryptic peptides and calculated using Protein Pilot (version 3.0, Applied Biosystem, Inc.). MS and MS/MS tolerances were set to 0.2 Da. The International Protein Index (IPI) (version 3.45, HUMAN) database was used to search for peptides. All proteins identified must have ≥ 95% confidence, and the protein confidence threshold cut-off was set at 1.3 (unused) with at least more than two peptides above the 95% confidence level. Fold-changes > 2.0 or < 0.5 were set as cut-off values to designate significant protein expression changes. The subcellular location and function of identified proteins were determined according to the UniProt knowledgebase (Swiss-Prot/UniProtKB) and Gene Ontology (GO) database.

2.5. *ELISA analysis of individual samples*

Differential proteins were measured in an independent sample set of subjects (25 patients with TBM, 25 patients with cryptococcal meningitis and 25 healthy volunteers) by commercially available ELISA in accordance with the manufacturer's instructions. Data were expressed as mean ± S.D. and statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, IL). $p < 0.05$ was considered statistically significant.

3. Results

3.1. *CSF proteomes in patients with TBM*

After LC-MS/MS analysis, 208 proteins of CSF in TBM were identified and quantified. According to the GO database, most of the proteins identified were located outside cells or in the membrane, cytoplasm, or cytoskeleton, and most function through binding, catalytic activity, enzyme regulator activity, and transporter activity. Most of the proteins identified may be involved in biological regulation, cellular processes, response to stimulus, metabolic process, or the like (Figure 1).

3.2. *Identification of differentially expressed proteins*

Nine differentially expressed proteins were identified: 6 proteins were up-regulated and 3 proteins were down-regulated. Representative MS/MS spectra for one peptide, identified from the APOB antigen, are shown in Figure 2.

Up-regulated and down-regulated proteins are showed in Tables 1 and 2. These proteins were related to cholesterol metabolism, response to stimulus, calcium ion binding, and prostaglandin-D synthase activity.

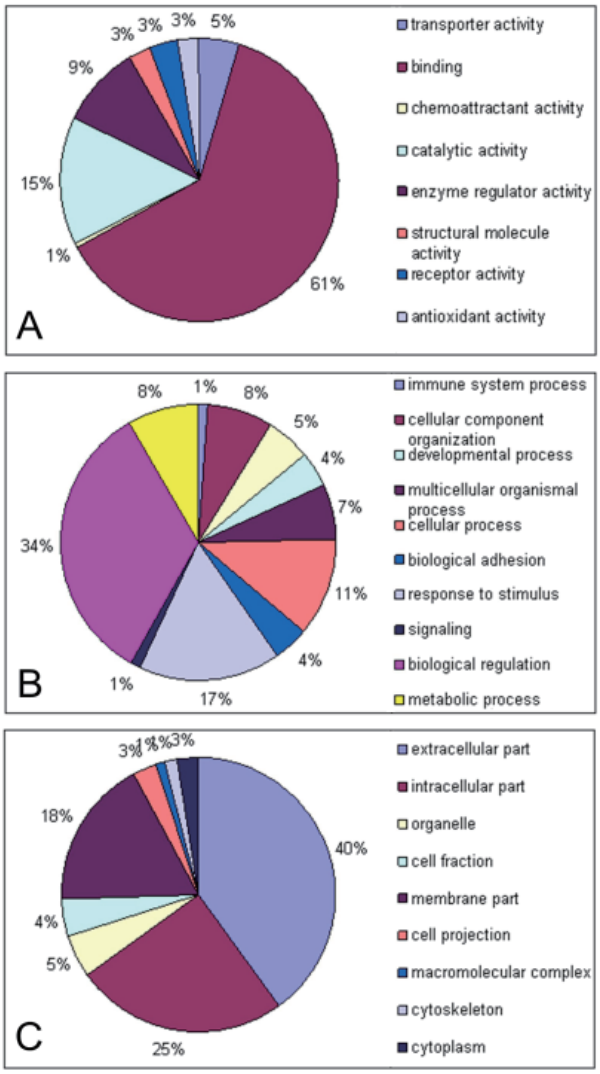

Figure 1. Gene ontology (GO) analysis of 208 proteins in CSF from patients with TBM. (A) molecular function; **(B)** biological process; **(C)** cellular component.

3.3. *Differential expression of S100A8 and APOB according to ELISA*

The levels of S100A8 and APOB expression in patients with TBM, patients with cryptococcal meningitis, and the control group were examined with ELISA to further ascertain differences in protein expression observed using iTRAQ. Differences between patients with TBM and patients with cryptococcal meningitis and the control group ($p < 0.05$) were noted. This result validates the current findings regarding the proteomic probing of differential CSF proteins (Table 3).

4. Discussion

The iTRAQ approach was first described in 2004. The method uses new isobaric reagents that consist of a charged reporter group, a peptide-reactive group, and a neutral balance portion, and the method can identify more proteins than other proteomic methods. It has

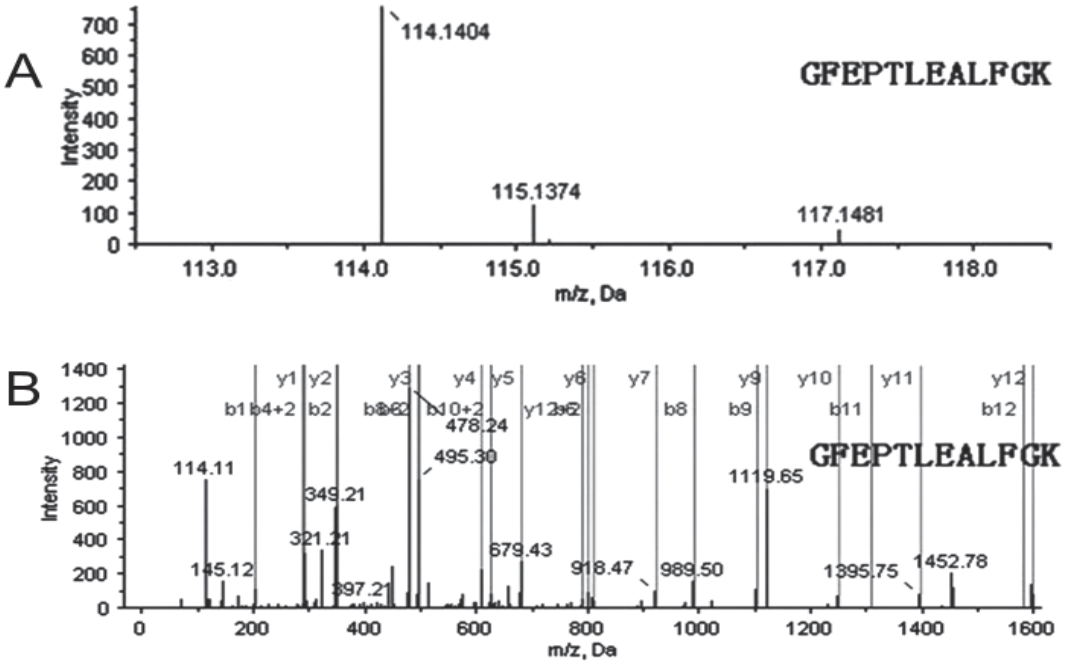

Figure 2. APOB Precursor MS region (GFEPTLEALFGK). The ion assignments were as follows: 114 for patients with TBM, 115 for patients with cryptococcal meningitis, and 117 for the control group. The intensity of reporter ions from precursor peptides indicates protein expression levels **(A)**. The MS/MS spectra revealed peptide sequences with GFEPTLEALFGK, leading to the identification of APOB **(B)**.

Table 1. Up-regulated proteins in CSF from individuals with TBM

IPI	Protein name	MW (D)	Peptide (95%)	Ratio 114:117	Ratio 114:115	Subcellular Location	Molecular function
IPI00847179.1	Apolipoprotein A-I (APOA4)	45372	8	4.4	2.2	secreted	cholesterol transporter activity, antioxidant activity, and copper ion binding
IPI00884926.1	Orosomucoid 1 (ORM1)	23540	20	9.8	2.1	secreted	regulation of immune system processes and protein binding
IPI00292532.6	Cathelicidin antimicrobial peptide (CAMP)	19591	2	2.9	7.4	secreted	antibacterial activity and antibiotic activity
IPI00007047.1	S100 Calcium-binding Protein A8 (S100A8)	10835	4	3.9	9.9	cytoplasm; cell membrane	calcium ion binding and protein binding
IPI00022229.1	Apolipoprotein B-100 (APOB)	515563	7	13.4	3.9	secreted	cholesterol transporter activity, enzyme binding, and low-density lipoprotein receptor binding
IPI00847635.1	a1-antichymotrypsin (SERPINA3)	47652	17	3.1	2.3	secreted	serine-type endopeptidase inhibitor activity and protein binding

CSF: cerebrospinal fluid; TBM: tuberculous meningitis; IPI: international protein index; MW: molecular weight; D: daltons.

Table 2. Down-regulated proteins in CSF from individuals with TBM

IPI	Protein name	MW (D)	Peptide (95%)	Ratio 114:117	Ratio 114:115	Subcellular location	Molecular function
IPI00514285.2	Prostaglandin D2 synthase (PTGDS)	21080	86	0.35	0.42	nucleus membrane	fatty acid binding, transporter activity, and prostaglandin-D synthase activity
IPI00021842.1	Apolipoprotein E (APOE)	36154	31	0.44	0.36	secreted	antioxidant activity, low-density lipoprotein particle receptor binding, and beta-amyloid binding
IPI00607600.1	Calsyntenin-1 (CLSTN1)	108643	6	0.11	0.48	postsynaptic cell membrane	calcium ion binding

CSF: cerebrospinal fluid; TBM: tuberculous meningitis; IPI: international protein index; MW: molecular weight; D: daltons.

Table 3. Differential expression of S100A8 and APOB in CSF

Protein	Patients with TBM (ng/mL)	Patients with cryptococcal meningitis (ng/mL)	Control group (ng/mL)
S100A8	$48.9 \pm 9.1^{*,\#}$	16.9 ± 5.2	13.9 ± 6.7
APOB	$480.1 \pm 215.2^{*,\#}$	166.5 ± 241.9	125.6 ± 131.2

Compared to patients with cryptococcal meningitis, $^{\#}p < 0.05$; Compared to the control group, $^{*}p < 0.05$.

been extensively used to identify biomarkers in various disease contexts (*13,14*).

The present study successfully used iTRAQ-LC-MS/MS to analyze the expression of proteins in CSF from individuals with TBM. A total of 208 proteins with a molecular weight ranging from 10 KD to 135 KD were identified through LC-MS/MS and a database search. GO analysis indicated that these proteins are involved in transport, biological regulation, cellular processes, immune response, metabolic process, and the like. Nine of the 208 proteins were found to be differentially expressed in patients with TBM compared to patients with cryptococcal meningitis and the control group. Six of the 9 proteins were up-regulated and three were down-regulated. Two differential proteins were identified using ELISA. The 9 differentially expressed proteins may be involved in the host response to TBM at the molecular level and may be potential diagnostic biomarkers for TBM.

S100A8 belongs to the S100 protein family and originates from neutrophil granulocytes and macrophages. This protein favors cell growth and restrains proliferation, has the ability to mediate apoptosis and enzymatic activity, promotes a dynamic cytoskeleton, maintains calcium ion stability, and stimulates the development of cancer (*15,16*). In recent years, S100A8 has been found to be able to regulate inflammatory activity and S100A8 is overexpressed in many infectious diseases (*17*). In the present study, patients with TBM had higher levels of S100A8 expression in CSF than did patients with cryptococcal meningitis and the control group, which implies that S100A8 may be related to the pathological and biological progression of TBM. CLSTN1 is allocated in the postsynaptic membrane of CNS synapses and is a proteolytically processed protein of the postsynaptic membrane with a calcium-binding cytoplasmic domain (*18*). It is a dynamic modulator of postsynaptic calcium *via* extracellular proteolysis and may modulate local Ca transport either beneath the postsynaptic membrane or around intracellular Ca stores. In the present study, CLSTN1 levels in the CSF of patients with TBM were lower than those in patients with cryptococcal meningitis and the control group. Symptoms such as loss of memory and impaired cognition are presumed to be related to the abnormal expression of CLSTN1. However, further research is needed to determine the exact mechanism of this process.

APOB, a component of low-density lipoprotein, can participate in endogenous cholesterol and triacylglycerol transport (*19*). Upregulation of APOB can cause arterial atherosclerosis, which is responsible for coronary disease and arteriosclerosis (*20*). APOA4 plays a key role in human cholesterol homeostasis, secretion of chylomicron particles, and antioxidant activity (*21*). The present study found that levels of APOB and APOA4 expression were higher in patients with TBM than in patients with cryptococcal meningitis and the control group, which suggests that the onset and development of TBM is related to abnormal cholesterol metabolism. Further studies are needed to prove this hypothesis. APOE in human brains is mainly synthesized and secreted by astrocytes, oligodendrocytes, neurons, and activated neuroglia cells (*22*). It modulates cholesterol and phospholipid homeostasis and adjusts the mobilization and redistribution of cholesterol and phospholipids during remolding of the neurilemma to mediate the maintenance of synapse flexibility and repair of impaired nerve cells (*23*). The *APOE* gene can affect memory and facilitate neuron maintenance and repair. Research has shown that APOE-knockout mice have learning and memory deficits compared to normal controls (*24*). In the present study, APOE levels in the CSF of patients with TBM were lower than those in patients with cryptococcal meningitis and the control group, presumably because astrocyte cells or neurons that have been attacked exhibit impeded composition and secretion of APOE. The lack of APOE degrades the repair function of nerve cells. An insufficient supply of acetylcholine leads to nervous system sequelae, such as memory loss and dementia. *APOE* gene polymorphism has been associated with Alzheimer's disease, ischemic stroke, hemorrhagic stroke, multiple sclerosis, Parkinson's disease, schizophrenia, and other diseases (*25-27*). Further studies are needed to determine the effects of *APOE* gene polymorphism on TBM.

ORM1, SERPINA3, and CAMP are proteins known to be related to acute inflammatory reactions. When MTB attacks meninges, it causes a severe inflammatory reaction. Large amounts of cell factors and mediators of inflammation are released and cause an acute reaction, resulting in an increase in ORM1 and SERPINA3 secretion. Several studies have found that SERPINA3 is overexpressed in CSF from patients with Alzheimer's disease and that it plays an important role in the pathology and pathogenesis of the disease (*28,29*). Presumably there are common factors involved in the pathogenesis of both TBM and Alzheimer's disease. CAMP plays an important part in natural immune systems. It functions as a antimicrobial, regulating the immune system and the release of inflammation mediators and accelerating revascularization (*30,31*). The present study found that MTB can cause high levels of CAMP expression, indicating that CAMP serves as an immediate and effective frontline defense.

Further studies are needed to clarify the effect of high levels of ORM1, SERPINA3, and CAMP expression on the onset of TBM and assessment of prognosis.

PTGDS is a member of the prostaglandin synthetases and a transporter of retinoids and thyroxine. PTGDS is expressed in tissues of the blood-brain barrier and secreted into the CSF. Changes in PTGDS expression may influence signal transmission and the activities of vitamin A and thyroxine (*32*). PTGDS may be involved in blood-brain development and maintenance. It may play an important role in the maturation and maintenance of both the CNS and the male reproductive system. A retrospective analysis of published data that quantified relative amounts of PTGDS in patients with multiple sclerosis, schizophrenia, and Parkinson's disease compared to controls revealed significant dysregulation (*33*). In the present study, levels of PTGDS in CSF from patients with TBM were lower than those in CSF from patients with cryptococcal meningitis and the control group. This implies that the pathogenesis of TBM may be related to the abnormal expression of PTGDS, although the exact mechanism of this process remains unclear.

In conclusion, 9 differentially expressed proteins associated with TBM were identified using an iTRAQ-based quantitative proteomic approach. Some proteins such as PTGDS, CLSTN1, and S100A8 were found to be expressed at high levels in TBM for the very first time. These promising potential markers warrant further study and evaluation in patients with TBM to determine their clinical utility. The present study also demonstrated that the differential protein profiles of diseases can be quickly determined by iTRAQ-LC-MS/MS, a commercially available method of quantitate comparative proteomics.

Acknowledgements

This work was supported by grants from the Shanghai Public Health Talent Cultivation Project, P.R. China, awarded to Qiang Ou (No. 08GWQ054) and the National Key Scientific Program, awarded to XJ Cheng (No. 2009ZX10004-104 & 2012ZX10004-220). The authors wish to thank the study participants and hospital staff for their contributions to this study.

References

1. Sheu JJ, Yuan RY, Yang CC. Predictors for outcome and treatment delay in patients with tuberculous meningitis. Am J Med Sci. 2009; 338:134-139.
2. Chugh A, Husain M, Gupta RK, Ojha BK, Chandra A, Rastogi M. Surgical outcome of tuberculous meningitis hydrocephalus treated by endoscopic third ventriculostomy: Prognostic factors and postoperative neuroimaging for functional assessment of ventriculostomy. J Neurosurg Pediatr. 2009; 3:371-377.
3. Pan S, Zhu D, Quinn JF, Peskind ER, Montine TJ, Lin B, Goodlett DR, Taylor G, Eng J, Zhang J. A combined dataset of human cerebrospinal fluid proteins identified by multi-dimensional chromatography and tandem mass spectrometry. Proteomics. 2007; 7:469-473.
4. Bai S, Liu S, Guo X, Qin Z, Wang B, Li X, Qin Y, Liu YH. Proteome analysis of biomarkers in the cerebrospinal fluid of neuromyelitis optica patients. Mol Vis. 2009; 15:1638-1648.
5. Blennow K. CSF biomarkers for Alzheimer's disease: Use in early diagnosis and evaluation of drug treatment. Expert Rev Mol Diagn. 2005; 5:661-672.
6. Pasinetti GM, Ungar LH, Lange DJ, Yemul S, Deng H, Yuan X, Brown RH, Cudkowicz ME, Newhall K, Peskind E, Marcus S, Ho L. Identification of potential CSF biomarkers in ALS. Neurology. 2006; 66:1218-1222.
7. Kumar GS, Venugopal AK, Mahadevan A, *et al*. Quantitative proteomics for identifying biomarkers for tuberculous meningitis. Clin Proteomics. 2012; 9:12.
8. Merchant M, Weinberger SR. Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis. 2000; 21:1164-1177.
9. Ross PL, Huang YN, Marchese JN, *et al*. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 2004; 3:1154-1169.
10. Ogata Y, Charlesworth MC, Higgins L, Keegan BM, Vernino S, Muddiman DC. Differential protein expression in male and female human lumbar cerebrospinal fluid using iTRAQ reagents after abundant protein depletion. Proteomics. 2007; 7:3726-3734.
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.
12. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, Hunter CL, Nuwaysir LM, Schaeffer DA. The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics. 2007; 6:1638-1655.
13. Latterich M, Abramovitz M, Leyland-Jones B. Proteomics: New technologies and clinical applications. Eur J Cancer.2008; 44:2737-2741.
14. Aggarwal K, Choe LH, Lee KH. Shotgun proteomics using the iTRAQ isobaric tags. Brief Funct Genomics. 2006; 5:112-120.
15. Seeliger S, Vogl T, Engels IH, Schröder JM, Sorg C, Sunderkötter C, Roth J. Expression of calcium-binding proteins MRP8 and MRP14 in inflammatory muscle diseases. Am J Pathol. 2003; 163:947-956.
16. Mueller A, O'Rourke J, Grimm J, Guillemin K, Dixon MF, Lee A, Falkow S. Distinct gene expression profiles characterize the histopathological stages of disease in Helicobacter-induced mucosa-associated lymphoid tissue lymphoma. Proc Natl Acad Sci U S A. 2003; 100:1292-1297.
17. Ehrchen JM, Sunderkötter C, Foell D, Vogl T, Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol. 2009; 86:557-566.
18. Vogt L, Schrimpf SP, Meskenaite V, Frischknecht R, Kinter J, Leone DP, Ziegler U, Sonderegger P. Calsyntenin-1, a proteolytically processed postsynaptic membrane protein with a cytoplasmic calcium-binding

domain. Mol Cell Neurosci. 2001; 17:151-166.
19. Rutledge AC, Su Q, Adeli K. Apolipoprotein B100 biogenesis: A complex array of intracellular mechanisms regulating folding, stability, and lipoprotein assembly. Biochem Cell Biol. 2010; 88:251-267.
20. Sniderman AD, Holme I, Aastveit A, Furberg C, Walldius G, Jungner I. Relation of age, the apolipoprotein B/apolipoprotein A-I ratio, and the risk of fatal myocardial infarction and implications for the primary prevention of cardiovascular disease. Am J Cardiol. 2007; 100:217-221.
21. Wong WM, Gerry AB, Putt W, Roberts JL, Weinberg RB, Humphries SE, Leake DS, Talmud PJ. Common variants of apolipoprotein A-IV differ in their ability to inhibit low density lipoprotein oxidation. Atherosclerosis. 2007; 192:266-274.
22. Gauthier S, Reisberg B, Zaudig M, *et al*. Mild cognitive impairment. Lancet. 2006; 367:1262-1270.
23. Masliah E, Samuel W, Veinbergs I, Mallory M, Mante M, Saitoh T. Neurodegeneration and cognitive impairment in apoE-deficient mice is ameliorated by infusion of recombinant apoE. Brain research. 1997; 751:307-314.
24. Hafezi-Moghadam A, Thomas KL, Wagner DD. ApoE deficiency leads to a progressive age-dependent blood-brain barrier leakage. Am J Physiol Cell Physiol. 2007; 292:C1256-1262.
25. Tzourio C, Arima H, Harrap S, Anderson C, Godin O, Woodward M, Neal B, Bousser MG, Chalmers J, Cambien F, MacMahon S. *APOE* genotype, ethnicity, and the risk of cerebral hemorrhage. Neurology. 2008; 70:1322-1328.
26. Huang X, Chen P, Kaufer DI, Tröster AI, Poole C. Apolipoprotein E and dementia in Parkinson disease: A meta-analysis. Arch Neurol. 2006; 63:189-193.
27. Kim J, Basak JM, Holtzman DM. The role of apolipoprotein E in Alzheimer's disease. Neuron. 2009; 63:287-303.
28. Padmanabhan J, Levy M, Dickson DW, Potter H. Alpha1-antichymotrypsin, an inflammatory protein overexpressed in Alzheimer's disease brain, induces tau phosphorylation in neurons. Brain. 2006; 129:3020-3034.
29. Baker C, Nielsen HM, Minthon L, Wright HT, Chappell S, Okyere J, May S, Morgan K, Kalsheker N, Janciauskiene SM. Effects of Alzheimer's peptide and alpha1-antichymotrypsin on astrocyte gene expression. Neurobiol Aging. 2007; 28:51-61.
30. Yamshchikov AV, Kurbatova EV, Kumari M, Blumberg HM, Ziegler TR, Ray SM, Tangpricha V. Vitamin D status and antimicrobial peptide cathelicidin (LL-37) concentrations in patients with active pulmonary tuberculosis. Am J Clin Nutr. 2010; 92:603-611.
31. Méndez-Samperio P. The human cathelicidin hCAP18/LL-37: A multifunctional peptide involved in mycobacterial infections. Peptides. 2010; 31:1791-1798.
32. Li W, Tao R, Zhang X, Ju G, Shi J, Liu S, Wang Z, Jin S, Guo Y, Wei J. A family-based study of genetic association of the *PTGDS* gene with schizophrenia in a Chinese population. Psychiatr Genet. 2008; 18:99.
33. arrington MG, Fonteh AN, Biringer RG, R Hühmer AF, Cowan RP. Prostaglandin D synthase isoforms from cerebrospinal fluid vary with brain pathology. Dis Markers. 2006; 22:73-81.

(Received June 21, 2013; Revised July 28, 2013; Accepted August 7, 2013)

Original Article

DOI: 10.5582/bst.2013.v7.4.193

Sub-acute exposure to the herbicide atrazine suppresses cell immune functions in adolescent mice

Shuhua Zhao[1], Jian Liu[1], Fan Zhao[3], Wei Liu[4], Na Li[5], Qi Suo[1], Jing Zhao[2], Lijing Zhao[2,*]

[1] *Department of Gynecology and Obstetrics, The Second Hospital Affiliated with Jilin University, Changchun, China;*
[2] *Department of Pathophysiology, School of Basic Medicine, Jilin University, Changchun, China;*
[3] *Department of Orthopedics, The Second Hospital Affiliated with Jilin University, Changchun, China;*
[4] *Jilin Province Academy of Environmental Science, Changchun, China;*
[5] *Changchun University of Chinese Medicine, Changchun, China.*

Summary **Atrazine (ATR), one of the most widely used herbicides worldwide, has caused a series of toxicological and environmental problems. This study sought to investigate the effects of ATR on the immune system of mice. Four-week-old female C57B l/6 mice were treated with 5, 25, and 125 mg/kg ATR for 28 days. On day 29, blood was collected and the spleen was harvested to detect lymphocyte transformation, natural killer (NK) cell activity, cellular phenotypes, and cytokines. Results indicated that the thymus and spleen weights decreased after ATR treatment, and the spleen was found to be more sensitive to ATR than the thymus. Decreases in lymphocyte transformation and NK cell activity were also observed in mice treated with 25 mg/kg ATR and 125 mg/kg ATR compared to the control group. In addition, there were also alterations of lymphocyte phenotypes in the spleen, and the percentages of CD3+ and CD4+ cells decreased in mice treated with 25 mg/kg ATR and 125 mg/kg ATR compared to the control group. Moreover, serum interleukin-4 level decreased significantly after treatment with 25 mg/kg and 125 mg/kg ATR, but ATR did not affect the expression of interleukin-2, interferon-γ, and tumor necrosis factor-α. These results suggest that ATR may have induced damage in spleen cells. As ATR is an environmental contaminant, its immunosuppressive action raises concerns that it may potentiate clinical conditions such as tumors, inflammation, and infections. Thus, it needs to be carefully monitored and studied.**

Keywords: Atrazine, sub-acute exposure, immunotoxic potential, adolescent mice

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, or ATR), is registered for the control of broadleaf weeds and certain grassy weeds. It has been used mostly to prevent weeds from growing in corn, sorghum, coffee, wheat, and sugarcane fields, conifer forests, Christmas tree farms, golf courses, and residential lawns (*1*). Since it first came on the market in 1958 in Switzerland, ATR has been one of the most commonly used herbicides worldwide (*2*). As a result, ATR has been found regularly in soil and surface moisture, where it tends to persist for months and migrate great distances from where it is used. ATR has even been found above the Arctic Circle, albeit at low concentrations (*3-5*). An estimated 2-3 million people who use groundwater as their primary drinking water are exposed to 0.2 ppb ATR.

The extensive use of ATR and its persistence underline the importance of understanding its general impacts on the environment. Many epidemiological studies have been performed, and some have indicated a possible correlation between atrazine exposure and an increased incidence of neoplastic diseases (*6*). Other studies have found that atrazine may act as an endocrine-disrupting compound (EDC) with effects on the central nervous system (*7*), endocrine system (*8*), and immune system (*9*).

Laboratory studies have revealed that exposure to environmental contaminants may suppress the amphibian immune system, rendering animals more susceptible to infection (*10-13*). Fatima found that herbicides at concentrations present in water in Europe caused

**Address correspondence to:*
Dr. Lijing Zhao, Department of Pathophysiology, School of Basic Medicine, Jilin University, Changchun, China.
E-mail: zhao_lj@jlu.edu.cn

immune suppression in goldfish (*11*). A meta-analysis reveals that ATR exposure consistently compromises the immune function of fish and amphibians (*5*). Brodkin *et al*. reported that in frogs ATR exposure suppressed the thioglycollate-stimulated recruitment of white blood cells to the peritoneal cavity compared to the background levels and also decreased the phagocytic activity of these cells (*10*). Langerveld *et al*. reported that ATR altered the expression of genes associated with growth and metabolism, proteolysis, fibrinogen complex formation, and immune regulation (*14*). A significant decrease in the number of intracelomatic cells and a significant decrease in the phagocytic index are observed after ATR exposure. In addition, silver catfish fingerlings exposed to ATR are more susceptible to intracelomatic challenge with the pathogen *Aeromonas hydrophila* (*15*).

Some studies have examined the immunotoxic potential of acute ATR exposure in adult animals. Exposure to ATR for 14 days can induce a significant decrease in the number of hematopoietic progenitors in murine bone marrow (*16*), cause a transient suppression of IgM production and T cell proliferation (*17*), decrease the CD4+ lymphocytes and MHC-II+ cells (*18*), and increase the mixed lymphocyte reaction (MLR) and cytotoxic T lymphocyte (CTL) response (*19*). Rajkovic *et al*. reported that ATR increased the number of degranulated mast cells of rats (*20*). Đikic *et al*. detected apoptotic cells in the thymus and lymph nodes of rats exposed to ATR (*21*).

Prenatal ATR exposure often causes substantially different effects in adult offspring from those noted in adult animals that are exposed (*22,23*). In one study, Rooney *et al*. orally administered ATR to Sprague-Dawley rats from day 10 of gestation to day 23 postnatally when pups were weaned. Two weeks after weaning, male pups that were inoculated with sheep red blood cells (SRBC) had a delayed hypersensitivity response that decreased in magnitude and a decreased IgM antibody response (*24*). Rowe *et al*. used time-release pellets to subcutaneously expose pregnant Balb/c dams to 700 μg/day of ATR for 21 days from day 10 and day 12 of pregnancy. At 3 months of age, male, but not female, mouse pups had increases in T cell proliferation, cytolytic activity, and the number of IgM-secreting B cells in the spleen, but there were no marked changes in the body weight, organ-to-body weight ratio of the spleen, and the numbers of CD8+ T cells, CD4+ T cells, and B220+ B cells in the spleen (*25*).

Previous studies described the toxic effects of atrazine on the adult and prenatal immune systems of animals. However, the immune system is still developing in the early postnatal period. The developing immune system is more sensitive to xenobiotic exposure than the adult immune system (*26-28*). A continuous remodeling of the primary and secondary immune organs occurs in mice throughout their lives, *i.e.*, a decline in the relative weight of the thymus, spleen, and lymph nodes occurs before puberty, while only the thymus continues to involute after puberty (*29*). One of the proposed windows of vulnerability for the developing rodent immune system occurs postnatally, which is from day 30 to sexual maturity (*30*). Combined with the limited immunotoxicity data in terms of age and with the possible impact and relevance of such data for immunotoxicological risk assessment, the current study sub-acutely exposed 4-week-old C57B l/6 mice to ATR for 28 days in order to assess the immunotoxic potential of ATR.

2. Materials and Methods

2.1. *Chemicals and reagents*

ATR (99% purity) and concanavalin A (ConA) were obtained from Sigma Chemical Company (St Louis, Missouri, USA). RPMI-1640 medium and fetal calf serum (FBS) were purchased from Gibco Laboratory (Gaithersburg, Maryland, USA). Interleukin-2 (IL-2), interleukin-4 (IL-4), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Minneapolis, Minnesota, USA). CD3, CD4, and CD8 fluorescence-conjugated antibody were from Ebioscience (San Diego, California, USA). ATR solutions (0.5 mg/mL, 2.5 mg/mL, and 12.5 mg/mL) were prepared by dissolving ATR in corn oil. These solutions were kept at 4ºC for a maximum of 1 week.

2.2. *Animals and treatment*

Male mice housed three or more per cage often fight, and this may cause unpredictable stress responses that affect the immunological parameters being studied. Thus, 4-week-old pathogen-free female C57B l/6 mice were used in this study. Animals were purchased from the Experimental Animal Center of Norman Bethune Medical College, Jilin University (Changchun, China). This study was conducted in accordance with internationally recognized guidelines and approved by the Animal Research Committee of Norman Bethune College of Medicine, Jilin University. Animals were housed in groups of five in a temperature- and humidity-controlled environment and given access to food and water *ad libitum*. These animals were randomly divided into 4 groups by body weight (ten/group) and were orally given 0, 5, 25, or 125 mg/kg ATR for 28 consecutive days. Blood and the spleen and thymus were collected 24 h after the final round of ATR treatment.

2.3. *Body weight and organ weight*

Mice were weighed once every 7 days starting on day 1 after ATR treatment. The organs were collected aseptically and weighed on day 29. The relative weights

of organs were calculated as organ weight (mg)/body weight (g).

2.4. *Pathological examination*

The spleen was collected, fixed in 10% buffered formaldehyde, embedded in paraffin, and then sectioned. The sections were stained with hematoxylin-eosin for histological assessment.

2.5. *Preparation of splenocytes*

Suspensions of single cells from the spleen were prepared by gently passing the spleen through a nylon mesh filter. Cellular debris was removed with a 400-μm stainless steel mesh. Red blood cells were lysed with a hypotonic buffered solution and lymphocytes were washed with PBS and re-suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Viable cells were counted using the trypan blue exclusion method with a hemocytometer.

2.6. *Lymphocyte transformation test (LTT)*

Freshly isolated splenocytes were suspended in complete RPMI-1640 medium supplemented with 10% FBS. The splenocytes were seeded into 6 wells in a 96-well plate (each well containing 2×10^4 cells). Cells in three wells were treated with PBS and served as controls, while cells in the remaining three wells were treated with 20 μg/mL ConA. After culturing in an incubator containing 5% CO_2 at 37ºC for 48 h, cell counting was done using a 3-(4,5-dimethylthiazol-2-yl) -2,5-diphe-nyltetrazolium bromide (MTT) assay. The optical density (OD) was quantified using a microplate reader. The following formula was used to calculate the stimulation index (SI): SI = OD of ConA-treated cells/OD of control cells.

2.7. *Natural killer (NK) cell cytotoxicity assay*

NK cells from mouse spleens were prepared as described above and used as effector cells. Mouse lymphoma YAC-1 cells, which are sensitive to NK cells, were used as target cells. Briefly, 1×10^4 YAC-1 cells and effector cells were separately added to a 96-well plate. After incubation for 48 h, NK cytotoxicity was assessed using the MTT assay described above. Cytotoxic activity was calculated using the following formula: NK cytotoxicity (%) = $[1 - (OD_{E+T} - OD_E)/OD_T \times 100\%]$. (E + T = a mixture of effector cells and target cells, E = effector cells, T = target cells).

2.8. *Flow cytometry*

For cell phenotype analysis, fluorescence-conjugated antibody was used in accordance with the manufacturer's instructions. Briefly, splenocytes were re-suspended in 100 μL of binding buffer followed by addition of 10 μL of anti-CD3-PE-Cy5, anti-CD4-FITC, or anti-CD8-PE. After incubation for 15 min at 37ºC, cells were washed thrice and then subjected to flow cytometry on the FAC Scan (Becton Dickinson Immuno-cytometry, San Jose, CA), in which 1×10^5 cells were counted. Analysis was performed using the CELL Quest software package (BDIS) in list mode and the lymphocyte gate as defined by forward/side scatter characteristics.

2.9. *Enzyme-linked immunosorbent assay (ELISA) of IL-2, IL-4, IFN-γ, and TNF-α*

After treatment with ATR for 28 days, animals were sacrificed and blood was collected. The serum was obtained by centrifugation at 3,000 g × for 10 min. The serum levels of murine IL-2, IL-4, IFN-γ, and TNF-α were detected with corresponding ELISA kits in accordance with the manufacturer's instructions.

2.10. *Statistical analysis*

Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 10. Data were expressed as means ± standard deviations (S.D.). Comparisons among multiple groups were done using two-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *General state and body weight*

All animals survived to the end of study. There were no overt changes in appearance and/or behaviors and no significant differences in body weight (Figure 1A) after ATR exposure.

3.2. *Toxic effects of ATR on the spleen*

The mean weights of the spleen and thymus decreased in mice treated with 25 mg/kg ATR and 125 mg/kg ATR ($p < 0.05$) compared to the control group (Figure 1B). Histopathological examination of the spleen revealed degenerative changes. The spleens appeared atrophic in mice treated with 25 mg/kg ATR and 125 mg/kg ATR and were characterized by effacement of germinal centers, diminution of white pulp, and congestion of red pulp (Figure 1C). There were no significant changes in mice treated with 5 mg/kg ATR in comparison to the control group.

3.3. *Lymphocyte transformation*

An MTT assay was performed to evaluate splenic lymphocyte proliferation in mice treated with different

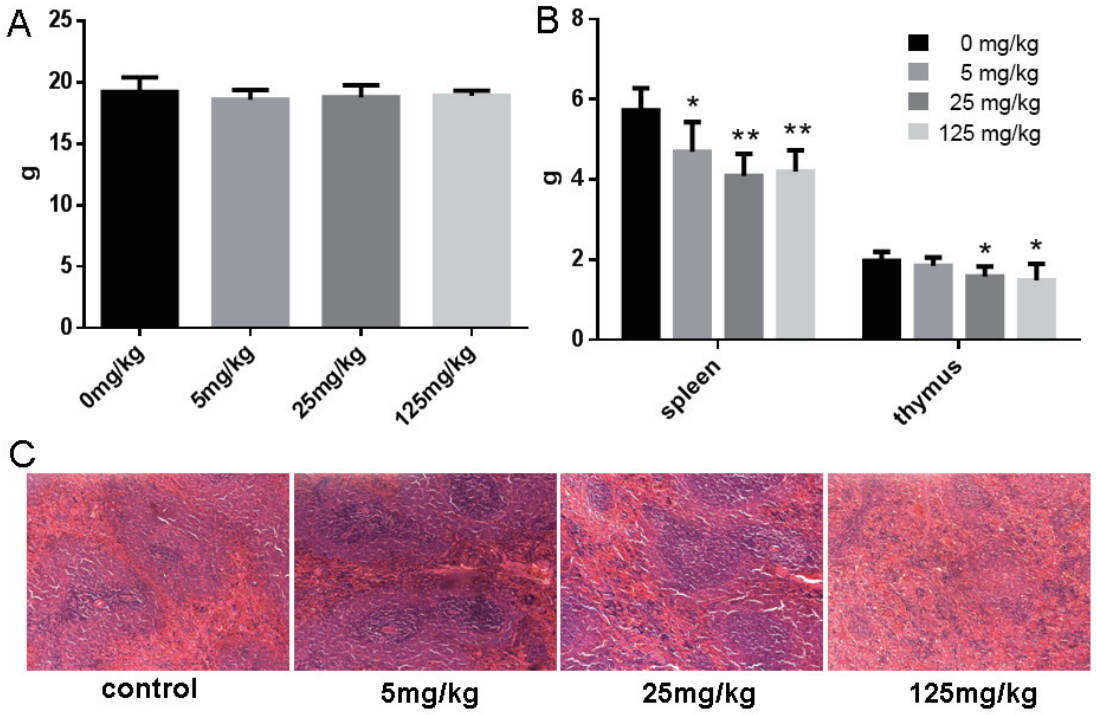

Figure 1. Toxic effects of ATR on the spleen. (A) Body weight was comparable among the 4 groups treated with ATR. **(B)** The mean weight of the spleen and thymus decreased in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR (** $p < 0.01$, * $p < 0.05$). **(C)** Histopathological examinations of the spleen revealed degenerative changes in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR.

concentrations of ATR. The SI was 2.69 ± 0.21 in mice treated with 25 mg/kg ATR and 1.64 ± 0.67 in mice treated with 125 mg/kg ATR, reflecting a significant decrease compared to the SI in the control group (3.19 ± 0.10) ($p < 0.05$, Figures 2A). The SI did not differ significantly in mice treated with 5 mg/kg ATR (2.66 ± 0.72) and the control group. These results suggest that ATR exposure reduced lymphocyte proliferation in mice.

3.4. *NK cell cytotoxicity assay*

To determine the impact of ATR on NK cell function, the activity of NK cells was measured *via* a cytotoxicity assay. Results indicated that NK cell cytotoxic activity was 71.16 ± 2.29% in mice treated with 125 mg/kg ATR, reflecting a significant decrease compared to the control group (85.08 ± 6.43%) ($p < 0.05$) (Figures 2B). Compared to the control group, there were no significant differences in the NK cell cytotoxic activity in mice treated with 25 mg/kg ATR (90.54 ± 3.23%) and 5 mg/kg ATR (84.04 ± 12.53).

3.5. *Lymphocyte phenotypes in the spleen*

Flow cytometry was used to detect the surface markers of lymphocytes. Results revealed that the percentage of CD3+ lymphocytes in the spleen decreased in mice treated with 25 mg/kg ATR (36.22 ± 2.62%) and 125 mg/kg ATR (34.80 ± 4.22%) compared to the control group (45.22 ± 0.55%) ($p < 0.05$) (Figure 3A). The percentage of CD4+ lymphocytes in the spleen decreased

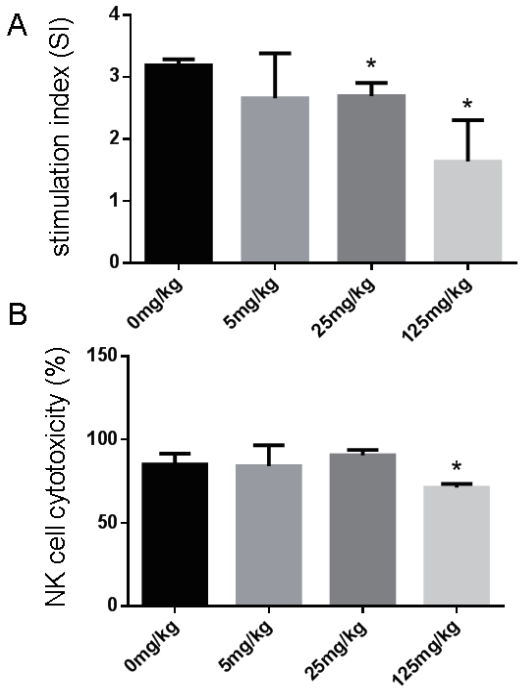

Figure 2. Toxic effects of ATR on splenocyte function. (A) Splenic lymphocyte proliferation decreased significantly ($p < 0.05$) in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR compared to the control group. **(B)** NK cell cytotoxic activity decreased significantly ($p < 0.05$) in mice treated with 125 mg/kg ATR compared to the control group ($p < 0.05$).

significantly in mice treated with 25 mg/kg ATR (8.39 ± 0.56%) and 125 mg/kg ATR (7.14 ± 0.88%) compared to the control group (11.08 ± 0.49%) ($p < 0.05$) (Figure 3B).

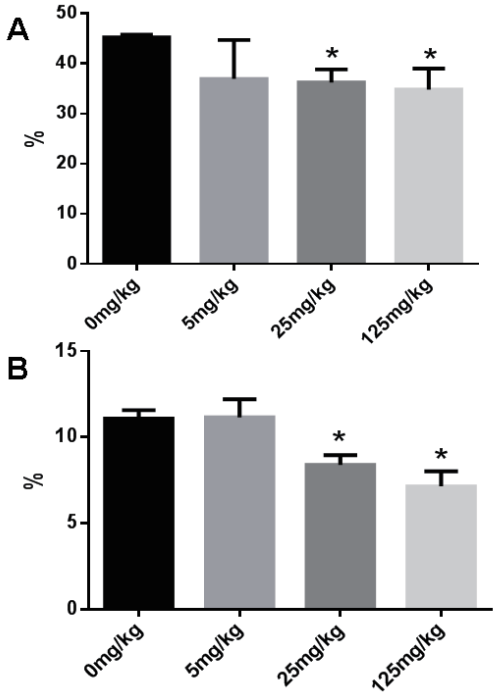

Figure 3. Effect of ATR on percentage of splenic lymphocyte phenotypes. (A) The percentage of CD3+ splenic lymphocytes decreased in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR compared to the control group ($p < 0.05$). **(B)** The percentage of CD4+ splenic lymphocytes decreased significantly in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR compared to the control group ($p < 0.05$). There were no significant changes in the percentage of CD8+ splenic lymphocytes in the four groups treated with ATR ($p > 0.05$).

Neither CD3+ (36.94 ± 7.79%) nor CD4+ (11.14 ± 1.16%) lymphocytes changed significantly in mice treated with 5 mg/kg ATR. There were no significant changes in the percentage of CD8+ lymphocytes in the groups treated with ATR and the control group. In fact, ATR treatment reduced the splenic CD4+/CD8+ ratio.

3.6. *IL-2, IL-4, IFN-γ, and TNF-α*

To further investigate the mechanisms by which ATR inhibits NK activation and decreases T lymphocytes, the serum levels of pro-inflammatory and immunosuppressive cytokines such as IL-2, IL-4, IFN-γ, and TNF-α were detected. As shown in Figure 4A, IL-2 levels in mice treated with ATR were comparable to those in the control group ($p > 0.05$), and the serum level of IL-2 was 330.88 ± 50.40 in mice treated with 0 mg/kg ATR, 307.24 ± 58.35 in mice treated with 5 mg/kg ATR, 322.70 ± 53.38 in mice treated with 25 mg/kg ATR, and 330.42 ± 53.05 in mice treated with 125 mg/kg ATR. As shown in Figure 4B, the IL-4 levels in mice treated with 25 mg/kg ATR (16.93 ± 1.14 ng/mL) and 125 mg/kg ATR (17.25 ± 1.98 ng/mL) had decreased about 15% from levels in the control group (20.45 ± 2.56 ng/mL) ($p < 0.05$), while there were no significant differences in those levels in mice treated with 5 mg/kg ATR (18.03 ± 2.50 ng/mL) and the control group. Figure 4C shows that IFN-γ levels in mice treated with ATR were similar to those in the control group ($p > 0.05$). The serum level of IFN-γ was 131.58 ± 40.95

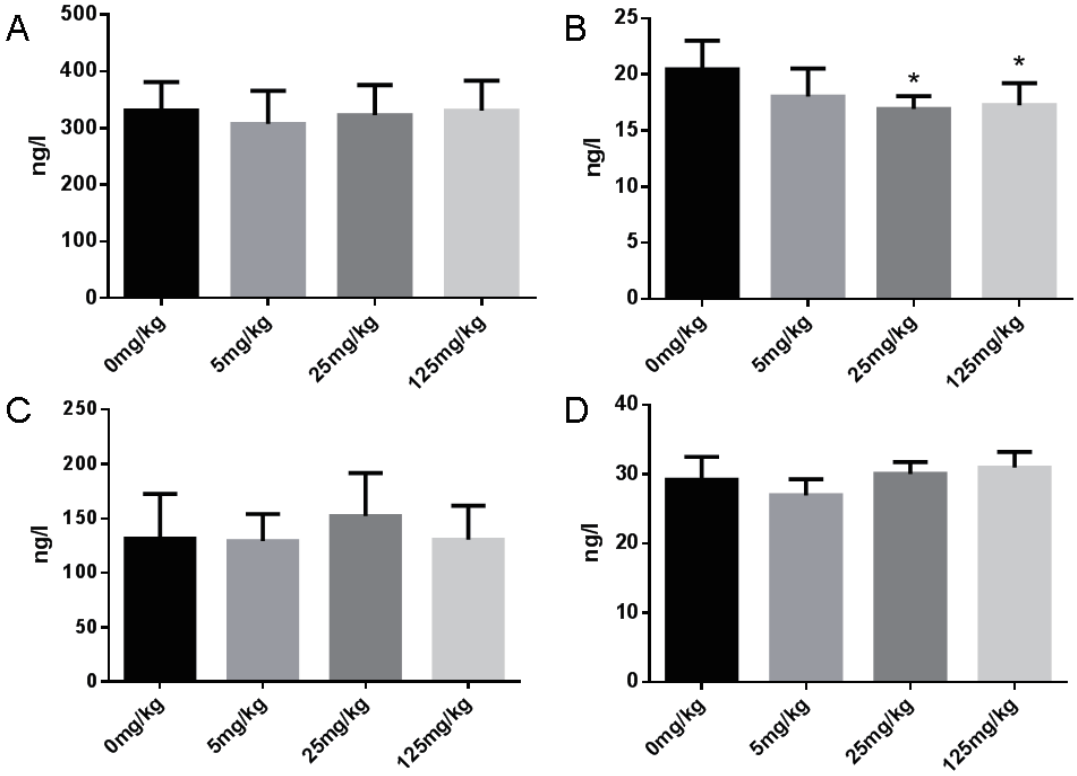

Figure 4. Effects of ATR on serum cytokine levels. (A) Serum IL-2 levels in groups of mice treated with ATR were comparable to those in the control group ($p > 0.05$). **(B)** Serum IL-4 levels decreased significantly ($p < 0.05$) in mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR. **(C)** The serum IFN-γ level in groups treated with ATR was similar to that in the control group ($p > 0.05$). **(D)** There were no significant changes in TNF-α in the four groups treated with ATR ($p > 0.05$).

in mice treated with 0 mg/kg ATR, 129.29 ± 24.77 in mice treated with 5 mg/kg ATR, 152.21 ± 39.59, in mice treated with 25 mg/kg ATR, and 130.54 ± 31.26 in mice treated with 125 mg/kg ATR. As shown in Figure 4D, there were no significant differences in TNF-α among the four groups treated with ATR ($p > 0.05$). The serum level of TNF-α was 29.45 ± 3.30 in mice treated with 0 mg/kg ATR, 26.99 ± 2.33 in mice treated with 5 mg/kg ATR, 30.10 ± 1.70 in mice treated with 25 mg/kg ATR, and 30.97 ± 2.28 in mice treated with 125 mg/kg ATR.

4. Discussion

The immune system is a complex network of interacting regulatory genes, hormones, and cells that has evolved in multicellular organisms for the purpose of maintaining homeostasis against a dynamic battery of foreign environmental agents and/or pathogens. Environmental pollutants can interfere with the normal operation of the immune system, leading to a broad range of disorders, including endocrine dysfunction, tumorigenesis, increased rates of inflammatory infections, and autoimmune diseases. Widespread use of the herbicide ATR increases the chance for and rate of exposure to other foreign agents that the immune system must contend with, and yet the immunotoxicological potential of ATR has not been studied extensively. The present study sought to examine the effects of ATR on selected immune parameters in juvenile mice. ATR (up to 125 mg/kg per day) was orally administered to 4-week-old female C57BL/6 mice for 28 days. One day after the final round of ATR treatment, mice were sacrificed and their serum and spleens were collected.

During the experiment, overt changes in the appearance and behaviors were not observed in mice treated with ATR, and no significant difference in body weight was noted after ATR exposure. However, relative spleen and thymus weights decreased significantly in mice treated with 25 mg/kg ATR and 125 mg/kg ATR compared to the control group, but there were no significant changes in those weights in mice treated with 5 mg/kg ATR. Since abnormal weights of the spleen and thymus are important indicators used to investigate immunotoxic potential (*9*), the current results indicate that ATR was likely to be immunotoxic to juvenile mice. The decrease in immune organ weight may be associated with the inhibition of lymphocyte proliferation and/or the increase in lymphocyte death in the spleen and thymus (*9*). In the current study, the histopathological features of the spleen were detected by light microscopy, and results revealed degenerative changes, compared to the control group, in the spleens of mice treated with 25 mg/kg ATR and 125 mg/kg ATR but not in mice treated with 5 mg/kg ATR. These results suggested that ATR exposure had an adverse impact on the immunity of the spleen in mice. This conclusion is supported by other studies reporting the adverse effects of ATR on the immune system (*18*).

T cells play a central role in organizing the immune defense and are practically involved in all types of immune reactions either by orchestrating the type of immune response or as effectors themselves (*31*). ConA is one of the biologically relevant stimuli applied to activate lymphocytes (*32*). In order to understand the effects of ATR induced T-cell response, the ability of ConA to stimulate the proliferation of T lymphocytes was investigated 1 day after the final round of ATR treatment. Results indicated that 5 mg/kg ATR induced similar T-cell proliferation in the control group while the SI of lymphocytes decreased significantly in mice treated with 25 mg/kg ATR and 125 mg/kg ATR in comparison to the control group. These findings suggest that ATR exposure for 28 days could lead to the inhibition of T-cell proliferation. NK cells play a central role in the immune defense against tumor development and viral infections. Thus, any agent that interferes with the ability of NK cells to lyse their targets may increase the risk for tumorigenesis and/or viral infections (*1*). To determine the impact of ATR on NK cell function, the antitumor activity of NK cells was detected *via* a cytotoxicity assay. Results showed the NK cell cytotoxic activity in mice treated with 125 mg/kg ATR was significantly lower than that in the control group, but 5 mg/kg ATR and 25 mg/kg ATR did not significantly affect NK cell cytotoxicity. These results suggest that treatment with 125 mg/kg ATR for 28 days may induce NK cell dysfunction.

A lymphoproliferative assay provides information only about overall proliferative responses without detailing the specific cell subset involved in these responses. Expression of activation molecules prior to proliferation, on the other hand, offers a useful method to predict lymphocyte proliferative activity (*33*). Exposure to foreign substances induces or stimulates a specific immune system. Some immune responses are directed to specific cells or cytokines that contribute to the immune response (*34*). To better understand the immune environment after ATR treatment, whether the cell phenotypes obtained in flow cytometry are modulated or whether they provide a true reflection of these cells must be determined.

Lymphocyte subsets are major cellular components of the adaptive immune response (*35*). CD3 is a signaling component of the TCR (T-cell receptor) complex, almost all T-lymphocytes express CD3, and therefore the CD3+ antibody is used as a genetic marker of T-lymphocytes. In addition, cytotoxic T lymphocytes express CD8 and recognize endogenous antigens by binding to MHC class I molecules; helper T-lymphocytes express CD4 and require the processing and presentation of antigens in association with MHC class II molecules by antigen-presenting cells and helper T-lymphocytes coordinate and assist other immune cells (*36*). In order to determine if ATR decreased the

yields of CD3+, CD4+, and CD8+ T lymphocytes, flow cytometry was used to detect surface markers of T lymphocyte subsets in this study. Interestingly, results indicated a significant decrease in the number of CD3+ T lymphocytes and a reduction in the ratio of CD4+/CD8+ T lymphocytes in the spleens of both mice treated with 25 mg/kg ATR and mice treated with 125 mg/kg ATR. A possible explanation for this is that ATR may affect T lymphocytes by decreasing CD4+ T lymphocytes mainly by affecting macrophage activation and production of cytokines (*37*).

CD4+ T lymphocytes can be further subdivided by the patterns of cytokine expression. Th1 CD4+ T lymphocytes, involved in cellular immunity, are known to produce IL-2, IFN-γ, and TNF-α and enhance macrophage function, cellular immunity, and synthesis of opsonizing and complement-fixing antibodies. Th2 CD4+ T lymphocytes involved in humoral responses are known to enhance the immunoglobulin G-mediated response and activation of eosinophils through the actions of IL-4, IL-5, and IL-13 (*36*). Numerous studies have found that the immune responses in mice and humans are critically influenced by the balance between Th1 and Th2 cytokines (*38*). Any absence in the cytokine network would cause an imbalance between Th1 and Th2 cytokines and potentially cause inflammation-related diseases. Inflammation-related cytokines have been regarded as either pro-inflammatory or anti-inflammatory according to their contribution to the inflammatory response. Th1 cells express mainly pro-inflammatory cytokines, and Th2 cells primarily secrete anti-inflammatory cytokines. The current results indicated that 25 mg/kg ATR and 125 mg/kg ATR reduced serum IL-4 levels while the serum levels of IFN-γ, IL-2, and TNF-α remained unchanged. IL-4, produced particularly during allergic, cellular, and humoral responses to selected pathogen infections, may modulate other lymphoid cell activities such as regulation of the differentiation of antigen-stimulated T lymphocytes and control of immunoglobulin class switching in B lymphocytes (*39*). Turnbull *et al*. found that IL-4 could inhibit macrophage cytokine production and restrain macrophage activation (40). The current results demonstrated that ATR at a concentration higher than 25 mg/kg could alter the production of inflammation-related cytokines in serum. As a result, the balance between Th1 and Th2 immune responses might be disrupted.

The immunotoxicity of ATR manifests as a decrease in immune response capacity, including the suppression of immune cell function and atrophy of immune organs (*10,18*), which were also observed in the present study. The ATR concentrations in this study ranged from 5 mg/kg to 125 mg/kg, which were determined based on findings in previous studies (*21,41*) and higher than what is expected in nature. However, these amounts ensured that the effects of ATR were noticeable in this study. In California, potential occupational exposure to ATR was assessed during mixing, loading, and application of ATR on field corn, and the absorbed daily dosage (ADD) of ATR for a mixer/loader/applicator was estimated to be 1.8-6.1 μg/kg/day in the event of short-term exposure (15-21 days) (*9, 21*). However, the ADD of ATR is expected to be higher for commercial applicators and farmers in developing countries due to inappropriate personal protective equipment and unintentional overspraying. In the surface water in the East Liaohe River Basin of Jinlin Province, the average ATR content was 9.7 μg/L in regions with agricultural plots and 8.854 μg/L in regions without agricultural plots; ATR content was as high as 18.93 μg/L in July (*42*). These data indicate that the daily ATR exposure for ordinary residents in the watershed is about 0.37-0.79 μg/kg/day, and this value is higher for farmers. ATR is not removed from the body within 24 h and its metabolites can still be detected in the urine 48 h after exposure (*9*). Thus, some effects may occur after repeated exposures and result in accumulation of substances above a critical threshold.

5. Conclusions

This study found that ATR interferes with immune function by inducing degenerative changes in the spleen, inhibiting the proliferation of T lymphocytes and NK cell cytotoxic activity, decreasing the percentage of CD3+ and CD4+ T lymphocytes, and reducing the serum levels of IL-4. Results indicated that ATR may be associated with inflammation, infections, and other diseases. However, the many molecular mechanisms involved in this process are still unclear. Therefore, further studies are needed to elucidate the exact mechanisms underlying the deleterious effects of ATR on the immune system and to investigate the effects of prolonged exposure to ATR under natural conditions.

Acknowledgements

The authors wish to thank Prof. Xuejian Zhao and Dr. Hejun Ren for their helpful suggestions. The authors also wish to thank Kun Li for his help critically reading and revising this manuscript. This work was supported by grants from the Jilin Provincial Science & Technology Department (grant No. 201215081) and the Jilin Provincial Department of Finance Projects (grant no. 2012845).

References

1. Whalen MM, Loganathan BG, Yamashita N, Saito T. Immunomodulation of human natural killer cell cytotoxic function by triazine and carbamate pesticides. Chem Biol Interact. 2003; 145:311-319.
2. Hayes TB, Khoury V, Narayan A, Nazir M, Park A,

Brown T, Adame L, Chan E, Buchholz D, Stueve T, Gallipeau S. Atrazine induces complete feminization and chemical castration in male African clawed frogs (Xenopus laevis). Proc Natl Acad Sci U S A. 2010; 107:4612-4617.
3. Dorfler U, Feicht EA, Scheunert I. S-triazine residues in groundwater. Chemosphere. 1997; 35:99-106.
4. Koskinen WC, Clay SA. Factors affecting atrazine fate in north central U.S. soils. Rev Environ Contam Toxicol. 1997; 151:117-165.
5. Rohr JR, McCoy KA. A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ Health Perspect. 2010; 118:20-32.
6. Simpkins JW, Swenberg JA, Weiss N, Brusick D, Eldridge JC, Stevens JT, Handa RJ, Hovey RC, Plant TM, Pastoor TP, Breckenridge CB. Atrazine and breast cancer: A framework assessment of the toxicological and epidemiological evidence. Toxicol Sci. 2011; 123:441-459.
7. Weselak M, Arbuckle TE, Wigle DT, Krewski D. In utero pesticide exposure and childhood morbidity. Environ Res. 2007; 103:79-86.
8. Lasserre JP, Fack F, Revets D, Planchon S, Renaut J, Hoffmann L, Gutleb AC, Muller CP, Bohn T. Effects of the endocrine disruptors atrazine and PCB 153 on the protein expression of MCF-7 human cells. J Proteome Res. 2009; 8:5485-5496.
9. Zhang X, Wang M, Gao S, Ren R, Zheng J, Zhang Y. Atrazine-induced apoptosis of splenocytes in BALB/C mice. BMC Med. 2011; 9:117.
10. Brodkin MA, Madhoun H, Rameswaran M, Vatnick I. Atrazine is an immune disruptor in adult northern leopard frogs (Rana pipiens). Environ Toxicol Chem. 2007; 26:80-84.
11. Fatima M, Mandiki SN, Douxfils J, Silvestre F, Coppe P, Kestemont P. Combined effects of herbicides on biomarkers reflecting immune-endocrine interactions in goldfish. Immune and antioxidant effects. Aquat Toxicol. 2007; 81:159-167.
12. Kiesecker JM. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? Proc Natl Acad Sci U S A. 2002; 99:9900-9904.
13. Koprivnikar J, Forbes MR, Baker RL. Contaminant effects on host-parasite interactions: Atrazine, frogs, and trematodes. Environ Toxicol Chem. 2007; 26:2166-2170.
14. Langerveld AJ, Celestine R, Zaya R, Mihalko D, Ide CF. Chronic exposure to high levels of atrazine alters expression of genes that regulate immune and growth-related functions in developing Xenopus laevis tadpoles. Environ Res. 2009; 109:379-389.
15. Kreutz LC, Barcellos LJ, Marteninghe A, Dos Santos ED, Zanatta R. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (Rhamdia quelen) to Aeromonas hydrophila challenge. Fish Shellfish Immunol. 2010; 29:694-697.
16. Mencoboni M, Lerza R, Bogliolo G, Flego G, Pannacciulli I. Effect of atrazine on hemopoietic system. *In Vivo*. 1992; 6:41-44.
17. Fournier M, Friborg J, Girard D, Mansour S, Krzystyniak K. Limited immunotoxic potential of technical formulation of the herbicide atrazine (AAtrex) in mice. Toxicol Lett. 1992; 60:263-274.
18. Filipov NM, Pinchuk LM, Boyd BL, Crittenden PL. Immunotoxic effects of short-term atrazine exposure in young male C57BL/6 mice. Toxicol Sci. 2005; 86:324-332.
19. Karrow NA, McCay JA, Brown RD, Musgrove DL, Guo TL, Germolec DR, White KL, Jr. Oral exposure to atrazine modulates cell-mediated immune function and decreases host resistance to the B16F10 tumor model in female B6C3F1 mice. Toxicology. 2005; 209:15-28.
20. Rajkovic V, Matavulj M, Johansson O. Combined exposure of peripubertal male rats to the endocrine-disrupting compound atrazine and power-frequency electromagnetic fields causes degranulation of cutaneous mast cells: A new toxic environmental hazard? Arch Environ Contam Toxicol. 2010; 59:334-341.
21. Dikić D, Zidovec-Lepej S, Remenar A, Bendelja K, Benković V, Horvat-Knežević A, Brozović G, Oršolić N. Effects of prometryne on apoptosis and necrosis in thymus, lymph node and spleen in mice. Environ Toxicol Pharmacol. 2009; 27:182-186.
22. Laws SC, Ferrell JM, Stoker TE, Cooper RL. Pubertal development in female Wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-S-chlorotriazine and hydroxyatrazine. Toxicol Sci. 2003; 76:190-200.
23. Rayner JL, Wood C, Fenton SE. Exposure parameters necessary for delayed puberty and mammary gland development in Long-Evans rats exposed in utero to atrazine. Toxicol Appl Pharmacol. 2004; 195:23-34.
24. Rooney AA, Matulka RA, Luebke RW. Developmental atrazine exposure suppresses immune function in male, but not female Sprague-Dawley rats. Toxicol Sci. 2003; 76:366-375.
25. Rowe AM, Brundage KM, Schafer R, Barnett JB. Immunomodulatory effects of maternal atrazine exposure on male Balb/c mice. Toxicol Appl Pharmacol. 2006; 214:69-77.
26. Blaylock BL, Holladay SD, Comment CE, Heindel JJ, Luster MI. Exposure to tetrachlorodibenzo-p-dioxin (TCDD) alters fetal thymocyte maturation. Toxicol Appl Pharmacol. 1992; 112:207-213.
27. Holladay SD. Prenatal immunotoxicant exposure and postnatal autoimmune disease. Environ Health Perspect. 1999; 107 (Suppl 5):687-691.
28. Theus SA, Tabor DR, Soderberg LS, Barnett JB. Macrophage tumoricidal mechanisms are selectively altered by prenatal chlordane exposure. Agents Actions. 1992; 37:140-146.
29. Domínguez-Gerpe L, Rey-Méndez M. Age-related changes in primary and secondary immune organs of the mouse. Immunol Invest. 1998; 27:153-165.
30. Dietert RR, Etzel RA, Chen D, Halonen M, Holladay SD, Jarabek AM, Landreth K, Peden DB, Pinkerton K, Smialowicz RJ, Zoetis T. Workshop to identify critical windows of exposure for children's health: Immune and respiratory systems work group summary. Environ Health Perspect. 2000; 108 (Suppl 3):483-490.
31. Pichler WJ, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. Allergy. 2004; 59:809-820.
32. Ruiz VE, Sachdev M, Zhang S, Wen S, Moss SF. Isolating, immunophenotyping and *ex vivo* stimulation of CD4(+) and CD8(+) gastric lymphocytes during murine Helicobacter pylori infection. J Immunol Methods. 2012; 384:157-163.
33. Almajid FM. Lymphocyte activation test for diagnosis

of seronegative brucellosis in humans. Indian J Pathol Microbiol. 2011; 54:775-781.
34. Da Rocha Piemonte M, De Freitas Buchi D. Analysis of IL-2, IFN-gamma and TNF-alpha production, alpha5 beta1 integrins and actin filaments distribution in peritoneal mouse macrophages treated with homeopathic medicament. J Submicrosc Cytol Pathol. 2002; 34:255-263.
35. Mazzoccoli G, Sothern RB, Parrella P, Muscarella LA, Fazio VM, Giuliani F, Polyakova V, Kvetnoy IM. Comparison of circadian characteristics for cytotoxic lymphocyte subsets in non-small cell lung cancer patients versus controls. Clin Exp Med. 2012; 12:181-194.
36. Roxburgh CS, McMillan DC. The role of the in situ local inflammatory response in predicting recurrence and survival in patients with primary operable colorectal cancer. Cancer Treat Rev. 2012; 38:451-466.
37. Yang DQ, You LP, Song PH, Zhang LX, Bai YP. A randomized controlled trial comparing total glucosides of paeony capsule and compound glycyrrhizin tablet for alopecia areata. Chin J Integr Med. 2012; 18:621-625.
38. Tedgui A, Mallat Z. Cytokines in atherosclerosis: Pathogenic and regulatory pathways. Physiol Rev. 2006; 86:515-581.
39. Varin A, Gordon S. Alternative activation of macrophages: Immune function and cellular biology. Immunobiology. 2009; 214:630-641.
40. Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L, Hernandez M, Colonna M. Cutting edge: TREM-2 attenuates macrophage activation. J Immunol. 2006; 177:3520-3524.
41. Pruett SB, Fan R, Zheng Q, Schwab C. Patterns of immunotoxicity associated with chronic as compared with acute exposure to chemical or physical stressors and their relevance with regard to the role of stress and with regard to immunotoxicity testing. Toxicol Sci. 2009; 109:265-275.
42. Yan DH, He Y, Wang H. Environmental characteristics of the atrazine in the waters in East Liaohe River Basin. Huan Jing Ke Xue. 2005; 26:203-208. (in Chinese)

(Received July 9, 2013; Revised August 27, 2013; Accepted August 28, 2013)

BST
BioScience Trends

Guide for Authors

1. Scope of Articles

BioScience Trends is an international peer-reviewed journal. BioScience Trends devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of 100 references. Mini reviews are also accepted.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 2,000 words in length (excluding references).

Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references).

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in BioScience Trends in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references.

3. Editorial Policies

Ethics: BioScience Trends requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to BioScience Trends, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter signed by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit http://www.biosciencetrends.com/downcentre.php (Download Centre).

Copyright: A signed JOURNAL PUBLISHING AGREEMENT (JPA) form must be provided by post, fax, or as a scanned file before acceptance of the article. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. A form facilitating transfer of copyright can be downloaded by clicking the appropriate link and can be returned to the e-mail address or fax number noted on the form (Please visit Download Centre). Please note that your manuscript will not proceed to the next step in publication until the JPA Form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in BioScience Trends.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in BioScience Trends and need assistance before submitting a manuscript. Authors can visit this organization directly at http://www.iacmhr.com/iac-eso/support.php?lang=en. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title Page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of

interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For article types including Original Article, Brief Report, Review, Policy Forum, and Case Report, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For News and Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Key words or phrases that do not occur in the title should be included in the Abstract page.

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

Materials and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al.* Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. Examples are given below:

Example 1 (Sample journal reference):
Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):
Darby S, Hill D, Auvinen A, *et al.* Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):
Ministry of Health, Labour and Welfare of Japan. Dietary reference intakes for Japanese. *http://www.mhlw.go.jp/houdou/2004/11/h1122-2a.html* (accessed June 14, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that the symbols and numbers appeared in the figures should be clear. Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays. All color figures will be reproduced in full color in the online edition of the journal at no cost to authors.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g.* mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide www.bipm.org/en/si/ for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and BioScience Trends accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g.*, Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to BioScience Trends for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to BioScience Trends online at http://www.biosciencetrends.com. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at office@biosciencetrends.com.

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author via e-mail. Corrections must be returned to the editor (proof-editing@biosciencetrends.com) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: Page charges will be levied on all manuscripts accepted for publication in BioScience Trends ($140 per page for black white pages; $340 per page for color pages). Under exceptional circumstances, the author(s) may apply to the editorial office for a waiver of the publication charges at the time of submission.

(*Revised February 2013*)

Editorial and Head Office:
Pearl City Koishikawa 603
2-4-5 Kasuga, Bunkyo-ku
Tokyo 112-0003 Japan
Tel: +81-3-5840-8764
Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com

BST
BioScience Trends

JOURNAL PUBLISHING AGREEMENT (JPA)

Manuscript No.:

Title:

Corresponding Author:

The International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) is pleased to accept the above article for publication in BioScience Trends. The International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) reserves all rights to the published article. Your written acceptance of this JOURNAL PUBLISHING AGREEMENT is required before the article can be published. Please read this form carefully and sign it if you agree to its terms. The signed JOURNAL PUBLISHING AGREEMENT should be sent to the BioScience Trends office (Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan; E-mail: **office@biosciencetrends.com**; Tel: **+81-3-5840-8764**; Fax: **+81-3-5840-8765**).

1. Authorship Criteria

As the corresponding author, I certify on behalf of all of the authors that:
1) The article is an original work and does not involve fraud, fabrication, or plagiarism.
2) The article has not been published previously and is not currently under consideration for publication elsewhere. If accepted by BioScience Trends, the article will not be submitted for publication to any other journal.
3) The article contains no libelous or other unlawful statements and does not contain any materials that infringes upon individual privacy or proprietary rights or any statutory copyright.
4) I have obtained written permission from copyright owners for any excerpts from copyrighted works that are included and have credited the sources in my article.
5) All authors have made significant contributions to the study including the conception and design of this work, the analysis of the data, and the writing of the manuscript.
6) All authors have reviewed this manuscript and take responsibility for its content and approve its publication.
7) I have informed all of the authors of the terms of this publishing agreement and I am signing on their behalf as their agent.

2. Copyright Transfer Agreement

I hereby assign and transfer to IACMHR Co., Ltd. all exclusive rights of copyright ownership to the above work in the journal BioScience Trends, including but not limited to the right 1) to publish, republish, derivate, distribute, transmit, sell, and otherwise use the work and other related material worldwide, in whole or in part, in all languages, in electronic, printed, or any other forms of media now known or hereafter developed and the right 2) to authorize or license third parties to do any of the above.

I understand that these exclusive rights will become the property of IACMHR Co., Ltd., from the date the article is accepted for publication in the journal BioScience Trends. I also understand that IACMHR Co., Ltd. as a copyright owner has sole authority to license and permit reproductions of the article.

I understand that except for copyright, other proprietary rights related to the Work (*e.g.* patent or other rights to any process or procedure) shall be retained by the authors. To reproduce any text, figures, tables, or illustrations from this Work in future works of their own, the authors must obtain written permission from IACMHR Co., Ltd.; such permission cannot be unreasonably withheld by IACMHR Co., Ltd.

3. Conflict of Interest Disclosure

I confirm that all funding sources supporting the work and all institutions or people who contributed to the work but who do not meet the criteria for authors are acknowledged. I also confirm that all commercial affiliations, stock ownership, equity interests, or patent-licensing arrangements that could be considered to pose a financial conflict of interest in connection with the article have been disclosed.

Corresponding Author's Name (Signature):

Date: