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Policy Forum

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Public policy response, aging in place, and big data platforms: Creating an effective collaborative system to cope with aging of the population

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Summary **The unprecedented rapid aging of the population is poised to become the next global public health challenge, as is apparent by the fact that 23.1% of the total global burden of disease is attributable to disorders in people aged 60 years and older. Aging of the population is the biggest driver of substantial increases in the prevalence of chronic conditions, and the prevalence of multi-morbidity is much higher in older age groups. This places a large burden on countries' health and long-term care systems. Many behavioral changes and public policy responses to aging of the population have been implemented to cope with these challenges. A system of "aging in place" has been implemented in some high-income countries in order to better provide coordinated and cost-effective health services for the elderly. This approach reduces institutional care while supporting home- or community-based care and other services. Advances in information and communications technology (ICT), assistive devices, medical diagnostics, and interventions offer many ways of more efficiently providing long-term care as part of aging in place. The use of big data on a web services platform in an effective collaborative system should promote systematic data gathering to integrate clinical and public health information systems to provide support across the continuum of care. However, the use of big data in collaborative system is a double-edged sword, as it also bring challenges for information sharing, standardized data gathering, and the security of personal information, that warrant full attention.**

Keywords: Older people, public health, institutional care, home-based care, community-based care, information platform

1. Introduction

Advances in medicine and socioeconomic development have substantially improved life expectancy worldwide. According to estimates from the World Health Organization (WHO), the worldwide average life expectancy was 72.7 years for females (ranging from 59.0 years in Africa to 82.0 years in high-income countries), and 68.1 years for males in 2012 (ranging from 56.3 years in Africa to 75.8 years in high-income countries) (Table 1) (*1*,*2*). Typically, women in Japan have the longest life expectancy in the world at 87 years (*2*).

There is no doubt that the increased longevity is one of the most remarkable success stories in human history. Coupled with decreased fertility rates, however, it is now ushering in unprecedented rapid aging of the population. Demographic projections suggest that the world's population aged 60 years and older is set to rise from 841 million in 2013 to more than 2 billion by 2050 and exceed the number of children by 2047 (*3*,*4*). By 2050, 21.1% of the world population will be 60 years or older, and 80% of this demographic group will live in low-income and middle-income countries, compared to about two-thirds at present (*4*,*5*).

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Table 1. Life expectancy (LE) and healthy life expectancy (HALE) by country-income group worldwide*

Country income group	Males				Females			
	LE at birth (years)		HALE at birth (years)		LE at birth (years)		HALE at birth (years)	
	2000	2010	2000	2010	2000	2010	2000	2010
High income countries	72.4	75.8	64.7	67.5	79.6	82.0	70.0	72.0
Low- and middle-income countries								
African Region	49.0	56.3	42.4	48.8	51.4	59.0	43.8	50.4
Region of the Americas	70.8	73.5	62.7	64.9	77.0	79.3	67.2	69.1
Eastern Mediterranean Region	63.6	66.1	54.8	57.4	66.4	69.7	56.1	59.2
European Region	68.2	72.4	60.7	64.2	76.7	79.6	67.1	69.6
South East Asian Region	61.6	65.7	53.5	57.4	64.3	69.4	55.0	59.7
Western Pacific Region	70.0	73.9	63.0	66.6	74.8	78.1	66.7	69.8
Worldwide	63.9	68.1	56.4	60.1	68.5	72.7	59.7	63.4

*Data are from WHO methods for life expectancy and healthy life expectancy 2014 (*1*), and WHO World Health Statistics 2014 (*2*).

2. Burden of disease as a result of aging of the population

Aging of the population is poised to become the next global public health challenge (*6*). An analysis of data from the Global Burden of Disease study shows that 23.1% of the total global burden of disease is attributable to disorders in people aged 60 years and older; 49.2% of the burden is found in high-income countries while 19.9% is found in low-income and middle-income countries (*7*).

The major causes of death and disability in older age (≥ 60 years) are non-communicable diseases, regardless of a country's income level (*8,9*). In addition, infectious disease morbidity and mortality in many low-income and middle-income countries increasingly affect older people because of the aging population and changes in the epidemiology of some diseases such as HIV/AIDS or tuberculosis (*10,11*). Globally, the leading contributors to disease burden in older people are cardiovascular diseases (30.3% of the total burden in people aged 60 years and older), malignant neoplasms (15.1%), chronic respiratory diseases (9.5%), musculoskeletal diseases (7.5%), and mental and neurological disorders (6.6%) (Figure 1) (*7,12*).

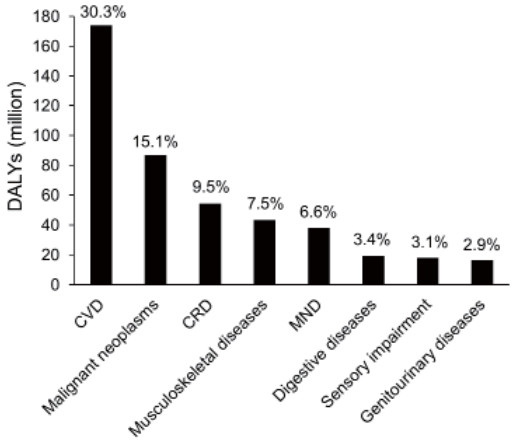

Figure 1. Leading contributors to the burden of non-communicable diseases in people aged 60 years and older in 2010 (IHME GBD) (*7,12*). Disability-adjusted life years (DALYs) data based on global burden of disease (GBD) estimates from the Institute of Health Metrics and Evaluation (IHME). CVD, cardiovascular diseases; CRD, chronic respiratory diseases; MND, mental and neurological disorders.

Aging of the population is the biggest driver of substantial increases in the prevalence of chronic conditions, such as dementia, stroke, chronic obstructive pulmonary disease, and diabetes, that are strongly associated with age (*6*). Furthermore, the prevalence of multi-morbidity is much higher in older age groups, with 65% of people aged 65-84 years and 82% of people aged at least 85 years affected (*13*). Therefore, the age-appropriate care for chronic diseases and the complexity of integrating care for complex multi-morbidity are sharp exemplars of the challenges faced by health-care systems across the world in the 21st century (*14*).

3. Public health response to aging of the population

The Madrid International Plan of Action on Ageing called for the elimination of social and economic inequalities in access to health care and the development of healthcare and long-term care to meet the needs of older people (*15*). Furthermore, the Post-2015 Development Agenda indicates that the goal of ensuring healthy lives and promoting wellbeing for everyone at all ages cannot be achieved without attention to the health of older people (*6*).

Over the past few decades, improvements in the effectiveness and coverage of health care and reduced exposure to environmental, behavioral, and biological risk factors have played a role in mitigating the disease burden posed by aging of the population. Data show that many behavioral changes and public policy responses to aging of the population have the potential to mitigate the disease burden (*16,17*). Smoking cessation, a reduction in excessive alcohol consumption, adherence to healthier diets, engagement

in more physical activity, and taking advantage of adult vaccines such as those for influenza, pneumococcal disease, human papillomavirus, and shingles, are behavioral responses that should promote improved health in older people (*18-21*). Regardless of how effectively non-communicable chronic diseases and even some communicable diseases are prevented or delayed, however, many older people will inevitably be affected. Therefore, continuous monitoring and interventions are urgently needed for older people.

Continuous monitoring and interventions for older people poses challenges for countries' economies and health systems. These approaches will place a large burden on health and long-term care systems since increased health-care spending at older ages is largely driven by much higher outlays in the final years of life (*22*). Health systems need to find cost-effective strategies to expand health care and to respond to the needs of older people. Health care that is effective, safe, efficient, and responsive and that avoids imposing an unbearable financial burden on older people will be central to achievement of the goal of universal health coverage. Additionally, older people are increasingly living alone or as part of a couple, rather than in the larger, multigenerational households of the past as a result of increased spatial mobility and changes in family structure. For example, in some European countries nearly 50% of women aged 65 years or older live alone (*23*). In addition, many older people want to continue to live in their home and their community where they have been living for a long time even if they are ill (*24*). These changes are stimulating increasing debate on the role of the government and family in providing long-term care to many older people who need it. Faced with this challenge, some high-income countries have been working to reduce institutional care while supporting home- or community-based care and other services that enable older people to remain in their own homes or a home-like environment. This approach is known as "aging in place".

4. Aging in place (home- or community-based care) to provide coordinated and informed geriatric services

An extension of basic packages of cost-effective interventions to match the needs of older people with appropriate technologies, effective treatment of chronic diseases is needed to reduce disability. To optimize their functioning, health systems could be redesigned to better provide coordinated and informed geriatric services that enable older people to age in place (*e.g.*, at home or in the community) to the extent possible (*25*). Ideally, these services would be seamlessly linked with social and long-term care to provide a continuum of care that extends from home or community to institutional care (*24,26*). Core services would include prevention and early detection of disease, acute and chronic care, rehabilitation, provision of assistive devices, and palliative care (*26*).

Many long-term care programs for aging in place have been implemented in high-income countries such as Australia, Canada, and the United States (*27-29*). Over the past decade, Japan's government-initiated, mandatory, public, long-term care insurance (LTCI) system has ushered in increased use of aging in place at a reduced cost to households (*30*). Unlike systems elsewhere that rely on cash allowances for long-term care of older people, the Japanese LTCI system only provides services and recipients can choose their services and providers, on the grounds that family caregivers benefit most from direct help with their tasks and that quality of care is best assured by relying on trained, licensed, and supervised caregivers. Many services are covered by the LTCI system in Japan (*31*): *i*) services in the home, including a home helper (housekeeping and personal care), a visiting nurse, bathing, remodelling to accommodate an elderly family member, and assistive devices; *ii*) services outside the home, including day care, day care with rehabilitation, and short-stay respite care; and *iii*) institutional services, including nursing homes, homes with more medical services, and chronic-care hospitals. Additionally, the costs of care in private nursing homes and group homes for individuals with dementia are covered.

Launched in 2000, the Japanese LTCI system has been in place for more than a decade and now serves nearly 5 million people (*32*). The number of beneficiaries in institutions increased by 83%, but more notable has been the 203% increase in those receiving home and community-based services in the program's first 10 years (*32*). Largely on the basis of the Japanese approach, the South Korean Government started long-term care programs that essentially opted for a services-only strategy, and personnel in Taiwan hope to implement a similar system (*33,34*).

5. Information and communications technology to integrate clinical and public health information systems to provide support across the continuum of care

The goal of aging in place is to improve health outcomes in older people and to improve the personalization of services while reducing inequalities in both health outcomes and responsiveness. Data show that aging in place can be simple and cost-effective while simultaneously offering substantial benefits to the individual (*24,35*). This approach can also meet the increasing need for improved quality of life for older people by emphasizing home- or community-based care for patients in the interim stage after the completion of acute-stage treatment or for end-of-life care (*36*). However, the needs of older people frequently blur the lines between disease prevention and health promotion as defined by public health practices in previous eras

(*37*,*38*). In addition, older people, family caregivers, clinicians, system designers, public health practitioners, and policymakers have different technology and information needs (*24*). Accordingly, the pressing issue is to use modern technology to deliver healthcare services. Specifically, this means developing systematic data gathering to integrate clinical and public health information systems to provide support across the continuum of care.

Advances in information and communications technology (ICT), assistive devices, medical diagnostics, and interventions offer many ways of more efficiently providing long-term care as part of aging in place. For example, the advent of wearable devices that can continuously monitor physical activity may rapidly transform our understanding of functional trajectories and their determinants. Other innovations use low-cost laptop computers with sensors to read vital signs and perform electrocardiograms, allowing images to be sent to trained physicians at other locations, and create electronic health records; this could avoid the need for travel and dealing with long and uncertain waiting times (*26*,*39*).

Furthermore, integrated platforms have been created with the aid of ICT in recent years to provide comprehensive health services to older people. For example, the Australian e-Health Research Centre and Queensland Health have developed an innovative Care Assessment Platform, an ICT-enabled home-care cardiac rehabilitation program (*40*). With the aid of mobile phones and the Internet, the Care Assessment Platform can provide all the elements of traditional cardiac rehabilitation for patients recovering from a myocardial infarction, including education, mentoring, goal-setting, personal feedback, and counseling over a 6-week period. Based on the latest advances in research on big data, a program named the Intelligent Aging-in-place Home care Web Services Platform is being implemented in Taiwan (*41*). This program has a cloud computing setting to offer personalized healthcare services everywhere to facilitate the most desirable and cost-efficient provision of care as part of aging in place.

6. Use of big data as a double-edged sword: Information sharing, standardized data gathering, and the security of personal information

The use of big data on a web services platform to facilitate aging in place should promote systematic data gathering to integrate clinical and public health information systems to provide support across the continuum of care. However, aging in place is a comprehensive system including public health, medical treatment, nursing care, and welfare work. Given that the availability of medical care resources varies considerably in different regions, an effective collaborative system should be established, and this system should be tailored to the region in order to capitalize on local characteristics. A point worth noting, however, is that such a collaborative system is a double-edged sword. The use of big data in a collaborative system also brings challenges in terms of information sharing, standardized data gathering, and the security of personal information.

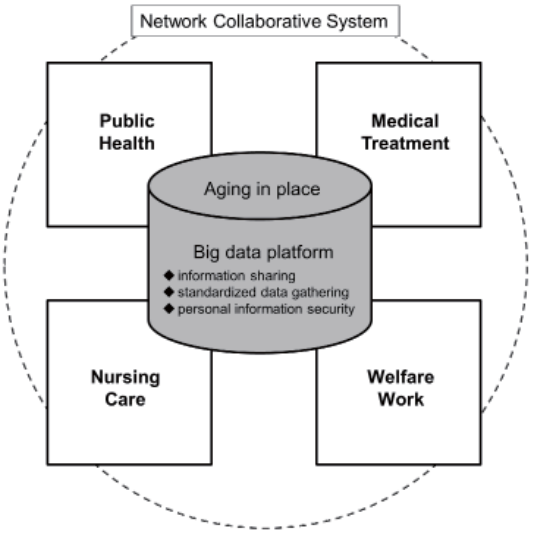

Figure 2. Exploration on an effective information network with standardized data gathering and management to integrate health services for the elderly in public health, medical treatment, nursing care, and welfare work in Japan.

In light of this challenge, Japan drafted "The Guideline to Promote the Appropriate Use of Information Systems in Care for the Elderly in Conjunction with At-home Care" in 2014 (*42*). The Guideline is intended to foster an effective information network with standardized data gathering and management to integrate health services for the elderly in public health, medical care, nursing care, and welfare work (Figure 2). This information network seeks to collect 237 pieces of information in the 5 categories of "patient attributes", "residence and family", "medical treatment", "nursing care and lifestyle," and "diagnosis, treatment and care". The aforementioned guideline has standardized data entry, changes to data, data use, and personnel responsible for the system. Such a collaborative system based on big data with standardized data gathering and management should promote clinical and public health information systems to provide support for the elderly across the continuum of care.

7. Conclusion

Age-appropriate care for chronic diseases in the elderly and the complexity of integrating care for complex multi-morbidity are sharp exemplars of the challenges faced by health-care systems across the world in the 21st century. Data show that aging in place could be a

simple and cost-effective approach that simultaneously offers substantial benefits to the individual. An example of that approach, the Japanese LTCI system was designed to help family caregivers by having the government handle some aspects of care. The LTCI system has ushered in the increased use of aging in place at a reduced cost to households.

Effective collaborative systems based on big data are now being examined and put into practice through the use of modern technology to deliver healthcare services and the development of systematic data gathering to integrate clinical and public health information systems to provide support across the continuum of care. However, the use of big data in a collaborative system is a double-edged sword. To cope with challenges in terms of information sharing, standardized data gathering, and the security of personal information, Japan drafted the guideline for creation of an effective information network with standardized data gathering and management. The resulting network should integrate health services for the elderly in hospitals, home-based care, community-based care, and institutional care.

References

1. WHO. WHO methods for life expectancy and healthy life expectancy. Geneva: World Health Organization. *www.who.int/healthinfo/statistics/LT_method.pdf* (accessed November 15, 2014).
2. WHO. World health statistics 2014. Geneva: World Health Organization. *http://www.who.int/gho/publications/world_health_statistics/2014/en/* (accessed November 17, 2014).
3. United Nations Department of Economic and Social Affairs Population Division. World Population Ageing 2013. *http://www.un.org/en/development/desa/population/publications/ageing/WorldPopulationAgeingReport2013.shtml* (accessed November 16, 2014).
4. Chatterji S, Byles J, Cutler D, Seeman T, Verdes E. Health, functioning, and disability in older adults - present status and future implications. Lancet. 2015; 385:563-575.
5. United Nations Department of Economic and Social Affairs, Population Division, population Estimates and Projections Section. World Population Prospects: The 2012 Revision. *http://esa.un.org/unpd/wpp/index.htm* (accessed November 19, 2014).
6. Suzman R, Beard JR, Boerma T, Chatterji S. Health in an ageing world - what do we know? Lancet. 2015; 385:484-486.
7. WHO. The global burden of disease. 2004 update. Geneva: World Health Organization. *http://www.who.int/healthinfo/global_burden_disease/GBD_report_2004update_full.pdf* (accessed December 2, 2014).
8. Wilmoth JR. Demography of longevity: Past, present, and future trends. Exp Gerontol. 2000; 35:1111-1129.
9. Fries JF. Aging, natural death, and the compression of morbidity. 1980. Bull World Health Organ. 2002; 80:245-250.
10. Adjuik M, Smith T, Clark S, et al. Cause-specific mortality rates in sub-Saharan Africa and Bangladesh. Bull World Health Organ. 2006; 84:181-188.
11. Aboderin IA, Beard JR. Older people's health in sub-Saharan Africa. Lancet. 2015; 385:e9-e11.
12. Prince MJ, Wu F, Guo Y, Gutierrez Robledo LM, O'Donnell M, Sullivan R, Yusuf S. The burden of disease in older people and implications for health policy and practice. Lancet. 2015; 385:549-562.
13. Barnett K, Mercer SW, Norbury M, Watt G, Wyke S, Guthrie B. Epidemiology of multimorbidity and implications for health care, research, and medical education: A cross-sectional study. Lancet. 2012; 380:37-43.
14. Banerjee S. Multimorbidity - older adults need health care that can count past one. Lancet. 2015; 385:587-589.
15. United Nations. Report of the Second World Assembly on Ageing. *http://www.un.org/en/events/pastevents/ageing_assembly2.shtml* (accessed December 4, 2014).
16. Christensen K, Doblhammer G, Rau R, Vaupel JW. Ageing populations: The challenges ahead. Lancet. 2009; 374:1196-1208.
17. Doyle Y, McKee M, Rechel B, Grundy E. Meeting the challenge of population ageing. BMJ. 2009; 339:b3926.
18. Do R, Xie C, Zhang X, et al. The effect of chromosome 9p21 variants on cardiovascular disease may be modified by dietary intake: Evidence from a case/control and a prospective study. PLoS Med. 2011; 8:e1001106.
19. Lorig KR, Holman H. Self-management education: history, definition, outcomes, and mechanisms. Ann Behav Med. 2003; 26:1-7.
20. Chakravarty EF, Hubert HB, Krishnan E, Bruce BB, Lingala VB, Fries JF. Lifestyle risk factors predict disability and death in healthy aging adults. Am J Med. 2012; 125:190-197.
21. He FJ, Pombo-Rodrigues S, MacGregor GA. Salt reduction in England from 2003 to 2011: Its relationship to blood pressure, stroke and ischaemic heart disease mortality. BMJ Open. 2014; 4:e004549.
22. Mcgrail K, Green B, Barer M, Evans R, Hertzman C, Normand C. Age, costs of acute and long-term care and proximity to death: Evidence for 1987-88 and 1994-95 in British Columbia. Age Ageing. 2000; 29:249-253.
23. Tomassini C, Glaser K, Wolf DA, Broese van Groenou MI, Grundy E. Living arrangements among older people: An overview of trends in Europe and the USA. Popul Trends. 2004; 115:24-34.
24. Reeder B, Meyer E, Lazar A, Chaudhuri S, Thompson HJ, Demiris G. Framing the evidence for health smart homes and home-based consumer health technologies as a public health intervention for independent aging: A systematic review. Int J Med Inform. 2013; 82:565-579.
25. Yarmo-Roberts D, Freak-Poli RL, Cooper B, Noonan T, Stolewinder J, Reid CM. The heart of the matter: Health status of aged care clients receiving home- and community-based care. J Aging Res. 2010; 2010:275303.
26. Beard JR, Bloom DE. Towards a comprehensive public health response to population ageing. Lancet. 2015; 385:658-661.
27. Horner B, Boldy DP. The benefit and burden of "ageing-in-place" in an aged care community. Aust Health Rev. 2008; 32:356-365.
28. Ouellet MC, Sirois MJ, Beaulieu-Bonneau S, Morin J, Perry J, Daoust R, Wilding L, Provencher V, Camden S, Allain-Boulé N, Émond M. Is cognitive function a concern in independent elderly adults discharged home from the emergency department in Canada after a minor injury? J Am Geriatr Soc. 2014; 62:2130-2135.

29. Satariano WA, Scharlach AE, Lindeman D. Aging, place, and technology: Toward improving access and wellness in older populations. J Aging Health. 2014; 26:1373-1389.
30. Campbell JC, Ikegami N. Long-term care insurance comes to Japan. Health Aff (Millwood) 2000; 19:26-39.
31. Tamiya N, Noguchi H, Nishi A, Reich MR, Ikegami N, Hashimoto H, Shibuya K, Kawachi I, Campbell JC. Population ageing and wellbeing: Lessons from Japan's long-term care insurance policy. Lancet. 2011; 378:1183-1192.
32. Ministry of Health, Labour and Welfare (MHLW). Report on various current states around LTCI (Kaigo hoken seido wo torimaku genjo). *http://www.mhlw.go.jp/stf/shingi/2r985200000123iu-att/2r985200000123se.pdf* (accessed December 10, 2014). (in Japanese)
33. Kim H, Kwon S, Yoon NH, Hyun KR. Utilization of long-term care services under the public long-term care insurance program in Korea: Implications of a subsidy policy. Health Policy. 2013; 111:166-174.
34. Hsieh HF, Chen KM, Lin MH, Wang YC, Huang HT. Social welfare utilization and needs of older adults in Taiwan: Survey research. Nurs Outlook. 2014; 62:459-468.
35. SArai Y, Iinuma T, Takayama M, et al. The Tokyo Oldest Old survey on Total Health (TOOTH): A longitudinal cohort study of multidimensional components of health and well-being. BMC Geriatr. 2010; 10:35.
36. Kubo M. Long-term care insurance and market for aged care in Japan: Focusing on thestatus of care service providers by locality and organisational nature based onsurvey results. Australas J Ageing. 2014; 33:153-157.
37. Stults BM. Preventive health care for the elderly. West J Med. 1984; 141:832-845.
38. Breslow L. Health measurement in the third era of health. Am J Public Health. 2006; 96:17-19.
39. Bloom DE, Chatterji S, Kowal P, Lloyd-Sherlock P, McKee M, Rechel B, Rosenberg L, Smith JP. Macroeconomic implications of population ageing and selected policy responses. Lancet. 2015; 385:649-657.
40. Varnfield M, Karunanithi MK, Särelä A, Garcia E, Fairfull A, Oldenburg BF, Walters DL. Uptake of a technology-assisted home-care cardiac rehabilitation program. Med J Aust. 2011; 194:S15-S19.
41. Su CJ, Chiang CY. IAServ: An intelligent home care web services platform in a cloud for aging-in-place. Int J Environ Res Public Health. 2013; 10:6106-6130.
42. The Institute of Gerontology of The University of Tokyo. The Guideline to Promote the Appropriate Use of Information Systems in Care for the Elderly in Conjunction with At-home Care. *http://www.iog.u-tokyo.ac.jp/wp-content/uploads/2014/05/5435d2ad3a28ce3767b71b2bfb764856.pdf* (accessed December 12, 2014).

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Review

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Food traceability systems in China: The current status of and future perspectives on food supply chain databases, legal support, and technological research and support for food safety regulation

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Summary **Over the past few decades, the field of food security has witnessed numerous problems and incidents that have garnered public attention. Given this serious situation, the food traceability system (FTS) has become part of the expanding food safety continuum to reduce the risk of food safety problems. This article reviews a great deal of the related literature and results from previous studies of FTS to corroborate this contention. This article describes the development and benefits of FTS in developed countries like the United States of America (USA), Japan, and some European countries. Problems with existing FTS in China are noted, including a lack of a complete database, inadequate laws and regulations, and lagging technological research into FTS. This article puts forward several suggestions for the future, including improvement of information websites, clarification of regulatory responsibilities, and promotion of technological research.**

Keywords: Food safety, sharing information, quality control, recall

1. Introduction

With the rapid development of China's economy, the food safety problem has garnered public attention since the 1990s. This problem harms public health and causes a wide range of diseases (*1*). As a result of the outbreak of bovine spongiform encephalopathy (BSE), commonly known as "mad-cow disease," in 1986, a food traceability system (FTS) was established to manage food safety (*2*). An FTS is an information-based proactive strategy to manage food safety that facilitates the identification of food risks and orderly recalls in the event of an incident to prevent food safety hazards. The roles of an FTS are to *i*) improve food supply management; *ii*) facilitate traceback for food safety and quality; *iii*) market foods with subtle quality attributes; *iv*) better use resources such as flora and fauna for food production, and *v*) establish long-term relationships in the food chain (*3,4*).

Since FTS was first used to control BSE, FTS has successively been used to manage food safety in the United States of America (USA) and many European countries, and structure of FTS has improved over the past decade. In 2002, the European Commission defined "traceability" as the ability to trace and follow a food, feed, food-producing animal, or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing, and distribution (*5,6*). In 2004, the 27th Session of the Codex Alimentarius Commission of the United Nations defined the general principles of an FTS: "Traceability/ product tracing: the ability to follow the movement of a food through specified stage(s) of production, processing, and distribution" (*7*). In 2005, Section 7.9 of International Standardization Organization (ISO) 22000:2005 (food safety management system) clearly indicated that an FTS should be able to identify a direct supplier of materials and the distribution of the end product and that records of traceability should be maintained for a defined period to allow system evaluation to enable handling of unsafe products and product withdrawal (*8*). In 2007, ISO 22005:2007 Food

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Table 1. Major food safety incidents in China from 2003 to 2014

Year	Incident	Content	Consequence
2003	Jinhua ham dichlorvos incident	Ham producers added the highly toxic pesticide dichlorvos when making ham.	Companies in Jinhua suffered huge economic losses.
2004	Fuyang, Anhui substandard infant milk powder incident	More than 100 babies suffer from severe malnutrition after eating substandard infant milk powder.	60 infants were injured, and at least eight babies died.
2005	"Sudan 1" at KFC fast food outlets in Shanghai	Sudan 1 was found in condiments of the New Orleans-style roasted wings at KFC.	The company suffers economic losses.
2006	Shanghai clenbuterol food poisoning incident	Clenbuterol in pork and viscera exceeded limits.	336 people suffered food poisoning in nine districts of Shanghai
2006	"Red-yolk salted duck eggs" incident	The eggs contained the industrial dye Sudan IV.	Harmed consumer health.
2008	"Melamine" incident	The chemical melamine was found in infant milk powder.	39,965 infants and toddlers were treated on an outpatient basis after consuming the infant formula.
2011	"Dyed" steamed bun incident in Shanghai	The sweetener sodium cyclamate and preservative potassium sorbate were added to steamed buns that were expired and then the buns were repackaged.	334,864 "dyed" steamed buns were resold, harming public health.
2012	Lipton "poisoned tea" incident	The tea contained a variety of harmful pesticides.	Harmed consumer health.
2013	Kung Fu Restaurant ice cube incident	The microbial colony count on ice cubes at the Kung Fu Restaurant exceeded the limit.	Loss of consumer trust.
2014	Hui Yuan "Rotten Fruit" incident	The factory bought rotten fruit to make juice	Massive drop in company stock.

Traceability Standard states that: "food safety is the joint responsibility of all the actors involved" (*9*).

As an agricultural country, China has witnessed numerous problems and incidents related to food safety (Table 1) that have captured public attention both nationally and internationally (*10,11*). During the Fuyang inferior infant milk powder incident, 229 Chinese babies suffered from severe malnutrition after eating inferior infant milk powder (*12*). During the "melamine" incident, 39,965 infants and toddlers received outpatient treatment as of September 2008 after eating infant formula (*13*). In the face of those incidents, China has realized the importance of the FTS and started to use an FTS to facilitate meat traceability in 2004 (*14*). This marked the creation of China's FTS. Overall, however, FTS databases, laws, and technological research in China need to be improved further.

The FTS has been established around the world to reduce the risk of food safety problems and ensure quality and supply-chain integrity (*15,16*). The current paper reviews the use of FTS in the USA, Europe, Japan, Australia, and other countries. This paper discusses the current state of the FTS in China, its achievements, its existing problems, and prospects for the future.

2. FTS around the world

2.1. *The characteristics of foreign FTS*

FTS has been established in many countries as a method of managing food quality and safety risks in order to improve the system for supervision and management of food safety (*17,18*).

Based on the FTS established in the Netherlands, United Kingdom (UK), European Union (EU), Japan, Australia, USA, Canada, India, South Korea, and Norway (Table 2) (*3,19-35*), the current FTS has the following characteristics:

Establishment of a database for the FTS. A complete database of the food supply chain has been created in some countries to facilitate a website providing information on food traceability (*36*), such as the Cattle Tracking System (CTS) established in the UK that stipulates all livestock born after July 1, 1996 have identification documents (*20*).

Legal support for the FTS. With the support of government, food safety laws and regulations have been enacted in many countries to promote clearly assigned responsibilities. For example, the Food Safety Modernization Act, the Food Safety Enhancement Act, and other comprehensive laws have been enacted in the USA to promote multi-sector management by the Food and Drug Administration (FDA) with the support of the Department of Agriculture, the Department of Health, and the Environmental Protection Agency (*24*). In the USA, a regulatory body has legal authority to oversee a specific product or resource with additional levels of supervision. This means that the Department of Agriculture is mainly responsible for the regulation of livestock and poultry products, the Department of Health is responsible for the regulation of the vast majority of food (in addition to the scope of the jurisdiction of the Department of Agriculture), and the Environmental Protection Agency is responsible for water quality security and pesticide registration and management (*36*).

Scientific research to provide technical support to

Table 2. National regulations regarding FTS

Region (Date)	Content	*Ref.*
Netherland (1992)	• In the early 1990s, Holland's Animal Husbandry Department implemented the Integrated Chain Control system (initials in Dutch: IKB) for "the overall control of the food chain" including food, meat, and egg products. The IKB requires controlled production, processing, and sales of meat and eggs. • In 1992, the IKB began with pork as a pilot product. In 1995, it expanded to beef production. So far, more than three-quarters of the pork and most beef are controlled in the IKB system. An identification and registration (I&R) system is also a part of the IKB system. The IKB system can quickly trace the source of animal products through the recognition and registration system. • In order to ensure the quality of dairy products and implement traceability based on the food chain, the Dutch implemented a Dairy Quality Control (KKM) system based on GMP and the dairy industry chain.	(*19*)
UK (1996)	• The UK established CTS which operates online. The system is one of the four core elements of an FTS. Livestock breeding records are recorded in the CTS system in order to facilitate positioning and tracking. The four elements of the livestock identification and registration system are identification, farm records, id cards, and a livestock tracking system.	(*20*)
EU (2000)	• On January 12, 2000, the European Commission formally issued the "White Paper on Food Safety" to introduce the concept of traceability "from farm to table" and to define the tasks and responsibilities of each participant in the food chain. • On January 28, 2002, the EU's 178th Council Regulation (2002) specified that sales of all food must be traceable.	(*21-23*)
Japan (2001)	• In 2001, a Japanese beef traceability system was established in order to deal with mad cow disease. • In May 2002, the Japanese Government formulated a "beef id card" system. Consumers can obtain information on the cattle breed, breeding, slaughtering, and distribution process information. • In June 2002, the Japanese FTS expanded to rice and oysters. Via an electronic tag on rice packaging, consumers can learn the rice's origin, producers, the pesticides and fertilizers used in the production process, and specific processing information. • In June 2003, the Japanese Diet passed a special act to identify cattle. This act has regulated beef sales and required all beef packing to identify the beef containing since December 1, 2003. • The Japan Agricultural Co-operatives decide to implement an "identity code recognition system" for vegetables, meat, and other agricultural products by the end of 2006. The system requires detailed information such as product origin, producers, and fertilizers and pesticides used to facilitate consumer queries.	(*24-26*)
Australia (2001)	• Australia began a National Livestock Identification Scheme (NLIS) in 2001.	(*27,28*)
USA (2002)	• The Bioterrorism Preparedness Act, *i.e.* the Public Health Security and Bioterrorism Preparedness and Response Act of 2002, designated food safety as a priority of national security and put forward traceability "from farm to table" for food risk management. • In May 2003, the FDA announced food safety regulations requiring that all food transportation, distribution, and import companies keep records of the food distribution process. • From 2005 to 2009, the USA gradually instituted nationwide animal breeding, processing, transportation, and traceability management through the National Animal Identification System (NAIS). The NAIS includes three key links: registered farms, animal identification, and information tracking.	(*3,29*)
Canada (2002)	• In 2002, Canada instituted a beef cattle identification system in order to facilitate the traceability. Live cattle are given an ear tag or brand for identification after NIXS certification. When cattle are moved to a new location, a radio frequency identification reader at the farm or abattoir will read and record that information in the NLIS database.	(*30-32*)
India (2006)	• In 2006, India enacted the Food Safety and Standards Act. This Act has defined the FTS, it requires that food producers provide information on the food production process, it requires that information on companies providing raw materials be indicated, and it requires that food on the market be labeled in order to ensure the traceability of food.	(*33*)
South Korea (2007)	• On December 21, 2007, South Korea's Parliament announced the Cattle and Beef Traceability Act. The act applies to cattle raised in South Korea and mainly specifies that the owner of the cow must report the cow's birth or death, import or export, transfer or processing (including slaughter) to the Food and Agriculture Forestry and Fisheries Department (MIFAFF). Owners must assign identification numbers to each head of cattle and report incidents. Meat processing plants and sellers must display the ID number on meat and beef products so that their source can be traced.	(*34*)
Norway (2010)	• In 2010, the Norwegian Seafood Export Council required that producers of marine products mark the country of origin on packaging and implement marine product traceability to promote global sales of marine products from Norway.	(*35*)

the FTS. The scientific research providing technical support to FTS is relatively advanced (*37*). Japan, as an example, has been attempting to use the latest scientific research to enhance the reliability of its FTS (*38*). One of Japan's approaches is to use DNA markers to trace animal products (*3*).

2.2. *The role of the FTS*

The series of food scandals around the world has resulted in reduced consumer confidence and has highlighted the lack of an integrated approach of the food chain. A traceability system emphasizing comprehensive control and continuous management of food production needs to be established, and legislation that covers all aspects of food production and distribution needs to be passed (*4*). In response to this situation, the EU's General Food Law came into effect in 2002; this law mandates traceability for all food and feed businesses. Traceability allows targeted product withdrawal and recall when necessary and the provision of accurate information to the public.

The benefits of FTS are numerous and diverse and they affect the whole supply chain. The role of an FTS is mainly reflected in the following aspects (Table 3):

Table 3. Benefits of traceability systems

Items	Qualitative benefits	Quantitative benefits
Supply	Food safety; Establishes long-term relationships.	Improves techniques; Allows selection of varieties.
Production	Better use of resources; Improves operational procedures.	Increased production; Increased productivity of employees; Efficient production process.
Distribution	Increases the trust of customers; Increases the breadth of customers.	Lower compensation fees.

i) establishing long-term relationships in supply; *ii*) promoting the efficiency of production processes; and *iii*) increasing the breadth of customers (*39*). Before the implementation of an FTS, suppliers were commonly chosen based only on price. With the traceability system, however, each supplier must be monitored in a way that allows a price-quality ratio to be calculated. In this sense, the FTS has helped suppliers to reduce their costs.

3. FTS in China

3.1. *Policy support for FTS*

In 2004, the creation of China's FTS began with a project to create a meat FTS proposed by the State Food and Drug Administration (SFDA) (*40*). In September 2004, the State Council published its "Decision on Further Enhancing Food Safety" that proposed a system of quality and safety standards for agricultural product and that established a routine monitoring and traceability system for the quality and safety of agricultural products (*41*).

China has implemented at least 52 laws and regulations on FTS, indicating that China has gradually enhanced the role of the system for tracing food products (Table S1, *http://www.biosciencetrends.com/docindex.php?year=2015&kanno=1*). The "State Council's Decision on Further Enhancing Food Safety" issued in 2004 proposed a system of quality and safety standards for agricultural products and suggested the establishment of a routine monitoring and traceability system for the quality and safety of agricultural products. In the aspect regulation of distribution/sales companies, the "Notice of the Ministry of Finance on printing and distribution of documents on the management of special funds for development of rural logistics systems" provides support to upgrade and transform facilities for the wholesale marketing of fresh agricultural products. The Food Safety Law of the People's Republic of China requires records that cover all of the links in the food supply chain to facilitate traceability and product recall in the future.

China also has issued 118 local regulations governing FTS that increase government inspections and financial investment. These regulations stipulate traceability technology, material support, pilot projects, and inspection results in detail (*42-48*).

3.2. *Use of FTS in practice in China*

In 2004, the "Pilot Project for a System to Trace the Quality of Beijing Vegetable Products" was launched by the Beijing Municipal Agriculture Bureau and the Department of Agriculture of Hebei Province. The earliest project to create an FTS in China, the project selected 6 counties in Hebei as a base of vegetable production at which to implement unified packaging and product traceability labels.

In 2007, Beijing Jinweifuren Halal Food Co., Ltd. developed software for a traceability system with the technical support of the Beef Cattle Research Center of China Agricultural University (CAU). The system covers abattoirs and fattening farms in Jinwei. With increasing government regulations on beef quality and safety, traceability is becoming an imperative requirement in the cattle industry (*49*).

In 2008, an FTS was created to uniformly track all food for the Olympic Games in Beijing. This system featured the integrated use of numerous technologies – radio frequency identification device (RFID), global positioning system (GPS), automatic temperature recording and control, humidity control, and encrypted communication – to track and record a host of information on food, including its production, processing, transportation, and storage (*50*). In addition, quality monitoring stations were established at key sites to inspect and record information on food quality and implementing complete monitoring from the site of food production to processing companies and distribution centers up to the final consumer. This allowed food to be tracked during the Olympics.

Since 2010, the Ministry of Commerce (MOC) and Ministry of Finance (MOF) have supported a pilot program to create a meat and vegetable distribution traceability system (MVDTS) in 50 cities in four batches (Figure 1) (*51*). In order to promote the MVDTS, the central government spent 1.86 billion yuan. This amount covered major municipalities, cities with independent planning, and provincial capitals. The MOC program seeks to have the MVDTS cover all cities with a population of more than million, and the MVDTS should cover meat, vegetables, livestock, marine products, fruit, edible fungi, soy products, and other types of food by the end of the ‘12th Five-Year Plan' (52)."

In addition, websites with information on food

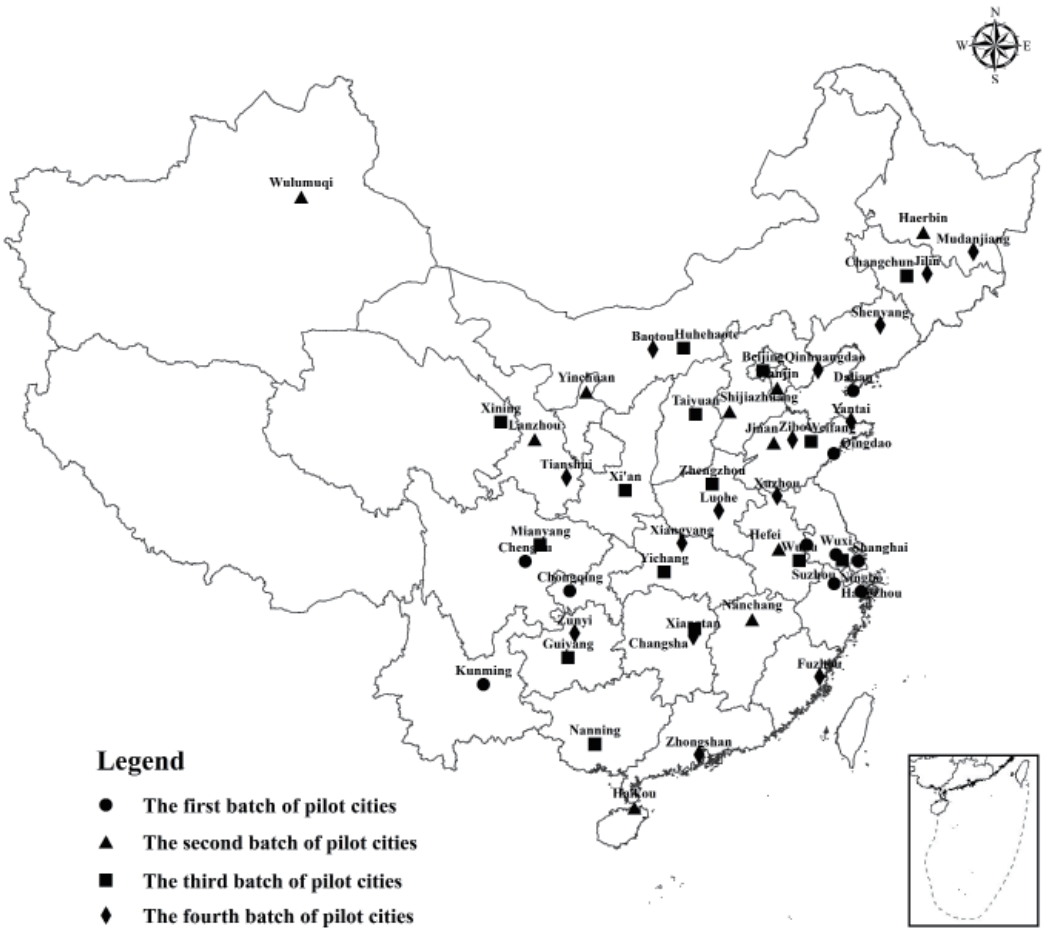

Figure 1. The pilot cites for creation of a Meat and Vegetable Distribution Traceability System in China since 2010

traceability have gradually been created. These include the National Platform for Tracking Food Safety (*http://www.chinatrace.org*), the Product Identification, Authentication, and Tracking System (*http://www.95001111.com*), and the Agricultural Product Quality and Safety Network (*http://www.safetyfood.gov.cn*). By November 2014, the National Platform for Tracking Food Safety will be able to track 34,364,423 items. This indicates that more manufacturers are aware of and participating in the system and that basic data are gradually being collected.

3.3. *Challenges facing FTS in China*

Although China's FTS has made some progress, including policy support, project implementation, and website design, these are still many challenges that need to be overcome.

3.3.1. *Lack of a complete database of the food supply chain*

The implementation of food traceability requires a substantial amount of valid information (*53*). The Japanese beef traceability system, as an example, was established in 2001 in response to mad cow disease. In May 2002, the Japanese government developed the "Beef ID Card" system. Consumers can use this card to learn information about the cattle breed, breeding, slaughtering, and distribution process online. In June 2002, the Japanese FTS extended to the rice and oyster industries. Consumers can learn specific information on a product's origin, producers, the pesticides and fertilizers used in the production process, and processing (*54*).

In contrast, data collection for an FTS in China faces several challenges: *i*) The supply chain is mainly based in small workshops, and numerous participants are widely distributed and highly mobility, so summarizing required information is very difficult; *ii*) The types of foods consumed and frequency with which they are consumed vary widely, and companies shift between upstream and downstream so each link is not closed, resulting in a lack of data on food raw materials, the production process, and storage and transportation; *iii*) Logistics information services and technology are lagging and there is a lack of a specialized platform on which to exchange food technology and no mechanism by which to exchange information technology, so establishing a complete database of the supply chain will be difficult. Given the severe shortage of basic data, a website providing information on food traceability cannot be created (*55*).

3.3.2. *An incomplete system of laws and regulations fails to clearly define responsibilities*

Laws must be improved and responsibilities for regulating food safety must be clarified to create an FTS. The EU implemented centralized management of food safety with separate decision-making sections, administrative departments, and departments for risk analysis. The Consumers, Health, and Food Executive Agency of the European Commission is a decision-making body that oversees food safety, and the Food and Veterinary Office (FVO) is a major driver of policies on food safety. In order to provide scientific support to the decision-making body, the EU set up the Food Safety Authority as an independent organization to transparently manage food safety and provide related technical support. The EU issued the White Paper on Food Safety and enacted food hygiene legislation, the General Food Law, as well as a variety of laws and regulations related to food and feed.

In China, supervision of food quality and safety is segmented into multiple sectors because of dispersed farming (*56*). Currently, the agencies regulating food safety in China have the following main features: *i*) most regulators track food "from farm to table" (*57*); *ii*) the administrative levels managing food safety are horizontal, performing their duties within respective areas with relative independence (*58*); *iii*) the delegation of regulatory authority is left to administrative agencies (*59*). As an example, the Ministry of Agriculture (MOA), Bureau of Quality Supervision, and MOC are all involved in creating systems for tracking food quality and safety (*60*), but the lack of unified direction has resulted in different departments creating traceability systems with differing hardware and technologies and poor compatibility. This hampers the exchange and sharing of information and prevents the traceability system from operating at its true capacity.

3.3.3. *Lagging technological research into FTS*

The West has advanced technical support for traceability systems (*37*). As an example, the EU has established a special system to track genetically modified food. This system requires participants to record information on the production and processing of genetically modified food and to retain that information for at least five years (*61*).

Although bar codes and RFID have long been used in industry, these approaches are not yet widely used in the food industry in China, limiting the ability of the Safety Inspection Bureau to perform sampling inspections. Furthermore, there is a lack of a standardized network transmission protocol for communication among departments, red tape hampering the documentation of testing information among related departments, information leaks, and a lack of detailed screening (*59*).

4. Future perspectives on the FTS in China

4.1. *The systematic creation of an FTS*

In light of the theoretical basis for and practical use of FTS in the USA and Europe, the following aspects of the FTS in China need to be improved.

i) *Gradually improve websites providing information on food traceability*. In China, the responsibility for regulating food safety is shared by multiple departments, so management of food safety must be planned and coordinated to ensure that departments are connected and share resources. The Government needs to establish a website providing information on food traceability. A network of information on food traceability is the only official channel to educate the public and disseminate information on food safety regulations, and such a network is also the best way to manage and share information on food safety. Such an approach is essential to food safety (*62*).

ii) *Improve the system of laws and regulations and clarify responsibilities for regulation of food safety*. China needs a way to establish and perfect a legal framework to implement a mandatory traceability system based on administrative regulations and rules (*63*). In addition, additional laws and regulations need to be enacted, particularly with regard to information on traceability, requirements for companies, and delineation of regulatory requirements and responsibilities. China's FTS will be successful only if it is based on laws and regulations clearly stipulating legal obligations and responsibilities for production companies and regulators (*64*).

iii) *Promoting technological research related to traceability systems*. China's FTS is still in its early phase, so the Government should enhance research into tracking technology (*65*). The implementation of an FTS involves various technologies, and a traceability system is based on unified standards (*66*). The accuracy of the information communicated and the seamless connection of different databases can be achieved using unified encoding and standards on food information in order to facilitate quick traceability. Based on information standardization, the following technologies may support the sharing of information: data sharing technology (*67*), coding technology (*68,69*), RFID (*70*), network technology, GPRS and GIS technology (*71*), and bioinformatics techniques (*11*). Information can be shared using the aforementioned technologies, allowing the creation of an FTS.

4.2. *Perspectives on the development of agriculture through use of an FTS*

An FTS is a tool for quality and safety management (*72*). The advantage of an FTS is that it prevents the incidence of food safety hazards and it reduces

the impact of such incidents when they occur (*73*). Once national and local laws and regulations on food traceability have been enacted, the food industry will be left with little option but to implement a traceability system as part of its efforts to manage food safety. This covers all movement and processing in the food chain. Interest in FTS will benefit the global trade in food. The search for cost-effective technological innovations to facilitate FTS is an important challenge facing agriculture in the new globalized economy.

An FTS is also essential factor to facilitate the establishment of common standards and guidelines for management of food safety and quality (*74,75*). These standards concern the following areas: management of food quality, management of food safety, traceability of products, and data capturing technology and exchange of electronic data in commerce, industry, and administration. Standards on internal traceability refer to records kept by a business and are not specifically required. External traceability is the sharing of information among the different stakeholders of the supply chain, and standards for and methods of exchanging data are needed. Thus, growers, processors, middlemen, policymakers, stakeholders, and consumers all need to be aware of the requirements for creation of an FTS.

In conclusion, the FTS plays an important role in ensuring food safety in the face of serious challenges to food safety. China implemented a system track meat products in 2004 and China's FTS is still in the pilot stage. China's FTS has several flaws, such as the lack of a complete database of the supply chain, vague responsibilities of food safety regulators, and a lag in technology. Based on the experiences of developed regions such as the EU, the USA, and Japan, China should take the following actions in the future: i) improving websites providing information on food traceability; ii) defining the responsibilities of food safety regulators, and iii) enhancing the technological capacity to create a traceability system in order to create a complete FTS based on the sharing of information.

References

1. Iyengar V. Food safety measurement issues: Way forward. J Radioanal Nucl Chem. 2013; 297:451-455.
2. Tong XS, Wu Y. A review of studies on food safety traceability systems. Logistics Engineering and Management. 2010; 1:126-138. (in Chinese)
3. Smith GC, Tatum JD, Belk KE, Scanga JA, Grandin T, Sofos JN. Traceability from a US perspective. Meat Sci. 2005; 71:174-193.
4. Opara LU, Mazaud F. Food traceability from field to plate. Outlook on Agriculture. 2001; 4:239-247.
5. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002. Official Journal of the European Communities. *http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:031:0001:0024:EN:PDF* (accessed November 1, 2014)
6. Fang Y, Gao G, Fan XL, Chen HN. Research on food safety traceability system in China. Agricultural Quality & Standards. 2005; 02:37-39. (in Chinese)
7. CAC/GL 60-2006. Principles for Traceability/ Product Tracing as a Tool within a Food Inspection and Certification System. *https://www.google.com/url?q=http://www.codexalimentarius.org/download/standards/10603/CXG_060e.pdf&sa=U&ei=pl41U_2rAYTLrQfAoYGwDg&ved=0CAUQFjAA&client=internal-uds-cse&usg=AFQjCNELJ8xxCjerW3lCZ-qmX9MQ-TE0hw* (accessed November 15, 2014)
8. ISO 22000:2005. Food safety management systems - Requirements for any organization in the food chain. *http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=35466* (accessed November 8, 2014)
9. ISO 22005:2007. Traceability in the feed and food chain -- General principles and basic requirements for system design and implementation. *http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=36297* (accessed November 8, 2014)
10. Hu JY, Zhang X, Moga LM, Neculita M. Modeling and implementation of the vegetable supply chain traceability system. Food Control. 2013; 30:341-353.
11. Liu JH, Wang YS, Du J, Tian ZH. Study of a food traceability system based on the sharing of information. World Standardization & Quality Management. 2006; 12:32-35. (in Chinese)
12. Yang SX. Legal analysis of the "Fuyang inferior infant milk powder incident." China Anti-Counterfeiting Report. 2004; 04:44-46. (in Chinese)
13. The contamination of Chinese dairy products. *http://www.baidu.com/link?url=r3DK_8KSgM-vB3VmBycqhkAAFx7Fh4pRRiqjs2psgKAevudl3B6c4J8y3V5Yv4OlFMKlP02FciOhnvIZkHW3Ha* (accessed December 8, 2014)
14. Wang FY, Zhao YM, Zhang XY, Shang MH. General status of the creation of food safety traceability systems in China. Agriculture Network Information. 2008; 10:134-137. (in Chinese)
15. Crandall PG, O'Bryan CA, Babu D, Jarvis N, Davis ML, Buser M, Adam B, Marcy J, Ricke SC. Whole-chain traceability, is it possible to trace your hamburger to a particular steer, a U. S. perspective. Meat Science. 2013; 95:137-44.
16. Yin JF, Tao YL, Liu TB, Chen SM, Wu YH, Dong J, Tian SR, Ke LL. A preliminary study of the creation of food traceability systems. Journal of Anhui Agri. 2008; 36:11985-11987. (in Chinese)
17. Liu SH, Zheng HG, Hu HY, He PJ, Guo YQ. Establishment of a quality-safety traceability system for navel orange. Sci Verse Science Direct. 2012; 1:569-575.
18. Lavelli V. High-warranty traceability system in the poultry meat supply chain: A medium-sized enterprise case study. Food Control. 2013; 33:148-156.
19. Valeeva NI, Meuwissen MP, Lansink AG, Huirne RB. Improving food safety within the dairy chain: An application of conjoint analysis. Journal of Dairy Science. 2005; 4:1601-12.
20. Chen HH, Tian ZG. Comparative study on agricultural products traceability system at home and abroad. Market Modernization. 2007; 21:5-6. (in Chinese)
21. Teresa P, Giovanni M, Miguel A, Fernando G. Food Track & Trace ontology for helping the food traceability control. Journal of Food Engineering. 2014; 120:17-30.
22. Ma HW, Wang SX. Food safety conditions for creation of a meat traceability system. China Safety Science Journal.

2006; 11:4-9 (in Chinese).
23. Schwägele F. Traceability from a European perspective. Meat Science. 2005; 1:164-73.
24. Yamanouchi K, Yoshikawa Y. Bovine spongiform encephalopathy (BSE) safety measures in Japan. J. Vet. Med. Sci. 2007; 69:1-6.
25. Onodera T, Kim CK. BSE situation and establishment of Food Safety Commission in Japan. Journal of Veterinary Science. 2006; 1:1-11.
26. Sugiura K, Onodera T. Cattle traceability system in Japan for bovine spongiform encephalopathy. Veterinaria Italiana. 2008; 3:519-26.
27. Hernández-Jover M, Schembri N, Toribio JA, Holyoake PK. Evaluation of the implementation of new traceability and food safety requirements in the pig industry in eastern Australia. Australian veterinary Journal. 2009; 10:387-96.
28. Britt AG, Bell CM, Evers K2, Paskin R. Linking live animals and products: traceability. Revue Scientifique Technique. 2013; 2:571-82.
29. Ammendrup S, Barcos LO. The implementation of traceability systems. Revue Scientifique et Technique. 2006; 2:763-73.
30. Bhatt T, Zhang JJ. Food product tracing technology capabilities and interoperability. Journal of food science. 2013; 10:1111/1750-3841.
31. Rajić A, Waddell LA, Sargeant JM, Read S, Farber J, Firth MJ, Chambers A. An overview of microbial food safety programs in beef, pork, and poultry from farm to processing in Canada. Journal of Food Protection. 2007; 5:1286-94.
32. Stanford K, Stitt J, Kellar JA, McAllister TA. Traceability in cattle and small ruminants in Canada. Revue Scientifique Technique. 2001; 2:510-522.
33. Chander M, Subrahmanyeswari B, Mukherjee R, Kumar S. Organic livestock production: an emerging opportunity with new challenges for producers in tropical countries. Revue Scientifique technique. 2011; 3:969-83.
34. Murata H, Babasaki K, Saegusa T, Takemoto K, Yamada A, Ohta A. Traceability of Asian Matsutake, specialty mushrooms produced by the ectomycorrhizal basidiomycete Tricholoma matsutake, on the basis of retroelement-based DNA markers. Applied Environmental Microbiology. 2008; 7:2023-31.
35. Donnelly KA, Karlsen KM, Olsen P. The importance of transformations for traceability - A case study of lamb and lamb products. Meat science. 2009; 1:68-73.
36. Zheng CM, Bai L. Study on creation of a food safety commission. Food and Nutrition in China. 2011; 04:10-13.
37. Zhang HL, Sun XD, Liu YD. Food safety and technological implications of food traceability systems. Computer and Computing Technologies in Agriculture IV, PT 2. 2011; 345:1-10.
38. Koji S. Traceability system for agricultural products using RF-ID and mobile phones. Tsukuba, Japan, 2009; pp 2293-2301.
39. Josa A, Luis A. Traceability as a strategic tool to improve inventory management: A case study in the food industry. Int. J. Production Economics. 2009; 118:104-110.
40. Xu YG. For an explanation of GB/T 6583 - ISO 8402 <Quality management and quality assurance terms>. China Standards Review. 1995; 03:17-18. (in Chinese)
41. The State Council. The State Council's decision to further enhance food safety efforts. *http://www.chinalaw.gov.cn/article/fgkd/xfg/xzfg/200504/20050400043097.shtml* (accessed November 26, 2014). (in Chinese)
42. Xiao L. The new policy of General Administration of Quality Supervision. Chian Fisheries. 2004; 10:5. (in Chinese)
43. Alfredo PM, Alejandro AM, Mira T, Piero F. Advanced traceability system in aquaculture supply chain. Journal of Food Engineering. 2014; 122:99-109..
44. Qi L, Zhang J, Mark X, Fu Z, Chen W, Zhang XS. Developing WSN-based traceability system for recirculation aquaculture. Mathematical and Computer Modelling. 2011; 53:2162-2172.
45. The NPC Standing Committee President. The People's Republic of China on animal products. *http://www.gov.cn/ziliao/flfg/2005-09/12/content_31030.htm* (accessed November 12, 2014) (in Chinese)
46. Yang ML, Liu J. The Comprehension on Food Safety Law of China. Chinese Journal of Food Hygiene. 2009; 3:193-197. (in Chinese)
47. Bai JF, Zhang CP, Jiang J. The role of certificate issuer on consumers' willingness-to-pay for milk traceability in China. Agricultural Economics. 2013; 44:537-544.
48. The State Council. The State Council's decision to enhance food safety efforts. *http://www.gov.cn/zwgk/2012-07/03/content_2175891.htm* (accessed November 18, 2014) (in Chinese)
49. Feng JY, Fu ZT, Wang ZQ, Xu M, Zhang XS. Development and evaluation on a REID-based traceability system for cattle/beef quality safety in China. Food Control. 2013; 31:314-325.
50. Lan HJ, Huang FQ, Lin ZK. The design of a food traceability system for 2008 Beijing Olympic Games. China Storage & Transport. 2008; 05:86-89. (in Chinese)
51. Ministry of Commerce. The Ministry of Commerce institutes a meat and vegetable distribution traceability system. *http://sczxs.mofcom.gov.cn/article/tupxw/201312/20131200409479.shtml* (accessed November 16, 2014) (in Chinese)
52. Ministry of Commerce. Speech by Chang XC at an on-the-spot meeting of the MVDTS. *http://sczxs.mofcom.gov.cn/article/cbw/ea/201303/20130300058312.shtml* (accessed November 18, 2014) (in Chinese)
53. Donnelly KAM, Thakur M, Sakai J. Following the mackerel – Cost and benefits of improved information exchange in food supply chains. Food Control. 2013; 33:25-31.
54. Yu WJ. Establish quality safety traceability system to improve the international competitiveness of agriculture products of China. Animal Science and Veterinary Medicine. 2004; 09:46-48. (in Chinese)
55. Liu XL, Guo B, Wei YM, Shi JL, Sun SM. Stable isotope analysis of cattle tail hair: A potential tool for verifying the geographical origin of beef. Food Chemistry. 2013; 140:135-140.
56. Zhang XS, Zhang J, Liu F, Fu ZT, Mu WS. Strengths and limitations on the operating mechanisms of traceability system in agro food, China. Food Control. 2010; 21:825-829.
57. Liu LM, Qian H, Gao YC, Wang D. Analysis and assessment of food traceability status in China. Advances in Chemical Engineering. 2012; 396:1353-1357.
58. Qian YZ, Wang F, Gao S, Gao G. Regulation of the quality and safety of agricultural products in the new period. Agricultural Quality & Standards. 2010; 01:13-15. (in Chinese)
59. Hu YZ, Zhang Y. Some thoughts on perfecting China's

food traceability system. Guide of Sci-tech Magazine. 2013; 03:72-105. (in Chinese)
60. Zhan JC, Zhu YF, Min XL, Yang XY. Views on a food safety traceability system. Journal of Anhui Agricultural Sciences. 2009; 28:13907-13909. (in Chinese)
61. She LN, Li ZM, Pan RC. Comparing evolution of the safety policy on genetically modified food in the United States and the European Union. Biotechnology Bulletin. 2011; 10:1-6. (in Chinese)
62. Fang C, Zhao LD. Meat traceability system based on iris recognition research. China Safety Science Journal. 2008; 7:11-17. (in Chinese)
63. Li W. The creation of food traceability systems in developed countries and their spread to China. China Anti-Counterfeiting Report. 2012; 09:26-29. (in Chinese)
64. Du WB. A study of the legal system for food safety traceability. Social Sciences. 2011; 1:1-31. (in Chinese)
65. Zan LS, Zheng TC, Shen GL, Wang LG, Zeng XH. Design and Development of Quality Traceability Information Management System and Safety of the Beef Production's Entire Processes. Scientia Agricultura Sinica. 2006; 10:2083-2088 (in Chinese).
66. Zhang TT. A primer on creation of a network for information on food safety. China Food. 2009; 17:66-67. (in Chinese)
67. Li DL. Computer and Computing Technologies in Agriculture III. (Zhao CJ, eds.) Third IFIP TC 12 International Conference, Beijing, China, 2009; pp. 1-561.
68. Kun L, Thomasson JA, Lee KM, Shen MX, Ge YF, Herrman TJ. Printing data matrix code on food-grade tracers for grain traceability. Sci Verse Science Direct. 2012; 113:395-401.
69. Liang K, Thomasson JA, Shen MX, Armstrong PR, Ge Y, Lee KM, Herrman TJ. Ruggedness of 2D code printed on grain tracers for implementing a prospective grain traceability system to the bulk grain delivery system. Food Control. 2013; 33:359-365.
70. Liu S, Zhang DY, Zhang R, Liu B. Analysis on RFID operation strategies of organic food retailer. Food Control. 2013; 33:461-466.
71. Xiong BH, Fu RT, Lin ZH, Luo QY, Yang L, Pan JR. A Solution on Pork Quality Traceability from Farm to Dinner Table in Tianjin City, in China. Agricultural Sciences in China. 2009; 9:147-156.
72. Banati D. European perspectives of food safety. J Sci Food Agric. 2014; 10:1002.
73. Fabrizio D, Paolo G. Food traceability systems: Performance evaluation and optimization. Computers and Electronics in Agriculture. 2011; 1:139-146.
74. Techane B, Girma G. Food traceability as an integral part of logistics management in food and agricultural supply chain. Food Control. 2013; 33:32-48.
75. Zhang XS, Lv SY, Xu M, M WS. Applying evolutionary prototyping model for eliciting system requirement of meat traceability at agribusiness level. Food Control. 2010; 21:1556-1562.

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Review

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The advantages of using traditional Chinese medicine as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer

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Summary **Recent studies indicate that Traditional Chinese medicine (TCM) can play an important role in the whole course of cancer treatment such as recovery stages of post-operative, radiotherapy or chemotherapy stages instead of only terminal stage of cancer. In this review, we have summarized current evidence for using TCM as adjuvant cancer treatment in different stages of cancer lesions. Some TCMs (*e.g.*, TJ-41, Liu-jun-zi-tang, PHY906, Coumarin, and Aescine) are capable of improving the post-operative symptoms such as fatigue, pain, appetite, diarrhea, nausea, vomiting, and lymphedema. Some TCMs (*e.g.*, Ginseng, Huang-Qi, BanZhiLian, TJ-48, Huachansu injection, Shenqi fuzheng injection, and Kanglaite injection) in combination with chemo- or radio-therapy are capable of enhancing the efficacy of and diminishing the side effects and complications caused by chemo- and radiotherapy. Taken together, they have great advantages in terms of suppressing tumor progression, relieving surgery complications, increasing the sensitivity of chemo- and radio-therapeutics, improving an organism's immune system function, and lessening the damage caused by surgery, chemo- or radio-therapeutics. They have significant effects on relieving breast cancer-related lymphedema, reducing cancer-related fatigue and pain, improving radiation pneumonitis and gastrointestinal side effects, protecting liver function, and even ameliorating bone marrow suppression. This review of those medicines should contribute to an understanding of Chinese herbal medicines as an adjunctive therapy in the whole course of cancer treatment instead of only terminal stage of cancer, by providing useful information for development of more effective anti-cancer drugs and making more patients "survival with cancer" for a long time.**

Keywords: Traditional Chinese medicine (TCM), adjunctive therapy, the whole course of cancer treatment, post-operation, chemotherapy, radiotherapy

1. Introduction

Cancer is redefined as a chronic health problem like hypertension and diabetes by the World Health Organization (WHO) and is increasing fast in incidence in all regions of the world. It is predicted to be a worldwide important cause of morbidity and mortality in the next few decades. By 2020 in the world approximately 24.6 million people will live with cancer with about 12.5% of all deaths attributable to cancer, and in China approximately 3.12 million per year or 8,550 new cancer cases per day will emerge with a death toll of 2.5 million; furthermore, the chance of suffering from a malignant tumor is about 22% for a person in his life (*1,2*). Thus, in the coming years it is still a huge challenge for cancer prevention and therapy in the world especially in low and middle-income countries such as China.

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Surgery, chemotherapy, radiotherapy, targeted therapy and immunotherapy are examples of anti-cancer therapies currently being utilized for controlling tumor growth, prolonging survival time, and improving quality of life to some extent. However, these therapies either alone or in combination have been shown to have numerous limitations and drawbacks: (*i*) Given poor diagnosis and other factors, most cancer patients are diagnosed too late to undergo surgery; even with the surgical indications, a series of complications might occur postoperatively such as bleeding, infection, bile reflux, and lymphedema, and postoperative recurrence and metastasis is quite common in patients who have had a resection. (*ii*) Chemotherapy and radiotherapy are still major postoperative adjunctive therapies or preferred therapies for patients with malignant tumors in middle and advanced stages, however, there are many side effects and complications such as myelosuppression, gastrointestinal tract reaction, cardiac damage, liver and renal function, or local radiation damage; in addition, cancer cells have ability to develop resistance to these conventional therapeutics over time and some cancers are insensitive to chemotherapy or radiotherapy. (*iii*) Targeted therapy as a newer type of cancer treatment which can more precisely identify and attack cancer cells, however, some drugs target substances that are more common on cancer cells but are also found on healthy cells and can affect healthy cells, too, causing side effects (*e.g.*, high blood pressure, damage to liver, kidneys, or heart, allergic reactions) (*3*); in addition, the price of targeted drugs is so high that most patients cannot afford them. (*iv*) Immunotherapy is another new approach including cytokine infusions, cancer vaccines, and T cell therapy. It can stimulate immune cells to enhance their anticancer activity (*4*); however, the advantages of immunotherapy on improving the patient's quality of life and prolonging survival time are still unclear currently; furthermore, in addition the price of immunotherapy is so high that most patients cannot afford it. Therefore, more effective or adjunctive therapies must be soon developed for cancer prevention and treatment.

With development of medicine and update of knowledge, cancer therapy has come into a diversified comprehensive treatment stage. Many scholars put forward the concept of "survival with cancer", and they insist controlling cancer and causing cancer cells to "be static" and "hibernate" for a long time, is better than striving to reduce the lump and completely kill all cancer cells (*5*). In the process of "survival with cancer", traditional Chinese medicine (TCM) might play an important role.

TCM, as an important component of complementary and alternative medicine, evolved over thousands of years with its own unique system of theories, diagnostics and therapies in Asian countries, especially China. In the world including Western countries, TCM has been increasingly used in the last decades and has become well known for its significant role in preventing and treating cancer. It is estimated that the United States National Cancer Institute (NCI) spends around $120 million each year on TCM related research projects (*6*). According to conventional views, we believe that TCM possesses advantages as an adjuvant therapy to alleviate cancer symptoms at terminal stages when Western medicine treatments cannot offer any other treatment options. However, recent studies indicate that TCM can play an important role in the whole course of cancer prevention and treatment such as recovery stages post-operation, and when undergoing radiotherapy or chemotherapy (*7*). It may be capable of preventing tumorigenesis, shrinking or stabilizing tumors, reducing tumor recurrence and metastasis. It may also be capable of protecting cancer patients from suffering from complications, increasing sensitivity or reducing side-effects of conventional treatment, and improving quality of life and survival (*8*).

Since cancer is defined as a chronic disease like hypertension and diabetes, the role of TCM in treating chronic diseases especially cancer should not be overlooked. Thus, an understanding of TCM is needed by physicians and other health care providers. In this review, we will summarize the current evidence for using TCM as adjuvant cancer treatment in different stages of cancer lesions. First, some single TCMs and traditional herbal formulations which are commonly prescribed by traditional Chinese physicians for cancer patients will be summarized. Second, some TCM preparations which possess properties such as anti-cancer, improving immunity and protecting bone marrow, and are commonly used in clinical cancer treatment will be summarized. Third, the advantages of TCMs in different stages of cancer treatment such as recovery stages post-operation, and when undergoing radiotherapy or chemotherapy will be summarized. In a word, we hope this review should contribute to an understanding of TCM as adjuvant treatment for different stages of cancer, providing useful information for development of more effective anti-cancer drugs and making more patients "survival with cancer" for a long time.

2. Single TCMs commonly prescribed by traditional Chinese physicians for cancer treatment

Based on traditional Chinese medicine classic theory, the formation of tumors are usually due to deficiency of vital energy or Qi and blood in the body, combined with some pathogenic factors such as external evil invading, emotional abnormality, overeating and so on, leading to Qi stagnation, blood stasis and heat- and dampness-induced toxicity blocking in the body, and then forming a lump or lumps for many days (*9,10*). According to the above theory, TCMs which are used for cancer treatment usually fall into three categories: the first one with the properties of spiriting vital energy (Qi and blood), the second one with the properties of promoting blood

circulation and removing blood stasis, and the third with properties of clearing heat and detoxifying (*11*). Numerous studies have shown that many TCMs (*e.g.*, *Ginseng Radix*, *Radix astragali*, *Radix Codonopsis*, and *Poria cocos*) with properties of spiriting vital energy play important roles in cancer treatment with the basis summarized as follows: (*i*) improving immune system function; (*ii*) protecting hematopoiesis of bone marrow; (*iii*) harmonizing gastrointestinal function (*12,13*). In addition, numerous studies have shown that many TCMs with properties of promoting blood circulation and removing blood stasis (*e.g.*, *Angelica sinensis Radix*, and *Curcuma longa*), or clearing heat and detoxifying (*e.g.*, *Hedyotis diffusa willd*, and *Scutellaria barbata*), also play important roles in cancer treatment involving anti-proliferation, anti-inflammation, anti-oxidation, anti-angiogenesis, anti-thrombotic, immune-modulation and so on (*7,11,12,14*). Thus, according to references and our clinical experience, we will choose some single TCMs commonly prescribed by traditional Chinese physicians for cancer treatment and give them a brief introduction especially regarding clinical studies (Table 1).

2.1. *Some single TCMs with properties of spiriting vital energy*

2.1.1. *Ginseng Radix*

Ginseng Radix (Ren-Shen in Chinese or Ginseng in Korea) is a well-known and popular TCM, which is believed to be the king of the herbs in the Orient particularly in China, Korea and Japan. It has been used for several thousand years with mysterious powers as a tonic, prophylactic and restorative agent (*15*). *Ginseng Radix* is reported to contain many active constituents including ginsenosides, essential oils, peptidoglycans, polysaccharides, nitrogen-containing compounds, fatty acids and phenolic compounds (*16*). Modern pharmacological studies have shown that *Ginseng Radix* and its active constituents have antitumor, antioxidant, immunomodulation, anti-ulcer, anti-adhesive, antioxidant, hepatoprotective, hypoglycemic activities, and so on (*17*). They have been proposed to have a chemopreventive effect on lung, gastric, liver, pancreas, and colon cancers and so on in cells, animals or humans (*17-19*). An epidemiological study indicated that patients taking ginseng had a 50% lower risk of cancer recurrence compared to patients not taking ginseng (*12*). In the following, we emphasize clinical research in recent years. An observational pilot study of cultivated wild Ginseng pharmaco-puncture (CWGP) was conducted at the East-West Cancer Center of Daejeon University (Daejeon, Korea) and the results indicated that CWGP showed potential as an effective treatment for advanced cancer patients on improving response and survival rate (*20*). A phase III trial was developed by Mayo Clinic Rochester (Rochester, USA) to evaluate the efficacy of Ginseng on cancer-related fatigue and the results indicated that 2,000 mg/daily of Ginseng showed great benefit for ameliorating cancer-related fatigue without any discernible toxicity (*21*). Moreover, Ginseng extract could help in protecting tissue damage from inflammatory cytokines (IL-2, IL-10, IL-12, TNF-alpha, and IFN-gamma) in children after chemotherapy, which might be associated with decreased late complications of childhood (*22*). In addition, Ginseng appears to be a promising radio-protector and is capable of attenuating the deleterious effects of radiation on normal human tissue, and especially for cancer patients undergoing radiotherapy, which might be associated with its anti-oxidation and immunomodulation properties (*23*).

2.1.2. *Radix astragali*

Radix Astragali (Huang-Qi in Chinese), is the dried root of *Astragalus membranaceus Bge. Var*. mongholicus, and one of the most famous and frequently used herbal medicines and healthy food supplements used as a tonic. It has been used for over 2000 years in TCM prescriptions for the treatment of animal bites and poisons, wounds and burns, nephritis, diabetes, albuminuria, hypertension, cirrhosis, and various cancers (*24*). Chemical constituent investigations on *Radix Astragali* indicated that it contains several bioactive constituents including, isoflavonoids, triterpenoid saponins, polysaccharides, amino butyric acids and various trace elements; of which triterpenoid saponins represent the major beneficial constituents responsible for the bioactivities and efficacies of *Radix Astragali* on human health (*25*). Modern pharmacological studies have shown that *Radix Astragali* and its active constituents possess antioxidant, antitumour, hepatoprotective, anti-diabetic, antimicrobial, antiviral and immune enhancement activities (*25,26*). They have been proposed to have an anticancer effect on breast, gastric, liver, colon, lung cancers and so on in cells, animals or humans. *Via* inhibiting cancer cell proliferation, regulating immunity, suppressing angiogenesis, or reducing side effects of chemotherapeutics, they play important roles in cancer therapy (*26-28*). In the following, we emphasize clinical research in recent years. Some studies indicated that decoctions of Huang-Qi compounds could reduce the proportion of patients who experienced nausea and vomiting and decrease the rate of leucopenia in colorectal cancer patients treated with chemotherapy. Huang-Qi compounds are also associated with increases in the proportions of T-lymphocyte subsets (CD3, CD4 and CD8). That is to say, Huang-Qi compounds may play important roles in stimulating immunocompetent cells and decreasing side effects in patients treated with chemotherapy (*29*). In addition, to investigate the effect of Huang-Qi injection on short-term prognosis in childhood with acute lymphoblastic leukemia (ALL), a retrospective analysis was performed

Table 1. Single TCMs commonly prescribed by traditional Chinese physicians for cancer treatment

Common name	Other names	Efficacy according to TCM theory	Major active ingredients	Biological activity	Preclinical and/or clinical evidence of anticancer activity	*Ref.*
Ginseng Radix	Ren-Shen in Chinese, Ginseng in Korea	As a tonic, prophylactic and restorative agent with the efficacy of spiriting vital energy	Ginsenosides, essential oil, peptidoglycans, polysaccharides, nitrogen-containing compounds, fatty acids and phenolic compounds	Antitumor, antioxidant, immunomodulation, anti-ulcer, anti-adhesive, antioxidant, hepatoprotective, hypoglycemic	*Clinical*: *i*) Ameliorate cancer-related fatigue without any discernible toxicity; *ii*) Protect tissue damage from inflammatory cytokines in children after chemotherapy; *iii*) Attenuating the deleterious effects of radiation	*15-23*
Radix Astragali	Huang-Qi in Chinese	As a tonic with the efficacy of spiriting vital energy	Isoflavonoids, triterpenoid saponins, polysaccharides, amino butyric acids and various trace elements	Antitumor, antioxidant, hepatoprotective, anti-diabetic, antimicrobial, antiviral, immunomodulation	*Clinical*: *i*) Improve gastrointestinal side effects, and ameliorate bone marrow suppression in colorectal cancer patients with chemotherapy; *ii*) Increase the proportions of T-lymphocyte subsets; *iii*) Decrease infectious rate in ALL children with chemotherapy	*24-31*
Radix Codonopsis	Dang-Shen in Chinese	As a tonic with the efficacy strengthening spleen and nourishing lung	Sterol, triterpenes, glycoside, alkaloid, and polysaccharide	Antioxidant, antimicrobial, antitumor, immunomodulation	*Clinical*: Improve clinical symptoms, signs and quality of life of liver cancer patients	*32-34*
Poria cocos	Fu-Ling in Chinese, Hoelen in Japanese	As a tonic with the efficacy of strengthening spleen	Triterpenes, polysaccharides, steroids, amino acids, choline, and histidine	Antitumor, anti-inflammatory, antioxidant, antiviral, immunomodulation	*Clinical*: *i*) Improve tumor response rate, one year survival and quality of life of patients when combined with FOLFOX4 regimen chemotherapy; *ii*) Alleviate chemotherapy-related adverse events including neutropenia, nausea and vomiting, and neurotoxicity	*32, 35-39*
Hedyotis diffusa willd	Bai-Hua-She-She-Cao in Chinese	With the efficacy of clearing heat and detoxifying	Triterpenes, polysaccharide and anthraquinones	Antitumor, chemopreventive, hepatoprotective, antiviral, antibacterial, antidiabetic, antioxidant, gastroprotective	*Preclinical*: *i*) Upregulate G0/G1 phase arrest and induce apoptosis in human leukemia cells; *ii*) inhibit colorectal cancer growth in mouse though STAT3 pathway; *Clinical*: Is the most commonly prescribed single Chinese herbs for colon cancer patients post-surgery in Taiwan	*11,32, 40-42*
Scutellaria barbata	Ban-Zhi-Lian in Chinese, Banjiryun in Korea	With the efficacy of clearing heat and detoxifying	Phenolic acids, flavonoids, triterpene acids and sterol glucosides	Antitumor, anti-inflammatory	*Preclinical:* Induce colon cancer cell apoptosis, inhibit cell proliferation and tumor angiogenesis via modulation of several pathways, including Akt, p53, STAT3, Erk, and p38 signaling pathways; *Clinical*: Stable disease and improve the side effects of chemo- or radio-therapy such as dysfunction of liver, diarrhea, fatigue, and pain in breast cancer patients	*32, 43-46*
Angelicae Sinensis Radix	Dang-Gui in Chinese, Dong Quai in English, Toki in Japanese, Tanggwi in Korea	With the efficacy of promoting blood circulation and removing blood stasis	Ligustilide, butylphthalide, senkyunolide A, phthalide dimers, ferulic acid, coniferyl ferulate, polyacetylenes	Antitumor, neuroprotective, immunomodulation, cardiovascular protective	*Preclinical:* Enhance radio-sensitivity of radiation in human liver cancer cells by modulating caspase-dependent apoptosis protein	*32, 47-49*
Curcuma longa	Jiang-Huang in Chinese	With the efficacy of promoting blood circulation and removing blood stasis	Curcumin, demethoxycurcumin, and bisdemethoxycurcumin,	Antitumor, anti-inflammatory, antioxidant	*Preclinical*: Inhibits carcinogenesis and modulates chemo-resistance and radio-resistance	*32, 50-51*

on the clinical data of 105 children newly diagnosed with ALL. The results indicated that Huang-Qi injection combined with chemotherapy had an enhanced anti-tumor effect and could improve the short-term prognosis and clinical outcome in children with ALL. Huang-Qi injection could reduce bone marrow suppression induced by chemotherapy or irritation of hematopoiesis of bone marrow and increase granulocyte level, and then decrease infectious rate in ALL children (*30*). In addition, it was reported that some TCM decoction of which Huang-Qi was a main ingredient could effectively prevent and reduce the occurrence and intensity of acute peripheral neuro-sensory toxicity caused by oxaliplatin (*31*).

2.1.3. *Radix Codonopsis*

Radix Codonopsis (Dang-Shen in Chinese) is the dried root of *Codonopsis pilosula* (*Franch*.) *Nannf*., and belongs to the family Campanulaceae. As one of the most popular TCMs in China, Japan and Korea, *Radix Codonopsis* has been used for thousands of years for treatment of dyspepsia, poor appetite, fatigue and psychoneurosis with properties of making the middle warmer, invigorating the spleen and nourishing the lung according to TCM theory (*32*). In many case, it was utilized primarily as a substitute of the much more costly *Panax ginseng* and was therefore called the poor man's ginseng. Some reports indicated that the main bioactive constituents were sterols, triterpenes, glycosides, alkaloids, polysaccharides and so on. Modern pharmacology research indicated that *Radix Codonopsis* and its active constituents had the functions of antioxidant, antimicrobial, antitumor and improving immunity (*33*). According to basic and clinical studies, they were reported to play important roles in cancer therapy *via* inhibiting cancer growth, regulating immunity, suppressing invasion and migration, or reducing side effects of chemotherapeutics. *Radix Codonopsis* could induce spleen lymphocyte proliferation, inhibit the decline of IL-2 levels in serum, and improve immune function in mice. Polysaccharides from *Radix Codonopsis* exhibited significant inhibitory effects on tumor cell growth, invasion, and migration of human epithelial ovarian cancer cells (*34*). Although *Radix Codonopsis* is commonly prescribed by traditional Chinese physicians for cancer treatment in the clinic, there are few clinical studies published currently in English. There was a report that some TCM decoction of which *Radix Codonopsis* was a main ingredient could effectively improve clinical symptoms, signs and quality of life of liver cancer patients. Thus, more rigorous trials are needed to confirm the efficacy of *Radix Codonopsis* and its active constituents on cancer therapy in the future.

2.1.4. *Poria cocos*

Poria cocos (Fu-Ling in Chinese or Hoelen in Japanese), is a kind of edible and pharmaceutical mushroom, and usually grows around the roots of old, dead pine trees. It is the dried sclerotium of the fungus *Poria cocos* (*Schw.*) *Wolf* (*Fam. Polyporaceae*). It is a well-known TCM used to treat diabetes, dysentery, chronic fatigue syndrome, diarrhea, dizziness, edema, insomnia, kidney problems, nervousness, urination problems, and weakness (*32*). According to the record of the Chinese Pharmacopoeia (2010 edition), approximately 10% of TCM preparations contain *Poria cocos*. The chemical composition of *Poria cocos* mainly includes triterpenes, polysaccharides, steroids, amino acids, choline, histidine, *etc* (*35*). Modern pharmacological studies showed that *Poria cocos* and some of its active constituents had functions of anticancer, anti-inflammatory, antioxidant, antiviral and improved immunity (*35,36*). According to basic and clinical studies, *Poria cocos* and some of its active constituents were reported to play important roles in cancer therapy *via* inhibiting cancer growth, regulating immunity, suppressing invasion and migration, or reducing side effects of chemotherapeutics in leukemia, lung, colorectal cancers and so on (*37-39*). Triterpenes from *Poria cocos* could induce apoptosis of leukemia (HL60) and lung (A549) cells *via* mitochondrial or death receptor pathways (*37*). Triterpenes from Poria cocos also could suppress growth and invasiveness of pancreatic cancer cells through the downregulation of MMP-7 (*38*). In addition, a systematic review evaluated clinical evidence for addition of herbal medicines to the FOLFOX 4 regimen chemotherapy for advanced colorectal cancer in the clinic. The results indicated that *Poria cocos* as one of the most frequently used herbs combined with the FOLFOX4 regimen chemotherapy could effectively improve tumor response rate, one year survival and quality of life of patients. It also could alleviate chemotherapy-related adverse events including neutropenia, nausea and vomiting, and neurotoxicity, compared to the FOLFOX4 regimen chemotherapy alone (*39*).

2.2. *Some single TCMs with properties of clearing heat and detoxifying*

2.2.1. *Hedyotis diffusa willd*

Hedyotis diffusa willd (Bai-Hua-She-She-Cao in Chinese) has been known as an ingredient of popular herbal teas and a famous TCM for a long time in the Orient and tropical Asia. Three major classes of compounds, including triterpenes, polysaccharides and anthraquinones, have been reported as the main bioactive compounds from this herb (*32*). *Hedyotis diffusa willd* and some of its active constituents have a wide variety of reported pharmacological activities, including anticancer, chemopreventive, hepatoprotective, antiviral, antibacterial, antidiabetic, antioxidant, and gastroprotective properties (*40*). It is widely applied in

the treatment of inflammations such as appendicitis, urethritis, and bronchitis, hepatitis, tonsillitis, and sore throat due to its antibacterial activity (*32*). Recently, *Hedyotis diffusa willd* and some of its active constituents have gained increasing attention as an antitumor herb in liver, lung, prostate, colon, brain, pancreas and other cancers (*40*). The ethanol extract of *Hedyotis diffusa willd* could upregulate G0/G1 phase arrest and induce apoptosis in human leukemia cells by modulating caspase cascade signaling and altering the gene levels related to cell growth, signal transduction, apoptosis, cell adhesion, cell cycle, DNA damage and repair, transcription and translation by cDNA microarrays (*41*). The ethanol extract of Hedyotis diffusa willd also could inhibit colorectal cancer growth in mice though the STAT3 pathway without apparent adverse effects (*42*). In addition, a cross-sectional study on the prescription patterns and reasons for the use of TCM for colon cancer patients post-surgery in Taiwan were analyzed and the results indicated that *Hedyotis diffusa willd* was the most commonly prescribed single Chinese herbs for colon cancer (*11*). However, more rigorous trials are needed to confirm the efficacy of *Hedyotis diffusa willd* and its active constituents on cancer therapy in the future.

2.2.2. *Scutellaria barbata*

Scutellaria barbata (Ban-Zhi-Lian in Chinese or Banjiryun in Korea) is a perennial herb which is natively distributed throughout Korea and southern China and as a well-known herb commonly used in folk medicines. It has been used as an anti-inflammatory and an antitumor agent to treat hepatitis, liver cirrhosis, pulmonary abscess, appendicitis, osteomyelitis, hematemesis, epistaxis, dysentery, jaundice, sore throat, carbuncle, scrofula and malignant tumors for many years. The chemical composition of *Scutellaria barbata* mainly includes phenolic acids, flavonoids, triterpene acids and sterol glucosides (*32*). Recently, *Scutellaria barbata* and its active constituents have gained increasing attention for its use as an anti-tumor herb in human breast cancer, prostate cancer, leukemia, hepatoma carcinoma, uterine carcinoma, cervical carcinoma, lung carcinoma, skin cancer, colorectal carcinoma, renal adenocarcinoma, nasopharyngeal carcinoma and oral epidermoid carcinoma (*43*). On the basis of cDNA microarray analysis, the mechanism underlying the anticancer activity of *Scutellaria barbata* appears to involve DNA damage, cell cycle control, nucleic acid binding, protein phosphorylation and dephosphorylation, and dendritic cell functions (*44*). The ethanol extract of *Scutellaria barbata* was able to induce colon cancer cell apoptosis, inhibit cell proliferation and tumor angiogenesis *via* modulation of several pathways, including Hedgehog, Akt, p53, STAT3, Erk, and p38 signaling pathways and alteration of the expression of multiple critical target genes such as, Bcl-2, Bax, Cyclin D1, CDK4, and p21 in *in vitro* and *in vivo* studies (*45*). In addition, a phase 1B dose escalation trial of *Scutellaria barbata* (BZL101) for patients with metastatic breast cancer was conducted by Memorial Cancer Institute (Hollywood, FL, USA). The results indicated that oral administration of BZL101 was safe, well tolerated, and showed promising clinical evidence of anticancer activity in patients. It could effectively stabilize disease and improve the side effects of chemo- or radio-therapy such as dysfunction of liver, diarrhea, fatigue, and pain (*46*).

2.3. *Some single TCMs with properties of promoting blood circulation and removing blood stasis*

2.3.1. *SAngelica sinensis Radix*

Angelicae Sinensis Radix (Dang-Gui in Chinese, Dong Quai in English, Toki in Japanese, or Tanggwi in Korea), is the root of *Angelica sinensis* (*Oliv.*) Diel and has been used for thousands of years worldwide. Since, it has both properties of nourishing blood and promoting blood circulation and removing blood stasis, we will give it a brief introduction in this part. It is usually used to strengthen heart, lung, and liver meridians, as well as lubricate the bowel (*32*). Furthermore, it is named "female ginseng" because of its use for various health conditions of women including dysmenorrhea, pelvic pain, recovery from childbirth and menopausal symptoms (*47*). Over 70 compounds have been identified from *Angelicae Sinensis Radix*, including essential oils such as ligustilide, butylphthalide and senkyunolide A, phthalide dimers, organic acids and their esters such as ferulic acid, coniferyl ferulate, polyacetylenes, vitamins and amino acids (*48*). *Angelicae Sinensis Radix* and some of its active constituents have a wide variety of reported pharmacological activities, including antitumor, neuroprotective, immunomodulatory and cardiovascular protective functions (*47*). Recently, some reports indicated that *Angelicae Sinensis Radix* and some of its active constituents *(e.g.*, Z-Ligustilide) exhibited great anticancer effects in liver, prostate, and oral cancers *via* inducing apoptosis, revising multidrug resistance or modulating lymphocyte activity and improving immunity (*47-49*). In addition, the decoction containing *Radix Angelicae Sinensis* could enhance radiosensitivity of radiation in human liver cancer cells by modulating caspase-dependent apoptosis protein (*49*). *Although Radix Angelicae Sinensis* is commonly prescribed by traditional Chinese physicians for cancer treatment in the clinic, there are few clinical studies published currently in English. Thus, more rigorous trials are needed to confirm the efficacy of *Radix Angelicae Sinensis* and its active constituents on cancer therapy in the future.

2.3.2. *Curcuma longa*

Curcuma longa (Jiang-Huang in Chinese), a member

of the ginger family and commonly known as turmeric, is a culinary spice and therapeutic used in Asia for thousands of years to induce color and flavor in food as well as to treat a wide array of diseases such as diabetes, atherosclerosis, acne, jaundice, dysmenorrheal, as well as, cancer (*32*). Some reports indicated that among all spices, *Curcuma longa* has been proven for its better anticancer potential. Curcuminoids, for example, curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are major components of *Curcuma longa* and they are reported to have numerous pharmacological activities including anti-inflammatory, antioxidant, and anticancer properties. Curcumin, a yellow natural polyphenol, is traditionally used as a spice and coloring in foods and is an important ingredient in curry. Recently, among curcuminoids, the anticancer properties of curcumin have been drawn more attention from researchers. Many studies indicated that its anticancer molecular mechanisms involved cell cycle arrest, angiogenesis and metastasis by regulating molecules such as, Cdk inhibitor, p21/WAF/CIPI, p53, NFκB, STAT-3, c-myc, COX-2, NOS, Cyclin D1, TNFα, MMP-9, bFGF, EGF, GCSF, IL-8, PDGF, TGFα, TNF, VEGF, fibronectin, vitronectin, and collagen. It also has a synergistic effect in combination with chemotherapeutics like cyclophosphamide, doxorubicin, mitomycin, *etc*. (*50*). In addition, extensive preclinical research within the last decade in cell culture and in animals has revealed that curcumin can sensitize tumors to different chemotherapeutic agents including doxorubicin, 5-FU, paclitaxel, vincristine, oxaliplatin, etoposide and so on in numerous cancers (*e.g.*, breast, colon, pancreas, gastric, liver, blood, lung, prostate, and ovary) (*51*). Similar studies have also revealed that curcumin sensitizes a variety of tumors including, glioma, neuroblastoma, cervical carcinoma, epidermal carcinoma, prostate cancer, and colon cancer to radiotherapy. Moreover, curcumin has also been shown to protect normal organs such as liver, kidney, oral mucosa, and heart from chemotherapy and radiotherapy-induced toxicity. However, there are few clinical studies about *Curcuma longa* and its active compound curcumin in cancer therapy published currently in English. Thus, more rigorous trials are needed to confirm the efficacy of *Curcuma longa* and its active constituents on cancer therapy in the future.

3. Traditional Chinese herbal formulations commonly prescribed by traditional Chinese physicians for cancer treatment

Traditional Chinese herbal formulations, or Kampo in Japanese, are a combination of compatible herbs in fixed dosages, most of which come from classical or well known Chinese textbooks of medicine like "Shang Han Lun" and "Jin Gui Yao Lue" (*52*). Currently, several traditional Chinese herbal formulations, such as Bu-zhong-yi-qi-tang, have been found to have a potentially beneficial effect for treating various cancers. A brief outline of the anticancer pharmacology of some traditional Chinese herbal formulations commonly prescribed by traditional Chinese physicians for cancer treatment is presented below (Table 2).

3.1. *Bu-zhong-yi-qi-tang*

Bu-zhong-yi-qi-tang (Hochuekki-to or TJ-41 in Japanese, or Bojungikki-Tang in Korean) is a classical formulation widely used in China, Japan, and South Korea for a long time. It was found by Dongyuan Li (A.D. 1,180-1,251, Jin and Yuan dynasties) as a tonic for the treatment of weakness including fatigue, visceroptosis, gastrointestinal motility disorder, and rectal prolapse due to chronic diarrhea and has been identified as an effective drug for the treatment of TCM spleen-qi deficiency in clinical practice. It contains 7 herbs including *Pinellia tuber*, *Scutellaria baicalensis*, *Zingiberis rhizoma*, *Zizyphi fructus*, *Coptidis rhizoma*, *Glycyrrhiza radix*, and *Panax ginseng* (*12*). Recently, much of the pharmacological research has shown that Bu-zhong-yi-qi-tang has potent immunomodulatory and anticancer properties. TJ-41 has a significant chemo-preventative effect on ovarian and liver cancer lines by inducing apoptosis or arresting the cell cycle (*12*). It could restore mitomycin C (MMC)-induced immunosuppression in mice by increasing the activity of bone-marrow cells and natural killer (NK) cells and preventing lethal HSV-1 infection (*53*). Preoperative administration of TJ-41 in patients with gastrointestinal malignancies showed that it could prevent surgical stress-induced immunosuppression by maintaining NK cell activity and inhibiting the elevation of stress mediators noradrenaline and IL-6 (*54*). In addition, TJ-41 might have beneficial effects on cancer-related fatigue and quality of life in cancer patients, and it also could reduce the extent of side effects such as leucopenia and intestinal damage and fatigue occurring as a result of radiation or chemotherapy to treat malignant tumors (*12,55*).

3.2. *Shi-quan-da-bu-tang*

Shi-quan-da-bu-tang (Juzentaiho-to or TJ-48 in Japanese) is a well-known Chinese herbal formulation first recorded in the Chinese Song Dynasty (about A.D. 1,200) and it comprises 10 herbs including *Ginseng radix*, *Astragali radix*, *Angelicae radix*, *Rehmanniae radix*, *Atractylodis lanceae rhizoma*, *Cinnamomi cortex*, *Poria, Paeoniae radix*, *Ligustici rhizoma* and *Glycyrrhizae radix* (*12*). It has been used for many years for the treatment of various kinds of disease such as anemia, rheumatoid arthritis, atopic dermatitis, chronic fatigue syndrome, and ulcerative colitis. Recently, TJ-48 has been reported to have antitumor effects and to modulate immune responses. It could reduce the side effects of

Table 2. Traditional Chinese herbal formulations commonly prescribed by traditional Chinese physicians for cancer treatment

Common name	Other names	Composition	Biological activity	Preclinical and/or clinical evidence of anticancer activity	*Ref.*
Bu-zhong-yi -qi-tang	Hochuekki-to or TJ-41 in Japanese, Bojungikki-tang in Korea	Includes 7 herbs: *Pinellia tuber, Scutellaria baicalensis, Zingiberis rhizoma, Zizyphi fructus, Coptidis rhizoma, Glycyrrhiza radix, Panax ginseng*	Antitumor, immunomodulation	*Preclinical*: *i*) Have significant chemo-preventative effects on ovarian and liver cancer lines by inducing apoptosis or arresting the cell cycle; *ii*) Restore mitomycin C-induced immunosuppression in mice; *Clinical*: *i*) Prevent surgical stress-induced immunosuppression; *ii*) Improve cancer-related fatigue and quality of life in cancer patients; *iii*) reduce side effects such as leucopenia, intestinal damage and fatigue induced by radiation or chemotherapy	*12, 53-55*
Shi-quan-da -bu-tang	Juzentaiho-to or TJ-48 in Japanese	Includes 10 herbs: *Ginseng radix, Astragali radix, Angelicae radix, Rehmanniae radix, Atractylodis lanceae rhizoma, Cinnamomi cortex, Poria, Paeoniae radix, Ligustici rhizoma Glycyrrhizae radix*	Antitumor, immunomodulation	*Preclinical*: Alleviate bone marrow suppression caused by TS-1 in mice; *Clinical*: *i*) Alleviating hematotoxicity among breast cancer patients undergoing chemotherapy; *ii*) Slow down the process of hepatocarcinogenesis and improve hepatic recurrence-free survival through the inhibition of Kupffer cell-induced oxidative stress in patients with HCC; *iii*) Regulate T cells through decreasing Foxp3+ Treg populations in advanced pancreatic cancer patients	*12, 56-59*
Huang-qin-tang	PHY906	Includes 4 herbs: *Scutellaria baicalensis Georgi, Paeonia lactiflora Pall, Glycyrrhiza uralensis Fisch, Ziziphus jujuba Mill*	Antitumor, anti-inflammatory	*Preclinical*: *i*) Enhance the antitumor efficacy of some anticancer drugs such as 5-FU and alleviate radio- or chemo-therapy induced side effects; *ii*) Regulate intestinal bacteria, alleviate diarrhea and reduce irinotecan-induced mortality in mice; *Clinical*: Provide a safe and feasible salvage therapy combined with capecitabine after gemcitabine failure for advanced pancreatic cancer	*12, 60-62*
Xiao-chai -hu-tang	Sho-sai-ko-to or TJ-9 in Japanese	Includes 7 herbs: *Bupleurum falcatum, Scutellaria baicalensis, Panax ginseng, Zizyphus jujube, Pinellia ternate, Zingiber officinale, Glycyrrhiza glabra*	Antitumor, anti-inflammatory antioxidant, immunomodulation, hepatoprotective, anti-hepatic fibrosis	*Preclinical*: Enhance immune regulation, inhibit angiogenesis and induce apoptosis of cancer cells *Clinical*: *i*) Prevent the development of HCC from hepatitis C virus-associated liver cirrhosis (HCV-LC) in the HCV-LC patients; *ii*) Decrease the incidence of stomatitis in cancer patients undergoing chemotherapy	*12, 63-67*

chemotherapy, radiation therapy and surgical treatment, and prevent various types of cancers (*e.g.*, breast, liver, brain and pancreatic cancer) or their metastasis according to numerous preclinical and clinical studies. TJ-48 was effective in alleviating bone marrow suppression caused by TS-1 (an oral anticancer drug containing a 5-fluorouracil derivative Tegafur) in mice (*56*). TJ-48 was also effective in alleviating hematotoxicity among patients with breast carcinoma receiving chemotherapy, without affecting the presentation of tumor markers (CEA and CA153) in the short term (*57*). Moreover, TJ-48 could slow down the process of hepatocarcinogenesis and improve hepatic recurrence-free survival through the inhibition of Kupffer cell-induced oxidative stress in patients with hepatocellular carcinoma (HCC) (*58*). TJ-48 also could increase regulatory activities in T cells through decreasing $Foxp3^+$ Treg populations in advanced pancreatic cancer patients, and this effect might lead to immune-augumentation for various combination therapies (*59*).

3.3. *Huang-qin-tang*

Huang-qin-tang is a classical traditional Chinese herbal formulation with four herbs (*Scutellaria baicalensis Georgi*, *Paeonia lactiflora Pall*, *Glycyrrhiza uralensis Fisch*, and *Ziziphus jujuba Mill*), and it was first recorded in "Shang Han Lun" which is one of the famous classics of TCM edited by a well-known Chinese physician during the Han Dynasty Zhongjing Zhang. It has been used for over 1800 years to treat a variety of gastrointestinal symptoms including diarrhea, nausea and vomiting, and abdominal cramps (*12,60*). PHY906 is a modified pharmaceutical preparation derived from the traditional herbal formulation Huang-qin-tang and it consists of the same four herbs as Huang-qin-tang at a relative weight ratio of 3:2:2:2 (*12*). To ensure standardization and maintain inter-batch reliability of PHY906, high performance liquid chromatography (HPLC) was used to establish a "chemical fingerprint" of PHY906 by Professor Yung-Chi Cheng and his team (Yale University School of Medicine). Furthermore, they have conducted a series of preclinical and clinical studies to investigate the anticancer activities of PHY906 in recent years. In vivo studies, the combination of PHY906 with irinotecan, capecitabine, 5-FU, and leucovorin (LV) resulted in significant improvement in gastrointestinal toxicities, antitumor activity, and overall survival with no increased host toxicity *versus* chemotherapeutics alone (*60*). In the case of irinotecan, for which severe delayed-onset diarrhea is the major dose-limiting toxicity, PHY906 could effectively regulate intestinal bacteria, alleviate diarrhea and reduce drug-induced mortality in mice. Moreover, treatment with PHY906 mitigated the intestinal injuries of fractionated whole abdomen irradiation and improved recovery from radiation injury, which indicated PHY906

as a potential adjunct to radiation therapy (*61*). In addition, a phase II study was conducted to explore the efficacy of capecitabine combined with PHY906 in patients with advanced pancreatic cancer who were previously treated with gemcitabine-based regimens. The results showed capecitabine plus PHY906 provided a safe and feasible salvage therapy after gemcitabine failure for advanced pancreatic cancer with six-month survival rate at 44% (*62*).

3.4. *Xiao-chai-hu-tang*

Xiao-chai-hu-tang (Sho-sai-ko-to or TJ-9 in Japanese), a classical traditional Chinese herbal formulation originally recorded in "Shang Han Lun", has been used to treat liver diseases especially chronic hepatitis and liver cancer for thousands of years in China and Japan. It consists of seven medicinal herbs (*Bupleurum falcatum*, *Scutellaria baicalensis*, *Panax ginseng*, *Zizyphus jujube*, *Pinellia ternate*, *Zingiber officinale*, and *Glycyrrhiza glabra*) (*63*). Much pharmacological research has shown that TJ-9 has potent antiinflammation, antioxidation, immunomodulation, hepatoprotective, anti-hepatic fibrosis, and antitumor properties. Recently, many basic or clinical studies have been conducted to assess the beneficial effects and safety of TJ-9 for cancer treatment. These studies have demonstrated that TJ-9 treats cancer by enhancing immune regulation, anti-angiogenesis and apoptosis of tumor cells (*64*). TJ-9 exhibited significant growth inhibition of ovarian cancer cell lines, and the mechanisms of the inhibitory effects can be attributed, in part, to apoptosis induced by TJ-9. TJ-9 could effectively inhibit the growth of H22 mouse solid liver cancer and improve the immune function of tumor-bearing mice by increasing NK cells, T lymphocytes and IL-2 levels (*65*). Furthermore, TJ-9 plays important roles in preventing hepatocarcinogenesis. A study was performed to find a way to prevent the development of HCC from hepatitis C virus-associated liver cirrhosis (HCV-LC) in HCV-LC patients who had received reduction ALT therapy such as TJ-9. The results indicated that chances of surviving for more than ten years without developing HCC for HCV-LC patients (*66*). In addition, TJ-9 gargle as a gargling agent for patients receiving chemotherapy showed a significantly decreased incidence of stomatitis, and a painkilling effect compared to gargling with providone-iodine and amphotericin B. Thus, TJ-9 gargle was considered to be a useful method against stomatitis prevention and sharp pain mitigation from chemotherapy (*67*).

4. Chinese medicine preparations commonly used in clinical practice for cancer treatment

Chinese medicine preparations are a form of Chinese herbal medicine that are isolated from single herbs or their active compounds or herbal formulations and prepared using modern advanced pharmaceutical technology. There are various dosage forms including injections, tablets, pills, capsules, and liquids. Compared to traditional decoctions, Chinese medicine preparations are safer, more effective, and easier to use (*12,32*). Thus, Chinese medicine preparations are becoming increasingly popular in China and are attracting worldwide attention.

Currently in China, some Chinese medicine preparations are derived from single TCMs or their active compounds or herbal formulations, which have the properties of spiriting vital energy and their anticancer molecular mechanisms mainly by improving immunity (*e.g.*, Shenqi fuzheng injection and Kanglaite injection). Some Chinese medicine preparations are derived from single TCMs or their active compounds or herbal formulations, which have properties of clearing heat and detoxifying, promoting blood circulation and removing blood stasis and their anticancer molecular mechanisms involving apoptosis, cell cycle arrest, angiogenesis and metastasis, immunoregulation and so on (*e.g.*, Huachansu injection and Cantharidin sodium injection). We want to stress that some TCMs and some natural compounds like Mylabris, Chansu, camptothecin derivatives, and vinca alkaloids are toxic. However, the application of these toxicants provides a magic power to deal with severe diseases like cancer, and this process might be described as "fighting fire with fire" (*68*). Thus, in the following, a brief outline of the oncologic pharmacology of the most commonly used Chinese medicine preparations including some toxicants that have been approved by the State Food and Drug Administration (FDA) of China are briefly presented below (Table 3).

4.1. *Shenqi fuzheng injection*

Shenqi fuzheng injection is an injectable traditional Chinese herbal formula comprised of two herbal medicines, *Radix Astragali* (Huang-Qi) and *Codonopsis pilosula* (Dang-Shen). The injection has been approved by China's FDA since the 1990s. It is commonly used to improve immune function against chronic diseases including cancer and cerebrovascular diseases such as, angina, coronary heart disease, heart failure, and so on (*32*). Recently, many trials have demonstrated that Shenqi fuzheng injection might play an important role in the treatment of various advanced cancers. It could improve immune response *via* raising activity of NK cell, macrophage and T-lymphocyte subgroups without any injuries of heart, liver and kidney function or other adverse reactions in cancers. It also could reduce the toxicity of radiation therapy and chemotherapy. A systematic meta-analysis involving thirteen randomized controlled trials and 860 patients was conducted on Shenqi fuzheng injection for advanced gastric cancer (*69*). It showed that the combination of chemotherapy with Shenqi fuzheng injection obtained a series of positive results including improving quality of life,

Table 3. Chinese medicine preparations commonly used in clinical for cancer treatment

Common name	Source or composition	Biological activity	Preclinical and/or clinical evidence of anticancer activity	*Ref.*
Shenqi fuzheng injection	Comprised of 2 herbs: *Radix Astragali* *Codonopsis pilosula*	Antitumor, immunomodulation	*Clinical*: Improve quality of life, increase complete remission and partial remission efficacy rate, enhance immunity, and decrease adverse events such as nausea, vomiting, oral mucositis, and leucopenia in cancer patients undergoing chemotherapy	*32, 69-71*
Kanglaite injection	Extracted from *Semen Coicis Yokuinin*	Antitumor	*Clinical*: *i*) Improve the short-term efficacy, improve the quality of life, and decrease the risk of gastrointestinal reaction and myelosuppression in cancer patients undergoing radio- or chemotherapy; *ii*) combined with hepatic arterial intervention could improve the short-term clinical efficacy, quality of life, and decrease the pain of patients with unresectable HCC	*12, 72-74*
Huachansu injection	Extracted from the skin and parotid venom glands of the toad *Bufo bufo gargarizans* Cantor	Antitumor, Anti-HBV immunomodulation	*Preclinical*: Inhibit cell proliferation, induce cell differentiation and apoptosis, disrupt cell cycle, inhibit cancer angiogenesis, reverse multi-drug resistance, and regulate immune response in cancer cells *Clinical*: *i*) Enhance the antitumor efficacy of gemcitabine and oxaliplatin and improve the quality of life of patients; *ii*) Postpone tumor recurrence and metastasis, prolong the survival time and increase the survival rate of post-surgical patients with HCC; *iii*) Decrease the quantity of pericardial effusion in cancer patient	*12, 75-80*
Cantharidin sodium injection	Extracted from *blister beetles*	Antitumor, immunomodulation	*Clinical*: *i*) Improve quality of life; *ii*) Reduce side effects induced by chemotherapy such as leukopenia and gastrointestinal reactions	*81-83*

increasing complete remission and partial remission efficacy rate, and decreasing adverse events such as nausea, vomiting, oral mucositis, and leucopenia. Moreover, Shenqi fuzheng injection intervention appeared to be useful to increase efficacy and reduce toxicity when combined with platinum-based chemotherapy for advanced non-small-cell lung cancer (NSCLC) and colorectal cancer (*12,70*). In addition, the combination of concurrent chemoradiotherapy with Shenqi fuzheng injection in patients with head and neck neoplasms could effectively enhance immunity with the levels of CD3, CD4, and CD4/CD8 increased, improve quality of life, and decrease adverse events such as nausea, vomiting, oral mucositis, skin lesions, and leucopenia (*71*).

4.2. *Kanglaite injection*

Kanglaite injection is an anti-tumor drug, which contains extracts from Chinese herbal medicine coix seed (*Semen Coicis Yokuinin*) using modern advanced pharmaceutical technology. In August 1997, Phase III clinical trials were completed and Kanglaite injection was officially launched in China after final approval from the Ministry of Public Health (*12*). Kanglaite injection is mainly used for the treatment of NSCLC, liver cancer, gastric cancer, *etc*. It has been found to significantly decrease cancer cachexy, improve quality of life of cancer patients, and may ameliorate multiple drug resistance of cancers when combined with radiotherapy and chemotherapy in clinical use. A systematic meta-analysis involving 34 clinical trials was conducted to assess the effects of Kanglaite injection combined with chemotherapy *versus* chemotherapy alone in the treatment of advanced non-small cell lung carcinoma (*72*). It showed that the combination could improve the short-term efficacy, and performance status and decrease the risk of gastrointestinal reaction compared with systematic chemotherapy alone. Another systematic meta-analysis involving 9 clinical trials indicated that Kanglaite injection combined with hepatic arterial intervention could improve the short-term clinical efficacy, quality of life, and decrease the pain of patients with unresectable HCC (*73*). Moreover, a network of meta-analysis involving 38 randomized controlled trials and 2,761 participants was conducted to compare which was the best Chinese herb injection based on the FOLFOX regimen for gastric cancer. Kanglaite injection exhibited greater effects than many other Chinese herb injections in clinical efficacy and safety for gastric cancer. It could strengthen the overall response rate, improve the quality of life, reduce nausea and vomiting, and reduce the incidence of leukopenia (III-IV) (*74*).

4.3. *Huachansu injection*

Huachansu injection or Cinobufacini injection is a water-soluble preparation extracted from the skin and parotid venom glands of the toad (*Bufo bufo gargarizans* Cantor) which contains Chansu. It has been approved by China's FDA since the 1990s and widely used to treat patients with lung, liver, colon, and pancreatic cancers at oncology clinics in China (*12*). Cardiac glycosides including bufalin, resibufogenin, and cinobufagin are the three major active constituents to which the antitumor activity of Huachansu injection may be attributed. Huachansu injection exhibited significant effects on inhibition cell proliferation, induction of cell differentiation and apoptosis, disruption of the cell cycle, inhibition of cancer angiogenesis, reversal of multi-drug resistance, and regulation of the immune response in cancer cells (*75,76*). It also could effectively

enhance physical immunity and improve the quality of life with little toxicity in cancer patients. A pilot study of Huachansu injection in patients with HCC, NSCLC, and pancreatic cancer showed that Huachansu injection could improve the quality of life of patients and even enhanced tumor shrinkage with little toxicity (*77*). Another clinical study using Huachansu injection in combination with gemcitabine and oxaliplatin in treating gallbladder carcinomas showed that Huachansu injection substantially enhanced the antitumor efficacy of gemcitabine and oxaliplatin and improved the quality of life of patients (*78*). Moreover, a case-control trial (n = 120) was conducted to assess the effects of Huachansu injection plus Jiedu granules (a Chinese medicine compound) *versus* transcatheter arterial chemoembolization (TACE) in post-surgical patients with HCC in Changhai Hospital (Shanghai, China). Huachansu injection plus Jiedu granules could postpone tumor recurrence and metastasis, prolong survival time and increase survival rate of post-surgical patients with HCC (*79*). In addition, Huachansu injection has been reported to be effective for treating malignant pericardial effusion, pleural effusions, and ascites. Sun *et al*. reported a case of advanced lung cancer with malignant pericardial effusion treated by intrapericardial Huachansu injection instillation (*80*). Huachansu injection could effectively relieve the patient's cardiac tamponade symptoms and improve the patient's quality of life with the levels of CA-125 in pericardial effusion decreased and the quantity of pericardial effusion significantly reduced. Furthermore, there were little gastrointestinal adverse reactions and myelosuppression in the patient after injection of Huachansu in the pericardial cavity.

4.4. *Cantharidin sodium injection*

Cantharidin is a sesquiterpene derivative extracted from blister beetles which has long been used as a poisonous TCM to treat life-threatening diseases such as cancer according to the principle "fighting fire with fire". Cantharidin is a potent and selective inhibitor of protein phosphatase 2A (PP2A). Recent pharmacologic studies proved that cantharidin could induce cell cycle arrest and apoptosis, and interfere with the metabolism of nucleic acids and of proteins in cancer cells, significantly inhibit the growth of various cancers including HCC, uterine cervix cancer, nasopharyngeal carcinoma, cutaneous cancer, leucemia, and so on (*81*). Cantharidin sodium injection is a semi-synthetic derivative of cantharidin. It has been approved by China's FDA since the 1990s and widely used to treat patients with lung, liver, colon, pancreatic, gastric and breast cancers at oncology clinics in China. It could reduce the uptake of amino acids in cancer cells, inhibit protein synthesis, stimulate macrophages, lymphocytes, cause polymorphonuclear cells to produce interleukin, and finally improve immunity and enhance anticancer efficacy (*82*). Several clinical studies showed that Cantharidin sodium injection combined with chemotherapy could effectively improve quality of life and reduce side effects induced by chemotherapy such as leukopenia and gastrointestinal reactions without any increase in toxicity in patients with NSCLC, colorectal, breast and gastric cancer (*81,83*). Although Cantharidin sodium injection is wildly used in cancer therapy in China, most of these studies are published in Chinese and little is known about use of the Cantharidin sodium injection outside of China. Thus, the mechanisms of the injection's action must be investigated and the injection must be clinically evaluated further.

5. Clinical trials of TCMs as adjuvant treatment in the whole course of cancer therapy

As is known, surgery, chemotherapy and radiotherapy are major conventional cancer therapies. Although these therapies are directed at killing or eradicating cancer cells, a series of complications and side effects will come along such as upper limb lymphedema, infection/fever, fatigue, pain, anemia, diarrhea, nausea and vomiting, hair loss, and bone marrow suppression. These complications and side effects inconvenience and cause discomfort to patients and they may also limit or prevent the delivery of therapy at its optimal dose and time, potentially causing fatalities. Thus, more effective therapies to help prevent and control complications and side effects of conventional cancer therapy must soon be developed. Some TCMs have been found to be adjunctive in cancer therapy. Here we will give a brief outline on the use of TCMs to reduce some complications and side effects associated with conventional cancer therapy in clinical studies (Table 4).

5.1. *Lymphedema*

Lymphedema is a serious medical complication commonly associated with breast cancer treatment, such as surgery and/or radiation, and it may occur more than 5 years after surgery. Lymphedema involves the accumulation of protein-rich fluid in the interstitial space, and occurs when the lymphatic system is damaged and no longer properly drains the lymph fluid back to the systemic circulation (*84*). When the lymphatic system is damaged, fluid accumulates in the affected limb, leading to swelling, fibrosis, reduced range of motion, decreased function, and, in later stages, infection and pain. However, it is difficult to cure once it occurs despite the various treatments which have been developed such as massage, compression garments, bandages, or sleeves, manual lymphatic drainage, individualized exercise, and education regarding skin care and infection prevention (*85*). In recent years, some reports related to the effectiveness of TCMs use among cancer patients with lymphedema have been reported.

Coumarin, a chemical compound derived from some

Table 4. Clinical trials of TCMs as adjuvant treatment in the whole course of cancer therapy

TCMs	Complications or side effects	Clinical trials of TCMs as adjuvant treatment in cancer therapy				*Ref.*
		Patients	Experimental group	Control group	Outcomes	
Coumarin	Lymphedema	*n* = 31 (post mastectomy)	Coumarin	Placebo	More effective than the placebo in reducing the limb volume and skin temperature and in increasing the softness of the limb tissue	*86*
Aescine	Lymphedema	*n* = 20	Sodium aescine + Diosmin	Physiotherapy	Compared to physiotherapy group, the limb volume and the softness of the limb tissue were significantly improved in the combination group	*88*
Liu-jun-zi-Tang	Gastrointestinal function	*n* = 19 (post proximal gastrectomy)	Liu-jun-zi-Tang + conventional treatment	Conventional treatment	Significantly improved the symptoms of postgastrectomy syndrome and long-term quality of life in patients with gastric cancer compared to the control group	*90*
PHY906	Gastrointestinal side effects	*n* = 24	PHY906 + chemotherapy	Chemotherapy (Capecitabine)	Some gastrointestinal side effects such as diarrhea, abdominal cramps, and vomiting were reduced	*60*
Compound kushen injection	Pain	*n* = 521	Compound kushen injection + radiotherapy or bisphosphonates	Radiotherapy or bisphosphonates	Exhibited significant effects on improving pain relief in patients with bone cancer pain	*93*
TJ-41	Fatigue	*n* = 751	TJ-41 + chemotherapy or supportive care	chemotherapy or supportive care	Significantly relieve cancer-related fatigue and improve quality of life compared to the control group	*94*
Shen-mai-san	Fatigue	*n* = 30	Shen-mai-san + chemotherapy or radiotherapy	Chemotherapy or radiotherapy	Effective for treating cancer-related fatigue and had anti-fatigue activity	*95*
Fufang E-Jiao Jiang	Bone marrow suppression	*n* = 64	Fufang E-Jiao Jiang + chemotherapy + rhIL-11 + rhG-CSF	Chemotherapy + rhIL-11 + rhG-CSF	Showed significant effects on relieving the myelosuppression caused by GP regimen chemotherapy and increasing white cell and blood platelets counts compared to the control group	*96*
TJ-48	Bone marrow suppression	*n* = 29	TJ-48 + FOLFOX4 regimen chemotherapy	FOLFOX4 regimen chemotherapy	Effectively increase white cell counts and improve the symptoms such as dizziness and fatigue	*97*
Shenqi fuzheng injection	Radiation pneumonitis	*n* = 48	Shenqi fuzheng injection + antibiotics + high efficient hormone	Antibiotics + high efficient hormone	Significantly improve respiratory symptoms and signs, and regulate subsets of T-lymphocytes, as well as increase patients' quality of life compared to the control group	*98*
Xiao-chai-hu-tang, Huang-lian-jie-du-tang or Yin-chen-wu-ling-san	Hepatotoxicity	*n* = 89	TCMs (*e.g.*, Xiao-chai-hu-tang, Huang-lian-jie-du-tang or Yin-chen-wu-ling-san) + chemotherapy	Chemotherapy	The serum levels of ALT and AST in combination group were lower than those in the control group	*100*

plants such as Tonka beans, might reduce lymphedema and the incidence of secondary infections because it decreases the volume of protein by stimulating proteolysis. A randomized, double-blind, placebo-controlled, cross-over trial to examine the effect of coumarin in 31 women with post-mastectomy lymphedema and 21 men and women with lymphedema of the lower extremity of various causes was conducted with the patients receiving 400 mg of coumarin or placebo for 6 months. The results indicated that coumarin was more effective than the placebo in reducing the limb volume and skin temperature and in increasing the softness of the limb tissue, with mild side effects, such as mild nausea and diarrhea arising in seven patients who took coumarin (*86*). However, Loprinzi *et al*. reported that coumarin had potential negative and even life-threatening side effects, such as hepatotoxicity (*87*). Therefore, further research with a rigorous design and larger sample size is needed to re-evaluate the effectiveness of coumarin in treating lymphedema.

Sodium aescine is a Chinese medicine preparation extracted from Horsechestnut seed and is widely used to treat chronic venous insufficiency. It is also widely used to treat soft tissue swelling caused by various reasons such as breast cancer related upper limb lymphedema. To observe the efficacy of sodium aescine combined with Diosmin on upper limb lymphedema in patients after breast cancer surgery, a randomized controlled study was conducted. The results indicated that compared to physiotherapy group (n = 16), the limb volume and the softness of the limb tissue were significantly improved in the combination group (n = 20) (*88*). Moreover, a possible mechanism of aescine may be related to inhibiting the activity of enzymes elastase and hyaluronidase (*85*). However, there are several limitations associated with the current published studies on sodium aescine such as indeterminate results, small sample sizes, and little examination of outcomes. Therefore, further research with rigorous design and larger sample size is needed to re-evaluate the effectiveness of sodium aescine in treating lymphedema.

5.2. *Gastrointestinal side effects*

Gastrointestinal side effects including loss of appetite, diarrhea, nausea, and vomiting are the most common symptoms occurring in cancer patients after surgery and/or receiving chemo- or radio-therapy. However, there is still no effective treatment to ameliorate these symptoms in cancer patients. Recently, many clinical trials have suggested that some TCMs may be effective at treating gastrointestinal side effects. Liu-jun-zi-Tang (or Rikkunshito in Japanese) is a famous Traditional Chinese herbal formulation including 6 herbs (*Ginseng Radix*, *Poria cocos*, *Rhizoma atractylodis macrocephalae*, *liquorice root*, *pinelliae tuber*, *pericarpium citri*, *common ginger*, and *Jujube*). It is widely used in China and Japan for the treatment of upper gastrointestinal symptoms of patients with functional dyspepsia, gastroesophageal reflux disease, dyspeptic symptoms of post-gastrointestinal surgery patients, and chemotherapy-induced dyspepsia in cancer patients. Some Japanese researchers found that Liu-jun-zi-tang as a ghrelin enhancer was effective for treating cisplatin-induced dyspepsia and cancer cachexia-anorexia syndrome (*89*). Liu-jun-zi-tang significantly improved the symptoms of postgastrectomy syndrome and long-term quality of life in patients with gastric cancer who had undergone proximal gastrectomy (*90*). In addition, some clinical studies have shown that PHY906 enhances the therapeutic indices of a broad spectrum of anticancer agents such as Capecitabine, 5-FU and irinotecan in colorectal, liver, and pancreatic cancers. PHY906 could reduce chemotherapy-induced toxicities especially gastrointestinal side effects (*e.g.*, diarrhea, abdominal cramps, and vomiting) and/or increase chemotherapeutic efficacy without affecting the pharmacokinetics of chemotherapeutic agents (*12,60*). Furthermore, advanced clinical trials are ongoing to demonstrate the effectiveness of PHY906 as adjuvant therapy for cancer patients undergoing chemotherapy.

5.3. *Pain*

Pain is a common and burdensome symptom of cancer and the causes of pain can be the cancer itself (the tumor pressed on bones, nerves, or other organs) or its treatment (*e.g.*, surgery, chemotherapy, or radiotherapy). It was reported that 75-90% cancer patients especially who have bone metastasis experienced pain during their illness (*91*). As indicated in current WHO guidelines, three step analgesic ladder therapies are the standard of care for cancer pain. However, up to 50% of cancer pain is still undertreated. In recent years, many clinical trials have suggested that some TCMs as adjunctive therapy may be effective at treating cancer related pain and that their effects are similar to those of Western analgesics. TCMs may reduce the side effects of conventional analgesics, and then enhance cancer patients' quality of life (*12*). To assess the effectiveness of oral TCMs in relieving pain secondary to bone metastases in patients, a meta-analysis enrolled a total of 16 randomized controlled trials and 1,008 patients were identified and analyzed (*92*). The results showed that TCMs plus conventional treatment increased the pain-relief rate compared with the conventional treatment alone. Another meta-analysis enrolled a total of 7 randomized controlled trials and 521 patients were identified and analyzed to assess the efficacy and safety of compound kushen injection (a preparation extracted from *Radix sophorae flavescentis*) for bone cancer pain. Compared with radiotherapy or bisphosphonates, compound kushen injection showed significant effects on improving pain relief in patients with bone cancer pain (*93*). In addition, some clinical

trials indicated that acupuncture might be beneficial for alleviating cancer pain. In all, TCM interventions appear to have beneficial effects on pain secondary to bone metastases in patients. However, there are several limitations associated with the current published studies on TCMs relieving pain such as indeterminate results, small sample sizes, and little examination of outcomes. Therefore, further research with rigorous design and larger sample size is needed to re-evaluate the effectiveness of TCMs in treating cancer related pain.

5.4. *Fatigue*

Cancer-related fatigue is a highly prevalent, persistent and subjective sense of tiredness related to cancer disease or cancer treatment which cannot be relieved by sleep or rest, and overall 50-90% of people with cancer experience fatigue. It significantly interferes with patients' daily activities and decreases their quality of life (*12,94*). However, it remains under-recognized and under-treated, partly because of limited understanding of its pathophysiology and lack of effective treatments. Several Chinese herbal medicines may have beneficial effects on cancer-related fatigue and quality of life for cancer patients. To assess the effectiveness and safety of Chinese herbal medicine for the treatment of cancer-related fatigue, a meta-analysis enrolled a total of 10 randomized controlled trials and 751 patients with various cancers such as liver, lung, and breast cancer were identified and analyzed (*94*). The results showed that some TCMs such as TJ-41 used alone or in combination with chemotherapy or supportive care showed significant relief in cancer-related fatigue and improved quality of life compared to chemotherapy or supportive care based on single trials. In addition, a randomized, double-blind, placebo-controlled clinical trial was conducted to evaluate the efficacy of Shen-mai-san (a famous Traditional Chinese herbal formulation composed of processed *Ginseng*, *Liriope spicata*, and *Schizandrae fructus*) in patients with cancer who were undergoing chemotherapy or radiotherapy (*95*). Shen-mai-san was found to be effective for treating cancer-related fatigue and had anti-fatigue activity. According to TCM theory, Shen-mai-san has a synergistic effect for qi and yin deficiency which are similar to chemotherapy- or radiotherapy-induced side effects and has the ability to prevent fatigue. These findings from a limited number of trials suggest that TCM seems to be effective and safe in the treatment of cancer-related fatigue. However, current evidence is insufficient to draw a confirmed conclusion due to the poor methodological quality of included trials. However, there are several limitations associated with the current published studies on TCMs relieving fatigue such as indeterminate results, small sample sizes, and little examination of outcomes. Thus, conducting rigorously designed trials on potential Chinese herbal medicine are warranted.

5.5. *Bone marrow suppression*

Bone marrow suppression is a reduction in the activity of bone marrow, resulting in decreased numbers of red blood cells, platelets, and white blood cells. One of the most common reasons for a patient to have this condition is chemotherapy treatment for cancer. While the bone marrow is functioning below normal levels, the patient is at risk, and needs to be monitored very closely. In some cases, hospitalization is recommended for people with Bone marrow suppression until their bone marrow is functioning normally. In recent years, some TCMs have been reported to have beneficial effects on chemotherapy-related bone marrow suppression. Colla corii asini (or E-Jiao in Chinese), donkey-hide gelatin prepared by stewing and concentrating from *Equus asinus* L. donkey hide, is a health-care food and traditional Chinese medicine widely used in life-nourishing and clinical hematic antanemic therapy for more than 2,000 years in China (*32*). Many studies indicated that E-Jiao and its preparations such as Fufang E-Jiao Jiang could effectively promote the recovery of bone marrow hemopoietic function in cancer patients with myelosuppression who were undergoing chemotherapy. Fufang E-Jiao Jiang in combination with conventional interleukin-11 (rhIL-11) and recombinant human granulocyte colony stimulating factor (rhG-CSF) in cancer patients showed significant effects on relieving the myelosuppression caused by GP (Gemcitabine + DDP) regimen chemotherapy and increasing white cell and blood platelet counts compared to using rhIL-11 and rhG-CSF groups alone (*96*). In addition, TJ-48 was reported to have significant effects on alleviating myelosuppression caused by FOLFOX4 regimen chemotherapy in patients with gastrointestinal malignant tumor. It could effectively increase white cell counts and improve symptoms such as dizziness and fatigue (*97*). In all, TCM interventions appear to have beneficial effects on alleviating myelosuppression caused by chemotherapy in cancer patients. However, there are several limitations associated with the current published studies on TCMs relieving myelosuppression such as indeterminate results, small sample sizes, and little examination of outcomes. Thus, conducting rigorously designed trials on potential Chinese herbal medicine are warranted.

5.6. *Radiation pneumonitis*

Radiation pneumonitis is one of the most common complications during radiotherapy of thoracic tumors. It impacts the quality of life of the patients and has a life-threatening danger. However, there is a lack of drugs for prevention and treatment of this disease. In recent years, some TCMs have been reported to have beneficial effects on radiotherapy-related radiation pneumonitis.

Shenqi fuzheng injection combined with antibiotics and short-term pulse therapy with highly efficient hormones had a good effect for radiation pneumonitis compared to the control group treated only by antibiotic and hormone pulse therapy. The combination could effectively improve respiratory symptoms and signs, and regulate subsets of T-lymphocytes (CD3+, CD4+, CD8+ and CD4+/CD8+ ratio), as well as increase patients' quality of life (98). In addition, a prospective randomized clinical study was conducted to assess the effect of a Traditional Chinese herbal formulation composed of Liangxue Jiedu Huoxue Decoction including 7 herbs (*Radix rehmanniae*, *Rhizoma chuanxiong*, *Cortex moutan*, *Peach seed*, *Flos carthami*, *Radix astragali*, and *Fructus forsythiae*). In this study, 100 lung cancer patients scheduled to receive radiotherapy were randomly divided into a treatment group (Liangxue Jiedu Huoxue Decoction+ radiotherapy) and control group (radiotherapy) with 50 patients in each group (*99*). Results showed that the incidence rate of radiation pneumonitis was lower in the treatment group than in the control group (13.04% *versus* 33.33%, $p < 0.05$). Furthermore, the extent of lung injures and the symptoms of radiation pneumonitis improved in the treatment group. In all, TCM interventions appear to have beneficial effects on alleviating radiation pneumonitis caused by radiotherapy in cancer patients. However, there are several limitations associated with the current published studies on TCMs relieving radiation pneumonitis such as indeterminate results, small sample sizes, and little examination of outcomes. Thus, conducting rigorously designed trials on potential Chinese herbal medicine are warranted.

5.7. *Hepatotoxicity*

Hepatotoxicity is a common side effect of chemotherapy. Its prevalence ranges from 33 to 65.6% among patients with cancer, and up to 30% of patients have grade III or IV hepatotoxicity. Although 90% of patients resumed normal liver function after stopping use of hepatotoxic drugs, current chemotherapeutic guidelines require stopping chemotherapy when hepatotoxicity as indicated by AST and ALT levels, reaches four times the upper normal limit. Moreover, there is a lack of drugs that effectively protect liver function and elevation of liver enzymes (*12*). In recent years, some TCMs have been reported to have beneficial effects on improving hepatotoxicity. To examine the effectiveness of TCM for protecting the liver from the toxic effects of chemotherapy among patients with cancer receiving joint TCM (*e.g.*, Xiao-chai-hu-tang, Huang-lian-jie-du-tang or Yin-chen-wu-ling-san) and chemotherapy, a case-control study involving 89 patients and 184 chemotherapy courses was conducted (*100*). Use of TCM with chemotherapy resulted in protection of the liver during chemotherapy, as manifested by lower serum AST and ALT levels. However, this study only examined a limited spectrum of patient outcomes. A future study with more-homogeneous samples and a larger sample size is needed.

6. Conclusion

In conclusion, TCM as adjuvant cancer treatment plays important roles in different stages of cancer lesions including post-operation, radiotherapy or chemotherapy stages. Some TCMs (*e.g.*, TJ-41, Liu-jun-zi-tang, PHY906, Coumarin, and Aescine) are capable of improving the post-operation symptoms such as fatigue, pain, appetite, diarrhea, nausea, vomiting, and lymphedema. Some TCMs (*e.g.*, Ginseng, Huang-Qi, BanZhiLian, TJ-48, Huachansu injection, Shenqi fuzheng injection, and Kanglaite injection) in combination with chemo- or radio-therapy are capable of enhancing the efficacy of and diminishing the side effects and complications caused by chemo- and radiotherapy. Taken together, they have great advantages in terms of suppressing tumor progression, relieving surgery complications, increasing the sensitivity of chemo- and radio-therapeutics, improving an organism's immune system function, and lessening the damage caused by surgery, chemo- or radio-therapeutics. They have significant effects on relieving breast cancer-related lymphedema, reducing cancer-related fatigue and pain, improving radiation pneumonitis and gastrointestinal side effects, protecting liver function, and even ameliorating bone marrow suppression. This review of those medicines should contribute to an understanding of Chinese herbal medicines as an adjunctive therapy in the whole course of cancer treatment instead of only the terminal stage of cancer, with providing useful information for development of more effective anti-cancer drugs and making more patients "survival with cancer" for a long time. However, rigorously designed trials on potential Chinese herbal medicine must be further examined involving cancer patients in the future.

References

1. WHO (2012). Non-Communicable Diseases. Burden: Mortality, Morbidity and Risk Factors. *http://www.who.int/nmh/publications/ncd_report_chapter1.pdf* (Accessed October, 24, 2014).
2. Hao J, Chen WQ. 2012 Chinese Cancer Registry Annual Report. National Cancer Center, Disease prevention and control bureau, Ministry of Health.
3. Targeted therapy. *http://en.wikipedia.org/wiki/Targeted_therapy* (Accessed October, 24, 2014).
4. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011; 480:480-489.
5. Chen M, Guo Y. Consideration about the concept of "tumor-bearing survival". Zhong Hua Zhong Yi Yao Za

Zhi. 2012; 27:3193-3194. (in Chinese)
6. Li X, Yang G, Li X, Zhang Y, Yang J, Chang J, Sun X, Zhou X, Guo Y, Xu Y, Liu J, Bensoussan A. Traditional Chinese medicine in cancer care: A review of controlled clinical studies published in Chinese. PLoS One. 2013; 8:e60338.
7. Zhou J, Zhou T, Jiang M, Wang X, Liu Q, Zhan Z, Zhang X. Research progress on synergistic anti-tumor mechanisms of compounds in traditional Chinese medicine. J Tradit Chin Med. 2014; 34:100-105.
8. Liang W, Yew DT, Hon KL, Wong CK, Kwok TC, Leung PC. Indispensable value of clinical trials in the modernization of traditional Chinese medicine: 12 years' experience at CUHK and future perspectives. Am J Chin Med. 2014; 42:587-604.
9. Ling CQ. My reviews on the etiology and pathogenesis of malignant cancer. Journal of Traditional Chinese Medicine. 2009; 50:952-953. (in Chinese)
10. Smith ME, Bauer-Wu S. Traditional Chinese Medicine for cancer-related symptoms. Semin Oncol Nurs. 2012; 28:64-74.
11. Chao TH, Fu PK, Chang CH, Chang SN, Chiahung Mao F, Lin CH; Evidence-based Chinese Medicine Research Group. Prescription patterns of Chinese herbal products for post-surgery colon cancer patients in Taiwan. J Ethnopharmacol. 2014; 155:702-708.
12. Qi F, Li A, Inagaki Y, Gao J, Li J, Kokudo N, Li XK, Tang W. Chinese herbal medicines as adjuvant treatment during chemo- or radio-therapy for cancer. Biosci Trends. 2010; 4:297-307.
13. Ling CQ, Yue XQ, Ling C. Three advantages of using traditional Chinese medicine to prevent and treat tumor. J Integr Med. 2014; 12:331-335.
14. Konkimalla VB, Efferth T. Evidence-based Chinese medicine for cancer therapy. J Ethnopharmacol. 2008; 116:207-210.
15. Xiang YZ, Shang HC, Gao XM, Zhang BL. A comparison of the ancient use of Ginseng in traditional Chinese medicine with modern pharmacological experiments and clinical trials. Phytother Res. 2008; 22:851-858.
16. Lee SY, Kim YK, Park N, Kim CS, Lee CY, Park SU. Chemical constituents and biological activities of the berry of *Panax ginseng*. J Med Plants Res. 2010; 5:349-353.
17. Sun YX. Structure and biological activities of the polysaccharides from the leaves, roots and fruits of *Panax ginseng* C.A. Meyer: An overview. Carbohydr Polym. 2011; 85:490-499.
18. Hofseth LJ, Wargovich MJ. Inflammation, cancer, and targets of ginseng. J Nutr. 2007; 137 (Suppl 1):183S-185S.
19. Yun TK. Experimental and epidemiological evidence on nonorgan specific cancer preventive effect of Korean ginseng and identification of active compounds. Mutat Res. 2003; 523-524:63-74.
20. Lee JH, Kwon KR, Cho CK, Han SS, Yoo HS. Advanced cancer cases treated with cultivated wild ginseng phamacopuncture. J Acupunct Meridian Stud. 2010; 3:119-124.
21. Barton DL, Liu H, Dakhil SR, Linquist B, Sloan JA, Nichols CR, McGinn TW, Stella PJ, Seeger GR, Sood A, Loprinzi CL. Wisconsin Ginseng (Panax quinquefolius) to improve cancer-related fatigue: A randomized, double-blind trial, N07C2. J Natl Cancer Inst. 2013; 105:1230-1238.
22. Lee JM, Hah JO, Kim HS. The effect of red ginseng extract on inflammatory cytokines after chemotherapy in children. J Ginseng Res. 2012; 36:383-390.
23. Lee TK, Johnke RM, Allison RR, O'Brien KF, Dobbs LJ Jr. Radioprotective potential of ginseng. Mutagenesis. 2005; 20:237-243.
24. Qi LW, Cao J, Li P, Yu QT, Wen XD, Wang YX, Li CY, Bao KD, Ge XX, Cheng XL. Qualitative and quantitative analysis of Radix Astragali products by fast high-performance liquid chromatography-diode array detection coupled with time-of-flight mass spectrometry through dynamic adjustment of fragmentor voltage. J Chromatogr A. 2008; 1203:27-35.
25. Yan MM, Liu W, Fu YJ, Zu YG, Chen CY, Luo M. Optimisation of the microwave-assisted extraction process for four main astragalosides in Radix Astragali. Food Chem. 119:1663-1670.
26. Li J, Bao Y, Lam W, Li W, Lu F, Zhu X, Liu J, Wang H. Immunoregulatory and anti-tumor effects of polysaccharopeptide and Astragalus polysaccharides on tumor-bearing mice. Immunopharmacol Immunotoxicol. 2008; 30:771-782.
27. Law PC, Auyeung KK, Chan LY, Ko JK. Astragalus saponins downregulate vascular endothelial growth factor under cobalt chloride-stimulated hypoxia in colon cancer cells. BMC Complement Altern Med. 2012; 12:160.
28. Lin YW, Chiang BH. Anti-tumor activity of the fermentation broth of Cordyceps militaris cultured in the medium of Radix astragali. Process Biochem. 2008; 43:244-250.
29. Taixiang W, Munro AJ, Guanjian L. Chinese medical herbs for chemotherapy side effects in colorectal cancer patients. Cochrane Database Syst Rev. 2005; 1:CD004540.
30. Yan PH, Yan M, Wang XM, Wang SH. Effect of Huangqi injection on short-term prognosis in children with acute lymphoblastic leukemia. Zhongguo Dang Dai Er Ke Za Zhi. 2014; 16:141-146. (in Chinese)
31. Li Y, Cui HJ, Huang JC, Wu XQ. Clinical study of Jiawei Huangqi Guizhi Wuwu Decoction in preventing and treating peripheral neuro-sensory toxicity caused by oxaliplatin. Chin J Integr Med. 2006; 12:19-23.
32. The State Pharmacopoeia Commission of the People's Republic of China. Pharmacopoeia of the People's Republic of China 2010. China Chemical Industry Press, Beijing, China, 2010.
33. Yang C, Gou Y, Chen J, An J, Chen W, Hu F. Structural characterization and antitumor activity of a pectic polysaccharide from Codonopsis pilosula. Carbohydr Polym. 2013; 98:886-895.
34. Xin T, Zhang F, Jiang Q, Chen C, Huang D, Li Y, Shen W, Jin Y, Sui G. The inhibitory effect of a polysaccharide from *Codonopsis pilosula* on tumor growth and metastasis *in vitro*. Int J Biol Macromol. 2012; 51:788-793.
35. Sun Y. Biological activities and potential health benefits of polysaccharides from Poria cocos and their derivatives. Int J Biol Macromol. 2014; 68:131-134.
36. Ríos JL. Chemical constituents and pharmacological properties of Poria cocos. Planta Med. 2011; 77:681-691.
37. Cheng S, Eliaz I, Lin J, Thyagarajan-Sahu A, Sliva D. Triterpenes from Poria cocos suppress growth and invasiveness of pancreatic cancer cells through the downregulation of MMP-7. Int J Oncol. 2013; 42:1869-1874.
38. Kikuchi T, Uchiyama E, Ukiya M, Tabata K, Kimura Y, Suzuki T, Akihisa T. Cytotoxic and apoptosis-inducing activities of triterpene acids from Poria cocos. J Nat Prod. 2011; 74:137-144.

39. Chen M, May BH, Zhou IW, Xue CC, Zhang AL. FOLFOX 4 combined with herbal medicine for advanced colorectal cancer: A systematic review. Phytother Res. 2014; 28:976-991.
40. Niu Y, Meng QX. Chemical and preclinical studies on Hedyotis diffusa with anticancer potential. J Asian Nat Prod Res. 2013; 15:550-565.
41. Kuo YJ, Yang JS, Lu CC, Chiang SY, Lin JG, Chung JG. Ethanol extract of *Hedyotis diffusa willd* upregulates G0/G1 phase arrest and induces apoptosis in human leukemia cells by modulating caspase cascade signaling and altering associated genes expression was assayed by cDNA microarray. Environ Toxicol. 2014; doi:10.1002/tox.21989.
42. Cai Q, Lin J, Wei L, Zhang L, Wang L, Zhan Y, Zeng J, Xu W, Shen A, Hong Z, Peng J. *Hedyotis diffusa Willd* inhibits colorectal cancer growth *in vivo via* inhibition of STAT3 signaling pathway. Int J Mol Sci. 2012; 13:6117-6128.
43. Lee SZ, Huang TC, Wei MC, Yang YC. Extraction of protocatechuic acid from Scutellaria barbata D. Don using supercritical carbon dioxide. J Supercrit Fluid. 2013; 81:55-66.
44. Nie XP, Qu GW, Yue XD, Li GS, Dai SJ. Scutelinquanines A-C, three new cytotoxic neo-clerodane diterpenoid from *Scutellaria barbata*. Phytochem Lett. 2010; 3:190-193.
45. Lin J, Chen Y, Cai Q, Wei L, Zhan Y, Shen A, Sferra TJ, Peng J. Scutellaria Barbata D Don Inhibits Colorectal Cancer Growth *via* Suppression of Multiple Signaling Pathways. Integr Cancer Ther. 2013; 13:240-248.
46. Perez AT, Arun B, Tripathy D, Tagliaferri MA, Shaw HS, Kimmick GG, Cohen I, Shtivelman E, Caygill KA, Grady D, Schactman M, Shapiro CL. A phase 1B dose escalation trial of Scutellaria barbata (BZL101) for patients with metastatic breast cancer. Breast Cancer Res Treat. 2010; 120:111-118.
47. Chen XP, Li W, Xiao XF, Zhang LL, Liu CX. Phytochemical and pharmacological studies on Radix Angelica sinensis. Chin J Nat Med. 2013; 11:577-587.
48. Chao WW, Lin BF. Bioactivities of major constituents isolated from *Angelica sinensis* (Danggui). Chinese Medicine. 2011; 6:29.
49. Kou W, Li YD, Liu K, Sun SB, Dong YM, Wu ZH. *Radix Angelicae Sinensis* and *Radix Hedysari* enhance radiosensitivity of 12C6+ radiation in human liver cancer cells by modulating apoptosis protein. Saudi Med J. 2014; 35:945-952.
50. Kumar P, Kadakol A, Shasthrula P, Mundhe NA, Jamdade VS, Barua CC, Gaikwad AB. Curcumin as an Adjuvant to Breast Cancer Treatment. Anticancer Agents Med Chem. 2015. [Epub ahead of print]
51. Goel A, Aggarwal BB. Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. Nutr Cancer. 2010; 62:919-930.
52. Qi FH, Wang ZX, Cai PP, Zhao L, Gao JJ, Kokudo N, Li AY, Han JQ, Tang W. Traditional Chinese medicine and related active compounds: A review of their role on hepatitis B virus infection. Drug Discov Ther. 2013; 7:212-224.
53. Kido T, Mori K, Daikuhara H, Tsuchiya H, Ishige A, Sasaki H. The protective effect of hochu-ekki-to (TJ-41), a Japanese herbal medicine, against HSV-1 infection in mitomycin C-treated mice. Anticancer Res. 2000; 20:4109-4113.
54. Kimura M, Sasada T, Kanai M, Kawai Y, Yoshida Y, Hayashi E, Iwata S, Takabayashi A. Preventive effect of a traditional herbal medicine, Hochu-ekki-to, on immunosuppression induced by surgical stress. Surg Today. 2008; 38:316-322.
55. Jeong JS, Ryu BH, Kim JS, Park JW, Choi WC, Yoon SW. Bojungikki-tang for cancer-related fatigue: A pilot randomized clinical trial. Integr Cancer Ther. 2010; 9:331-338.
56. Ogawa K, Omatsu T, Matsumoto C, Tsuchiya N, Yamamoto M, Naito Y, Yoshikawa T. Protective effect of the Japanese traditional medicine juzentaihoto on myelosuppression induced by the anticancer drug TS-1 and identification of a potential biomarker of this effect. BMC Complement Altern Med. 2012; 12:118.
57. Huang SM, Chien LY, Tai CJ, Chiou JF, Chen CS, Tai CJ. Effectiveness of 3-week intervention of Shi Quan Da Bu Tang for alleviating hematotoxicity among patients with breast carcinoma receiving chemotherapy. Integr Cancer Ther. 2013; 12:136-144.
58. Tsuchiya M, Kono H, Matsuda M, Fujii H, Rusyn I. Protective effect of Juzen-taiho-to on hepatocarcinogenesis is mediated through the inhibition of Kupffer cell-induced oxidative stress. Int J Cancer. 2008; 123:2503-2511.
59. Ikemoto T, Shimada M, Iwahashi S, Saito Y, Kanamoto M, Mori H, Morine Y, Imura S, Utsunomiya T. Changes of immunological parameters with administration of Japanese Kampo medicine (Juzen-Taihoto/TJ-48) in patients with advanced pancreatic cancer. Int J Clin Oncol. 2014; 19:81-86.
60. Liu SH, Cheng YC. Old formula, new Rx: The journey of PHY906 as cancer adjuvant therapy. J Ethnopharmacol. 2012; 140:614-623.
61. Rockwell S, Grove TA, Liu Y, Cheng YC, Higgins SA, Booth CJ. Preclinical studies of the Chinese herbal medicine formulation PHY906 (KD018) as a potential adjunct to radiation therapy. Int J Radiat Biol. 2013; 89:16-25.
62. Saif MW, Li J, Lamb L, Kaley K, Elligers K, Jiang Z, Bussom S, Liu SH, Cheng YC. First-in-human phase II trial of the botanical formulation PHY906 with capecitabine as second-line therapy in patients with advanced pancreatic cancer. Cancer Chemother Pharmacol. 2014; 73:373-380.
63. Qi FH, Wang ZX, Cai PP, Zhao L, Gao JJ, Kokudo N, Li AY, Han JQ, Tang W. Traditional Chinese medicine and related active compounds: A review of their role on hepatitis B virus infection. Drug Discov Ther. 2013; 7:212-224.
64. Zheng CS, Wu YS, Bao HJ, Xu XJ, Chen XQ, Ye HZ, Wu GW, Xu HF, Li XH, Chen JS, Liu XX. Understanding the polypharmacological anticancer effects of Xiao Chai Hu Tang *via* a computational pharmacological model. Exp Ther Med. 2014; 7:1777-1783.
65. Li J, Xie M, Gan Y. Effect of Xiaochaihu decoction and different herbal formulation of component on inhibiting H22 liver cancer in mice and enhancing immune function. Zhongguo Zhong Yao Za Zhi. 2008; 33:1039-1044. (in Chinese)
66. Rino Y, Tarao K, Morinaga S, Ohkawa S, Miyakawa K, Hirokawa S, Masaki T, Tarao N, Yukawa N, Saeki H, Takanashi Y, Imada T. Reduction therapy of alanine aminotransferase levels prevent HCC development in patients with HCV-associated cirrhosis. Anticancer Res. 2006; 26:2221-2226.

67. Matsuoka H, Mizushima Y, Kawano M, Tachibana N, Sawada Y, Kato S, Nagakura H, Tanaka M, Suzuki K, Tadanobu K. Clinical availability of the herbal medicine, SYOUSAIKOTOU, as a gargling agent for prevention and treatment of chemotherapy-induced stomatitis. Gan To Kagaku Ryoho. 2004; 31:2017-2020. (in Japanese)
68. Wang S, Wu X, Tan M, Gong J, Tan W, Bian B, Chen M, Wang Y. Fighting fire with fire: Poisonous Chinese herbal medicine for cancer therapy. J Ethnopharmacol. 2012; 140:33-45.
69. Li J, Wang JC, Ma B, Gao W, Chen P, Sun R, Yang KH. Shenqi Fuzheng Injection (SFI) for advanced gastric cancer: A systematic review of randomized controlled trials. Chin J Integr Med. 2015; 21:71-79.
70. Dong J, Su SY, Wang MY, Zhan Z. Shenqi fuzheng, an injection concocted from Chinese medicinal herbs, combined with platinum-based chemotherapy for advanced non-small cell lung cancer: A systematic review. J Exp Clin Cancer Res. 2010; 29:137.
71. Guo ML, Chen HY, Han XL, Zhang HZ. Influence of Shenqi fuzheng injection combined with radio-chemotherapy on quality of life in head and neck cancer patients. Modern Journal of Integrated Traditional Chinese and Western Medicine. 2014; 23:1273-1275. (in Chinese)
72. Liu X, Yang Q, Xi Y, Yu K, Wang W, Zhao X, Kou X. Kanglaite injection combined with chemotherapy *versus* chemotherapy alone in the treatment of advanced non-small cell lung carcinoma. J Cancer Res Ther. 2014; 10 (Suppl 1):46-51.
73. Fu F, Wan Y, Mulati, Wu T. Kanglaite injection combined with hepatic arterial intervention for unresectable hepatocellular carcinoma: A meta-analysis. J Cancer Res Ther. 2014; 10 (Suppl 1):38-41.
74. Wang JC, Tian JH, Ge L, Gan YH, Yang KH. Which is the best Chinese herb injection based on the FOLFOX regimen for gastric cancer? A network meta-analysis of randomized controlled trials. Asian Pac J Cancer Prev. 2014; 15:4795-4800.
75. Qi F, Li A, Inagaki Y, Kokudo N, Tamura S, Nakata M, Tang W. Antitumor activity of extracts and compounds from the skin of the toad Bufo bufo gargarizans Cantor. Int Immunopharmacol. 2011; 11:342-349.
76. Qi F, Li A, Inagaki Y, Xu H, Wang D, Cui X, Zhang L, Kokudo N, Du G, Tang W. Induction of apoptosis by cinobufacini preparation through mitochondria- and Fas-mediated caspase-dependent pathway. Food Chem Toxicol. 2012; 50:295-302.
77. Meng Z, Yang P, Shen Y, Bei W, Zhang Y, Ge Y, Newman RA, Cohen L, Liu L, Thornton B, Chang DZ, Liao Z, Kurzrock R. Pilot study of huachansu in patients with hepatocellular carcinoma, nonsmall-cell lung cancer, or pancreatic cancer. Cancer. 2009; 115:5309-5318.
78. Qin TJ, Zhao XH, Yun J, Zhang LX, Ruan ZP, Pan BR. Efficacy and safety of gemcitabine-oxaliplatin combined with huachansu in patients with advanced gallbladder carcinoma. World J Gastroenterol. 2008; 14:5210-5216.
79. Chen Z, Chen HY, Lang QB, Li B, Zhai XF, Guo YY, Yue XQ, Ling CQ. Preventive effects of jiedu granules combined with cinobufacini injection *versus* transcatheter arterial chemoembolization in post-surgical patients with hepatocellular carcinoma: A case-control trial. Chin J Integr Med. 2012; 18:339-344.
80. Sun T, Zhang Y, Shen Y, Hu K, Zuo M. A case of advanced lung cancer with malignant pericardial effusion treated by intrapericardial Cinobufacini injection instillation. Biosci Trends. 2014; 8:235-239.
81. Wang MJ, Nan X, Feng G, Yu HT, Hu GF, Liu YQ. Design, synthesis and bioactivity evaluation of novel acylthiourea derivatives of cantharidin. Ind Crop Prod. 2014; 55:11-18.
82. Han L, Sun YJ, Pan YF, Ding H, Chen X, Zhang X. Cantharidin combined with chemotherapy for Chinese patients with metastatic colorectal cancer. Asian Pac J Cancer Prev. 2014; 15:10977-10979.
83. Ji ZQ, Huang XE, Wu XY, Liu J, Wang L, Tang JH. Safety of Brucea javanica and cantharidin combined with chemotherapy for treatment of NSCLC patients. Asian Pac J Cancer Prev. 2014; 15:8603-8605.
84. Patricolo GE, Armstrong K, Riutta J, Lanni T. Lymphedema care for the breast cancer patient: An integrative approach. Breast. 2015; 24:82-85.
85. Wanchai A, Armer JM, Stewart BR. Complementary and alternative medicine and lymphedema. Semin Oncol Nurs. 2013; 29:41-49.
86. Casley-Smith JR, Morgan RG, Piller NB. Treatment of lymphedema of the arms and legs with 5,6-benzo-[alpha]-pyrone. N Engl J Med. 1993; 329:1158-1163.
87. Loprinzi CL, Kugler JW, Sloan JA, Rooke TW, Quella SK, Novotny P, Mowat RB, Michalak JC, Stella PJ, Levitt R, Tschetter LK, Windschitl H. Lack of effect of coumarin in women with lymphedema after treatment for breast cancer. N Engl J Med. 1999; 340:346-350.
88. Jiang ZM. Upper extremity lymphedema treated with compound Diosmin and Sodium aescine after breast cancer surgery. World Journal of Integrated Traditional and Western Medicine. 2010; 5:341-342. (in Chinese)
89. Takeda H, Muto S, Nakagawa K, Ohnishi S, Sadakane C, Saegusa Y, Nahata M, Hattori T, Asaka M. Rikkunshito as a ghrelin enhancer. Methods Enzymol. 2012; 514:333-351.
90. Gunji S, Ueda S, Yoshida M, Kanai M, Terajima H, Takabayashi A. Effects of rikkunshito, a kampo medicine, on quality of life after proximal gastrectomy. J Surg Res. 2013; 185:575-580.
91. Chen HY, Li SG, Cho WC, Zhang ZJ. The role of acupoint stimulation as an adjunct therapy for lung cancer: A systematic review and meta-analysis. BMC Complement Altern Med. 2013; 13:362.
92. Wang SJ, Xu J, Gong DD, Man CF, Fan Y. Meta-analysis of oral Chinese herbal medicine as an adjuvant treatment in relieving pain secondary to bone metastases. Chin J Integr Med. 2013 Oct 14. [Epub ahead of print]
93. Yanju B, Yang L, Hua B, Hou W, Shi Z, Li W, Li C, Chen C, Liu R, Qin Y, Lv W. A systematic review and meta-analysis on the use of traditional Chinese medicine compound kushen injection for bone cancer pain. Support Care Cancer. 2014; 22:825-836.
94. Su CX, Wang LQ, Grant SJ, Liu JP. Chinese herbal medicine for cancer-related fatigue: A systematic review of randomized clinical trials. Complement Ther Med. 2014; 22:567-579.
95. Lo LC, Chen CY, Chen ST, Chen HC, Lee TC, Chang CS. Therapeutic efficacy of traditional Chinese medicine, Shen-Mai San, in cancer patients undergoing chemotherapy or radiotherapy: Study protocol for a randomized, double-blind, placebo-controlled trial. Trials. 2012; 13:232.
96. Fu L, Fu H, Liu LQ, Huo ZJ, Yu YH, Yu JM. Efficacy of donkey-hide gelatin mixture for gemcitabine and cisplatin chemotherapy regimen induced myelosuppression.

Chinese Clinical Oncology. 2014; 19:739-742. (in Chinese)
97. Zhang PY, Qiu N. The clinical observation on Shi-quan-da-bu-tang relieving myelosuppression caused by chemotherapy. Modern Journal of Integrated Traditional Chinese and Western Medicine. 2009; 18:2157-2158. (in Chinese)
98. Zheng W, Gao ZH, Wu LN. Clinical observation on treatment of radiative pneumonia in patients with lung cancer by integrative Chinese and Western medicine. Zhongguo Zhong Xi Yi Jie He Za Zhi. 2007; 27:1121-1123. (in Chinese)
99. Xiao C, Ding HJ, Feng LC, Qu BL, Dou YQ. Efficacy of Liangxue Jiedu Huoxue Decoction in prevention of radiation pneumonitis: A randomized controlled trial. Zhong Xi Yi Jie He Xue Bao. 2010; 8:624-628. (in Chinese)
100. Liu ML, Chien LY, Tai CJ, Lin KC, Tai CJ. Effectiveness of Traditional Chinese Medicine for Liver Protection and Chemotherapy Completion among Cancer Patients. Evid Based Complement Alternat Med. 2011; 2011:291843.

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Original Article

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Perinatal outcomes of pregnancies complicated by preterm premature rupture of the membranes before 34 weeks of gestation in a tertiary center in China: A retrospective review

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Summary **Preterm premature rupture of the membranes (PPROM) remains the leading cause of preterm deliveries and neonatal mortality and morbidity. The current cohort study sought to retrospectively examine perinatal outcomes in cases of PPROM < 34 weeks' gestation that were managed conservatively from 2010 to 2012 and to identify risk factors for short-term neonatal outcomes. Subjects were 510 pregnancies consisting of 114 twin and 396 singleton pregnancies. Clinical chorioamnionitis occurred in 17.8% of the pregnancies. Neonatal mortality was 7.4%, the rate of major neonatal conditions was 40%, and the rate of NICU admission was 72.9%. The latency period exceeded 48 h in 62.5% of the pregnancies and 7 days in 24.3% of the pregnancies. Twin pregnancies had a shorter latency period than singleton pregnancies (median of 2 days versus 4 days, $p < 0.001$). Pregnancies complicated with early vaginal bleeding had a higher neonatal mortality (13.95% *vs*. 6.36%, $p = 0.013$) and morbidity (51.16% *vs*. 38.32%, $p = 0.024$), fewer weeks of gestation at PPROM ($p = 0.029$). Multivariate logistic regression analysis revealed that weeks of gestation at PPROM (OR: 0.953, 95% CI: 0.939-0.966, $p < 0.001$) and a latency period (OR: 0.948, 95%CI: 0.926-0.970, $p < 0.001$) were associated with neonatal mortality or morbidity. A twin pregnancy (OR: 0.319, 95% CI: 0.17-0.6, $p < 0.001$) and weeks of gestation at PPROM (OR: 0.737, 95% CI: 0.66-0.822, $p < 0.001$) were associated with the latency period. Gestational age at PPROM, a twin pregnancy, and the latency period are associated with neonatal mortality and morbidity.**

Keywords: Preterm premature rupture of the membranes, neonatal morbidity, neonatal mortality, latency period

1. Introduction

Preterm birth occurs in approximately 12% of pregnancies (*1*). Preterm premature rupture of the membranes (PPROM), a subtype of preterm labor, is defined as spontaneous membrane rupture without onset of labor before 37 weeks of gestation. PPROM occurs in approximately 3% of pregnancies and results in one-third of preterm births. It remains the leading cause of preterm deliveries and neonatal mortality and morbidity (*2,3*).

Conditions due to prematurity include respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), necrotising enterocolitis (NEC), a prolonged stay in the neonatal intensive care unit (NICU), neonatal sepsis, required use of positive pressure ventilation (PPV), cardiac abnormalities, hyperbilirubinemia and hemolytic anemia, as well as cerebral palsy, and other long-term outcomes (*4*). Therefore, appropriate management of PPROM is crucial to improving neonatal and maternal outcomes. Obstetrical strategies to treat PPROM remain controversial and there is no consensus regarding active or conservative management of PPROM within 30 and 34 weeks' gestation. Conservative management to prolong a pregnancy is a classical approach to treating PPROM before 34 weeks' gestation in association with antibiotic therapy and corticosteroids. Delivery is recommended when PPROM occurs at or beyond 34 weeks' gestation (*5-7*).

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This Hospital, a tertiary care hospital in southwest China, conservatively manages PPROM < 34 weeks' gestation. This Hospital manages the majority of complicated pregnancies in this area. The aim of the present study was to evaluate neonatal and maternal outcomes in women with conservatively managed PPROM < 34 weeks' gestation and to identify risk factors for short-term neonatal outcomes.

2. Methods

This was a retrospective cohort study. Approval was obtained from the Institutional Review Board. Medical charts of women with spontaneous PPROM < 34 weeks' gestation who were admitted to Obstetrics at West China Second University Hospital from 2010 to 2012 were reviewed.

2.1. *Subjects*

Cases of triplets or higher order pregnancies, congenital malformations incompatible with life, a twin pregnancy with a delayed-interval delivery, fetal death before PPROM, and maternal and/or fetal indications for immediate delivery after admission were excluded.

2.2. *PPROM diagnosis*

Diagnosis of PPROM was based on the patient's history of watery discharge and leakage of amniotic fluid from the cervical os during a sterile speculum examination. Gestational age was determined based on the patient's last period or by ultrasound performed during the first trimester or early second trimester.

2.3. *Management of PPROM*

Couples were counseled by obstetricians about the adverse outcomes of an intentional delivery and conservative management and they selected conservative management when the patient was not in labor at admission. Dexamethasone was given for maturation of the fetal lungs, and the complete course was 4 doses of 5 mg *i.m*. within a 48-h interval. A broad-spectrum antibiotic (ampicillin, ceftezole, or clarithromycin) was administered from admission until delivery or for up to 7 days to prevent infection. Magnesium sulfate, a calcium channel blocker (nifedipine), or a betamimetic agent (ritodrine) was used as a tocolytic agent depending on the patient's status.

The status of the mother and fetus was closely monitored until delivery. Patients were checked by obstetricians daily. Maternal vital signs were monitored every 6 hours, and serum C-reactive protein (CRP) levels and the white blood cell count were checked weekly. Ultrasound scans were performed once a week to evaluate the status of the fetus. CTGs were performed daily to assess the status of the fetus. The treatment was promptly adjusted when necessary depending on the status of the mother and fetus. None of the pregnant women in this study underwent amniocentesis to assess infection or fetal lung maturity.

Clinical chorioamnionitis was performed if the patient presented with three or more of the following criteria: maternal body temperature ≥ 38°C, maternal tachycardia (≥ 110 beats/min), persistent fetal tachycardia (> 160 beats/min) or bradycardia (< 120 beats/min), presence of uterine tenderness, elevated CRP, malodorous vaginal discharge, and leukocytosis (≥ 15,000 cells/mm^3) in maternal blood.

When clinical chorioamnionitis was diagnosed during prolongation of a pregnancy, or there were maternal and/or fetal indications for delivery, or gestation reached 34 weeks, the pregnancy was managed with active induction of labor or a Cesarean delivery depending on the patient's obstetric history and the fetus' status.

2.4. *Data collection*

Maternal parameters including maternal age, parity, spontaneous or *in vitro* fertilization, singleton or twin pregnancy, and gestational age at PPROM were reviewed. Furthermore, parameters including weeks of gestation at delivery, mode of delivery, clinical chorioamnionitis, and obstetric complications were investigated. Recent studies of early vaginal bleeding in singleton and twin pregnancies have cited an increased risk of PPROM, preterm birth, preeclampsia, and placental abruption (*8,9*). Therefore, early vaginal bleeding during the pregnancy was reviewed during this study. Such bleeding was defined as the presence of blood in the vagina in the first or second trimester. Fetal parameters including fetal distress, birth weight, neonatal death, admission to the NICU, and major neonatal conditions (including patent ductus arteriosus (PDA), RDS, IVH, PVL, NEC, and sepsis) were also studied.

2.5. *Statistical analyses*

Maternal, fetal, and neonatal parameters were analyzed using a Chi-squared test, Fisher's exact test, a two-tailed student's *t*-test, and Mann-Whitney U test. Multivariate logistic regression was used to correlate the latency period, neonatal mortality, and neonatal morbidity with the parameters collected from medical charts. A p value < 0.05 was considered statistically significantly. Analysis was performed with the statistical software SPSS version 17.0.

3. Results

3.1. *Maternal characteristics*

Subjects were 510 patients who were diagnosed with

Table 1. Maternal characteristics of cases of PPROM < 34 weeks of gestation

Characteristics	PPROM, *n* = 510
Maternal age (mean ± S.D.)	28.4 ± 5.6
Maternal age > 35years, *n* (%)	72 (14.1%)
Weeks of gestation at admission (mean ± S.D.)	31.6 ± 1.9
Nulliparous, *n* (%)	361 (70.8%)
Conception	
Natural, *n* (%)	473 (92.8%)
IVF, *n* (%)	37 (7.3%)
Pregnancy	
Singleton, *n* (%)	396 (77.7%)
Twin, *n* (%)	114 (22.4%)
Previous cesarean section, *n* (%)	58 (11.4%)
History of preterm delivery, *n* (%)	6 (1.2%)
Early vaginal bleeding, *n* (%)	69 (13.5%)

PPROM < 34 weeks' gestation from 2010 to 2012. Of the 510 pregnancies, 396 were singleton pregnancies and 114 were twin pregnancies. The mean maternal age was 28.4 years (range: 17-45 years). The mean weeks of gestation at PPROM was 31.6 weeks (range: 20^{+2} -33^{+6} weeks). Of the 510 patients, 11.4% (58/510) had a previous Cesarean section, 1.2% (6/510) had one or more preterm births, and 13.5% (69/510) had a history of vaginal bleeding in early stages of the current pregnancy. The maternal characteristics of all patients in the study are shown in Table 1. There were no maternal deaths or severe morbidity noted in the current study.

3.2. *Clinical outcomes*

The mean weeks of gestation at delivery were 32.5 weeks (range 27^{+2} -34^{+3} weeks). Obstetrical complications such as hypertensive disorders of pregnancy, diabetes, intrahepatic cholestasis of pregnancy, umbilical prolapse, and placenta abruption were recorded based on medical charts and are shown in Table 2. The Cesarean section was performed in 37.1% of pregnancies with a medical complication such as breech presentation, fetal distress, or failure of labor to progress. During prolongation of a pregnancy, 284 patients (55.7%) went into labor spontaneously and delivered before 34 weeks. Indications for active induction of labor or a Cesarean delivery were as follows: 91 patients (17.8%) presented with clinical chorioamnionitis, 29 (5.7%) had maternal or fetal complications during prolongation of the pregnancy, and 106 (20.8%) had a pregnancy that lasted 34 weeks.

Fetal and neonatal data were reviewed. The mean neonatal birth weight was 1815.1 ± 462.6 g and 162 neonates (26.1%) were delivered with a birth weight < 1,500g (VLBW). Death of one twin occurred in three twin pregnancies that were conservatively managed. The neonatal mortality rate was 7.4% (46 cases) and the rate of major neonatal conditions was 40% (249 cases). Furthermore, the rate of NICU admission was 72.9%. Major neonatal conditions noted in the current study were IVH in 197 pregnancies (31.7%), RDS in 72 (11.6%), PDA in 10 (1.6%), PVL in 7 (1.1%), sepsis in 7 (1.1%), and NEC in 3 (0.5%). Data on these conditions are shown in Table 2.

Table 2. Clinical outcomes in cases of PPROM

Characteristics	PPROM, *n* = 510
Weeks of gestation at delivery (mean ± S.D.)	32.5 ± 1.7
Cesarean delivery, *n* (%)	189 (37.1%)
Obstetrical complications	
Diabetes[a], *n* (%)	109 (21.4%)
Intrahepatic cholestasis of pregnancy, *n* (%)	48 (9.4%)
Hypertensive disorders of pregnancy[b], *n* (%)	22 (4.3%)
Clinical chorioamnionitis, *n* (%)	91 (17.8%)
Fetal distress, *n* (%)	17 (3.3%)
Placenta abruption, *n* (%)	11 (2.2%)
Umbilical prolapse, *n* (%)	16 (3.1%)
Fetal death, *n* (%)	3 (0.6%)
Indications for termination	
Clinical chorioamnionitis, *n* (%)	91 (17.8%)
Maternal or fetal complications	29 (5.7%)
Pregnancy lasted 34 weeks	106 (20.8%)
Neonatal outcomes	
Birth weight (mean ± S.D.)	1815.1 ± 462.6
VLBW, *n* (%)	162 (26.1%)
NICU admission, *n* (%)	453 (72.9%)
Neonatal death, *n* (%)	46 (7.4%)
Major neonatal condition	
Intraventricular hemorrhage, *n* (%)	197 (31.7%)
Respiratory distress syndrome, *n* (%)	72 (11.6%)
Patent ductus arteriosus, *n* (%)	10 (1.6%)
Periventricular leukomalacia, *n* (%)	7 (1.1%)
Sepsis, *n* (%)	7 (1.1%)
Necrotizing enterocolitis, *n* (%)	3 (0.5%)

[a] Diabetes: includes type 1 diabetes, type 2 diabetes, gestational diabetes; [b] Hypertensive disorders of pregnancy: includes chronic hypertension, gestational hypertension, and preeclampsia.

3.3. *Neonatal mortality, major neonatal conditions, and NICU admission by gestational age at PPROM*

The gestational age at PPROM was divided into four periods to indicate perinatal outcomes by different weeks of gestation: < 28 weeks, 28-29^{+6} weeks, 30-31^{+6} weeks, and 32-33^{+6} weeks. The groups were analyzed in terms of the neonatal mortality, the rate of NICU admission, major neonatal conditions, and the latency period.

As gestational age at PPROM increased, the risk of an adverse neonatal outcome declined. The neonatal mortality rate was 7.4% (46 cases) in cases of PPROM, 50% in pregnancies < 28 weeks, 16.1% in pregnancies of 28-29^{+6} weeks, 5.7% in pregnancies of 30-31^{+6} weeks, and 2.2% in pregnancies of 32-33^{+6} weeks. The differences among the groups were statistically significantly (Table 3). As gestational age at PPROM increased, the rate of neonatal mortality significantly decreased.

The rate of major neonatal conditions was 40% (249 cases) in cases of PPROM, 57.1% in pregnancies < 28 weeks, 63.44% in pregnancies of 28-29^{+6} weeks,

Table 3. Neonatal mortality, major neonatal conditions, and NICU admission by gestational age at PPROM

Gestational age at PPROM	major neonatal condition, *n* (%)	neonatal mortality, *n* (%)	NICU admission, *n* (%)
< 28 w (*n* = 28)	16 (57.1)	14 (50)[a]	26 (92.9)[b]
28-29^{+6} w (*n* = 93)	59 (63.4)	15 (16.1)[a]	84 (90.3)[b]
30-31^{+6} w (*n* = 177)	71 (40.1)[c]	10 (5.7)[a]	149 (84.2)[b]
32-33^{+6} w (*n* = 323)	103 (31.9)[c]	7 (2.2)[a]	194 (60.1)
Total (*n* = 621)	249 (40)	46 (7.4)	453 (72.9)

Comparisons made by chi-square or Fisher's exact test, $p < 0.05$ is significant. [a] compared to each other, [b] compared to 32^{+0}-33^{+6} w, [c] compared to 28-29^{+6} w. Major neonatal condition: 28-29^{+6} w *vs*. 30-31^{+6} w, $p < 0.001$; 28-29^{+6} w *vs*. 32-33^{+6} w, $p < 0.001$; < 28 w *vs*. 32-33^{+6} w, $p = 0.007$. Neonatal mortality: < 28 w *vs*. 28-29^{+6} w, $p < 0.001$; < 28 w *vs*. 30-31^{+6} w, $p < 0.001$; < 28 w *vs*. 32-33^{+6} w, $p < 0.001$; 28-29^{+6} w *vs*. 30-31^{+6} w, $p = 0.005$; 28-29^{+6} w *vs*. 32-33^{+6} w, $p < 0.001$; 30-31^{+6} w *vs*. 32-33^{+6} w, $p = 0.04$. NICU admission: 32-33^{+6} w *vs*. < 28 w, $p = 0.001$; 32-33^{+6} w *vs*. 28-29^{+6} w, $p < 0.001$; 32-33^{+6} w *vs*. 30-31^{+6} w, $p < 0.001$.

Table 4. Perinatal outcomes in cases of PPROM with early vaginal bleeding

Characteristics	Present (*n* = 69)	Absent (*n* = 441)	*p*
Weeks of gestation at PPROM (mean ± S.D.)	31.1 ± 2.2	31.7 ± 1.8	0.029
Weeks of gestation at delivery (mean ± S.D.)	32.1 ± 2.0	32.6 ± 1.7	0.059
Neonatal birth weight (mean ± S.D.)	1766.2 ± 440.9	1822.9 ± 465.9	0.292
VLBW, %	27.9	25.4	0.625
Neonatal mortality, %	13.95	6.36	0.013
Neonatal morbidity, %	51.16	38.32	0.024

Comparisons done using a Student's *t*-test, chi-square test, or Fisher's exact test, $p < 0.05$ is significant.

40.11% in pregnancies of 30-31^{+6} weeks, and 31.9% in pregnancies of 32-33^{+6} weeks. The rate of major neonatal conditions decreased as the gestational age at PPROM increased (Table 3).

The rate of NICU admission was 60.1% in pregnancies of 32-33^{+6} weeks. This rate was significantly lower than that in pregnancies < 28 weeks, pregnancies of 28-29^{+6} weeks, and pregnancies of 30-31^{+6} weeks (Table 3).

3.4. *Perinatal outcomes in cases of early vaginal bleeding*

Perinatal outcomes were investigated in cases of early vaginal bleeding and cases where it was absent (Table 4). Cases of early vaginal bleeding involved a significantly higher rate of neonatal mortality (13.95% *vs*. 6.36%, $p = 0.013$) and morbidity (51.16% *vs*. 38.32%, $p = 0.024$). Cases of early vaginal bleeding were associated with significantly fewer weeks of gestation at PPROM (31.1 ± 2.2 weeks *vs*.31.7 ± 1.8 weeks, $p = 0.029$).

3.5. *Clinical chorioamnionitis*

The latency period and clinical chorioamnionitis were significant related. The latency period in cases of clinical chorioamnionitis was longer than that in cases where clinical chorioamnionitis was absent (median 5 days *vs*. 3 days, $p = 0.001$). Cases of clinical chorioamnionitis were associated with fewer weeks of gestation at PPROM than were cases where clinical chorioamnionitis was absent (31.2 ± 2.1 *vs*. 31.7 ± 1.9, $p = 0.014$). There was no significant correlation between clinical chorioamnionitis and neonatal mortality and morbidity.

3.6. *Latency period*

The latency period was defined as the time from membrane rupture to delivery. In this study, the median latency period was 4 days (range 0-56 days). The latency period was divided into three periods: ≤ 48 h, 3-7 days, and > 7 days. The latency period exceeded 48 h in 62.5% of pregnancies and 7 days in 24.3%. Moreover, twin pregnancies were associated with a shorter latency period than singleton pregnancies (median latency period of 2 days versus 4 days, $p < 0.001$, Mann-Whitney U test).

There was a strong inverse correlation between gestational age at PPROM and the latency period. PPROM at < 28 weeks was associated with a significantly higher rates of latency > 7 days than other groups (66.7% *vs*. 30.8%, 66.7% *vs*. 28.2%, 66.7% *vs*. 17.1%). PPROM at 32-33^{+6} weeks was associated with a significantly higher rates of latency ≤ 48 h than other groups (43.9% *vs*. 14.3%, 43.9% *vs*. 29.5%, 43.9% *vs*. 33.1%).

3.7. *Analysis of composite factors for the latency period*

Multivariate logistic regression was used to examine the correlation between the latency period and potential risk factors such as maternal age, method of conception, parity, singleton or twin pregnancy, history of early vaginal bleeding, weeks of gestation at PPROM, and clinical chorioamnionitis. Results indicated that a twin pregnancy (OR: 0.319, 95% CI: 0.17-0.6, $p < 0.001$) and weeks of gestation at PPROM were associated with

the latency interval (OR: 0.737, 95% CI: 0.66-0.822, $p < 0.001$).

3.8. *Analysis of composite factors for neonatal mortality or morbidity*

Multivariate logistic regression was used to examine the correlation between neonatal mortality or morbidity and potential risk factors such as maternal age, method of conception, parity, singleton or twin pregnancy, history of early vaginal bleeding, weeks of gestation at PPROM, clinical chorioamnionitis, and the latency period. Results indicated that weeks of gestation at PPROM (OR: 0.953, 95% CI: 0.939-0.966, $p < 0.001$) and the latency period (OR: 0.948, 95% CI: 0.926-0.970, $p < 0.001$) were associated with neonatal mortality or morbidity.

4. Discussion

One-third of preterm births are the result of PPROM. PPROM remains the leading cause of preterm deliveries and adverse neonatal outcomes. The etiology of PPROM remains elusive.

Obstetrical strategies to treat PPROM remain controversial. Intentional delivery should not be an option for women with PPROM between 28 and 34 weeks of gestation in the absence of other indications for early delivery since it increases the incidence of neonatal deaths and the rate of Cesarean sections (*10*). Expectant management is a classical approach to managing PPROM before 34 weeks' gestation, including admission to the hospital, administration of corticosteroids, and amniocentesis to exclude intra-amniotic infection and/or broad-spectrum antibiotics prophylaxis (*11*). Corticosteroids for fetal maturation have been proven to improve neonatal outcomes in preterm births and reduce perinatal mortality, RDS, and IVH (*12*). According to the guidelines of the SOGC, following PPROM at ≤ 32 weeks' gestation, antibiotics should be administered to women not in labor to prolong pregnancy and decrease maternal and neonatal morbidity; for PPROM at > 32 weeks' gestation, antibiotics should be administered to prolong a pregnancy if fetal lung maturity is not evident and/or delivery is not planned (*13*). Although there is no consensus regarding tocolysis, it may be used in women presenting with uterine contractions or can be used prophylactically to capitalize on the benefit of corticosteroids. Delivery is recommended when PROM occurs at or beyond 34 weeks' gestation (*14*). In the current study, management depended on Chinese guidelines for preterm labor and PPROM, which also recommend the conservative management of PPROM before 34 weeks' gestation in combination with antibiotic therapy and corticosteroids. Indications for active induction of labor or a Cesarean delivery during prolongation of a pregnancy were as follows: 17.8% of patients presented with clinical chorioamnionitis, 5.7% had maternal or fetal complications during prolongation of a pregnancy, and 20.8% had a pregnancy lasting 34 weeks.

During prolongation of a pregnancy, maternal and fetal infections can occur. This is especially true for chorioamnionitis, which is identified by means of clinical signs, histologic evidence, and the culture of microorganisms; chorioamnionitis is the most obvious infection associated with early labor and delivery (*15*). The current patients were treated with antibiotics. There were no maternal deaths or severe neonatal conditions noted in this study. In this study, clinical chorioamnionitis was noted in 17.8% of patients. This figure disagrees with those reported elsewhere due to differences in inclusion criteria. Ehsanipoor *et al*. studied cases of PPROM between 24 and $31^{6/7}$ weeks' gestation and noted clinical chorioamnionitis in 9.8% of twins and 23.2% of singletons; chorioamnionitis in the placenta was noted in 35.9% of twins and 67.7% in singletons (*16*). Goya *et al*. reported that clinical chorioamnionitis occurred in 23.1% of cases of expectantly managed PPROM < 34 weeks, and they diagnosed subclinical chorioamnionitis with amniocentesis in 4.6% of cases (*17*). In a retrospective study of women with singleton and twin pregnancies and PPROM between 24^{+0} and 36^{+6} weeks of gestation, 7.5% were found to have clinical chorioamnionitis (*18*). A disadvantage of the current study is that it did not include histopathological chorioamnionitis due to incomplete data on placental pathology, so the perinatal outcomes in cases of PPROM with histopathological chorioamnionitis could not be determined and the diagnosis of clinical chorioamnionitis could not be confirmed with histopathological results.

PPROM is responsible for 30% of the neonatal morbidity and mortality in premature births. In the current study, the fetal death rate was 0.6% and the neonatal mortality rate was 7.41%. These figures agree with those found in the literature (*17*). As expected, the risk of adverse neonatal outcomes declined with a higher gestational age at PPROM; this finding agrees with the results of a study by Pasquier *et al*. (*19*). In the current study, the neonatal mortality rate was 50% at < 28 weeks of gestation, 16.13% at 28-29^{+6} weeks, 5.65% at 30-31^{+6} weeks, and 2.17% at ≥ 32 weeks. Another study reported that the neonatal mortality rate was 53.6% at ≤ 28 weeks of gestation, 8.4% at 28-32 weeks, and 3.4% at ≥ 33 weeks (*4*). The rate of major neonatal conditions in the current study was 40%. These conditions consisted of IVH in 31.7% of cases, RDS in 11.6%, PDA in 1.6%, PVL in 1.1%, sepsis in 1.1%, and NEC in 0.5%. These figures agree with those reported by Gezer *et al*., who noted RDS in 30.7% of cases, IVH in 11.4%, sepsis in 13.6%, NEC in 0.4%, PDA in 4.4%, and leukomalacia in 0.4% (*4*).

In the present study, the rate of NICU admission was 72.9%, which is the same as in other studies. Walker *et al*. reported that PPROM for > 28 days was associated with an increased risk of death and morbidity (*20*). The current results indicated that weeks of gestation at PPROM (OR: 0.953, 95% CI: 0.939-0.966, $p < 0.001$) and the latency period (OR: 0.948, 95% CI: 0.926-0.970, $p < 0.001$) were significantly associated with neonatal mortality or morbidity. Thus, the contention by Walker *et al*. is correct. In light of maternal and fetal findings, a conservative approach can prove beneficial in cases of PPROM at < 34 weeks.

Vaginal bleeding in either the first or second trimester increased the risk of preterm delivery, PPROM, histological chorioamnionitis, and abruption. Ascending infection has been implicated as a possible etiology for PPROM. Data on early vaginal bleeding in cases of PPROM were analyzed to determine if early vaginal bleeding in cases of PPROM is associated with neonatal mortality and morbidity. Perinatal outcomes were compared in cases of early vaginal bleeding and cases where it was absent. Cases of early vaginal bleeding involved significantly fewer weeks of gestation at PPROM (31.07 ± 2.23 weeks *vs*. 31.69 ± 1.84 weeks, $p = 0.029$). Cases of early vaginal bleeding also involved a higher neonatal mortality and morbidity. Thus, early vaginal bleeding is a risk factor for fewer weeks of gestation at PPROM and adverse neonatal outcomes.

Some factors are known to affect the latency period, including gestational age, oligohydramnios, number of fetuses, and complications of pregnancy such as intra-amniotic infection, placental abruption, and active labor (*21*). In the current study, 37.5% of women with PPROM delivered within 48 h and 24.3% had a latency period of > 7 days. Gopalani *et al*. reported 38.8% of patients with PPROM at 24 to 31.9 weeks delivered within 48 h, but 32% had a latency period of > 7 days (*22*). The current study found that a younger gestational age at PPROM was associated with a longer latency period and that twin pregnancies were associated with a shorter latency period than singleton pregnancies (median latency period of 2 days versus 4 days, $p < 0.001$). Multivariate logistic regression analysis was performed after adjusting for confounding factors to determine risk factors for the latency period. Gestational age at PPROM ($p < 0.001$) and a twin pregnancy ($p < 0.001$) were found to be inversely correlated with the latency period. This finding agrees with the results of other studies (*18,23,24*).

The rate of Cesarean sections in this study was 37.06%. Other studies have reported a rate from 10% to 53% (*25,26*). The indications for a Cesarean section in the current cases were fetal distress, repeated Cesarean sections, abruptio placenta, failure of labor to progress, and breech presentation.

A limitation of this study was its retrospective nature. In addition, this study did not include histopathological chorioamnionitis due to incomplete data on placental pathology. Perinatal outcomes in cases of PPROM with histopathological chorioamnionitis were unable to be determined. Further prospective studies will focus on the associations revealed by this analysis.

In conclusion, the current results revealed that weeks of gestation at PPROM and the latency period were significantly associated with neonatal mortality or morbidity. A twin pregnancy and weeks of gestation at PPROM were significantly correlated with the latency period. Although early vaginal bleeding was not significantly associated with neonatal mortality or morbidity according to multivariate logistic regression, it may also be a risk factor for perinatal outcomes in cases of PPROM due to fewer weeks of gestation at PPROM and a higher neonatal mortality and morbidity.

Acknowledgements

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References

1. Martin JA, Kung HC, Mathews TJ, Hoyert DL, Strobino DM, Guyer B, Sutton SR. Annual summary of vital statistics. Pediatrics. 2008; 121:788-801.
2. Mercer BM. Preterm premature rupture of the membranes: Current approaches to evaluation and management. Obstet Gynecol Clin North Am. 2005; 32:411-428.
3. Ye G, Jiang Z, Lu S, Le Y. Premature infants born after preterm premature rupture of membranes with 24-34 weeks of gestation: A study of factors influencing length of neonatal intensive care unit stay. J Matern Fetal Neonatal Med. 2011; 24:960-965.
4. Gezer A, Parafit-Yalciner E, Guralp O, Yedigoz V, Altinok T, Madazli R. Neonatal morbidity mortality outcomes in pre-term premature rupture of membranes. J Obstet Gynaecol. 2013; 33:38-42.
5. Baud O, Fontaine RH, Olivier P, Maury L, El Moussawi F, Bauvin I, Arsac M, Hovhannisyan S, Farnoux C, Aujard Y. Premature rupture of membranes: Pathophysiology of neurological impact. Arch Pediatr. 2007; 14:S49-S53.
6. Kenyon SL, Taylor DJ, Tarnow-Mordi W, ORACLE Collaborative Group. Broadspectrum antibiotics for preterm, prelabour rupture of fetal membranes: The ORACLE I randomised trial. Lancet. 2001; 357:979-988.
7. Harding JE, Pang J, Knight DB, Liggins GC. Do antenatal corticosteroids help in the setting of preterm rupture of membranes? Am J Obstet Gynecol. 2001; 184:131-139.
8. Velez Edwards DR, Baird DD, Hasan R, Savitz DA, Hartmann KE. First-trimester bleeding characteristics associated with increased risk of preterm birth: Data from a prospective pregnancy cohort. Hum Reprod. 2012;

27:54-60.
9. McPherson JA, Odibo AO, Shanks AL, Roehl KA, Macones GA, Cahill AG. Adverse outcomes in twin pregnancies complicated by early vaginal bleeding. Am J Obstet Gynecol. 2013; 208:56.e1-5.
10. Al-Mandeel H, Alhindi MY, Sauve R. Effects of intentional delivery on maternal and neonatal outcomes in pregnancies with preterm prelabour rupture of membranes between 28 and 34 weeks of gestation: A systematic review and meta-analysis. J Matern Fetal Neonatal Med. 2013; 26:83-89.
11. Di Renzo GC, Roura LC, Facchinetti F, *et al*. Guidelines for the management of spontaneous preterm labor: Identification of spontaneous preterm labor, diagnosis of preterm premature rupture of membranes, and preventive tools for preterm birth. J Matern Fetal Neonatal Med. 2011; 24:659-667.
12. Vidaeff AC, Ramin SM. Antenatal corticosteroids after preterm premature rupture of membranes. Clin Obstet Gynecol. 2011; 54:337-343.
13. Yudin MH, Van Schalkwyk J, Van Eyk N, Boucher M, *et al*. Antibiotic therapy in preterm premature rupture of the membranes. J Obstet Gynaecol Can. 2009; 31:863-867.
14. ACOG practice bulletin no. 80: Premature rupture of membranes. Clinical management guidelines for obstetrician-gynecologists. Obstet Gynecol. 2007; 109:1007-1019.
15. Muglia LJ, Katz M. The enigma of spontaneous preterm birth. N Engl J Med. 2010; 362:529-535.
16. Ehsanipoor RM, Arora N, Lagrew DC, Wing DA, Chung JH. Twin versus singleton pregnancies complicated by preterm premature rupture of membranes. J Matern Fetal Neonatal Med. 2012; 25:658-661.
17. Goya M, Bernabeu A, Garcia N, Plata J, Gonzalez F, Merced C, Llurba E, Suy A, Casellas M, Carreras E, Cabero L. Premature rupture of membranes before 34 weeks managed expectantly: Maternal and perinatal outcomes in singletons. J Matern Fetal Neonatal Med. 2013; 26:290-293.
18. Dagklis T, Petousis S, Margioula-Siarkou C, Mavromatidis G, Kalogiannidis I, Prapas N, Mamopoulos A, Rousso D. Parameters affecting latency period in PPROM cases: A 10-year experience of a single institution. J Matern Fetal Neonatal Med. 2013; 26:1455-1458.
19. Pasquier JC, Picaud JC, Rabilloud M, Claris O, Ecochard R, Moret S, Mellier G. Neonatal outcomes after elective delivery management of preterm premature rupture of the membranes before 34 weeks' gestation (DOMINOS study). Eur J Obstet Gynecol Reprod Biol. 2009; 143:18-23.
20. Walker MW, Picklesimer AH, Clark RH, Spitzer AR, Garite TJ. Impact of duration of rupture of membranes on outcomes of premature infants. J Perinatol. 2014; 34:669-672.
21. Caughey AB, Robinson JN, Norwitz ER. Contemporary diagnosis and management of preterm premature rupture of membranes. Rev Obstet Gynecol. 2008; 1:11-22.
22. Gopalani S, Krohn M, Meyn L, Hitti J, Crombleholme WR. Contemporary management of preterm premature rupture of membranes: Determinants of latency and neonatal outcome. Am J Perinatol. 2004; 21:183-190.
23. Melamed N, Hadar E, Ben-Haroush A, Kaplan B, Yogev Y. Factors affecting the duration of the latency period in preterm premature rupture of membranes. J Matern Fetal Neonatal Med. 2009; 22:1051-1056.
24. Trentacoste SV, Jean-Pierre C, Baergen R, Chasen ST. Outcomes of preterm premature rupture of membranes in twin pregnancies. J Matern Fetal Neonatal Med. 2008; 21:555-557.
25. Kwak HM, Shin MY, Cha HH, Choi SJ, Lee JH, Kim JS, Roh CR, Kim JH, Oh SY. The efficacy of cefazolin plus macrolide (erythromycin or clarithromycin) versus cefazolin alone in neonatal morbidity and placental inflammation for women with preterm premature rupture of membranes. Placenta. 2013; 34:346-352.
26. Lieman JM, Brumfield CG, Carlo W, Ramsey PS. Preterm premature rupture of membranes: Is there an optimal gestational age for delivery? Obstet Gynecol. 2005; 105:12-17.

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Original Article

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L-carnitine affects osteoblast differentiation in NIH3T3 fibroblasts by the IGF-1/PI3K/Akt signalling pathway

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Summary **Fibroblasts in soft tissues are one of the progenitors of ectopic calcification. Our previous experiment found that the serum concentrations of small metabolite L-carnitine (LC) decreased in an ectopic calcification animal model, indicating LC is a potential calcification or mineralization inhibitor. In this study, we investigated the effect of LC on NIH3T3 fibroblast osteoblast differentiation, and explored its possible molecular mechanisms. Two concentrations of LC (10 μM and 100 μM) were added in Pi-induced NIH3T3 fibroblasts, cell proliferation was compared by MTT assays, osteoblast differentiation was evaluated by ALP activity, mineralized nodules formation, calcium deposition, and expressions of the osteogenic marker genes. Our results indicated that 10 μM LC increased the proliferation of NIH3T3 cells, but 100 μM LC slightly inhibited cell proliferation. 100 μM LC inhibits NIH3T3 differentiation as evidenced by decreases in ALP activity, mineralized nodule formation, calcium deposition, and down-regulation of the osteogenic marker genes *ALP*, *Runx2* and *OCN*, meanwhile 10 μM of LC exerts an opposite effect that promotes NIH3T3 osteogenesis. Mechanistically, 100 μM LC significantly inhibits IGF-1/PI3K/Akt signalling, while 10 μM LC slightly activates this pathway. Our study suggests that a decease in LC level might contribute to the development of ectopic calcification in fibroblasts by affecting IGF-1/PI3K/Akt, and addition of LC may benefit patients with ectopic calcification.**

Keywords: Ectopic calcification, fibroblast, L-carnitine, IGF-1/PI3K/Akt, proliferation, osteoblastic differentiation

1. Introduction

Ectopic calcification is defined as inappropriate deposition of calcium/phosphate complexes in connective tissues in aberrant locations (*1*). Pseudoxanthoma elasticum (PXE) is a prototype of multisystem ectopic mineralization disorders characterized by calcium phosphate deposition in various tissues (*2*). PXE is caused by mutations in the *ABCC6* gene which encodes for a putative transmembrane transporter protein, ABCC6 (*3-5*). The *Abcc6*$^{-/-}$ mouse which recapitulates the features of PXE, is a mouse model of PXE including extensive mineralization in the arterial blood vessels, skin and Bruch's membrane in the eyes (*6,7*). Fibroblasts are present in all connective tissues, which are the main component of dense connective tissue and the progenitors of ectopic calcification (*8*). Osteoblasts and fibroblasts are both of mesenchymal origin. In cell morphology, osteoblasts are nearly indistinguishable from fibroblasts, except for the formation of mineralized extracellular matrix which locates outside the cells. Additionally, all the genes expressed in fibroblasts are also expressed in osteoblasts, but osteoblasts express only two osteoblast-specific transcripts: one encoding runt-related transcription factor 2 (Runx2), a transcription factor, and the other encoding osteocalcin (OCN), a secreted molecule (*9*). Thus, fibroblasts and osteoblasts may express mutual transformation behavior due to the same origin, partial overlap of phenotype, and similarity of differentiation. Previous studies have indicated that fibroblasts can convert to osteoblasts and form bone

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in vitro through induction of inflammatory mediators, TGF-β, *etc*. (*10*). Furthermore, *in vivo* experiments also confirmed that when there was an appropriate stimulus condition (*e.g*. bone chips), fibroblasts became active, could be transformed into osteoblasts, and finally formed mineralized nodules (*11*).

Cellular metabolic activity plays an important role in regulating cell survival, differentiation and tissue growth (*12*). L-carnitine (LC) is a trimethylated amino acid which is an essential cofactor for the transport of long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix for their subsequent β-oxidation (*13*). LC facilitates energy availability, and is especially important for those tissues with high energy requirements. Studies indicated that cells of the osteoblastic lineage generate 40% to 80% of their energy demands through fatty acid β-oxidation (*14*). It has also been demonstrated that LC could protect osteoblastic cells from apoptosis (*15,16*), increased metabolic activity and protein production of porcine osteoblast-like cells (*17*), as well as affected osteoblastic activity (*15*).

Further studies *in vivo* and *in vitro* have suggested that proliferating and differentiating factors which affect osteoblastic activity exert their roles through the involvement of insulin-like growth factor (IGF) expression (*18*). IGF-1 is produced and stored in the bone matrix which stimulates proliferation and differentiation of osteoblasts (*19-21*). Moreover, studies in animals and humans have shown that supplementation with LC increased plasma concentrations of IGF-1 (*22-24*). IGF-1 plays an important role in the activation of the IGF-1/PI3K/Akt signaling pathway. Binding of IGF-1 to its receptor results in a multiple auto-phosphorylation cascade. As a consequence, phosphoinositide-3-kinase (PI3K) is activated, and then its downstream Akt translocates to the membrane, where it becomes phosphorylated (at threonine 308 and serine 473) and is thereby activated by PI3K (*25,26*). Considerable evidence collected *in vitro* and *in vivo* substantiated that the activation of the IGF-1/PI3K/Akt pathway could effectively increase osteoblast differentiation and calcification (*27,28*).

Our understanding of the effects of LC on osteoblastic differentiation and mineralization has been advanced by our previous study on the metabonomics analysis of *Abcc6*$^{-/-}$ knock-out mice whose LC concentration was decreased in plasma. Therefore, we hypothesized that LC may have a negative impact on osteoblastic differentiation and mineralization. To investigate this hypothesis, we performed an experiment with a cell model of ectopic calcification, cultured NIH3T3 cells in a Pi-inducing medium which allowed mineralization to occur. To elucidate the effects on osteoblast differentiation and mineralization *in vitro*, the results were evaluated on mineralized nodule formation, calcium deposits, ALP activity and expression of osteogenic marker genes.

2. Materials and Methods

2.1. *Cell culture and treatment*

Mouse embryonic fibroblast cells NIH 3T3 cells, obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were routinely cultured in DMEM (Gibco, Carlsbad, CA, USA) normal growth medium supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Invitrogen, Carlsbad, CA, USA). At about 80% confluence, cells were switched to Pi-inducing medium consisting of the normal growth medium described above supplemented with 2 mM Na_3PO_4 (*29*) which represented the control, or to Pi-inducing medium supplemented with 10 μM LC (Santa Cruz, California, USA) or 100 μM LC. Cells were then continued to be cultured at 37°C in a humidified atmosphere containing 5% (v/v) CO_2 for up to 7 days or 12 days. The medium was replaced every 2 days and the first day of culture in Pi-inducing medium was defined as day 0.

2.2. *Cell proliferation analysis*

Cell proliferations of NIH3T3 cells undergoing osteoblast differentiation with different LC concentrations were investigated by 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay, which is a sensitive quantitative colorimetric assay (*30*). Cells were seeded on 96-well plates and treated for 24 h, 48 h and 72 h. The effects of LC on osteoblast proliferation were evaluated every 24 hours for 3 days. MTT reagent (5 mg/mL) was added to each well after the medium had been aspirated, and incubated for 4 h at 37°C , then formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 490 nm using Bio-Tek Synergy HT.

2.3. *Mineralization analysis*

To measure mineralized nodule formation, the cellular matrix was stained using a special, calcium-specific stain, Alizarin Red S (AR-S) dye, which is an indicator of mineralization (*31*). NIH3T3 cells were seeded on 24-well plates and treated for 12 days, after that the cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, and then were stained with 0.5% (w/v) AR-S solution for 30 min at room temperature. Dye was thoroughly washed three times with PBS and the images of the stained cells were captured. For quantitative analyses of the mineralization indicated by AR-S, the cells were incubated in 10% (w/v) cetylpyridium chloride at 37°C for 1 h. The absorbance

of the supernatant was measured at 562 nm (*32*).

2.4. *Calcium deposition analysis*

NIH3T3 cells were seeded on 24-well plates, after 12 days of treatment the cells were decalcified with 0.6 mol/L HCl at 37°C for 24 h. The calcium content of the HCl supernatant was determined using a Calcium Assay Kit (Sigma, St. Louis, MO, USA) (*33*). After decalcification, cells were washed three times with PBS and solubilized with 0.1 mol/L NaOH/0.1% SDS at 4°C for 1 h. The protein content was measured with a BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA), and the calcium content of the cell layer was normalized by protein content.

2.5. *Alkaline phosphatase activity analysis*

LabAssay™ ALP kit (Wako, Osaka, Japan) was used to measure the expression of ALP. This kit uses p-nitrophenylphosphate as a substrate, and released p-nitrophenol measured at 405 nm as the enzyme activity. After 12 days of culture, NIH3T3 cells were lysed by addition of 200 μL buffer containing 25 mM Tris-HCl (pH 7.4) and 0.5% Triton X-100 (*34*), using three cycles of freezing and thawing to verify that the cells were completely lysed. Then, 20 μL of cell lysate was mixed with 100 μL working assay solution and incubated for 15 min at 37°C. The reaction was stopped by addition of 80 μL stop solution and the absorbance at 405 nm was measured by microplate reader. The ALP activity was normalized by the total protein concentration for each sample using the BCA method.

2.6. *RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR)*

RNA levels were analyzed by real-time PCR in cells treated for 7 days or 12 days. Total RNA was extracted from 24-well plates using Trizol reagent (Gibco, Carlsbad, CA, USA) and the purified total RNA was used for cDNA synthesis with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After the RT reaction, cDNA was used as the template for RT-qPCR of *Runx2*, *ALP*, *OCN* and *IGF-1*. Glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) served as the internal control. RT-qPCR was performed using a SYBR Green qPCR Kit (Toyobo, Osaka, Japan) in a real-time PCR detection system, LightCycler 480 thermocycler (Roche Applied Science, Mannheim, Germany) with gene-specific primers: 5'-TGG CTC TGC CTT TAT TCC CTA GT-3' and 5'-AAA TAA GGT GCT TTG GGA ATC TGT-3' for *ALP*, 5'-AAG TGC GGT GCA AAC TTT CT-3' and 5'-TCT CGG TGG CTG GTA GTG A-3' for *Runx2*, 5'-TGC TTG TGA CGA GCT ATC AG-3' and 5'-GAG GAC AGG GAG GAT CAA GT-3' for *OCN*, 5'-GCT CTG CTT GCT CAC CTT C-3' and 5'-TCA GTG GGG CAC AGT ACA TC-3' for *IGF-1* and 5'-ACC ACA GTC CAT GCC ATC AC-3' and 5'-TCC ACC ACC CTG TTG CTG TA-3' for *GAPDH*. The transcript levels were normalized using the *GAPDH* transcript levels.

2.7. *Western blotting*

NIH3T3 cells were collected after 7 days of treatment, and the cells were homogenized in cell lysis buffer for Western and IP (Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitors on ice for 60 min and centrifuged at 13,000 g for 15 min at 4°C. Total protein concentration of the cell lysate was determined by the BCA method. Forty micrograms of the total protein from each sample was suspended in Laemmli loading buffer and incubated at 95°C for 5 min. Proteins were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene fluoride (PVDF) membrane. After 1 h of blocking with 5% low fat milk in TBST (10 mM Tris, 100 mM NaCl, and 0.05% Tween-20), membranes were incubated overnight at 4°C with the specific antibodies for goat polyclonal anti-mouse Runx2 (C-19) (1:500) (Santa Cruz Biotechnology, Carlsbad, CA, USA), rabbit polyclonal anti-mouse Akt (1:1,000) (Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-mouse Phospho-Akt (Ser473) (1:2000) (Cell Signaling Technology, Danvers, MA, USA), and mouse monoclonal anti-mouse GAPDH (G-9) (1:1,000) (Santa Cruz Biotechnology, Carlsbad, CA, USA). Primary antibodies were immunostained with the appropriate peroxidase-conjugated secondary antibodies. Finally, the blots were developed with enhanced chemiluminescence (ECL) (Millipore Corporation, Billerica, MA, USA) and exposed to X-ray film.

2.9. *Statistical analysis*

Statistical analyses were performed using SPSS-17.0 software (SPSS Inc., Chicago, IL, USA). Results are expressed as the mean ± S.D. All data were analyzed by analysis of variance (ANOVA) and unpaired Student's *t*-test. Statistical significance between groups was defined as $p < 0.05$.

3. Results

3.1. *Effects of LC on the proliferation of NIH3T3 cells*

MTT assay is a main application that allows for proliferation of cells to be assessed. The absorbance of each well at 490 nm was measured after treatment for 24, 48 and 72 h. To compare with the control group, no significant differences were observed between them at 24 and 48 h, but after 72 h, the Absorbance values of the

10 μM LC group were slightly higher, while the 100 μM LC group were slightly reduced (Figure 1). Therefore, the MTT assay indicated that 10 μM LC increased the proliferation of NIH3T3 cells, but 100 μM LC slightly inhibited cell proliferation.

3.2. *Effects of LC on the osteogenic activities in NIH3T3 cells*

First, we investigated whether ALP activity was altered during LC treatment in NIH3T3 cells. In this study, we found that the ALP activity in the 10 μM LC group was much higher, but in the 100 μM LC group was lower than the control group ($p < 0.05$, Figure 2A). Therefore, this indicates that 10 μM LC can promote ALP activity, while 100 μM LC suppresses the ALP activity in NIH3T3 cells. Next, the expression of Runx2 was determined by Western blotting analysis. The result showed that the expression of Runx2 was upregulated in 10 μM LC treated cells and was markedly dowregulated in 100 μM LC treated cells compared with the control cells at 7 days (Figure 2B). Together, these results suggest that LC has alterable effects on the osteogenic activities in NIH3T3 cells; either increased or decreased depending on the levels of specific concentration. Low concentration of LC facilitates osteoblast differentiation, while high concentration of LC counteracts the process in NIH3T3 cells.

3.3. *Effects of LC on the mineralization in NIH3T3 cells*

In this study, we confirmed the mineralization of NIH3T3 cells by AR-S stain. As shown in Figure 3A, obvious mineralized nodules were noted in 10 μM LC, but decreased in 100 μM LC in comparison to the control. To evaluate calcium deposition in the NIH3T3 cell matrix, quantitative assays of mineralization were carried out after incubation in cetylpyridium chloride. The results revealed that calcium deposition under the 10 μM LC condition was increased, whereas the 100 μM LC condition was decreased ($p < 0.05$, Figure 3B). To further examine the mineralization, the calcium depositions were also carried out using a Calcium Assay Kit. The data were consistent with the AR-S stain ($p < 0.05$, Figure 3C). These results provided further evidence that 10 μM LC had a positive effect on osteoblast differentiation and mineralization in NIH3T3 cells, whereas 100 μM LC had an opposite effect.

3.4. *Effects of LC on the expression of osteogenic marker genes in NIH3T3 cells*

To gain further insight into the molecular mechanism of LC function in osteoblast differentiation, the expression of typical osteogenic marker genes (*Runx2*, *ALP*, and *OCN*) were examined by realtime PCR. The expression level of *Runx2*, which regulates osteoblast

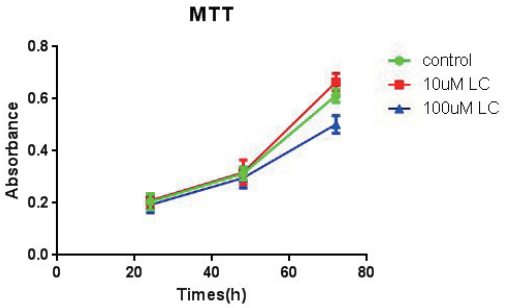

Figure 1. Effects of LC on the proliferation of NIH3T3 cells. The proliferation of NIH3T3 cells was measured by MTT assays after treatment with 10 or 100 μM LC or no treatment (control) for 24 h, 48 h and 72 h. Data are expressed as the mean ± S.D., $n = 6$. $^*p < 0.05$, *vs*. control.

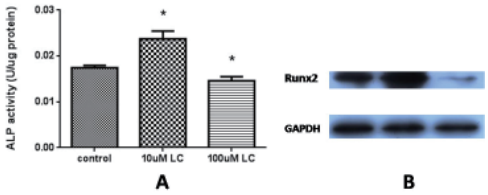

Figure 2. Effects of LC on osteogenic activities in NIH3T3 cells. The ALP activity (A) and Runx2 expression (B) were determined by LabAssay™ ALP kit and Western blotting analysis, respectively. NIH3T3 cells induced with Pi-inducing medium after treatment with 10 or 100 μM LC or no treatment (control) for 7 or 12 days are shown. Bars are shown as the mean ± S.D., $n = 3$. $^*p < 0.05$ *vs*. control.

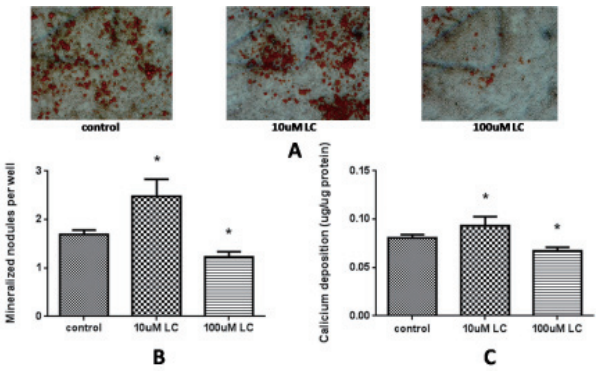

Figure 3. Effects of LC on matrix mineralization in NIH3T3 cells. The calcium deposits in the mineralized matrix were analyzed by Alizarin Red S staining **(A)** which was quantified at 562 nm **(B)** and Calcium Assay Kit **(C)**. NIH3T3 cells induced with Pi-inducing medium after treatment with 10 or 100 μM LC or no treatment (control) for 12 days are shown. Bars are shown as the mean ± S.D., $n = 3$. $^*p < 0.05$, *vs*. control.

differentiation at the early stages, was markedly upregulated in the 10 μM LC group, and downregulated in the 100 μM LC group compared with the control at 7 days (Figure 4A). The expressions of *ALP* and *OCN* in the 10 μM LC group and 100 μM LC group were significantly higher or lower than the control group at 12 days (Figures 4B and 4C). These results also suggest that 10 μM LC can facilitate osteoblast differentiation, while 100 μM LC inhibits osteoblast differentiation in NIH3T3 cells which is consistent with mineralized

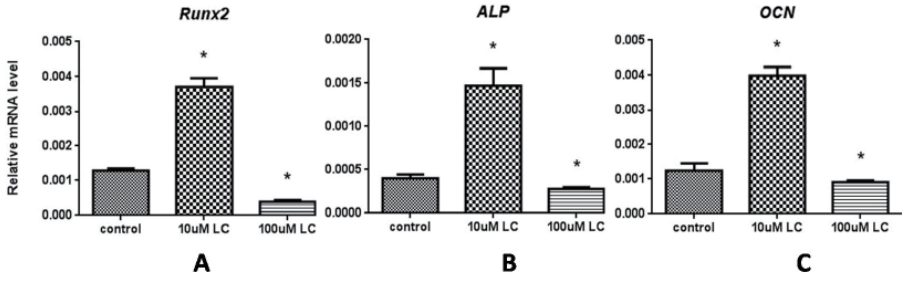

Figure 4. Effects of LC on expression levels of different osteoblast differentiation marker genes in NIH3T3 cells. Real-time PCR analyses of the *Runx2* **(A)**, *ALP* **(B)**, and *OCN* **(C)** mRNA levels in NIH3T3 cells induced with Pi-inducing medium after treatment with 10 or 100 μM LC or no treatment (control) for 7 or 12 days are shown. The results were normalized by the mRNA levels of *GAPDH* as a housekeeping gene. Results are the mean ± S.D., $n = 3$. $^{*}p < 0.05$ *vs*. control.

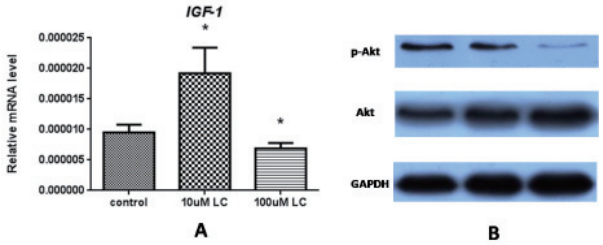

Figure 5. Effects of LC on the IGF-1/PI3K/Akt signaling pathway in NIH3T3 cells. Real-time PCR analyses of *IGF-1***(A)** and Western blotting analysis of p-Akt and Akt proteins **(B)** in NIH3T3 cells induced with Pi-inducing medium after treatment with 10 or 100 μM LC or no treatment (control) for 7 days are shown. Data are expressed as the mean ± S.D., $n = 3$. $^{*}p < 0.05$, *vs*. control.

nodule formation, calcium deposition, ALP activity and the expression of Runx2 shown above.

3.5. *Effects of LC on the IGF-1/PI3K/Akt signaling pathway in NIH3T3 cells*

In order to investigate whether LC could have an influence on the IGF-1/PI3K/Akt signaling pathway, we used RT-PCR and Western blot analysis. RT-PCR showed that the mRNA expression of *IGF-I*, was upregulated in the 10 μM LC group, and downregulated in the 100 μM LC group compared with the control at 7 days (Figure 5A). This demonstrated that 10 μM LC increases expression of *IGF-1*, but 100 μM LC decreases expression. Moreover, we identified the protein expression of Phospho-Akt (p-Akt) by Western blot analysis after 7 days treatment. As shown in Figure 5B, although the expression level of p-Akt protein had no significant difference when treated with 10 μM LC, the 100 μM LC was significantly lower than the control at 7 days. As well as the total mass of Akt protein had no significant difference between the three groups. This suggested that supplementation of 100 μM LC leads to an inactivation of the IGF-1/PI3K/Akt signalling pathway.

4. Discussion

Ectopic calcification is a common problem associated with several clinical conditions, such as aging, organ injury, and autoimmune diseases. Studies have noted that several factors, either systemic or local, can antagonize the aberrant mineralization of connective tissues (*35*). In spite of the fact that molecular mechanisms underlying the regulation of ectopic calcification are unclear, some evidence has emerged in support of the concept that ectopic calcification is a cell regulated process (*36*). To test the possible roles of LC in ectopic mineralization, we cultured NIH3T3 cells in Pi-inducing medium and supplemented with LC. Cells undergo a phenotypic transition into osteoblast cells, evidenced by an increase in mineralized nodule formation and calcium deposits.

During the early stage of osteoblast differentiation, osteoblasts synthesize Runx2, ALP and other osteoblastic differentiation markers, ultimately leading to the induction of extracellular matrix calcification (*37,38*). Runx2 is a crucial transcription factor which regulates the expression of major bone matrix protein genes and determines osteoblast differentiation (*39*). Runx2 has been shown to regulate the expression of OCN (*40*). OCN is essential for hydroxyapatite binding and deposition in the extracellular matrix of bone, whose synthetic peak is consistent with the peak of ALP activity (*41*). ALP is a well-established phenotypic marker of osteoblast differentiation and a critical enzyme in calcification (*42*). In this study, we found that both the mRNA and protein levels of Runx2 were increased in the 100 μM LC group and decreased in the 10 μM LC group compared with the control. It demonstrated that LC could affect the differentiation of osteoblasts by regulating the level of Runx2 expression. The results were parallel to the gene expression of *ALP*, *OCN* and ALP activity. Most importantly, the formation of mineralized nodules in NIH3T3 cells evaluated by AR-S and calcium deposits provided powerful evidence. Taken together, these results suggest that LC exerted a dominant effect on osteoblast differentiation and mineralization in NIH3T3 cells.

Some studies suggest that LC promoted osteoblast proliferation and differentiation *in vitro* (*43,44*), while other studies indicated that LC inhibited proliferation in VSMCs (*45*). In this study, we found that the 10 μM LC positively affected osteoblast proliferation,

but the higher concentration of LC (100 μM) slightly decreased osteoblast proliferation compared with the control. Meanwhile, it has recently been demonstrated that supplementation of LC lead to an activation of the IGF-1/PI3K/Akt signalling pathway (*46*). Therefore, we investigated whether the effect of LC on proliferation and osteoblast differentiation could be mediated by the IGF-1/PI3K/Akt signalling pathway. To this end, we tested the effects of LC on *IGF-1*, p-Akt and Akt expression by RT-qPCR and Western blot analysis. The results showed that the gene expression of *IGF-1* and the protein level of p-Akt were downregulated in the 100 μM LC treated cells. Consequently, we postulate that the proliferation and osteoblast differentiation effects on fibroblasts could be in turn responsible for the IGF-1/PI3K/Akt signalling pathway induced by LC. Taking all the experiments together, our study indicates that the higher concentration of LC (100 μM) slightly inhibits osteoblast proliferation, and plays negative roles in osteoblast differentiation and mineralized bone matrix formation in fibroblast cells, which is consistent with our previous study on the metabonomics analysis of *Abcc6*$^{-/-}$ knock-out mice whose LC concentration was decreased in plasma.

In conclusion, our data have demonstrated for the first time that supplementation of 100 μM LC leads to an inactivation of the IGF-1/PI3K/Akt signalling pathway, and slightly inhibits the proliferation and osteoblast differentiation of fibroblast cells. So far there is no effective treatment for the systemic manifestations of ectopic mineralization disorders. From this point of view, we have probably provided a reasonable basis for the potential utility of LC in the prevention and treatment of ectopic calcification, although gaining a better understanding of the mechanism should lead to improved prevention and treatment of ectopic calcification in the future. In a follow-up study, we will continue to study the mechanism underlying ectopic calcification prevention and treatment by LC.

Acknowledgements

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References

1. Cotran RS, Kumare V, Robbins SL. Cellular injury and cellular death. In: Pathological Basis of Disease. 5th ed., WB Saunders Press, Philadelphia, PA, USA, 1994; pp. 1-35.
2. Uitto J, Li Q, Jiang Q. Pseudoxanthoma elasticum: Molecular genetics and putative pathomechanisms. J Invest Dermatol. 2010; 130:66.
3. Ringpfeil F, Lebwohl MG, Christiano AM, Uitto J. Pseudoxanthoma elasticum: Mutations in the *MRP6* gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc Natl Acad Sci U S A. 2000; 97:6001-6006.
4. Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, de Paepe A, Boyd CD. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet. 2000; 25:223-227.
5. Bergen AA, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, de Jong PT. Mutations in *ABCC6* cause pseudoxanthoma elasticum. Nat Genet. 2000; 25:228-231.
6. Gorgels TG, Hu X, Scheffer GL, van der Wal AC, Toonstra J, de Jong PT, van Kuppevelt TH, Levelt CN, de Wolf A, Loves WJ, Scheper RJ, Peek R, Bergen AA. Disruption of *Abcc6* in the mouse: Novel insight in the pathogenesis of pseudoxanthoma elasticum. Hum Mol Genet. 2005; 14:1763-1773.
7. Klement JF, Matsuzaki Y, Jiang QJ, Terlizzi J, Choi HY, Fujimoto N, Li K, Pulkkinen L, Birk DE, Sundberg JP, Uitto J. Targeted ablation of the *Abcc6* gene results in ectopic mineralization of connective tissues. Mol Cell Biol. 2005; 25:8299-8310.
8. Friedenstein AY. Induction of bone tissue by transitional epithelium. Clin Orthop Relat Res. 1968; 59:21-37.
9. Ducy P, Schinke T, Karsenty G. The osteoblast: A sophisticated fibroblast under central surveillance. Science. 2000; 289:1501-1504.
10. Rutherford RB, Moalli M, Franceschi RT, Wang D, Gu K, Krebsbach PH. Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone *in vivo*. Tissue Eng. 2002; 8:441-52.
11. Reddi AH, Huggins C. Biochemical sequences in the transformation of normal fibroblasts in adolescent rats. Proc Natl Acad Sci U S A. 1972; 69:1601-1605.
12. Zhang J, Khvorostov I, Hong JS, *et a*l. UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. EMBO J. 2011; 30: 4860-4873.
13. Borum PR. Regulation of carnitine concentration in plasma. In: Carnitine biosynthesis, metabolism and functions (Frenkel RA, McGarry JD, eds.). Academic Press, New York, USA, 1980; pp. 115-126.
14. Adamek G, Felix R, Guenther HL, Fleisch H. Fatty acid oxidation in bone tissue and bone cells in culture. Biochem J. 1987; 242:129-137.
15. Colucci S, Mori G, Vaira S, Brunetti G, Greco G, Mancini L, Simone GM, Sardelli F, Koverech A, Zallone A, Grano M. L-Carnitine and isovaleryl L-carnitine fumarate positively affect human osteoblast proliferation and differentiation *in vitro*. Calcif Tissue Int. 2005; 6:458-465.
16. Xie H, Tang SY, Li H, Luo XH, Yuan LQ, Wang D, Liao EY. L-Carnitine protects against apoptosis of murine MC3T3-E1 osteoblastic cells. Amino Acids. 2008; 2:419-423.
17. Chiu KM, Keller ET, Crenshaw TD, Gravenstein S. Carnitine and dehydroepiandrosterone sulphate induced protein synthesis in porcine osteoblast-like cells. Calcif Tissue Int. 1999; 64:527-533.
18. Mohan S, Baylink DJ. Insulin-like growth factor system components and the coupling of bone formation to

resorption. Horm Res. 1996; 45(Suppl 1):59-62.
19. Jonsson KB, Ljunghall S, Karlström O, Johansson AG, Mallmin H, Ljunggren O. Insulin-like growth factor I enhances the formation of type I collagen in hydrocortisone-treated human. Biosci Rep. 1993; 13:297-302.
20. Gangji V, Rydziel S, Gabbitas B, Canalis E. Insulin-like growth factor II promoter expression in cultured rodent osteoblasts and adult rat bone. Endocrinology. 1998; 139:2287-2292.
21. Langdahl BL, Kassem M, Møller MK, Eriksen EF. The effects of IGF-I and IGF-II on proliferation and differentiation of human osteoblasts and interactions with growth hormone. Eur J Clin Invest. 1998; 28:176-183.
22. Heo YR, Kang CW, Cha YS. L-Carnitine changes the levels of insulin-like growth factors (IGFs) and IGF binding proteins in streptozotocin-induced diabetic rat. J Nutr Sci Vitaminol (Tokyo). 2001; 47:329-334.
23. Kita K, Kato S, Amanyaman M, Okumura J, Yokota H. Dietary L-carnitine increases plasma insulin-like growth factor-I concentration in chicks fed a diet with adequate dietary protein level. Br Poult Sci. 2002; 43:117-121.
24. Doberenz J, Birkenfeld C, Kluge H, Eder K. Effects of L-carnitine supplementation in pregnant sows on plasma concentrations of insulin-like growth factors, various hormones and metabolites and chorion characteristics. J Anim Physiol Anim Nutr (Berl). 2006; 90:487-499.
25. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002; 296:1655-1657.
26. Hofler A, Nichols T, Grant S, Lingardo L, Esposito EA, Gridley S, Murphy ST, Kath JC, Cronin CN, Kraus M, Alton G, Xie Z, Sutton S, Gehring M, Ermolieff J. Study of the PDK1/AKT signaling pathway using selective PDK1 inhibitors, HCS, and enhanced biochemical assays. Anal Biochem. 2011; 414:179-186.
27. Chen LL, Huang M, Tan JY, Chen XT, Lei LH, Wu YM, Zhang DY. PI3K/AKT pathway involvement in the osteogenic effects of osteoclast culture supernatants on preosteoblast cells. Tissue Eng Part A. 2013; 19:2226-2232.
28. Suzuki E, Ochiai-Shino H, Aoki H, Onodera S, Saito A, Saito A, Azuma T. Akt activation is required for TGF-β1-Induced osteoblast differentiation of MC3T3-E1 pre-osteoblasts. PLoS One. 2014; 9:e112566.
29. Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Morii H, Giachelli CM. Phosphate regulation of vascular smooth muscle cell calcification. Circ Res. 2000; 87:e10-17.
30. Castiglioni S, Casati S, Ottria R, Ciuffredav P, Maier JA. N6-isopentenyladenosine and its analogue N6-benzyladenosine induce cell cycle arrest and apoptosis in bladder carcinoma T24 cells. Anticancer Agents Med Chem. 2013; 13:672-678.
31. Yamakawa K, Iwasaki H, Masuda I, Ohjimi Y, Honda I, Saeki K, Zhang J, Shono E, Natio M, Kikuchi M. The utility alizarin red s staining in calcium pyrophosphate dihydrate crystal deposition disease. J Rheumatol. 2003; 30: 1032-1035.
32. Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J Biol Chem. 1995; 270:9420-9428.
33. Hernandez L, Park KH, Cai SQ, Qin L, Partridge N, Sesti F. The antiproliferative role of ERG K+ channels in rat osteoblastic cells. Cell Biochem Biophys. 2007; 47:199-208.
34. Zhang YY, Cui YZ, Luan J, Zhou XY, Zhang GL, Han JX. Platelet-derived growth factor receptor kinase inhibitor AG-1295 promotes osteoblast differentiation in MC3T3-E1 cells via the Erk pathway. Biosci Trends. 2012; 6:130-135.
35. Rutsch F, Nitschke Y, Terkeltaub R. Genetics in arterial calcification: Pieces of a puzzle and cogs in a wheel. Circ Res. 2011; 109:578-592.
36. Giachelli CM. Ectopic calcification: Gathering hard facts about soft tissue mineralization. Am J Pathol. 1999; 154:671-675.
37. Beresford JN, Graves SE, Smoothy CA. Formation of mineralized nodules by bone derived cells *in vitro*: A model of bone formation? Am J Med Genet. 1993; 45:163-178.
38. Stein GS, Lian JB, Owen TA. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J. 1990; 4:3111-3123.
39. Ji C, Casinghino S, Chang DJ, Chen Y, Javed A, Ito Y, Hiebert SW, Lian JB, Stein GS, McCarthy TL, Centrella M. CBFa(AML/PEBP2)-related elements in the TGF-beta type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem. 1998; 69:353-363.
40. Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res. 2010; 339:189-195.
41. Patti A, Gennari L, Merlotti D, Dotta F, Nuti R. Endocrine actions of osteocalcin. Int J Endocrinol. 2013; 2013:846480.
42. Weinreb M, Shinar D, Rodan GA. Different pattern of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. J Bone Miner Res. 1990; 5:831-842.
43. Keller J, Couturier A, Haferkamp M, Most E, Eder K. Supplementation of carnitine leads to an activation of the IGF-1/PI3K/Akt signaling pathway and down regulates the E3 ligase MuRF1 in skeletal muscle of rats. Nutr Metab (Lond). 2013; 10:28.
44. Colucci S, Mori G, Vaira S, Brunetti G, Greco G, Mancini L, Simone GM, Sardelli F, Koverech A, Zallone A, Grano M. L-carnitine and isovaleryl L-carnitine fumarate positively affect human osteoblast proliferation and differentiation of adult stem cells. Calcif Tissue Int. 2005; 76:458-465.
45. Lu Q, Zhang Y, Elisseef JH. Carnitine and accetylcarnitine modulate mesenchymal differentiation of adult stem cells. J Tissue Eng Regen Med. 2013; 1747. (doi: 10.1002/term.1747)
46. Xie H, Yang B, Zhou XM, Song FL, Li JM, Zhou K, Hu W, Peng YQ, Tang SY, Yuan LQ, Xiong SY, Liao XB. L-carnitine and taurine synergistically inhibit the proliferation and osteoblastic differentiation of vascular smooth muscle cells. Acta Pharmacol Sin. 2010; 31:289-296.

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Original Article

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Susceptibility to proteases of anti-Tn-antigen MLS128 binding glycoproteins expressed in human colon cancer cells

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Summary **Anti-Tn antigen MLS128 monoclonal antibody was produced two decades ago by immunizing mice with "cancerous antigens" derived from LS180 colon cancer cells. Previous studies demonstrated that MLS128 bound to 110 kDa glycoprotein (GP) in colon cancer cells, thereby inhibiting cell growth. Extensive attempts have been made towards understanding the inhibitory action of MLS128 on colon cancer cell growth and solving the primary structure of 110 kDa GP. Since limited proteolysis of 110 kDa GP was observed in microdomain fractions that had been kept frozen for several years, susceptibility of 110 kDa GP to trypsin and other proteases as well as N-glycosidase F has been investigated. Furthermore, 110 kDa GP expression was examined in colon cancer cells independently cultured in Akiyama laboratory. In summary, 110 kDa GP contains N-glycans. It does not contain inter-disulfide bonds but appears to have intra-disulfides. It must contain multiple cleavage sites for trypsin and thermolysin since these proteases digested 110 kDa GP to MLS128-undetectable small fragments. It seems to contain cleavage sites for cathepsin D which could cause limited digestion. LS180 cells derived from Akiyama laboratory produced a limited proteolysis product-like 75 kDa GP. This study provides a structural basis for developing cancer diagnostics and therapeutics.**

Keywords: Mucin-type O-glycans, N-glycans, limited proteolysis, colon cancer cell lines

1. Introduction

Glycosylation is the major post-translational modification of proteins, which is usually required for their functions. Glycoproteins in humans contain two main types of glycans, N-linked and O-linked glycans. Mucin type O-linked glycosylation is the most common form of O-glycosylation which is covalently bound *via* O-linked N-acetylgalactosamine (GalNAc) to serine or threonine residues of glycoproteins. GalNAcα-Ser/Thr, known as Tn antigen, is thus the precursor for all mucin-type O-glycans. Extended glycosylation shields Tn-antigen in healthy and benign tissues. Tn antigens are, however, uncovered in approximately 90 % of carcinomas (*1*). Tn antigen is thus considered as a cancer-specific biomarker and a potential target for cancer therapeutics.

Anti-Tn antigen MLS128 monoclonal antibody (mAb) was produced two decades ago by immunizing mice with "cancerous antigens" derived from LS180 colon cancer cells (*2,3*). Previous studies demonstrated that MLS128 bound to 110 kDa glycoprotein (GP) in colon cancer cells, thereby inhibiting cell growth (*4,5*). To further understand the inhibitory action of MLS128 on colon cancer cell growth, the primary structure of 110 kDa GP must be identified. Various attempts have been made to isolate and identify the 110 kDa GP. MLS128-stainable spots separated by two-dimensional gel electrophoresis (2D EP) were subjected to in-gel digestion with trypsin. Tryptic peptides excised

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from the gel were then analyzed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry along with software to search databases. Despite repeated attempts, the 110 kDa protein could not have been successfully identified. This failure may be due to the 110 kDa GP's extremely low susceptibility to trypsin as the result of interference by abundant O-glycosylation, the lack of a database for glycosylated peptides, or the limited availability of samples (*6*).

An important finding from the previous study though was the limited proteolysis of 110 kDa GP observed in microdomain fractions that had been kept frozen for several years (*6*). Conformational relaxation during storage, freezing, and thawing must have exposed the cleavage site(s) to contaminating proteases, resulting in limited proteolysis as was observed. Although protease inhibitor cocktails were added to cell lysates and during sucrose gradient fractionation, degradation of 110 kDa still occurred, suggesting that unidentified proteases that are resistant to the added inhibitors are responsible for such a limited proteolysis of 110 kDa GP.

Based on the above-mentioned observations, susceptibility of 110 kDa GP to trypsin and other proteases as well as N-glycosidase F has been investigated. Furthermore, to examine whether or not colon cancer cells generally express the 110 kDa GP, cell lysates from 6 colon cancer and other cell lines derived from Akiyama laboratory, were subjected to Western blotting which revealed that 110 kDa GP is commonly expressed in colon cancer cells, and that the limited proteolysis product similar to that observed (*6*) seems to be produced in LS180 cells. This manuscript describes the results of further characterization of the 110 kDa GP which may likely lead to identification of the receptor for MLS128 in colon cancer cells.

2. Materials and Methods

2.1. *Materials*

Production and characterization of MLS128 were previously described (*2,3*). Goat anti-mouse IgG labeled with horseradish peroxidase (HRP) was from Jackson ImmunoResearch Lab. (West Grove, PA, USA). Cell culture media (DMEM and McCoy's 5A) were purchased from Gibco (Grand Island, NY, USA). Protease Inhibitor Cocktail (P2714), trypsin, and thermolysin were obtained from Sigma-Aldrich (St Louis, MO, USA). N-Glycosidase F and cathepsin D were purchased from Roche Applied Science (Indianapolis, IN, USA) and Merck Millipore (Temecula, CA, USA), respectively.

2.2. *Cell culture*

In Yamaguchi laboratory, LS180 colon cancer cells were cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 4.5 mg/mL D-glucose and 110 μg/mL sodium pyruvate. HT29 cells were cultured in McCoy's 5A containing 10% FBS. In Akiyama laboratory, LS180 and WiDr colon cancer cells and 293FT cells were cultured in DMEM supplemented with 10% FBS. HT29 and HCT116 colon cancer cells were cultured in McCoy's 5A supplemented with 10% FBS. RKO colon cancer cells were cultured in MEM supplemented with 10% FBS, MEM NEAA (Gibco) and sodium pyruvate. DLD1 colon cancer cells and H1299 human non-small cell lung carcinoma cells were cultured in RPMI1640 supplemented with 10%FBS. All culture media included 1% Penicillin-Streptomycin solution (Sigma-Aldrich).

2.3. *Preparation of cell lysates*

LS180 and HT-29 cells as well as other cells were cultured in their respective media. Cells were collected by scraping, followed by centrifugation at 200 × g for 5 min, and then solubilized in 50 mM Tris-HCl buffer, pH 7.4, containing 1% NP40, 2 mM EDTA, 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM PMSF and protease inhibitors (P2714, Sigma-Aldrich) (lysis buffer A) on ice for 15 min. Supernatants were obtained from solubilized cells by centrifugation at 17,000 × g for 10 min. To examine effects of protease inhibitors, LS180 and HT29 cell lysates were also prepared in the absence of 10 mM sodium orthovanadate, 1 mM PMSF and protease inhibitors (P2714, Sigma-Aldrich). Protein concentrations were measured by the Bradford method.

2.4. *Western blotting analyses*

Solubilized proteins (10 μg) from each cell line were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 3% BSA in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1% Tween 20 (TBST) for 1 h at room temperature. Western blotting was carried out with MLS128 as a primary antibody, and then bound primary antibody was detected with HRP-conjugated anti-mouse IgG and color development using Ez West blue (ATTO Co., Tokyo, Japan).

2.5. *Treatment of LS180 and HT29 cell lysates with enzymes and Western blotting*

N-Glycosidase F treatment was carried out using 200 μg of cell lysates in 40 μL of PBS containing 0.5% SDS and 1% β-mercaptoethanol. Cell lysates were incubated at 90°C for 3 min, then cooled down to room temperature to which 0, 1, 3 U of N-Glyosidase F and 10 μL of 5% NP-40 was added. After overnight incubation at 37°C, the reaction was stopped by addition of 5× SDS-PAGE

sample buffer. Ten µg of cell lysates from each reaction mixture were applied to SDS-PAGE and immunoblotted with MLS128 as described above. For digestions of 110 kDa GP by trypsin, thermolysin, and cathepsin D, cell lysates were denatured as described above. Cell lysates (200 µg) were digested with trypsin in 50 µL of 0.2 M Tris-HCl buffer, pH 8.0, at 37°C overnight whereas 100 µg of cell lysates were digested with cathepsin D in 25 µL of 0.1 M sodium acetate buffer, pH 3.5, at 37°C overnight. Furthermore, cell lysates (100 µg) were digested with thermolysin in 25 µL of 50 mM Tris-HCl buffer, pH 8.0, at 30°C overnight.

2.6. *Sucrose gradient fractionation of HT29 and LS180 cell lysates*

HT29 and LS180 cells grown in 3~5 150 mm-dishes were washed with chilled PBS and lysed in 2 mL lysis buffer B (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, containing protease inhibitors and 1 mM PMSF, 10 mM sodium vanadate, and 0.1% NP40) on ice for 20 min. After centrifugation for 5 min at 1,300 × g, supernatants (2 mL) were diluted with 2 mL of 85% (w/v) sucrose in 10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 5 mM EDTA (TNE buffer). The diluted lysates were overlaid with 4 mL of 30% (w/v) sucrose and then with 4 mL of 5% (w/v) sucrose in TNE buffer in an ultracentrifuge tube. The samples were centrifuged at 39,000 rpm for 18 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, USA), and fractions were collected from the top for immunoblot analysis. Fractions were subjected to SDS-PAGE and immunoblotting with MLS128 as described bove.

3. Results

3.1. *Structural characterization*

3.1.1. *The 110 kDa GP contains N-glycans in addition to abundant O-glycans*

MLS128 binds to a 110 kDa glycoprotein expressed in LS180, HT29, and LS174T colon cancer cells as previously reported (*5*). Since it is known that MLS128 binds Tn-antigen motifs such as two or three consecutive Tn-antigens (Tn2 or Tn3) (*3,7*), 110 kDa GP is expected to contain abundant O-glycans, many of which must be clustered. In order to determine whether or not N-glycans exist in 110 kDa GP, cell lysates prepared from LS180 and HT29 cells were treated with N-glycosidase F, and subjected to Western blotting with anti-Tn antigen mAb (MLS128). The results clearly demonstrated production of one smaller 100 kDa GP from 110 kDa GP at 1 and 10 U doses in both LS180 and HT29 cells (Figure 1), which indicated that 110 kDa GP contains N-glycans.

3.1.2. *The 110 kDa GP does not contain inter-disulfide bonds but appears to have intra-disulfides*

SDS-PAGE analyses of 110 kDa GP derived from LS180 and HT29 cells under reducing and non-reducing conditions revealed a molecular size of nearly 110 kDa, which indicated that 110 kDa GP does not seem to contain inter-disulfide bonds (Figure 2). Under non-reducing conditions, however, MLS128-stained bands were broader and smaller, approximately 100 kDa, than those seen under reducing conditions, which indicated that 110kDa GP contains intra-disulfide bonds.

3.2. *Sensitivity to proteases*

3.2.1. *Effects of endogenous proteases on 110 kDa GP during preparation of cell lysates*

In the experiments shown in Figure 2, cell lysates prepared in the presence or absence of protease inhibitors were used to compare the effect of reduction on the structure of 110 kDa GP. In both LS180 and HT29 cells, proteolytic degradation of 110 kDa GP did not seem to occur during preparation of cell lysates since an intact 110 kDa GP was observed in both cell

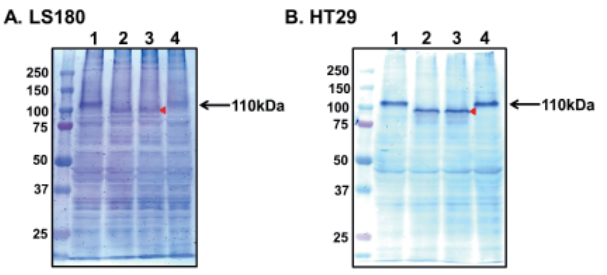

Figure 1. N-Glycosidase F digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by 0 U (lane 1 & 4), 1 U (lane 2) and 3 U (lane 3) of N-glycosidase F at 37°C overnight, and then 10 µg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow heads indicate a 100 kDa fragment.

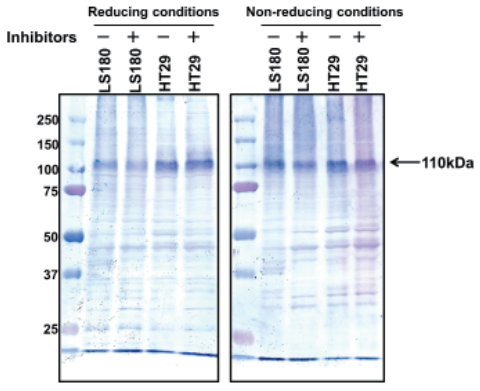

Figure 2. Comparison of non-reduced and reduced 110 kDa GP. Cell lysates were prepared from LS180 and HT29 cells in the presence (+) or absence (-) of inhibitors. Cell lysates, 10 µg each, were analyzed under reducing or non-reducing conditions by SDS-PAGE and immunoblotting as described in the Methods.

lysates prepared in the presence of protease inhibitors (Figure 2, lanes indicated with "Inhibitors +") and those prepared in the absence of them (Figure 2, lanes indicated with "Inhibitors −"). Such results suggested that 110 kDa GP is not particularly sensitive to endogenous proteases at least during the preparation of cell lysates.

3.2.2. *Digestions of 110 kDa GP by exogenous proteases*

We earlier suspected that the recovery of MLS128-detectable proteins from 2D EP gels may have been insufficient due to insensitivity of 110 kDa GP to trypsin digestion (*6*). Results that 110 kDa GP from both LS180 and HT29 cells were completely digested by trypsin at a concentration of 1 μg/μL (Figure 3B, lane 6 for HT29 cell lysates; data not shown for LS180 cell lysates) demonstrated that the low recovery of 110 kDa GP peptides from 2D EP gels was not likely due to its insensitivity to trypsin digestion. The results shown in Figures 3A and 3B were carried out to determine whether trypsin at lower concentrations is able to produce limited proteolysis fragments of 110 kDa GP as previously observed in microdomain fractions (*6*). Since LS180 lysates-derived 110 kDa GP was more sensitive to trypsin digestion than HT29 cell lysates-derived 110 kDa GP, different ranges of trypsin concentrations were used to establish partial digestions. Interestingly, trypsin at a concentration of 33 ng/μL produced a faint ~90 kDa fragment from LS180 cell lysates-derived 110 kDa GP (Figure 3A, lane 6).

Although effects of endogenous proteases on 110 kDa GP appear to be negligible while cell lysates were prepared, contamination of endogenous proteases must have caused limited proteolysis during the long-time storage of cell lysates in their partially-purified fractions. Since protease inhibitors used during sucrose gradient fractionation of LS180 and HT29 cell lysates did not include inhibitors for aspartyl proteases, there is a possibility that aspartic proteases such as cathepsin D may be responsible for the limited proteolysis observed in the microdomain fractions during 3-4 years of storage at –80°C. Results shown in Figure 4 indicated that cathepsin D treatment in fact produced a smaller size with approximately 90 kDa GP from both LS180 and HT29 cell lysates-derived 110 kDa GP. Although the 90 kDa is not exactly the same size as previously observed in the microdomain fractions, this result suggests a possible involvement of cathepsin D-like protease in the limited proteolysis of 110 kDa GP. Furthermore, although thermolysin at a concentration of 400 ng/μL completely digested LS180 and HT29 cell lysates-derived 110 kDa GP (Figures 5A and 5B, lane 3), it partially digested LS180 cell lysates-derived 110 kDa GP to about 90 kDa at a concentration of 40 ng/μL (Figure 5A, lane 2).

In summary, these results suggested that although 110 kDa GP is heavily O-glycosylated, there must be amino acid sequences accessible to those proteases, which may contribute to production of limited proteolysis as observed (*6*). In addition to cathepsin D which produced

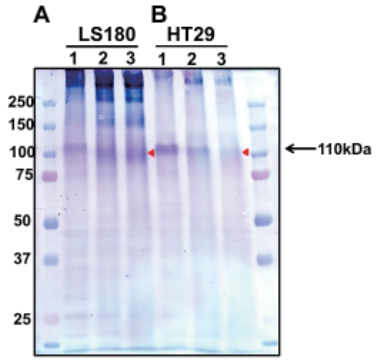

Figure 4. Cathpsin D digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by 0 U (lane 1), 1 U (lane 2) and 10 U (lane 3) of cathepsin D at 37°C overnight, and then 10 μg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow heads indicate a 90 kDa fragment.

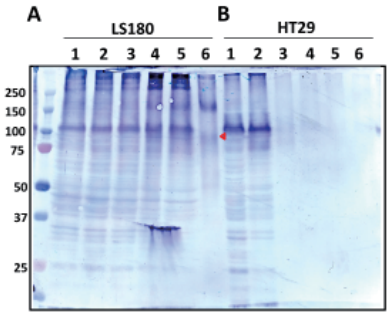

Figure 3. Trypsin digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by trypsin ; 0 ng/μL (**A** & **B**, lane 1), 2 ng/μL (**A**, lane 2), 5 ng/μL (**A**, lane 3), 11 ng/μL (**A**, lane 4), 25 ng/μL (**A**, lane 5), 33 ng/μL (**A**, lane 6 & **B**, lane 2), 100 ng/μL (**B**, lane 3), 200 ng/μL (**B**, lane 4), 500 ng/μL (**B**, lane 5), and 1000 ng/μL (**B**, lane 6) at 37°C overnight, and then 10 μg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow head indicates a 90 kDa fragment.

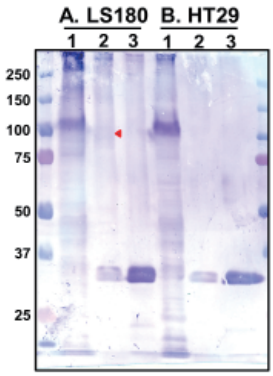

Figure 5. Thermolysin digestion of 110 kDa GP. LS180 **(A)** and HT29 **(B)** cell lysates were digested by 0 μg/μL (lane 1), 40 ng/μL (lane 2) and 400 ng/μL (lane 3) of thermolysin at 30°C overnight, and then 10 μg of cell lysate per lane were immunoblotted as described in the Methods. The red arrow head indicates a 90 kDa fragment.

the 90 kDa fragment in 110 kDa GP derived from both LS180 and HT29 cells, susceptibility of LS180 cell lysates to trypsin and thermolysin which appeared to produce a transient 90 kDa fragment was noticeable.

3.3. *Expression of 110 kDa GP in various colon cancer and control cancer cell lines*

3.3.1. *110 kDa GP is expressed in two additional colon cancer cell lines*

Six colon cancer and other types of cell lines cultured in Akiyama laboratory were analyzed together with LS180 and HT29 cells cultured in Yamaguchi laboratory to examine whether or not those cells cultured in Akiyama laboratory express 110 kDa GP (Figure 6A). In addition to LS180 and HT29 cells, DLD1 and WiDr colon cancer cells expressed 110 kDa GP at levels lower than those of LS180 and HT29 cells. In contrast, 110 kDa GP was not significantly expressed in two other colon cancer cell lines, RKO and HCT116, as well as non-colon cancer cell lines such as H1299 human non-small cell lung carcinoma cells and 293FT human embryonal kidney cells. This study thus revealed that two additional colon cancer cell lines expressed 110 kDa GP in addition to three colon cancer cell lines, LS180, LS174T, and HT29, previously reported (*5*). Five out of seven colon cancer cell lines examined have been found positive for the expression of 110 kDa GP. These results indicated a rather high incidence of 110 kDa GP expression in colon cancer cell lines.

3.3.2. *Limited proteolysis seems to occur in LS180 cells derived from Akiyama laboratory*

A notable difference was observed in the result shown in Figure 6A, that is, in contrast to 110 kDa GP that was clearly detected in LS180 cells cultured in Yamaguchi laboratory, an approximately 75 kDa GP was immunostained as a second major band in LS180 cells cultured in Akiyama laboratory. Western blotting was carried out again to closely compare the molecular sizes and expression levels of 110 kDa GP in LS180 and HT29 cells cultured in two independent laboratories. Results shown in Figure 6B confirmed the above-mentioned observation, that is, 110 kDa GP was found as the major binding protein for MLS128 in LS180 and HT29 cells cultured in either laboratory.

The fact that an additional 75 kDa fragment was found in LS180 cells cultured in Akiyama laboratory is intriguing. Proteolysis fragments with 70~75 kDa were previously identified by immunoblotting by MLS128 of sucrose density gradient fractions of both LS180 and HT29 cell lysates (*6*). The detection of such molecular masses was not definite in the previous experiments using biotin-labeled antibodies as secondary antibodies because endogenous biotin-containing enzymes with 125, 75, and 73 kDa in cell lysates were unavoidably stained (*8*). An alternative staining procedure was thus applied to distinguish MLS128 stainable fragment with 75 kDa, if it exists, from 75, and 73 kDa proteins immunostained in the previous study. Fractions 1-12 from sucrose density gradient fractionation of HT29 cell lysates were analyzed by loading 5 times more sample to SDS-PAGE gel than the previous experiments. MLS128-bound GPs blotted to a PVDF membrane was visualized by EZ-West staining (Figure 7A). The results demonstrated that two

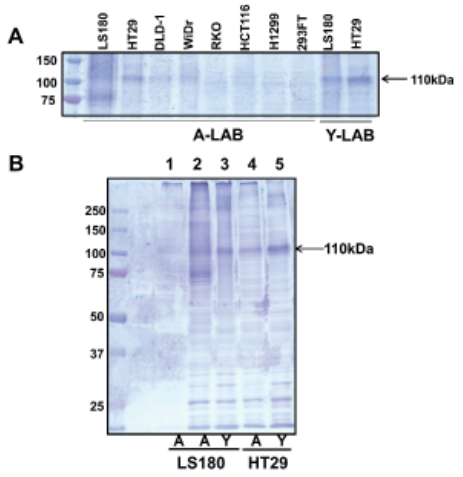

Figure 6. Binding of MLS128 to various colon cancer and other cell lines. In **A**, six colon cancer and other cell lines as indicated from Akiyama laboratory (A-LAB) were examined for expression of 110 kDa GP together with LS180 and HT29 cells cultured in Yamaguchi laboratory (Y-LAB). Other cell lines used were human non-small cell lung carcinoma cells (H1299) and human embryonal kidney cells (293FT). Ten μg of cell lysates per lane were immunoblotted as described in the Methods. In **B**, 2 and 10 μg of cell lysate prepared from LS180 cells from Akiyama (A) laboratory (lanes 1 and 2, respectively) were immunoblotted together with 10 μg of LS 180 cell lysate from Yamaguchi (Y) laboratory (lane 3) to closely compare expression levels of 110 kDa and 75 kDa immunoreactive bands. Likewise, 10 μg each of HT29 cell lysate from Akiyama and Yamaguchi laboratories were immunoblotted next each other (lanes 4 and 5, respectively).

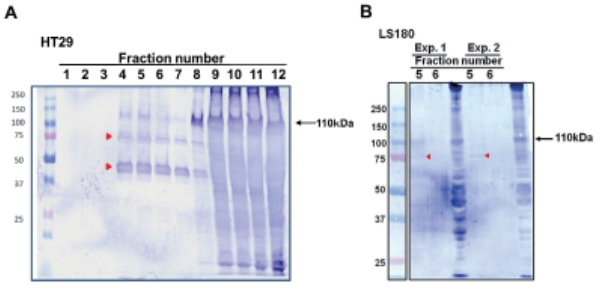

Figure 7. Immunoblotting of HT29 and LS180 cell lysates fractionated by sucrose gradient centrifugation. In **A**, fractions kept frozen for 4 years were analyzed by Western blotting and EZ-West staining using 5 times more sample than those used for the previous study (*6*). Red arrow heads indicate postions of 75 and 45 kDa fragments immunostained. In **B**, fraction numbers 5 and 6 in the microdomain from two independent sucrose gradient fractionation of LS180 cell which had been kept frozen for 4 years were analyzed by Western blotting and EZ-West staining using the same amount of sample as used for the previous study (*6*). The red arrow head indicates a 75 kDa fragment immunostained.

fragments with ~75 kDa and 45 kDa in addition to the intact 110 kDa GP were clearly seen in fractions 4-7. It is notable that fraction 8-12 in which most cellular proteins recovered contained an intact 110 kDa. This suggests so-called microdomain fractions 4-7 did not contain high levels of proteins including scaffolds which may protect 110 kDa from degradation. Furthermore, sucrose density gradient fractions 5 and 6 derived from LS180 cell lysates from two independent experiments were subjected to Western blotting and EZ-West staining. The results shown in Figure 7B demonstrated that MLS128 stainable 75 kDa fragments similar to that found in HT29 cell lysates were visible.

4. Discussion

Towards identification of 110 kDa GP, different strategies have been taken to date. After affinity purification of LS180 cell lysates by MLS128-immobilized agarose chromatography, the eluates were subjected to SDS-PAGE. The 110 kDa GP areas were analyzed by LS-MS/MS, which only revealed contaminated proteins (data not shown). Two-dimensional gel electrophoresis carried out using immunoprecipitation (IP) from HT29 cell lysates showed three distinctive MLS128-stainable spots. Tryptic peptides derived from these spots were analyzed using MALDI-TOF along with software to search data bases, which did not lead to identification of 110 kDa GP (*6*). In contrast, we found that those three spots were not detected in 2D EP of IP from LS180 cell lysates although they were visible by immunoblotting of 2D EP to which cell lysates were applied. Therefore, we speculated that possible differences exist in the distribution of Tn antigen clusters on 110 kDa GP derived from LS180 and HT29 cells. For example, if Tn antigens on 110 kDa GP derived from LS180 cells are less dense than those on 110 kDa GP derived from HT29 cells, IP with MLS128 would result in a low recovery of 110 kDa GP from LS180 cells. To further gain the structural information of 110 kDa GP, in this study, we took advantage of MLS128 by which 110 kDa GP and its fragments can be detected as long as consecutive Tn-antigen epitopes exist even after treatments with enzymes such as N-glycosidase F and proteases.

The present study provided the following structural information on 110 kDa GP. *i*) 110 kDa GP contains N-glycans. *ii*) It does not contain inter-disulfide bonds but appears to have intra-disulfides. *iii*) It must contain multiple cleavage sites for trypsin and thermolysin since these proteases digested 110 kDa GP to MLS128-undetectable small fragments. *iv*) It seems to contain cleavage sites for cathepsin D which could cause limited digestion. *v*) LS180 cells derived from Akiyama laboratory produced a limited proteolysis product-like 75 kDa GP.

First of all, this study revealed that 110 kDa GP derived from LS180 and HT29 cells contains some N-glycans in addition to many O-glycans, most of which are expected to form Tn-antigen clusters. The result suggests that 110 kDa GP belongs to well-known mucin-type glycoproteins such as glycophorin A and leukosialin (*9,10*). Glycophorin A consisting of 131 amino acid residues contains 15 O-glycans and one N-glycan at Asn^{45} whereas leukosialin consists of 381 amino acids in which 29 Ser/Thr are O-glycosylated and Asn^{239} is N-glycosylated. Apparent molecular masses of glycophoryn A and leukosialin were reported to be 36 and 50-150 kDa (*9-12*). Size wise, leukosialin is close to a 110 kDa GP. Different apparent molecular masses of 110-130 kDa have been reported for leukosialin expressed in various leukemic cell lines. The different molecular masses were shown to be caused by expression of distinct patterns of O-linked oligosaccharides that are specific to each cell type (*12*). Leukosialin, also known as CD43, was originally found in hematopoietic cells (*12*), but later identified in a variety of nonhematopoietic cancers including lung, breast, and colon (*11*). Aberrant glycosylation of leukosialin/CD43 in various cancers must have resulted in apparent molecular masses of 50-150 kDa (*11*). Monoclonal antibody called UN1 recognizes a heavily sialylated and O-glycosylated protein with apparent molecular weight of 100-120 kDa. The tumor antigen UN1 was later identified to be a different glycoform of leukosialin/CD43 (*13*). Based on these previous studies, it is probable that 110 kDa GP may share the same protein backbone as leukosialin/CD43 consisting of 381 amino acids. It should be noted, however, that 110 kDa GP is heavily O-glycosylated with GalNAc, but that extension of further glycosylation to GalNAc including sialylation does not often seem to occur. In fact, digestion of 110 kDa GP derived from LS180 and HT29 cells by sialidase did not change the molecular mass or the intensity of the 110 kDa band as judged by Western blotting (data not shown), suggesting a minimum involvement of sialic acid residues in 110 kDa GP. Earlier, immunoblotting with anti-CD43 antibody was carried out using LS180 and LS174T cell lysates, which resulted in several immunostained bands but did not recognize 110 kDa GP. To determine the amino acid sequence of 110 kDa GP, a new strategy has been initiated which takes advantage of a smaller fragment, 75 kDa GP, derived from LS180 cells cultured in Akiyama laboratory.

Since limited proteolysis was observed in the microdomain fractions after 3-4 years of storage at –80°C (*6*), susceptibility of 110 kDa GP to endogenous and exogenous proteases was analyzed. Although similar sizes of limited proteolysis fragmentation was not produced, an around 90 kDa GP was detected from LS180 and HT29 cell lysates digested by cathepsin D. Such a similar size fragment was observed in LS180 cell lysates, but not in HT29 cell lysates, digested by trypsin (Figure 3A, line 6) or thermolysin (Figure 5A, line 2). These results are consistent with our previous

results with IP using MLS128 which did not seem to precipitate LS180-derived 110 kDa GP as efficiently as HT29-derived 110 kDa GP, suggesting a possibility that differential O-glycosylations to 110 kDa GP may exist in two cell lines (*6*).

The most intriguing result in the present study was the finding of 75 kDa GP in LS180 cells cultured in Akiyama laboratory in addition to 110 kDa GP. LS180 cells originally obtained from ATCC were independently cultured in Yamaguchi and Akiyama laboratories. Over the years, differences in cell constituents must have developed in LS180 cells in Akiyama laboratory. It is possible that the region susceptible to contaminating endogenous proteases in 110 kDa GP is somehow cleaved in the cell or during lysate preparations from LS180 cells from Akiyama laboratory. The size of 75 kDa GP found in LS180 cells cultured in Akiyama laboratory (Figure 6) is very similar to the limited proteolysis fragments observed in newly immunoblotted microdomain fractions shown in Figure 7. This interesting result has given a new direction as to how to identify the primary structure of 110 kDa GP. Thus we are now analyzing the smaller size MLS128-stainable fragment using 2D EP, enzyme digestion, and sophisticated mass spectrometry available at City of Hope Mass Spectrometry & Proteomics Core equipped with an Orbitrap Fusion (Thermo) and a Triple Quadrupole (Agilent).

The present study also revealed that 110 kDa GP may be an oncoprotein expressed mainly in colon cancer cells. In addition to LS180, LS174T, and HT29 cell lines previously reported (*5*), the current study found that two out of four colon cancer cell lines newly examined expressed 110 kDa GP, which sums up to 71% of colon cancer cell lines being positive for the expression of 110 kDa GP. This is consistent with an earlier study on normal and malignant tissues by MLS128 histochemistry which revealed that positive immunostaining was detected with high frequency (75-100%) in carcinomas of the esophagus, stomach, colon, biliary tract and pancreas (*14*). Further studies on 110 kDa GP expressed in colon cancer cells would provide a fundamental basis for developing cancer diagnostics and therapeutics.

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References

1. Springer GF. Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy. J Mol Med. 1997; 75:594-602.
2. Numata YY, Nakada H, Fukui S, Kitagawa H, Ozaki H, Inoue K, Kawasaki M, Funakoshi T, Yamashina I. A monoclonal antibody directed to Tn antigen. Biochem Biophys Res Commun. 1990; 170:981-985.
3. Nakada H, Numata Y, Inoue M, Tanaka N, Kitagawa H, Funakoshi I, Fukui S, Yamashina I. Elucidation of an essential structure recognized by an anti-GalNAc α-Ser(Thr) monoclonal antibody (MLS 128). J Biol Chem. 1991; 266:12402-12405.
4. Morita N, Yajima Y, Asanuma H, Nakada H, Fujita-Yamaguchi Y. Inhibition of cancer cell growth by anti-Tn monoclonal antibody MLS128. Biosci Trends. 2009; 3:32-37.
5. Zamri N, Masuda N, Oura F, Yajima Y, Nakada H, and Fujita-Yamaguchi Y. Effects of two monoclonal antibodies, MLS128 against Tn-antigen and 1H7 against insulin-like growth factor-I receptor, on the growth of colon cancer cells. Biosci Trends. 2012; 6:303-312.
6. Zamri N, Masuda N, Oura F, Kabayama K, Yajima Y, Nakada H, Yamamoto K, Fujita-Yamaguchi Y. Characterization of anti-Tn-antigen MLS128 binding proteins involved in inhibiting the growth of human colorectal cancer cells. Biosci Trends. 2013; 7:221-229.
7. Matsumoto-Takasaki A, Hanashima S, Aoki A, Yuasa N, Ogawa H, Sato R, Kawakami H, Mizuno M, Nakada H, Yamaguchi Y, Fujita-Yamaguchi Y. Surface plasmon resonance and NMR analyses of anti Tn-antigen MLS128 monoclonal antibody binding to two or three consecutive Tn-antigen clusters. J Biochem. 2012; 151:273-282.
8. Robinson BH, Oei J, Saunders M, Gravel R. [^{3}H]Biotin-labeled proteins in cultured human skin fibroblasts from patients with pyruvate carboxylase deficiency. J Biol Chem. 1983; 247:6660-6664.
9. Chasis JA, Mohandas N. Red blood cell glycophorins. Blood. 1992; 80:1869-1879.
10. Pallant A, Eskenazi A, Mattei MG, Fournier RE, Carlsson SR, Fukuda M, and Frelinger JG. Characterization of cDNAs encoding human leukosialin and localization of the leukosialin gene to chromosome 16. Proc Natl Acad Sci U S A. 1989; 86:1328-1332.
11. Tuccillo FM, de Laurentiis A, Palmieri C, Fiume G, Bonelli P, Borrelli A, Tassone P, Scala I, Buonaguro FM, Quinto I, Scala G. Aberrant glycosylation as biomarker for cancer: Focus on CD43. Biomed Res Int. 2014; 2014:742831.
12. Carlsson SR, Sasaki H, Fukuda M. Structural variations of O-linked oligosaccharides present in leukosialin isolated from erythroid, myeloid, and T-lymphoid cell lines. J Biol Chem. 1986; 261:12787-12795.
13. de Laurentiis A, Gaspari M, Palmieri C, *et al*. Mass spectrometry-based identification of the tumor antigen UN1 as the transmembrane CD43 sialoglycoprotein. Mol Cell Proteomics. 2011; 10:M111.007898.
14. Ohshio G, Imamura T, Imamura M, Yamabe H, Sakahara H, Nakada H, Yamashina I. Distribution of Tn antigen recognized by an anti-Tn monoclonal antibody (MLS128) in normal and malignant tissues of the digestive tract. J Cancer Res Clin Oncol. 1995; 121:247-252.

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Original Article

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Roles of the highly conserved amino acids in the globular head and stalk region of the Newcastle disease virus HN protein in the membrane fusion process

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Summary **Newcastle disease virus (NDV), an avain paramyxovirus, has been assigned to the genus *Avulavirus* within the family *Paramyxoviridae*. It causes Newcastle disease (ND) that is a highly contagious and fatal viral disease affecting poultry and most species of birds. The hemagglutinin-neuraminidase (HN) protein of NDV has multiple functions including mediating hemadsorption (HAD), neuraminidase (NA), and fusion promotion activities affecting the process of viral attachment, entry, replication and dissemination. Fusion ability of the NDV was highly correlated to its virulence. Mutations in the HN globular head and headless HN of NDV were constructed to determinate the impact of highly conserved amino acids in the globular head of paramyxovirus HN proteins and the roles of the stalk region of HN in the fusion process. It was found that the interaction between F and HN mutants E401A, G402A, G468A, V469A, Y526A, and T527A was equal to that in F and wt HN. The mutations of G402A, G468A, V469A, and T527A had various effects on cell fusion promotion, receptor binding ability, and NA activity, but the membrane merging rate was comparable to wt HN. The elimination of hemadsorption ability and NA activity of E401A and Y526A resulted in the loss of the fusion promotion function of HN. The conclusion was that receptor binding and NA had a common active site and E401 and Y526 amino acids were essential for virus attachment, entry, and dissemination. In addition, G468A mutation made different contributions to HAD and NA, which indicated that G468 was one of the potential key amino acids in switching the two functions between receptor binding and sialic acid destruction of HN. It was also proven that the headless HN of NDV could promote the fusion event mediated by F. Thus, it revealed a novel mechanism in F activation of NDV.**

Keywords: NDV, HN protein, Fusion promotion, HN-F interaction

1. Introduction

The Paramyxovirus family has many pathogenic members in humans including measles virus, mumps virus, respiratory syncytial virus (RSV), human meta pneumovirus (hMPV), and human parainfluenza virus 1-5 (hPIV3), while others are zoonotic members such as Newcastle disease virus (NDV), Nipah virus, Hendra virus, Sendai virus and avian pneumovirus (APV).

HN proteins and F proteins co-exist on the surface of most paramyxoviruses. HN proteins are responsible for virus attachment, host-cell membrane fusion promotion, and virus dissemination. HN proteins are composed of four sections, the globular head, stalk, transmembrane region and cytotail, and N-terminus inserts into the membrane (*1,3,7*). It has multiple functions including receptor binding, neuraminidase activity, and fusion promotion. Hemagglutinin-

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neuraminidase is involved in attachment to sialic acid receptors and removal of sialic acids from infected cells. But the details of modulation between these two processes are obscure. The single F protein can not activate the fusion process in NDV. Homologous F and HN proteins of NDV are needed for the fusion process with few exceptions in most paramyxoviruses. In Nipah and Hendra viruses, attachment proteins can be used interchangeably, which suggests slightly different F and HN interaction mechanisms among these paramyxoviruses (*3*). However, no final conclusion has yet been reached on the pattern of interaction between HN and F proteins (*4,11,12,14*).

When NDV infects the host cells, the attachment protein first binds to the sialic acid on the cell surface, and then triggers the fusion (*4,5*). It has been reported that behavior of the receptor-binding promotes dramatic conformational changes of the F protein, which facilitate the membrane fusion process. There are two models of the fusion of paramyxoviruses (*2*). In the first, the HN tetramer is not tightly associated with the F protein trimer on the virus surface. Following the receptor-binding, F protein conformational changes are promoted and convert it to a pre-hairpin and then to trimer-of-hairpins formation. In the dissociation mode, some substantial evidence indicates that there are physical associations between the HN protein and F protein. In the Nipah virus and measles virus, if the interaction between the receptor-binding and the fusion protein is too tight, fusion is blocked (*12,19-22*). However, the mutations in the NDV HN weaken its association with F protein and inhibited the fusion event (*29*). The interaction of the HN proteins and F proteins varies in different stages of the paramyxoviruses membrane fusion and the exact manner remains to be determined. In addition, the conserved amino acids in the HN stalk region impact its NA activity (*23*).

In this study, highly conserved amino acids in the NDV HN globular domain were selected to determinate the variations of biological function in HN. The 401-403, 468-470, and 526-528 amino acids are extensively homologous in many paramyoxviruses (Figure 1A), which suggests they may play important roles in the virus life cycle. Site-directed mutations were constructed and the receptor-binding ability, neuraminidase activity, and cell fusion promotion capacity were detected. A headless mutation of NDV HN was also constructed to determinate its F promotion ability.

2. Materials and Methods

2.1. *Cells and viruses*

BHK-21 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, San Jose, CA, USA) supplemented with 1% glutamine, 1% penicilin-streptomycin (Invitrogen, California, USA), and 10% fetal calf setum (Gibco, California, USA). Wild-type (wt) vaccinia virus was produced in BHK-21 cells and used to quantify cell fusion. Recombinant vaccinia virus vTF7-3 was used to provide T7 RNA polymerase in the vaccinia-T7 RNA polymerase expression system (*25*). Recombinant vaccinia virus vTF7-3 was also maintained in BHK-21 cells.

2.2. *Recombinant Plasmid Vectors and the Transient Expression System*

NDV HN and F genes were inserted into pBluescript(+) (pBSK+) at the BamHI site, as previously described (*26*). The stalk region of the NDV HN gene (HNs) was amplified by PCR and inserted into the pEGFP-N3 vector. Wt or mutated HN proteins were co-expressed in BHK-21 cells with the F protein by the vaccinia-T7 RNA polymerase expression system. All experiments were performed in 35-mm plates seeded a day earlier with the same amount of cells.

2.3. *Site-directed mutagenesis*

Oligonucleotide primers (Sangon Biotech Co. Ltd., Shanghai, China) complementary with appropriate sequence of the NDV HN gene were designed to mutate the amino acids in the conserved domains of the NDV HN globular head. Overlapping PCR was used to product each pair of the recombinant plasmids. Two products with a short homologous sequence were co-transformed into *Escherichia coli* TG1 cells, and they recombined to form a complete plasmid. All mutants were sequenced to verify the proper mutation.

2.4. *Expression eEfficiency of HN glycoprotein on the cell surface*

The expression levels of mutant HN proteins on the cell surface were assayed with Fluorescence-activated Cell Sorter (FACS) (Roche, Indianapolis, IN, USA) analysis with a mixture of two mouse MAbs (Chemicon International, Inc., Temecula, California, USA) specific for the NDV HN protein, as previously described (*28*). The secondary antibody was rabbit anti-mouse IgG (ZSGB-BIO, Beijing, China). Cell surface expression efficiency was quantified as the mean fluorescence intensity per cell. Medium fluorescence intensities were normalized to the medium fluorescence intensities of NDV wt HN. Cells transfected with only the vector and wt HN genes co-expressed with the F gene were also incubated as negative and positive controls, respectively.

2.5. *Indirect immunofluorescence assay (IFA)*

IFA was used to detect whether the R403A, Y470A, and T528A mutants were intracellularly expressed

(Figure 2A). BHK-21 cells were plated on 35-mm plates containing a cover glass and transfected as previously described (*33*). The primary antibody and the secondary antibody were the same as in the FACS assay. The monolayers were fixed with acetone 24 h post co-expression F and mutated HN. Photographs of the cells were immediately taken by fluorescence microscope (OLYMPUS, Japan).

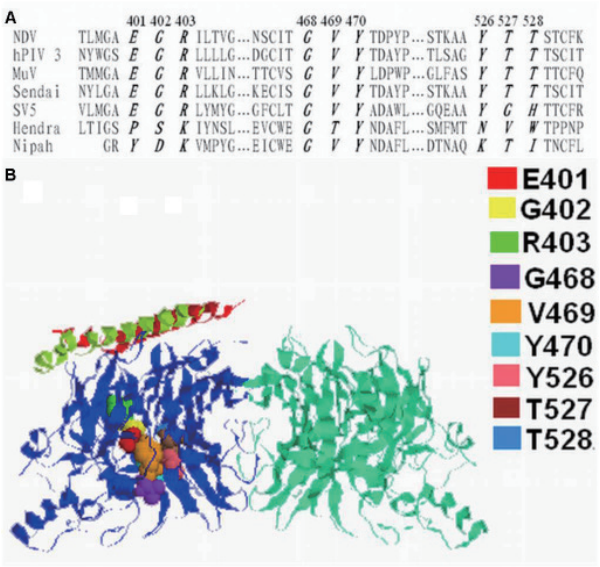

Figure 1. The position of conserved amino acids mutated in the NDV HN global head. (A) The selected amino acid positions of HN in NDV, hPIV 3, MuV, Sendai virus, SV5, Hendra virus, and Nipah virus. The conserved amino acids E401, G402, R403, G468, V469, Y470, Y526, T527, and T528 to be mutated are marked in italic and bold. **(B)** The 3-dimensional structure of NDV HN and locations of mutated residues. The 401-403, 468-470, and 526-528 amino acids are in β4-S1, β5-S1, and β6-S1 sheets, respectively. The mutants are marked in different colors. The figure was generated by RasMol, using the structure of Yuan et al (PDB IDs, 3T1E).

2.6. *Quantification of cell fusion*

Monolayers of BHK-21 seeded 1 day earlier with 4 × 10^5 cells/35 mm plate were co-transfected with the desired HN and F genes, washed with PBS, fixed with methanol for 5 min, and stained with Giemsa stain. The Reporter Gene Method was used to quantify the cell fusion (*34,35*). The plasmid pG1NT7β-gal encoding β-galactosidase was transfected into BHK-21 cells. At 16 h post transfection, these cells were mixed with those transfected with F and HN genes. After 5 h incubation at 37°C, monolayers were lysed with lysis buffer and the extract was assayed for β-galactosidase activity using high sensitivity β-galactosidase assay kit (Stratagene, La Jolla, CA, USA). The level of fusion was quantified by determination of the absorbance at 590 nm with an automated ELISA reader. The cells transfected with only the F gene were used as a negative control to eliminate the background fusion.

2.7. *Neuraminidase (NA) assay*

Transfection was preformed as previously described. To each well 0.5 mL of 625 mg/mL of neuramin-lactose (Sigma, St. Louis, USA) in 0.1 M sodium acetate (pH 6.0) was added and the plates were incubated at 37°C for 1 h. NA activity was quantified by measuring the intensities of the colors in the butanol layer at 549 nm minus the background of cells expressing only the vector (*29-32*).

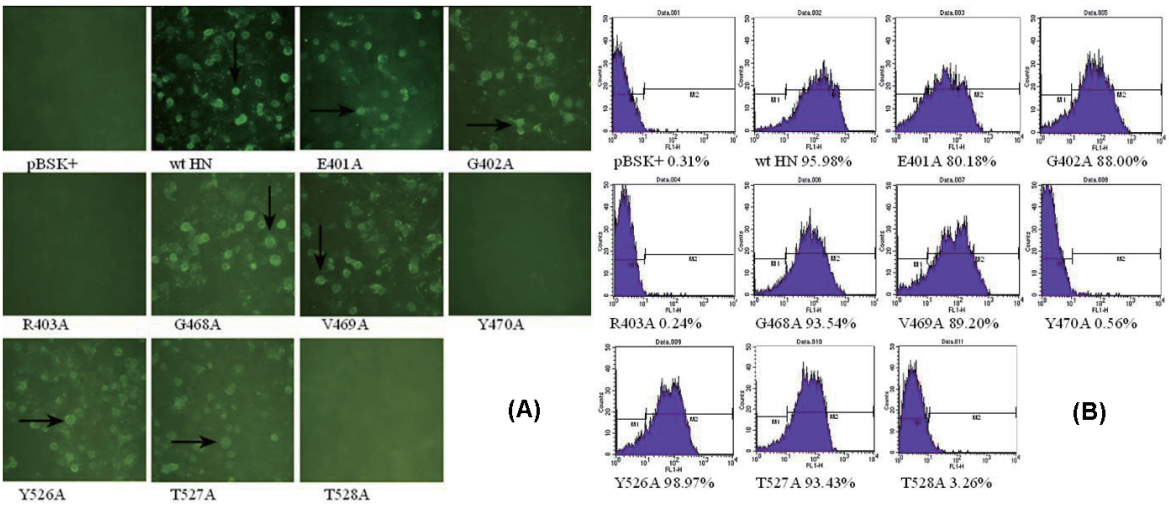

Figure 2. Expression efficiency of mutations in NDV HN head. (A) Indirect immunofluorescent assay (IIFA) of NDV HN mutations. BHK-21 cells were plated on 35-mm plates containing a cover glass and transfected as previously described. The primary antibody and the FITC labelled secondary antibody were the same as in the FACS assay. The monolayers were fixed with acetone 24h post co-expression F and mutated HN. Photographs of the cells were immediately taken by fluorescence microscope (OLYMPUS, Japan). The arrows point to the HN expression cells. **(B)** FACS data shows the expression efficiency of HN mutations by fluorescence intensity. The mutations of E401A, G402A, G468A, V469A, Y526A, and T527A were successfully expressed on the cell surface. The mutations R403A, Y470A, and T528A were not detected by FACS or IIFA. A mixture of monoclonal antibodies for NDV HN was used as primary antibodies in IIFA and FACS assays. The second antibody was fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG with a 1:200 dilution.

2.8. *Hemadsorption (HAD) assay*

The receptor-binding activity of mutant genes was detected by the ability to absorb guinea pig erythrocytes (*26*). The monolayers of BHK-21 that expressed NDV HN proteins were incubated for 30 min at 4°C with 2% guinea pig erythrocytes diluted with PBS supplemented with 1% of each $CaCl_2$ and $MgCl_2$. Then the cells were treated with 50 mM NH4Cl at 4°C until all erythtocytes were lysed, and the lysate was clarified by centrifugation. The absorbance density was determined at 540 nm on the UV spectrophotometer (Shimadzu, Kyoto, Japan) minus the background which was obtained with cells expressing only the vector. All work with animals followed National Institutes of Health guidelines and was approved by the Animal Care Committee of Shandong University (Approved protocol no. 20130305).

2.9. *Hemifusion assay*

The lipid probe octadecyl rhodamine B (R18) (Invitrogen, California, USA) was used to determine the ability of HN mutations to promote hemifusion from RBCs to BHK-21 cells co-transfected with HN and F genes as the protocol previously described (*29*). RBCs were washed and re-suspended with PBS, then labeled with R18 (1 mg/mL in ethanol) at room temperature for 30 min in the dark. RBCs were washed 5 times with ice-cold PBS to remove the uncombined R18 and re-suspended in PBS (0.1% hematocrit). Labeled RBCs were added to co-transfected monolayers and incubated at 4°C for 30 min. Cells were washed with PBS to remove unbonded RBCs, then incubated and the fluorescence was visualized using a fluorescence microscope (OLYMPUS, Japan) at 37°C.

2.10. *Co-immunoprecipitation (Co-Ip) assay*

Twenty-four hours after transfection, the interaction between mutated HN and F at the surface of BHK-21 cells was detected by the co-IP assay as previously described (*29,34*). The wt F proteins and a cleavage site mutant form of F proteins were used. The F (Fcsm) proteins that efficiently interacted with HN proteins without fusion activity were used to avoid the impact of HN and F interaction in or after the fusion process. The monolayers were co-transfected with 1 μg HN and 1 μg Fcsm for each well. Cells were washed 3 times with cold PBS. The AminoLink Plus Coupling Resin (Thermo Scientific, San Jose, CA, USA) were incubated with MAb specific for NDV F (AbBioSci Inc., Changzhou, China) for 2 h at room temperature. Cells were lysed by ice-cold IP lysis buffer on ice for 5 min with periodic mixing. The lysate was added into the resins and incubated with gentle mixing and at 4°C overnight. The proteins were eluted by elution buffer and heated at 100°C for 5 min for Western blot.

2.11. *Fusion promotion of the HN stalk region*

The stalk region (HNs) of NDV HN was constructed by PCR amplification from the full-length of NDV HN in pBSK(+)-HN. The PCR products were cloned into the pEGFP-N3 vector. The nucleotide sequences were verified by BIOSUNE (Beijing, China). The NDV wt F and HNs were expressed by transient transfection of the pBSK-F and HNs vectors in BHK-21 cells. Transfected BHK-21 cells were placed at 37 or 42°C to allow fusion. At 24 h post-transfection, the monolayers were washed with PBS, fixed by methanol, stained using Giemsa stain, and then photographed.

3. Results

3.1. *Site-directed mutation*

The selected amino acids were highly conserved in paramyxovirus (Figure 1A) and mutations were successfully constructed. Alanine substitution was individually introduced into each amino acid position. Each mutation was inserted into pBluescript(+)(pBSK+) and sequence analysis confirmed no additional mutations in the recombinant plasmids. Hydrophilic amino acids E401, G402, R403, G468, Y470, Y526, T527, and T528 were mutated to hydrophobic amino acid alanine. V469 is a hydrophobic amino acid, but it was changed to alanine.

3.2. *Cell surface expression efficiency of mutant NDV HN proteins*

IFA was used to determine HN mutant proteins expression qualitatively intracellular or extracellular. There was no detectable fluorescence in substitutions of R403A, Y470A, or T528A mutants. Green fluoresence was detected in E401, G402, G468, V469, Y526, and T527 mutants (Figure 2A). The FACS was used to determine expression efficiency of mutant NDV HN proteins. The results of FACS indicated that mutants were successfully expressed on the cell surface except for R403A, Y470A, and T528A mutants. To some extent, expression efficiency of the rest of the mutants was similar to wt HN from 69.3% to 102.9% (Figure 2B and Table 1) on average of three independent experiments.

3.3. *Fusion promotion ability of individual mutants*

Giemsa staining (Figure 3A) and the reporter gene method were used to test the qualitative and quantitative effect of the individual mutation, respectively. The cell fusion promotion activities of all mutants decreased, to some extent, compared with the wt HN and the fusion activity of mutated protein E401A and Y526A almost disappeared (Figure 4). The mutations of G402A, G468A, V469A, and T527A exhibited various effects

Table 1. Functional profile of the mutants in the globular head of NDV HN

Amino acid substitutions	Position of β-Sheet	Avg cell surface expression (% of wt)	Avg cell fusion (% of wt)	Avg NA (% of wt)	Avg HAd (% of wt)
E401A	B4-S1	69.3 ± 13.4	2.43 ± 1.9	10.1 ± 0.6	7.3 ± 1.0
G402A	B4-S1	101.4 ± 8.5	53.4 ± 1.8	72.8 ± 1.9	71.5 ± 1.8
R403A	B4-S1	—	—	—	—
G468A	B5-S1	94.6 ± 2.6	70.0 ± 3.0	35.3 ± 0.9	93.9 ± 9.1
V469A	B5-S1	92.0 ± 4.7	12.4 ± 0.8	68.1 ± 2.0	46.5 ± 1.6
Y470A	B5-S1	—	—	—	—
Y526A	B6-S1	84.1 ± 25.7	3.2 ± 0.4	0.4 ± 0.5	7.9 ± 1.7
T527A	B6-S1	102.9 ± 23.0	54.2 ± 4.3	35.5 ± 2.9	49.2 ± 7.7
T528A	B6-S1	—	—	—	—

The average of cell surface expression, cell fusion, NA activity, and HAD ability were determined by FACS, Report Gene Method, NA assays, and HAD assays, respectively. The data are averages of three independent experiments.

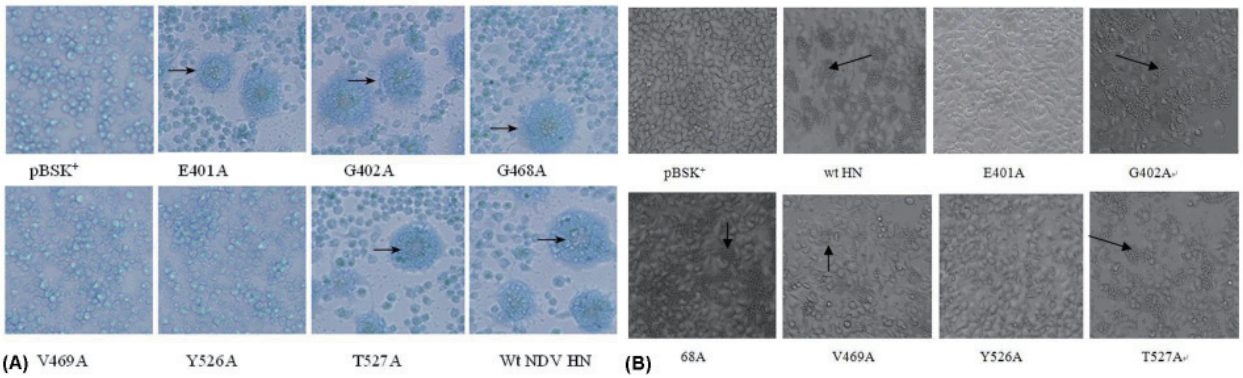

Figure 3. Detection of cell fusion ability and hemadsorption ability of mutation proteins in the NDV HN global head. (A) Giemsa staining results of NDV HN mutations in the presence with F co-transfection (24h post-transfection) in BHK-21 monolayers. The arrows point to the syncytia. **(B)** The monolayers that expressed HN were incubated with 2% guinea pig erythrocytes suspension at 4°C for 30 min. The redundant erythrocytes were removed by washing with PBS 3 times and photomicrographs were taken. The attached guinea pig erythrocytes are indicated by arrows.

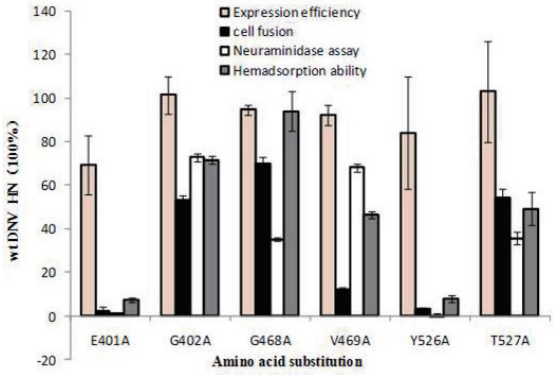

Figure 4. Expression efficiency and function of mutational NDV HN proteins. Comparison of mutant NDV HN expression efficiency, cell fusion activity, NA assay, and HAD ability (% of wt HN). The data represent the means of three independent determinations plus standard deviations.

on cell fusion promotion, receptor binding ability, and NA activity.

3.4. *HAD ability and NA activity of HN mutants*

The HAD assay was confirmed by the ability to adsorb guinea pig erythrocytes at 4°C to reveal the receptor binding ability (Figure 3B). Mutant G468A retained a similar HAD ability as the wt NDV HN (93.9%). G402A decreased to 71.5% of wt NDV HN. The hemabsorption abilities of V469A and T527A were 46.5% and 49.1% respectively. E401A and Y526A significantly affected the receptor binding ability (7.3% and 7.9%, respectively). Sialyllactose colorimetrically was used to quantify the NA activity of each mutated protein. Compared with wt NDV HN, mutated proteins G402A, V469A, G468A and T527A had 72.7%, 68.1%, 35.3%, and 35.5% of the NA activity, respectively. Neuraminidase activity of E401A and Y526A was almost undetectable, less than 5% (1.09%, and 0.38%) of the wt HN protein (Figure 4 and Table 1).

3.5. *Hemifusion assay*

R-18 labeled RBCs were used to detect the lipid-mixing rate and fusion efficiency (Figure 5). The BHK-21 monolayers of wt HN or E401A, G402A, G468A, V469A, and T527A co-transfection with F obtained the maximum fluorescence almost at the same time at 37°C. We attempted to quantify the lipid-mixing rate for these mutations, but the lipid-mixing process was accomplished in a few seconds. The time of fusion promotion was too short to distinguish the variation among wt HN and HN mutations. The number of syncytia of E401A, G402A, V469A, and T527A was

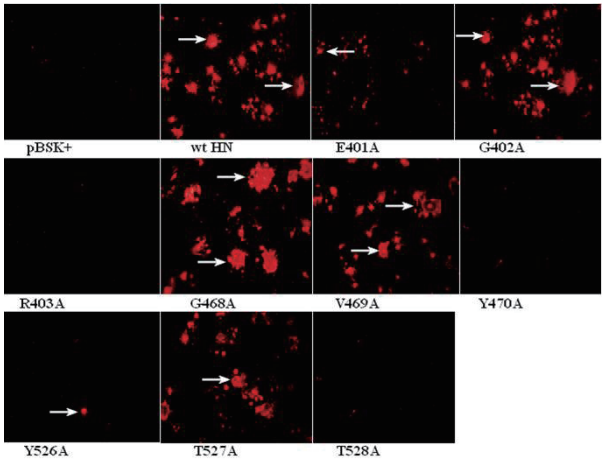

Figure 5. Cell fusion of R18-labeled RBCs with HN and F protein co-transfection BHK-21 cells. The labeled RBCs were incubated with HN and F co-expressed monolayers at 4°C for 30 min. Monolayers were washed with ice-cold PBS to remove the unbound RBCs, incubated at 37°C for 5 min, and detected using fluorescence microscope. Arrows indicate lipid mixing.

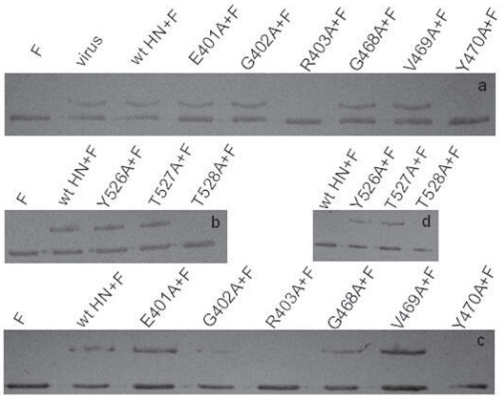

Figure 6. Co-immunoprecipitation of NDV HN and NDV F. The AminoLink Plus Coupling Resin was incubated with MAb specific for NDV F for 2 h at room temperature. The monolayers co-expressed with F and HN genes were lysed by ice-cold IP Lysis Buffer on ice for 5 min with periodic mixing. The lysate was added into the resins and incubated for overnight at 4°C. The proteins were eluted by Elution Buffer and heated at 100°C for 5 min for Western Blot. A mixture of an anti-F MAb and two anti-HN MAbs was used for Western Blot. Monolayers transfected F alone, co-transfected with wt HN and F, and virus supernatant were used as controls. **a** and **b** show the wt HN or HN mutations co-expression with Fcsm in BHK-21 monolayers. The wt HN or HN mutations and wt F were co-expressed in BHK-21 monolayers in **c** and **d**. The grayscale of the photograph was analyzed by Image J 1.48V.

less than that in wt HN and the diameter of the syncytias for HN mutations was smaller. It was proven that the decrease of receptor-binding ability in HN had no impact on the rate of lipid-mixing in the fusion promotion process but the fusion efficiency declined at the end of the lipid-mixing event.

3.6. *Co-IP assay*

If the fusion could not be promoted, the interaction between HN and F was not impacted with co-expression

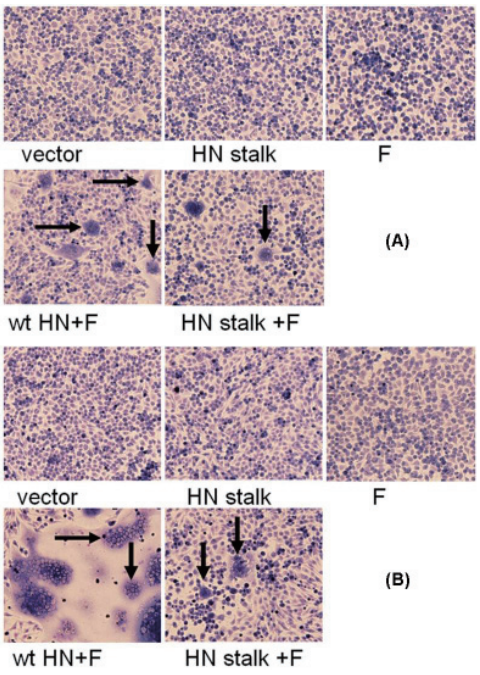

Figure 7. The F promotion capacity of the HN stalk by co-expression F and HN stalk genes. BHK-21 monolayers were infected with recombinant vaccina virus and co-transfected with wt HN or HNs and F genes. At 24 h post-transfection, the monolayers were fixed with methanol for 10 min and stained with Giemsa stain. Syncytias are indicated by arrows. BHK-21 monolayers were incubated at 37°C **(A)** and 42°C **(B)**.

Fcsm and wt HN or HN mutations (Figures 6a and 6b). The monolayers co-expressed wt F and wt HN or mutant HN detected varying degrees of fusion (Figures 6c and 6d). Nevertheless, the interaction between Fcsm and HN mutations were comparable to that in wt HN. It was concluded that the interaction of F and HN proteins was not related with the receptor binding ability of HN. The globular head of HN did not participate in the interaction and the interaction of HN and F disappeared in the stage of post-fusion.

3.7. *Fusion promotion of NDV HNs*

In the monolayers co-transfacted with F and HNs, syncytia were detected at 37°C and 42°C (Figures 7A and 7B). But the number of syncytia of HNs was less than that in wt HN and the size of syncytia in HNs was smaller. It indicated that the headless HN could promote the fusion process in NDV, but the fusion efficiency declined. It was inconsistent with the theory that the receptor binding event promotes the fusion process mediated by F. However, there is no convincing explanation for the promotion mechanism of HNs in NDV.

4. Discussion

The head region of NDV HN has three functions, receptor binding ability, fusion promotion, and NA activity. We determined the roles of the highly

conserved amino acids in the head region of NDV HN proteins. Some amino acids changes in the HN globular head removed the F promotion ability of HN protein and restrained syncytia formation. Fewer and smaller syncytias were detected in the HN mutations. It is thought that the disappearance of receptor binding ability is responsible for these changes based on previous studies. However, a headless HN recombinant protein was also constructed to test the roles of the HN stalk region in the F promotion process. It was found that the headless HN promoted the fusion process though the fusion extent was weaker than wt HN.

The expression efficiency of nine NDV HN mutants was determined by FACS. The mutant proteins E401A, G402A, G468A, V469A, Y526A, and T527A were successfully expressed, but the mutants R403A, Y470A, and T528A were not detected by FACS. Indirect immunofluorescence assay was also used to determine expression of R403A, Y470A, and T528A mutants and no intracellular or extracellular fluorescence was found. It is possible that the mutants affected the protein translation or transport process of the recombinant proteins. No reasonable reason was established in this study. It can not be excluded that the monoclonal antibody binding site was changed so that the MAbs could not bind to the NDV HN proteins.

NDV, SV5, and hPIV3 are members of paramyxoviridae virus with six β-sheets in the globular head of the HN protein. Highly conserved animo acids found in the S1 region of each β-sheet in these viruses suggested that HAD ability and NA activity of HN protein are related to these conserved amino acids in paramyxoviruses. For the mutants used in this study, 401-403 amino acids are in β4-S1 sheet, 468-470 amino acids are in β5-S1 sheet, and 526-528 amino acids are in β6-S1 sheet. E401, G468, and Y526A are in the external part of the pocket-like structure in globular head of the HN protein that are necessary for the receptor-binding ability and neuraminidase activity. The G402, V469A, and T527A on the inner side of E401A, G468A, and Y526A are less important for the receptor-binding ability and neuraminidase activity. The R403, Y470, and T528 in the internal part of the receptor-binding motif of the NDV HN protein are not expressed on the cell surface. It is possible that the highly conserved amino acids in β-sheet of the external part of the globular head of the HN are vitally important for the receptor-binding ability and neuraminidase activity. It has been confirmed that both the receptor binding and nueraminidase activity are mediated by the same activity site in the NDV HN protein globular head. The conformation change of the NDV HN protein is able to switch between the two different states (*6,18*). The virus attachment process is implemented by HN protein binding with the sialic acids on the host cell surface and the virus dissemination depends on the removal of sialic acids on the cell surface by HN nueraminidase activity. There may be something balancing mechanism between the virus switching these two functions of the HN protein and the conserved amino acids may provide some novel information.

Murrell *et al*. reported that in 4-GU-DANA-resistant HPIV3 virus variant (ZM1) the receptor-binding ability and NA activity were higher than that in the parent virus. It suggested that the receptor-binding ability was closely related with neuraminidase activity. The virus increased neuraminidase activity to promote virus release when its hemagglutinin ability was improved. In the same mutant, the impact on receptor-binding ability, neuraminidase activity, and cell fusion is different (*36*). The mutations in NDV HN demonstrated that R174L, R416L, R498L, E547Q, Y526L, and I175E impacted the receptor binding ability and NA activity. We have proved that decrease of hemadsorption ability for the mutants G402A (71.5%), V469A (46.5%), and T527A (49.1%) correlated with the loss of neuraminidase activity (72.7%, 68.1%, and 35.5%, respectively). However, G468A had a different result from other mutants. The hemadsorption ability of G468A was 93.9% of the wt HN but neuraminidase activity was only 35.3% of the wt HN proteins. A conclusion can be drawn that E401, Y526, G402, V469, and T527 play the same role in the receptor-binding and receptor remove process, but G468 plays a different role in these two processes. It may be one of the key amino acids in switching the functions between the receptor binding and destruction process. Previous studies have shown that G403A in NDV HN did not impact the biological function of HN, but we found that the G403A mutation did not express intracellularly or on the cell surface.

The 3D structure of HN global head demonstrated that side chains of E401 and Y526 are hydrogen bonding to sialic acid ligands. Changing the side chains of E401 and Y526 to E401A and Y526A almost eliminated the receptor binding ability and NA activity of the HN protein in this study. The hemadsorption ability and neuraminidase activity of mutants E401A (2.43%, 3.17%) and Y526A (1.09%, 0.38%) almost disappeared compared with the wt HN. It could indicate that E401 and Y526 are essential for both virus attachment and dissemination.

The structure of the globular head in NDV HN proteins revealed that the β-sheet propeller motif forms a NA activity site. It has been reported that K147, Y526, and E547 constituted the NA activity domain in accordance with the conclusion in this research (*6*). The NA activity of Y526A declined to 0.38% of the wt NDV HN. Reduction of HAD ability and cell fusion were comparable in mutants G402A, G468A, and T527A. It can be assumed that the decline of cell fusion promotion was caused by the decrease of receptor recognition ability. However, the decline of cell fusion promotion ability (12.4 ± 0.8%) of V469A was lower than that of receptor-binding ability (46.5 ± 1.6%) and NA activity (68.1 ± 2.0%). Thus, V469A impacts the hemagglutinin,

sialidase, and fusion promotion activity in the virus life cycle.

However, there was a contradiction that the headless HN of NDV promoted the fusion process. Related theories agree that the head of HN is necessary for the F protein promotion in the membrane fusion process. Some conserved amino acids (E401A and Y526A) change in the HN protein of NDV which lead to incapacity of receptor binding, and also loss of the fusion promotion function and syncytia formation ability. In these mutations, the whole stalk region of HN exists and did nothing for the F promotion. It has been reported that the head of HN was responsible for the virus attachment and useless for the F promotion process. However, we think that the globular head of HN suppresses the F promotion and the stalk region of HN has the ability to promote the F conformation change. It possibly revealed a new model of the HN and F interaction. In the family of paramyxovirus, there is a similar flexible linker structure between the globular head and stalk region that was the potential for the movement of the globular head in HN proteins. The globular head of HN binding to sialic acids is separated from F by the flexible linker structure between the globular head and stalk region of HN. Then, the stalk region of HN promoted the conformation changes of F protein to activate the fusion process.

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References

1. Weissenhorn W, Hinz A, Gaudin Y. Virus membrane fusion. Federation of European Biochemical Societies. 2007; 518:2150-2155.
2. White JM, Delos SE, Brecher M, Schornberg K. Structures and mechanisms of viral membrane fusion proteins: Multiple variations on a common theme. Crit Rev Biochem Mol Biol. 2008; 43:189-219.
3. Dutch RE. Entry and fusion of emerging paramyxoviruses. Plos Pathogens. 2010; 6:e1000881.
4. Murrell M, Porotto M, Weber T, Greengard O, Moscona A. Mutations in human parainfluenza virus type 3 hemagglutinin-neuraminidase causing increased receptor binding activity and resistance to the transition state sialic acid analog 4-GU-DANA (Zanamivir). J Virol. 2003; 77:309-317.
5. Yuan P, Thompson TB, Wurzburg BA, Paterson RG, Lamb RA, Jardetzky TS. Structural studies of the parainfluenza virus 5 hemagglutinin-neuraminidase tetramer in complex with its receptor: Sialyllactose. Structure. 2005; 13:803-815.
6. Lawrence MC, Borg NA, Streltsov VA, Pilling PA, Epa VC, Varghese JN, McKimm-Breschkin JL, Colman PM. Structure of the haemagglutinin-neuraminidase from human parainfluenza virus Type III. J Mol Biol. 2004; 335:1343-1357.
7. Yu M, Wang E, Liu Y, Cao D, Jin N, Zhang CW, Bartlam M, Rao Z, Tien P, Gao GF. Six-helix bundle assembly and characterization of heptad repeat regions from the F protein of Newcastle disease virus. J Gen Vriol. 2002; 83:623-629.
8. Lamb RA, Paterson RG, Jardetzky TS. Paramyxovirus membrane fusion: Lessons from the F and HN atomic structures. Virology. 2006; 344:30-37.
9. Russell CJ, Luque LE. The structural basis of paramyxovirus invasion. Trends Microbiol. 2006; 14:243-246.
10. Aguilar HC, Matreyek KA, Choi DY, Filone CM, Young S, Lee B. Polybasic KKR motif in the cytoplasmic tail of nipah virus fusion protein modulates membrane fusion by inside-out signaling. J Virol. 2007; 81:4520-4532.
11. Aguilar HC, Matreyek KA, Filone CM, Hashimi ST. N-Glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. J Virol. 2006; 80:4878-4889.
12. Corey EA, Iorio RM. Mutations in the stalk of the measles virus hemagglutinin protein decrease fusion but do not interfere with virus-specific interaction with the homologous fusion protein. J Virol. 2007; 81:9900-9910.
13. Lawrence MC, Borg NA. Structure of the haemagglutinin-neuraminidase from human parainfluenza virus type III. J Mol Biol. 2003; 335:1343-1357.
14. Peisajovich SG, Shai Y. New insights into the mechanism of virus-induced membrane fusion. Trends Microbiol. 2002; 27:183-190.
15. Peisajovich SG, Shai Y. Viral fusion proteins: Multiple regions contribute to membrane fusion. Biochim Biophys Acta. 2003; 1614:122-129.
16. Baker KA, Dutch RE, Lamb RA, Jardetzky TS. Structural basis for paramyxovirus-mediated membrane fusion. Mol Cell. 1999; 3:309-319.
17. Morrison TG. Structure and function of a paramyxovirus fusion protein. Biochim Biophys Acta. 2003; 1614:73-84.
18. Zaitsev V, von Itzstein M, Groves D, Kiefel M, Takimoto T, Portner A, Taylor G. Second sialic acid binding site in Newcastle disease virus hemagglutinin-neuraminidase: Implications for fusion. J Virol. 2004; 78:3733-3741.
19. Aguilar HC, Matreyek KA, Filone CM, Hashimi ST, Levroney EL, Negrete OA, Bertolotti-Ciarlet A, Choi DY, McHardy I, Fulcher JA, Su SV, Wolf MC, Kohatsu L, Baum LG, Lee B. N-Glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. J Virol. 2006; 80:4878-4889.
20. Aguilar HC, Lee B. Emerging paramyxoviruses: Molecular mechanisms and antiviral strategies. Expert Rev Mol Med. 2011; 13:1-19.
21. Melanson VR, Iorio RM. Amino acid substitutions in the F-specific domain in the stalk of the Newcastle disease virus HN protein modulate fusion and interfere with its interaction with the F Protein. J Virol. 2004; 78:13053-13061.
22. Plemper RK, Hammond AL, Gerlier D, Fielding AK, Cattaneo R. Strength of envelope protein interaction modulates cytopathicity of Measles virus. J Virol. 2002; 76:5051-5061.

23. Wang ZY, Iorio RM. Amino acid substitutions in a conserved region in the stalk of the Newcastle disease virus HN glycoprotein spike impair its neuraminidase activity in the globular domain. J Gen Virol. 1999; 80:749-753.
24. Chizmadzhev YA. The mechanisms of lipid-protein rearrangements during viral infection. Bioelectrochemistry. 2004; 63:129-136.
25. Fuerst TR, Niles EG, Studier FW, Moss B. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA poly-merase. Proc Natl Acad Sci U S A. 1986; 83:8122-8126.
26. Deng R, Wang Z, Mirza AM, Iorio RM. Lo-calization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. Virology. 1995; 209:457-469.
27. Mirza AM, Deng R, Iorio RM. Site-directed mutagenesis of a conserved hexapeptide in the paramyxovirus hemagglutinin-neuraminidase glycoprotein: Effects on antigenic structure and function. J Virol. 1994; 68:5093-5099.
28. Gravel KA, Morrison TG. Interacting domains of the HN and F proteins of Newcastle disease virus. J Virol. 2003; 77:11040-11049.
29. Morris SJ, Sarkar DP, White JM, Blumenthal R. Kinetics of pH-dependentfusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells measurement by dequenching of fluorescence. J Bio Chem. 1989; 264:3972-3978.
30. Li J, Quinlan E, Mirza A, Iorio RM. Mutated form of the Newcastle disease virus hemagglutinin-neuraminidase interacts with the homologous fusion protein despite deficiencies in both receptor recognition and fusion promotion. J Virol. 2004; 78:5299-5310.
31. Corey EA, Mirza AM, Levandowsky E, Iorio RM. Fusion deficiency induced by mutations at the dimer interface in the Newcastle disease virus hemagglutinin-neuraminidase is due to a temperature-dependent defect in receptor binding. J Virol. 2003; 77:6913-6922.
32. Melikyan GB, Chernomordik LV. Membrane rearrangements in fusion mediated by viral proteins. Trends Microbiol. 1997; 5:349-354.
33. Mirza AM, Deng R, Iorio RM. Site-directed mutagenesis of a conserved hexapeptide in the paramyxovirus hemagglutinin-neuraminidase glycoprotein: Effects on antigenic structure and function. J Virol. 1994; 68:5093-5099.
34. Deng R, Wang Z, Mahon PJ, Marinello M, Mirza A, Iorio RM. Mutations in the Newcastle disease virus hemagglutinin-neuraminidase protein that interfere with its ability to interact with the homologous F protein in the promotion of fusion. Virology. 1999; 253:43-54.
35. Corey EA, Mirza AM, Levandowsky E, Iorio RM. Fusion deficiency induced by mutations at the dimer interface in the Newcastle disease virus hemagglutinin-neuraminidase is due to a temperature-dependent defect in receptor binding. J Virol. 2003; 77:6913-6922.
36. Yuan P, Swanson KA, Leserb GP, Paterson RG, Lambb RA, Jardetzky TS. Structure of the Newcastle disease virus hemagglutinin-neuraminidase (HN) ectodomain reveals a four-helix bundle stalk. Proc Natl Acad Sci U S A. 2011; 108:14920-14925.

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Original Article

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Short- and long-term outcomes of hepatectomy with or without radiofrequency-assist for the treatment of hepatocellular carcinomas: a retrospective comparative cohort study

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Summary **The objective of this study was to compare the short- and long-term outcomes of radiofrequency-assisted liver resection (RFLR) and conventional clamp-crushing liver resection (CCLR) and to evaluate the safety and efficiency of RFLR. Between January 2008 and December 2012, a total of 597 patients with hepatocellular carcinoma (HCC) who underwent curative hepatectomy were identified. A total of 272 patients underwent RFLR, and 325 patients received CCLR. The short- and long-term outcomes were compared. The patients in the RFLR and CCLR groups showed similar baseline characteristics. The RFLR group showed less intraoperative blood loss (485.5 *vs.* 763.2 mL, $p = 0.003$), a lower transfusion requirement rate (19.1 *vs.* 31.7%, $p \leq 0.01$), shorter surgery duration (211 *vs.* 296 min, $p \leq 0.01$) and a lower vascular inflow occlusion rate (25.7 *vs.* 33.8%, $p = 0.032$). No significant postoperative changes in bilirubin or liver enzymes were observed in the two groups. The degree of postoperative complications and morbidity did not significantly differ between the two groups. There were no significant differences in the 1-, 2- and 3-year overall survival rates (73.8%, 58.5%, and 55.7% *vs.* 80.8%, 65.8%, and 56.2%, respectively) or disease-free rates (51.9%, 47.2%, and 46.0% *vs.* 54.5%, 44.9%, and 38.5%, respectively) between the RFLR and CCLR groups. These results suggested RFLR was a safe and efficient method for patients with HCC. RFLR was associated with decreased blood loss, fewer blood transfusions, shorter surgery times and less vascular inflow occlusion application. The RFLR group did not show increased liver injury or postoperative morbidity or mortality.**

Keywords: Hepatocellular carcinoma, radiofrequency-assisted, hepatectomy, comparative study, blood loss

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common primary malignant cancer of the liver and the third leading cause of cancer-related deaths worldwide (*1,2*). Curative liver resection is widely accepted as the initial and first-line therapeutic strategy for patients with early-stage and advanced-stage HCC (as defined in the Barcelona Clinic Liver Cancer (BCLC) staging system, according to the guidelines of the American Association for the Study of Liver Disease and European Association for the Study of the Liver) (*3*). Liver resection has been increasingly performed in most specialized centers and has undergone remarkable improvement over recent decades. This has resulted in improvements in diagnosis, surgical techniques, anesthetic management and postoperative care, thereby reducing morbidity and mortality following hepatic resection (*4*).

Despite improvements in the safety of liver resection, the procedure continues to present a risk of massive intraoperative bleeding. Recent technological innovations and improved techniques, including vascular control, low central venous pressure, various hemostatic agents and multiple parenchymal

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transection techniques aimed at controlling and minimizing hemorrhaging during the transection of liver parenchyma in haptic resections have been applied in clinical practice (*5*). Radiofrequency (RF)-assisted liver resection devices have been developed that employ a bipolar needle and utilize the RF energy to pre-coagulate the liver transection plane. The heat produced by the microwaves seals the vessels and enables the bloodless resection of parenchyma transections. This method was first introduced by Habib's group at Hammersmith Hospital, London, UK and has been shown to effectively reduce intraoperative bleeding (*6*). Although this device is promising, severe adverse events have been reported, especially bile leakage and severe liver damage, which could increase postoperative morbidity and mortality (*7,8*).

Our study compared RF-assisted liver resection (RFLR) with conventional clamp-crushing liver resection (CCLR) in HCC patients and investigated whether RFLR could reduce intraoperative bleeding, vascular inflow occlusion and postoperative complications. Our study also analyzed RFLR damage to liver function and postoperative morbidity in HCC concomitant cirrhosis in the short-term and compared the long-term results with those of CCLR.

2. Methods

2.1. *Patients*

From January 2008 to December 2012, on a series of 597 patients diagnosed with HCC hepatectomy was performed at Institute of Hepatobiliary Surgery, Southwest Hospital. Patients who received CCLR or RFLR were identified. Data comprising demographic information, perioperative parameters and complications of all participants were prospectively collected and retrospectively analyzed from a review of medical charts and a computerized database. The study protocol was approved by the Clinical Trial Ethics Committee of Southwest Hospital, Third Military Medical University, and informed consent for the study was obtained from the participants prior to treatment.

Patients who fulfilled the following criteria were included in the study: *i*) a clinical diagnosis of resectable HCC; *ii*) 18 to 65 years of age; *iii*) preoperative liver function tests showing Child-Pugh Classification A or B with no encephalopathy or upper gastrointestinal bleeding history; *iv*) 15-min indocyanine green retention (ICG-R15) of < 30%; *v*) acceptable clotting profile [platelet count (PLT) > 50×10^9/L and a prolonged prothrombin time of < 5 seconds]; *vi*) sufficient relative residual liver volume (% RLV) ≥ 40%; *vii*) no tumor invasion in the primary branch of the portal vein, hepatic vein, or inferior vena cava; *viii*) no metastasis in lymph nodes or other organs; and *ix*) written informed consent.

2.2. *Diagnosis and definition*

The diagnosis of HCC was preoperatively confirmed based on the criteria of the practice guidelines of the American Association for the Study of Liver Disease (AASLD) and was confirmed *via* a pathological specimen test after surgery (*9*). Ultrasonic (US) contrast, tri-phasic abdominal contrast-enhanced spiral computed tomography (CT) scanning and abdominal magnetic resonance imaging (MRI) were applied to detect liver lesions. Either *i*) two imaging techniques showing typical HCC features or *ii*) positive findings on imaging together with or without an alpha-fetoprotein (AFP) level > 400 ng/mL was considered to indicate HCC.

Liver resection was classified based on Couinaud's classification system. Major liver resection was defined as the resection of three or more liver segments according to Couinaud's classification, whereas minor liver resection was defined as the resection of two segments or less (*10*). Operative mortality was defined as any death within 90 days after surgery or during the hospital stay. The severity of postoperative complications was estimated according to the Clavien-Dindo classification system (*11*). The postoperative peak of alanine aminotransferase (ALT) level and total bilirubin (TIBL) level were chosen to be accurate markers of hepatocellular injury and recovery following hepatic resection. Liver failure was diagnosed using the "50-50 criteria" (*12*). Bile leakage was defined as the drainage of 50 mL or more of bile from the surgical drain or drainage from an abdominal collection lasting 3 days or more (*13*).

2.3. *Preoperative management*

All patients underwent careful preoperative assessment, including laboratory tests (*e.g.*, blood biochemistry, alpha-fetoprotein assay, and hepatitis B virus DNA PCR test), chest X-ray, electrocardiogram (ECG), contrast enhanced ultrasonography (CEUS), tri-phasic abdominal contrast-enhanced spiral CT scanning and abdominal MRI. Liver function was assessed with Child-Pugh grading, indocyanine green retention at 15 minutes (ICG-R15) and/or an oral glucose tolerance test (OGTT). The indication for surgery for each patient was discussed at the Multi-Disciplinary Treatment and Pathway Meeting.

2.4. *Surgical procedure*

A team of four senior hepatobiliary surgeons who had more than 10 years of experience and had independently conducted standard anatomical hepatectomy in more than 100 patients performed all hepatic resections. The surgical procedures were selected according to a previously described algorithm (*14,15*). All hepatic

resection surgeries were carried out under general anesthesia using a "J" right subcostal incision with a midline extension. Intraoperative ultrasound (IOUS) was routinely applied to confirm the tumor location and size. Then, the resection was delineated 1 cm from the edge of the tumor using an electrosurgical knife. RFLR was performed using a bipolar radiofrequency device (Habib™ 4X, Generator 1500X RITA Medical Systems, Inc., California, USA). The device consisted of two pairs of opposing electrodes with an active end which was 6-10 cm in length. RFLR was conducted by inserting the electrode into the liver parenchyma and parallel to the delineated line. Coagulation desiccation was performed and induced pale tissue coloration due to coagulation necrosis. The electrodes were withdrawn 1-2 cm in preparation for the next session. After the treatment, the liver parenchyma was dissected using a surgical scalpel along the desiccated line. Electric scalpel or water dissector (Jet2, Erbe Corp., Germany), together with a Kelly clamp were used to dissect the liver parenchyma in both RFLR and CCLR. After crush-clamping, small vessels (< 2 mm) were sealed and divided using the electric scalpel. Large vessels and major intrahepatic bile ducts were ligated and divided. In CCLR, the tumor was resected only using the clamp-crushing method without RFA pre-coagulation treatment. The Pringle maneuver (15 min of occlusion and 5 min of reperfusion) was applied in both groups to achieve intermittent inflow occlusion to control massive bleeding, if necessary.

2.5. Statistical methods

Statistical analyses were performed using the Statistical Package for the Social Sciences version 19.0 software (SPSS, Chicago, Illinois, USA). Continuous variables were expressed as the means ± standard deviation or median with interquartile range and compared using an independent sample *t* test and the Mann-Whitney U test. For categorical variables, comparisons were made using chi-square analysis and a Fisher's exact test. All differences were examined using a two-tailed test, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Patient characteristics*

A total of 597 HCC patients (528 males and 69 females) who had surgical liver resection were enrolled and analyzed in this study. A total of 272 (45.6%) and 325 (54.4%) patients received RFLR and CCLR, respectively. The baseline characteristics of the patients receiving RFLR and CCLR are shown in Table 1. Most patients in both groups suffering from HCC were males (89.3% *vs*. 87.7%, respectively, $p = 0.531$). The mean age was 49.0 years in the RFLR group compared with 49.5 years in the CCLR group ($p = 0.568$). Hepatitis B viral infection was common in both groups (88.2% *vs*. 84.3%, respectively, $p = 0.167$). There were no significant differences between the two groups regarding age, gender, HBV infection, tumor size, tumor number, ICG-R15, TIBL, ALT, AST, platelet count, or perioperative AFP level. In both groups, most liver resections were carried out for HCC with Child-Pugh A.

Table 1. Baseline demographic in HCC patients undergoing RFLR or CCLR

Items	RFLR	CCLR	*p value*
No. of patients, *n* (%)	272 (45.6%)	325 (54.4%)	
Gender			0.531
Male	243 (89.30%)	285 (87.70%)	
Female	29 (10.80%)	40 (12.30%)	
Age (years)	48.98 ± 11.48	49.51 ± 10.99	0.568
HBsAg, n (%)			0.167
Positive	240 (88.24%)	274 (84.31%)	
Negative	32 (11.76%)	51 (15.69%)	
Serum Biochemistry			
PLT count($\times 10^9$/L)	135.92 ± 79.06	139.49 ± 78.97	0.583
ALT level	46.83 ± 38.16	56.64 ± 65.88	0.03
AST level	50.81 ± 34.51	64.53 ± 115.32	0.059
Total Bilirubin	18.08 ± 22.59	22.41 ± 37.62	0.097
Albumin	41.16 ± 8.89	40.30 ± 9.94	0.272
AFP level			0.831
≤ 400	192 (70.59%)	232 (71.38%)	
> 400	80 (29.41%)	93 (28.62)	
ICG-R15 (%)			1.000
≤ 20	259 (98.90%)	322 (99.07%)	
> 20	3 (1.10%)	3 (0.93%)	
Tumor Size max (cm)	6.09 ± 3.23	6.24 ± 3.63	0.583
Tumor Number, *n* (%)			0.604
Single	206 (75.74%)	252 (77.54%)	
Multiple	66 (24.26%)	73 (22.46%)	
Child-Pugh A/B/C	258/14/0	298/27/0	0.128

HBAg: hepatitis B virus surface antigen, PLT: platelet, ALT: alanine aminotransferase, AST: aspartate aminotransferase, ICG-R15: indocyanie green retention test at 15 min, AFP: alpha-fetoprotein.

3.2. *Intraoperative outcomes*

The surgical variables and perioperative outcomes for the 597 patients are shown in Table 2. The surgical procedures in the RFLR group comprised 108 (39.7%) major resections and 164 (60.3%) minor resections. There were 146 (44.9%) major resections and 179 (55.1%) minor resections in the CCLR group. There was no intraoperative death in either group. The average duration of the operation was significantly shorter in the RFLR group compared with the CCLR group (211 *vs*. 296 min, respectively, $p \leq 0.01$). Significantly reduced blood loss was observed in the RFLR group compared with the CCLR group (485.5 *vs*. 763.2 mL, respectively, $p = 0.003$). The RFLR group also required less operative blood transfusions than the CCLR group (128.8 *vs*. 312.1 mL, respectively, $p \leq 0.01$). The patient transfusion requirement was also significantly less in the RFLR group compared with the CCLR

group (52 *vs*. 103, 19.1% *vs*. 31.7%, respectively, $p \leq 0.01$). Additionally, the Pringle maneuver was less frequently required in the RFLR group compared with the CCLR group (25.7% *vs*. 33.8%, respectively, $p = 0.032$). However, there were no significant differences in occlusion times between the two groups (30.3 *vs*.33.3min, respectively, $p = 0.303$).

3.3. *Postoperative morbidity and mortality*

The mean hospital stay time after surgery was 16.4 days in the RFLR group and 16.3 days in the CCLR group ($p = 0.838$). The length of ICU stay did not differ between the patients who underwent RFLR and those who underwent CCLR (1.6 ± 1.4 *vs*. 1.6 ± 1.7 days, respectively, $p = 0.808$).

Postoperative complications are displayed in Table 3. The Clavien-Dindo Classification of Surgical Complications was used to evaluate the severity of postoperative complications (*11*). The mortality rate seemed to be higher in the RFLR group compared with the CCLR group (4.8% *vs*. 2.5%, respectively), however this difference was not significant ($p = 0.126$). The overall postoperative morbidity rate was 28.6% (171/597), and there was no significant difference between the two groups in postoperative morbidity (30.5% (83/272) in the RFLR group versus 27.1% (88/325) in the CCLR group ($p = 0.355$)). The most severe complications were liver failure, sepsis, MODS and portal vein thrombosis, which could result in death. A total of 16 (5.9%) patients who underwent RFLR and 8 (2.5%) patients who underwent CCLR suffered from liver failure; however, only 6 patients in the RFLR group and 4 patients in the CCLR group progressed to MODS and death and there was no significant difference ($p = 0.286$). The kinetics of postoperative ALT and TIBL in each group is shown in Figure 1. The ALT peak on postoperative day (POD) 1 in the RFLR group was not significantly different from that in the CCLR group (500.5 ± 429.7 *vs*. 490.3 ± 464.6 IU/L, $p = 0.317$) and had a tendency to return to normal values by POD 10. The TIBL was similar for both groups with an increase until POD 3 followed by a slow decrease to preoperative values on POD 7 ($p = 0.64$).

Table 2. Comparison of intraoperative data of patients in RFLR and CCLR groups

Items	RFLR	CCLR	*p value*
Type of resection (%)			0.199
Major	108 (39.7%)	146 (44.9%)	
Minor	164 (60.3%)	179 (55.1%)	
Duration of operation (min)			0.000
Mean (S.D.)	211.2 (63.2)	295.9 (107.3)	
Median (range)	203 (85-532)	285 (120-738)	
Blood Loss (mL)			0.003
Mean (S.D.)	485.54 (465.8)	763.2 (1154.8)	
Median (range)	350 (50-4500)	400 (50-12000)	
Transfusion (mL)			0.000
Mean (S.D.)	128.8 (308.8)	312.1 (745.0)	
Median (range)	600 (0-2280)	540 (0-7000)	
No. of transfused patients	52 (19.1%)	103 (31.7%)	0.000
Pringle maneuver (%)	70 (25.7%)	110 (33.8%)	0.032
Pringle time (min)			0.303
Mean (S.D.)	30.33 (13.33)	33.25 (21.18)	
Median (range)	30 (0-60)	30 (0-123)	

S.D.: standard deviation.

Table 3. Baseline demographic in HCC patients undergoing RFLR or CCLR

Items	RFLR ($n = 272$)	CCLR ($n = 325$)	*p value*
Hospital Stay (days)	16.4 ± 8.4	16.3 ± 7.3	0.838
ICU stay (days)	1.6 ± 1.4	1.6 ± 1.7	0.808
Dindo-Clavien morbidity	83 (30.5%)	88 (27.1%)	0.355
Classification			
I	20 (7.4%)	11 (3.4%)	
II	16 (5.9%)	20 (6.2%)	
III	28 (10.3%)	37 (11.4%)	
IV	6 (2.2%)	12 (3.7%)	
V	13 (4.8%)	8 (2.5%)	
Type of complication			
Bile leakage	22 (8.1%)	21 (6.5%)	0.444
Gastrointestinal bleeding	0	1 (0.3%)	1.000
Abdominal bleeding	2 (0.7%)	1 (0.3%)	0.877
Portal vein thrombosis	2 (0.7%)	1 (0.3%)	0.877
Wound infection	10 (3.7%)	7 (2.2%)	0.265
Abdominal infection	8 (2.9%)	11 (3.4%)	0.942
Lung infection	11 (4.0%)	27 (8.3%)	0.759
Ileus	1 (0.4%)	0	0.929
Pleural effusion	13 (4.8%)	27 (8.3%)	0.086
Intra-abdominal abscess need tapping	20 (7.4%)	16 (4.9%)	0.214
Sepsis	2 (0.7%)	4 (1.2%)	0.847
Respiratory distress	3 (1.1%)	11 (3.4%)	0.118
Renal failure	4 (1.5%)	5 (1.5%)	1.000
Liver failure	16 (5.9%)	12 (3.7%)	0.286
MODS	6 (2.2%)	8 (2.5%)	1.000
Death	13 (4.8%)	8 (2.5%)	0.126

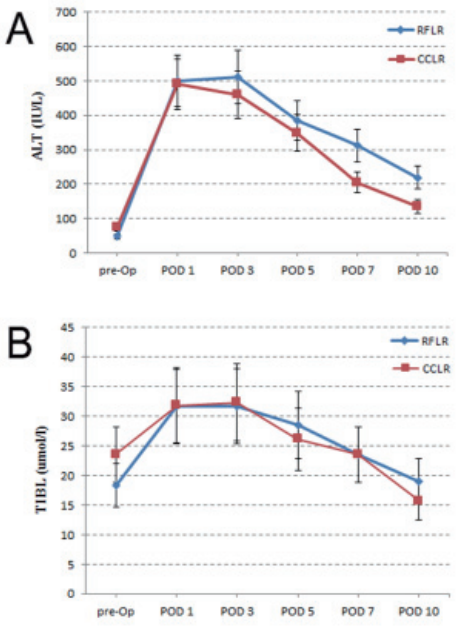

Figure 1. Postoperative liver injury and recovery assessed by serial measurement of (A) alanine aminotransferase (ALT) and (B) total bilirubin (TIBL) level in RFLR and CCLR groups.

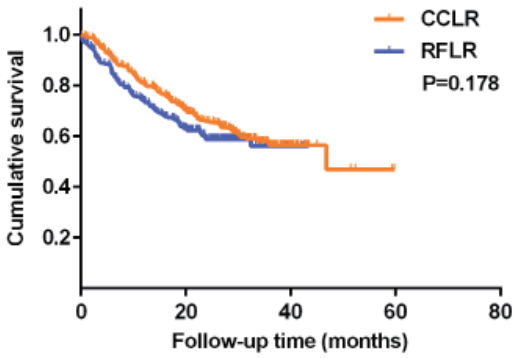

Figure 2. Overall survival for the patients in RFLR and CCLR groups.

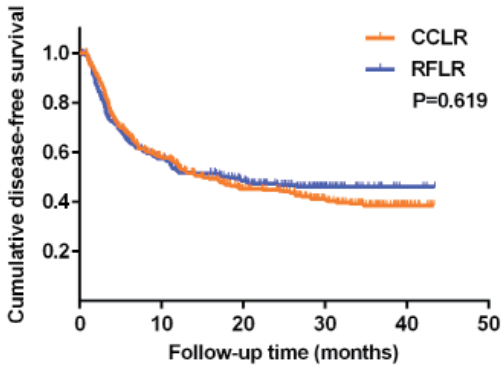

Figure 3. Recurrence-free survival for the patients in RFLR and CCLR groups.

3.4. *Disease-free survival and overall survival*

The median follow-up duration in the RFLR patients was 21.9 months (range 1-43 months) and 26.7 months (range 1-60 months) in the CCLR patients. The 1-, 2-, and 3-year overall survival rates were 73.8%, 58.5%, and 55.7% in the RFLR group and 80.8%, 65.8% and 56.2% in the CCLR group, respectively (Figure 2). There were no significant differences between the two groups (p = 0.178). The 1-, 2-, and 3-year disease-free survival rates were 51.9%, 47.2%, and 46.0% in the RFLR group, and 54.5%, 44.9% and 38.5% in the CCLR group, respectively (Figure 3). No significant differences between the two groups were observed (p = 0.619).

4. Discussion

The results of this retrospective study revealed that the efficacy of RFLR was superior to that of CCLR (without RF assistance) during operations for patients with HBV-related and cirrhotic HCC. The amount of blood loss, blood transfusion frequency, operation duration and vascular inflow occlusion were significantly reduced in the RFLR group compared with those in the CCLR group. Additionally, RFLR did not induce further damage or increase mortality/morbidity after hepatic resection compared with CCLR. The 1-, 2-, and 3-year overall survival and disease-free rates were similar between the two groups.

Controlling blood loss and minimizing bleeding during liver parenchyma transection are primary concerns of every hepatic surgeon (*16*). Previous studies have shown that increased intraoperative blood loss and increased blood transfusions are critical factors associated with perioperative morbidity, mortality and tumor recurrence for patients with HCC (*17-20*). Accordingly, efforts have been made to minimize intraoperative blood loss. A RF device for liver parenchyma transection has been recently introduced and has met with great interest due to its ability to minimize bleeding and reduce surgery duration (*6*). The central finding of this study included significantly reduced blood loss as well as a reduced need for blood transfusion in patients undergoing RFLR compared with those undergoing CCLR. These apparent benefits compensated for the RFA coagulate necrosis effect, which inhibited blood flow to small vessels and surrounding tissues during liver parenchyma transection. Our results were similar to those of Arita (*21*) and Li *et al*. (*22*) and showed RFLR achieved less blood loss. In this large-scale retrospective study, more than 90% of the patients had HBV-related cirrhosis concomitant with HCC. Liver tissue hardens and loses elasticity in these patients. This consequently adds difficulty in separating or dissecting the liver parenchyma and increases the risk of massive bleeding. Therefore, RFLR was an effective method to reduce blood loss and blood transfusion during liver parenchyma transection in HBV-related cirrhosis.

Another substantial advantage of the RFLR compared with CCLR is that RFLR reduced the frequency and duration of hepatic vascular inflow occlusion, which maximally protected hepatocellular function and reduced postoperative liver dysfunction or liver failure. The Pringle maneuver (PM) was the most commonly used and traditional hepatic vascular inflow occlusion technique to control massive bleeding in hepatic resection. However, recent studies have suggested that the Pringle maneuver may promote ischemia-reperfusion injury in the liver and induce postoperative liver dysfunction or liver failure (*23-25*). Our results demonstrated that the application of PM in RFLR patients was less than that in CCLR patients. We also observed a slightly higher postoperative ALT level, longer fall back in the RFLR group, and slightly higher postoperative liver failure in the RFLR group; however, these differences were not significant. This finding is important for HCC with cirrhosis because it is better to avoid unnecessary vascular inflow occlusion and maintain the vascular inflow stability of the liver during surgery. It has been known that RF coagulation induced necrosis of the tumor tissue and normal tissue of the remnant liver (*26*). Reducing the application of PM allows more remnant liver preservation and a lowered

incidence of postoperative liver failure.

We also observed that the postoperative 90-day overall morbidity rate of 28.6% was in line with previously reported rates of 16.7% to 54%, and the overall mortality rate (within 30 days after operation) of 3.5% was consistent with previously reported rates of 8.9% to 19.6% for liver resection (*27-29*). The overall morbidity and mortality rates did not differ between the patients who underwent RFLR and those who underwent CCLR in this study. Bile leakage has been traditionally regarded as a major complication after hepatectomy. The incidence of postoperative bile leakage after RFLR was 7.2% in this study, which is consistent with or even better than in most reported series ranging from 0% to 12.5% (*30-32*). In the present study, the postoperative bile leakage rate in the RFLR group was comparable to that in the CCLR group (8.1% *vs*. 6.5%, $p = 0.444$). Our results were better than those of Li (*22*) and Lupo *et al*. (*33*), showing that RFLR did not cause additional bile leakage. This could be explained by the following: *i*) We started the RF device at 60 W for 10-30 sec per each coagulation and adjusted the power based on the severity of liver cirrhosis; and *ii*) We rarely exceeded 80 W, whereas Lupo's (*33*) group used 100 W for 3-6 min per application. More conservative energy use decreased damage to remnant liver parenchymal function, avoided necrosis on the surgical surface and decreased biliary damage (*26*). We noticed a gradual lowering of the incidence of bile leakage in the RFLR group during the study period (over a period of years) due to a thorough understanding of the RF device and improved knowledge of intrahepatic anatomy.

The overall survival and disease-free survival rates were comparable and did not significantly differ between the two groups. A previous study reported overall 3-year survival rates ranging from 30% to 63% and a 3-year disease-free survival range of 24%-54% (*29,34,35*). Our results are in line with their data. Prassas *et al*. (*36*) reported longer overall survival and longer disease-free survival rates for RFLR. They hypothesized that tissue ablation generated by the RF device induced tumor cell death beyond the histological margin and enabled a more complete R0 resection (*37*). However, our study did not provided robust evidence to support this. This could be because tumor prognosis is dependent on numerous factors including preoperative tumor stage and grade, intraoperative bleeding, postoperative complications, sample size and follow-up duration in selected patients (*38*). Thus, a longer follow-up is required to confirm the superiority of RFLR.

Although this report represents a large, single center comparative study to evaluate the effectiveness and safety of RFLR for the treatment of patients with HCC, it has some limitations. First, the retrospective, non-randomized, non-blinded nature of the study unavoidably induced bias in selecting patients to receive either RFLR or CCLR. However, the preoperative parameters or baselines of the two groups were comparable, the two groups of patients were well matched, and a large sample size was obtained. These may compensate for the aforementioned limitation. Second, different surgeons and surgical habits were possible confounders. However, the team consisted of four senior hepatobiliary surgeons who independently conducted standard anatomical hepatectomy in more than 100 patients in our unit performed the surgeries based on standard algorithms. Finally, fully understanding the long-term outcomes would require a longer follow-up. Our results could only report on the 1-, 2-, and 3-year overall survival and disease-free survival rates. The follow-up of some patients was limited and insufficient to estimate 5-year overall survival and disease-free survival. Therefore, a further randomized controlled trial comparing these two groups of patients is currently ongoing in our institute. This study has been registered on Clinicaltrials.gov (identifier: NCT 01992978), and we are recruiting patients. We look forward to the publication of this trial and others.

5. Conclusion

RFLR was shown to be a safe and effective method for selected patients undergoing liver resection. Compared with CCLR, RFLR is advantageous with reduced intraoperative bleeding, decreased operative duration, and resulted in fewer blood transfusions and less vascular inflow occlusion. RFLR did not either worsen liver function recovery or increase complications or mortality rates. However, RFLR should be performed by experienced hepatobiliary surgeons who have a thorough understanding of intrahepatic anatomy and are proficient with the RF device. Further large-sample, multicenter, randomized and controlled studies are necessary to assess the long-term effects of RFLR and determine the most suitable method for patients with cirrhosis and HCC.

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References

1. DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. Cancer treatment and survivorship statistics, 2014. CA Cancer J

Clin. 2014; 64:252-271.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011; 61:69-90.
3. Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. CA Cancer J Clin. 2012; 62:394-399.
4. Bruix J, Sherman M. Management of hepatocellular carcinoma. Hepatology. 2005; 42:1208-1236.
5. Huntington JT, Royall NA, Schmidt CR. Minimizing blood loss during hepatectomy: A literature review. J Surg Oncol. 2014; 109:81-88.
6. Weber JC, Navarra G, Jiao LR, Nicholls JP, Jensen SL, Habib NA. New technique for liver resection using heat coagulative necrosis. Ann Surg. 2002; 236:560-563.
7. Tepetes K. Risks of the radiofrequency-assisted liver resection. J Surg Oncol. 2008; 97:193; author reply 194-195.
8. Mitsuo M, Takahiro T, Yasuko T, Masayasu A, Katsuya O, Nozomi S, Yoshihide O, Isamu K. Radiofrequency (RF)-assisted hepatectomy may induce severe postoperative liver damage. World J Surg. 2007; 31:2208-2212; discussion 2213-2204.
9. Lin MT, Chang KC, Tseng PL, Yen YH, Wang CC, Tsai MC, Cheng YF, Eng HL, Wu CK, Hu TH. The validation of 2010 AASLD guideline for the diagnosis of hepatocellular carcinoma in an endemic area. J Gastroenterol Hepatol. 2014.
10. Couinaud C. Anatomic principles of left and right regulated hepatectomy: Technics. J Chir. 1954; 70:933-966.
11. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: A new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg. 2004; 240:205-213.
12. Balzan S, Belghiti J, Farges O, Ogata S, Sauvanet A, Delefosse D, Durand F. The "50-50 criteria" on postoperative day 5: An accurate predictor of liver failure and death after hepatectomy. Ann Surg. 2005; 242:824-828, discussion 828-829.
13. Capussotti L, Ferrero A, Vigano L, Sgotto E, Muratore A, Polastri R. Bile leakage and liver resection: Where is the risk? Arch Surg. 2006; 141:690-694; discussion 695.
14. Pai M, Jiao LR, Khorsandi S, Canelo R, Spalding DR, Habib NA. Liver resection with bipolar radiofrequency device: Habib 4X. HPB (Oxford). 2008; 10:256-260.
15. Pai M, Navarra G, Ayav A, Sommerville C, Khorsandi SK, Damrah O, Jiao LR, Habib NA. Laparoscopic Habib 4X: A bipolar radiofrequency device for bloodless laparoscopic liver resection. HPB (Oxford). 2008; 10:261-264.
16. Belghiti J, Hiramatsu K, Benoist S, Massault P, Sauvanet A, Farges O. Seven hundred forty-seven hepatectomies in the 1990s: An update to evaluate the actual risk of liver resection. J Am Coll Surg. 2000; 191:38-46.
17. Sasaki K, Matsuda M, Ohkura Y, Kawamura Y, Inoue M, Hashimoto M, Ikeda K, Kumada H, Watanabe G. Factors associated with early cancer-related death after curative hepatectomy for solitary small hepatocellular carcinoma without macroscopic vascular invasion. J Hepatobiliary Pancreat Sci. 2014; 21:142-147.
18. Kooby DA, Stockman J, Ben-Porat L, Gonen M, Jarnagin WR, Dematteo RP, Tuorto S, Wuest D, Blumgart LH, Fong Y. Influence of transfusions on perioperative and long-term outcome in patients following hepatic resection for colorectal metastases. Ann Surg. 2003; 237:860-869; discussion 869-870.
19. Pessaux P, van den Broek MA, Wu T, Olde Damink SW, Piardi T, Dejong CH, Ntourakis D, van Dam RM. Identification and validation of risk factors for postoperative infectious complications following hepatectomy. J Gastrointest Surg. 2013; 17:1907-1916.
20. Katz SC, Shia J, Liau KH, Gonen M, Ruo L, Jarnagin WR, Fong Y, D'Angelica MI, Blumgart LH, Dematteo RP. Operative blood loss independently predicts recurrence and survival after resection of hepatocellular carcinoma. Ann Surg. 2009; 249:617-623.
21. Arita J, Hasegawa K, Kokudo N, Sano K, Sugawara Y, Makuuchi M. Randomized clinical trial of the effect of a saline-linked radiofrequency coagulator on blood loss during hepatic resection. Br J Surg. 2005; 92:954-959.
22. Li M, Zhang W, Li Y, Li P, Li J, Gong J, Chen Y. Radiofrequency-assisted versus clamp-crushing parenchyma transection in cirrhotic patients with hepatocellular carcinoma: A randomized clinical trial. Dig Dis Sci. 2013; 58:835-840.
23. Ypsilantis P, Lambropoulou M, Anagnostopoulos C, Tsigalou C, Vasiliadis C, Kortsaris A, Papadopoulos N, Simopoulos C. Pringle maneuver exacerbates systemic inflammatory response and multiple-organ injury induced by extended liver radiofrequency ablation. Hum Exp Toxicol. 2011; 30:1855-1864.
24. Torzilli G, Procopio F, Donadon M, Del Fabbro D, Cimino M, Montorsi M. Safety of intermittent Pringle maneuver cumulative time exceeding 120 minutes in liver resection: A further step in favor of the "radical but conservative" policy. Ann Surg. 2012; 255:270-280.
25. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol. 2003; 284:G15-26.
26. Daylami R, Kargozaran H, Khatri VP. Liver resection using bipolar InLine multichannel radiofrequency device: Impact on intra- and peri-operative outcomes. Eur J Surg Oncol. 2012; 38:531-536.
27. Xiao WK, Chen D, Hu AB, Peng BG, Guo YZ, Fu SJ, Liang LJ, Li SQ. Radiofrequency-assisted versus clamp-crush liver resection: A systematic review and meta-analysis. J Surg Res. 2014; 187:471-483.
28. Peng ZW, Lin XJ, Zhang YJ, Liang HH, Guo RP, Shi M, Chen MS. Radiofrequency ablation versus hepatic resection for the treatment of hepatocellular carcinomas 2 cm or smaller: A retrospective comparative study. Radiology. 2012; 262:1022-1033.
29. Kamiyama T, Nakanishi K, Yokoo H, Kamachi H, Tahara M, Yamashita K, Taniguchi M, Shimamura T, Matsushita M, Todo S. Perioperative management of hepatic resection toward zero mortality and morbidity: Analysis of 793 consecutive cases in a single institution. J Am Coll Surg. 2010; 211:443-449.
30. Cescon M, Vetrone G, Grazi GL, Ramacciato G, Ercolani G, Ravaioli M, Del Gaudio M, Pinna AD. Trends in perioperative outcome after hepatic resection: Analysis of 1500 consecutive unselected cases over 20 years. Ann Surg. 2009; 249:995-1002.
31. Torzilli G, Donadon M, Montorsi M, Makuuchi M. Concerns about ultrasound-guided radiofrequency-assisted segmental liver resection. Ann Surg. 2010; 251:1191-1192; author reply 1192-1193.
32. Pai M, Frampton AE, Mikhail S, Resende V, Kornasiewicz O, Spalding DR, Jiao LR, Habib NA.

Radiofrequency assisted liver resection: Analysis of 604 consecutive cases. Eur J Surg Oncol.2012; 38:274-280.
33. Lupo L, Gallerani A, Panzera P, Tandoi F, Di Palma G, Memeo V. Randomized clinical trial of radiofrequency-assisted versus clamp-crushing liver resection. Br J Surg. 2007; 94:287-291.
34. Jaeck D, Bachellier P, Oussoultzoglou E, Weber JC, Wolf P. Surgical resection of hepatocellular carcinoma. Post-operative outcome and long-term results in Europe: An overview. Liver Transpl. 2004; 10:S58-63.
35. Allemann P, Demartines N, Bouzourene H, Tempia A, Halkic N. Long-term outcome after liver resection for hepatocellular carcinoma larger than 10 cm. World J Surg. 2013; 37:452-458.
36. Prassas E, Petrou A, Kontos M, Rizos D, Neofytou K, Pikoulis E, Diamantis T, Felekouras E. Radiofrequency ablation assisted resection for hepatocellular carcinoma: Morbidity, mortality and long term survival. J BUON. 2014; 19:256-262.
37. Agrawal S, Belghiti J. Oncologic resection for malignant tumors of the liver. Ann Surg. 2011; 253:656-665.
38. Eisele RM, Zhukowa J, Chopra S, Schmidt SC, Neumann U, Pratschke J, Schumacher G. Results of liver resection in combination with radiofrequency ablation for hepatic malignancies. Eur J Surg Oncol. 2010; 36:269-274.

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Original Article

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Fatal cases of human infection with avian influenza A (H7N9) virus in Shanghai, China in 2013

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Summary **We retrospectively reviewed the medical records of 17 fatal H7N9 cases in Shanghai in 2013, analyzed clinical variables and described their clinical and epidemiologic characteristics. The median age was 73 years, and 82.4% had underlying medical conditions. The most frequent symptoms were fever (100%), followed by productive cough (47.1%) and dry cough (35.5%). Thirteen (76.5%) patients had dyspnea or respiratory distress, five (29.4%) had shock, and four (23.5%) had acute kidney injury. Seventeen (100.0%) patients had lymphopenia. Involvement of both lungs was found by chest radiography in 14 (82.4%) patients at presentation. Fifteen (88.2%) patients were hospitalized. The median times from illness onset to hospitalization and to diagnosis confirmation were both six days. Eleven (64.7%) patients were admitted to the intensive care unit. Sixteen (94.1%) patients were treated with oseltamivir. The median time from illness onset to oseltamivir treatment was six days. Among six patients for whom the duration of viral shedding was available, the median duration of viral shedding after oseltamivir treatment was 17 days. The median time from illness onset to death was 11 days. Refractory hypoxemia accounted for most deaths. The clinical and epidemiologic characteristics in the Shanghai fatal series of patients do not differ from other reports of H7N9 patients in China. This investigation reflects a delay in the diagnosis and antiviral treatment of H7N9 patients in the early stage of the epidemic in Shanghai. Late antiviral treatment and a long duration of viral shedding may be associated with a fatal outcome in these patients.**

Keywords: Avian influenza A (H7N9) virus, death, diagnosis, antiviral treatment

1. Introduction

The subtype H7N9 avian influenza virus has not been known to infect humans until only recently. On March 31, 2013, China confirmed the first three human cases of novel avian influenza A (H7N9) virus infection in Shanghai and Anhui, two of them have died (*1,2*). Novel reassortant H7N9 viruses were associated with severe and fatal respiratory disease in humans, most persons with confirmed H7N9 virus infection were critically ill (*1,2*). This increasing number of new H7N9 cases and high mortality has caused global concern and worries of spread outside of China (*3*). Since Shanghai reported the first case of human infection with H7N9

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virus at the end of March 2013, a total of 33 laboratory-confirmed human cases have been reported in Shanghai in 2013; 18 patients (54.5%) died while 15 were discharged from the hospital with full recovery (*4*). The majority of the Shanghai cases occurred between February and early April 2013 (*4*). To better understand the characteristics of severe influenza caused by this virus, we describe here the epidemiologic and clinical features in the fatal cases of human infection with avian influenza A (H7N9) virus in Shanghai, China in 2013.

2. Methods

2.1. *Ethics statement*

The study protocol was approved by the Shanghai Public Health Clinical Center Ethics Committee (SPHCCEC). Informed consent was waived by the SPHCCEC.

2.2. *Patients*

In our study, we used the case definitions of confirmed human infection with the novel H7N9 virus, which have been described by Li *et al*. (*2*). Laboratory confirmation of the novel H7N9 virus was performed with the use of the same protocols published previously (*1,2,5*). Only patients with laboratory-confirmed infection were enrolled in this study. In 2013, 18 fatal cases of human infection with avian influenza A (H7N9) virus have been reported in Shanghai. Clinical data were available for 17 of the 18 patients. The study population included the 17 fatal cases of laboratory-confirmed influenza A (H7N9) infection diagnosed in 2013. The 17 patients received treatment in nine hospitals in Shanghai. Most patients were treated in local hospitals, but some were referred to the special hospital (Shanghai Public Health Clinical Center, Fudan University) by first medical cares after the diagnosis was confirmed.

2.3. *Data collection*

Data were collected through a review of medical records. Clinical data for confirmed cases were abstracted from original medical records with use of a data abstraction sheet. We collected information on demographic characteristics, underlying medical conditions, clinical presentation, laboratory findings, the date of illness onset, visits to clinical facilities, hospitalization, treatment and clinical outcomes.

2.4. *Laboratory evaluation*

In this study, acute kidney injury (AKI) is defined as any of the following: increase in serum creatinine (SCr) by ≥ 0.3 mg/dL (≥ 26.5 μmol/L) within 48 h; or increase in SCr to ≥ 1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or urine volume < 0.5 mL/kg/h for 6 h.

Blood cultures were obtained from all patients on admission to the hospital. Blood cultures were performed for patients presenting with chills and shivering. Sputum or endotracheal aspirates were sent for identification of possible causative bacteria or fungi.

2.5. *Statistical analysis*

SPSS software for Windows (Version 11.5; SPSS Inc., Chicago, IL) was used for statistical analysis. Continuous variables were computed with standard methods and are presented as mean and standard deviations (SD) or medians (interquartile range, IQR). For categorical variables, the percentages of patients in each category were calculated.

3. Results

3.1. *Epidemiologic characteristics*

The epidemiologic characteristics of the 17 patients at presentation are shown in Table 1. In Patients 3, 7, 11 and 12, the diagnosis was confirmed by means of virus isolation. All the other diagnoses were confirmed by means of nucleic acid detection. The median age of the patients was 73 years (range, 27 to 88). Most confirmed cases occurred in males (76.5%), and 76.5% of the case patients were retirees. A total of 82.4% of the patients had one or more underlying medical conditions. Hypertension (58.8%) and diabetes (35.3%) were the most common underlying medical conditions. Four (23.5%) patients reported a history of recent live poultry exposure. The time of illness onset in nine (52.9%) patients was on or before the day when the first human infections with the novel influenza A/H7N9 virus were reported in Eastern China.

3.2. *Clinical and other features*

The most commonly reported symptoms were fever or history of fever (100.0%), followed by productive cough (47.1%), dry cough (35.5%), fatigue (17.6%) and sore throat (11.8%) (Table 1). Muscle pain, hemoptysis, runny nose and altered consciousness were reported in one patient, respectively. No patient had diarrhea, conjunctivitis, or a rash. Physical examination revealed crackles in 13 (76.5%) patients and wheezing in two (11.8%) patients on chest examination. Thirteen (76.5%) patients had dyspnea or respiratory distress, five (29.4%) had shock, and four (23.5%) had AKI. In all patients, there were marked abnormalities on chest radiography; involvement of both lungs was found by chest radiography in 14 (82.4%) patients at presentation. Bilateral ground-glass opacities and consolidation were the most common radiologic findings.

Table 1. Epidemiologic and clinical characteristics of the patients at presentation

Patient	Age, y/sex	Occupation	Time of illness onset	Underlying conditions	Exposure to poultry	Symptoms	Crackles	Shock	AKI	Abnormalities On chest radiography	Cause of death
1	87/M	Retiree	April 2	Smoker	No	Fever, fatigue, dyspnea	Yes	No	No	Both lungs	Hypoxemia
2	73/M	Farmer	March 31	None	Yes	Fever, dry cough, dyspnea	Yes	Yes	Yes	Both lungs	Hypoxemia
3	27/M	Butcher	February 27	Hepatitis B	No	Fever, productive cough	Yes	No	No	Both lungs	Hypoxemia
4	74/M	Retiree	April 5	HT, diabetes	Yes	Fever, fatigue, dry cough, dyspnea, alteredconsciousness	Yes	No	Yes	Both lungs	Multi-organ failure
5	49/M	Poultry worker	March 29	None	Yes	Fever, dyspnea, productive cough	Yes	No	No	Both lungs	Hypoxemia
6	77/M	Retiree	April 3	HT, CHD	Yes	Fever	Yes	Yes	No	Left lung	Hypoxemia
7	87/M	Retiree	February 19	HT, COPD	No	Fever, dyspnea, productive cough	Yes	No	No	Both lungs	Hypoxemia
8	63/M	Retiree	April 1	HT, diabetes	No	Fever, dyspnea	No	No	No	Right lung	Hypoxemia
9	51/F	Retiree	March 27	Diabetes	No	Fever, muscle pain, dry cough, dyspnea	Yes	Yes	No	Both lungs	Hypoxemia
10	67/F	Retiree	March 22	HT, CHD	No	Fever, dyspnea, productive cough	No	Yes	Yes	Right lung	Hypoxemia
11	63/M	Retiree	March 4	HT, smoker	No	Fever, dry cough, sore throat	No	Yes	No	Both lungs	Hypoxemia, AHF
12	74/M	Retiree	February 20	HT,CHD,COPD, diabetes, smoker	No	Fever, productive cough, sore throat, dyspnea	Yes	No	No	Both lungs	Hypoxemia
13	88/M	Retiree	April 10	HT, CHD, COPD, diabetes	No	Fever, productive cough, hemoptysis, fatigue, dyspnea	Yes	No	No	Both lungs	Multi-organ failure
14	83/F	Retiree	April 2	HT, diabetes	No	Fever, dry cough, dyspnea	Yes	No	No	Both lungs	Multi-organ failure
15	58/M	Worker	March 17	COPD, smoker, HT, hepatitis B	No	Fever, dyspnea, productive cough	Yes	No	Yes	Both lungs	Multi-organ failure
16	56/M	Retiree	April 2	None	No	Fever, dry cough	No	No	No	Both lungs	DIC, Shock
17	79/F	Retiree	April 11	CHD,COPD	No	Fever, productive cough, dyspnea, runny nose	Yes	No	No	Both lungs	AHF

NOTE: HT, hypertension; COPD, chronic obstructive pulmonary disease; CHD, coronary heart diseases; AHF: acute heart failure; AKI: acute kidney injury; DIC, disseminated intravascular coagulation

3.3. *Laboratory and microbiologic assessment*

The laboratory values of the 17 patients at presentation are shown in Table 2. Leukopenia was found in five (29.4%) patients, 17 (100.0%) had lymphopenia, two (11.8%) had neutropenia, and eight (47.1%) patients had thrombocytopenia. The ratios of CD4-positive cells to CD8-positive cells in Patients 2, 11, 13, 15, 16 and 17 were 8.8, 0.75, 2.04, 2.29, 3.10 and 2.20, respectively.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine levels were elevated in seven (41.2%), 14 (82.4%) and five (29.4%) of the 17 patients, respectively. Measurements of creatine kinase (CK) and lactate dehydrogenase (LDH) levels at presentation were available in 16 patients. CK levels were elevated in 12 (75.0%) of the 16 patients. LDH levels were elevated in 16 (100%) of them. C-reactive protein (CRP) levels were available in 13 patients and were elevated in 12 (92.3%) of them.

Blood cultures in Patient 6 were positive for *Candida albicans*, all the other blood cultures were negative. Sputum cultures on admission were all negative.

3.4. *Diagnosis and treatment*

The diagnosis and treatment of the patients with avian influenza A (H7N9) are summarized in Table 3. The median time from onset of symptoms to first medical care was two days (range, 0 to 5). Fifteen (88.2%) patients were hospitalized. The median time from illness onset to hospitalization was six days (range, 2 to 19). Eleven (64.7%) patients were admitted to the ICU (intensive care unit). The median time from illness onset to ICU admission was six days (range, 5 to 21). Patients 3 and 7 were the first two cases confirmed on March 31, 2013. The time from illness onset to diagnosis confirmation was 31 days in Patient 3 and 39 days in Patient 7. Patients 11 and 12 were retrospectively confirmed cases. The time from illness onset to diagnosis confirmation was 40 days in Patient 11 and 52 days in Patient 12. Among the other 13 patients, the median time from illness onset to diagnosis confirmation was six days (range, 5 to 21). Among all 17 patients, the median time from illness onset to diagnosis confirmation was six days (range, 5 to 52).

Sixteen (94.1%) patients were treated with the neuraminidase (NA) inhibitor oseltamivir. The median time from illness onset to oseltamivir treatment was six days (range, 2 to 19). Only two (12.5%) patient received oseltamivir within 48 h after illness onset. The median duration of oseltamivir treatment was six days (range, 1 to 30). The median dosage of oseltamivir was 150 mg per day (range, 150 to 300).

The duration of viral shedding after oseltamivir treatment was 9 days, ≥ 6 days, ≥ 13 days, 29 days, 30

Table 2. Laboratory values at presentation

Variable	Patient																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Leukocyte (per mm^3)	5380	5410	2100	2700	2900	7100	4670	5620	3290	3680	6210	7110	7050	4500	5380	5480	6590
Lymphocyte (per mm^3)	410	360	230	410	700	380	530	1490	180	210	750	170	840	410	130	1140	260
Neutrophil (per mm^3)	4330	4890	1900	2620	2000	6560	4110	3430	3040	2140	5270	6810	5870	4030	5090	3730	6190
Platelet (10^3 per mm^3)	186	71	58	192	71	102	78	94	155	162	390	67	166	121	75	137	79
Hemoglobin (g/liter)	110	147	143	124	168	149	131	118	136	131	114	129	135	113	119	130	129
CK (U/liter)	1883	170	2932	3889	1600	681	501	537	351	54	187	811	391	NA	772	290	170
LDH (U/liter)	911	886	1683	NA	2150	640	480	433	525	444	620	651	505	1529	906	146	1218
Creatinine (μmol/liter)	81.6	159.6	53	195	116	81	73	102	33	611	51	102	106.2	55	176.4	88.7	150.8
ALT (U/liter)	27	20	71	103	76	132	31	54	80	33	34	33	41	54	35	25	37
AST (U/liter)	110	86	156	519	258	239	77	159	100	38	57	35	57	137	74	19	202
CRP (mg/liter)	194	47	32	160	NA	192	114	NA	NA	4	134	220	196	NA	80.5	115	114
CD4:CD8 ratio	NA	8.80	NA	NA	NA	NA	NA	NA	NA	NA	0.75	NA	2.04	NA	2.29	3.10	2.20
Myoglobulin (ng/ml)	270	NA	119	NA	442	391	NA	229.4	54	NA	NA	NA	NA	NA	232	313	231
BNP (pg/ml)	1524	NA	158	301	871	423	7480	NA	327	NA	3000	10300	NA	NA	1309	194	7286
Serum amylase(U/liter)	198	NA	NA	240	74	NA	NA	NA	NA	NA	224	NA	122	NA	204	NA	76
Blood culture	–	–	–	–	–	CA	–	–	–	–	–	–	–	–	–	–	–
Sputum culture	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

NOTE: ALT, alanine aminotransferase; LDH, serum lactate dehydrogenase; AST, aspartate aminotransferase; CK, serum creatine kinase; CRP, C-reactive protein; BNP, brain natriuretic peptide; CA: *Candida albicans*; NA, not available; A plus sign denotes positive, and a minus sign negative.

Table 3. Diagnosis and treatment of patients infected with avian influenza A (H7N9) virus

Variable	Patient																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Days from onset of symptoms to first medical care	4	0	5	0	5	2	4	2	1	2	3	5	0	0	3	0	4
Hospitalization	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Days from illness onset to hospitalization	8	6	5	5	N/A	4	7	N/A	6	9	5	7	2	5	19	2	6
ICU admission	Yes	Yes	Yes	Yes	No	No	Yes	No	No	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Days from illness onset to ICU admission	8	6	7	5	N/A	N/A	8	N/A	N/A	9	N/A	N/A	6	5	21	6	6
Days from illness onset to diagnosis confirmation	8	6	31#	5	5	5	39#	6	5	10	40*	52*	6	8	21	6	5
Oseltamivir treatment	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Days from illness onset to oseltamivir treatment	8	5	7	4	N/A	5	7	2	3	8	6	7	6	7	19	2	5
Duration of oseltamivir treatment	9	5	4	2	N/A	6	6	4	1	11	2	4	13	7	29	30	21
Dosage of oseltamivir treatment (mg/d)	150	150	150	300	N/A	300	150	150	150	300	150	300	300	150	150	150	150
Days of viral shedding after oseltamivir treatment	9	≥ 6	NA	NA	N/A	NA	NA	NA	NA	NA	NA	NA	≥ 13	NA	29	30	21
Oxygen therapy on admission	Yes	Yes	No	Yes	N/A	No	Yes	N/A	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Mechanical ventilation on admission	Yes	Yes	No	Yes	N/A	No	Yes	N/A	Yes	Yes	No	Yes	No	Yes	Yes	No	No
Non-invasive mechanical ventilation during follow-up	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Invasive mechanical ventilation during follow-up	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ECMO during follow-up	No	No	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	Yes	No
Renal failure any time during follow-up	No	Yes	No	Yes	No	No	No	No	No	Yes	No	No	Yes	Yes	Yes	No	No
Continuous renal replacement therapy	No	No	No	Yes	No	No	No	No	No	Yes	No	No	Yes	Yes	Yes	No	No
Use of antibiotics before admission	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Use of antibiotics during hospitalization	Yes	Yes	Yes	Yes	N/A	Yes	Yes	N/A	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Use of corticosteroids any time during follow-up	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Intravenous immunoglobulin any time during follow-up	Yes	Yes	Yes	Yes	No	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Vasopressors any time during follow-up	No	Yes	No	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes	No	No	No	No
Days from onset of symptoms to death	19	11	11	6	5	11	13	6	7	22	8	11	19	38	75	85	109

NOTE: NA, not available; N/A, not applicable; # the first two cases; *cases confirmed retrospective; ECMO: extracorporeal membrane oxygenation.

days and 21 days in Patients 1, 2, 13, 15, 16 and 17, respectively. Influenza A (H7N9) RNA was detected in the clinical specimens using real-time reverse transcription polymerase chain reaction (rRT-PCR) 6 days and 13 days after oseltamivir treatment was intiated in Patients 2 and 13, respectively. The patients' samples were still positive for influenza H7N9 virus when the two patients died. Among the six patients for whom the duration of viral shedding was available, the median duration of viral shedding after oseltamivir treatment was 17 days.

Among the 15 hospitalized patients, 12 (80.0%) required oxygen therapy, and nine (60.0%) required mechanical ventilation on admission.

Fifteen (88.2%) patients were treated empirically with broad-spectrum antibiotics both before admission and during hospitalization. Sixteen (94.1%) patients received methylprednisolone during follow-up. Thirteen

(76.5%) patients received treatment with intravenous immunoglobulin, and vasopressors were used in eight (47.1%) patients during follow-up. Patients 2, 4, 10, 13, 14 and 15 developed renal failure during follow-up, and Patients 4, 10, 13, 14 and 15 received continuous renal replacement therapy.

The median time from onset of symptoms to death was 11 days (range, 5 to 109). Refractory hypoxemia accounted for most deaths (Table 1). Neither during the period when these patients were hospitalized nor subsequently was any illness reported in a health care worker or laboratory staff member.

4. Discussion

This report describes 17 fatal cases of human infection with avian influenza A (H7N9) virus in Shanghai. The investigation shows that the clinical and epidemiologic characteristics in the Shanghai fatal series of patients do not differ from larger reports of H7N9 patients in China (*2,5*). The majority of the patients were older men with preexisting medical conditions. The prominent clinical features on admission were those of a severe influenza syndrome with fever, cough, fatigue, and shortness of breath. The most striking laboratory findings were marked lymphopenia and thrombocytopenia. These clinical presentations were similar to those in the 2004 outbreak of influenza A (H5N1) in Vietnam, although diarrhea was a more prominent feature in the H5N1 patients (*6*). Our study shows that avian influenza A (H7N9) virus infection, characterized by multiple organ dysfunction, carries a high risk of death. The findings in this series of patients further demonstrates that novel H7N9 virus can cause severe and fatal disease in humans.

This investigation reflects a delay in the diagnosis of H7N9 patients in Shanghai. The H7N9 subtype virus has not been known to infect humans until only recently. A delayed diagnosis may be due to a weak knowledge among medical staff and the general public towards H7N9 virus associated diseases. This delay may compromise early management of patients with avian influenza A (H7N9).

This investigation also reflects a significant delay in antiviral treatment of H7N9 patients. Our previous report found that the risk of death was increased among H7N9 patients in whom antiviral therapy was initiated more than five days after illness onset (*5*). Our previous study also showed that the median time from the initiation of antiviral therapy to a negative test result on daily real-time RT-PCR assay was six days (*5*). In the current study, the median duration of viral shedding after oseltamivir treatment was 17 days. A study showed that reduction of viral load following antiviral treatment correlated with improved outcome of H7N9 patients (*7*). A study on timing of oseltamivir administration and outcomes in hospitalized adults with pandemic 2009 influenza A (H1N1) virus infection showed that time from onset of symptoms to oseltamivir administration was associated with a prolonged duration of fever, prolonged hospital length of stay, and higher mortality (*8*). A similar study showed that early oseltamivir treatment as an independent variable associated with reduced ICU mortality in critically ill patients with 2009 pandemic influenza A (*9*). Together with our study, these findings suggest that late antiviral treatment and a long duration of viral shedding may be associated with a fatal outcome in our case series. Therefore, early treatment of suspected or confirmed cases of avian influenza A (H7N9) is strongly recommended.

Most patients received methylprednisolone during follow-up in the current study. A study showed that emergence of NA Arg292Lys mutation in two avian influenza A (H7N9) patients who also received corticosteroid treatment led to treatment failure and a poor clinical outcome (*7*). The emergence of antiviral resistance in H7N9 viruses, especially in patients receiving corticosteroid therapy, is a concern. Controlled clinical studies are needed to assess the role of corticosteroids in the treatment of influenza A (H7N9) virus infections.

More aggressive treatments including oxygen therapy, mechanical ventilation, intravenous immunoglobulin, vasopressors, and continuous renal replacement therapy, have been used in patients with illness of greater severity. Most patients were treated empirically with broad-spectrum antibiotics. Antibiotic chemoprophylaxis should not be used where bacterial infection is not suspected. However, when pneumonia is present, antibiotic treatment is appropriate initially for community-acquired pneumonia.

There are some limitations to this study. First, the small case number prevents us from drawing any more conclusions. The study population is not representative of the entire H7N9 population and the results may not be generalizable. Second, the design of the study was observational, we were able to examine potential associations but were unable to assess causation. Further prospective clinical studies are needed for a better understanding of avian influenza A (H7N9) deaths.

In conclusion, the investigation shows that the clinical and epidemiologic characteristics in a Shanghai fatal series of patients do not differ from other reports of H7N9 patients in China. This investigation reflects a delay in the diagnosis and antiviral treatment of H7N9 patients in the early stage of the epidemic in Shanghai. Late antiviral treatment and a long duration of viral shedding may be associated with a fatal outcome in these patients. Strategies to facilitate rapid identification of cases and early antiviral treatment are urgently required.

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References

1. Gao R, Cao B, Hu Y, *et al*. Human Infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013; 368: 1888-1897.
2. Li Q, Zhou L, Zhou M, *et al*. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med. 2014, 370: 520-532.
3. Uyeki TM, Cox NJ. Global concerns regarding novel influenza A (H7N9) virus infections. New Engl J Med. 2013; 368: 1862-1864.
4. Shen Y, Lu H. Human infection with avian influenza A(H7N9) virus in Shanghai: current status and future trends. Chin Med J (Engl). 2014; 127: 1973-1976.
5. Gao HN, Lu HZ, Cao B, *et al*. Clinical findings in 111 cases of influenza A (H7N9) virus infection. N Engl J Med. 2013; 368: 2277-2285.
6. Tran TH, Nguyen TL, Nguyen TD, *et al*. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004; 350: 1179-1188.
7. Hu Y, Lu S, Song Z, *et al*. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. Lancet. 2013; 381: 2273-2279.
8. Viasus D, Paño-Pardo JR, Pachón J, Riera M, López-Medrano F, Payeras A, Fariñas MC, Moreno A, Rodríguez-Baño J, Oteo JA, Ortega L, Torre-Cisneros J, Segura F, Carratalà J; Novel Influenza A(H1N1) Study Group of the Spanish Network for Research in Infectious Diseases (REIPI). Timing of oseltamivir administration and outcomes in hospitalized adults with pandemic 2009 influenza A(H1N1) virus infection. Chest. 2011; 140: 1025-1032.
9. Rodríguez A, Díaz E, Martín-Loeches I, *et al*. Impact of early oseltamivir treatment on outcome in critically ill patients with 2009 pandemic influenza A. J Antimicrob Chemother. 2011; 66: 1140-1149.

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Brief Report

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Possible relationship between the heart rates and serum amyloid A in a hyperglycemic population

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Summary **Hyperglycemia predicts cardiovascular disease (CVD)-related outcomes. The resting heart rates (HRs) and serum amyloid A (SAA), an inflammatory marker, are respectively factors associated with CVD-related outcomes; however, little is known regarding the associations between these two factors. This study aimed to investigate the correlation between the HRs and SAA levels under hyperglycemic conditions. This study included 298 subjects (males, 44%; mean age, 61.1 years) without a history of CVD and/or hypertensive levels. Clinical data, including general laboratory measurements, HRs and SAA, were measured. The analyses were performed after dividing all of the subjects into two groups based on the blood glucose level (< or ≥ 6.1 mmol/L). There was a higher SAA level in the hyperglycemic group (n = 143; median [interquartile range] 6.1 [4.1-10.6] μg/mL) than in the counterpart group (n = 155; 6.0 [3.5-8.5] μg/mL; $p < 0.01$). There was a trend toward increased HRs in the hyperglycemic group (mean [standard deviation] 65.3 [11.2] bpm) compared to the counterpart group (63.2 [9.4] bpm; $p = 0.08$). In the hyperglycemic group, there was a significant positive correlation between the HRs and SAA levels (multiple variables-adjusted analysis: β = 0.21, $p = 0.02$), while no correlation was found in the counterpart group (β = 0.06, $p = 0.50$). In summary, a positive correlation between the HRs and SAA levels can present under hyperglycemic conditions. These findings may provide relevant insights into the CVD-related pathologies associated with hyperglycemia. Further studies are warranted.**

Keywords: Amyloid A protein, diabetes mellitus, glucose intolerance, heart rhythm, inflammation.

1. Introduction

Hyperglycemia (even when the plasma glucose concentration is at a prediabetic level) predicts cardiovascular disease (CVD)-related outcomes (*1,2*). An increase in the resting heart rates (HRs) also predicts CVD-related outcomes (*3*), while abnormal HRs are often seen in hyperglycemic subjects (*4-6*). Recently, low-grade chronic inflammation has been reported to be a crucial player under hyperglycemic conditions (*7-9*). Therefore, knowledge about the association between the heart rhythmicity and chronic inflammation would be helpful for understanding and managing the pathologies associated with hyperglycemia.

Serum amyloid A (SAA) is induced by various proinflammatory cytokines (*e.g.*, interleukin 6) and is clinically used as an inflammatory marker of CVD (*10*). The SAA level is sometimes increased in hyperglycemic subjects (*11,12*). Several prior studies have indicated a positive correlation between the HRs and C-reactive protein (CRP), another inflammatory marker (*13,14*). However, these studies did not focus on hyperglycemia, and there has been an observation that SAA is closer to CVD-related pathologies than other markers, such as CRP (*15-17*). Therefore, this study aimed to investigate the association between the HRs and SAA levels under conditions of hyperglycemia.

2. Materials and Methods

A total of 298 subjects who visited our University Hospital were enrolled in this study. The subjects

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Table 1. The characteristics of the subjects according to the blood glucose levels (< *vs.* ≥ 6.1 mmol/L)

Items	All subjects (*n* = 298)	Subjects < 6.1 mmol/L (*n* = 155)	Subjects ≥ 6.1 mmol/L (*n* = 143)	*p*-value
Age, years old	61.1 ± 10.8	60.4 ± 9.8	62.0 ± 11.8	0.21
Males/females, *n*	131/167	55/100	76/67	< 0.01*
Current smokers, *n* (%)	42 (14.1%)	18 (11.6%)	24 (16.8%)	0.20
Body mass index, kg/m^2	24.0 ± 3.5	23.9 ± 3.2	24.1 ± 3.7	0.74
Heart rates, bpm	64.2 ± 10.4	63.2 ± 9.4	65.3 ± 11.2	0.08
Systolic BP, mmHg	122.5 ± 9.3	123.8 ± 8.9	121.1 ± 9.4	0.01*
Diastolic BP, mmHg	75.3 ± 7.8	76.3 ± 8.0	74.3 ± 7.4	0.03*
Total cholesterol, mmol/L	5.3 ± 0.9	5.3 ± 0.9	5.1 ± 0.9	0.06
Triglyceride, mmol/L	1.2 (0.9-1.7)	1.2 (0.9-1.7)	1.2 (0.9-1.6)	0.88
HDL-cholesterol, mmol/L	1.5 ± 0.5	1.5 ± 0.4	1.5 ± 0.5	0.54
Glucose, mmol/L	6.0 (5.3-7.7)	5.3 (4.9-5.7)	7.7 (6.7-9.6)	< 0.01*
Serum amyloid A, μg/mL	6.1 (3.8-9.3)	6.0 (3.5-8.5)	6.1 (4.1-10.6)	< 0.01*

BP: blood pressure, HDL: high-density lipoprotein. The data are shown as the means ± standard deviations, medians (interquartile ranges) or subject numbers (%). *p*-values: $p < 0.05$ was set as the significance level in the comparison between the subjects with glucose levels of < and ≥ 6.1 mmol/L (*t*-tests).

included those without acute infectious diseases and severe liver or kidney diseases (these are possible diseases that are associated with unstable glucose levels (*18*)). The excluded subjects were those with a history of CVD events and those who showed a hypertensive level of systolic/diastolic blood pressure (SBP/DBP) of ≥ 140/90 mmHg (*19*), because these subjects could receive drugs affecting their HRs. Current smokers were defined as those who reported a current smoking habit.

The institutional ethics committee approved this study, and informed consent was obtained from all subjects. The body mass index (BMI) was determined while the subjects were wearing light clothes without shoes. Physiological variables, such as the HRs and SBP/DBP in the subject's right arm, were measured after a five-minute rest at room temperature. After blood was sampled while fasting, the concentrations of serum lipids (total cholesterol [TC], triglyceride [TG], high-density lipoprotein cholesterol [HDL-C]) and plasma glucose were enzymatically measured. The SAA concentrations were measured by an ELISA format.

The differences between the groups were examined using t-tests and Chi-square tests. Correlations between the HRs and SAA levels were examined using Pearson's correlation tests and multiple regression analyses adjusted for confounding variables (we entered the SBP only into the adjusted models due to the collinearity of the SBP and DBP). Because of their skewed distribution, the TG, glucose and SAA levels were log-transformed in all analyses. A subgroup analysis was performed after dividing all of the subjects into two groups based on their blood glucose level (< and ≥ 6.1 mmol/L), because this glucose level reflects impaired glucose tolerance (*20,21*) A p-value of < 0.05 was considered to be statistically significant.

3. Results and Discussion

The clinical data of all subjects to be included in the study are shown in Table 1. The data regarding the age, current smoking status, BMI, HRs and lipid concentrations did not show any relative differences between the subject groups divided according to their glucose levels (Table 1). Compared with the subjects with a glucose level of < 6.1 mmol/L, those with a glucose level of ≥ 6.1 mmol/L included more males, and exhibited a lower SBP/DBP, as well as higher glucose and SAA levels.

In all of the subjects, the correlation between the HRs and SAA levels was significant but weak ($r = 0.14$, $p = 0.02$). When the data were adjusted for confounding factors (age, gender, smoking, BMI, SBP, lipids and glucose), the adjustment did not largely affect the correlation between the HRs and SAA levels ($\beta = 0.15$, $p = 0.02$). The subgroup analyses were performed using similar analyses, and found a significant and greater correlation between the HRs and SAA levels ($r = 0.18$, $p = 0.03$; $\beta = 0.21$, $p = 0.02$) in the subjects with the blood glucose level of ≥ 6.1 mmol/L, while there was a nonsignificant correlation between the HRs and SAA levels ($r = 0.04$, $p = 0.67$; $\beta = 0.06$, $p = 0.50$) in the subjects with the glucose level of < 6.1 mmol/L.

Thus, the present study noted a significantly positive correlation between the HR and SAA levels in the hyperglycemic population. There has been no study on the association between the HRs and SAA with special reference to hyperglycemia, although these factors are known to have value for predicting the outcomes related to CVD, such as ischemic heart disease and acute coronary syndrome (*1-3*,*15-17*). Therefore, the present study findings can offer relevant information for the CVD-related pathologies associated with hyperglycemia.

There are two possible explanations for the present study results. First, increased HRs are often seen in hyperglycemic subjects (this trend was also observed in the present study), who may have impaired autonomic nervous system function, such as activated sympathetic and decreased parasympathetic modulation of the heart (*4-6*). The sympathetic activation itself promotes the secretion of pro-inflammatory cytokines, leading to increased inflammation (*4,6*). Another idea can also be considered. Hyperglycemia is an inflammatory condition

(*7-9*), and in fact, the present study demonstrated a higher SAA level in the hyperglycemic population than in the counterpart population, in line with the prior studies (*11,12*). This inflammatory condition creates sympathetic activation, leading to increased HRs (*4,6*).

The present study is associated with some limitations. First, the study design was cross-sectional; accordingly, the causality of the results could not completely be determined. Second, subjects with obvious hypertension and/or CVD-related outcomes were not evaluated. The other relevant measurements, such as hemoglobin A1c, CRP and autonomic nervous system (as expressed as body temperature, thermal and vibratory perception), were also not evaluated. The present study should only be considered a preliminary study at this point. Prospective evaluations of various populations and other relevant measurements are required to further confirm our findings.

In summary, a positive correlation between the HRs and SAA levels can present under hyperglycemic conditions. The findings may be useful for understanding and managing the CVD-related pathologies associated with hyperglycemia. Further studies are warranted to further explore this topic.

References

1. Hopper I, Billah B, Skiba M, Krum H. Prevention of diabetes and reduction in major cardiovascular events in studies of subjects with prediabetes: Meta-analysis of randomised controlled clinical trials. Eur J Cardiovasc Prev Rehabil. 2011; 18:813-823.
2. Blendea MC, McFarlane SI, Isenovic ER, Gick G, Sowers JR. Heart disease in diabetic patients. Curr Diab Rep. 2003; 3:223-229.
3. Gus Q. Zhang, Weiguo Zhang. Heart rate, lifespan, and mortality. Ageing Res Rev. 2009; 8:52-60.
4. Vinik AI, Maser RE, Ziegler D. Autonomic imbalance: Prophet of doom or scope for hope? Diabet Med. 2011; 28:643-651.
5. Spallone V, Ziegler D, Freeman R, Bernardi L, Frontoni S, Pop-Busui R, Stevens M, Kempler P, Hilsted J, Tesfaye S, Low P, Valensi P; Toronto Consensus Panel on Diabetic Neuropathy. Cardiovascular autonomic neuropathy in diabetes: Clinical impact, assessment, diagnosis, and management. Diabetes Metab Res Rev. 2011; 27:639-653.
6. Vinik AI, Erbas T, Casellini CM. Diabetic cardiac autonomic neuropathy, inflammation and cardio vascular disease. J Diabetes Invest. 2013; 4:4-18.
7. Sjöholm A, Nyström T. Inflammation and the etiology of type 2 diabetes. Diabetes Metab Res Rev. 2006; 22:4-10.
8. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol. 2009; 5:219-226.
9. Kolb H, Mandrup-Poulsen T. The global diabetes epidemic as a consequence of lifestyle-induced low-grade inflammation. Diabetologia. 2010; 53:10-20.
10. Kotani K, Yamada T, Gugliucci A. Paired measurements of paraoxonase 1 and serum amyloid A as useful disease markers. Biomed Res Int. 2013; 2013:481437.
11. Kumon Y, Suehiro T, Itahara T, Ikeda Y, Hashimoto K. Serum amyloid A protein in patients with non-insulin-dependent diabetes mellitus. Clin Biochem. 1994; 27:469-473.
12. Hatanaka E, Monteagudo PT, Marrocos MS, Campa A. Interaction between serum amyloid A and leukocytes - a possible role in the progression of vascular complications in diabetes. Immunol Lett. 2007; 108:160-166.
13. Hamaad A, Sosin M, Blann AD, Patel J, Lip GY, MacFadyen RJ. Markers of inflammation in acute coronary syndromes: Association with increased heart rate and reductions in heart rate variability. Clin Cardiol. 2005; 28:570-576.
14. Haensel A, Mills PJ, Nelesen RA, Ziegler MG, Dimsdale JE. The relationship between heart rate variability and inflammatory markers in cardiovascular diseases. Psychoneuroendocrinology. 2008; 33:1305-1312.
15. Johnson BD, Kip KE, Marroquin OC, Ridker PM, Kelsey SF, Shaw LJ, Pepine CJ, Sharaf B, Bairey Merz CN, Sopko G, Olson MB, Reis SE; National Heart, Lung, and Blood Institute. Serum amyloid A as a predictor of coronary artery disease and cardiovascular outcome in women: The National Heart, Lung, and Blood Institute-Sponsored Women's Ischemia Syndrome Evaluation (WISE). Circulation. 2004; 109:726-732.
16. Kosuge M, Ebina T, Ishikawa T, Hibi K, Tsukahara K, Okuda J, Iwahashi N, Ozaki H, Yano H, Kusama I, Nakati T, Umemura S, Kimura K. Serum amyloid A is a better predictor of clinical outcomes than C-reactive protein in non-ST-segment elevation acute coronary syndromes. Circ J. 2007; 71:186-190.
17. Uurtuya S, Kotani K, Koibuchi H, Taniguchi N, Yamada T. Serum amyloid A protein and carotid intima-media thickness in healthy young subjects. J Atheroscler Thromb. 2009; 16:299-300.
18. Fischer KF, Lees JA, Newman JH. Hypoglycemia in hospitalized patients. Causes and outcomes. N Engl J Med. 1986;315:1245-1250.
19. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL Jr, Jones DW, Materson BJ, Oparil S, Wright JT Jr, Roccella EJ; Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. National Heart, Lung, and Blood Institute; National High Blood Pressure Education Program Coordinating Committee. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. Hypertension. 2003; 42:1206-1252.
20. Suzuki H, Fukushima M, Usami M, Ikeda M, Taniguchi A, Nakai Y, Matsuura T, Yasuda K, Hosokawa M, Seino Y, Yamada Y. IGT with fasting hyperglycemia is more strongly associated with microalbuminuria than IGT without fasting hyperglycemia. Diabetes Res Clin Pract. 2004; 64:213-219.
21. Enkhmaa B, Shiwaku K, Anuurad E, Nogi A, Kitajima K, Yamasaki M, Oyunsuren T, Yamane Y. Prevalence of the metabolic syndrome using the Third Report of the National Cholesterol Educational Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) and the modified ATP III definitions for Japanese and Mongolians. Clin Chim Acta. 2005; 352:105-113.

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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.

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