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Original Articles

Brief Reports

Policy Forum

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Developmental Origins of Health and Disease (DOHaD): Implications for health and nutritional issues among rural children in China

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Summary **In China, with fast economic growth, health and nutrition status among the rural population has shown significant improvement in the past decades. On the other hand, burden of non-communicable diseases and prevalence of related risk factors such as overweight and obesity has also increased. Among rural children, the double burden of malnutrition and emerging overweight and obesity has been neglected so far. According to the theory of Developmental Origin of Health and Diseases (DOHaD), malnutrition, including both undernutrition (stunting and wasting) and over-nutrition (overweight and obesity) during childhood is closely related to worsened health outcomes during adulthood. Such a neglected problem is attributable to a complicated synergy of social and environmental factors such as parental migration, financial situation of the household, child-rearing knowledge and practices of the primary caregivers, and has implications for public health. Based on literature review of lessons from the field, intervention to address malnutrition among rural children should be a comprehensive package, with consideration of their developmental environment and geographical and socioeconomic diversity. The scientific evidence on DOHaD indicates the probability and necessity of prevention of adult disease by promotion of maternal and child health and reducing malnutrition by provision of high-quality complementary foods, promotion of a well-balanced dietary pattern, and promotion of health literacy in the public would bring a potential benefit to reduce potential risk of diseases.**

Keywords: Malnutrition, developmental origins of health and disease (DOHaD), non-communicable diseases (NCDs), children, rural, China

1. Introduction

In general, patterns of disease burden and malnutrition parallels and changes with socioeconomic development. Due to the socioeconomic gap, a huge difference between urban and rural settings regarding children's health, nutrition and development has been widely observed in China. Traditionally, prevalence of overweight and obesity in the urban area is higher than that in the rural area; while nutrient deficiencies and wasting among rural children are much more common compared to their urban counterparts. In China, the geographical and socioeconomic situation diversifies the pattern of malnutrition: in some poor and remote rural areas of Midwestern provinces, the major nutritional problems among children tend to be nutrient deficiencies such as anemia and stunting (*1-3*), while overweight and obesity among rural children have also reported, especially

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in developed provinces (*4,5*). The double burden of malnutrition and emerging overweight and obesity among rural children has been neglected so far. Although compared to that in urban children, the prevalence of anemia and stunting in rural children is higher, overweight and its risk factors among rural children are emerging as well. In our interventional program, we also found that both wasting and overweight are relevantly prevalent among rural children in Shandong Province, a developed region in the country, and in particular those left behind by rural-to-urban migrant parents had a higher prevalence compared to their non-left-behind counterparts (Figure 1). Such a neglected problem is attributable to complicated social and environmental factors and has implications for public health.

Since the 1980s, when retrospective cohort studies implemented by David Barker and colleagues indicated that the incidence of certain adult diseases such as cardiovascular disease and type II diabetes may be linked to early-age development, the hypothesis "Developmental Origins of Health and Disease (DOHaD)" and its conceptual framework has been established (*6*). Based on the theory of DOHaD, the beginning stages of life including pregnancy, neonatal period and childhood provides an essential opportunity to mitigate the environmental insults that may increase an individual's sensitivity to, or risk of developing diseases later in life (*7*). In this brief review, we examine the current epidemiological characteristics of malnutrition among rural children, their developmental environment and possible interventions to address the double burden of malnutrition and potential disease burden of non-communicable diseases (NCDs) in view of the theory of DOHaD.

2. Interpretation of causes and prevention of NCDs based on the theory of DOHaD

Although it generated considerable skepticism and related mechanisms remain unclear, Barker's hypothesis has established an association between developmental origins and health at later life stages. Since then, more robust evidence from epidemiological work based on large cohorts of the population and laboratory studies to explore epigenetic mechanisms confirmed that nutritional and toxicant exposure in the early stages of life have a profound impact on the development of NCDs in adulthood and led to widespread acceptance of the association (*8,9*).

Recently, it has been widely recognized that NCDs are the long-term outcome of physiological adaptations to the environment, and the complicated process referred to as "programming" (*10*). The process of programming is a complex interaction between genetic make-up and environmental adaptation. During early stages of development, when cells are differentiating and tissues are developing, the organism responds to exposure in the environment such as nutrient and physiological factors, drugs and environmental chemicals, by molecular pathways including DNA methylation, histone covalent modification and non-coding RNA expression, and such epigenetic modification can be passed from one cell generation to the next (*11*). The mechanisms result in permanent changes in physiology and metabolism of the infant. The early stages of development, including intrauterine, neonatal and infant life and early childhood, is the time when epigenetic marks undergo critical modifications, and the epigenetic system gradually becomes less plastic and sensitive to environmental alterations as the process of development finishes (*12*). Epigenetic mechanisms allow the organism to adapt to changing environments in order to maintain or improve reproductive capability; on the other hand, developmentally plastic processes can also have adverse consequences on disease risks later in life (*13*). Animal models provided robust evidence on epigenetic mechanisms involved in the theory of DOHaD.

It has been proven that malnutrition, not only over-nutrition (overweight and obesity) but also undernutrition (stunting and wasting) during childhood are closely related to worsened health outcomes in adulthood (*14*). Maternal malnutrition, such as under-nutrition, obesity, excessive weight gain and gestational diabetes in pregnancy influence disease risks in the next generation (*13*). Besides antenatal issues, growth in childhood is another major influence on programming, as recent studies also suggested that epigenetic changes in metabolic pathways may not be limited to malnutrition in the prenatal stage, but also in the postnatal period. Lower birth weight coupled with a higher body-mass index in childhood, which is now common in developing countries undergoing the nutritional and epidemiologic transition to Western styles of diet, sedentary behavior, obesity, and chronic diseases, appears to be associated with the highest risks of obesity, insulin resistance, the metabolic syndrome, type II diabetes, and ischemic cardiovascular disease (*15*). Stunting and growth failure due to malnutrition, which remains at a high level in developing countries, is difficult to reverse after 36 months of age and rapid weight gain in later childhood increases the risk of metabolic symptoms (*14,16*). In developing countries such as Pakistan, it has been observed that poor early growth due to malnutrition and later obesity often co-exist (*17*), and looking back to the nutritional and growth status of childhood, those who developed NCDs in adulthood tended to have below-average height, weight and BMI in early life with rapid weight gain in later childhood (*14*). Based on findings in epidemiological studies so far, the relevantly high burden of malnutrition in early childhood would increase the risk of epidemic and a high burden of NCDs in their adulthood in the future.

3. The developmental environment for children's health and development in rural China

The rural area of China recently has experienced an epidemiological and nutritional transition. With the rapid economic growth, health and nutrition status among the rural population has shown significant improvement in the past decades. On the other hand, NCDs such as cardiovascular diseases (CVD), type II diabetes mellitus and hypertension, as well as a prevalence of related risk factors such as overweight and obesity in adults and children have also increased in rural China (*1,18,19*). The shift of the disease and nutrition pattern is attributable to life style, health-related behaviors and diet (*20*). Recently in the rural area, health-related behaviors including diet and physical activities of adults, particularly the primary caregivers of children, affects health and nutritional status of not only themselves, but also children. A matter of great concern requiring urgent action is to prevent and control NCDs by removing unhealthy various risk factors in daily life by both population-wide interventions and targeted measures for individuals at high risk (*21*).

Social and environmental factors contributing to the emerging nutritional problem of rural children are complicated, and the underlying common background is a profound social mobilization with rapid economic growth. During the past decades a relevant amount of the rural migrant population moved to the urban area and left their children behind to grandparents. It is estimated that the amount of rural-to-urban migrant population has increased from 70 million to 221 million during the period from 1990 to 2010, accounting for 16.5% of the overall population in China, and continues to increase. Population mobilization has put huge pressure on public services in the urban area such as, education, medical insurance, employment, and social welfare. A relevantly large number of rural-to-urban migrant populations tend to not be covered by those public services and left their children at the villages. Consequently, the population of rural left-behind children increased rapidly as well, accounting approximately for 30% of rural children and 22% of the total number of children in the country. Having separated from their parents, more than half of those left-behind children are raised by their elderly grandparents.

A child's quality of life and developmental outcomes are affected by the environment where he or she grows up (*22*). Parental migration tends to have a profound impact on both positive and negative aspects of a child's growth. Positively, parental migration from rural areas to urban areas often leads to a higher income and enhanced socioeconomic status (*23*). On the other hand, under a special developmental environment of lack of parents' involvement, such a large population suffers from various problems in their physical and psychological health, nutritional status and development (*24-26*). A further concern is that ‘left-behind' children will also be at risk of inappropriate food intake and malnutrition because of lack of nutritional knowledge among elderly caregivers (*27*), rather than lack of access to foods. In the rural area, when the financial situation of the household is improved, children can easily access high-fat or non-nutrition-based foods including snacks and sugar-sweetened beverages, which significantly affect overall energy intake and nutrient balance (*28*). In our surveyed rural children, inappropriate consumption of snacks and sugar-sweetened beverages, picky eating and unbalanced nutrient intake in meals is prevalent, particularly among those left-behind children raised by their elderly grandparents. Such a dietary pattern exposes children to the double burden of malnutrition. For most rural households, recently when food availability is no longer a problem with improved income, how to eat healthily tends to be a new issue.

Previous studies suggested that the impact of parenting style on a child's health and development often depends on the characteristics of the primary caregiver, such as education, income, child-rearing beliefs, and behavior, rather than status of separation from parents or not (*29*). Most primary caregivers of left-behind children are their grandparents, whose educational level tends to be low and knowledge related to children's health, nutrition and development are poor, profoundly affecting child-rearing beliefs and practices. This kind of population often tends to be exposed to high risks for NCDs as well, as a national surveillance showed that those who are older, who live in eastern or central China or who are poorly educated tended to more likely be exposed to multiple risk factors for NCDs, such as insufficient intake of fruit and vegetables, overweight and obesity, raised blood pressure, physical inactivity, raised total serum cholesterol, and raised blood glucose (*30*). Therefore, in rural China, it should be highlighted that in such a population it is crucial to not only control NCDs at the community level, but also to address the double burden of malnutrition of rural children. Promotion of health-related behaviors including diet, physical activity and child-rearing beliefs and practices is expected to bring benefit to the population for different generations.

4. Effective solutions for double-burden of malnutrition among rural children in China

To tackle both nutrient deficiency and overweight among rural children, effective health and nutritional intervention for rural children in China is urgently needed. So far, previous studies implemented in developing countries suggested some effective interventions to solve specific problems of children's malnutrition and growth, such as multiple micronutrient supplementation (including iron, zinc, and vitamins), behavior change for complementary

feeding, and universal salt iodization (2031). Most of those interventions proven effective and considered cost-effective are for prevention of undernutrition, while the impact of those to prevent overweight and obesity and related NCDs remain uncertain (*32*). Moreover, what is already known is that comprehensive and multifaceted interventions rather than a single intervention would be more effective to tackle various nutritional problems among children living in developing countries. In a population with sufficient food, education about complementary feeding improves children's growth; while in a population with insufficient food provision of food supplements works. (2031). As young children's nutritional status results from a complex interaction of various factors such as food consumption, knowledge and practices of caregivers, and health system strengthening, an integrated strategy is necessary for their growth and development (*31,33*).

It should be highlighted that the malnutrition issue faced by children living in rural China is unique and within the country nutritional epidemiology is diversified by regions. Except those living in remote and poor western regions, food is now sufficient for a relevantly large amount of the population and even a rapid nutrition transition to a high-fat, high-energy-density and low-fiber diet is ongoing (*34,35*). Therefore, interventions targeting the undernutrition issue among vulnerable children in less developed countries are suggestive for those poor regions where underweight and stunting is still common, but may be not an appropriate solution for affluent regions. Both at the global and national level, strategies to prevent and control malnutrition have focused on nutritional deficiency and need to be re-examined and aligned with measures to fight against overweight and obesity (*26*).

To promote child-rearing practices of the primary caregivers, we implemented an interventional program during the past year targeting primary caregivers of 735 rural children aged 3 to 6 years old who have not yet been admitted to the preliminary school in two counties of Shandong Province, a developed region in China. The program aimed to improve rural children's health and growth status, especially that of left-behind children, involving primary caregivers (including children's parents and grandparents). Left-behind children's parents who were working out of the villages were invited to the intervention when they went back during a vacation as well. The intervention was conducted every two-months, consisting of a set of free-style educational and consulting programs on children's health and nutrition targeting primary caregivers, and an interaction of children, primary caregivers, kindergarten teachers and local community administrators. Such intervention was developed and provided by academic and clinical experts in various fields such as pediatrics, nutrition, and developmental psychology, with the main components focusing on children's growth and development, nutrition

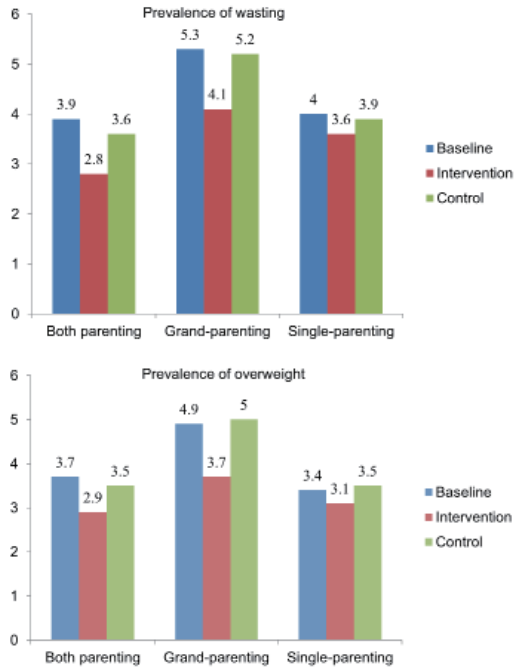

Figure 1. Prevalence of overweight and wasting among rural children, in the field of Shandong Province, China.

and feeding, prevention and treatment of common diseases, vaccination, and education and communication with children. In the results, when knowledge and feeding practices of the primary caregivers and the dietary pattern of children (assessed by 3-day food records and factor analysis) were improved after the intervention, the prevalence of both wasting and overweight was reduced significantly in the intervention group compared to that in the control group, and the program particularly showed a positive effect for the left-behind children (Figure 1). Improvement of health literacy and feeding practices of the primary caregivers contributed to alleviation of the double burden of malnutrition among rural children.

Our field attempt provided a preliminary solution to empower the primary caregivers and to improve the developmental environment of rural children. The approach is especially appropriate for affluent rural regions in China, where household income is able to ensure availability of food. For poor and under developed regions, provision of complementary food supplements combined with knowledge enhancing the primary caregivers still plays a major role for improving feeding practices. Taking these facts into consideration, interventions for Chinese rural children should be a comprehensive package, with consideration of their developmental environment and geographical and socioeconomic diversity.

Similar to other developed and developing countries of the world, emerging nutritional problems and non-communicable diseases occurred first in higher socioeconomic groups and later shifted to the less advantaged in China. The rural area is a high priority for prevention and control of NCDs in the country. Recently,

the incidence of NCDs and the disease burden in the rural area has been much higher than that in the urban area. Among rural residents, major risk factors such as overweight and obesity, inadequate physical exercise, and high-fat and energy-dense diet are also emerging and prevalent. So far, most community-based interventions such as promotion of diet and lifestyle have focused on adults and most investment has been injected into secondary prevention and treatment, whereas the major disease burden caused by non-communicable diseases such as cardiovascular disease and type II diabetes have their origins in transition from malnutrition in early childhood (*9*). The latest evidence shows overweight or obese children who were obese as adults had increased risk of type II diabetes, hypertension, dyslipidemia, and carotid-artery atherosclerosis and the risk is not changed whether they became obese or not by adulthood (*26*), suggesting early intervention during childhood could be more effective (*38*). The scientific evidence on DOHaD indicates the probability and necessity of prevention of adult disease by promotion of maternal and childhood health (*9*).

Without effective measures to tackle the double burden of malnutrition among rural children, it is not possible to attack the epidemic of NCDs in the near future. Although the long-term outcome of maternal and infant intervention to prevent related NCDs remain uncertain because of the absence of high-quality cohort data in a short time period, the current recommendations based on the theory of DOHaD presume that reducing malnutrition by provision of high-quality complementary foods, promotion of a well-balanced dietary pattern, and promotion of health literacy in the public would bring potential benefits to reduce the potential risk of diseases. In this respect, a cross-sectional endeavor to improve the developmental environment of rural children will have a vital role to play.

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References

1. Yang W, Li X, Li Y, Zhang S, Liu L, Wang X, Li W. Anemia, malnutrition and their correlations with socio-demographic characteristics and feeding practices among infants aged 0-18 months in rural areas of Shaanxi province in northwestern China: A cross-sectional study. BMC Public Health. 2012; 12:1127.
2. Chen C, He W, Wang Y, Deng L, Jia F. Nutritional status of children during and post-global economic crisis in China. Biomed Environ Sci. 2011; 24:321-328.
3. Wang YY, Chen CM, Wang FZ, Jia M, Wang KA. Effects of nutrient fortified complementary food supplements on anemia of infants and young children in poor rural of Gansu. Biomed Environ Sci. 2009; 22:194-200.
4. Lyu Y, Ouyang F, Ye XY, Zhang J, Lee SK, Li Z. Trends in overweight and obesity among rural preschool children in southeast China from 1998 to 2005. Public Health. 2013; 127:1082-1089.
5. Xie S, Wang J, Li N, Jiang W, Yang S, Li X, Ling Z, Zhang J. Survey on overweight and obesity of preschool children in rural areas from ten provinces of China. Zhonghua Liu Xing Bing Xue Za Zhi. 2014; 35:425-428.
6. Barker DJP. Mothers, babies and health in later life (2nd edition). Churchill Livingstone: Edinburgh, UK, 1998.
7. Macaulay EC, Donovan EL, Leask MP, Bloomfield FH, Vickers MH, Dearden PK, Baker PN; Gravida International College Summit Team. The importance of early life in childhood obesity and related diseases: A report from the 2014 Gravida Strategic Summit. J Dev Orig Health Dis. 2014; 5:398-407.
8. McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: Prediction, plasticity, and programming. Physiol Rev 2005; 85:571-633.
9. Gluckman P, Hanson M. Developmental origins of health and disease. Cambridge University Press, New York, USA, 2006.
10. Moore V, Davies M. Early life influences on later health: The role of nutrition. Asia Pacific J Clin Nutr. 2001; 10:113-117.
11. Skinner MK, Manikkam M, Guerrero-Bosagna C. Epigenetic transgenerational actions of endocrine disruptors. Reprod Toxicol. 2011; 31:337-343.
12. Grun F, Blumberg B: Endocrine disrupters as obesogens. Mol Cell Endocrinol 2009, 304:19-29.
13. Barouki R, Gluckman PD, Grandjean P, Hanson M, Heindel JJ. Developmental origins of non-communicable disease: Implications for research and public health. Environ Health. 2012; 11:42.
14. DeBoer MD, Lima AA, Oría RB, Scharf RJ, Moore SR, Luna MA, Guerrant RL. Early childhood growth failure and the developmental origins of adult disease: Do enteric infections and malnutrition increase risk for the metabolic syndrome? Nutr Rev. 2012; 70:642-653.
15. Gillman MW. Developmental origins of health and disease. N Engl J Med 2005; 353:1848-1850.
16. Victora CG, Adair L, Fall C, Hallal PC, Martorell R, Richter L, Sachdev HS; Maternal and Child Undernutrition Study Group. Maternal and child undernutrition: Consequences for adult health and human capital. Lancet. 2008; 371:340-357.
17. Jafar TH, Qadri Z, Islam M, Hatcher J, Bhutta ZA, Chaturvedi N. Rise in childhood obesity with persistently high rates of under nutrition among urban school-aged Indo-Asian children. Arch Dis Child. 2008; 93:373-378.
18. Yan S, Li J, Li S, Zhang B, Du S, Gordon-Larsen P, Adair L, Popkin B. The expanding burden of cardiometabolic risk in China: The China Health and Nutrition Survey. Obes Rev. 2012; 13:810-821.
19. Zhang X, Sun Z, Zhang X, Zheng L, Liu S, Xu C, Li J, Zhao F, Li J, Hu D, Sun Y. Prevalence and associated factors of overweight and obesity in a Chinese rural population. Obesity. 2008; 16:168-171.
20. Amuna P, Zotor FB. Epidemiological and nutrition transition in developing countries: Impact on human health and development. Proc Nutr Soc. 2008; 67:82-90.
21. Robinson HM, Hort K. Non-communicable diseases and health systems reform in low- and middle-income

countries. Pac Health Dialog. 2012; 18:179-190.
22. Bronfenbrenner U. The ecology of human development. Harvard University Press: Cambridge, MA, USA, 1979.
23. Toyota, M., Yeoh, B. S. A., & Nguyen, L. Editorial introduction: Bringing the "left-behind" back into view in Asia: A framework for understanding the "migration-left behind nexus." Population, Space and Place. 2007; 13:157-161.
24. Ma S. China's "left behind" children often suffer health consequences. CMAJ. 2010; 182:E731-732.
25. Wen M, Lin D. Child development in rural China: Children left behind by their migrant parents and children of nonmigrant families. Child Dev. 2012; 83:120-136.
26. Tao XW, Guan HY, Zhao YR, Fan ZY. Mental health among left-behind preschool-aged children: Preliminary survey of its status and associated risk factors in rural China. J Int Med Res. 2014; 42:120-129.
27. Ding G, Bao Y. Editorial Perspective: Assessing developmental risk in cultural context: The case of 'left behind' children in rural China. J Child Psychol Psychiatry. 2014; 55:411-412.
28. Rodríguez-Artalejo F, García EL, Gorgojo L, Garcés C, Royo MA, Martín Moreno JM, Benavente M, Macías A, De Oya M; Investigators of the Four Provinces Study. Consumption of bakery products, sweetened soft dringks and yogurt among children aged 6-7 years: Association with nutrient intake and overall diet quality. Br J Nutr. 2003; 89:419-429.
29. Clarke-Stewart KA, Vandell DL, McCartney K, Owen MT, Booth C. Effects of parental separation and divorce on very young children. J Fam Psychol. 2000; 14:304.
30. Li Y, Wang L, Jiang Y, Zhang M, Wang L. Risk factors for non-communicable chronic diseases in women in China: Surveillance efforts. Bull World Health Organ. 2013; 19:650-660.
31. Bhutta ZA, Ahmed T, Black RE, Cousens S, Dewey K, Giugliani E, Haider BA, Kirkwood B, Morris SS, Sachdev HP, Shekar M; Maternal and Child Undernutrition Study Group. What works? Interventions for maternal and child undernutrition and survival. Lancet. 2008; 371:417-440.
32. Garmendia ML, Corvalan C, Uauy R. Assessing the public health impact of developmental origins of health and disease (DOHaD) nutrition interventions. Ann Nutr Metab. 2014; 64:226-230.
33. Bhutta ZA, Das JK, Rizvi A, Gaffey MF, Walker N, Horton S, Webb P, Lartey A, Black RE; Lancet Nutrition Interventions Review Group; Maternal and Child Nutrition Study Group. Evidence-based interventions for improvement 5maternal of maternal and child nutrition: What can be done and at what cost? Lancet 2013; 382:452-477.
34. Du S, Lu B, Zhai F, Popkin BM. A new stage of the nutrition transition in China. Public Health Nutr. 2002; 5:169-174.
35. Cui Z, Dibley MJ. Trends in dietary energy, fat carbohydrate and protein intake in Chinese children and adolescents from 1991 to 2009. Br J Nutr 2012; 108:1292-1299.
36. Zong XN, Li H. Physical growth of children and adolescents in China over the past 35 years. Bull World Health Organ. 2014; 92:555-564.
37. Juonala M, Magnussen CG, Berenson GS, Venn A, Burns TL, Sabin MA, Srinivasan SR, Daniels SR, Davis PH, Chen W, Sun C, Cheung M, Viikari JS, Dwyer T, Raitakari OT. Childhood adiposity, adult adiposity, and cardiovascular risk factors. N Engl J Med. 2011, 365:1876-1885.
38. Hanson MA, Gluckman PD, Ma RCW, Matzen P, Biesma RG. Early life opportunities for prevention of diabetes in low and middle income countries. BMC Public Health. 2012; 12:1025.

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Policy Forum

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Towards government-funded special biomedical research programs to combat rare diseases in China

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Summary **Rare diseases are rarely conditions that are often debilitating and even life-threatening, which was identified by the World Health Organization (WHO) with a prevalence of 0.65-1‰. 5,000-7,000 rare diseases are thought to exist, which account for around 10% of diseases for individuals worldwide. It is estimated that over 10 million people were patients with rare disease in China. During the past years, public awareness of rare diseases has in fact heightened with the launching of campaigns by patients' organizations and spontaneous efforts by members of the public, not only in developed countries and regions including United States of America (USA), the European Union (EU), and in Japan, but also in China. However, the features of missed or delayed diagnosis, shortage of effective drugs, and the high cost of currently available drugs for rare diseases make it an important public health issue and a challenge to medical care worldwide. To combat rare disease, the government should assume the responsibility of taking on the important task of promoting the sustained development of a system of medical care for and research into rare diseases. Government-funded special biomedical research programs in the USA, EU, and Japan may serve as a reference for China coping with rare diseases. The government-funded special biomedical research programs consisting of leading clinicians and researchers to enhance basic and applied research on rare diseases were expected to be launched in China.**

Keywords: Rare diseases, orphan drugs, public health, medical care

1. Introduction

February 28, 2015 marks the eighth international "Rare Disease Day" coordinated by EURORDIS. On and around this day, over 650 awareness campaigns have been held by patients' organizations in more than 80 countries and regions worldwide in line with this year's theme, "Day-by-day, hand-in-hand" (*1*), with the purpose of launching a blast of upsurge for the attention on patients with rare diseases.

Public awareness of rare diseases has in fact heightened over the past ten years with the launching of campaigns by patients' organizations and spontaneous efforts by members of the public. One example is a charity effort known as the "Ice Bucket Challenge" that went "viral" via social media in the United States of America (USA) and then spread to the rest of the world in the summer of 2014 (*2*). This effort firmly established awareness of a rare disease known as "amyotrophic lateral sclerosis (ALS)" in the public consciousness. Even though "Rare Disease Day" campaigns are held every February since 2008 and events like the "Ice Bucket Challenge" occasionally surface like a flash in the pan, the serious challenge begins when the fever of spontaneous non-governmental efforts eventually dies down, who will fill the "vacancy" and maintain attention on and provide support to patients with ALS or other rare diseases?

2. Rare disease as an important medical and social issue

Rare diseases are rarely conditions that are often debilitating and even life-threatening, which was

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identified by the World Health Organization (WHO) with a prevalence of 0.65-1‰. It is estimated that the combined number of patients suffering from rare diseases in USA and the European Union (EU) exceed 55 million, and 5,000-7,000 rare diseases are thought to exist, which account for around 10% of diseases for individuals worldwide (*3,4*). It is well-known that rare diseases are an important medical and social issue (*5*). With the features of missed or delayed diagnosis, shortage of effective drugs, and the high cost of currently available drugs (*6,7*), the non-governmental spontaneous activities are clearly not enough to improve the plight for patients with rare diseases. Given this reality, the government should assume the responsibility of taking on the important task of promoting the sustained development of a system of medical care for and research into rare diseases.

In addition to efforts to specifically define and classify rare diseases, specific legislation has been drafted to encourage discovery and development of orphan drugs, and the health insurance system for rare diseases has been improved in many countries and regions, such as the USA, EU, Australia, Japan, and South Korea (*8*). Government-funded special biomedical research programs to enhance basic and applied research on rare diseases should also receive full attention. Evidence has shown that biomedical research on rare diseases could provide insight into the pathologies of these diseases and revealed their underlying mechanisms (*9-11*). Such work may ultimately reveal avenues to potential therapeutics. Moreover, once biomedical research identifies suitable drug candidates and becomes more translational, it will garner industry attention and potentially lead to safe and effective orphan drugs.

3. Government-funded special biomedical research programs in USA, EU, and Japan

In Western countries, many research centers or projects have been established to support special biomedical research programs on rare diseases and development of orphan drugs, such as the Office of Rare Diseases Research (ORDR) established in the USA in 1993 within the National Institutes of Health (NIH) and the Rare Disease Task Force (RDTF) established in the EU in 2004 within the European Commission Public Health Directorate (*8*). In Asian countries, biomedical research on rare diseases has made great advances in Japan due to the system known as the Specified Disease Treatment Research Program established in 1972 with the support of the Ministry of Health, Labor, and Welfare (MHLW) (*12*).

Over the past 5 year, new efforts have been instituted to combat rare diseases through government-support biomedical research in Japan. A project entitled the "Early Exploratory Clinical Trial Bases for Specific Research Areas" was launched in 2011 in order to promote innovative drugs and medical devices from Japan for treatment of rare diseases. Pursuant to this project, 5 academic institutions were selected as national early exploratory clinical trial bases for specific research areas including cancer, cerebral and cardiovascular diseases, neuropsychiatric disorders, and immunological rare diseases. Two of the bases - the School of Medicine in Keio University and the University of Tokyo Hospital - have formulated specific plans to develop new drugs to treat immunological and neuropsychiatric rare diseases, promoting the translation of orphan drugs from basic studies to clinical use. For each base, the Japanese government invests up to 500 million yen for infrastructure construction and 150 million yen for clinical trial research led by physicians (*13*). Furthermore, the "Revision of Measures to Combat Intractable Diseases" was approved by the MHLW on January 25, 2013. This ordinance highlights "government-funded special biomedical research programs to enhance basic and applied research on rare diseases" as one of three pillars with which to combat rare diseases in Japan. One hundred and thirty diseases have been targeted by special research programs and research grants from the government, and allocated funds increased to 11.3 billion yen in 2013 (*14*).

4. Rare diseases research in China: current status and future perspectives

In China, although rare diseases have yet to be officially defined due to a delay in legislation, over 10 million patients have rare diseases based on to the WHO definition of rare diseases and the incidence of rare diseases in other countries (*15*). During the past ten years, with the efforts from patients with rare diseases and their families, patients' advocacy organizations, health-care professionals, lawyers, and representatives of the People's Congress, the public awareness of rare diseases has indeed increased in China, however, the official definition of rare diseases, the specific legislation to encourage research of rare diseases and development of orphan drugs have not been established until now.

In China, support for special biomedical programs on rare disease research comes mainly from the National Natural Science Foundation of China (NSFC). Data show that 366 projects (involving 32 rare diseases) were funded by the NSFC from 1999 to 2007 with total funding of 89.358 million RMB and annual funding of about 10 million RMB; this amount represents just 1/10th of similar funding in the USA (*16*), which lead to the lag in benefiting Chinese patients with rare diseases through better diagnosis and more treatment choices.

To combat rare diseases in China, the measures of legislation to confirm the definition and classification of rare diseases, assembling accurate epidemiological data

on rare diseases, incentives to encourage manufacturers to develop orphan drugs are urgently needed. The government-funded special biomedical research programs performed in USA, EU, and Japan have showed that it could benefit to prompt research on the prevalence, diagnosis, treatment, and management of rare diseases, then to improve the quality of life for patients with rare diseases. The government-funded special biomedical research programs consisting of leading clinicians and researchers to enhance basic and applied research on rare diseases were expected to be launched in China.

5. Conclusion

The features of rare diseases make these conditions an important public health issue and a challenge to medical systems worldwide. To combat rare diseases, the government should assume the responsibility of taking on the important task of promoting the sustained development of a system of medical care for and research into rare diseases. Government-funded special biomedical research programs in the USA, EU, and Japan may serve as a reference for China coping with rare diseases. The government-funded special biomedical research programs consisting of leading clinicians and researchers to enhance basic and applied research on rare diseases were expected to be launched in China.

References

1. Rare Diseases Europe. Rare Disease Day 2015. *http://www.rarediseaseday.org* (accessed March 20, 2015).
2. The Lancet Neurology. The bucket list for amyotrophic lateral sclerosis. Lancet Neurol. 2014; 13:1061.
3. Luzzatto L, Hollak CE, Cox TM, Schieppati A, Licht C, Kääriäinen H, Merlini G, Schaefer F, Simoens S, Pani L, Garattini S, Remuzzi G. Rare diseases and effective treatments: Are we delivering? Lancet. 2015; 385:750-752.
4. Stolk P, Willemen MJ, Leufkens HG. Rare essentials: Drugs for rare diseases as essential medicines. Bull World Health Organ. 2006; 84:745-751.
5. Schieppati A, Henter JI, Daina E, Aperia A. Why rare diseases are an important medical and social issue. Lancet. 2008; 371:2039-2041.
6. O'Sullivan BP, Orenstein DM, Milla CE. Pricing for orphan drugs: Will the market bear what society cannot? JAMA. 2013; 310:1343-1344.
7. Picavet E, Annemans L, Cleemput I, Cassiman D, Simoens S. Market uptake of orphan drugs - a European analysis. J Clin Pharm Ther. 2012; 37:664-667.
8. Song P, Gao J, Inagaki Y, Kokudo N, Tang W. Rare diseases, orphan drugs, and their regulation in Asia: Current status and future perspectives. Intractable Rare Dis Res 2012; 1:3-9.
9. Remuzzi G, Garattini S. Rare diseases: What's next? Lancet. 2008; 371:1978-1979.
10. WästfeltM, Fadeel B, Henter JI. A journey of hope: Lessons learned from studies on rare diseases and orphan drugs. J Intern Med. 2006; 260:1-10.
11. Bayrakli F, Erkek E, Kurtuncu M, Ozgen S. Intraventricular hemorrhage as an unusual presenting form of Sneddon syndrome. World Neurosurg. 2010; 73:411-413.
12. Hayashi S, Umeda T. 35 years of Japanese policy on rare diseases. Lancet. 2008; 372:889-890.
13. The Ministry of Health, Labour and Welfare. The Results of Early Exploratory Clinical Trial Bases for Specific Research Areas. *http://www.mhlw.go.jp/stf/houdou/2r9852000001jym4.html* (accessed March 21, 2015).
14. The Japanese Ministry of Health, Labor, and Welfare. Reform of measures to combat intractable diseases. *http://www.mhlw.go.jp/stf/shingi/2r9852000002n6ro-att/2r9852000002n6uj.pdf* (accessed February 15, 2015). (in Japanese)
15. Wang JB, Guo JJ, Yang L, Zhang YD, Sun ZQ, Zhang YJ. Rare diseases and legislation in China. Lancet. 2010; 375:708-709.
16. Ding JX, Sun XD, Ji N, Shao WJ. The accessibility assessment on orphan drugs and legal system research in China and America. Chinese Pharmaceutical Journal. 2011; 46:1129-1132. (in Chinese)

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Original Article

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A cross-sectional study of leukopenia and thrombocytopenia among Chinese adults with newly diagnosed HIV/AIDS

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Summary **We conducted a cross-sectional study to determine the prevalence and risk factors of leukopenia and thrombocytopenia among Chinese adults with newly diagnosed HIV/AIDS. One thousand nine hundred and forty-eight newly diagnosed HIV-infected patients were enrolled between 2009 and 2010. Serum samples obtained from each individual were collected for complete blood count. Factors associated with the presence of leukopenia and thrombocytopenia were analyzed by multiple logistic regression. The overall prevalence of leukopenia and of thrombocytopenia was 33.2% and 15.6%, respectively. The prevalence of leukopenia was higher among females than among males (39.4% *versus* 31.2%). The prevalence of leukopenia increased with decreasing CD4 count (8.2%, 26.5%, 33.4%, and 41.5% among patients with CD4 count of ≥ 350, 200-349, 50-199, and < 50 cells/mm^3 respectively). The prevalence of thrombocytopenia also showed an increasing trend with decreasing CD4 count (5.8%, 12.2%, 17.8%, and 17.5% among patients with CD4 count of ≥ 350, 200-349, 50-199, and < 50 cells/mm^3, respectively). Logistic analysis showed that female sex, lower CD4 count, and Han ethnicity were significantly associated with an increased risk of leukopenia, and that lower CD4 count, and HIV transmission by blood were significantly associated with an increased risk of thrombocytopenia. The study reflects that leukopenia and thrombocytopenia are common among Chinese adults with newly diagnosed HIV/AIDS; and lower CD4 count is associated with an increased risk of both leukopenia and thrombocytopenia. We propose that a routine assessment of these parameters is necessary for timely and adequate clinical management.**

Keywords: Acquired immune deficiency syndrome, leukopenia, thrombocytopenia, prevalence, risk factor, CD4$^+$T lymphocyte count

1. Introduction

Hematologic abnormalities, which involved all lineages of blood cells and include anemia, leukopenia as well as thrombocytopenia, are among the most common complications of HIV infection (*1-3*). Among these hematologic disorders, anemia is the most common hematologic manifestation (*2,4,5*). In different studies, the prevalence of anemia in individuals with AIDS has been reported at 63% to 95%, making it more common than thrombocytopenia or leukopenia in AIDS patients (*6*). Hematological abnormalities, mainly anemia and leukopenia, in antiretroviral-naive HIV-infected patients result in poor antiretroviral treatment outcome and otherwise strongly predict mortality (*7,8*). Our previous study (*9*) showed that anemia is highly prevalent among adults with newly diagnosed HIV/AIDS in China. The overall prevalence of anemia among antiretroviral-naive HIV-infected patients was 51.9%. We found that older age, lower CD4 count and minority ethnicity are associated with an increased risk of anemia in antiretroviral-naive HIV-infected patients. However, so far the prevalence of leukopenia and thrombocytopenia in the Chinese population

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has not been well characterized. Considering such information for newly diagnosed HIV-infected patients may help to optimize antiretroviral treatment of HIV-infected individuals, we conducted a cross-sectional study to determine the prevalence of leukopenia and thrombocytopenia among Chinese adults with newly diagnosed HIV/AIDS, and to identify demographic and HIV-related factors associated with the presence of leukopenia and thrombocytopenia.

2. Methods

2.1. *Study population*

The cross-sectional survey was conducted among antiretroviral-naive HIV-infected patients from China's provinces and municipalities including Xinjiang, Jiangxi, Henan, Heilongjiang, Guangdong, Shaanxi, Guangxi, Hunan, Shanghai, and Yunnan during 2009 and 2010. The details of the study population have been described previously (*9*). In brief, antiretroviral-naive HIV-infected patients aged 18 years or more at the time of enrolment with confirmed HIV infection were eligible for this study. Patients who were on antiretroviral therapy (ART) were excluded.

2.2. *Blood samples*

Three-milliliter venous blood samples were collected from each patient. Full blood-count analyses were performed at the study laboratories in each province using a CELL DYN 3200 hematology analyser (Abbott Laboratories, USA). All the study laboratories successfully completed a standardization and certification program.

2.3. *Data collection*

Data were collected according to standardized criteria. All participants provided information on demographic characteristics, risk-behavior, and laboratory test results. Variables in the study included age, sex, HIV transmission route, and CD4 count. Age was denoted as < 40, 40-59, or ≥ 60 years. HIV transmission route was categorized as sexual contact (including homosexual or heterosexual), blood (including blood transfusion or injection drug use), or unknown transmission risk. CD4 count was denoted as < 50, 50-199, 200-349 or ≥ 350 cells/mm^3.

2.4. *Statistical analysis*

Statistical analysis was done using IBM SPSS Statistics version 19. Leukopenia was defined as total white blood cell (WBC) count $< 4 \times 10^9$/L while thrombocytopenia was defined as total platelet $< 100 \times 10^9$/L. Continuous variables were computed with standard methods and were expressed as mean and standard deviations (SD). Continuous variables were compared using *t* tests and analysis of variance. Categorical variables are reported as frequencies and percentage of each category. A chi-square test was applied for categorical attributes. The odds ratio and 95% confidence intervals were calculated to assess the relationship between each risk factor and the risk of leukopenia and thrombocytopenia; to adjust for the effects of potential confounders, we used multiple logistic regression models. All variables included in the models were determined a priori based on epidemiological importance and biological plausibility. Variables included in the models were age, sex, ethnicity, CD4 count, and HIV transmission route. The statistical test was two-tailed and performed at a level of statistical significance of 0.05.

2.5. *Ethics statement*

Written informed consent was obtained from all participants in accordance with this study's protocols and procedures approved by the Shanghai Public Health Clinical Center Ethics Committee. No patient identifiers were included in the dataset used for this analysis.

3. Results

3.1. *Demographic characteristics*

The study population is consisted of a total of 1,948 adults with newly diagnosed HIV/AIDS, of which the detailed information has been described in our previous survey (*9*). The study sample was primarily male (75.8%) (n = 1,476), the mean age was 40 years, 24.1% (n = 470) were ethnic minorities, and the mean CD4 count was 136 cells/mm^3. Most patients (74.2%) acquired HIV through sexual contact (n = 1,446).

3.2. *WBC counts among newly diagnosed HIV/AIDS patients*

The mean WBC count was $(5.37 \pm 3.04) \times 10^9$/L, which was higher among males than females (p = 0.001), and was also higher among ethnic minority patients than the Han patients (p = 0.001) (Table 1). The mean WBC count did not differ by CD4 count, age or HIV transmission route (p = 0.108, p = 0.239, p = 0.220).

3.3. *Platelet counts among newly diagnosed HIV/AIDS patients*

The mean platelet count was $(186.74 \pm 97.26) \times 10^9$/L, which was higher among ethnic minority patients than the Han patients ($p < 0.001$). The mean platelet count differed by CD4 count and HIV transmission route, the mean platelet count was highest among both patients with CD4 counts of < 50 cells/mm^3 (p = 0.002) and

Table 1. The WBC and platelet counts among newly diagnosed HIV/AIDS patients

Cohort	WBC (mean ± SD)	PLT (mean ± SD)
Number	1,948	1,948
Overall	5.37 ± 3.04	186.74 ± 97.26
Range	(0.53, 37.70)	(1, 626)
Sex		
Male	5.49 ± 3.02	186.05 ± 96.99
Female	4.98 ± 3.09	188.89 ± 98.19
p value for difference	0.001	0.581
Ethnicity		
Han	5.22 ± 2.85	181.99 ± 92.79
Minority ethnicity	5.83 ± 3.55	201.67 ± 108.90
p value for difference	0.001	< 0.001
CD4 count, cells/mm^3		
< 50	5.39 ± 3.59	196.42 ± 108.08
50-199	5.26 ± 2.95	178.54 ± 96.36
200-349	5.24 ± 2.18	178.87 ± 82.11
≥ 350	5.87 ± 1.95	188.00 ± 70.35
p value for difference	0.108	0.002
Age, years		
18-39	5.44 ± 3.26	187.57 ± 100.39
40-59	5.21 ± 2.61	184.92 ± 93.63
≥ 60	5.51 ± 3.23	189.21 ± 91.80
p value for difference	0.239	0.807
HIV transmission route		
Sexual contact	5.33 ± 3.00	192.06 ± 96.22
Blood	5.61 ± 3.28	168.75 ± 98.39
Unknown transmission risk	5.19 ± 2.91	177.01 ± 99.45
p value for difference	0.220	< 0.001

WBC: white blood cell; PLT: platelet; SD: standard deviation.

Table 2. Prevalence of leukopenia and thrombocytopenia among newly diagnosed HIV/AIDS patients

Cohort	Leukopenia	Thrombocytopenia
Number	1,948	1,948
Overall	33.2%	15.6%
Sex		
Male	31.2%	15.5%
Female	39.4%	15.7%
p value for difference	0.001	0.932
Ethnicity		
Han	34.1%	15.4%
Minority ethnicity	30.2%	16.0%
p value for difference	0.119	0.782
CD4 count, cells/mm^3		
< 50	41.5%	17.5%
50-199	33.4%	17.8%
200-349	26.5%	12.2%
≥ 350	8.2%	5.8%
p value for difference	< 0.001	< 0.001
Age, years		
18-39	32.3%	16.6%
40-59	34.6%	14.5%
≥ 60	32.9%	12.7%
p value for difference	0.585	0.284
HIV transmission route		
Sexual contact	33.0%	13.6%
Blood	32.1%	22.9%
Unknown transmission risk	37.0%	17.3%
p value for difference	0.521	< 0.001

patients infected with HIV through sexual contact ($p < 0.001$) (Table 1). The mean platelet count did not differ by sex or age ($p = 0.581$, $p = 0.807$).

3.4. *Prevalence of leukopenia among newly diagnosed HIV/AIDS patients*

Among the 1,948 patients, 646 (33.2%) had leukopenia (Table 2). The prevalence of leukopenia was higher among females than among males (39.4% versus 31.2%) ($p = 0.001$). The prevalence of leukopenia was 8.2%, 26.5%, 33.4%, and 41.5% among patients with CD4 counts of ≥ 350, 200-349, 50-199, and < 50 cells/mm^3, respectively. The prevalence of leukopenia increased with decreasing CD4 count ($p < 0.001$). The prevalence of leukopenia did not differ by ethnicity, age or HIV transmission route ($p = 0.119$, $p = 0.585$, $p = 0.521$).

3.5. *Prevalence of thrombocytopenia among newly diagnosed HIV/AIDS patients*

Among the 1,948 patients, 303 (15.6%) had thrombocytopenia (Table 2). The prevalence of thrombocytopenia was 5.8%, 12.2%, 17.8%, and 17.5% among patients with CD4 counts of ≥ 350, 200-349, 50-199, and < 50 cells/mm^3, respectively. The prevalence of thrombocytopenia showed an increasing trend with decreasing CD4 count ($p < 0.001$). The prevalence of thrombocytopenia was 13.6%, 22.9%, and 17.3% among patients infected with HIV through sexual contact, blood, and unknown transmission risk, respectively. The prevalence of thrombocytopenia differed by HIV transmission route, it was highest among patients infected with HIV through blood ($p < 0.001$). The prevalence of thrombocytopenia did not differ by sex, ethnicity or age ($p = 0.932$, $p = 0.782$, $p = 0.284$).

3.6. *Risk factors for leukopenia among newly diagnosed HIV/AIDS patients*

In a multivariate analysis using a logistic regression model, we analyzed factors associated with the presence of leukopenia. Female sex, Han ethnicity and lower CD4 count were significantly associated with an increased risk of leukopenia. HIV transmission route and age failed to show an association with the presence of leukopenia (Table 3).

3.7. *Risk factors for thrombocytopenia among newly diagnosed HIV/AIDS patients*

In a multivariate analysis using a logistic regression model, we analyzed factors associated with the presence of thrombocytopenia. Lower CD4 count and HIV transmission by blood were significantly associated with an increased risk of thrombocytopenia. Sex, ethnicity and age failed to show an association with the presence of thrombocytopenia (Table 3).

Table 3. Identification of risk factors for the presence of leukopenia or thrombocytopenia among newly diagnosed HIV/AIDS patients, results of the regression model

Cytopenia/Risk factor	*p* value	Odds ratio	95% CI
Leukopenia			
Female sex	0.001	1.471	(1.180, 1.835)
Minority ethnicity	0.010	0.738	(0.585, 0.930)
Age,per 20-year increment	0.570	1.045	(0.897, 1.218)
CD4 count, per increase of 150 cells/mm^3	< 0.001	0.610	0.547, 0.681)
HIV transmission route	0.418	-	-
Sexual contact	-	1.000	-
Blood	0.212	1.184	(0.908, 1.542)
Unknown transmission risk	0.555	1.111	(0.784, 1.573)
Thrombocytopenia			
Female sex	0.789	1.040	(0.778, 1.392)
Minority ethnicity	0.689	0.942	(0.703, 1.262)
Age,per 20-year increment	0.193	0.873	(0.711, 1.071)
CD4 count, per increase of 150 cells/mm^3	< 0.001	0.725	(0.630, 0.834)
HIV transmission route	< 0.001	-	-
Sexual contact	-	1.000	-
Blood	< 0.001	2.123	(1.571, 2.869)
Unknown transmission risk	0.210	1.322	(0.854, 2.045)

CI: Confidence interval.

4. Discussion

In present study, we observed a high prevalence of leukopenia and thrombocytopenia among newly diagnosed, antiretroviral-naive HIV-infected Chinese adults. Together with our previous study (*9*), the data indicate that hematologic abnormalities are relatively prevalent among antiretroviral-naive HIV-infected patients. The results from our studies further demonstrate that antiretroviral-naive HIV-infected patients exhibit a wide range of hematologic abnormalities. Anemia is the most common hematological abnormality, followed by leukopenia and thrombocytopenia. Cytopenias often cause symptoms and contribute to the complications suffered by AIDS patients like infections, anemia and bleeding. Medical professionals across all disciplines need to be aware of the hematological complications of HIV infection. Our findings constitute further evidence of the need for monitoring hematologic parameters of HIV-infected patients, both before ART initiation and routinely during treatment. Routine blood tests are currently recommended for HIV-infected patients both before and after initiating ART by HIV care and treatment guidelines.

In addition, our study showed that the overall prevalence of both leukopenia and thrombocytopenia increased with decreasing CD4 count, and that lower CD4 count was associated with an increased risk of both leukopenia and thrombocytopenia. Together with our previous investigation on anemia among the same population (*9*), these associations suggest that the stage of HIV-infection is an important determinant to pretreatment hematologic abnormalities. Hematologic manifestations of HIV infection are common and more frequent with progression of disease. A similar study demonstrated that blood cytopenias mainly occur in HIV patients with advanced immunosuppression and clinical stages (*10*). Another study found an association between CD4 count and hemoglobin level, neutrophil count, and platelet count (*7*). These results suggest that the presence of hematologic abnormalities in newly diagnosed HIV-infected patients is related to HIV infection itself. Therefore, it is important to identify patients with hematologic abnormalities and to consider HIV as a possible underlying cause.

The origin of hematological disorders in HIV infection remain to be studied. Current observations suggest that HIV infection may affect processes important during early stages of hematopoiesis or stem cell differentiation (*11*). Both a direct cytopathic effect of HIV on haemopoietic progenitors and an immune system mediated mechanism are involved in hematological abnormalities (*1*). Multiple interacting factors contribute to the hematological manifestations of HIV disease, it could be due to direct effects of HIV infection, opportunistic infections, lymphomas, malignancy or side effects of therapy. A study isolated the impact of HIV infection alone on hematologic manifestations and confirmed that these changes were reversible by ART (*12*). Therefore, control of the HIV infection will have the main role in the management of hematological manifestations of HIV.

Similar to our previous study on anemia in antiretroviral-naive HIV-infected patients, both demographic and HIV-related factors were associated with leukopenia and thrombocytopenia (*9*). In our study population, hematologic abnormalities were significantly associated with the factors of sex (only for leukopenia), age (only for anemia), ethnicity (except for thrombocytopenia), and HIV transmission route (only for thrombocytopenia). These findings provide focused targets for improving routine screening for hematologic

abnormalities in order to reduce the morbidity of the HIV-infected patients. Our findings have implications for the optimal choice of initial antiretroviral agents, monitoring of ART toxicities, and improvement of ART programs, especially in resource-limited countries.

Our findings are consistent with several published studies, which also indicate high leukopenia and thrombocytopenia prevalence and similar associated risk factors in HIV-infected patients. A study conducted among adult Zimbabweans showed that the prevalence of leukopenia and of thrombocytopenia was 11.7% and 24.7%, respectively (*13*). Leukopenia and thrombocytopenia were seen in 26.8% and 21.7% of HIV patients with CD4 counts less than 200 cells/mm^3 in India, respectively (*14*). Leukopenia and thrombocytopenia occurred in 24.3% and 8.3% of adult AIDS patients at initiation of ART in Uganda, respectively (*15*). The study in Uganda showed that the presence of any cytopenia was associated with female sex, decreasing CD4 count and decreasing body mass index. An investigation in South Korea found that the leading risk factor for cytopenia was AIDS status at initial presentation (*12*). Another study showed that neutropenia was associated with CD4 and platelet counts; and that thrombocytopenia was associated with country, gender, and chronic hepatitis B infection (*16*). However, studies in India (*17*) and Rwanda (*5*) did not find a significant correlation between thrombocytopenia and low CD4 count. The differences in the prevalence and risk factors could be attributed to demographic characteristics, geographical location, cut-off values used to define the cytopenias, and different stages of HIV illness in the study populations.

However, there are several limitations with our study. First, potential sample selection bias may have affected the findings. The HIV epidemic is serious in some provinces and among some most-at-risk populations in China. The study population is not representative of the entire HIV-positive population in China and so the results may not be generalizable. Second, this was an observational study, we were able to examine potential associations but were not able to assess causation, so it is not clear if the cytopenias preceded the HIV infection or *vice versa*. Third, cytopenias are associated with several factors including geographical location and comorbidities such as tuberculosis, hepatitis B infection, fever and oral candidiasis (*15*), these factors were not assessed in our study. Therefore, we were not able to determine the association between these factors and the prevalence of cytopenias. Furthermore, if these variables had been controlled for, some variables such as CD4 count might not have remained significant in the logistic regression model. In addition, we did not collect the information about concomitant medication use and were not able to assess its impact on cytopenias in this population.

In conclusion, leukopenia and thrombocytopenia are common among Chinese adults with newly diagnosed HIV/AIDS. Lower CD4 count is associated with an increased risk of both leukopenia and thrombocytopenia in antiretroviral-naive HIV-infected patients. We propose here that a routine assessment of these parameters is necessary for timely and adequate clinical management.

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References

1. Brizzi MF, Porcu P, Porteri A, Pegoraro L. Haematologic abnormalities in the acquired immunodeficiency syndrome. Haematologica. 1990; 75:454-463.
2. Erhabor O, Ejele OA, Nwauche CA, Buseri FI. Some haematological parameters in human immunodeficiency virus (HIV) infected Africans: The Nigerian perspective. Niger J Med. 2005; 14:33-38.
3. Bello JL, Burgaleta C, Magallon M, Herruzo R, Villar JM. Hematological abnormalities in hemophilic patients with human immunodeficiency virus infection. Am J Hematol. 1990; 33:230-233.
4. Meidani M, Rezaei F, Maracy MR, Avijgan M, Tayeri K. Prevalence, severity, and related factors of anemia in HIV/AIDS patients. J Res Med Sci. 2012; 17:138-142.
5. Munyazesa E, Emile I, Mutimura E, Hoover DR, Shi Q, McGinn AP, Musiime S, Muhairwe F, Rutagengwa A, Dusingize JC, Anastos K. Assessment of haematological parameters in HIV-infected and uninfected Rwandan women: A cross-sectional study. BMJ Open. 2012; 2:e001600.
6. Sullivan PS, Hanson DL, Chu SY, Jones JL, Ward JW. Epidemiology of anemia in human immunodeficiency virus (HIV)-infected persons: Results from the multistate adult and adolescent spectrum of HIV disease surveillance project. Blood. 1998; 91:301-308.
7. De Santis GC, Brunetta DM, Vilar FC, Brandão RA, de Albernaz Muniz RZ, de Lima GM, Amorelli-Chacel ME, Covas DT, Machado AA. Hematological abnormalities in HIV-infected patients. Int J Infect Dis. 2011; 15:e808-e811.
8. Anastos K, Shi Q, French AL, Levine A, Greenblatt RM, Williams C, DeHovitz J, Delapenha R, Hoover DR. Total lymphocyte count, hemoglobin, and delayed-type hypersensitivity as predictors of death and AIDS illness inHIV-1-infected women receiving highly active antiretroviral therapy. J Acquir Immune Defic Syndr. 2004; 35:383-392.
9. Shen Y, Wang Z, Lu H, Wang J, Chen J, Liu L, Zhang

R, Zheng Y. Prevalence of anemia among adults with newly diagnosed HIV/AIDS in China. PLoS One. 2013; 8:e73807.
10. Wankah PN, Tagny CT, Mbanya DN. Profile of blood cell abnormalities among antiretroviral therapy naïve HIV patients attending the Yaounde University Teaching Hospital, Cameroon. BMC Hematol. 2014; 14:15.
11. Koka PS, Reddy ST. Cytopenias in HIV infection: Mechanisms and alleviation of hematopoietic inhibition. Curr HIV Res. 2004; 2:275-82.
12. Choi SY, Kim I, Kim NJ, Lee SA, Choi YA, Bae JY, Kwon JH, Choe PG, Park WB, Yoon SS, Park S, Kim BK, Oh MD. Hematological manifestations of human immunodeficiency virus infection and the effect of highly active anti-retroviral therapy on cytopenia. Korean J Hematol. 2011; 46:253-257.
13. Adewuyi JO, Coutts AM, Latif AS, Smith H, Abayomi AE, Moyo AA. Haematologic features of the human immunodeficiency virus (HIV) infection in adult Zimbabweans. Cent Afr J Med. 1999; 45:26-30.
14. Parinitha S, Kulkarni M. Haematological changes in HIV infection with correlation to CD4 cell count. Australas Med J. 2012; 5:157-162.
15. Kyeyune R, Saathoff E, Ezeamama AE, Löscher T, Fawzi W, Guwatudde D. Prevalence and correlates of cytopenias in HIV-infected adults initiating highly active antiretroviral therapy in Uganda. BMC Infect Dis. 2014; 14:496.
16. Firnhaber C, Smeaton L, Saukila N, Flanigan T, Gangakhedkar R, Kumwenda J, La Rosa A, Kumarasamy N, De Gruttola V, Hakim JG, Campbell TB. Comparisons of anemia, thrombocytopenia, and neutropenia at initiation of HIV antiretroviral therapy in Africa, Asia, and the Americas. Int J Infect Dis. 2010; 14:e1088-e1092.
17. Dikshit B, Wanchu A, Sachdeva RK, Sharma A, Das R. Profile of hematological abnormalities of Indian HIV infected individuals. BMC Blood Disord. 2009; 9:5.

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Original Article

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Hepatitis C virus infection in the general population: A large community-based study in Mianyang, West China

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Summary **Hepatitis C virus (HCV) infection remains a major public health problem. The objective of the current study was to reveal the seroepidemiology of HCV in the general population in Mianyang City. This study collected 438,575 blood samples from participants who had enrolled in the National Science and Technology Development Project and their demographic information, and then evaluated HCV antibody and alanine aminotransferase (ALT) levels. The overall anti-HCV positive rate was 0.80% (3,491/438,575) in the Mianyang general population, and it was 1.19% in rural population and 0.20% in urban. Anti-HCV positive rate increased with age, peaked at 45-54 years (2.01%), and then decreased. Anti-HCV prevalence was higher in males (0.89%) than that in females (0.73%). The prevalence of anti-HCV in participants with a history of blood transfusion, surgery, or with a previous diagnosis of hypertension was higher. The abnormal ALT levels (> 40 IU/L) were observed in 50.11% and 7.74% of anti-HCV positive and negative groups, respectively. In anti-HCV positive groups, the rate of abnormal ALT levels was higher in 55-64 age groups, male, and rural population. Though Mianyang was a low endemic area for HCV infection, the alarming fact was the large number of abnormal ALT levels in patients related to hepatitis C. This revealed delayed diagnosis and treatment of HCV infections. It is a necessity to promote early diagnosis and timely treatment of HCV infections.**

Keywords: Seroepidemiology, hepatitis C virus antibody, alanine aminotransferase, general population, China

1. Introduction

Hepatitis C is an infectious liver disease, caused by the hepatitis C virus (HCV) (*1*). The infection is often asymptomatic especially in its early stages, but once established, 74% to 86% of newly infected persons will develop chronic infection and the chronic stage constitutes one of the leading causes of cirrhosis and hepatocellular carcinoma (HCC) (*2,3*). HCV infection is a major public health problem in both developing and developed countries. The World Health Organization estimated that about 130 to 170 million people, 2% to 3% of the world's population, currently are HCV infected. Moreover, there are about 3 to 4 million new cases every year (*4*).

In 2012, HCV infection was the fourth most commonly reported infectious disease in China following hepatitis B virus infection, pulmonary tuberculosis and dysentery (*5*). Recent data estimated that up to 25 million Chinese were HCV infected (*6*). The prevalence of HCV infection ranged from 0.43% to 3.2%, and varied geographically and temporally in China (*7-9*). During the past decades, most of studies related to HCV infection in China focused on hospital based and high risk population groups including blood donors and receivers, drug abusers, individuals with HIV, and sex workers (*10-13*); while there were only a few large-scale studies in China which concentrated on HCV prevalence in the general population (*8,9,14*).

On the basis of the availability of new, direct-acting antiviral medications, we have entered a new era of hepatitis C therapeutics with an enormous opportunity to prevent morbidity and mortality among

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people with HCV infection (*15*). In this regard, a good understanding of HCV prevalence is important for prevention and treatment of HCV. Here, using blood samples and data that had been collected from the National Science and Technology Development Project for infectious disease prevention and control in Mianyang, Sichuan province, we conducted a seroepidemiology study to explore the HCV prevalence and the alanine aminotransferase (ALT) level to investigate the clinical and virological characteristics as well as treatment outcomes in the general population in Mianyang. We believe this study will help to devise strategies by health policy makers for control of hepatitis C disease.

2. Materials and Methods

2.1. *Survey design*

The National Science and Technology Development Project which was carried out from February 2010 to June 2012 had been initiated to collect data on hepatitis B virus (HBV), HCV and tuberculosis infection status in the general population in Mianyang, the second largest city of Sichuan province (Figure 1). The project used a cluster sampling design. There are nine administrative divisions: two districts and seven countries. One district of Fucheng and one country of Anxian, were selected randomly (Figure 1). Then, 8 sub-districts and 14 towns from Fucheng, and 18 towns from Anxian were randomly selected. Finally, within each town/sub-district, two villages/communities were selected according to economic level. In these participant sites that we selected, the study included all local residents and migrant workers who had lived there for 6 months.

After the staff received appropriate training from the lead researchers for this project, data collection was conducted in examination centers at local health stations and community clinics that were located in sub-district/towns that the project had chosen. According to a standard protocol, trained staff conducted face-to-face interviews *via* questionnaires after obtaining written consent from the participants. The questionnaire collected demographic information (including gender, age, and area of residence) and medical history information (including history of diabetes, history of hypertension, family history of hepatocellular carcinoma, blood transfusion and surgical intervention); and each copy of the questionnaire had a unique identification number. After the interview, a 5-mL serum sample, labeled with the same identification number as questionnaire, was collected from each participant. Blood samples were properly stored in a low temperature container (controlled from 4-8°C), and were transported daily to laboratory test hospitals for sample processing and serological testing.

For HCV and ALT testing, available blood samples after HBV testing were used. The study was approved by the Ethics Committee of West China Hospital, Sichuan University.

Figure 1. Map of Mianyang, China, showing the location of Mianyang (the star) and the two counties (black areas) selected for sampling in the study.

2.2. *Laboratory testing*

Commercial third-generation enzyme immunoassay kits (ELISAs) (Xinchuang Core Anti-HCV ELISA kit, Xiamen, China) were used for HCV antibody testing. Verification of positive samples was carried out by retesting the samples using the same kits. Only samples that were positive on both tests were considered to be true positive, which was the indicator of previous HCV contact history.

Serum ALT quantitation was performed after being screened for anti-HCV antibodies. Quantitation was achieved using a coupled enzyme and indicator reaction that utilizes pyruvate for a kinetic determination of NADH consumption measured by an automated biochemical analyzer (CS-T300, Dirui, China). Abnormal ALT levels were defined as greater than 40 IU/L.

2.3. *Statistical analysis*

We used Chi-square test to compare anti-HCV positive rates by different characteristics. We also used Chi-square test to compare the ALT level by age, sex and area. SPSS 17.0 software located in the Epidemiology and Biostatistics Department of West China School of Public Health at Sichuan University was used for analysis. p value < 0.05 was considered to be statistically significant.

3. Results

3.1. *Demographic characteristics*

A total of 438,575 serum samples were available for HCV screening from the participants. Of these participants, 175,161 (39.94%) were from the urban area and 263,414 (60.06%) from rural area. There were 200,942 (45.8%) male and 237,633 (54.2%) female participants. The age of the participants ranged from 1 to 90 years, with a mean of 35.91 ± 21.15 years old.

Table 1. Prevalence of anti-HCV stratified by different characteristics in Mianyang

Characteristics	Total samples	Anti-HCV positive case	Anti-HCV positive rate (%)	χ^2	*p* value
Age groups					
1-14	94349	71	0.08	2623.10	< 0.001
15-24	75145	138	0.18		
25-34	29949	154	0.51		
35-44	72393	941	1.30		
45-54	60676	1243	2.01		
55-64	62015	767	1.24		
≥ 65	42628	177	0.41		
Sex					
Female	237633	1725	0.73	32.26	< 0.001
Male	200942	1766	0.89		
Area					
Urban	175161	345	0.20	1325.25	< 0.001
Rural	263414	3146	1.19		
HBsAg positivity					
No	407239	3331	0.82	34.81	< 0.001
Yes	31336	160	0.51		
Blood transfusion					
No	435999	3434	0.79	65.87	< 0.001
Yes	2576	57	2.21		
Surgery					
No	409317	3085	0.75	138.98	< 0.001
Yes	29258	406	1.39		
History of hypertension					
No	420783	3318	0.79		
Yes	17792	173	0.97	7.30	0.007
History of diabetes					
No	436683	3474	0.80	0.25	0.62
Yes	1892	17	0.90		
Family history of HCC					
No	437857	3489	0.80	2.44	0.12
Yes	718	2	0.28		

Moreover, all the individual samples were categorized into seven age groups: 1-14, 15-24, 25-34, 35-44, 45-54, 55-64, ≥ 65. The proportions of these age groups were 21.5%, 17.1%, 6.8%, 16.5%, 14.1%, 14.1%, 9.8%, respectively.

3.2. *Anti-HCV positive rate*

3,491 were anti-HCV positive by ELISA, and the overall anti-HCV prevalence was 0.80% (3491/438,575). Statistical differences were observed considering the age group criterion. Anti-HCV prevalence was lowest in the 1-14 years old group (0.08%, 71/94,349). Anti-HCV prevalence increased with age, peaked at the 45-54 years old group (2.01%, 1,243/60,676), and then decreased. The individuals in age group 45-54 years showed an about 25-fold higher rate of anti-HCV seropositivity when compared with the individuals in age group 1-14 years. Overall, the individuals aged between 35-64 years represented 85% (2,951/3,491) of the anti-HCV seropositive subjects (Table 1, Figure 2).

The prevalence of anti-HCV was 0.89% in males (1,766/200,942) and 0.73% in females (1,725/235,908). The sex-specific prevalence of anti-HCV showed higher rates in male patients in each age group, except for individuals aged 25 to 34 years. Both males and females showed the highest anti-HCV prevalence in the age group 45-54 years. The difference in sex-specific prevalence widened with age and reached a maximum in the 45-54 year-old age group, followed by a decrease with advancing age (Table 1, Figure 3).

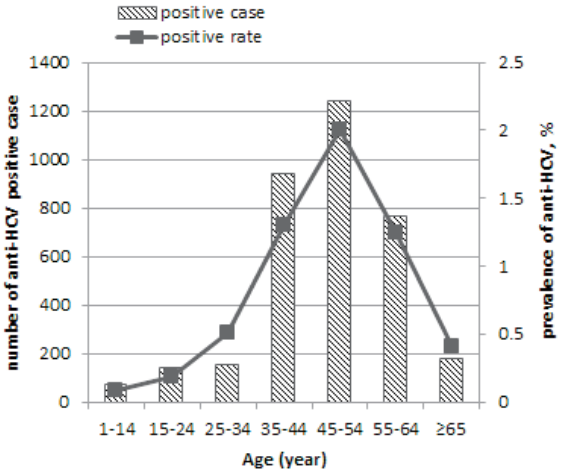

Figure 2. Overall characterization of anti-HCV by age group. Bars represent the number of anti-HCV positive cases in each group indicated by left vertical axis, and the squares represent the prevalence in each group indicated by right vertical axis. The horizontal axis represents the age ranges.

The overall prevalence of anti-HCV in rural areas was

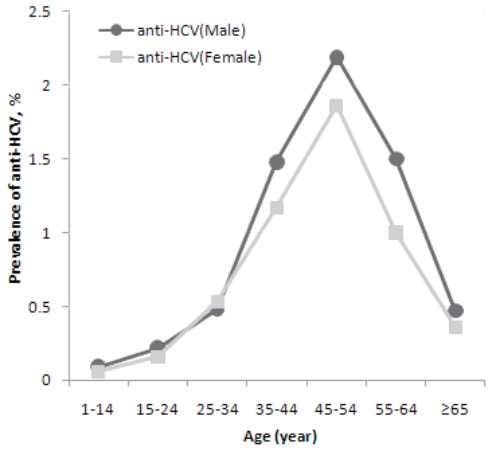

Figure 3. Sexual characterization of anti-HCV by age group. The left vertical axis represents the prevalence of anti-HCV. The dots represent the prevalence in males in each group, and the squares represent the prevalence in females in each group. The horizontal axis represents the age ranges.

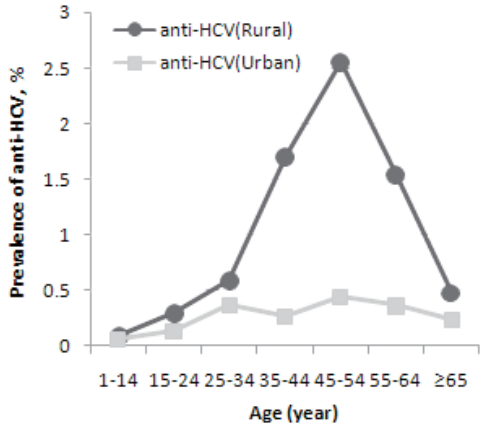

Figure 4. Regional characterization of anti-HCV by age group. The left vertical axis represents the prevalence of anti-HCV. The dots represent the prevalence in rural areas in each group, and the squares represent the prevalence in urban areas in each group. The horizontal axis represents the age ranges.

1.19% (3,146/263,414), which was much higher than that in urban areas, 0.20% (345/175,161). The individuals living in rural areas represented 90% of the anti-HCV seropositive subjects. The disparity in prevalence between rural areas and urban areas increased with age such that the largest differences were apparent in individuals of 45-54 years of age. In individuals aged 45-54 years, the prevalence of HCV infection was 4.8 times higher among rural areas than urban areas (Table 1, Figure 4).

There were 31,336 people out of the population of 438,575 in Mianyang whose serum tested positive for HBsAg, and the HBV infection rate was 7.15%. There were 160 people who were anti-HCV positive among the 31,336 HBsAg-positive patients. The HCV concurrent infection rate was 0.51%, slightly lower than the HCV infection rate (0.80%) of the local general population. Significant differences were observed for blood transfusion, surgery and history of hypertension regarding the prevalence of anti-HCV. The prevalence of anti-HCV in participants with a history of blood transfusion, surgery, or with a previous diagnosis of hypertension was higher (Table 1). However, a previous diagnosis of hypertension might not be truly related to HCV prevalence. Because the sample size of the study was large, any small difference could be detected.

3.3. *ALT levels*

There were 13 missing ALT values in the anti-HCV positive group. The overall prevalence of abnormal ALT level was 8.07%. It was higher in the anti-HCV positive group (50.12%) than in the anti-HCV negative group (7.74%).

In the anti-HCV positive group, the prevalence of abnormal ALT levels increased with age, from 14.08% in the 1-14 year group to 57.92% in the 55-64 year group, and then decreased. The prevalence of abnormal ALT was higher in males (57.25%) than in females (42.82%), and it was also higher in rural areas (52.50%) than in urban areas (28.28%). See Table 2.

Table 2. Serological features of abnormal ALT prevalence in anti-HCV positive group

Items	NO. anti-HCV positive	NO. ALT > 40 IU/L	Abnormal ALT rate (%)	χ^2	*p* value
Total	3478	1743	50.12		
Age groups				190.34	< 0.001
1-14	71	10	14.08		
15-24	137	29	21.17		
25-34	153	31	20.26		
35-44	939	482	51.33		
45-54	1238	689	55.65		
55-64	763	442	57.92		
≥ 65	177	60	33.90		
Sex				72.44	< 0.001
Female	1719	736	42.82		
Male	1759	1007	57.25		
Area				72.57	< 0.001
Urban	343	97	28.28		
Rural	3135	1646	52.50		

4. Discussion

As the fourth most commonly reported infectious disease in China, HCV infection can be expected to make up an increasingly large portion of the disease burden in the future. A seroepidemiology survey of hepatitis C infection is important for prevention and treatment of the disease (*16*). In 1992, the first nationwide cross-sectional seroepidemiologic survey of hepatitis C infection with a total of approximately 68,000 participants showed the prevalence of anti-HCV was 3.2% in the general population aged from 1 to 59 years (*8*). However, two recent studies reported a lower prevalence in the general population. In 2006, China Center for Disease Control and Prevention (CCDC) reported that the overall prevalence of anti-HCV was 0.43% among the population of 1 to 59 years old on the basis of 78,746 blood samples (*10*); in 2007, another study with 9538 serum samples collected from 6 regions reported that the overall positive rate of anti-HCV was 0.58% (*9*). New regulations on forbidding paid blood donations and the reuse of unsterilized needles for medical injections, increased health education, and more accurate diagnostic technologies in recent years might explain differences among these findings.

Our study reported that the positive rate of anti-HCV in the general population of Mianyang was 0.80% (3,491/438,575), which is higher than the previously reported rates in 2006 and 2007. The rate was very close to the result of a screening of 157,168 people in Jiangsu province where the total positive rate of anti-HCV was 0.79% (*15*).

Each age group contained at least one HCV infected person in Mianyang, but the anti-HCV positive rates differed. In spite of different gender and residential areas, the anti-HCV positive rate increased with age, reached a peak at 54 years old, and then declined. This implied a steady cumulative increase in incidence, probably caused by the sporadic transmission of virus persisting in the community/village through the years, and the high rate of chronic infection related to the natural history of HCV infection. After 54 years old, an increase in mortality among HCV-infected people may explain the decrease in prevalence of HCV infection.

Our analysis also found that individuals aged from 35 to 64 years represented 85% of the anti-HCV seropositive subjects. This finding was similar to a report from the United States that individuals born between 1945 and 1965 (aged from 40 to 59) comprised 81% of all chronic HCV infections (*17*). Given that, the Centers for Disease Control and Prevention of the U.S recommended a 1-time HCV test for all people born during that period (*18*). This recommendation could help to identify most individuals living with HCV. With implementation of this strategy, the U.S potentially averted approximately 120,000 deaths caused by HCV infection (*19*).

Interestingly, HCV infection disproportionately affected men more than women in this survey. Males showed a 1.2 times higher HCV infection rate than females, whereas other studies have reported equality between sexes or even female predominance in HCV infection (*8,20-22*). The underlying reason of male predominance in this study is still not clear. However, studies have demonstrated that, on average, about 20% of HCV infected individuals would spontaneously clear the virus after initial infection, and women were more likely to clear the virus spontaneously (*23,24*). In addition, we hypothesized that there may be more chances for men to be infected by HCV through unhealthy lifestyles or behaviors, such as smoking, drinking, poor hygiene, and unhealthy sexual activities.

We also found significant regional differences in the anti-HCV positive rate in Mianyang. Most of the HCV-infected persons resided in rural areas, which was inconsistent with previous studies in China (*16,25*). We suppose that this phenomenon was related to commercial blood selling in rural areas. Some studies have demonstrated that paid blood donors living in villages have comparatively higher anti-HCV rates (*26,27*). Before 1998, there were a number of commercial blood donors living in rural areas of China. These blood donors sold blood to unlicensed private blood collection centers for payment (*28*). Some of these illegal centers used unsafe blood collection methods, such as the reuse of none-sterilized needles and reinfusion of pooled red blood cells from multiple donors, which could easily lead to HCV infection. Given the greater burden of infection in rural areas, appropriate prevention measures to control the transmission of virus in rural areas were urgent. Health education, more thorough screening for HCV infection and early link of age-to-care and treatment initiation in rural areas were critical.

Although hepatitis B and hepatitis C have similar transmission routes, and although there was no vaccine for prevention of HCV, the study found that the HCV and HBV infection rates were significantly different in the general population in Mianyang (0.80% *vs*. 7.15%). Among the HBsAg-positive patients, the HCV concurrent infection rate was 0.51%, which is lower than that reported in other literature (*29,30*). Though there was a small amount of HBV and HCV coinfection, patients with dual HBV/HCV infection have a higher risk of progression to cirrhosis and decompensated liver disease than those in patients with monoinfection (*31,32*). So it is important for early detection and treatment of coinfection.

Participants with a history of blood transfusion had a higher prevalence of HCV infection in this study. In China, several studies have found that a history of blood transfusion was the most prevalent risk factor for HCV infection (*14,21,25,33*). This may be associated with commercial plasma donation in rural areas of China in the 1980s. Our study also found that participants with a history of surgery had a higher prevalence of HCV. In

the past, in some township hospitals, there were poor sterilization procedures and no HCV detection before surgery, which could easily lead to HCV infection.

ALT levels were related to HCV infection status in Mianyang. The anti-HCV positive group had a higher prevalence of abnormal ALT compared with the anti-HCV negative group. The association between abnormal ALT and gender was consistent with previous studies, which reported that males were associated with a higher prevalence of abnormal ALT than females (*34,35*). However, unlike previous studies, we found that individuals ≥ 65 years did not have a higher prevalence of abnormal ALT in our study (*36*). Because ALT was presumed to be a marker of hepatic inflammation, our finding demonstrated that HCV infection could lead to chronic necro-inflammatory hepatic damage. Moreover, some prospective studies showed that persistently abnormal serum ALT levels were strongly associated with high incidences of HCC in individuals positive for anti-HCV (*37,38*). However, there usually were no symptoms in HCV-infected individuals when ALT was slightly elevated. Once symptoms appear, most HCV infected persons have already developed HCC (*38*). Since abnormal ALT comprised a large proportion of individuals infected with HCV in the current study, it is important for these people to receive early antiviral treatment and regular follow-up to reduce the risk of developing HCC.

There are several limitations that should be considered in this study. First, HCV virological assessments including HCV RNA levels and HCV genotypes were not performed. Our conclusions drawn from HCV seroprevalence estimates was weakened by the limitation of antibody testing for distinguishing between past and current infection. Library testing was conducted in local township hospitals or community hospitals. However, HCV RNA and HCV genotypes tests require a full set of equipment and complicated techniques, which could not be achieved in these hospitals. Further studies need to be done to calculate the current HCV infection rate and HCV genotype distribution in Mianyang. Second, as this was a cross-sectional survey, we could only find that anti-HCV prevalence was associated with the age at the time of survey, but can't determine whether it is related to the year of birth or not. To better explore birth cohort effects, further research requires direct measurements or estimation of more representative birth-specific prevalence rates. Third, we did not collect enough information on the potential exposure to HCV infection, such as blood donation, family history of HCV infection, and dental therapy, so we could not determine how HCV spread in Mianyang. Despite these disadvantages, to the best of our knowledge, this is the largest seroepidemiological study concerning the prevalence of anti-HCV in the general population.

In conclusion, this large-scale cross-sectional study shows that anti-HCV prevalence among the general population in Mianyang is slightly higher than the national average prevalence. Insights from the analysis of this large-scale HCV seroepidemiological survey are fundamental to guide future HCV research and other interventions. Information from this study will also be useful for government to make policy for HCV treatment and prevention scientifically.

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References

1. Chen SL, Morgan TR.The natural history of hepatitis C virus (HCV) infection. Int J Med Sci. 2006; 3:47-52.
2. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med. 2001; 345:41-52.
3. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis. 2005; 5:558-567.
4. World Health Organization. Hepatitis C. *http://www.who.int/mediacentre/factsheets/fs164/en/* (accessed 27 April 2013).
5. China National Health and Family Planning Commission. 2012 Annual Mandatory Reportable Infectious Disease Summary. People's Republic of China National Health and Family Planning Commission, 2013. (in Chinese)
6. Sievert W, Altraif I, Razavi HA, *et al*. A systematic review of hepatitis C virus epidemiology in Asia, Australia and Egypt. Liver Int. 2011; 31:61-80.
7. Xia GL, Liu CB, Cao HL, Bi SL, Zhan MY, Su CA, Nan JH, Qi XQ. Prevalence of hepatitis B and C virus infections in the general Chinese population. Results from a nationwide cross-sectional seroepidemiologic study of hepatitis A, B, C, D, and E virus infections in China, 1992. Int Hepatol Commun. 1996; 5:62-73.
8. Lu J, Zhou YD, Lin XJ, Jiang YZ, Tian R, Zhang YH, Wu J, Zhang FW, Zhang Y, Wang Y, Bi S. General Epidemiological Parameters of Viral Hepatitis A, B, C, and E in Six Regions of China: A Cross-Sectional Study in 2007. PLoS ONE. 2009; 4:e8467.
9. Chen YS, Li L, Cui FQ, Xing WG, Wang L, Jia ZY, Zhou MG, Gong XH, Wang FZ, Zheng H, Luo HM, Bi SL, Wang N, Yang WZ, Liang XF. A sero-epidemiological study on hepatitis C in China. Zhonghua Liu Xing Bing XueZaZhi.2011; 32:888-891. (in Chinese)
10. Su Y, Norris JL, Zang C, Peng Z, Peng Z, Wang N. Incidence of hepatitis C virus infection in patients on haemodialysis: A systematic review and meta-analysis. Hemodial Int. 2013; 17:532-541.
11. Gao X, Cui Q, Shi X, Su J, Peng Z, Chen X, Lei N, Ding K, Wang L, Yu R, Wang N. Prevalence and trend of hepatitis C virus infection among blood donors in Chinese mainland: A systematic review and meta-analysis. BMC Infect Dis. 2011; 11:88.
12. Xia X, Luo J, Bai J, Yu R. Epidemiology of hepatitis C virus infection among injection drug users in China:

Systematic review and meta-analysis. Public Health. 2008; 122:990-1003.
13. Chen Y, Shen Z, Morano JP, Khoshnood K, Wu Z, Lan G, Zhu Q, Zhou Y, Tang S, Liu W, Chen J, Tang Z. Bridging the Epidemic: A Comprehensive Analysis of Prevalence and Correlates of HIV, Hepatitis C, and Syphilis, and Infection among Female Sex Workers in Guangxi Province, China. PLoS One. 2015; 10:e0115311.
14. Xu K, Zhu LG, Tang F, Bao CJ, Zhu YF, Cao MQ, Du GM, Xu JF, Peng H, Zhai XJ. Rate of infection and related risk factors on hepatitis C virus in three counties of Jiangsu province. Chin J Epidemiol. 2014; 35:1212-1217. (in Chinese)
15. Fox AN, Jacobson IM. Recent successes and noteworthy future prospects in the treatment of chronic hepatitis C. Clin Infect Dis. 2012; 55 (Suppl 1):S16-S24.
16. Qin Q, Smith MK, Wang L, Su Y, Wang L, Guo W, Wang L, Cui Y, Wang N. Hepatitis C virus infection in China: An emerging public health issue. J Viral Hepat. 2015; 22:238-244.
17. Denniston MM, Jiles RB, Drobeniuc J, Klevens RM, Ward JW, McQuillan GM, Holmberg SD. Chronic hepatitis C virus infection in the United States, National Health and Nutrition Examination Survey 2003 to 2010. Ann Intern Med. 2014; 160:293-300.
18. Smith BD, Morgan RL, Beckett GA, Falck-Ytter Y, Holtzman D, Teo CG, Jewett A, Baack B, Rein DB, Patel N, Alter M, Yartel A, Ward JW; Centers for Disease Control and Prevention. Recommendations for the identification of chronic hepatitis C virus infection among persons born during 1945-1965. MMWR Recomm Rep. 2012; 61:1-32.
19. Smith BD, Morgan RL, Beckett GA, Falck-Ytter Y, Holtzman D, Ward JW. Hepatitis C virus testing of persons born during 1945-1965: Recommendations from the Centers for Disease Control and Prevention. Ann Intern Med. 2012; 157:817-822.
20. Kim do Y, Kim IH, Jeong SH, *et al*. A nationwide seroepidemiology of hepatitis C virus infection in South Korea. Liver Int. 2013; 33:586-594.
21. Liu F, Chen K, He Z, Ning T, Pan Y, Cai H, Ke Y. Hepatitis C seroprevalence and associated risk factors, Anyang, China. Emerg Infect Dis. 2009 ; 15:1819-1822.
22. Uhanova J, Tate RB, Tataryn DJ, Minuk GY. A population-based study of the epidemiology of hepatitis C in a North American population. J Hepatol. 2012; 57:736-742.
23. Grebely J, Page K, Sacks-Davis R, *et al*. The effects of female sex, viral genotype, and IL28B genotype on spontaneous clearance of acute hepatitis C virus infection. Hepatology. 2014; 59:109-120.
24. Bakr I, Rekacewicz C, El Hosseiny M, Ismail S, El Daly M, El-Kafrawy S, Esmat G, Hamid MA, Mohamed MK, Fontanet A. Higher clearance of hepatitis C virus infection in females compared with males. Gut. 2006; 55:1183-1187.
25. Zhao Y, Shen L, Ma J, Gao Z, Han X, Qi S, Li Q. Epidemiology of Hepatitis C Virus Infection and Risk Factor Analysis in the Hebei Province, China. PLoS ONE. 2013; 8:e75586.
26. Qian HZ, Yang Z, Shi X, Gao J, Xu C, Wang L, Zhou K, Cui Y, Zheng X, Wu Z, Lu F, Lai S, Vermund SH, Shao Y, Wang N. Hepatitis C virus infection in former commercial plasma/blood donors in rural Shanxi Province, China: The China Integrated Programs for Research on AIDS. J Infect Dis. 2005 ; 192:1694-1700.
27. Huang C, Qiu F, Guo M, Yi Y, Shen L, Wang F, Jia Z, Ma J, Zhao Y, Zhang S, Zhang Y, Bi S. Prevalence and risk factors of hepatitis C among former blood donors in rural China. Int J Infect Dis. 2012; 16:e731-e734.
28. Shan H, Wang JX, Ren FR, Zhang YZ, Zhao HY, Gao GJ, Ji Y, Ness PM. Blood banking in China. Lancet. 2002; 360:1770-1775.
29. Chakravarti A, Verma V, Jain M, Kar P. Characteristics of dual infection of hepatitis B and C viruses among patients with chronic liver disease: A study from tertiary care hospital. Trop Gastroenterol. 2005; 26:183-187.
30. Mekky Ma, Nasr AM, Saleh MA, Wasif NK, Khalaf M, Aboalam H, Haredy M. Virologic and histologic characterisation of dual hepatitis B and C co-infection in Egyptian patients. Arab J Gastroenterol. 2013; 14:143-147.
31. Lee LP, Dai CY, Chuang WL, Chang WY, Hou NJ, Hsieh MY, Lin ZY, Chen SC, Hsieh MY, Wang LY, Chen TJ, Yu ML. Comparison of liver histopathology between chronic hepatitis C patients and chronic hepatitis B and C coinfected patients. J Gastroenterol Hepatol. 2007; 22:515-517.
32. Sagnelli E, Coppola N, Pisaturo M, Masiello A, Tonziello G, Sagnelli C, Messina V, Filippini P. HBV superinfection in HCV chronic carriers: A disease that is frequently severe but with the eradication of HCV. Hepatology. 2009; 49:1090-1097.
33. Prati D. Transmission of hepatitis C virus by blood transfusions and other medical procedures: A global review. J Hepatol. 2006; 45:607-616.
34. Hayashi J, Kashiwagi S, Ikematsu H, Nomura H, Kajiyama W, Noguchi A, Ikeda K. Sex- and age-specific prevalences of HBeAg and anti-HBe among HBsAg carriers with or without liver function abnormalities in Okinawa, Japan. MicrobiolImmunol. 1988; 8:843-850.
35. Chu CM, Sheen IS, Lin SM, Liaw YF. Sex difference in chronic hepatitis B virus infection: Studies of serum HBeAg and alanine aminotransferase levels in 10,431 asymptomatic Chinese HBsAg carriers. Clin Infect Dis. 1993; 16:709-713.
36. Chen P, Yu C, Ruan B, Yang S, Ren J, Xu W, Luo Z, Li L. Prevalence of hepatitis B in insular regions of southeast China: A community-based study. PLoS One. 2013; 8:e56444.
37. Suruki R, Hayashi K, Kusumoto K, Uto H, Ido A, Tsubouchi H, Stuver SO. Alanine aminotransferase level as a predictor of hepatitis C virus-associated hepatocellular carcinoma incidence in a community-based population in Japan. Int J Cancer. 2006; 119:192-195.
38. Tanaka H, Tsukuma H, Yamano H, Oshima A, Shibata H. Prospective study on the risk of hepatocellular carcinoma among hepatitis C virus-positive blood donors focusing on demographic factors, alanine aminotransferase level at donation and interaction with hepatitis B virus. Int J Cancer. 2004; 112:1075-1080.

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Original Article

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A comparative study of contrast enhanced ultrasound and contrast enhanced magnetic resonance imaging for the detection and characterization of hepatic hemangiomas

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Summary **This study aims to compare contrast enhanced ultrasound (CEUS) and contrast enhanced magnetic resonance imaging (CEMRI) for the detection and characterization of hepatic hemangiomas. Included in this retrospective study were 83 histopathologically confirmed lesions of hemangioma in 66 hospitalized patients who underwent both CEUS and CEMRI and received surgery. The enhancement patterns on CEUS and CEMRI in each lesion were compared and analyzed. In addition, data obtained by the two modalities were then compared with the pathological findings to determine their value in differential diagnosis of hepatic hemangiomas. CEUS diagnosed 78 lesions of hemangioma against 80 by CEMRI. There were no statistical significant differences in the diagnostic value between CEUS and CEMRI in terms of sensitivity (88.0% *vs*. 92.8%), specificity (99.0% *vs*. 99.4%), accuracy (97.3% *vs*. 98.4%), positive predictive value (93.6% *vs*. 96.3%), and negative predictive value (98.0% *vs*. 98.8%) ($p > 0.05$, all). In the arterial phase, the main enhancement pattern on both CEUS and CEMRI was peripheral nodular enhancement (73 *vs*. 76), but lesions with diffuse enhancement on CEUS outnumbered those on CEMRI (3 *vs*. 1) and lesions with circular enhancement on CEMRI outnumbered those on CEUS (3 *vs*. 2). In the portal venous phase and delayed phase, the main enhancement pattern was hyperechoic change on CEUS and hyperintense on CEMRI (66 *vs*. 65), some lesions presented isoechoic change (12 *vs*. 15). These results suggested CEUS, an equivalent to CEMRI, may have an added diagnostic value in hemangiomas.**

Keywords: Contrast enhanced ultrasound (CEUS), hepatic hemangiomas, contrast enhanced magnetic resonance imaging (CEMRI)

1. Introduction

Hepatic hemangioma, the most common benign hepatic tumor (*1*), accounts for 0.4-20% of all hepatic tumors (*2*). The vast majority of hemangioma cases present no specific clinical manifestations, and its diagnosis has depended mainly on such imaging tests as B-mode ultrasound scans (US), contrast enhanced ultrasound (CEUS), contrast enhanced computed tomography (CECT), and contrast enhanced magnetic resonance imaging (CEMRI).

B-mode US, noninvasive, economical, convenient and non-radioactive, has been found effective in detecting hepatic hemangiomas. On B-mode US, hemangiomas appear typically as a hyperechoic and

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well-defined lesion with or without small central regions of decreased echogenicity (*3*). However, the diagnostic accuracy of B-mode US in detecting hepatic hemangioma is low, usually only 46-60% (*3-7*), either because of its limitations in distinguishing different types of organization (*3,8*) or because of easy misdiagnosis of hypoechoic or mixed hyperechoic hepatic hemangioma as hepatic cancer (*5*) or because of the significantly increased difficulty of the ultrasound in detecting hepatic focal lesions in fatty liver (*9,10*). Color Doppler US can visualize intratumoral and peritumoral blood flow in 10-50% of hemangiomas (*4,11*). Since other focal liver lesions have the same characteristics as hemangioma, color Doppler does not improve the diagnostic sensitivity of the ultrasound. Its accuracy may also be limited by motion artifacts, inappropriate color scale settings, interference of heart beats in detecting lesions located in the left lobe, or inability to display gas-covered lesions in the right lobe. Therefore, B-mode US and color Doppler are limited in the characterization of hepatic hemangiomas (*4*).

CECT can accurately characterize most hemangiomas but has limitations in relation to the radiation exposure and its contraindications (*12*). Considered the gold standard in the diagnosis of hepatic hemangioma with a sensitivity of 90-100% and a specificity of 91-99% (*13,14*), CEMRI is also limited by extended scanning time. CEUS is a technically simple imaging modality that allows real-time acquisition without any of the drawbacks of contrast-enhanced MRI. CEUS with microbubble contrast agents and contrast-specific US modes have been introduced to overcome the limitations of B-mode and color Doppler US. Several studies compared the diagnostic value of CEUS and CEMRI in focal liver lesions (*15,16*), but reports about their use in specifying hepatic hemangiomas are yet to be found. In some other studies, CEMRI rather than pathological evaluation was used as the gold standard for the final diagnosis (*17,18*).

The objective of this study was to investigate, by comparing with pathological findings, the sensitivity and specificity of CEUS and CEMRI in hepatic hemangiomas.

2. Materials and Methods

2.1. *Subjects*

A retrospective review was performed on 763 focal liver lesions from 413 consecutive Chinese inpatients from January 2011 to July 2014. We identified a total of 83 histopathologically confirmed hemangioma lesions in 66 hospitalized patients (12 male and 54 female; aged 31-77 years old with an average of 51.3 ± 14.5 years) who underwent both CEUS and CEMRI and received surgical treatment. We excluded 3 lesions from 3 patients which were located at the subphrenic liver and could not be visualized with baseline US and CEUS. All patients included did not suffer from severe cardiovascular and/or cerebrovascular diseases and/or lung diseases. Among those patients, 31 had abdominal pain, 5 had acute abdominal pain resulting from bleeding within the tumor, and 22 had nausea, anorexia and early satiety. Eight patients were mistaken as malignant liver lesions. Of all the 66 patients, 55 had a single lesion, 7 had 2, 2 had 3, and 2 had 4, with their sizes ranging from 18.9 × 16.3 mm to 129.3 × 109.7 mm. As shown in Table 1, the characteristics of the size and location of 83 hemangiomas lesions were presented. After examination, the patients underwent sonographically guided core biopsies, regular or irregular hepatectomy or local lesion resection for all the target lesions for pathological diagnosis. Chronic liver diseases were observed in 4 patients, while liver tissues were found normal in all other patients.

The study was conducted under the approval and supervision of the ethics committee of Fudan University and the procedure followed was in accordance with the Declaration of Helsinki. After informed consent was obtained, CEUS followed by CEMRI were performed and all patients were monitored for adverse events for four hours after the procedure. The clinical status, blood pressure and heart rate were followed up.

2.2. *Ultrasound examinations*

Color Doppler ultrasound was performed using the Philips iU22 (Philips Ultrasound, Bothell, Washington, USA), ACUSON S2000 (Siemens Medical Solutions, Mountain View, CA, USA) and LOGIC E9 (GE, Healthcare, Milwaukee, WI, USA) ultrasound system, which is capable of real-time contrast-enhanced imaging. The 3.5 MHz transducer was used with a mechanical index (MI) of 0.06-0.09. The contrast agent used was SonoVue (Bracco, SpA, Milan, Italy), which was formulated into a suspension of sulphur

Table 1. Characteristics (location, number and size) of 83 hemangioma lesions

Lexicon	Lesion Number (*n*)
Anatomic site	
S II, S III	7
S IV	13
S I	3
S V, S VIII	21
S VI, S VII	25
S IV, S V, S VIII	4
S II, S III, S IV	2
S IV, S V, S VIII	4
S I, S IV	4
Size	
< 2 cm	3
2-5 cm	6
5-10 cm	20
> 10 cm	54

hexafluoride microbubbles (8 μL/mL) by adding 5 mL of physiological saline. A baseline ultrasound examination was performed to detect lesions and images were saved on a hard disk. For each lesion, we measured its size, position, border, shape, echogenicity, and blood flow. CEUS was performed with bolus administration, *via* cubital vein, with the contrast agent at a dose of 1.5-2.0 mL flushed with 5 mL saline. In patients with multiple lesions, an additional bolus of SonoVue (1.5-2.0 mL) was administered for each lesion at an interval of at least 15 min to allow for the clearance of the previous contrast injection. No contrast agent was appreciable either in the liver parenchyma or hemangiomas before starting a new examination. All the CEUS examinations were digitally recorded. The hemodynamic contrast enhancement was evaluated during three phases as defined by Guidelines and Good Clinical Practice Recommendations for Contrast Enhanced Ultrasound (CEUS) – Update 2008 (*19*): the arterial phase (within 40 sec), portal venous phase (40-120 sec) and delayed phase (120-300 sec).

2.3. *MRI examinations*

MRI was performed with a 1.5 T MR scanner (Siemens Magnetom Avanto, Germany) in combination with 8-channel phase array surface coils. Unenhanced fat-suppressed fast spin-echo (FS-FSE) T2WI was done with a slice thickness of 5.0 mm and a slice gap of 2.0 mm: one pre-contrast scan and three post-contrast scans. Gadobenate-dimeglumine (Gd-DTPA; MultiHance, Bracco, Milan, Italy) were administered through cubital vein at a dose of 0.2 mmol/kg at 3 mL/s. Multiple breath-hold contrast-enhanced imaging was performed at 20-25 s, 70-90 s and 120-180 s after the contrast injection.

2.4. *Image analysis and data evaluation*

All the conventional ultrasound images and CEUS video clips were reviewed independently by two experienced radiologists blinded to the final diagnosis and not involved in the scanning reviewed all cineloops off-line. They had respectively 6 and 9 years of experience in conventional liver US and more than 3 years of experience in liver CEUS interpretation. The other two experienced radiologists in CEMRI studies of the liver, blinded to the final diagnosis, recorded and analyzed changes in the dynamic enhanced images at different phases and made independent diagnoses and conclusions. In case of inconsistent conclusions, a mutually accepted final conclusion was made *via* consultation. Examiners engaged in CEUS and CEMRI were blind to each other's diagnosis. The echotexture or signal intensity from the lesion was identified as hyperechoic, isoechoic or hypoechoic in contrast with the surrounding hepatic parenchyma. Perfusion uniformity was observed to determine the homogeneity of the echoes. The filling defect referred to an area of non-perfusion in the lesion.

2.5. *CEUS criteria*

The characteristic findings of grayscale sonography in hepatic hemangioma included the regular shape, well-defined border, hyperechoic mass with/without the internal hypoechoic area, and presence/absence of posterior acoustic enhancement. Color Doppler US presented color flow inside or around the tumor lesions with RI < 0.6 (*20*). Based on the literature (*19,21*) and clinical experience, CEUS findings in hepatic hemangiomas were classified into three categories: *i*) nodular enhancement in arterial phase with gradual centripedal filling (Figure 1) and hyperechoic/isoechoic change in the portal venous phase and delayed phase; *ii*) peripheral circular enhancement in the arterial phase with continuous centripedal filling (Figure 2) and hyperechoic/isoechoic change in the portal venous phase and delayed phase; *iii*) diffuse enhancement in the arterial phase with hyperechoic change (Figure 3) in the portal venous phase and delayed phase. Diagnostic criteria for hepatic hemangioma were: A) focal liver lesion as indicated by the presence of CEUS features in categories *i* or *ii*; B) focal liver lesion as indicated by the presence of CEUS

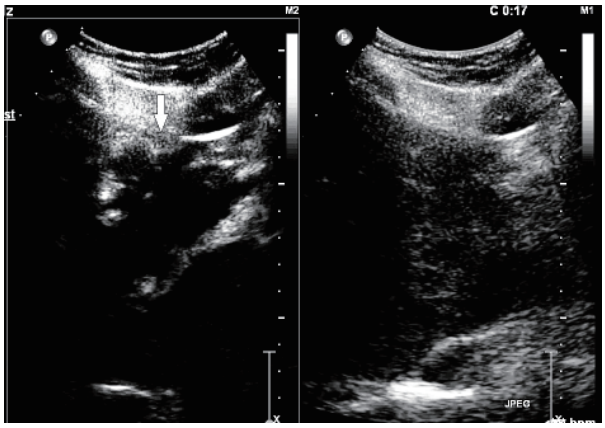

Figure 1. Category 1 of the enhancement pattern on CEUS: Peripheral nodular enhancement with continuous centripedal filling in arterial phase (arrow).

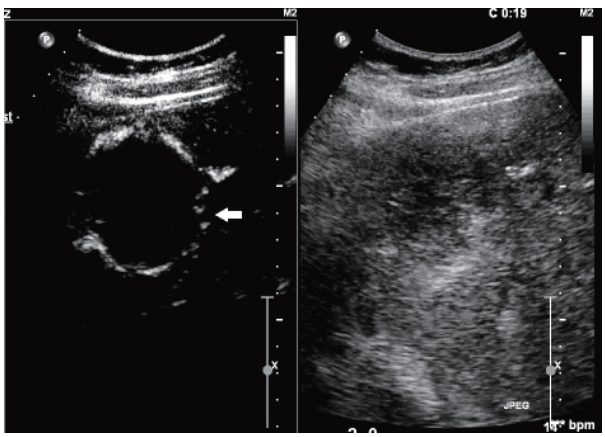

Figure 2. Category 2 of the enhancement pattern on CEUS: Peripheral circular enhancement with continuous centripedal filling in the arterial phase (arrow).

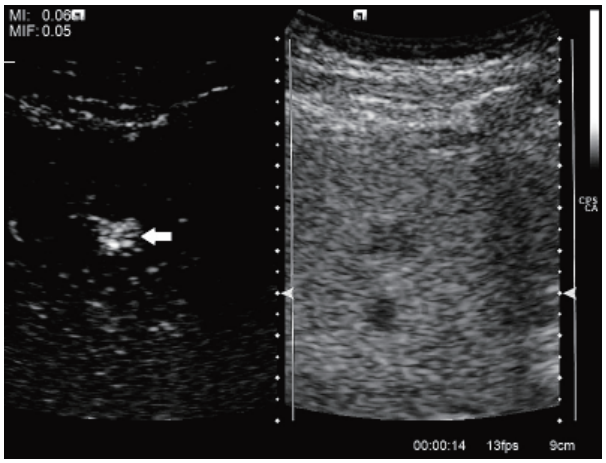

Figure 3. Category 3 of the enhancement pattern on CEUS: Diffuse enhancement with fast filling in the arterial phase (arrow).

features in categories *iii* and at least more than 5 features on conventional US.

2.6. *CEMRI criteria*

Unenhanced MRI features included regular shape, well-defined border, low signal intensity on T1WI and high signal intensity on T2WI. Characteristic CEMRI findings in hepatic hemangiomas were classified into three categories (*22,23*): *i*) nodular enhancement in the arterial phase with gradual cetripedal filling and hyperintense/isointense change in the portal venous phase and delayed phase; *ii*) peripheral circular enhancement in the arterial phase with continuous centripedal filling and hyperintense/isointense change in the portal venous phase and delayed phase; *iii*) diffuse enhancement in the arterial phase with hyperintense change in the portal venous phase and delayed phase. Diagnostic criteria for hepatic hemangioma were: A) focal liver lesion as indicated by the presence of CEMRI features in categories *i* or *ii*; B) focal liver lesion as indicated by the presence of CEUS features in category *iii* and at least more than 2 features on unenhanced MRI.

2.7. *Statistical analysis*

Chi-square tests were performed to analyze the sensitivity, specificity, accuracy, and the positive and negative predictive values of CEUS and CEMRI. The difference was considered statistically significant at a 2-tailed $p < 0.05$. The SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

3. Results

3.1. *CEUS and CEMRI findings*

CEUS showed peripheral nodular enhancement in the arterial phase in 73 confirmed lesions, among which 61 presented hyperechoic change and 12 presented isoechoic change in the portal venous phase and delayed phase. In 2 lesions, CEUS showed peripheral nodular enhancement in the arterial phase with continuous centripedal filling and hyperechoic change in the portal venous phase and delayed phase. In 3 lesions, CEUS showed diffuse enhancement in the arterial phase with hyperechoic change in the portal venous phase and delayed phase.

CEMRI showed nodular enhancement in the arterial phase with gradual cetripedal filling in 76 lesions, and 62 presented hyperechoic change and 15 isoechoic change in the portal venous phase and delayed phase. On CEMRI, 3 lesions showed peripheral circular enhancement in the arterial phase with continuous cetripedal filling and hyperechoic areas in the portal venous phase and delayed phase. One lesion presented diffuse enhancement in the arterial phase with hyperechoic change in the portal venous phase and delayed phase.

With CEUS, homogeneous perfusion was observed in 35 lesions, heterogeneous perfusion in 48, and filling defect in 46 against 42, 41, and 51, respectively, with CEMRI.

Table 2. Diagnostic performances of the CEUS and CEMRI as compared with histopathology

Modality	SE	SP	AC	PPV	NPV
CEUS	88.0% (73/83)	99.0% (475/480)	97.3% (548/563)	93.6% (73/78)	98.0% (475/485)
CEMRI	92.8% (77/83)	99.4% (477/480)	98.4% (554/563)	96.3% (77/80)	98.8% (477/483)
p	0.293	0.478	0.216	0.446	0.317

SE, sensitivity; SP, specificity; AC, accuracy; PPV, positive predictive value; NPV, negative predictive value.

3.2. *Diagnostic performance of CEUS and CEMRI against histopathology in hemangiomas*

Of all the 763 lesions recorded from January 2011 to July 2014, CEMRI identified 80 as hepatic hemangiomas and CEUS identified 78 as hepatic hemangiomas. The performance of CEUS in hepatic hemangiomas was close to that of CEMRI in terms of sensitivity (88.0% *vs*. 92.8%), specificity (99.0% *vs*. 99.4%), accuracy (97.3% *vs*. 98.4%), positive predictive value (93.6% *vs*. 96.3%), and negative predictive value (98.0% *vs*. 98.8%). There was no statistically significant difference between the two modalities ($p > 0.05$ for all) (Table 2).

4. Discussion

Imaging such as US, CT, and MRI are currently the most often used diagnostic modalities for hepatic hemangioma. In recent years, CEUS has been increasingly used in clinical medicine. The European Guidelines state that typical features of hepatic hemangiomas on CEUS include continuous centripetal filling in arterial phase

and hyperechoic change in the portal venous phase and delayed phase, a pattern which can be summed as "fast in slow out". In our current study, CEUS showed continuous centripetal filling with peripheral nodular and circular enhancement in 81 lesions of which 75 were pathologically confirmed as hemangiomas, showing a strong agreement with the European Guidelines. Of 81 lesions in question, 61 presented hyperechoic change in the portal venous phase and delayed phase, an observation attributable to the histological similarity between the benign tumor and normal liver parenchyma: presence of the blood sinus in both. However, isoechoic change in the portal venous phase and delayed phase was observed in 12 lesions, which differed from the European Guidelines. This might be accounted for by the rupture of the microbubbles caused by the prolonged exposure to sound waves of the lesions and their vicinity in particular. In addition, a small number (n = 3) of lesions presented diffuse enhancement in the arterial phase on CEUS, which is an atypical perfusion pattern. Some authors thought that diffuse enhancement in the arterial phase was associated with tumor size (usually < 3 cm) (*24*), while others thought that dynamic contrast enhancement patterns were associated with size of the blood vessels rather than tumor size (*25*). E. Quaia suggested that diffuse enhancement on CEUS occurs more easily in cirrhotic patients (*21*). The small number of the lesions with diffuse enhancement warrants further investigation using bigger samples. Filling defect was observed in 46 lesions on CEUS and 51 on CEMRI. Filling defect may be due to tumor sizes (all > 4 cm) and presence of thrombosis or fibrosis.

Similar enhancement patterns, featuring mainly peripheral nodular enhancement, were observed in 83 lesions on CEUS (88.0%, 73/83) and on CEMRI (92.8%, 77/83). There was no significant difference between the two modalities. More lesions, however, presented circular enhancement on CEMRI rather than CEUS (3 *vs*. 2). This may be due to the nature of the contrast agent used. SonoVue, a blood pool contrast agent, does not extravasate into the extravascular space and is, therefore, capable of reflecting the blood supply to and hemodynamics of the tumor lesion. Varied blood supply to different types of tumor results in different patterns of enhancement.

Gd-DTPA used in dynamic CEMRI is a non-tissue-specific extracellular contrast agent whose distribution in a tissue depends on the blood supply to the tissue and microvascular permeability. The principles of the two imaging modalities are different and the distribution and metabolism of the contrast agents in body tissues are also different. CEUS enjoys an edge over CEMRI for its real-time operation where the sonographer is able to observe the whole process of the contrast agent entering and leaving the lesion, while enhancement information on CEMRI may be missed by the radiologist as it is collected dynamically at the different phases. However, CEUS is limited in that observation of the enhancement and decline can be made only on one plane of the lesion after a single injection of the contrast agent, a disadvantage for CEMRI.

CEMRI, with a sensitivity of more than 90% and a specificity as high as 91-99%, is now considered the most accurate noninvasive imaging modality in diagnosing hepatic hemangioma (*13*). The characteristic manifestations of the hepatic hemangioma on CEMRI slices include low signal intensity on T1WI and high signal intensity on T2WI, peripheral nodular enhancement in the arterial phase with gradual centripedal filling, which is in agreement with the findings in this current study. Recently, CEUS has been playing an increasingly important role in the diagnosis of focal liver lesions. CECT or CEMRI or liver biopsy was taken as the gold standard in a multicenter study by Tranquart F. *etc*., in which CEUS was performed in 874 patients with 1,034 liver focal lesions (*26*). The study found that CEUS showed a sensitivity of 85.4% and a specificity of 93.7% against a sensitivity of 94.0% and a specificity of 96.4% respectively in this study, ours being obviously higher. This may be accounted for by the fact that, in the French study, pathological findings were not used as the gold standard in all patients, and using findings on CECT or CEMRI as the gold standard decreased sensitivity and specificity. In a comparative study of CEUS and CEMRI, Kristina Žvinienė (*18*) reported that CEUS as a diagnostic imaging modality for hepatic hemangioma was comparable to CEMRI in terms of specificity and positive predictive value but obviously underperformed the latter in terms of sensitivity and negative predictive value. However, the study in question was limited because evaluation of the two modalities could not be objective when pathological findings were not employed for the final assessment. This current study, employing pathological findings as the gold standard, compared CEUS and CEMRI in terms of sensitivity (88.0% *vs*. 92.8%), specificity (99.0% *vs*. 99.4%), accuracy (97.3% *vs*. 98.4%), positive predictive value (93.6% *vs*. 96.3%), and negative predictive value (98.0% *vs*. 98.8%). There were no statistical differences among the five value pairs. Therefore, CEUS is very likely to become an independent diagnostic imaging modality for hepatic hemangioma. However, lesions located deep at the subphrenic liver can still present a diagnostic dilemma even for CEUS. We excluded 3 lesions from 3 patients because of their location. Also, the typical characteristics of liver hemangiomas on CEUS were lost when cirrhosis was present, especially in small lesions (*24*). In our study, 2 lesions presented diffuse enhancement in the arterial phase with hypoechoic change in the portal venous phase and delayed phase on CEUS (Figures 4A-4D). This was explained by the presence of fibrosclerosis and vascular wall structure in the lesion on histologic analysis.

Two limitations are mentionable. First, our study was

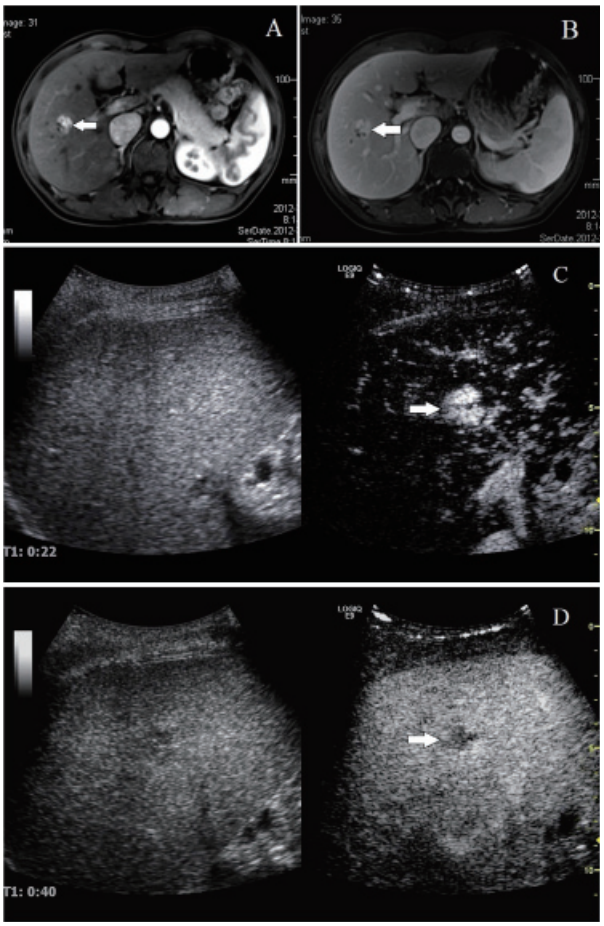

Figure 4. Liver hemangiomas on CEMRI, but malignant tumor on CEUS. (A) MRI: Peripheral enhancement with centripedal filling in the arterial phase (arrow). **(B)** MRI: High signal intensity with low-intensity signals from vascular structure in the portal venous phase (arrow). **(C)** CEUS: Diffuse enhancement in the arterial phase with fast filling (arrow). **(D)** CEUS: Hypoechoic change in the arterial phase (arrow).

retrospective in nature, and we will proceed with a future prospective analysis for the same purpose. Second, we did not explore the relationship between the determined sizes and contrast-enhancement patterns, which warrants further investigation.

In conclusion, CEUS and CEMRI play an equally important diagnostic role for hepatic hemangioma. CEUS serves as a substitute for CEMRI when the latter is impossible in claustrophobic patients or in patients with a pacemaker or metal foreign bodies or metal implants in the body. A small number of cases of atypical hepatic hemangioma require employment of the two imaging modalities as justified by the patient's medical history, clinical manifestations and findings in laboratory tests. Such comprehensive judgment by the radiologist, sonographer, and clinician improves the diagnosis of hepatic hemangioma.

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References

1. Feldman M. Hemangioma of the liver; special reference to its association with cysts of the liver and pancreas. Am J Clin Pathol. 1958; 29:160-162.
2. Caturelli E, Pompili M, Bartolucci F, Siena DA, Sperandeo M, Andriulli A, Bisceglia M. Hemangiomalike lesions in chronic liver disease: Diagnostic evaluation in patients. Radiology. 2001; 220:337-342.
3. Vilgrain V, Boulos L, Vullierme MP, Denys A, Terris B, Menu Y. Imaging of atypical hemangiomas of the liver with pathologic correlation. Radiographics. 2000; 20:379-397.
4. De Franco A, Monteforte MG, Maresca G, De Gaetano AM, Manfredi R, Marano P. Integrated diagnosis of liver angioma: Comparison of Doppler color ultrasonography, computerized tomography, and magnetic resonance. Radiol Med. 1997; 93:87-94.
5. Leifer DM, Middleton WD, Teefey SA, Menias CO, Leahy JR. Follow-up of patients at low risk for hepatic malignancy with a characteristic hemangioma at US. Radiology. 2000; 214:167-172.
6. Vilgrain V, Uzan F, Brancatelli G, Federle MP, Zappa M, Menu Y. Prevalence of hepatic hemangioma in patients with focal nodular hyperplasia: MR imaging analysis. Radiology. 2003; 229:75-79.
7. Quaia E, Bertolotto M, Dalla Palma L. Characterization of liver hemangiomas with pulse inversion harmonic imaging. Eur Radiol. 2002; 12:537-544.
8. Weskott HP. Emerging roles for contrast-enhanced ultrasound. Clin Hemorheol Microcirc. 2008; 40:51-71.
9. Bartolotta TV, Taibbi A, Galia M, Runza G, Matranga D, Midiri M, Lagalla R. Characterization of hypoechoic focal hepatic lesions in patients with fatty liver: Diagnostic performance and confidence of contrast-enhanced ultrasound. Eur Radiol. 2007; 17:650-661.
10. Wang ZL, Tang J, Weskott HP, Li JL, Wang W, Luo YK, An LC, Xu JH. Undetermined focal liver lesions on gray-scale ultrasound in patients with fatty liver: Characterization with contrast-enhanced ultrasound. J Gastroenterol Hepatol. 2008; 23:1511-1519.
11. Perkins AB, Imam K, Smith WJ, Cronan JJ. Color and power Doppler sonography of liver hemangiomas: A dream unfulfilled? J Clin Ultrasound. 2000; 28:159-165.
12. Chan MG, Cassidy FH, Andre MP, Chu P, Aganovic L. Delayed imaging in routine CT examinations of the abdomen and pelvis: Is it worth the additional cost of radiation and time? AJR Am J Roentgenol. 2014; 202:329-335.
13. Lee MG, Baker ME, Sostman HD, Spritzer CE, Paine S, Paulson EK, Keogan MT. The diagnostic accuracy/efficacy of MRI in differentiating hepatic hemangiomas from metastatic colorectal/breast carcinoma: A multiple reader ROC analysis using a jackknife technique. J Comput Assist Tomogr. 1996; 20:905-913.
14. Taseva A, Tasev V, Bulanov D, Dimitrov K, Popov V, Zivkov E, Dimitrova V. Diagnosis of liver hemangioma. Khirurgiia (Sofiia). 2013; 3:8-13.
15. Sirli R, Sporea I, Popescu A, Dănilă M, Martie A, Bota S, Jurchis A, Sendroiu M. Contrast enhanced ultrasound for the diagnosis of liver hemangiomas in clinical practice. Med Ultrason. 2011; 13:95-101.
16. Ryu SW, Bok GH, Jang JY, *et al*. Clinically useful diagnostic tool of contrast enhanced ultrasonography for

focal liver masses: Comparison to computed tomography and magnetic resonance imaging. Gut Liver. 2014; 8:292-297.
17. Bartolotta TV, Taibbi A, Galia M, Lo Re G, La Grutta L, Grassi R, Midiri M. Centrifugal (inside-out) enhancement of liver hemangiomas: A possible atypical appearance on contrast-enhanced US. Eur J Radiol. 2007; 64:447-455.
18. Zviniene K, Zaboriene I, Basevicius A, Jurkiene N, Barauskas G, Pundzius J. Comparative diagnostic value of contrast-enhanced ultrasonography, computed tomography, and magnetic resonance imaging in diagnosis of hepatic hemangiomas. Medicina (Kaunas). 2010; 46:329-335.
19. Claudon M, Cosgrove D, Albrecht T, *et al*. Guidelines and good clinical practice recommendations for contrast enhanced ultrasound (CEUS) – Update 2008. Ultraschall Med. 2008; 29:28-44.
20. Wang Y, Wang WP, Ding H, Huang BJ, Mao F, Xu ZZ. Resistance index in differential diagnosis of liver lesions by color doppler ultrasonography. World J Gastroenterol. 2004; 10:965-967.
21. Quaia E, Bartolotta TV, Midiri M, Cernic S, Belgrano M, Cova M. Analysis of different contrast enhancement patterns after microbubble-based contrast agent injection in liver hemangiomas with atypical appearance on baseline scan. Abdom Imaging. 2006; 31:59-64.
22. Semelka RC, Brown ED, Ascher SM, Patt RH, Bagley AS, Li W, Edelman RR, Shoenut JP, Brown JJ. Hepatic hemangiomas: A multi-institutional study of appearance on T2-weighted and serial gadolinium-enhanced gradient-echo MR images. Radiology. 1994; 192:401-406.
23. Leslie DF, Johnson CD, MacCarty RL, Ward EM, Ilstrup DM, Harmsen WS. Single-pass CT of hepatic tumors: Value of globular enhancement in distinguishing hemangiomas from hypervascular metastases. AJR Am J Roentgenol. 1995; 165:1403-1406.
24. Brancatelli G, Federle MP, Blachar A, Grazioli L. Hemangioma in the cirrhotic liver: Diagnosis and natural history. Radiology. 2001; 219:69-74.
25. Takayasu K, Moriyama N, Shima Y, Muramatsu Y, Yamada T, Makuuchi M, Yamasaki S, Hirohashi S. Atypical radiographic findings in hepatic cavernous hemangioma: Correlation with histologic features. AJR Am J Roentgenol. 1986; 146:1149-1153.
26. Tranquart F, Le Gouge A, Correas JM, *et al*. Role of contrast-enhanced ultrasound in the blinded assessment of focal lesions in comparison with MDCT and CEMRI: Results from a multicentre clinical trial. EJC SUPPL. 2008; 6:9-15.

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Brief Report

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Hepatitis B virus promotes autophagic degradation but not replication in autophagosome

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Summary **In this study, we investigate the relationship of hepatitis B virus (HBV) infection and autophagy. HepG2 cells and HepG2 cells infected with HBV (HepG2.2.15) were transfected with GFP-LC3 (green fluorescence protein conjugated with microtubule-associated protein 1 light chain 3) expression vector and autophagy status was then examined with confocal microscope. HepG2.2.15 cells were further treated with serum-free medium or 3-methyladenine (3-MA), and subjected to Hepatitis B core antigen (HBcAg), Hepatitis B surface antigen (HBsAg), or hepatitis B polymerase protein detection by immunohistochemistry. Localization of the GFP-LC3 and the HBV proteins was observed by confocal fluorescence microscope. The level of SQSTM1/p62 protein was also evaluated by Western blot analysis. In contrast to a diffuse distribution in HepG2 cells, GFP-LC3 formed distinct punctate dots, which were further enhanced by nutritional starvation, in HepG2.2.15 cells. The expression of hepatitis B polymerase and HBcAg, but not HBsAg, was positively correlated with the autophagic intensity. However, no co-localizations were observed between HBV proteins and autophagosomes. Suppression of autophagy reduced the expression of hepatitis B polymerase and HBcAg, but not HBsAg. Western blot showed that SQSTM1/p62 protein level was declined in HepG2.2.15 cells comparing HepG2 cells, and further reduced while upon serum starvation. In conclusion, HBV infection induces autophagic degradation and autophagy. Autophagy is critical for HBV replication. However HBV replication does not take place in autophagosomes.**

Keywords: Hepatitis B virus, autophagy, virus replication, HepG2 cell

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers worldwide, ranking the 5th highest in morbidity and the 3rd highest in mortality among various cancers (*1*). Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection is found to be associated with nearly 80% of HCC (*2*). HCC is particularly troublesome in China since over 55% new HCC cases worldwide were discovered in China, strongly due to the presence of 93 million HBV carriers (*3,4*). However, the pathogenesis of HBV and its underlying mechanisms for carcinogenesis is not well understood. Thus, this lack of understanding hinders the effective therapeutic approaches for curing HBV infections or decreasing its contribution to carcinogenesis.

Autophagy is a conserved cellular process in which double-membraned vesicles are formed, containing excess or dysfunctional proteins as well as cellular

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organelles, and delivered to lysosomal machinery for degradation. It is a highly regulated process that is essential for maintaining cellular homeostasis in response to multiple stress signals, including nutrient starvation, growth factor deprivation, genotoxic stress, and hypoxia. Autophagy is also known as one of the host defense responses against infections. It has been shown that autophagy is involved in selectively targeting intracellular pathogens, leading to their direct elimination (*5-9*). It also plays a role in the MHC class II antigen presenting for foreign antigens (*10*). Thus, some bacteria and viruses developed strategies to suppress or bypass cellular autophagy to ensure their survival (*11*). Some viruses, such as poliovirus, rhinoviruses, mouse hepatitis virus, SARS-CoV, even hijacked the components of autophagic pathway to facilitate their own replication (*12,13*).

It has been demonstrated that HBV induces autophagy and uses it to facilitate its DNA replication. This process is mediated by HBV X (HBx) protein, which binds to and activates class III phosphatidylinositol-3-kinase (C3-PI3K), an enzyme important for autophagy initiation (*14*). There is also evidence showing that HBx protein can up-regulate the expression of Beclin 1, the mammalian orthologue of the yeast autophagy protein Apg6 (*15*). However, the mechanism of how the autophagy contributes to viral DNA replication remains unclear.

In this study, we explored the role of autophagosomes in HBV replication by observation of autophagosomes and HBV with a confocal microscope. Meanwhile, SQSTM1/p62, one of the proteins involved in autophagy, was analyzed by western blotting for further understanding the function of autophagy.

2. Materials and Methods

2.1. *Strain, plasmid, and cell lines*

Competent *Escherichia coli* strain DH5α was purchased from Transgen Biotech Corp. (Beijing, China). Expression plasmid pEGFP-C1-LC3 and the HepG2 cell line were kindly provided by Prof. Hongbing Zhang. The HepG2.2.15 cell line was obtained from Peking University Hepatology Institute, which was transfected with HBV genome and thus stably express HBV proteins and produce virions.

2.2. *Cell culture and transfection*

Both HepG2 and HepG2.2.15 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. The HepG2.2.15 cultural medium has additions of G418 (Wako, Japan) for a final concentration of 380 μg/mL. The pEGFP-C1-LC3 transfection was performed using Lipofectamine 2000 (Invitrogen, CA, USA).

2.3. *Immunofluorescence*

HepG2.2.15 cells plated on coverslips were washed with PBS and fixed with 4% (v/v) formaldehyde in PBS for 10 min at room temperature. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature followed by blocking in TBS-T solution (25 mM Tris, pH 7.4, 3.0 mM KCl, 140 mM NaCl and 0.05% Tween 20) containing 5% (w/v) BSA (FirstLink, UK) for 30 min. Cells were incubated with one of the primary antibodies, anti-HBcAg (Abcam, CA, USA), anti-HBsAg (Abcam), or anti-hepatitis B polymerase antibody (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Goat anti-mouse IgG antibody conjugated to Cy3 (Beyotime Institute of Biotech, China) was used as a secondary antibody. Post-staining images were taken with FLUOVIEW10-ASW laser scanning confocal microscope (Olympus, Japan).

2.4. *Western blot analysis*

Cell lysates were subjected to SDS/PAGE on a 4-15% gradient gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat dry milk in Tris-buffered saline, the membrane was incubated with primary rabbit monoclonal antibody against P62 protein (Santa Cruz Biotechnology), followed by a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). Subsequently, the blot was developed using an ECL detection kit (Thermo, CA, USA).

3. Results and Discussion

3.1. *HBV replication induces autophagic vacuole formation*

Autophagy initiates with the formation of phagophore (an isolation membrane). One of the components involved in this early event is the protein LC3, which becomes lipidated (LC3-II) and associates with the newly formed double-membrane vesicles. It has been shown that exogenous GFP-LC3 (green fluorescence protein conjugated with microtubule-associated protein 1 light chain 3) behaves in a similar fashion as does endogenous LC3, upon autophagy induction. We took advantage of the GFP-LC3 fusion protein, and studied whether continual expression of HBV proteins leads to autophagy induction in HepG2 cells. We transfected the plasmid expressing GFP-LC3 into both HepG2 and HepG2.2.15 cells, and studied the distribution of the GFP signals in the cells through fluorescence microscopy. A diffused distribution of GFP signal was observed in HepG2 cells (Figure 1A) that was consistent with the distribution of the soluble form of LC3 in the absence of autophagy. The GFP signal in HepG2.2.15 cells, however, appeared as punctuated dots much

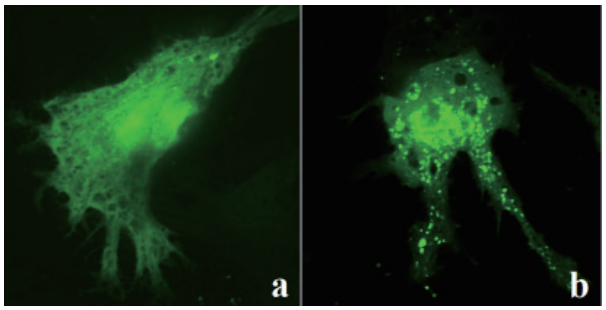

Figure 1. Induction of autophagic vacuoles by HBV. HepG2 cells (**panel a**) and HepG2.2.15 cells (**panel b**) were transfected with pEGFP-C1-LC3. Images were taken at 24 h post-transfection. The Green color was shown in a diffused pattern in HepG2 cells and in punctate dots in HepG2.2.15 cells. The punctate dots represent formation of autophagic vacuoles.

different than what was observed in HepG2 cells (Figure 1B). The punctuated localization is consistent with the observation that during autophagy the lipidated LC3 is associated with the autophagosomal membrane. The production of HBV by HepG2.2.15 cells was verified by FQ-PCR (data not shown). These observations indicated that the presence of HBV proteins induce autophagy in host cells.

This autophagy can be further augmented by nutrient starvation, one of the many common autophagy-inducing signals. HepG2.2.15 cells expressing GFP-LC3 were cultured in serum-free DMEM medium for 24 h. As shown in Figure 2, the intensity of green fluorescence produced from GFP-LC3 became stronger and more punctuated than those observed in cells cultured under normal conditions (comparing panels **a** and **d**). This indicated that nutrient starvation could further induce autophagy process in cells with an already elevated autophagy level due to virus expression.

HBV induced autophagy can be abolished by 3-methyladenine (3-MA), an inhibitor for type III PI3 kinase Vps34. When GFP-LC3 expressing HepG2.2.15 cells were treated with 10 mM 3-MA (Sigma, CA, USA), the distribution of green fluorescence became more diffused than that of the untreated control, suggesting localization of LC3 is more cytosolic (Figure 2 comparing panels **g** and **d**).

3.2. *The expression of HBsAg, HBcAg, and hepatitis B polymerase and their localization*

We then used immunofluorescence to study whether autophagy is required for the expression of HBV proteins, specifically that of hepatitis B polymerase, HBcAg, and HBsAg.

We did not find significant changes in the expression of hepatitis B polymerase or HBcAg when autophagy is further enhanced by starvation. The intensities in fluorescence reflecting the abundances of protein were not significantly different in cells growing in normal conditions or starved conditions (Figures 2A and 2B,

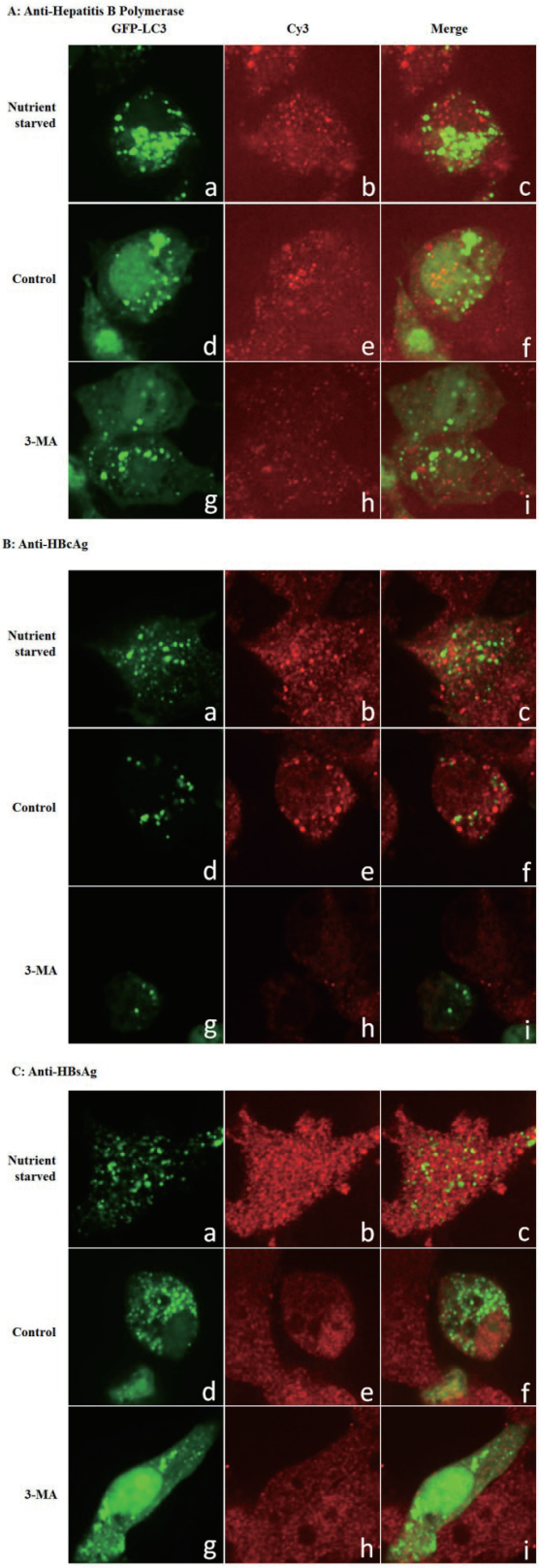

Figure 2. The effect of autophagy on HBV protein expressions. HepG2.2.15 cells transfected with pEGFP-C1-LC3 were cultured in serum-free DMEM medium, normal DMEM medium as control, or with 3-MA in DMEM medium. HBV protein expressions were detected with antibodies against hepatitis B polymerase **(A)**, HBcAg **(B)** or HBsAg **(C)** and subsequent with the Cy3-conjugated goat anti-mouse secondary antibody. Localization of autophagic vacuoles and HBV was observed by merging panels between GFP-LC3 (green) and Cy3 (red) signals.

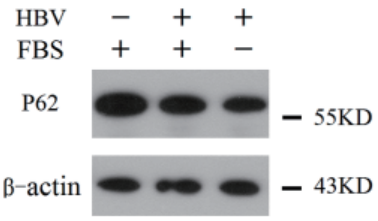

Figure 3. The autophagy induced by HBV leads autophagic degradation. HepG2 cells were cultured in normal DMEM medium (**lane 1**), while HepG2.2.15 cells were cultured either in normal DMEM medium (**lane 2**) or serum-free DMEM medium (**lane 3**). Cells were lysed for Western-blot analysis using the rabbit monoclonal antibody against P62 protein antibody (top panel). Actin (bottom panel) was also shown as a loading control.

comparing panels **b** and **e**). However, the abundances of both proteins reduced in cells where autophagy is inhibited by 3-MA (Figures 2A and 2B, comparing panels **e** and **h**). HBsAg expression seems to be different. We found that the fluorescence intensity of HBsAg is increased during starvation, when autophagy is enhanced in addition to the induction by HBV. Inhibition of autophagy by 3-MA has little effect on its expression (Figure 2C, comparing panel **e** and **h**).

It is reported that several human viruses, such as HCV, use autophagic vesicles as membranous support for translation of viral protein and enhancement of viral RNA replication (*12,13,16*). We investigated whether HBV similarly uses the autophagic vesicles as its location of replication. The merged images between GFP-LC3 and antibody staining for viral proteins, HBV polymerase, HBcAg, or HBsAg showed that the membrane-associated LC3 does not co-localize with viral proteins, suggesting that HBV may not occupy the autophagic vesicles as its location for viral protein translation and DNA replication (Figure 2, panels **c**, **f**, and **i**).

3.3. *The autophagy induced by HBV leads autophagic degradation*

SQSTM1/p62 protein is an ubiquitin-binding scaffold protein that binds directly to LC3 *via* the LC3 interacting region (LIR), and interacts with ubiquitinated proteins through the C-terminal ubiquitin associated (UBA) domains. SQSTM1/p62 can link ubiquitinated proteins to autophagosome for degradation, and itself can also be degraded through autophagy (*17*). The cellular levels of SQSTM1/p62 vary with the induction or inhibition of autophagy; it therefore often serves as a marker for autophagic degradation. We studied whether autophagy induced by HBV leads to autophagic degradation by detecting the SQSTM1/p62 protein level with Western blot. Analysis showed that SQSTM1/p62 protein level was decreased in HepG2.2.15 cells comparing to HepG2 cells, and it was further reduced in HepG2.2.15 cells cultured in serum-free DMEM medium (Figure 3). This indicated that autophagy induced by HBV leads to autophagic degradation.

Autophagy is a protective process that recycles damaged organelles and unwanted proteins to ensure cell viability under starvation and stress conditions. Increasing amounts of evidence has shown that it is also involved in regulating innate and adaptive immunity. It is particularly important in the cellular defense against intracellular pathogens, in which xenophagy recognizes intracellular microbes and targets them to the autophagy pathway for degradation. Thus, many pathogens, including viruses, have developed strategies for self-protection and further manipulate or utilize autophagy to their own advantages. For example, Epstein-Barr virus (EBV) and Kaposi's Sarcoma-Associated Herpes Virus (KSHV) negatively regulate autophagy during latency, whereas poliovirus (*18,19*), porcine reproductive and respiratory syndrome virus (PRRSV) (*20*), encephalomyocarditis virus (*21*), and picornavirus (*22*), utilize components of the autophagy pathway to promote viral replication. Some of the viruses use the autophagosome as a membrane structure to support its replication as well as to hijack it as a mechanism for releasing the packaged virions in a non-lytic manner. This promotes the fusion of the autophagosome with the plasma membrane (*12,19,21,23,24*). While viruses promote autophagy induction, it has been shown that they have developed mechanisms to inhibit the maturation of autophagosomes by preventing the fusion between the autophagosome and the lysosome (*19,20*). However, recent findings also indicate that some RNA viruses benefit from autophagosome maturation. For example, acidification of the autophagasome is required for poliovirus particle assembly and virion maturation (*18*).

Our study showed that autophagy could be induced in HepG2 cells when the HBV replication became persistent in the cells, and this autophagy could be further enhanced by nutrient starvation. This is consistent with the previous findings that HBV induces autophagy in host cells *via* its HBx protein (*14*). Several studies showed that HBx interacts with C3-PI3K to initiate the autophagy process and the induction of autophagy is required for HBV DNA replication *in vitro* (*14*). *In vivo*, blocking autophagy by knocking out Atg5 greatly reduced HBV DNA replication (*25*). Our data also suggested that the induction of autophagy was necessary for HBV gene expression. The expressions of HBcAg and Hepatitis DNA polymerase were reduced when the autophagy was blocked by 3-MA, although they were not further increased when the enhanced autophagy was induced by starvation in addition to HBV. However, the expression of HBsAg was a little different. We found that inhibition of autophagy by 3-MA had little effect on the expression of HBsAg, whereas starvation led to the increase of HBsAg expression. It is not clear why the modulation of HBsAg expression is different. As

it is known that HBsAg expressions in active hepatitis patients were found in a few discrete hepatocytes with a diffuse cytoplasmic distribution, it is possible that HBsAg expression is not directly related to viral replication cycle (*26*).

Unlike several RNA viruses like HCV, HBV does not appear to replicate in autophagosome. We found that viral proteins, such as HBcAg, hepatitis B polymerase, or HBsAg did not localize in the autophagosome; *i.e*. there was no co-localization between viral proteins and autophagosome marker LC3. This observation suggests that HBV may not replicate and assemble in the autophagosome.

In contrast to previous observations, we found that SQSTM1/p62 level was reduced in HepG2.2.15 cells, and was further reduced in cells cultured in starvation condition (*14,27*). Our data indicated that the fusion of autophagosome with lysosome was not blocked in HBV infected cells. Part of the reason for this discrepancy may possibly due to the use of different hepatoma cell lines in our study. It is also possible, particularly in this case that HBV does not need use autophagosome as replication location, that there might be certain mechanisms that virus can selectively block protein degradation in the autophagosome based on its own needs.

This study reconfirmed several previous findings but the exact role of autophagy in HBV life cycle is still not clear. Sir *et al*. reported that autophagy could be induced by HBx protein and was required for efficient viral DNA replication; however, blocking the fusion of autophagosome and lysosome lead to reduced level of viral RNA but had little effect on viral DNA production (*14*). Li *et al*. showed that autophagy was needed for enveloped virion production at the ER, but had only slight effect on HBV DNA replication (*27*). Lazar C *et al*. reported that ER degradation-enhancing, mannosidase-like proteins (EDEM) were up-regulated in HepG2.2.15 cells, and the presence of viral surface proteins and EDEM1 led to the degradation of envelope proteins through autophagy. They suggested that it might be the mechanism for HBV to control the level of virions produced in infected cells and to establish chronic infection (*28*).

In summary, our study suggested that autophagy was induced by HBV, and was required for the viral proteins HBcAg and hepatitis B polymerase expression. However, HBV did not block the protein degradation through an autophagy pathway, and neither did it use the autophagosome as its site for DNA replication or virion assembly. Whether the protein degradation through autophagy pathway plays any role in facilitating the HBV life cycle needs further investigation.

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References

1. Su IJ, Hsieh WC, Tsai HW, Wu HC. Chemoprevention and novel therapy for hepatocellular carcinoma associated with chronic hepatitis B virus infection. Hepatobiliary Surg Nutr. 2013; 2:37-39.
2. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology. 2012; 142:1264-1273 e1.
3. 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.
4. Miao R, Luo H, Zhou H, *et al*. Identification of prognostic biomarkers in hepatitis B virus-related hepatocellular carcinoma and stratification by integrative multi-omics analysis. J Hepatol. 2014; 61:840-849.
5. Huang J, Brumell JH. Autophagy in immunity against intracellular bacteria. Curr Top Microbiol Immunol. 2009; 335:189-215.
6. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell. 2004; 119:753-766.
7. Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S, Yoshimori T. Autophagy defends cells against invading group A Streptococcus. Science. 2004; 306:1037-1040.
8. Cao X, Liu B, Cao W, Zhang W, Zhang F, Zhao H, Meng R, Zhang L, Niu R, Hao X, Zhang B. Autophagy inhibition enhances apigenin-induced apoptosis in human breast cancer cells. Chin J Cancer Res. 2013; 25:212-222.
9. Facciorusso A, Antonino M, Del Prete V, Neve V, Scavo MP, Barone M. Are hematopoietic stem cells involved in hepatocarcinogenesis? Hepatobiliary Surg Nutr. 2014; 3:199-206.
10. Schmid D, Munz C. Innate and adaptive immunity through autophagy. Immunity. 2007; 27:11-21.
11. Kirkegaard K, Taylor MP, Jackson WT. Cellular autophagy: Surrender, avoidance and subversion by microorganisms. Nat Rev Microbiol. 2004; 2:301-314.
12. Jackson WT, Giddings TH, Jr., Taylor MP, Mulinyawe S, Rabinovitch M, Kopito RR, Kirkegaard K. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol. 2005; 3:e156.
13. Prentice E, Jerome WG, Yoshimori T, Mizushima N, Denison MR. Coronavirus replication complex formation utilizes components of cellular autophagy. J Biol Chem. 2004; 279:10136-10141.
14. Sir D, Tian Y, Chen WL, Ann DK, Yen TS, Ou JH. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proc Natl Acad Sci U S A. 2010; 107:4383-4388.
15. Tang H, Da L, Mao Y, Li Y, Li D, Xu Z, Li F, Wang Y,

Tiollais P, Li T, Zhao M. Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy *via* up-regulation of beclin 1 expression. Hepatology. 2009; 49:60-71.
16. Suhy DA, Giddings TH, Jr., Kirkegaard K. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: An autophagy-like origin for virus-induced vesicles. J Virol. 2000; 74:8953-8965.
17. Moscat J, Diaz-Meco MT. p62 at the crossroads of autophagy, apoptosis, and cancer. Cell. 2009; 137:1001-1004.
18. Richards AL, Jackson WT. Intracellular vesicle acidification promotes maturation of infectious poliovirus particles. PLoS Pathog. 2012; 8:e1003046.
19. Taylor MP, Jackson WT. Viruses and arrested autophagosome development. Autophagy. 2009; 5:870-871.
20. Sun MX, Huang L, Wang R, Yu YL, Li C, Li PP, Hu XC, Hao HP, Ishag HA, Mao X. Porcine reproductive and respiratory syndrome virus induces autophagy to promote virus replication. Autophagy. 2012; 8:1434-1447.
21. Zhang Y, Li Z, Ge X, Guo X, Yang H. Autophagy promotes the replication of encephalomyocarditis virus in host cells. Autophagy. 2011; 7:613-628.
22. Klein KA, Jackson WT. Picornavirus subversion of the autophagy pathway. Viruses. 2011; 3:1549-1561.
23. Le Sage V, Banfield BW. Dysregulation of autophagy in murine fibroblasts resistant to HSV-1 infection. PLoS One. 2012; 7:e42636.
24. Weiskirchen R, Tacke F. Cellular and molecular functions of hepatic stellate cells in inflammatory responses and liver immunology. Hepatobiliary Surg Nutr. 2014; 3:344-363.
25. Tian Y, Sir D, Kuo CF, Ann DK, Ou JH. Autophagy required for hepatitis B virus replication in transgenic mice. J Virol. 2011; 85:13453-13456.
26. Nakopoulou L, Adraskelas N, Stefanaki K, Zacharoulis D, Hadziyannis S. Expression of HBsAg and HBcAg in liver tissue: Correlation with disease activity. Histol Histopathol. 1992; 7:493-499.
27. Li J, Liu Y, Wang Z, Liu K, Wang Y, Liu J, Ding H, Yuan Z. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J Virol. 2011; 85:6319-6333.
28. Lazar C, Macovei A, Petrescu S, Branza-Nichita N. Activation of ERAD pathway by human hepatitis B virus modulates viral and subviral particle production. PLoS One. 2012; 7:e34169.

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Brief Report

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C35 is overexpressed in colorectal cancer and is associated tumor invasion and metastasis

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Summary **The aim of this study was to investigate the expression of *C35*, an oncogene previously found in breast and prostate cancers, and its clinicopathological significance in colorectal cancer (CRC). Qualitative and quantitative detection of C35 mRNA expression was performed using reverse transcription-PCR (RT-PCR) and real-time PCR. C35 protein expression was determined using immunohistochemistry. C35 mRNA was detected in none of 10 normal colorectal tissue samples, 55 of 65 (84.6%) CRC tissue samples, and 43 of 55 (78.2%) adjacent non-cancerous tissue samples. In addition, the level of C35 mRNA in CRC tissue samples was markedly higher than that in tumor adjacent non-cancerous tissue samples. C35 protein expression was detected in 58 of 80 (72.5%) CRC tissue samples and was closely associated with tumor serosal invasion, lymphnode metastasis, and an advanced Dukes stage. These results suggest that C35 might serve as a biomarker or therapeutic target for management of CRC.**

Keywords: Therapeutic target, biomarker, invasion and metastasis, cancer

1. Introduction

According to statistics from the World Health Organization published in 2012, colorectal cancer (CRC) was the third most common form of cancer in men (746,000 patients, 10.0% of all patients with cancer) and the second in women (614,000 patients, 9.2% of all patients with cancer) worldwide (*1*). In China, the incidence of CRC has been increasing in recent years as living conditions improve and eating habits change, with both sexes accounting for an estimated 253,000 patients in 2012 (*1*). Early detection and treatment of CRC is critical and is significantly associated with patient prognosis (*2*). Cancers that are confined within the wall of the colon may be cured with surgery while a tumor that has already spread is usually not curable and chemotherapy instead focuses on improving the patient's quality of life and symptoms (*3-5*). Individualized treatment with targeted agents such as cetuximab has many advantages over traditional cytotoxic agents and represents the future of CRC treatment (*6*). Accordingly, identification and characterization of cancer-specific biomarkers has enormous potential to facilitate detection and treatment of CRC.

The *C35* gene, located on human chromosome 17q12, encodes a 12 kDa membrane-anchored protein that functions as an oncogene in several types of cancer (*7-10*). Evans *et al*. found that C35 was highly expressed in breast carcinoma in comparison to adjacent normal breast epithelium (*11*). The same study also examined its expression in 38 normal human tissue samples, including blood, bone marrow, and gastrointestinal tissue, and it found that none of the normal tissue samples were positive for C35 expression, with the exception of Leydig cells in the testes (*11*). Similarly, a study by Vishwanatha *et al*. found that C35 was highly expressed in prostate cancer cell lines and tumors but minimally expressed in normal prostate cells and tissues (*8*). The difference in expression in cancerous tissue and normal tissue suggests that C35 may play a role in transforming healthy cells in cancer initiation and progression and thus has potential value in cancer management (*11*). The expression profile of C35 in CRC is not yet known. The present study examined C35 expression in CRC and investigated its clinicopathological significance.

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2. Materials and Methods

2.1. *Materials*

Cancer tissue samples and tumor-adjacent tissue samples were obtained via open surgery or endoscopic surgery from the Third Affiliated Hospital of Shandong Academy of Medical Sciences with the informed consent of patients. These samples were subjected to reverse transcription-PCR (RT-PCR) and real-time PCR. Cancer tissue samples and normal colorectal tissue samples were also obtained from King Med Diagnostics (Ji'nan, Shandong, China). These samples were subjected to immunohistochemistry. The study protocol was approved by the Hospital Ethics Review Committee. None of the patients received preoperative treatment.

2.2. *RT-PCR*

Total RNA was isolated using an RNAprep pure Tissue Kit (Tiangen Biotechco., Beijing, China). The OD260/OD280 ratio of total RNA extracted from tissue samples ranged from 1.8 to 2.0. First-strand cDNA synthesis was done using TIANScript RT Kit (Tiangen Biotechco.). Primer sequences were as follows: for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), sense, 5'-GACTCACCCTGCCCTCAATA-3'; antisense, 5'-CCCTGTAGCCTGGACCTGAT-3'; for C35, sense, 5'-GCCATCCGAAGAGCCAGTA-3'; antisense, 5'-ATTACCGAGGCGAAGAGTGG-3'. PCR cycling parameters were: at 95°C for 3 min for pre-denaturation, followed by 40 cycles at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, and a final cycle at 72°C for 5 min for extension. GAPDH was used as an internal control. PCR products (10 μL) were analyzed electrophoretically using 2% agarose gel electrophoresis and viewed under ultraviolet (UV) light. The remaining products were sent to Shanghai Sangon Biotech for sequencing.

2.3. *Real-time PCR*

Real-time PCR was performed on all of the cDNA synthesized from samples with *C35* gene expression. Primers were the same as used in RT-PCR. Real-time PCR was performed using a SYBR Green Kit (Takara Biotechco., Dalian, Liaoning, China). The reaction system included 10 μL of SYBR® Premix Ex Taq (2×) (Tli RnaseH Plus), 0.4 μL of PCR forward primer, 0.4 μL of PCR reverse primer, 0.4 μL of ROX Reference Dye (50×) II, 2 μL of sample, and 6.8 μL of dH2O. PCR cycling parameters were: at 95°C for 3 min for pre-denaturation, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec. Amplification was performed with an ABI 7500 Sequence Detection System. All reactions were performed in triplicate, and GAPDH served as an internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which the amplified product is first detected and the values are expressed as the ratio of the target to the control.

2.4. *Immunohistochemistry*

In total, 80 paraffin-embedded colorectal carcinoma (grade 1, 2, and 3) and 10 normal tissue samples were received from King Med Diagnostics. None of the patients were undergoing chemotherapy or radiotherapy before radical surgery. Patient age ranged from 46 to 87 years, with a median of 67 years and a mean age of 69 years. Sections were viewed using light microscopy and staging was confirmed independently by two qualified pathologists. Mouse anti-XTP4 monoclonal antibody (Abcam, Cambridge, MA, USA) and a GTvisionTMIII anti-mouse universal immunohistochemical detection kit (Gene Tech Company, Shanghai) were used in this study. Immunohistochemistry studies were performed using a Maxvision two-step method. Consecutive 3-μm sections from paraffin-embedded tissue were deparaffinized, hydrated, and rinsed in distilled H_2O. The sections were then boiled in citrate buffer (pH 6.0) in a high pressure cooker for 5 min and cooled to room temperature. Afterwards, they were washed 4 times with phosphate-buffered saline (PBS; pH 7.4) for 3 min. All of the sections were then incubated with the primary mouse anti-human monoclonal anti-XTP4 antibody (1:100 diluted) overnight at 4°C. The next day, the sections were washed 4 times in PBS for 3 min, and they were then incubated with anti-mouse IgG antibody for 25 min. Afterwards, the sections were washed 4 times with PBS for 3 min. DAB chromogenic reagent was dropped on sections and staining was observed under a light microscope. After staining, the sections were rinsed in distilled water to stop the reaction. After counterstaining with hematoxylin, the sections were dehydrated and mounted. PBS instead of the primary antibody was used as a negative control.

A staining index was devised by multiplying the intensity of positive staining by the proportion of stained cells. The staining intensity was graded using the following standard: 0 for unstained cells, 1 for cells stained yellow, 2 for cells stained orange, and 3 for cells stained brown. The proportion of stained cells was based on the following criteria: 0 for ≤ 5% staining, 1 for 6-25%, 2 for 26-50%, 3 for 51-75%, and 4 for > 75% staining. The staining scores of 0-1 point were considered negative, scores of 2-4 points were considered weakly positive, scores of 5-8 points were considered moderately positive, and scores of 9-12 points were considered strongly positive.

2.5. *Statistical analyses*

Statistical analysis was performed using the Statistical

Package for the Social Sciences (SPSS), version 17.0 (USA). Statistical analysis of RT-PCR results was performed using a Mann-Whitney U test. A possible association between positive expression of C35 and clinicopathological parameters was compared using a χ^2 test or Fisher's exact test. Differences with a p less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. *Qualitative detection of C35 mRNA expression*

RT-PCR was used to examine C35 mRNA expression in normal colorectal tissue samples (n = 10), colorectal carcinoma tissue samples (n = 65), and tumor-adjacent tissue samples (n = 55). Results indicated that 55 of 65 (84.6%) colorectal carcinoma tissue samples and 43 of 55 (78.2%) tumor-adjacent tissue samples were positive for C35 expression (Figure 1). In contrast, none of the normal colorectal tissue samples were positive for C35 expression (Figure 1).

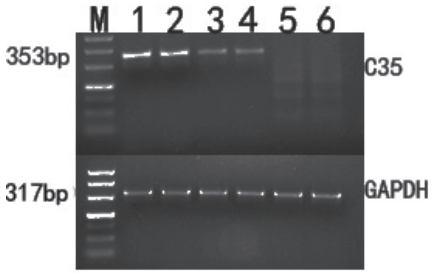

Figure 1. RT-PCR analysis of C35 mRNA expression. M, DNA marker; **1** and **2**, colorectal cancer tissue; **3** and **4**, paired adjacent-tumor tissue; **5** and **6**, normal colorectal tissue. GAPDH was used as internal control for parallel analysis of the same sample. Products from amplification of target mRNA (about 353 bp) and GAPDH mRNA (about 317 bp) were analyzed electrophoretically using 2% agarose gel electrophoresis followed by gel extraction and sequencing. Products from amplification of target mRNA were consistent with the C35 gene sequence.

3.2. *Quantitative detection of C35 mRNA expression*

Real-time PCR was performed to further explore whether *C35* gene expression differs in cancer tissue and adjacent tissue. Specimens that contained both cancer tissue and adjacent tissue (n = 50) were selected, and mRNA expression was determined and compared in cancerous and adjacent non-cancerous tissues. Results indicated that the level of C35 mRNA in cancer tissue was 5- to 10-fold higher than that in adjacent tissue. The level of C35 mRNA differed significantly in cancerous and adjacent non-cancerous tissues (p < 0.05).

3.3. *C35 protein expression and its clinicopathological significance*

Immunohistochemistry was used to examine C35 protein expression in 80 CRC tissue samples and 10 normal colorectal tissue samples. Results indicated that there was no C35 expression in normal colorectal tissue. In contrast, 58 of 80 CRC tissue samples (72.5%) displayed varying levels of C35 expression (Figure 2). C35 protein was mainly expressed in the cytoplasm, appearing as brown granules (Figure 2). The association between C35 expression and clinicopathological parameters was analyzed. C35 expression was more prevalent in CRC with serosal

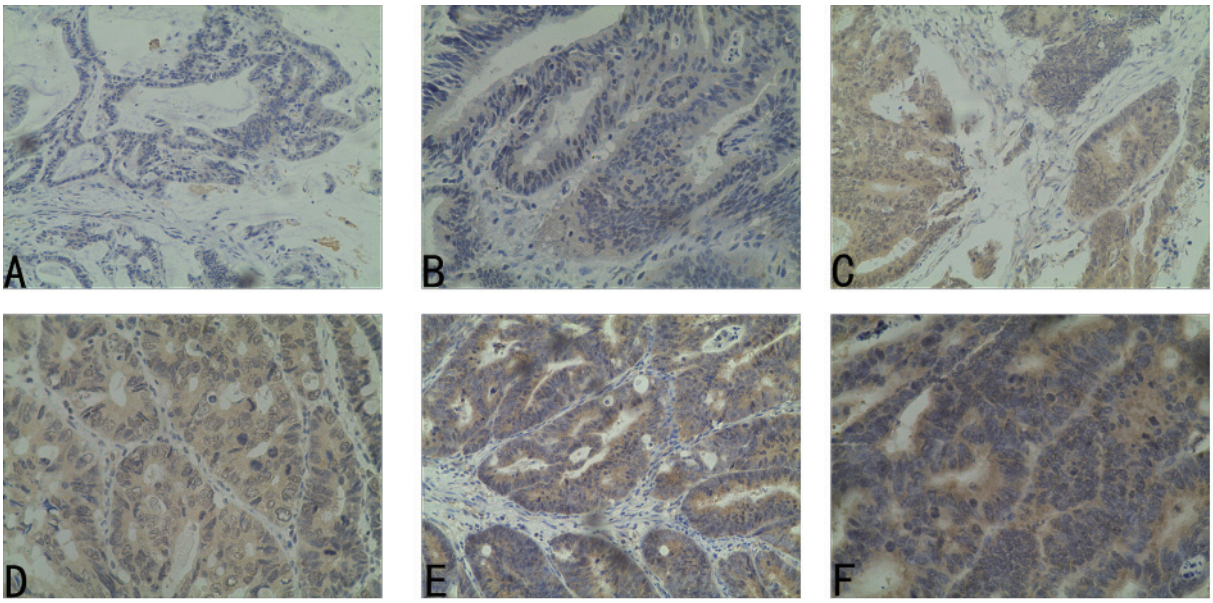

Figure 2. Expression of positivity for the C35 protein as determined by immunohistochemical staining of CRC tissue. A and **B**, weakly positive expression in CRC tissue samples (SP ×100, SP ×200); **C** and **D**, moderately positive expression in CRC tissue samples (SP ×100, SP ×200); **E** and **F**, strongly positive expression in CRC tissue samples (SP ×100, SP ×200).

Table 1. Association between C35 expression and clinicopathological parameters for colorectal cancer

Clinicopathological parameters	C35			Positive rate (%)	χ^2	*p* value
	n	+	-			
Gender						
Male	44	32	12	79.5	0.003	0.960
Female	36	26	10	72.2		
Age (years)						
< 60	18	16	2	88.9	2.158	0.142
≥ 60	62	42	20	67.7		
Tumor location						
Colon	42	32	10	76.2	0.604	0.437
Rectum	38	26	12	68.4		
Tumor diameter (cm)						
< 4	24	18	6	75.0	0.107	0.743
≥ 4	56	40	16	71.4		
Differentiation						
Well differentiated	49	34	15	69.1	0.615	0.433
Poorly differentiated	31	24	7	77.4		
Serosal invasion						
No	35	20	15	57.1	7.36	0.007**
Yes	45	38	7	84.4		
Lymphnode metastasis						
No	58	39	19	67.2	4.615	0.032*
Yes	22	20	2	90.9		
Dukes stage						
A+B	36	22	14	61.1	4.258	0.039*
C+D	44	36	8	81.8		

*$p < 0.05$; **$p < 0.01$

invasion (84.4%) than in CRC without serosal invasion (57.1%) (p = 0.007). In addition, CRC with lymphnode metastasis tested positive for C35 expression (90.9%) at a significantly higher rate than did CRC without lymphnode metastasis (67.2%) ($p = 0.032$). A higher level of C35 expression was noted with an advanced Dukes stage (C + D, 81.8%) in comparison to an early Dukes stage (A + B, 61.1%) ($p = 0.039$). There was no significant association between C35 expression and other clinicopathological parameters such as gender, age, tumor location, tumor diameter, and tumor differentiation ($p > 0.05$) (Table 1).

The present study investigated the expression profile of C35 at both the mRNA and protein levels in CRC tissue, adjacent non-cancerous tissue, and normal colorectal tissue. C35 mRNA was detected in none of 10 normal colorectal tissue samples, 55 of 65 (84.6%) CRC tissue samples, and 43 of 55 (78.2%) adjacent non-cancerous tissue samples. The level of C35 mRNA in CRC tissue was markedly higher than that in adjacent non-cancerous tissue. C35 protein expression was detected in 58 of 80 CRC tissue samples and was associated with tumor serosal invasion, lymphnode metastasis, and an advanced Dukes stage. These results suggest that C35 might have potential to serve as a biomarker or therapeutic target for CRC management.

The present study found that C35 expression was associated with invasion and metastasis of CRC, which suggests that C35 might play a role in this process. Previous studies indicated that overexpression of C35 in prostate cancer functionally enhanced migration and invasion of prostate cells (*8*). In contrast, downregulation of C35 by RNA interference reduced migration and invasion of prostate cells (*8*). The underlying mechanism for changes in C35 expression are a result of C35 acting as a signaling molecule to increase the invasive potential of prostate cancer cells by activating NF-κB-mediated downstream target genes, including matrix metalloproteinase 9, urokinase plasminogen activator, and vascular endothelial growth factor (*8*). These findings may explain why C35 expression is more prevalent in invasive CRC and they may provide clues for further research into the role of C35 and its underlying mechanism in the progression of CRC.

The present results indicated that C35 is predominantly expressed in CRC tissue in comparison to normal colorectal tissue. Thus, C35 may serve as a target for therapy. Sequencing of C35 revealed a 'CaaX' prenylation motif consisting of the last four amino acids, 'CVIL,' at the C-terminal end (*8*). The 'CaaX' group of proteins are known to be farnesylated by the enzyme farnesyltransferase or geranylgeranylated by the enzyme geranylgeranyl transferase type I (GGTase-I) (*12*). If 'X' is leucine, then protein is preferentially modified by the enzyme GGTase-I, suggesting C35 is prenylated preferentially by the enzyme GGTase-I (*12*). Key aspects of the prenylation of a 'CaaX'-type protein are its location at the cytoplasmic face of cellular membranes and its action to promote cell proliferation, migration, invasion, and metastasis (*13*). Therefore, interference with C35 prenylation may suppress that action and thus inhibit the progression of CRC. This indicates the potential for using GGTase-I inhibitors in CRC therapy. A point worth noting is that stains like simvastatin are capable of inhibiting the proliferation and migration of colon cancer cells (*14,15*), while geranylgeraniol, which can be taken up by cells and converted to geranylgeranyl diphosphate, may reverse the anti-tumor effects of stains (*15*). These findings suggest that the effects of stains on colon cancer cells might be mediated by interfering with geranylgeranylation of certain proteins. The effect of statins on C35 and the efficacy of these drugs on CRC should be studied in the future.

In conclusion, the present results indicated that C35 is predominantly expressed in CRC tissue in comparison to normal colorectal tissue and tumor-adjacent non-cancerous tissue. Furthermore, its expression is closely associated with invasive behavior by CRC. These results suggest the potential for utilizing C35 as a biomarker or therapeutic target for CRC management. C35 expression should be analyzed in a larger sample, and its biological action in CRC progression and drugs targeting this molecule should be studied in the future.

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References

1. GLOBOCAN 2012. World Health Organization. *http://globocan.iarc.fr/Default.aspx* (accessed February 21, 2015).
2. De Divitiis C, Nasti G, Montano M, Fisichella R, Iaffaioli RV, Berretta M. Prognostic and predictive response factors in colorectal cancer patients: Between hope and reality. World J Gastroenterol. 2014; 20:15049-15059.
3. Nieder C, Haukland E, Mannsaker B, Pawinski A, Dalhaug A. Early palliative radiation therapy in patients with newly diagnosed cancer: Reasons, clinical practice, and survival. Pract Radiat Oncol. 2015. DOI:10.1016/j.prro.2015.02.008.
4. Zhang W, Song T. The progress in adjuvant therapy after curative resection of liver metastasis from colorectal cancer. Drug Discov Ther. 2014; 8:194-200.
5. Saiura A, Yamamoto J, Hasegawa K, Oba M, Takayama T, Miyagawa S, Ijichi M, Teruya M, Yoshimi F, Kawasaki S, Koyama H, Makuuchi M, Kokudo N. A combination of oral uracil-tegafur plus leucovorin (UFT + LV) is a safe regimen for adjuvant chemotherapy after hepatectomy in patients with colorectal cancer: Safety report of the UFT/LV study. Drug Discov Ther. 2014; 8:48-56.
6. Duffy MJ. Personalized treatment for patients with colorectal cancer: Role of biomarkers. Biomark Med. 2015; 9:337-347.
7. Katz E, Dubois-Marshall S, Sims AH, Faratian D, Li J, Smith ES, Quinn JA, Edward M, Meehan RR, Evans EE, Langdon SP, Harrison DJ. A gene on the HER2 amplicon, C35, is an oncogene in breast cancer whose actions are prevented by inhibition of Syk. Br J Cancer. 2010; 103:401-410.
8. Dasgupta S, Wasson LM, Rauniyar N, Prokai L, Borejdo J, Vishwanatha JK. Novel gene C17orf37 in 17q12 amplicon promotes migration and invasion of prostate cancer cells. Oncogene. 2009; 28:2860-2872.
9. Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N. Colorectal cancer. Lancet. 2010; 375:1030-1047.
10. Liu QQ, Yin K, Zhu S, Zhang L, Wen PE, Li CL, Zhang DB, Liu M, Yan G. Inhibition of C35 gene expression by small interfering RNA induces apoptosis of breast cancer cells. Biosci Trends. 2010; 4:254-259.
11. Evans EE, Henn AD, Jonason A, Paris MJ, Schiffhauer LM, Borrello MA, Smith ES, Sahasrabudhe DM, Zauderer M. C35 (C17orf37) is a novel tumor biomarker abundantly expressed in breast cancer. Mol Cancer Ther. 2006; 5:2919-2930.
12. Fu HW, Casey PJ. Enzymology and biology of CaaX protein prenylation. Recent Prog Horm Res. 1999; 54:315-342; discussion 342-313.
13. Winter-Vann AM, Casey PJ. Post-prenylation-processing enzymes as new targets in oncogenesis. Nat Rev Cancer. 2005; 5:405-412.
14. Cho SJ, Kim JS, Kim JM, Lee JY, Jung HC, Song IS. Simvastatin induces apoptosis in human colon cancer cells and in tumor xenografts, and attenuates colitis-associated colon cancer in mice. Int J Cancer. 2008; 123:951-957.
15. Al-Haidari AA, Syk I, Thorlacius H. HMG-CoA reductase regulates CCL17-induced colon cancer cell migration via geranylgeranylation and RhoA activation. Biochem Biophys Res Commun. 2014; 446:68-72.

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Brief Report

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Upregulation of Bcl-2 enhances secretion of growth factors by adipose-derived stem cells deprived of oxygen and glucose

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Summary **There is an increasing recognition that beneficial effects of adipose-derived stem cell (ADSC) therapy may depend largely on the secretion of multiple growth factors. This study modified ADSCs with the *Bcl-2* gene in order to increase the secretion of growth factors during oxygen-glucose deprivation (OGD). The phenotypes of human ADSCs that were passaged 4 times were analyzed using flow cytometry. Then, ADSCs were genetically modified with *Bcl-2* and *Bcl-2* gene transduction was verified with Western blotting. Proliferative capacity and multipotent differentiation properties were evaluated in Bcl-2-modified ADSCs. Secretion of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) was evaluated using an enzyme-linked immunosorbent assay (ELISA) during OGD. Human ADSCs that were passaged 4 times expressed stem cell-associated markers but not a fibroblast marker or a hematopoietic stem cell marker. The *Bcl-2* gene was efficiently transfected into ADSCs; Bcl-2 modification did not affect the proliferative and multilineage differentiation capacity of ADSCs. In addition, Bcl-2 overexpression enhanced the secretion of VEGF, bFGF, and HGF by 14.47%, 16.9%, and 91%, respectively, compared to ADSCs alone that were deprived of oxygen and glucose. These data suggest that Bcl-2 overexpression enhances secretion of angiogenic growth factors by ADSCs deprived of oxygen and glucose.**

Keywords: Adipose-derived stem cells (ADSCs), Bcl-2, gene transduction, growth factor

1. Introduction

Human adipose-derived stem cells (ADSCs) are adult pluripotent stem cells. Their use in stem cell therapy has attracted increasing attention in recent years (*1-3*). ADSCs have the potential to differentiate into multiple lineages, such as bone, cartilage, tendons, nerves, and fat when cultivated under lineage-specific conditions (*4-5*). Compared to bone marrow mesenchymal stem cells, ADSCs can be easily obtained in large amounts with less donor site morbidity and discomfort (*6-8*). Because of their convenient isolation, high level of auto-reproducibility, pluripotentiality, and immunosuppressive properties, ADSCs are increasingly recognized as a promising source of human stem cells for cell therapy. ADSCs have been used in basic and clinical studies for the treatment of many diseases (*9-13*). The therapeutic mechanism of ADSCs in cell therapy is based on their ability to directly differentiate into tissue specific cells as well as the paracrine actions of factors released from ADSCs (*14-16*). However, the harsh microenvironment results in poor viability for transplanted cells, and this may significantly limit the paracrine capacity of ADSCs. Therefore, protecting ADSCs from apoptosis and improving their paracrine function is critical to a successful therapy based on ADSCs.

The Bcl-2 antiapoptotic protein is a member of the Bcl-2 family, which plays an important role in regulating the mitochondrial apoptotic pathway to inhibit cell death (*17*). Bcl-2 overexpression can delay the occurrence of cell death (*18*). Bcl-2 deficiency leads to a loss of a death repressor in specific cells (*19*). As indicated by a previous study (*20*), Bcl-2 overexpression significantly reduces ADSC apoptosis. ADSCs are known to enhance

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the secretion of angiogenic and antiapoptotic factors under hypoxic conditions (*16*). The present study sought to investigate the secretion of growth factors by Bcl-2-modified ADSCs under ischemic conditions induced by oxygen-glucose deprivation (OGD) in an *in vitro* model.

2. Materials and Methods

2.1. *Isolation and culturing of human ADSCs*

Samples of human adipose tissue were obtained from three healthy female donors undergoing thigh liposuction. Informed consent was obtained from these donors and the study protocol was approved by an institutional review board. The aspirated fat was washed with PBS and then digested with 0.1% collagenase type I (Gibco, Life Technologies, CA, USA). The digested tissue was centrifuged to separate the stromal cell fraction from adipocytes and connective tissues. The cells were resuspended with complete Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Keygentec, Nanjing, China). The cells were passed through a 200-μm mesh and then centrifuged. The isolated cells were resuspended in complete DMEM, placed into 25-cm^2 flasks, and cultured at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced every 3 days. Cells were passaged at a ratio of 1:2 per week. Only cells that were passaged 4 times were used in this study.

2.2. *Characterization of human ADSCs*

ADSCs were harvested by treatment with 0.25% trypsin/EDTA (Gibco) and then resuspended at a total number of 2×10^6 cells in 500 μL PBS. ADSCs were labeled for 15 min at 4°C with the following monoclonal antibodies: anti-CD29-FITC (eBioscience, San Diego, CA, USA), anti-CD34-FITC (BD Biosciences, San Diego, CA, USA), anti-CD44-PE (BD Biosciences), anti-CD90-FITC (BD Biosciences), anti-CD133 (293C3)-PE (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), or HLA-DR-FITC (BD Biosciences). The cells were thoroughly washed with PBS before analysis. FITC- or PE-labeled mouse IgG (Biolegend, San Diego, CA, USA) was used as the isotype control. The cells were then analyzed with a BDFACSCanto flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data were analyzed with the Cell Quest software package (Becton Dickinson, Bedford, Mass, USA).

2.3. *Gene transduction in ADSCs*

ADSCs that had been passaged 4 times were seeded in a six-well plate at 1×10^5 cells/well. At 80% confluence, the ADSCs were infected with an adenovirus encoding the enhanced green fluorescent protein gene (*Ad/EGFP*) at different multiplicities of infection (MOI = 0, 100, 200, and 500). At 48 h post-transduction, the transduction efficiency of the *EGFP* gene was examined under a fluorescence microscope (ZEISS, Germany) and with flow cytometry. The optimal MOI was used for *Bcl-2* gene delivery into ADSCs. ADSCs without gene transduction and those transfected with adenovirus carrying *Bcl-2* (Ad/Bcl-2) or adenovirus alone were termed "ADSCs", "Bcl-2-ADSCs", and "vector-ADSCs", respectively. Levels of Bcl-2 protein expression were evaluated using Western blotting.

2.4. *Measurement of doubling time*

ADSCs, vector-ADSCs, and Bcl-2-ADSCs were seeded at a density of 1×10^4 cells per cm^2 in six-well plates and incubated until they reached their logarithmic growth phase. The cells were then harvested at intervals of 48 h and counted with a cell counting plate. Doubling time was calculated according to a formula from a previous study (*21*). Doubling time = 48 h/$\log_2$ (N_2/N_1), where N1 is the first cell count and N2 is the cell count 48 h later.

2.5. *Differentiation of genetically modified human ADSCs*

To induce adipogenic differentiation, Bcl-2-ADSCs were seeded onto plates at 1×10^4 cells/cm^2 in complete DMEM. After culturing for 24 h, the medium was changed to adipogenic induction medium using an Adipogenesis Differentiation Kit (Gibco) and the cells were cultured for 14 days. Then, Oil Red O staining was used to observe oil droplets in the cytoplasm of cells. For chondrogenic differentiation, 5-μL droplets of Bcl-2-ADSCs in complete DMEM (1×10^7 cells/mL) were seeded onto plates. After culturing for 48 h, the medium was changed to chondrogenic induction medium using a Chondrogenesis Differentiation Kit (Gibco). Chondrogenic differentiation was induced for 14 days. Chondrogenic differentiation was assessed using Alcian Blue staining. For osteogenic differentiation, Bcl-2-ADSCs (5×10^3/cm^2) were seeded onto plates in complete DMEM. After culturing for 48 h, the medium was changed to osteogenic induction medium using an Osteogenesis Differentiation Kit (Gibco) and cells were cultured for 24 days. The cells were then stained with a 1% Alizarin Red S solution.

2.6. *Western blotting*

Equal amounts of denatured proteins were loaded into each lane of a gel. After electrophoresis, the resolved proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with PBS containing 0.2% Tween 20 and 5% skim milk for 2 h

at room temperature and then incubated with an anti-human Bcl-2 antibody (Keygentec) overnight at 4°C. The membrane was subsequently incubated with a horseradish peroxidase-conjugated secondary antibody, and specific bands were detected with an ECL kit (Keygentec).

2.7. *Oxygen-glucose deprivation*

ADSCs, vector-ADSCs, and Bcl-2-ADSCs were seeded at a density of 2.5×10^5 cells per well on three six-well plates in complete DMEM and then cultured for 24 h. OGD was then performed as described previously (*22*). Briefly, cells were cultured in glucose-free DMEM (Gibco) and exposed to hypoxic conditions in an airtight chamber with 95% N_2 and 5% CO_2 at 37°C.

2.8. *Determination of levels of growth factors*

Cells were cultured and either deprived of oxygen and glucose or not deprived of oxygen and glucose for up to 48 h. The conditioned media with the three groups of cells (ADSCs, vector-ADSCs, and Bcl-2-ADSCs) were collected to evaluate the levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) using a human VEGF enzyme immunoassay kit (Keygentec), human bFGF (Keygentec), or human HGF enzyme immunoassay kit (Keygentec) in accordance with the manufacturer's protocol.

2.9. *Statistical analysis*

Data are expressed as mean ± S.D. Statistical analyses were performed using the statistical software SPSS (version 13.0; IBM, USA). Differences among the 3 groups of cells (ADSCs, vector-ADSCs, and Bcl-2-ADSCs) were compared using one-way ANOVA. Comparisons between 2 groups were performed with an unpaired Student's t test. A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. *Characterization of human ADSCs*

Flow cytometric analysis indicated that ADSCs expressed stem cell-associated markers CD29, CD44, and CD90 but lacked expression of CD34, the fibroblast marker HLA-DR, and the hematopoietic stem cell marker CD133 (Figure 1).

3.2. *Bcl-2 overexpression in genetically modified human ADSCs*

At MOIs of 0, 100, 200, and 500, Ad/EGFP transduction efficiency was 0, 49%, 80%, and 80.5%, respectively, according to flow cytometry. Representative GFP expression is shown in Figure 2A. Based on these results, the transduction efficiency reached its maximum at a MOI of 200 (Figure 2B). After transduction, Bcl-2-ADSCs and vector-ADSCs were morphologically indistinguishable from ADSCs. Thus, an optimal MOI of 200 was chosen for subsequent experiments. Detectable protein expression of Bcl-2 was observed in Bcl-2-

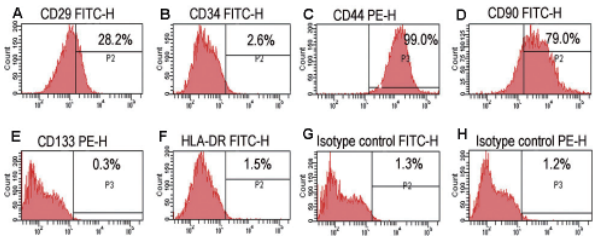

Figure 1. Representative data on the immunophenotypes of human ADSCs that were passaged 4 times. Fluorescence-activated cell sorter analysis indicating the expression of selected surface molecules. **(A)** CD29; **(B)** CD34; **(C)** CD44; **(D)** CD90; **(G** and **H)** Control cells labeled with non-immunoreactive isotype control antibodies. ADSCs expressed the stem cell-associated markers CD29, CD44, and CD90 and did not express CD34, the fibroblast marker HLA-DR, or the hematopoietic stem cell marker CD133.

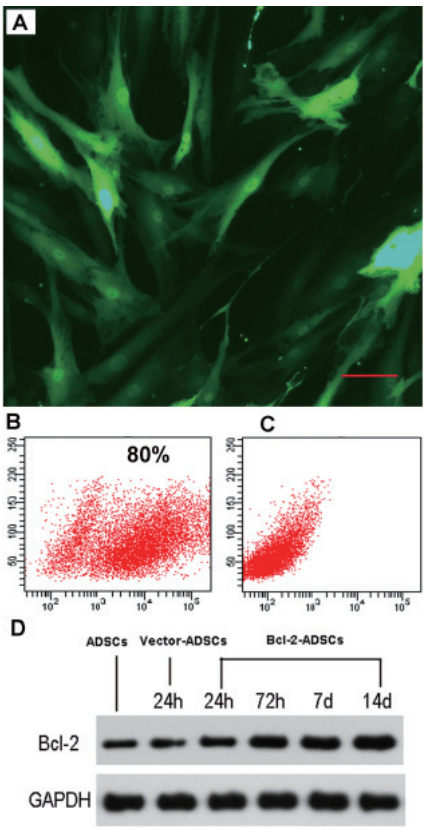

Figure 2. Transduction of ADSCs with an adenoviral vector. **(A)** Representative photomicrograph of ADSCs transfected with Ad/EGFP. Fluorescence-activated cell sorter analysis indicating the transduction efficiency of *EGFP*-transfected ADSCs **(B)** compared to untransfected ADSCs **(C)**. **(D)** Representative western blots showing overexpression of Bcl-2 protein in Bcl-2-ADSCs, which remained at a high level for 14 days post-transduction. GAPDH served as the loading control. Scale bar = 100 μm.

ADSCs as early as 24 h and that level of expression remained high as of day 14 (Figure 2C). In contrast, weak expression of Bcl-2 was detected in vector-ADSCs 24 h post-transduction and in ADSCs, indicating a low level of endogenous Bcl-2 protein in ADSCs.

3.3. *Bcl-2-modified human ADSCs retained their proliferative and multipotent capacity*

ADSCs, vector-ADSCs, and Bcl-2-ADSCs had comparable doubling times (Figure 3A). Bcl-2-ADSCs differentiated into adipocytes, as indicated by Oil Red O staining (Figure 3B, left). Chondrocytes were apparent according to staining with Alcian Blue (Figure 3B, center), and osteoblasts were apparent according to Alizarin Red S staining (Figure 3B, right).

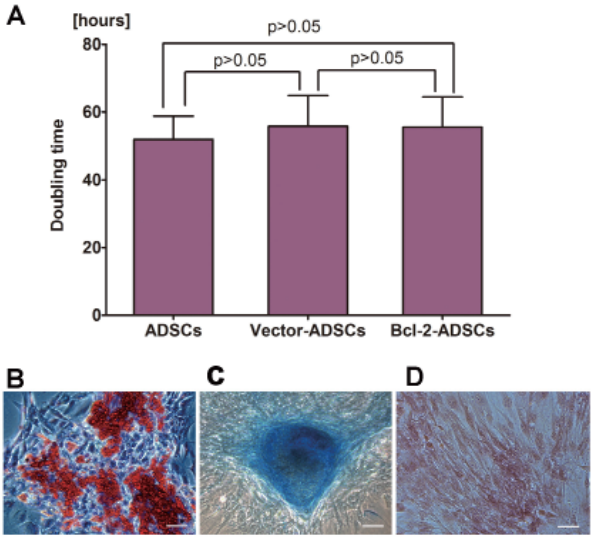

Figure 3. Doubling time and differentiation capacity of Bcl-2-ADSCs. (A) The rate of cell proliferation by ADSCs, vector-ADSCs, and Bcl-2-ADSCs was determined, and their doubling times were determined. There were no significant differences in the doubling time of ADSCs, vector-ADSCs, and Bcl-2-ADSCs. Bcl-2-ADSCs were cultured in adipogenic, chondrogenic, and osteogenic medium. **(B)** Adipogenic differentiation. Staining with Oil Red O. **(C)** Chondrogenic differentiation. Staining with Alcian Blue. **(D)** Osteogenic differentiation. Staining with Alizarin Red S. Values are presented as the mean ± S.D. Scale bar = 50 μm.

3.4. *Bcl-2-modified human ADSCs upregulated growth factors while deprived of oxygen and glucose*

During deprivation of oxygen and glucose, Bcl-2-ADSCs secreted a significantly higher level of VEGF than ADSCs alone (720.83 ± 90.02 versus 629.67 ± 20.45 pg/mL, $p < 0.05$) or than vector-ADSCs (622.50 ± 17.82 pg/mL, $p < 0.05$). Bcl-2-ADSCs secreted a significantly higher level of bFGF than ADSCs alone (383.83 ± 13.86 versus 328.33 ± 4.32 pg/mL, $p < 0.05$) or than vector-ADSCs (332.50 ± 4.85 pg/mL, $p < 0.05$). Bcl-2-ADSCs secreted a significantly higher level of HGF than ADSCs alone (3943.80 ± 260.78 versus 2064.70 ± 387.16 pg/mL, $p < 0.05$) or than vector-ADSCs (1952.00 ± 128.54 pg/mL, $p < 0.05$). Bcl-2-ADSCs secreted a 14.47% higher level of VEGF, a 16.9% higher level of bFGF, and a 91% higher level of HGF compared to ADSCs alone that were cultured while deprived of oxygen and glucose. There were no significant differences in the levels of these three factors in ADSCs and vector-ADSCs deprived of oxygen and glucose ($p > 0.05$). The levels of VEGF and HGF were higher during deprivation of oxygen and glucose than during normal culturing ($p < 0.001$). However, the level of bFGF during deprivation of oxygen and glucose was comparable to that during normal culturing. There were no significant differences in the levels of these three factors in ADSCs, vector-ADSCs, and Bcl-2-ADSCs during normal culturing (Figure 4).

Use of cell-based therapy to treat a number of diseases is a rapidly developing trend in tissue engineering and regenerative medicine. Availability of appropriate cell types remains a crucial issue for therapeutic efficacy and safety. There are several different types of stem cells that have been considered. Although embryonic stem cells have the greatest regenerative potential, their use is limited by ethical reasons, potential tumorigenicity, and immunological rejection (*23*). Use of endothelial progenitor cells from peripheral blood and bone marrow is hampered by expensive procedures of isolation and difficulties in obtaining a sufficient

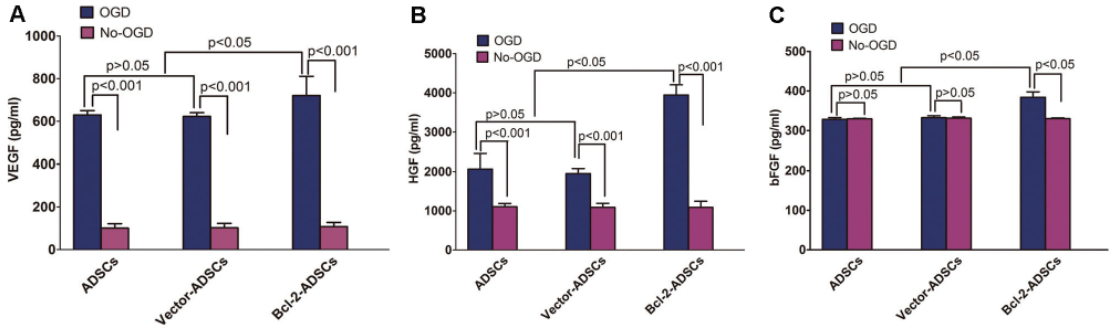

Figure 4. VEGF, HGF, and bFGF secretion by ADSCs, vector-ADSCs, and Bcl-2-ADSCs while deprived of oxygen and glucose or not deprived of oxygen and glucose. (A-C) After incubation for 48 h, the secretion of VEGF, HGF, and bFGF by cells that were not deprived of oxygen and glucose and cells that were deprived of oxygen and glucose ($n = 8$ in each group) in conditioned medium was measured using ELISA. VEGF, HGF, and bFGF concentrations are presented as the mean ± S.D

amount of cells. In addition, *in vitro* cell expansion over multiple passages increases the potential risk of malignancies and chromosomal abnormalities. The harvesting of bone marrow to obtain mesenchymal stromal cells is an invasive and painful procedure. ADSCs have the advantages of easy availability from subcutaneous adipose tissue in large quantities with minimal donor site morbidity and simple collection procedures compared to those for other types of stem cells. Therapy based on ADSCs has emerged as a novel approach to treat a wide range of medical conditions. Thus, ADSCs appear to be favorable candidates for practical stem cell-based therapy.

ADSCs play important roles in cell therapy through multidifferentiation as well as through secretion of growth factors. There is an increasing recognition that the benefits of cell therapy may be mostly attributed to the secretion of multiple complementary growth factors (*24-26*). Numerous studies have examined the paracrine mechanism of ADSCs. *In vitro*, ADSCs can secrete a variety of angiogenic, antiapoptotic, and mitogenic factors, including VEGF, HGF, bFGF, and transforming growth factor-β. Furthermore, the secretion of these growth factors is significantly enhanced when ADSCs are exposed to hypoxic conditions (*16,27*). These growth factors possess angiogenic potential and work collaboratively (*15,28,29*). Angiogenesis involves activation, migration, and proliferation of endothelial cells and is regulated by certain growth factors. These growth factors promote therapeutic angiogenesis, possibly by the combination of direct effects of some growth factors on endothelial cells and indirect effects, including paracrine upregulation of other growth factors (*30*). However, marrow-derived mesenchymal stromal cells have a poor survival once implanted and exposed to ischemic conditions; previous studies revealed that only a small amount of grafted cells had survived *in situ* 4 days after injection (*31-33*). Therefore, ADSC-based therapy may be hindered by low cell survival rates. Previous findings have indicated that Bcl-2 overexpression significantly reduces ADSC apoptosis under harsh conditions (*20*). Using Bcl-2 overexpression to protect ADSCs from apoptosis would establish a good foundation for the paracrine function of ADSCs.

In the present study, flow cytometric analyses revealed that ADSCs that were passaged 4 times expressed the stem cell-associated surface markers CD29, CD44, and CD90 but not the fibroblast marker HLA-DR or the hematopoietic stem cell marker CD133. A point worth mentioning is that ADSCs that were passaged 4 times did not express CD34. The expression profiles coincided with those in previous studies (*21,34,35*). Expression of CD34 and CD34 by ADSCs declined with additional passages. Western blotting indicated that the exogenous *Bcl-2* gene was efficiently transferred into ADSCs and resulted in a high of Bcl-2 expression at the protein level. ADSCs that were genetically modified with Bcl-2 retained their differentiation and proliferative capacity. Bcl-2 overexpression significantly increased the secretion of growth factors when the cells were cultured while deprived of oxygen and glucose. The present study, to the extent known, is the first to find that Bcl-2-ADSCs release higher levels of multiple growth factors, such as VEGF, bFGF, and HGF, than do vector-ADSCs and ADSCs alone while deprived of oxygen and glucose. Previous studies have indicated that the use of single angiogenic growth factors to strengthen neovascularization in patients with atherosclerotic disease results in only modest success (*36,37*). Angiogenesis and collateral growth are likely to require multiple growth factors acting in concert (*38,39*). The present study provides the first direct evidence that Bcl-2 upregulation enhances the potential secretion of growth factors by ADSCs under ischemic conditions. Bcl-2 modification has the advantage of enhancing the levels of a variety of growth factors instead of a single factor when cells are exposed to an ischemic environment early on. Bcl-2-modifid ADSCs can secrete a range of angiogenic growth factors that act in a synergistic manner to promote angiogenesis. This is highly preferable to the increased expression of a single angiogenic factor. The underlying mechanism by which Bcl-2 upregulation enhances the potential secretion of growth factors by ADSCs under ischemic conditions needs to be studied.

In conclusion, the present results indicated that Bcl-2 genetically modified ADSCs retain their proliferative and multilineage differentiation potential. In addition, Bcl-2 upregulation *via* gene transduction significantly enhanced the levels of paracrine factors secreted by ADSCs under ischemic conditions. These findings provide an experimental basis on which to enhance the potential secretion of growth factors by ADSCs under ischemic conditions.

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References

1. Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL. Paschalis EP, Wilkison WO, Gimble JM. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng. 2001; 7:729-741.
2. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem

cells. Mol Biol Cell. 2002; 13:4279-4295.
3. Prunet-Marcassus B, Cousin B, Caton D, André M, Pénicaud L, Casteilla L. From heterogeneity to plasticity in adipose tissues: Site-specific differences. Exp Cell Res. 2006; 312:727-736.
4. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng. 2001; 7:211-228.
5. Mizuno H. Adipose-derived stem and stromal cells for cell-based therapy: Current status of preclinical studies and clinical trials. Curr Opin Mol Ther. 2010; 12:442-449.
6. Karaçal N, Cobanoğlu U, Ambarcioğlu O, Kutlu N. The effect of fibrin glue on fat graft survival. J Plast Reconstr Aesthet Surg. 2007; 60:300-303.
7. Chajchir A, Benzaquen I. Fat-grafting injection for soft-tissue augmentation. Plast Reconstr Surg. 1989; 84:921-934.
8. Ahmed Ali. Contouring of the gluteal region in women: Enhancement and augmentation. Ann Plast Surg. 2011; 67:209-214.
9. Matsumoto D, Sato K, Gonda K, Takaki Y, Shigeura T, Sato T, Aiba-Kojima E, Iizuka F, Inoue K, Suga H, Yoshimura K. Cell-assisted lipotransfer: Supportive use of human adipose-derived cells for soft tissue augmentation with lipoinjection. Tissue Eng. 2006; 12:3375-3382.
10. Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, Harii K. Cell-assisted lipotransfer for cosmetic breast augmentation: Supportive use of adipose-derived stem/stromal cells. Aesth Plast Surg. 2008; 32:48-55.
11. Lendeckel S, Jödicke A, Christophis P, Heidinger K, Wolff J, Fraser JK, Hedrick MH, Berthold L, Howaldt HP. Autologous stem cells (adipose) and fibrin glue used to treat widespread traumatic calvarial defects: Case report. J Craniomaxillofac Surg. 2004; 32:370-373.
12. Fang B, Song Y, Lin Q, Zhang Y, Cao Y, Zhao RC, Ma Y. Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. Pediatr Transplant. 2007; 11:814-817.
13. Léobon B, Roncalli J, Joffre C, Mazo M, Boisson M, Barreau C, Calise D, Arnaud E, André M, Pucéat M, Pénicaud L, Prosper F, Planat-Bénard V, Casteilla L. Adipose-derived cardiomyogenic cells: *In vitro* expansion and functional improvement in a mouse model of myocardial infarction. Cardiovasc Res. 2009; 83:757-767.
14. Planat-Bénard V, Menard C, André M, Puceat M, Perez A, Garcia-Verdugo JM, Pénicaud L, Casteilla L. Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res. 2004; 94:223-229.
15. Yoshimura K, Eto H, Kato H, Doi K, Aoi N. *In vivo* manipulation of stem cells for adipose tissue repair/reconstruction. Regen Med. 2011; 6(6 Suppl):33-41.
16. Rehman J, Traktuev D, Li J, Merfeld-Clauss S, Temm-Grove CJ, Bovenkerk JE, Pell CL, Johnstone BH, Considine RV, March KL. Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. Circulation. 2004; 109:1292-1298.
17. Reed JC. Bcl-2 family proteins. Oncogene. 1998; 17:3225-3236.
18. Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell. 1993; 75:229-240.
19. Shelat PB, Plant LD, Wang JC, Lee E, Marks JD. The membrane-active tri-block copolymer pluronic F-68 profoundly rescues rat hippocampal neurons from oxygen-glucose deprivation-induced death through early inhibition of apoptosis. Neurosci. 2013; 30:12287-12299.
20. Cui Z, Shen L, Lin Y, Wang S, Zheng D, Tan Q. Inhibition of oxygen-glucose deprivation-induced apoptosis of human adipose-derived stem cells by genetic modification with antiapoptotic protein bcl-2. Aesthetic Plast Surg. 2014; 38:779-787.
21. Gonda K, Shigeura T, Sato T, Matsumoto D, Suga H, Inoue K, Aoi N, Kato H, Sato K, Murase S, Koshima I, Yoshimura K. Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. Plast Reconstr Surg. 2008; 121:401-410.
22. Li J, Qu Y, Chen D, Zhang L, Zhao F, Luo L, Pan L, Hua J, Mu D. The neuroprotective role and mechanisms of TERT in neurons with oxygen-glucose deprivation. Neuroscience. 2013; 252:346-358.
23. Calderon D, Planat-Benard V, Bellamy V, Vanneaux V, Kuhn C, Peyrard S, Larghero J, Desnos M, Casteilla L, Pucéat M, Menasché P, Chatenoud L. Immune response to human embryonic stem cell-derived cardiac progenitors and adipose-derived stromal cells. J Cell Mol Med. 2012; 16:1544-1552.
24. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005; 11:367-368.
25. Kim WS, Park BS, Sung JH. Protective role of adipose-derived stem cells and their soluble factors in photoaging. Arch Dermatol Res. 2009; 301:329-336.
26. Rubina K, Kalinina N, Efimenko A, Lopatina T, Melikhova V, Tsokolaeva Z, Sysoeva V, Tkachuk V, Parfyonova Y. Adipose stromal cells stimulate angiogenesis *via* promoting progenitor cell differentiation, secretion of angiogenic factors, and enhancing vessel maturation. Tissue Eng Part A. 2009; 15:2039-2050.
27. Li W, Ma N, Ong LL. Nesselmann C, Klopsch C, Ladilov Y, Furlani D, Piechaczek C, Moebius JM, Lützow K, Lendlein A, Stamm C, Li RK, Steinhoff G. Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. Stem Cells. 2007; 25:2118-2127.
28. Hisaka Y, Ieda M, Nakamura T, Kosai K, Ogawa S, Fukuda K. Powerful and controllable angiogenesis by using gene-modified cells expressing human hepatocyte growth factor and thymidine kinase. J Am Coll Cardiol. 2004; 43:1915-1922.
29. Suga H, Eto H, Shigeura T, Inoue K, Aoi N, Kato H, Nishimura S, Manabe I, Gonda K, Yoshimura K. IFATS collection: Fibroblast growth factor-2-induced hepatocyte growth factor secretion by adipose-derived stromal cells inhibits postinjury fibrogenesis through a c-Jun N-terminal kinase-dependent mechanism. Stem Cells. 2009; 27:238-249.
30. Folkman J, Shing Y. Angiogenesis. J Biol Chem. 1992; 267:10931-10934.
31. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and antideath strategies. J Mol Cell Cardiol. 2001; 33:907-921.
32. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart.

Circulation. 2002; 105:93-98.
33. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res. 2004; 95:9-20.
34. Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, Sato K, Inoue K, Nagase T, Koshima I, Gonda K. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol. 2006; 208:64-76.
35. Mizuno H, Tobita M, Uysal AC. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. Stem Cells. 2012; 30:804-810.
36. Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, Udelson JE, Gervino EV, Pike M, Whitehouse MJ, Moon T, Chronos NA. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: Double-blind, randomized, controlled clinical trial. Circulation. 2002; 105:788-793.
37. Henry TD, Annex BH, McKendall GR, *et al*. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation. 2003; 107:1359-1365.
38. Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med. 2003; 54:17-28.
39. Jiang M, Wang B, Wang C, He B, Fan H, Shao Q, Gao L, Liu Y, Yan G, Pu J. *In vivo* enhancement of angiogenesis by adenoviral transfer of HIF-1α-modified endothelial progenitor cells (Ad-HIF-1α-modified EPC for angiogenesis). Int J Biochem Cell Biol. 2008; 40:2284-2295.

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Brief Report

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The impact of intra-abdominal pressure on the stroke volume variation and plethysmographic variability index in patients undergoing laparoscopic cholecystectomy

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Summary **The purpose of the present study was to evaluate the effect of increasing intra-abdominal pressure (IAP) on stroke volume variation (SVV) and plethysmographic variability index (PVI) in patients undergoing laparoscopic cholecystectomy. PVI examined by Masimo Radical 7 pulse oximeter and SVV determined using FloTrac/Vigileo were monitored simultaneously in forty-five patients undergoing laparoscopic cholecystectomy (LC). Mean arterial blood pressure (MAP), heart rate (HR), cardiac index (CI), perfusion index (PI), airway pressures (P), SVV, and PVI were also recorded at the following predetermined time: 5 min after endotracheal intubation (T_1), 5 min after pneumoperitoneum at 5 mmHg (T_2), 5 min after pneumoperitoneum at 10 mmHg (T_3), 5 min after pneumoperitoneum at 15 mmHg (T_4), and 5 min after the termination of pneumoperitoneum (T_5). Forty-five patients with a total of 225 pairs of measurements were included in the analysis. Compared with the values at T_1, both SVV and PVI showed significant progressive increases as the IAP was adjusted from 5 to 10, 15 mmHg at T_2, T_3, and T_4, respectively. No significant difference was found when the pneumoperitoneum was terminated at T_5. Further regressive analysis indicated strong relationships between SVV and IAP ($r = 0.8118$, $p < 0.001$), PVI and IAP($r = 0.8876$, $p < 0.001$) respectively. Both PVI and SVV showed rapid and IAP correlative changes with increasing intra-abdominal pressure in patients undergoing laparoscopic cholecystectomy.**

Keywords: Intra-abdominal pressure, plethysmographic variability index, stroke volume variation

1. Introduction

With the development of mini-invasive technique, laparoscopic operations have been the mainstay of general surgery. Usually artificial pneumoperitoneum is established with carbon dioxide insufflation and the intra-abdominal pressure (IAP) is frequently maintained at 10 to 15 mmHg. Given that the preload is significantly influenced by the increase of IAP, it is important to evaluate the influence of IAP on the volume status of those patients undergoing laparoscopic surgery in clinical practice (*1*).

Currently there are two ways to monitor the state of volume. The first includes some traditional static parameters such as central venous pressure (CVP) and pulmonary capillary wedge pressure (PCWP). Both indices are invasive and have been shown to reflect the patient's volume status poorly and predict a cardiac response to fluid therapy incompetently (*2,3*). By contrast, dynamic variable such as stroke volume variation (SVV) has been shown to be a good predictor of volume responsiveness in mechanically ventilated patients undergoing open chest surgery, liver transplantation, and abdominal surgery (*4-6*). However, it was controversial whether SVV changed when IAP was increased in patients undergoing laparoscopic surgery (*7,8*). On the other hand, noninvasive plethysmographic variability index (PVI) is a new parameter used for the purpose of fluid responsiveness in patients receiving several kinds of surgeries (*9-14*). In patients undergoing laparoscopic surgery, a previous study has suggested that PVI increased significantly after pneumoperitoneum was established (*7*). However, it is still elusive whether PVI and SVV could convincingly reflect the changes

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of intra-abdominal pressure in mechanically ventilated patients.

Therefore, the aim of the present study was to investigate the responsiveness and the relationships between PVI and SVV with the increasing intra-abdominal pressure in patients undergoing laparoscopic cholecystectomy (LC).

2. Materials and Methods

2.1. *Subjects*

The Institutional Review Board of Jinling Hospital approved the present study and written informed consent was obtained from each patient. Forty five patients undergoing elective LC were included in this prospective observational study from October 2013 to May 2014. Exclusion criteria were arrhythmias, diabetes, pulmonary diseases, intracardiac shunts, valvular heart disease, peripheral vascular disease, long-term taking oral vascular active drugs, body mass index (BMI) smaller than 18 or greater than 30, the blood loss over 100 mL during operation and the duration of surgery over 60 min.

2.2. *Anesthesia and monitors*

After arriving the operation room, all patients received routine monitors including electrocardiogram (ECG), pulse oximetry, capnography, and noninvasive blood pressure. General anesthesia was induced by the same anesthesiologist with midazolam (0.05 mg/kg), propofol (2.0-2.5 mg/kg), fentanyl (3 μg/kg) and rocuronium (0.6 mg/kg) to facilitate tracheal intubation. Anesthesia was maintained with continuous infusion of propofol and cis-atracurium supplemented with a bolus of fentanyl 0.3 μg/kg to keep the bispectral index monitor (BIS, Aspect 1000, Aspect Medical System Inc., Natick, MA, USA) between 40 and 60. Mechanical ventilation was adjusted to maintain end-tidal pressure of CO_2 ($P_{ET}CO_2$) between 30-35 mmHg.

According to the operation manual, SVV was determined by inserting an arterial catheter (20 gauge, Surflo, Terumo, Japan) preoperatively, then connecting *via* a prepackaged arterial pressure tubing set (VAMP Plus, Edwards Lifesciences) to a FloTrac™ sensor (Edwards Lifesciences) and a Vigileo™ monitor (software version 01.12, Edwards Lifesciences). Meanwhile, PVI was measured by a Masimo Radical 7 pulse oximeter with PVI software (version 7.6.2.1) through a pulse oximeter probe placed on the index finger of the other hand and wrapped to prevent outside light from interfering with the signal. Both values were continuously measured.

2.3. *Study design*

All patients were studied immediately after induction of anesthesia. We avoided any stimulation to the patients for 5 min before data collection in order to minimize the change of vasomotor tone. PVI and SVV were recorded at a predetermined set of the 5 time points by the other same anesthesiologist during the end-expiratory phase: T_1, 5 min after endotracheal intubation; T_2, 5 min after pneumoperitoneum at 5 mmHg; T_3, 5 min after pneumoperitoneum at 10 mmHg; T_4, 5 min after pneumoperitoneum at 15 mmHg; T_5, 5 min after the termination of pneumoperitoneum. The mean arterial blood pressure (MAP), heart rate (HR), cardiac index (CI = cardiac output/body surface area), SVV, PVI, perfusion index (PI), BIS, airway pressures (P), and $P_{ET}CO_2$ were also recorded at the aforementioned time points.

To exclude the impact of fluid on the SVV and PVI, 8 mL/Kg balanced solution was infused before anesthesia induction to supplement the insufficient induced by fast, then balanced solution was given at the rate of 4 mL/kg/h.

2.4. *Statistical methods*

Statistical analysis was performed using the software program Prism version 5.0 (Graphpad, San Diego, CA, USA). Descriptive statistics of the variables are presented as means ± SD. Normality of the distributions was tested using a Kolmogorov-Smirnov test. Data analysis was performed using repeated measurement of analysis of variance (ANOVA) followed by the Bonferroni test for post hoc comparisons. Linear regression was used to measure the relationship between IAP and SVV, IAP and PVI respectively. A p value of less than 0.05 was regarded as statistically significant difference.

3. Results and Discussion

In the present study, 45 patients receiving LC were enrolled (24 males and 21 females), with a mean age of 46 years (range, 24-68). No patient met the exclusion criteria, thus 45 patients with a total of 225 pairs of measurements were included in the analysis. The general characteristics of patients were listed in Table 1.

As shown in Table 2, HR, CI and BIS did not alter significantly during the observed period. However, P and MAP increased significantly with the elevation of IAP.

Table 1. Patients characteristics

Parameters	
Gender (male/female)	24/21
Age (year)	46 ± 12
Height (cm)	168 ± 8
Weight (kg)	68 ± 9
ASA (I/II)	19/26
Operation time (min)	37 ± 5
Anesthesia time (min)	57 ± 13

Data are presented as the mean ± standard deviation, or as actual numbers, when appropriate.

Table 2. Values of mean arterial blood pressure (MAP), heart rate (HR), cardiac index (CI), perfusion index (PI), airway pressures (P), and bispectral index (BIS) at five time points

Parameters	T_1	T_2	T_3	T_4	T_5
MAP (mm Hg)	79 ± 7	80 ± 7	90 ± 8*	102 ± 10*	80 ± 8
HR (beats/min)	78 ± 10	77 ± 7	77 ± 7	76 ± 11	77 ± 6
CI ($L \cdot min^{-1} \cdot m^{-2}$)	2.7 ± 0.7	2.6 ± 0.6	2.5 ± 0.9	2.4 ± 0.6	2.9 ± 0.7
PI (%)	5.0 ± 0.4	3.7 ± 0.3*	2.1 ± 0.2*	1.2 ± 0.1*	4.8 ± 0.4
P (cmH_2O)	15.0 ± 2.6	16.6 ± 3.1	19.3 ± 3.4*	22.0 ± 3.9*	15.5 ± 2.7
BIS	51 ± 5	51 ± 4	50 ± 5	51 ± 5	51 ± 4

Data are presented as the mean ± standard deviation. T_1: 5 min after endotracheal intubation; T_2: 5 min after pneumoperitoneum at 5 mmHg; T_3: 5 min after pneumoperitoneum at 10 mmHg; T_4: 5 min after pneumoperitoneum at 15 mmHg; T_5: 5 min after the termination of pneumoperitoneum. * $p < 0.05$ *vs*. T_1.

In contrast, PI decreased significantly with the increase of IAP. After termination of pneumoperitoneum, these parameters returned to the level before insufflation.

As shown in Figure 1A, SVV measured at T_2 (13.73 ± 2.84%), T_3 (19.20 ± 2.84%) and T_4 (24.67 ± 3.93%) were significantly increased when compared with the value at T_1 (12.47 ± 3.13%). No significant difference was found between SVV at T_5 (11.39 ± 3.96%) with SVV at T_1. Similarly, the levels at T_2 (17.96 ± 3.02%), T_3 (26.07 ± 4.22%) and T_4 (34.67 ± 5.56%) was significantly enhanced compared with the PVI at T_1 (13.09 ± 3.36%). There was no significant difference between PVI at T_5 (14.64 ± 4.24%) with PVI at T_1.

As shown in Figure 2, both SVV and PVI correlated significantly with the level of IAP (between SVV and IAP, $r = 0.8118$, $p < 0.001$; between PVI and IAP, $r = 0.8876$, $p < 0.001$).

The major finding of the present study was that increasing IAP induced rapid and correlative changes for both SVV and PVI. After termination of insufflation, both SVV and PVI returned to the level without pneumoperitoneum.

Estimating the volume status of patients is a frequent problem for the anesthesiologist and surgeons. The estimation is usually obtained by means of static as well as dynamic variables. Frequently used static preload variables, including CVP and PCWP, often fail to provide reliable information on volume status and are not capable of predicting a cardiac response to fluid therapy (*2,3*). By contrast, dynamic variables such as PVI and SVV could reliably predict the response to fluid administration during mechanically ventilation (*6,9,11,15*). Although both SVV and PVI could reflect the fluid loading, it is known that both are disturbed by several confounder factors such as: arrhythmia, vasomotor tone, vasoactive drugs, pleural effusion, intra-abdominal pressure and so on (*16-18*).

In patients undergoing laparoscopic surgery, intra-abdominal pressure is deliberatively increased to 10-15mmHg by carbon dioxide insufflation. It is well known that pneumoperitoneum could induce significant disturbance of hemodynamics, which are manifested as decreases in cardiac output, increases of systemic and pulmonary vascular resistances, elevated arterial

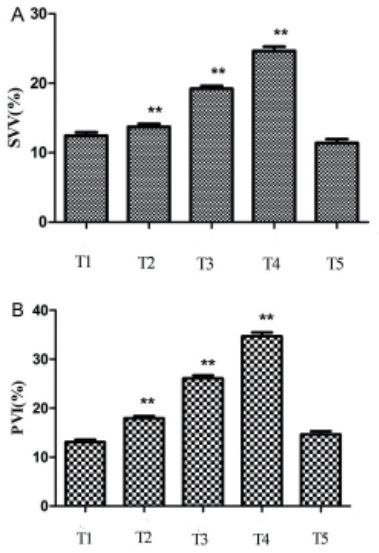

Figure 1. The values of stroke volume variation (SVV) and plethysmographic variability index (PVI) at different levels of intra-abdominal pressure (IAP). T_1: IAP = 0 mmHg before pneumoperitoneum; T_2: IAP = 5 mmHg after pneumoperitoneum; T_3: IAP = 10 mmHg after pneumoperitoneum; T_4: IAP = 15 mmHg after pneumoperitoneum; T_5: IAP = 0 mmHg termination of pneumoperitoneum. ** $p < 0.01$, compared with the level of SVV **(A)** or PVI **(B)** at the T_1, $n = 45$.

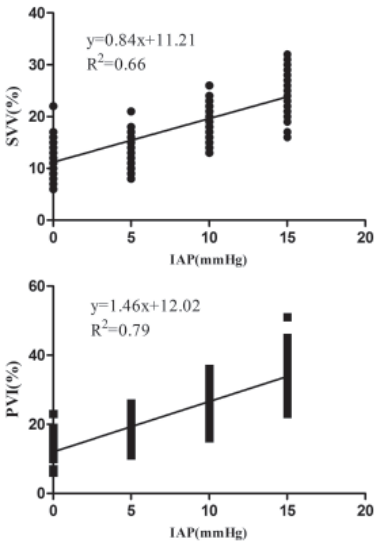

Figure 2. Correlation between stroke volume variation (SVV) and plethysmographic variability index (PVI) with the different levels of intra-abdominal pressure (IAP) from the data of 45 patients.

pressure and decreases in venous return (*1*). In the present study, we also found the increase of MAP and the decrease of PI. Using SVV and PVI, we further observed the effect of IAP on the volume status. In 2012, Høiseth *et al*. reported that pneumoperitoneum (10-12 mmHg) only changed the PVI significantly, but did not change the SVV (*7*). However, a recent study by Wajima *et al*. indicated that SVV was significantly increased at 2 to 5 min after pneumoperitoneum, and significantly decreased at 1 to 5 min after release of pneumoperitoneum (*8*). Compared with those studies, our work had three features that the level of IAP was not sole, but adjusted to three stages as 5, 10 and 15 mmHg; the values of SVV and PVI were recorded simultaneously at 5 min after the establishment of the different levels of pneumoperitoneum and the infused fluid was restricted at low speed to avoid the impact on the SVV and PVI. We found that both SVV and PVI increased progressively as the level of IAP was adjusted from 0 to 5, 10 and 15 mmHg. After termination of pneumoperitoneum, both SVV and PVI showed no difference with the level before pneumoperitoneum. Furthermore, the regressive analysis demonstrated that there existed positive regression between SVV and IAP, PVI and IAP, respectively.

Although both SVV and PVI could reflect the influence of IAP effectively, PVI might be the better choice for clinical setting. Firstly, PVI could be continuously and noninvasively monitored. As for SVV, it was invasive and not routinely available in daily clinical practice. Secondly, PVI showed a more consistent response to the IAP. In the present study, we found that the individual values of SVV and PVI at T_2 were actually smaller than those at T_1 in 17.8% (8/45) patients for SVV and 4.4% (2/45) patients for PVI. When IAP was 10 or 15 mmHg, there were no exceptions for both indices. Lastly, PVI showed a more steep response to the increase of IAP compared with the SVV.

There also existed some limitations in our study. Firstly, the IAP before pneumoperitoneum was arbitrarily determined as 0 mmHg, but it was well known that the IAP correlated with BMI in the healthy people (*19*). Thus the inclusion criteria for BMI was between 18 and 30. Furthermore our repeated measure study design should greatly overcome this shortcoming. Secondly, BIS could not effectively measure the degree of stress response, which would affect both SVV and PVI significantly. Unfortunately, the devices that could monitor the stress response are not available in the current clinical setting. Lastly, in the present study increasing IAP was induced by carbon dioxide insufflation, which might be different from conditions occured secondary to abdominal compression in critically ill patients. Therefore, future studies are warranted to determine the change of SVV and PVI with increased intra-abdominal pressure induced by different circumstances.

In conclusion, our data suggested that both SVV and noninvasive PVI demonstrated rapid and correlative changes with increased intra-abdominal pressure resulting from carbon dioxide insufflations. Furthermore, it seemed that PVI was more sensitive to reflect the change of IAH.

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References

1. Koivusalo AM, Lindgren L. Effects of carbon dioxide pneumoperitoneum for laparoscopic cholecystectomy. Acta Anaesthesiol Scand. 2000; 44:834-841.
2. Kumar A, Anel R, Bunnell E, Habet K, Zanotti S, Marshall S, Neumann A, Ali A, Cheang M, Kavinsky C, Parrillo JE. Pulmonary artery occlusion pressure and central venous pressure fail to predict ventricular filling volume, cardiac performance, or the response to volume infusion in normal subjects. Crit Care Med. 2004; 32:691-699.
3. Michard F, Teboul JL. Predicting fluid responsiveness in ICU patients: A critical analysis of the evidence. Chest. 2002; 121:2000-2008.
4. Wyffels PA, Sergeant P, Wouters PF. The value of pulse pressure and stroke volume variation as predictors of fluid responsiveness during open chest surgery. Anaesthesia. 2010; 65:704-709.
5. Su BC, Tsai YF, Cheng CW, Yu HP, Yang MW, Lee WC, Lin CC. Stroke volume variation derived by arterial pulse contour analysis is a good indicator for preload estimation during liver transplantation. Transplant Proc. 2012; 44:429-432.
6. Liu X, Fu Q, Mi W, Liu H, Zhang H, Wang P. Pulse pressure variation and stroke volume variation predict fluid responsiveness in mechanically ventilated patients experiencing intra-abdominal hypertension. Biosci Trends. 2013; 7:101-108.
7. Høiseth LØ1, Hoff IE, Myre K, Landsverk SA, Kirkebøen KA. Dynamic variables of fluid responsiveness during pneumoperitoneum and laparoscopic surgery. Acta Anaesthesiol Scand. 2012; 56:777-786.
8. Wajima Z, Shiga T, Imanaga K. Pneumoperitoneum affects stroke volume variation in humans. J Anesth. 2014. (DOI 10.1007/s00540-014-1963-y).
9. Fu Q, Mi WD, Zhang H. Stroke volume variation and pleth variability index to predict fluid responsiveness during resection of primary retroperitoneal tumors in Hans Chinese. Biosci Trends. 2012; 6:38-43.
10. Byon HJ, Lim CW, Lee JH, Park YH, Kim HS, Kim CS, Kim JT. Prediction of fluid responsiveness in mechanically ventilated children undergoing neurosurgery. Br J Anaesth. 2013; 110:586-591.
11. Zimmermann M, Feibicke T, Keyl C, Prasser C, Moritz S, Graf BM, Wiesenack C. Accuracy of stroke volume variation compared with pleth variability index to predict fluid responsiveness in mechanically ventilated patients undergoing major surgery. Eur J Anaesthesiol. 2010; 27:555-561.
12. Bagci S, Müller N, Müller A, Heydweiller A, Bartmann P,

Franz AR. A pilot study of the pleth variability index as an indicator of volume-responsive hypotension in newborn infants during surgery. J Anesth. 2013; 27:192-198.
13. Nilsson LM, Lindenberger DM, Hahn RG. The effect of positive end-expiratory pressure and tripled tidal volume on pleth variability index during hypovolaemia in conscious subjects: A volunteer study. Eur J Anaesthesiol. 2013; 30:671-677.
14. Haas S, Trepte C, Hinteregger M, Fahje R, Sill B, Herich L, Reuter DA. Prediction of volume responsiveness using pleth variability index in patients undergoing cardiac surgery after cardiopulmonary bypass. J Anesth. 2012; 26:696-701.
15. Cannesson M, Desebbe O, Rosamel P, Delannoy B, Robin J, Bastien O, Lehot JJ. Pleth variability index to monitor the respiratory variations in the pulse oximeter plethysmographic waveform amplitude and predict fluid responsiveness in the operating theatre. Br J Anaesth. 2008; 101:200-206.
16. Broch O, Gruenewald M, Renner J, Meybohm P, Schöttler J, Heß K, Steinfath M, Bein B. Dynamic and volumetric variables reliably predict fluid responsiveness in a porcine model with pleural effusion. PLoS One. 2013; 8:e56267.
17. Sandroni C, Cavallaro F, Marano C, Falcone C, De Santis P, Antonelli M. Accuracy of plethysmographic indices as predictors of fluid responsiveness in mechanically ventilated adults: A systematic review and meta-analysis. Intensive Care Med. 2012; 38:1429-1437.
18. Jacques D, Bendjelid K, Duperret S, Colling J, Piriou V, Viale JP. Pulse pressure variation and stroke volume variation during increased intra-abdominal pressure: An experimental study. Crit Care. 2011; 15:R33.
19. Cobb WS, Burns JM, Kercher KW, Matthews BD, James Norton H, Todd Heniford B. Normal intraabdominal pressure in healthy adults. J Surg Res. 2005; 129:231-235.

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Commentary

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Retraction of a study on genetically modified corn: Expert investigations should speak louder during controversies over safety

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Summary **Over the past few years, genetically modified organisms (GMO) have gradually become more familiar after numerous reports of problems with GMO safety, such as genetically modified (GM) potatoes disrupting immunity, GM corn inducing tumors, and GM rice being fed to unwitting Chinese children. Every time, these reports cause panic among the population and lead to objections to GMO in various fora. After each incident, the scientific community has delivered its academic appraisal and refuted rumors through slow and cautious investigations and evaluations. Unfortunately, during each event media outlets quickly scare the public about food safety and ignore the ensuing comments from scientists. Although scientists have investigated each GMO crisis and reached scientific and rational conclusions, they have less ability to disseminate information than the media, so the public is not promptly informed of their rational and objective viewpoints as experts. Thus, scientists need greater ability to disseminate information from scientific investigations and evaluations in order to correct the intemperate reporting by attention-seeking media.**

Keywords: Genetically modified organisms (GMO), safety, toxicity, allergy, media

Over the past few years, alarm about the safety of genetically modified (GM) foods has spread around the world over Facebook, Twitter, WeChat, and Line. After scientists publish striking research conclusions, these findings are widely disseminated to the public by the media. These research conclusions are then subsequently investigated, evaluated, and corrected by other scientists (Table 1). One alarming report originated with a controversial study on GM corn that was retracted in 2013 after many scientists challenged the scientific rationality of its findings. The study, first published in 2012 in the journal Food and Chemical Toxicology, announced that GM corn engineered to be tolerant to Monsanto's herbicide

Roundup caused health hazards in rats (*1*). The study reported that the GM corn promoted liver congestions and necrosis, tumor growth, and carried the risk of death.

The study captured headlines around the world with its gruesome pictures of rats that were apparently more likely to develop severe tumors and die earlier after being fed Monsanto's GM corn, regardless of whether or not the corn was cultivated with the herbicide Roundup. In November 2013, the publisher of Food and Chemical Toxicology, Elsevier, explained that it was retracting the study due to concerns about the research methodology following a "thorough and time-consuming analysis". Although a statement from Elsevier emphasized that there was no evidence of fraud or intentional misrepresentation of the data (*2*), the small number of rats used in the study meant that its conclusions were not definitive (*3,4*). The Sprague-Dawley strain of rats used in the study are known to have a high morbidity of tumors, but this factor alone was not sufficient to cause the higher incidence and mortality observed in the treated groups (*5-8*). One year later, the study's authors republished the study in a lesser-known journal, Environmental Sciences Europe (*9*). However, the story did not end there.

In 2014, Delaney *et al*. fed processed fractions from herbicide-tolerant (DP-Ø73496-4) canola to rodents for 13 weeks to verify the contention that GM foods are

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Table 1. Famous incidents in GM food safety and related controversies

Initiator (Year)	GM foods	Journals	Viewpoints	Criticisms	*Ref.*
Pusztai A (1999)	Potato	Lancet	Causes abnormalities in development and immunity	Rats were fed raw potatoes and given additional protein, which may have caused the observed effect	(*13*)
Losey JE (1999)	Corn pollen	Nature	Kills the larvae of the monarch butterfly	Pollen deposition decreases sharply a short distance from cornfields; butterflies were only fed milkweed dusted with corn pollen; numbers of butterflies did not decrease	(*3*)
Quist D and Chapela IH (2001)	Corn	Nature	Gene enters traditional local crops	Missampling; insufficient data	(*4*)
Séralini GE (2012)	Corn	Food Chem Toxicol	Promotes liver congestions and necrosis, tumor growth, and carries risk of death	Small number of rats; high morbidity of tumors in SD rats	(*1*)
Cui YY (2013)	Corn and rice	Documentary film	Toxic and carcinogenic	Statistical errors; subjective bias; weak scientific grounds	(*12,13*)

as safe as non-GM foods. This study was published in the journal Food and Chemical Toxicology (*10*). That same year, Séralini, the author of the retracted paper mentioned earlier, wrote a letter to the editor suggesting retraction of Delaney's paper. In his letter, Séralini pointed that the "uncontrolled presence of pesticide residues and other GMOs make the study inconclusive" (*11*). The publisher Elsevier was once again embroiled in a crisis.

Séralini's study was exaggerated by the media and was subsequently criticized by many scientists. A similar incident occurred in China at the same time. Yongyuan Cui, a former host of China's state broadcaster China Central Television (CCTV) who is noted for challenging authorities and mainstream society, questioned the safety of GM foods. Last year, he spent 500,000 yuan ($82,342) of his own money to travel to the United States (US) to shoot a documentary investigating American attitudes towards GM foods and controversies over genetically modified organisms (GMOs) in American academic circles. The documentary soon reached every media outlet in China. The documentary interviewed Americans in different occupations and reached the conclusion that GM foods were harmful to human health. Cui's considerable influence among the public in China led a large number of Chinese to believe that GM foods were toxic and carcinogenic. However, a few scientists have noted obvious errors in his documentary, such as statistical errors, subjective bias, and weak scientific backgrounds, that render the film unscientific and misleading (*12,13*). Cui garnered a great deal of attention from the media, but he was dismissed as a source of misinformation by the scientific community in China.

In fact, there are a number of similar arguments emerging every day in the field of GMOs. Problems occurring in the field of GMOs are, to a large extent, the result of the gap in scientific rationality and the common wisdom of the public and the media. This gap has placed scientists in a weaker position to educate the public than media reports. In 2013, Flipse and Osseweijer (*14*) examined reports on GM foods in major English-language media over the previous 15 years, and they found that GMO-related reports had increased during GMO-related incidents, such as the study in the Lancet by Pusztai, a Scottish scientist, suggesting that GM potatoes were harmful to health (*15*); a study by Losey *et al*. in Nature indicating that the death of monarch butterfly larvae was related to GM corn (*3*); and a study by Quist and Chapela indicating that GM corn with transgenic DNA had contaminated traditional corn in Mexico (*4*). Although all these studies were not accepted by most scientists, they made a great impact on the media. Scientists and biotechnology companies are always slower at disseminating information than the media since they have to spend more time investigating in order to avoid criticism. As a result, the media had lost interest when scientific conclusions were ultimately reached. Thus, there is an imbalance in the influence that information from the media and scientists has. Solitary reports usually deliver incomplete information to the public, and thus lead to misinformation.

During the incidents mentioned earlier, numerous media reports overwhelmed the faint voice of the scientific community. However, the media cannot provide solid evidence to solve problems with GMO safety. To the contrary, experts following scientific methods supply evidence. Here, previous crises concerning GM foods will be reviewed and how scientists investigated these incidents and what scientific conclusions they reached will be described.

The earliest study to question GMO safety was one by Pusztai *et al*. (*15*). Pusztai announced the study's results on a TV show in 1998. The study fed rodents unaltered potatoes, similar potatoes laced with lectins,

and GM potatoes (2 lines with the same gene added) producing their own lectin, and results indicated that rodents fed GM potatoes had abnormalities in development and immunity. However, this finding was questioned by a committee of researchers from the Rowett Institute and the Royal Society. The committee pointed out the following problems: the rats were fed raw potatoes, which contain various toxins; and the protein content in the 2 lines of GM potatoes differed, so protein content had to be supplemented. Thus, the committee suggested that Pusztai's data did not support his conclusions. Although Pusztai published his paper in the Lancet, the journal included a letter citing the doubts of the committee in the same issue. Several other research groups subsequently reported that they were unable to verify Pusztai's findings.

In 1999, John Losey, an entomologist from Cornell University, published a study in the journal Nature (*3*). The study reported that corn pollen containing the *Bacillus thuringiensis* (Bt) gene can kill the larvae of the monarch butterfly. This study induced an intense discussion about the ecological safety of GMOs. The study's findings were scrutinized by fellow scientists and the U.S. Environmental Protection Agency (USEPA) since the study was performed in the lab and not in the field. Thus, the US EPA asked several entomologists to evaluate this study. They concluded that GM corn pollen did not harm the larvae of the monarch butterfly for three reasons: *i*) corn pollen is too heavy to spread over 5 meters from cornfields, *ii*) monarch butterflies do not usually eat corn pollen and they lay their eggs after pollen is released by corn, and *iii*) there are actually large quantities of monarch butterflies in fields in the eastern and central US. Following the study, research groups studied the butterflies further and observed them in the field, and their findings agreed with the conclusions of the US EPA.

Soon after the monarch butterfly incident, David Quist and Ignacio H. Chapela, scientists from University of California at Berkeley, published a study in the journal Nature (*4*). They reported that GM crops had been detected in areas where GMOs were prohibited in southern Mexico. If true, the study indicated that genes from commercial GM crops were introgressing into traditional local crops. However, their study was questioned by several fellow scientists. After other researchers pointed out several mistakes in the study, such as missampling and insufficient data, Nature retracted the paper.

A point worth mentioning is that Losey's and Quist's papers were tentative in tone. If not for the intervention of the media, these studies would only have occasioned an internal discussion within the scientific community. Even though the media intervened and fanned a furor that eclipsed an academic discussion, the scientific community followed its own logic to discuss, evaluate, and ultimately refute those studies. Interestingly, news about Quist's paper is still on the website of the University of California at Berkeley, but the news also mentions that the study was retracted and feature links to sources contradicting its findings.

As Flipse and Osseweijer pointed out, the process of academic evaluation is too slow and judicious to match the pace of the media, but academics have sufficient power as experts to influence decision-makers to follow rational and empirical principle when formulating policy. When considering the introduction of GM crops, decision-makers should also be sure to take the public's will into account. Therefore, the scientific and expert opinions of the scientific community should be effectively conveyed to the public and information from scientists should be disseminated as widely and as loudly as that from the media. This will help the public to receive balanced information to make rational decisions should controversial issues arise again in the future.

References

1. Séralini GE, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, Hennequin D, de Vendômois JS. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem Toxicol. 2012; 50:4221-4231.
2. Wallace Hayes A. Editor in Chief of Food and Chemical Toxicology answers questions on retraction. Food Chem Toxicol. 2014; 65:394-395.
3. Losey JE, Rayor LS, Carter ME. Transgenic pollen harms monarch larvae. Nature. 1999; 399:214.
4. Quist D, Chapela IH. Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. Nature. 2001; 414:541-543.
5. Sanders D, Kamoun S, Williams B, Festing M. Re: Séralini, G.-E., *et al*. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem. Toxicol (2012). Food Chem Toxicol. 2013; 53:450-453.
6. de Souza L, Macedo Oda L. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem Toxicol. 2013; 53:440.
7. Butler D. Hyped GM maize study faces growing scrutiny. Nature. 2012; 490:158.
8. Arjó G, Portero M, Piñol C, Viñas J, Matias-Guiu X, Capell T, Bartholomaeus A, Parrott W, Christou P. Plurality of opinion, scientific discourse and pseudoscience: An in depth analysis of the Séralini *et al*. study claiming that Roundup™ Ready corn or the herbicide Roundup™ cause cancer in rats. Transgenic Res. 2013; 22:255- 267.
9. Séralini GE, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, Hennequin D, de Vendômois JS. Republished study: Long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Environmental Sciences Europe. 2014; 26:14.
10. Delaney B, Appenzeller LM, Roper JM, Mukerji P, Hoban D, Sykes GP. Thirteen week rodent feeding study with processed fractions from herbicide tolerant (DP-Ø73496-4) canola. Food Chem Toxicol. 2014; 66:173-184.
11. Mesnage R, Defarge N, Spiroux de Vendômois J, Séralini GE. Letter to the Editor regarding "Delaney *et al*., 2014": Uncontrolled GMOs and their associated pesticides make

the conclusions unreliable. Food Chem Toxicol. 2014; 72:322.
12. China News Service. Yongyuan Cui was criticized by the President of China Agricultural University for his documentary film on genetically modified foods. *http://www.chinanews.com/cul/2014/06-18/6292872.shtml* (accessed February 3, 2015).
13. Article by a doctoral student in plant molecular biology. Scientific mistakes in Yongyuan Cui's documentary film on genetically modified foods. *http://www.guokr.com/article/438078/* (accessed February 4, 2015).
14. Flipse SM, Osseweijer P. Media attention to GM food cases: An innovation perspective. Public Underst Sci. 2013; 22:185-202.
15. Ewen SW, Pusztai A. Effect of diets containing genetically modified potatoes expressing Galanthus nivalis lectin on rat small intestine. Lancet. 1999; 354:1353-1354.

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