ISSN 1881-7815 Online ISSN 1881-7823

BST BioScience Trends

Volume 13, Number 4
August, 2019

IRCA BSSA

IACMHR

www.biosciencetrends.com

BST
BioScience Trends

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

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

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Editorial

DOI: 10.5582/bst.2019.01241

Challenges and advances in stem cell therapy

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Summary **After decades of research, stem cells have shown tremendous promise to treat a range of diseases, such as heart disease, diabetes, and neurodegenerative disease. However, many challenges remain in their transformation from bench to bedside, such as the potential risk of tumorigenicity from undifferentiated human induced pluripotent stem cells (hiPSC), the lack of large-scale culture systems for clinical treatment, and the inconvenience of cell therapy itself. "Cell sheet technology" and secretome therapy represent advances in these regards. With gradual breakthroughs in theory and technology, stem cell therapy will lead to a new medical revolution in the coming era.**

Keywords: Stem cell, tissue engineering, cell sheet, secretome, regenerative medicine

Stem cells have the potential to differentiate into more than 200 types of adult cells in the body (*1*). They provide new cells for the body as it grows and substitute for target cells that have been damaged (*2*). Stem cell therapy, also known as regenerative medicine, uses stem cells and their derivatives to promote repair of damaged, diseased, or dysfunctional tissue. Heart disease, diabetes, neurodegenerative disease, hair loss, and even aging are all expected to benefit from this therapy (*3*).

Although stem cell therapy has shown considerable promise, in reality many challenges remain in its transformation from bench to bedside, such as the potential risk of tumorigenicity from undifferentiated human induced pluripotent stem cells (hiPSC), the lack of large-scale culture systems for clinical treatment, and the inconvenience of cell therapy itself. "Cell sheet technology" for tissue engineering represents an advance in overcoming the drawbacks of traditional transplantation techniques. In this issue of *BioScience Trends*, Gao *et al*. (*4*). described their progress in combining "cell sheet technology" and hiPSC-derived cardiac cells to fabricate functional human cardiac tissues. Another advance in stem cell-related therapies is secretome therapy. Secretome therapy has several advantages that stem cell-based therapy lacks, such as the fact that secretomes can be manufactured and transported more easily and there is no need to match the donor and recipient to avoid rejection, so secretome therapy has attracted a lot of attention. A review from Xia *et al*. (*5*) describes more detailed merits and applications of stem cell secretomes in this issue.

Stem cell therapy is at the forefront of life science. With gradual breakthroughs in theory and technology, stem cell therapy will provide new possibilities in the treatment of diseases and disorders that yet cannot be or that are hard to cure and lead to a new medical revolution in the coming era.

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(Received August 26, 2019; Accepted August 31, 2019)

Policy Forum

DOI: 10.5582/bst.2019.01213

China has faster pace than Japan in population aging in next 25 years

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Summary **The aging of a population structure is an inevitable path of demographic transition, as an inescapable result of decline in fertility rate and extension in life expectancy. Although demographic transition occurred earlier in Japan than in China, the two countries had similar patterns, both of which took a much shorter period of time than Western countries to complete demographic transition, as well as have been aging at a rapid speed that has rarely been seen in the world. Japan has the highest level of population aging in the world, and China has been experiencing a very fast pace of the population aging process and has the largest older population. Drawing upon data from World Population Prospects (2019), this paper compares changes in population aging in both China and Japan. Findings show that Japan's aging process is 30 years ahead of China, but China has been changing in a similar way as Japan. To be specific, both countries experienced four phases of the population aging process: accelerated development period, rapid development period, slow down period and high-level maintained period. In addition, both countries had a quick growing rate of population aging. It will take China 23 years and 10 years respectively for the aging rate increasing from 7% to 14% and then to 20%, while Japan took 24 and 11 years respectively, which is much shorter than developed countries in the West. Furthermore, China has a faster pace than Japan in population aging in the next 25 years. We found that from 2019-2044, China's aging rate, elderly dependency ratio, oldest-old coefficient and median age of population will increase 13.24 percent points, 24.21 percent points, 8.33 percent points, and 8.47 years, while the four indicators of Japan will increase 8.38 percent points, 22.52 percent points, 8.29 percent points, and 6.20 years, respectively.**

Keywords: Population aging, China, Japan, comparative study

1. Introduction

Population aging is a dynamic process of increasing proportion of older adults in the total population, which is a result of low fertility rate and high life expectancy (*1*). The United Nations and the World Health Organization defines "aging society" as older population aged 65 years and over taking up 7% of the total population. When the percentage reaches 14%, it is called "aged society", where "super-aged society" refers to more than 20% of the population is over 65 (*2*). Population aging is now a global trend. World Population Prospects (2019) (hereafter as WPP2019) (*3*) reported that since 2002, the proportion of older adults aged 65 and over in the global population has exceeded 7%. The rate is expected to reach 14.14% in 2040, 20.04% in 2079, and 22.59% by the end of this century. The population aging process in Asian countries draws special attention due to its large total population. According to WPP2019, the proportion of older adults aged 65 and over in Asia reached 7.11% in 2013, and is predicted to exceed 14% and 20% in 2036

Released online in J-STAGE as advance publication August 21, 2019.

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and 2056 respectively (will be 14.10% and 20.13%, respectively). In particular, countries and regions in East Asia have entered into "aging society" in as early as 1996. They are expected to become "aged society" in 2022, and become "super-aged society" in 11 years (in 2033) (Figure 1).

China and Japan are the most important countries in East Asia, and share similar culture as well due to historical reasons. Although demographic transition in Japan happened earlier than in China, the two countries had similar patterns, both of which took a much shorter period of time than Western developed countries to complete demographic transition (*4*). Japan is the first country in Asia to enter into aging society with a rapid process of population aging, and it still has the highest level of population aging in the world (*3,5-7*). China is about 30 years later than Japan entering into aging society. However, China has been experiencing a very fast pace of the population aging process (*8*), and it will be faster than Japan is the coming period (*9*)

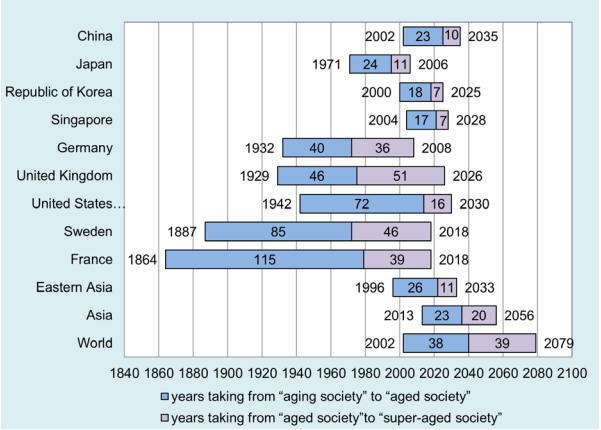

Figure 1. The year and time taken from "aging society" to "aged society", and to "super-aged society" in China, Japan, and other countries/regions. Data sources: The data before 1950 are from the 2018 edition of the white paper of elderly society (*10*), and the data during 1950-2100 are from WPP2019.

2. A Comparative study of the population aging in China and Japan

2.1. *Comparison of aging process in the two countries after entering into "aging society"*

It is generally accepted that Japan entered into "aging society" in 1970 (*10*), and China entered into "aging society" in 2000 (*11*). Japan was 30 years ahead of China. The data used in WPP2019 is slightly different from the domestic statistics of the two countries. WPP2019 (*3*) shows that Japan's aging rate was 6.88% in 1970, and 7.05% in 1971, China's aging rate reached 6.81% in 2000, 6.94% in 2001 and 7.08% in 2002. Therefore, according to WPP2019, the two countries entered into "aging society" in 1971 and 2002, respectively. In order to compare the patterns and future trend of population aging between the two countries, and with other countries and regions, this paper uses WPP2019 data. This section compares the speed and process of population aging between the two countries after the year when both entered into "aging society".

First of all, we compared the speed of population aging in China and Japan by measuring how long it takes for the two countries to become "aged society" from "aging society"; and to become "super-aged society" from "aged society", respectively. China is predicted to enter into "aged society" in 2025, and become "super-aged society" in 2035. It will take 23 years for China to complete the process from an "aging society" to an "aged society", and 10 years from an "aged society" to "super-aged society". Japan has already entered into "aged society" and "super-aged society" in 1995 and 2006, respectively. It took 24 years and 11 years for Japan to complete these two processes. China and Japan are both aging at a much faster rate (Figure 1).

In contrast, it took western developed countries longer to complete these two processes. For instance, France took 115 and 39 years, Sweden took 85 and 46 years, and Germany took 40 and 36 years. The United States of America and the United Kingdom took 72 years and 46 years to complete the process from 7% to 14%, respectively. The United States of America entered "aged society" in 2014 and is predicted to become "super-aged society" in 2030. It will take 16 years. Also, The United Kingdom has already entered "aged society" in 1975 and is predicted to become "super-aged society" in 2026.

We further analyzed the process of population aging during the 100 years since the two countries entered into "aging society". The time span of WPP2019 data is 1950-2100. We compared the changes of aging rate between 1971-2069 in Japan and 2002-2100 in China. *i*) The first stage: the first 16 years of entering into "aging society" (corresponding to the data in China from 2002 to 2017 and Japan from 1971 to 1986), the level of aging in China is close to, but slightly lower than of that in Japan. At this stage, both countries aged fast. *ii*) The second stage: 26 years later (corresponding to China's data in 2018-2044 and Japan's data in 1987-2013), China's aging level is close, but slightly higher than Japan's. At this stage, the aging rate of the two countries is faster than that of the first stage. China's aging rate is predicted to rise from 10.92% in 2018 to 24.71% in 2044, and Japan's aging rate rose from 10.71% in 1987 to 24.63% in 2013. *iii*) The third stage: for the remaining 56 years (corresponding to the data of 2045-2100 in China and 2014-2069 in Japan), China's aging level is lower than that of Japan. Especially after 55 years entering into "aging society", Japan's aging rate is significantly higher than that of China. At this stage, the speed of population aging in China is generally slower than that of Japan. China's speed of aging is predicted

to slow down in 55 years after entering into "aging society", and the aging rate will reach 30%. Since then, China's aging level will get into a plateau period, maintaining at a level of 31%. The speed of aging in Japan might slow down in 70 years after stepping into "aging society". About 80 years later, Japan's aging process is predicted to reach a plateau period, and the level of aging basically will maintain at 38%. Overall, the two countries both experience the acceleration period, the very rapid period, the slowdown period and the high level platform period of aging during one hundred years after entering into "aging society".

2.2. *China has a faster pace in population aging than Japan in the next 25 years*

The year 2018 is the time when the process of population aging in China turns from an accelerated development period to a rapid development period. And China will be experiencing the fastest speed of population aging in the next 25 years. In the above section, we have compared the aging rate in China and Japan in the same period of time when both entered into aging society. It was found that the aging rate was slightly higher in China than that in Japan during this rapid development period. In this section, we will compare the aging process between China and Japan over the next 25 years.

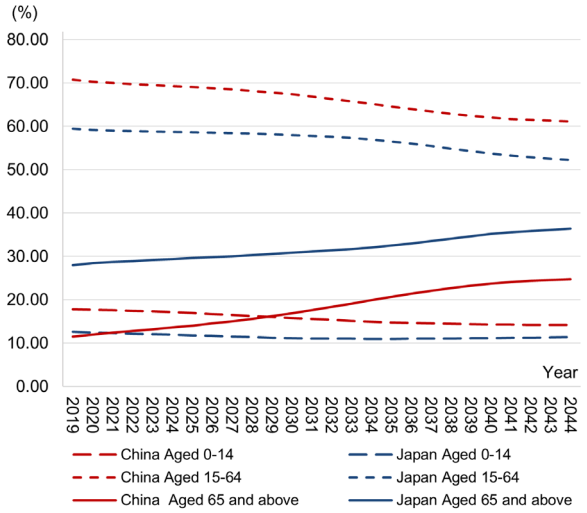

Figure 2. Population age structure changes in China and Japan 2019-2044.

Figure 2 presents the changes in age composition in China and Japan from 2019 to 2044. The proportion of children aged 0-14 years in China is higher than that in Japan, however, the rate will decrease faster in China than that in Japan, resulting in a narrower gap between these two countries. The proportion of working-age population aged 15-64 in both countries is declining. Although this proportion in China has been greater in China than that in Japan, the decline rate is larger than that in Japan as well. Moreover, the proportion of older population aged 65 and over in both countries is increasing, and China has a more rapid pace of aging than that in Japan. All of these numbers demonstrate that China's population aging process will be faster than Japan's in the next 25 years.

In addition to the aging rate, certain other indicators have also been commonly used to measure the process of population aging, including the elderly dependency ratio (refers to the ratio of older dependents (people aged 65 and above) to the working-age population (those aged 15-64)), the oldest-old coefficient (refers to the proportion of people aged 80 and over in the population of people aged 65 and over), and the median age of total population (*6,12-13*). Table 1 shows the aging process of China and Japan between 2019 and 2044 using these indicators. To be specific, in the next 25 years, China's aging rate will increase from 11.47% to 24.71%, an increase of 13.24 percent points; where Japan's aging rate will increase from 28.00% to 36.38%, an increase of 8.38 percent points. Regarding the elderly dependency ratio, China will raise from 16.22% to 40.43%, an increase of 24.21 percent points; where Japan will raise from 47.12% to 69.64%, an increase of 22.52 percent points. With respect to the proportion of the elderly aged 80 and above in the elderly, China will climb from 15.90% to 24.23%, an increase of 8.33 percent points; while Japan will increase from 31.02%

Table 1. Comparison on Selected Indicators of Population Aging in China and Japan 2019-2044

Year	Aging rate		Elderly dependency ratio		Oldest-old coefficient		Median age of the total population	
	China	Japan	China	Japan	China	Japan	China	Japan
2019	11.47	28.00	16.22	47.12	15.90	31.02	—	—
2020	11.97	28.40	17.02	48.01	15.45	31.61	38.42	48.36
2025	14.03	29.63	20.32	50.57	15.40	35.64	40.23	50.48
2030	16.87	30.87	25.04	53.22	16.70	41.50	42.65	52.07
2035	20.68	32.51	32.02	57.52	19.84	42.82	44.99	53.28
2040	23.73	35.17	38.27	65.52	20.92	40.00	46.32	54.12
2044	24.71	36.38	40.43	69.64	24.23	39.31	47.16	54.56
Change	13.24	8.38	24.21	22.52	8.33	8.29	8.74	6.20

Data sources: WPP2019.
Note: Due to the median age of total population in WPP2019 are separated by 5 years, the median age of total population of 2009 in Table1 is vacant, and the median age of total population of 2044 in Table1 is the data of 2045 in WPP2019 database.

to 39.31%, an increase of 8.29 percent points. Lastly, the median age of total population in China will rise from 38.42 years in 2020 to 47.16 years, an increase of 8.74 years, and the media age of total population in Japan will rise from 48.36 years to 54.26 years, an increase of 6.20 years. In sum, these four indicators show that the population aging process in China will be quicker than that in Japan in about 25 years.

2.3. *Reasons for population aging in China and Japan*

Population aging process is earlier in Japan than that in China, however, the common feature in both countries is the vast rapid speed (*14*). The aging of population structure is an inevitable path of demographic transition, as the inescapable result of decline in fertility rate and extension in life expectancy (*15*).

Low fertility rate has been considered as the most important determinant for population aging (*16*). China's total fertility rate (the number of children born to each woman in her lifetime) was 6 in 1950s and 1960s. Due to the development of social economy and the implementation of the family planning policy, the total fertility rate has dropped significantly in 1970s (*17*). It dropped to 3 at the end of 1970s, to 2.5 in 1980s, below the threshold of 2.1 in 1990s, and has been below 2.1 for three decades since then. It reached an historical low of 1.6 in 2000. The estimation in WPP2019 should take into account the impact of family planning policy on fertility in China, therefore, the birth rate was set to 1.70~1.75 between 2020-2050. Japan is the first non-Western country that was found to track the typical transition pattern (*18*). The total fertility rate has fallen much earlier in Japan than that in China. It has dropped to around 2.5 in 1950s, to about 2 in 1960s, and slightly rose to 2.13 in 1970-1975, then dropped back to 1.83 in 1975-1980. Japan's total fertility rate continued to decline in 1980s and in 1990s, and reached the lowest point of 1.3 during 2000-2005. Though it has increased a bit since 2005, the average is still lower than 1.4. The predication in WPP2019 should consider the effect of policy in encouraging giving birth in Japan, and set the total fertility rate in Japan as a slow increase in 2020-2050, reaching 1.55 in 2050 (Figure 3). In sum, China's fertility rate dropped sharply in the second half of the 20th century, which was far greater than Japan's. While in the first half of the 21st century, the two countries' fertility rates did not change much. China was relatively higher than Japan, but was still below the replacement level.

Another critical factor for population aging is the decline of mortality, especially among older adults (*16*). In Japan, since low fertility rates have been maintained for a long time, the more prominent reason for the growing number of aging population is low death rate of the elderly (*18*). In the second half of last century, life expectancy has greatly increased in both China and

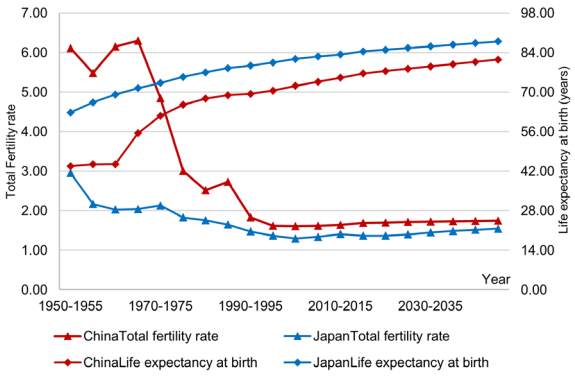

Figure 3. Fertility decline and life expectancy increase in China and Japan (1950-2050). Data sources: WPP2019.

Japan. China's average life expectancy has improved from 43.83 to 70.58, an increase of 26.75 years; Japan's average has advanced from 62.80 to 80.51, an increase of 17.71 years. During the same time period, the world's average life expectancy has increased from 46.96 to 65.63, an increase of 18.67 years, and Asia's has extended from 42.30 to 66.55, an increase of 24.25 years. We can see that China has the greatest growth rate in terms of life expectancy. Japan has already had the highest life expectancy, and its growth rate is not as great as China's. However, Japan's life expectancy is still about 10 years higher than China's at the end of 1990s. Since 2000, although the two countries' life expectancies continued to grow, the gap between them became narrower, reaching 76.62 years and 84.43 years in China and Japan respectively during 2015-2020. It is estimated by WPP2019 that life expectancy will reach 81.52 years and 87.94 years in China and Japan respectively by 2050. Similarly, the world's average life expectancy at birth will increase from 67.05 to 76.77 years during the same period, an increase of 8.44 years. Asian's average life expectancy at birth will increase from 68.33 to 77.89, an increase of 9.56 years. Overall, China has a much faster improvement rate in life expectancy than that of Japan, worldwide and Asia. China's mortality rate has declined more obviously than Japan's; however, China's life expectancy has always been lower than Japan's (Figure 3).

3. Conclusion

Due to declining fertility rate and extended life expectancy, China and Japan have experienced and will continue to encounter a rapid population aging process. Japan is about 30 years earlier than China entering into aging society, and has a higher level of aging than that of China as well; nevertheless, China will run into a faster pace than Japan in aging process in the coming 25 years. Population aging and its consequences raise challenges and concerns for society, family, and older adults. Compared to China, Japan has more advanced

social-economic development and policies and strategies to deal with population aging, as well as has greater capacity for coping with challenges. China has a lower level of population aging than that of Japan, however, the number of older adults in China is several times more than that of Japan (4.6 times in 2019 and 8.8 times in 2044). Therefore, China will face even more severe issues and challenges because of its rapid process of population aging and continued increase in the size of older population over a long period of time. The outpaced growth of aging population over that of economic development and social security system in China may put tremendous pressures on older individuals, their families, as well as on society (*19-21*).

Acknowledgements

This work was supported by the National Natural Science Foundation of China 71490734 (to Rong Chen and Meifeng Wang).

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(Received June 26, Revised August 12, 2019; Accepted August 18, 2019)

Review

DOI: 10.5582/bst.2019.01227

Recent progress in induced pluripotent stem cell-derived cardiac cell sheets for tissue engineering

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Summary **The past decade has witnessed remarkable development in tissue engineering technologies and stem cells. Our lab has developed a novel technology — "cell sheet technology" for tissue engineering. After the confluent cells are cultured on an innovative temperature-responsive culture dish, the cells can be harvested as an intact sheet by lowering temperature. We have successfully created multiple cell sheet-based tissues for therapies of a vast variety of diseases, in particular, myocardial diseases. On the other side, the discovery of human induced pluripotent stem cells (hiPSC) enables stable production of defined tissue-specific cell types and thus makes it possible to regenerate tissues or even organs for clinical application and in vitro drug screening/disease modeling. Recently, we have combined cell sheet technology and hiPSC-derived cardiac cells for fabrication of functional human cardiac tissues. This review summarizes ongoing challenges in this field and our progresses in solving issues, such as large scale culture of hiPSC-derived cardiac cells, elimination of undifferentiated iPSCs to decrease the risk of tumor formation as well as myocardial tissue fabrication technologies.**

Keywords: Cell sheet engineering, Induced pluripotent stem cell-derived cardiac cells, Large-scale suspension culture systems

1. Introduction

Various multipotent adult stem cells have been studied for repairing cardiac tissues, such as mesenchymal stem cells (*1,2*), bone marrow stem cells (*3*), skeletal myoblasts (*4*), and cardiac stem cells (5,6). However, low cardiogenic ability, insufficient survival rate and unestablished strategy to produce a large amount of cardiac cells limit the clinical application of adult stem cells. As a landmark breakthrough in science, human induced pluripotent stem cells (hiPSC) (*7,8*), which are able to differentiate into almost any tissue specific cell type, have received tremendous concern. When compared to human embryonic stem cells (ESC), hiPSCs circumvent ethical problems and are easily accessible. Technologies to induce differentiation of hiPSCs into various human somatic cells have been established (*9-13*). In particular, hiPSCs have been demonstrated to be able to differentiate into cardiac cells at high efficiency (*14*). Thus, human cardiac cells, which have been quite difficult to obtain previously, become available for research. Therefore, hiPSC-derived cardiac cells are considered to be the most promising cell source for heart regeneration. However, there are a number of factors that have hindered breakthroughs in clinical application of hiPSC-derived cardiac cells. These include the potential risk of tumorigenicity from undifferentiated hiPSCs and the lack of large-scale culture systems for clinical treatment (*15,16*).

Besides stem cells, transplantation techniques are also important for successful cell-based therapies. For example, dissociated cell injection has been demonstrated to be difficult to survive and retain cells around the target tissue (*17,18*). To solve this problem, we have pioneered scaffold-free 'cell sheet-based tissue engineering' (*19*). Multilayered *in vivo*-like cell-dense tissues, such as heart (*20-25*), have been constructed by using this technology. When compared to scaffold-

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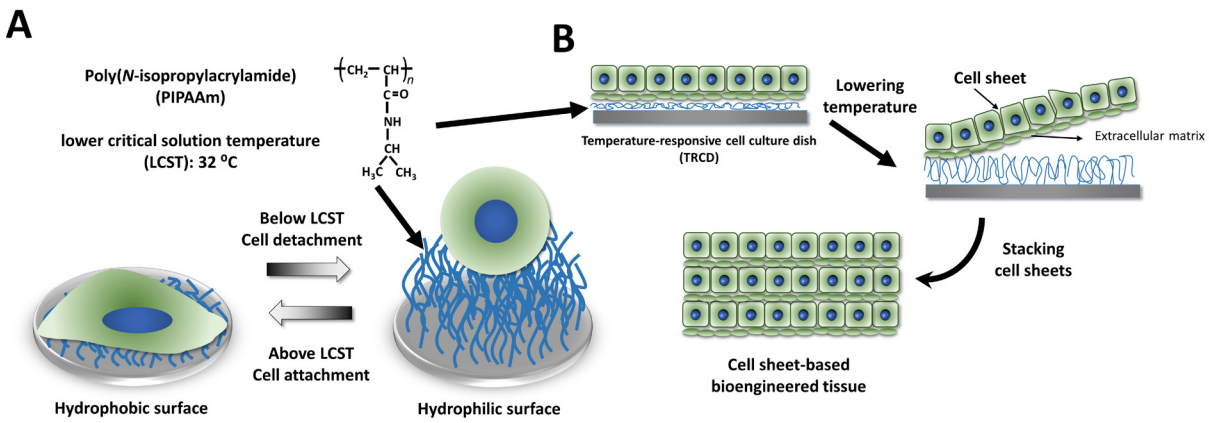

Figure 1. (A) Temperature-dependent changes of hydrophilicity/hydrophobicity of PIPAAm coated temperature-responsive cell culture dishes (TRCDs) allow controllable cell attachment/detachment. **(B)** Confluently cultured cells on a TRCD can be harvested as a free cell sheet, which retains the extracellular matrix and cell-cell junctions. Cell sheets can be further stacked to fabricate 3D bioengineered tissues.

based tissue engineering, scaffold free cell sheet-based tissue engineering does not have the problem of inflammatory responses, which are usually found during biodegradation of the scaffolds and lead to the failure of transplantation (*26*).

We have developed an innovative temperature-responsive cell culture dish (TRCD) to harvest cell sheets by reducing temperatures (*27,28*). The TRCDs are conventional culture dishes that are covalently immobilized with temperature-responsive polymer poly(N-isopropylacrylamide) (PIPAAm) at nanometer level thickness. As shown in Figure 1A, the TRCDs are hydrophobic when the temperature is above PIPAAm's lower critical solution temperature (LCST) of 32°C, so cells adhere to TRCDs stably. However, when the temperature is reduced below 32°C, the TRCDs become hydrophilic and enable cell detachment. If cells are confluently cultured on TRCDs, an intact sheet can be obtained by lowering the temperature (Figure 1B). A 'cell sheet' is composed of a sheet-like monolayer of cells and attached extracellular matrix (ECM) layer below the cells. The cell sheets can be stacked to form thick, multilayered tissues, mimicking stratified structure of the *in vivo* environment (Figure 1B). Without using enzymes, cell-cell junction and ECM proteins can be retained in the cell sheet, which preserve cell functions and facilitate adhesion of cell sheets onto target sites (*29,30*).

Until now, we have published many reviews to introduce our work on both basic research and clinical application of cell sheet-based tissue engineering (*31-41*). In this review, we mainly focus on our recent progresses relevant to hiPSCs.

2. Large-scale culture systems for cardiac differentiation from hiPSCs

When fabricating human tissues with hiPSCs for transplantation, a huge amount of cells is required. For example, 1-2 × 10^9 cardiac cells are necessary to repair the cardiac tissue of one patient after myocardial infarction (*42*). Therefore, establishment of large-scale culture methods of hiPSCs is an indispensable technology for their clinical application. Due to limited culture space, conventional two-dimensional (2D) culture systems do not allow large-scale production of hiPSCs, whereas three-dimensional (3D) suspension culture systems have demonstrated their ability to scale up hiPSCs production (*43,44*).

We have reported that a stirred suspension culture bioreactor combined with appropriate growth factors enables effective and easy production of mouse ESC-derived cardiomyocytes (*45*). However, the number of cardiomyocytes produced by this bioreactor is relatively low due to the change in culture conditions, such as lactate accumulation and pH decreases in the medium, which limit cell proliferation and differentiation. In order to maintain suitable culture conditions, we have integrated a continuous perfusion system into the stirred suspension culture bioreactor. This novel bioreactor, which allows embryoid body (EB) formation of mouse ESCs, is capable of expanding the cell number by up to 300-fold of the initial seeding cell number and efficiently promoting cardiac differentiation as well (*46*).

Compared with mouse ESCs, hiPSCs die easier in the single cell state. EB formation is indispensable for 3D suspension culture and low shear stress agitation is the key point for high density large-scale culture. Expansion of hiPSCs in the undifferentiated state through EB formation when using a suitable commercially available spinner flask at the optimal agitation rate has been reported (*47*). However, shear stress in 3D suspension culture is a bigger hurdle for the process of differentiation. Since hiPSCs just proliferate and secrete various types of extracellular matrix in the process of the undifferentiated expansion process, it

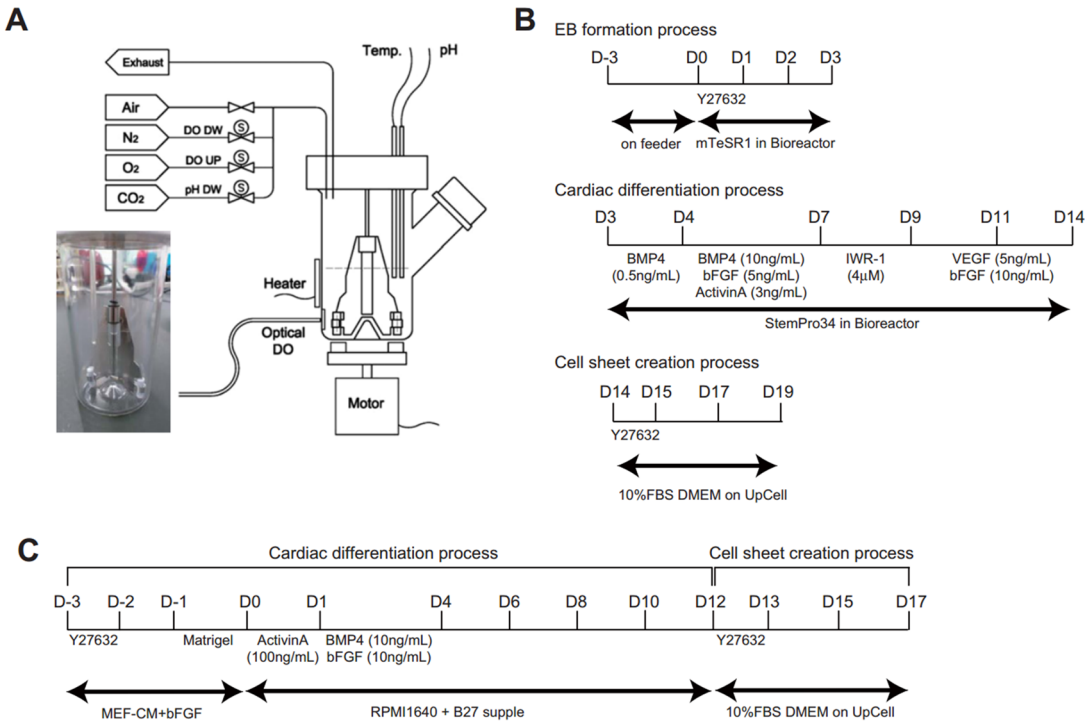

Figure 2. Scheme of the bioreactor and culture process (A) Schematic of the bioreactor system. The photograph shows the impeller. **(B)** Schematic of the culture process for cardiac differentiation in the bioreactor system. **(C)** Schematic of the culture process for cardiac differentiation in monolayer culture. This figure and the figure legend are reproduced with permission from ELSEVIER. (*48*)

might be easier to regulate EB formation. On the other hand, according to the changes of the characteristics of hiPSC-derived cells in the process of differentiation, EBs become fragile and a lower shear stress bioreactor is necessary. Recently we have developed a new type of bioreactor with a delta shaped impeller and it has enabled us to generate robust numbers of cardiomyocytes (> 1.0×10^8 cells/100mL bioreactor) from hiPSCs through an optimized concentration of growth factors, a small compound and dissolved oxygen concentration in 2 weeks (Figure 2) (*48*). This culture strategy has been already applied for not only cardiomyocyte production for preclinical study in a large animal model (*49*), but also other types of hiPSC-derived cells such as pancreatic progenitor cells (*50*), pancreatic exocrine cells (*51*), thyroid follicular cells (*52*) and vascular endothelial cells (*53*).

3. Elimination of undifferentiated hiPSC-derived cardiac cells

Any remaining undifferentiated hiPSCs may generate tumors after transplantation, but there is no differentiation protocol to promise 100% cardiac differentiation efficiency. Therefore, it is necessary to purify the differentiated cardiomyocytes. We have reported several protocols to eliminate undifferentiated hiPSCs (*54-57*).

First, we have demonstrated for the first time that methionine free culture medium is capable of efficiently eliminating undifferentiated hiPSCs without affecting viabilities of differentiated hiPSCs, such as cardiomyocytes and fibroblasts (*54*).

Second, we have demonstrated that TRPV-1 activation through transient culture at 42°C and an agonist is able to eliminate undifferentiated hiPSCs from bioengineered cardiac cell sheet tissues (*55*). TRPV-1 expression levels are significantly higher in hiPSCs than that in hiPSC-derived cardiomyocytes and apoptosis of hiPSCs at 42°C is TRPV-1-dependent. At the same time, this thermal change does not affect gene and protein expression levels of hiPSC-derived cardiomyocytes and fibroblasts. By using this approach, the final undifferentiated iPSCs in the cardiac tissues is only 0.4%.

Both of the above two methods do not bring in extra substances, so there is no worry about the effects of remaining materials in the regenerative medicine final product. However, more quantitative data about the elimination effects of these methods is needed. In addition, their impact on subsequent tumor prevention needs to be examined. In our recent study (*56*), we have demonstrated that culture at 42°C for 2 days eliminates 1×10^2 hiPSCs in fibroblast cell sheets and successfully prevents tumor formation in a nude rat model (Figure 3). Furthermore, the combination of methionine-free medium and 42°C culture sufficiently eliminates remaining robust hiPSCs (1×10^4 hiPSCs) in fibroblast

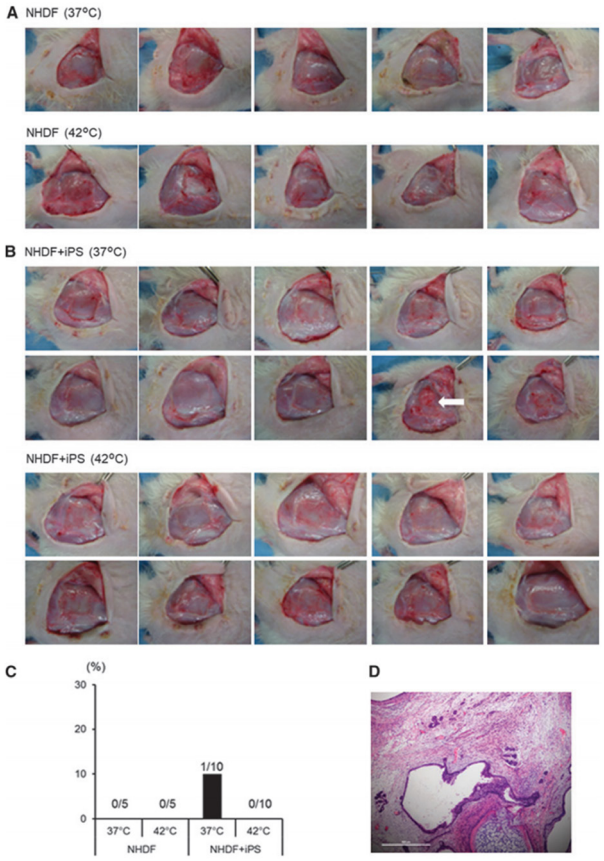

Figure 3. Evaluation of tumorigenicity of fibroblast sheets with 1 × 10^2 iPS cells. One day after starting co-culture of human dermal fibroblasts (1 × 10^5 cells) and human iPS cells [0 (A) and 1 × 10^2 cells (B)] on 24-well temperature-responsive culture plates, cells were cultured at 37°C or 42°C for 2 days until day 3 and then cultured further at 37°C until day 5. Monolayered cell sheets were then transplanted onto subcutaneous tissues of nude rats. **(A, B)** Macroscopic images of transplanted regions at 3 months after transplantation (A, *n* = 5 for each condition; B, *n* = 10 for each condition). Arrow indicates a tumor. **(C)** Percentages of tumor formation. **(D)** Representative image of a HE-stained tumor. Scale bar, 500 mm. HE, hematoxylin and eosin. This figure and the figure legend are reproduced with permission from Mary Ann Liebert, Inc. (*56*)

cell sheets and prevents tumor formation and tumor-related death.

In another study, we demonstrated that dinaciclib, the cyclin-dependent kinase 1/9 inhibitor, selectively eliminates undifferentiated hiPSCs without affecting the viability of cardiac cells (*57*). Nanomolar concentrations of dinaciclib is enough to induce DNA damage and upregulation of p53 protein levels in undifferentiated hiPSCs but not hiPSC-derived cardiomyocytes, this is because of a difference in MCL-1 transcriptional downregulation and MCL-1 degradation between hiPSCs and hiPSC-derived cardiomyocytes through the treatment with dinaciclib. A beating hiPSC-derived cardiac cell sheet can be fabricated after treatment with dinaciclib. In addition, combining dinaciclib with methionine-free medium and 42°C may further enhance the ability to prevent tumor formation (*56*).

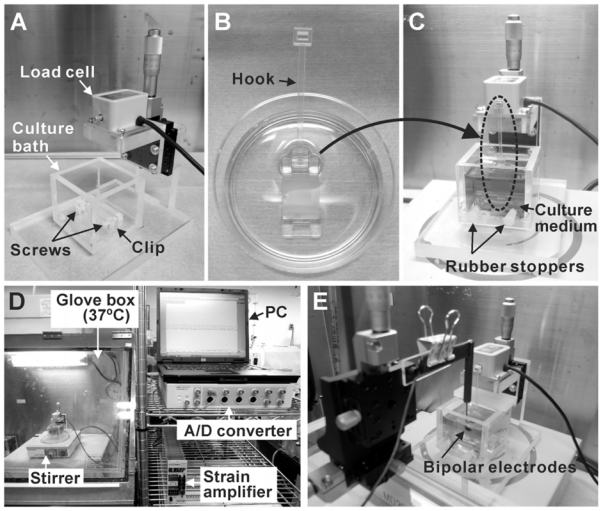

Figure 4. Configuration of contractile force measurement system. (A) The appearance of a contractile force measurement device. **(B)** The hook made by a 3D printer was fixed to the handle. **(C)** The cardiac cell sheet-tissue was mounted to the force measurement device vertically and fresh medium (Medium D) was poured. **(D)** The entire appearance of a contractile force measurement system. **(E)** The appearance of electrical pacing system. This figure and the figure legend are reproduced under the terms of the Creative Commons Attribution License, which permits unrestricted use. (*62*)

4. Cardiac tissue fabrication using hiPSC-derived cells

Previously, we have succeeded in fabrication of beating myocardial tissue *in vivo* by stacking neonatal rat cardiomyocyte sheets (*24*). Further, millimeter scale cardiac tissues are successfully fabricated *via* repeated transplantation of triple-layer cardiomyocyte sheets (*23*). By using the same polysurgery strategy, we recently have succeeded in fabrication of thick, functional human cardiac tissues from hiPSC-derived cardiac cells in a nude rat (*58*). hiPSC-derived cardiac cells are able to proliferate and become mature *in vivo*. The transplanted hiPSC-derived myocardial grafts survive, show spontaneous beating *in vivo* and demonstrate well-organized vascular networks.

In addition to sheet-like grafts, we have fabricated tubular tissues *via* wrapping neonatal rat cardiomyocyte sheets (*59,60*). The myocardial tubes are capable of generating measurable inner pressure changes through myocardial tube contraction *in vitro* (*59*). Furthermore, *in vivo* myocardial tubes have been successfully fabricated by wrapping rat cardiac cell sheets around resected adult rat thoracic aorta and transplanting the tube grafts in place of the abdominal aorta of athymic rats (*60*). The *in vivo* myocardial tubes generate inner pressure changes of about 6mm Hg according to their contraction. Recently, we reported a successful fabrication of human tubular cardiac tissues derived from hiPSCs (*61*). We wraped triple-layered cell sheets around the inferior vena cava of nude rats. Two months

later, the maximum inner pressure changes were around 9.1mm Hg under electrical stimulation. The mRNA expression of several contractile proteins in cardiac tissues at two months *in vivo* were significantly higher than that at one month. These tubular human cardiac tissues that can generate pulse pressure *in vivo* may contribute to the development of a bioengineered heart assist pump.

Besides cell-based therapies, hiPSCs can be used in development of human cell-based models for drug discovery. In addition, patient-specific hiPSCs allow customized *in vitro* models, which may be helpful in screening drugs that are effective on specific patients. Recently, we have developed a novel measuring system to dynamically detect the contractile force of hiPSC-derived cardiomyocyte sheets (Figure 4) (*62*). The cell sheets are attached to a thin fibrin gel for stable conduction of contractile force. Then the cell sheet/gel tissues are adapted to the measuring system and the real-time contractile force can be monitored clearly. The contractile force of the beating tissues are around 1 mN, and the mean force value per cross-sectional area is 3.3 mN/mm^2. The generated contractile forces are equal or higher than that of previous reported values. To verify whether this system can be used for drug screening, adrenaline is administered to hiPSC-derived cardiac tissues. As expected, both the contractile force and the beating rate significantly increase, reproducing the same behavior as *in vivo* cardiac tissues. Therefore, this force measurement system has great potential to be applied for *in vitro* drug testing.

In addition, we have recently reported an *in vitro* human non-alcoholic steatohepatitis (NASH) model by co-culture of primary human hepatocytes (PHH) and human fibroblasts in a cell sheet-based bioengineered tissue (*63*). As far as we know, this model is the first *in vitro* model that recapitulates hepatocellular ballooning, which is the key histological feature of NASH and used for the diagnosis of NASH. Although PHHs are the gold standard of *in vitro* human liver model, they are scarce, expensive and short-lived *in vitro*, limiting their application. Therefore, we are now considering development of hiPSC-derived hepatocytes in place of PHHs for building a cell sheet-based human NASH model.

5. Conclusion

We have done a lot of work in development of hiPSC technology and hiPSC-derived cardiac tissues, but still many issues need to be solved. For example, although we have succeeded in creating thick, vascularized cardiac tissues *in vitro* using neonatal rat cardiomyocytes (*64,65*), it is a big challenge to reproduce the same result using hiPSC-derived cardiac cells. Therefore, we are currently developing suitable perfusion systems for vascularization of hiPSC-derived cardiac tissues *in vitro*. Another issue is an unclear maturation mechanism of hiPSC-derived cardiac cells. When hiPSC-derived cardiac cells just finish differentiation, they are still immature and very different from cardiomyocytes *in vivo*. Study of the maturation process needs to be investigated. In addition to cardiomyocytes, functions of other cell types, such as cardiac fibroblasts, need to be cleared more. For example, we have found that cardiac fibroblasts possess anti-angiogenic properties mediated by Ly6/Plaur domain-containing 1 (LYPD1) (*66*). Thus, inhibition of LYPD1 may contribute to the fabrication of vascularized functional bioengineered tissues. We believe that unremitting efforts on development and integration of advanced technologies, such hiPSC technology, cell sheet technology, CRISPR technology and so on, will help us to achieve our final goal – regeneration of human hearts to save lives.

Conflict of Interest: Tatsuya Shimizu is a member of the scientific advisory board and a stakeholder of CellSeed Inc. Tatsuya Shimizu and Katsuhisa Matsuura are inventors of a bioreactor system for PSC culture, the patent of which is held by Able Co. and Tokyo Women's Medical University (US9574165B2). Tokyo Women' s Medical University receives research funds from CellSeed Inc. Able Co. and Nihon Kohden Co.

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(Received August 13, 2019; Accepted August 31, 2019)

Review

DOI: 10.5582/bst.2019.01226

Stem cell secretome as a new booster for regenerative medicine

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Summary **Stem cells are an undifferentiated cell population that has the ability to develop into many different cell types and also has the ability to repair damaged tissues in some cases. For a long time, the stem cell regenerative paradigm has been based on the assumption that progenitor cells play a critical role in tissue repair by means of their plasticity and differentiation potential. However, recent works suggest that the mechanism underlying the benefits of stem cell transplantation might relate to a paracrine modulatory effect rather than the replacement of affected cells at the site of injury. This paracrine modulatory effect derives from secretome which comprises a diverse host of growth factors, cytokines, chemokines, angiogenic factors, and exosomes which are extracellular vesicles that are produced in the endosomal compartment of most eukaryotic cells and are from about 30 to several hundred nanometers in diameter. The role of these factors is being increasingly recognized as key to the regulation of many physiological processes including leading endogenous and progenitor cells to sites of injury as well as mediating apoptosis, proliferation, migration, and angiogenesis. In reality, the immunomodulatory and paracrine role of these factors may mainly account for the therapeutic effects of stem cells and a number of *in vitro* and *in vivo* researches have proved limited stem cell engraftment at the site of injury. As a cell-free way for regenerative medicine therapies, stem cell secretome has shown great potential in a variety of clinical applications including prevention of cardiac disfunction, neurodegenerative disease, type 1 diabetes, hair loss, tumors, and joint osteoarthritis.**

Keywords: Secretions, stem cells, regenerative medicine, exosomes, conditioned medium

1. Introduction

Study results of the application of stem cells in various diseases are accumulating. Some researches showed beneficial effects of stem cell therapy in degenerative diseases such as myocardial infarction and revealed that stem cells cause tissue repair due to their ability to secrete molecules that perform a beneficial impact on the damaged tissue, rather than their capacity to differentiate into the necessary cells (*1*). Various studies on stem cell-derived molecules showed that the secreted factors alone without the stem cell itself may cause tissue repair in various diseases. The secreted factors are referred to as secretome, including growth factors, cytokines, chemokines, angiogenic factors, and exosome and can be found in the medium where the stem cells are cultured. The medium is called conditioned medium (*2*).

The application of secretions from medium has several merits compared to the application of stem cells, for medium can be manufactured, freeze-dried, packaged, and transported more easily. Moreover, as it is cell-free, there is no need to match the donor and the recipient to avoid a rejection reaction. Therefore, stem cell secretome has a promising role for regenerative medicine.

2. Brief overview of stem cell secretome

2.1. *What is the stem cell secretome*

Stem cell secretome is a collective term for the soluble factors produced by stem cells and utilized for their inter-cell communications (*3*). The secretome is thought to be encoded by approximately 10% of

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the human genome and includes a diverse array of serum proteins, growth factors, angiogenic factors, hormones, cytokines, extracellular matrix proteins, extracellular matrix proteases, hormones, and even, in low abundance, lipid mediators and genetic material (*4,5*). These secreted molecules are released by stem cells through classical and non-classical secretion mechanisms, including protein translocation, exocytosis, and vesicle or exosome encapsulation (*6,7*). The soluble factors and vesicles secreted by stem cells may act directly by mediating intracellular pathways in injured cells, or indirectly, by inducing the secretion of functionally active products from adjacent tissues.

2.2. *Why will we use secretome?*

The utility of cell-free therapies in regenerative medicine has more merits than conventional stem cell based therapies. The utility of secreted molecules could potentially avoid immune compatibility, tumorigenicity, and transmission of infections in stem cell therapies. Secretome could also largely reduce the cost and time associated with expansion and maintenance of cell lines because secretome could be prepared beforehand in large quantities and could be promptly available for treatment when needed. This makes their application suitable for emergencies such as myocardial infarction, cerebral ischemia, or trauma.

3. Various roles and mechanisms of secretome

3.1. *Tissue repair*

Stem cells showed advantageous therapeutic effects on tissue repair and wound healing without a significant degree of tissue engraftment at the site of injury (*8*). This research result in a number of animal models of multiple diseases led to the hypothesis that secretome of stem cells rather than direct tissue differentiation and engraftment may play a leading role in tissue repair. This assumption has been tested in a number of different clinical therapies *via* the utility of stem cell conditioned medium. The study of cell-specific secretions often begins from cell culture. However, *in vitro* researches can't totally simulate and test the full view of secretions in the microenvironment *in vivo*, studies quest to replicate the effects of the stem cell secretome *via* the utility of medium conditioned by stem cells (*9*). Study has demonstrated that the utility of stem cell conditioned media alone can replicate the therapeutic effects previously observed with the utility of stem cells directly (*1,10,11*).

3.2. *Angiogenesis*

Stem cells and their secretome play an important part in regulation of angiogenesis that has been validated both *in vitro* and *in vivo*. Great interest emerges in the role of stem cells in angiogenesis for there are a lot of clinical diseases related to insufficient angiogenesis, including atherosclerotic diseases like coronary artery disease and peripheral vascular disease, and wound healing disorders, as well as a great number of diseases related to pro-angiogenic factors such as chronic kidney disease, tumor growth and metastasis, and proliferative retinopathy (*12*). Angiogenesis is defined as the physiological process through which new blood vessels form from pre-existing vessels, formed in the earlier stage of vasculogenesis. Angiogenesis continues the growth of the vasculature by processes of sprouting and splitting [wikipedia]. A great deal of secreted molecules such as growth factors, chemokines, enzymes, matrix metallopeptidases, and adhesion molecules strictly regulate this process (*13*).

A great number of angiogenesis related molecules have been identified in stem cell secretome by antibody-based assays like ELISA assays, and immunohistochemistry methods. The secretions include vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), interleukin-6 (IL-6), and placental growth factor (PGF), angiopoietin-1, monocyte chemoattractant protein-1 (MCP-1), and cysteine-rich angiogenic inducer 61 (Cyr61) (*14-18*). Investigations have also found that the secretion of these angiogenic factors can be regulated by numerous chemokines and hypoxic conditions. For example, research by De Luca *et al*. showed that transforming growth factor alpha (TGF-α) had the ability to up-regulate the level of a few growth factors (VEGF, HGF, platelet-derived growth factor BB, IL6- and IL-8) in the stem cell secretome (*19*). Conditioned medium treated with TFG-α induced much more blood vessel growth compared to control medium *in vivo*. A few *in vitro* studies have validated the effects of stem cell secretome on each key step in angiogenesis. For instance, some mesenchymal stem cells like adipose-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells have the ability to induce endothelial cell proliferation, migration, and tube formation, as well as prevent endothelial cell apoptosis *in vitro* (*20-23*). Successful application of stem cells to increase angiogenesis has been demonstrated in animal models for peripheral artery disease, myocardial infarction, cerebral ischemia/stroke, stress urinary incontinence, and neurogenic bladder disease among other diseases (*24-26*).

3.3. *Anti-apoptosis*

Apoptosis is a form of programmed cell death that occurs in multicellular organisms (*27*). Stem cells play a role of preventing cell death not only *via* the restoration of the local niche but also by specifically secreting molecules that have been identified as inhibitors

of apoptosis and by down-regulating expression of apoptotic proteins. Li *et al*. reported a study which demonstrated that stem cells decreased apoptosis of alveolar macrophages when co-cultured at particular ratios (*28*). Moreover, the stem cells decreased expression of the pro-apoptotic factors Bax and cleaved caspase-3 while increasing expression of anti-apoptotic protein Bcl-2. A study by Tang *et al*. similarly showed that Bax expression was decreased while expression of pro-angiogenic factors, including basic FGF and VEGF, and stem cell homing factor CXCL-12 were increased in stem cells-treated hearts compared to medium-treated hearts (*29*). Whereafter, stem cells-treated hearts demonstrated increased capillary density and improved left ventricular contractility two months after treatment, which presumed to indicate improved function. A study by Gnecchi *et al*. additionally found that Akt up-regulated stem cells prohibit ventricular remodeling and restore cardiac function in less than 72 hours, an effect that they hypothesized was most likely attributable to the secretion of paracrine factors rather than myocardial regeneration (*30,31*). They also found that the conditioned medium derived from stem cells distinctly inhibited hypoxia induced apoptosis in adult rat cardiomyocytes *in vitro*. Furthermore, the utility of conditioned medium *in vivo* led to an obvious decrease in infarct size and increased ventricular function compared to controls.

3.4. *Immunoregulation effect*

The innate immune response plays an important role of being the body's frontline to infection or tissue damage, and severe immune and inflammatory responses to tissue injury can often have adverse effects. Stem cells possess immunomodulatory and immunological tolerance inducing effects that have been revealed to relieve and modulate possible damaging inflammatory reactions. Interestingly, these stem cells typically express MHC-I but have an absence of MHC-II, CD40, CD80, and CD86. On account of the absence of co-stimulatory cell surface molecules, stem cells often can't induce an immune response by the transplant host (*32*). Moreover, stem cells have been shown to work inhibiting immune responses by three main mechanisms: first, by mediating interaction between cells; second, through the action of soluble factors; and third, *via* induction of regulatory T cells. The first report of suppression of cell-mediated immune responses by stem cells was by Di Nicola *et al*., who found that stem cells inhibit proliferation of CD4+ and CD8+ T cells even in the absence of direct cell-cell contact (*33*). Immunomodulatory abilities of stem cells have been validated to work in each of the three main steps of the immune response: first, antigen recognition and presentation; second, T cell activation, proliferation, and differentiation; and third, the T-cell effector stage (*34*).

4. Effects of secretome on various diseases

4.1. *Cardiac*

Stem cells have been reported to promote cardiomyocyte recovery after myocardial ischemia. Originally this was supposed to be due to their capability to differentiate into cardiomyocytes to substitute for damaged tissue. Nevertheless, recent papers suggest that differentiation into tissue alone is not adequate to explain the beneficial effects seen after stem cell therapy. Moreover, a few researchers have reported that the stem cell secretome alone is sufficient to improve functional recovery. In a study by Uemura *et al*., bone marrow stem cells were injected into the left ventricle of mice following coronary artery ligation (*35*). Twenty eight days after myocardial infarction, stem cells were found in very low numbers in the mice cardiac tissue. But the good effects of treatment were still observed including induction of infarct area compared to control group and increased left ventricular ejection fraction. This result suggested that expansion and differentiation of stem cells were not adequate to explain the tissue functional improvements. The researchers also studied the role of preconditioning stem cells. A group of mice was treated with preconditioned bone marrow-derived stem cells that experienced hypoxia to up-regulate several secretions; these mice subsequently obtained the greatest functional recovery. These findings suggest possible future study in which secretome could be justified by cellular preconditioning to increase particular factors and to develop a more effective secretome therapy.

Other work by Kshitiz *et al*. reinforced the idea that secretome of bone marrow-derived stromal cells revealed a cardioprotective biochemical cocktail (*36*). In this study, researchers reconstructed dynamic secretory signatures of cells based on a very limited number of time points. By using this method, they demonstrated that the secretory signatures of CD133-positive bone marrow stem cells are uniquely defined by distinct biological contexts, including signals from injured cardiac cells undergoing oxidative stress, characteristic of cardiac infarction. Moreover, they showed that the mixture of recombinant factors reproducing the dynamics of bone marrow stem cell secretome can mediate a highly effective rescue of cells injured by oxidative stress and an improved cardiac output.

Besides adult stem cell secretome, umbilical cord stem cell secretome also has been researched by a few of laboratories. Han *et al*. reported that human umbilical cord stem cell derived exosomes encapsulated in functional peptide hydrogels promote cardiac repair (*37*). In a rat myocardial infarction model, these researchers demonstrated that the PA-GHRPS peptide protected H9C2 cells from H_2O_2-

induced oxidative stress and the gelatinization ability of PA-GHRPS can be enhanced by peptide NapFF. Therefore, these two peptides were mixed to form a PGN hydrogel, which was used to encapsulate exosomes. Their results showed that the PGN hydrogel was able to encapsulate exosomes effectively and ensured a stable and sustained release of exosomes. The exosome/PGN hydrogel mixture was injected into the infarcted border zone of rat hearts. Compared to the exosome treatment alone, the mixture improved myocardial function by reducing inflammation, fibrosis and apoptosis, and by promoting angiogenesis. A similar study was also published by Zhou *et al*. (*38*). In addition, Lazzarini *et al*. from another team reported that human amniotic fluid stem cell secretome effectively counteracts doxorubicin-induced cardiotoxicity (*39*). In this research, they showed that, following hypoxic preconditioning, amniotic fluid stem cell conditioned medium antagonizes senescence and apoptosis of cardiomyocytes and cardiac progenitor cells, two major features of doxorubicin, a conventional chemotherapeutic medicine, causing cardiotoxicity. Mechanistic studies with mouse neonatal ventricular cardiomyocytes reveal that medium inhibition of doxorubicin-elicited senescence and apoptosis is associated with decreased DNA damage, nuclear translocation of NF-kB, and upregulation of the NF-kB controlled genes, *Il6* and *Cxcl1*, promoting mouse neonatal ventricular cardiomyocyte survival. Furthermore, medium induces expression of the efflux transporter, *Abcb1b*, and doxorubicin extrusion from neonatal ventricular cardiomyocytes. The PI3K/Akt signaling cascade, upstream of NF-kB, is potently activated by medium and pre-treatment with a PI3K inhibitor, which abrogates NF-kB accumulation into the nucleus, modulation of *Il6*, *Cxcl1* and *Abcb1b*, and prevention of doxorubicn-initiated senescence and apoptosis in response to medium.

4.2. *Oncology*

There is a lot of interest in leading tissue regenerative strategies to restore function in the wake of cancer treatment and remission (*40*). Nevertheless, a serious concern is the oncologic potential surrounding stem cell therapy and the possibility of triggering a cancer recurrence. Unfortunately, many of the properties unique to stem cells such as tissue revascularization, multipotentiality, immunomodulatory effects, and cell homing and migration are also characteristics that exist in tumor progression and metastasis. However, utility of a cell-free therapy such as stem cell secretome could further avoid these risks.

A few models have been established for studying stem cell-cancer cell interactions. The effect of stem cells on tumor growth remains controversial and is a complex field of current study. Conflicting research results have been shown such as that stem cells have been shown to have both pro- and anti-tumorigenic effects, even in the same cancer model, and, sometimes, even using the same cancer cell lines (*41,42*). Some potentially concerning oncogenic effects of stem cells include modulation of paracrine activities resulting in local immunosuppression, angiogenesis, promotion of tumor growth and invasion *via* remodeling of the extracellular matrix, promotion of the epithelial to mesenchymal transition of tumor cells necessary for invasion and later metastasis, and inhibition of tumor necrosis/apoptosis. Secretions such as VEGF, TGF-β, and IL-6 which are normally secreted by stem cells are expressed in increased quantities by stem cells that have been recruited by tumor cells, supporting tumor growth and invasion (*3*).

4.3. *Diabetes*

Stem cells have gained attention due to their potential for providing a limitless source of glucose responsive insulin-producing β cells as well as their ability to enhance the survival and function of transplanted islets. This holds potential to solve the problem of limited availability of suitable donor islets, and can also enhance the therapeutic outcome of islet transplantation in T1D patients (*43*).

In a recent study, Mahdipour *et al*. demonstrated that stem cell-derived exosomes lead to regeneration of β islets through a Pdx-1 dependent mechanism in a rat model of type 1 diabetes (*44*). In their study, exosomes were intravenously injected into animals at different time points and in single or repeated therapeutic doses. After about 6 weeks, animals were euthanized and the pancreas was analyzed for the presence of regenerated β islets as well as insulin secretion. Non-fasting blood glucose and serum insulin level were also monitored during the study. The results represented that menstrual blood-derived mesenchymal stem cell-derived exosomes enhance the β cell mass and insulin production in the pancreas of diabetic animals that received repeated doses of exosomes. Immunohistochemistry analysis also confirmed the presence of insulin in the islets of treated animals. Further investigations proposed that exosomes induce islet regeneration through the pancreatic and duodenal homeobox 1 pathway. Exosome tracking also revealed homing of injected exosomes to the pancreas.

Clinical studies on prevention of diabetes-associated complications also are growing vigorously. Reports have indicated that T1DM patients have an approximately fivefold higher risk of hip fracture compared with individuals without diabetes, which is partly due to reduced bone mineral density and bone quality (*45*). In a rat calvarial defect model, Zhu *et al*. demonstrated that *in vitro*, bone marrow-derived stem cell exosomes enhanced osteogenic differentiation

of BMSCs and promoted the angiogenic activity of HUVECs (*46*). Similarly, *in vivo*, exosomes promoted bone regeneration and neovascularization in rat calvarial defects. In addition, diabetic nephropathy is another serious complication of diabetes mellitus and a common cause of end-stage renal disease. Also autophagy has a defensive role against kidney damage caused by hyperglycemia. Ebrahim *et al*. reported that stem cell-derived exosomes ameliorated diabetic nephropathy by autophagy induction through the mTOR signaling pathway (*47*). The research data shows that exosomes markedly improved renal function and showed histological restoration of renal tissues, with significant increase of LC3 and Beclin-1, and a significant decrease of mTOR and fibrotic marker expression in renal tissue. All previous effects were partially abolished by the autophagy inhibitors chloroquine and 3-MA.

4.4. *neurodegenerative disease*

Neurologic complications are commonly regarded as irreversible impairments that stem from limited potential of regeneration of the central nervous system (CNS). On the other side, the regenerative potential of stem cells has been evaluated in basic research, as well as in preclinical studies. Since exosomes can be obtained from different cell sources to mediate neuroprotective and neurotherapeutic functions, investigations have been focusing on the best cell source to generate and deliver exosomes to the CNS niche (*48*).

Exosomes have produced beneficial effects in a variety of models of neurodegenerative diseases, such as Parkinson's disease. 6-Hydroxydopamine (6-OHDA) is commonly used as an *in vivo* and *in vitro* model of Parkinson's disease because it triggers selective apoptosis of dopaminergic neurons. Jarmalavičiūtė *et al*. reported that exosomes obtained from human dental pulp stem cells were able to suppress apoptosis of dopaminergic neurons following treatment with 6-OHDA (*49*). However, if the same stem cells were cultured through normal settings, the exosomes failed to inhibit apoptosis, demonstrating that culture situation has an important impression on the properties of the exosomes. 6-OHDA induces apoptosis through the generation of reactive oxygen species (ROS) (*50*), suggesting that exosomes can decrease the sensitivity of dopaminergic neurons to oxidative stress. Future investigations are required to determine the specific proteins or miRNAs, which account for these neuroprotective characteristics of exosomes. In an experimental autoimmune encephalomyelitis mouse model, Riazifar *et al*. found that intravenous administration of exosomes produced by stem cells stimulated by IFNγ(IFNγ-Exo) *i*) reduced the mean clinical score of mice compared to PBS control, *ii*) reduced demyelination, *iii*) decreased neuroinflammation, and iv) up-regulated the number of CD4+CD25+FOXP3+ regulatory T cells (Tregs) within the spinal cords of mice (*51*) [Nuro].

4.5. *Anti-aging*

Aging is a biological process that induces changes to the structural integrity and physiological function of skin (*52*), such as the development of dyschromia, roughness, and fine rhytids followed by persistent deeper folds. Structural changes are a result of dermal atrophy, decreased collagen, the loss of subcutaneous fat, the loss of inherent elasticity, and increased melanogen (*53*). In the present study, researchers focused on fibroblasts because they are the main source of dermal extracellular matrix (ECM) proteins, mainly are collagens and elastins that are processed to assemble fibers conferring tensile strength and resilience to skin, and consequently maintain the homeostasis and juvenscence of skin (*54,55*).

In a skin recovery study, Wang *et al*. irradiated human dermal fibroblasts with ultraviolet radiation B at different senescent levels, and then treated them with stem cell conditioned medium (*56*). Then they found medium therapy can slightly or significantly improve cellular proliferative activity and restore functions both in irradiated and non-irradiated HDFs. Besides, medium therapy decreased cellular apoptosis and senescence induced by UVB. A similar finding was also reported by Kim *et al*. who found that medium stimulated both collagen synthesis and migration of dermal fibroblasts, which improved the wrinkling and accelerated wound healing in animal models, as well as protected dermal fibroblasts from oxidative stress induced by chemicals and UVB irradiation (*57*).

In a few studies, effective factors in the secretome were screened and analyzed. Proteomic analysis of human bone marrow stem cell secretome identifies nineteen secreted proteins, including extracellular matrix structural proteins, collagen processing enzymes, pigment epithelium-derived factor (PEDF) and cystatin C. Immunodepletion and reconstitution experiments show that PEDF is the predominant fibroblast chemoattractant in the conditioned medium (*58*) [Skin-D]. This stimulatory effect of PEDF on fibroblast chemotaxis is in contrast to the PEDF-mediated inhibition of endothelial cell migration, reported previously. These differential functional effects of PEDF toward fibroblasts and endothelial cells may serve to program an ordered temporal sequence of scaffold building followed by angiogenesis during wound healing.

4.6. *Hair loss*

Hair loss, also known as alopecia or baldness, refers to

a loss of hair from part of the head or body (*59*). Hair loss in some people causes psychological distress (*60*). Pattern hair loss by age 50 affects about half of males and a quarter of females (*61*). Interventions that can be tried include the medications minoxidil (or finasteride) and hair transplant surgery (*62,63*).

In a number of researches, several growth factors from stem cells and fibroblasts conditioned medium were demonstrated to have a beneficial effect on hair growth. VEGF has been proven to affect hair growth and follicular size by angiogenesis (*64*). Hepatocyte growth factor and IGF also activate hair growth through various pathways (*65,66*). Platelet-derived growth factor induces and maintains anagen hair in murine hair follicles (*67*). In a recent study, Shin *et al.* (*68*) found that after treatment with stem cell conditioned medium for 12 weeks hair density increased from 105.4 to 122.7 hairs/cm^2 ($p < 0.001$). Hair thickness increased from 57.5 μm to 64.0 μm ($p < 0.001$). None of the patients reported severe adverse reactions. Another research performed by Yan *et al.* (*69*) suggested that exosomes from dermal papilla cells (DPCs) have ability to mediate hair follicle stem cells (HFSCs) proliferation and differentiation [hair-b]. They cultured hair follicle stem cells with DPCs and found exosomes from DPCs attached to the surface of HFSCs. Using micro RNA (miRNA) high-throughput sequencing, they identified miR-22-5p-LEF1 was a novel axis regulating HFSCs proliferation.

5. Outlooks

Although the utility of secretome as a cell-free way could be a promising substitute for stem cell therapies, numerous challenges remain to be dealt with before clinical translation. One of the most serious challenges is to determine a therapeutic schedule in consideration of a great number of complex interactions of secreted molecules during tissue damage (*70*). To illuminate how cytokines are expressed during tissue damage and wound healing and how they impair therapeutic effects will be helpful for developing more effective therapeutic methods (*11*). Further research is required to exactly analyze the secretome and to identify which molecules are responsible for its therapeutic effects. Better understanding of both signaling pathways that regulate secretome expression and of secretome constitution itself will be beneficial for improving regulation of its secretion, a major barrier for translating secretome into clinical application.

The utility of conditioned medium as a form of application of stem cell secretome also exists with a few deficiencies. First, stem cells possess a dynamic expression profile that is difficult to capture with the utility of conditioned medium, which only has a static composition. Second, conditioned medium can't reflect all stages that stem cells experience and thus some secretions will be absent in a given medium. Therefore, the production of conditioned medium requires careful collection for each cell type to avoid omitting intracellular secretions from dead cells or from cells undergoing apoptosis (*71*). Finally, the preparation and concentration of secretome in quantities sufficient for clinical administration are also a problem.

6. Conclusions

Since the discovery of stem cells in the 1960s, many kinds of stem cells have been identified and used for various diseases such as cardiovascular, neurodegenerative, autoimmune, and diabetes. One of the most important functions of stem cells is the paracrine/autocrine action of stem cell secretome including its imunomodulatory effects and ability to promote tissue regeneration. Nevertheless, exploration of the stem cell secretome is only in its nascent stages for most diseases. Advances in high-throughput detective technologies and bioinformatics have already benefited analysis of the complex secreted factors that constitute the secretome and will continue to be beneficial for validation of secretome ingredients of various stem cell kinds under different conditions. So far, stem cell secretome has been proven to play a helpful role in a number of cellular processes including angiogenesis, autoimmune and inflammatory modulation, and tissue repair. Stem cell secretome has been studied mainly in the form of conditioned medium and has shown effectiveness in cell and animal experiments for various pathologic processes including those in cardiac, neurologic, diabetic, dermal, and hair fields. However, up to now a lot of barriers remain to be surmounted before making secretome a clinically useful method for regenerative therapies. More studies on secretome component analysis, composition dynamic change, molecular mechanisms, and production of conditioned medium are still needed.

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(Received July 17, 2019; Revised August 21, 2019; Accepted August 31, 2019)

Review

DOI: 10.5582/bst.2019.01178

Lab on the eye: A review of tear-based wearable devices for medical use and health management

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Summary **Wearable sensors have garnered considerable interest because of their great promise in terms of personalized health and disease management. Tears are a superior target for wireless, non-invasive wearable devices, and tear-based platforms have developed rapidly over the past decade. Although an increasing number of tear analytes have been found to be associated with multiple diseases, glucose still serves as a main target for tear-based wearable devices. There has been much investment and efforts to develop tear-based wearable biosensors, with contact lens-based and spring-like sensors flourishing commercially. Current efforts have moved past ocular and systematic disease markers to nutrients and chemicals. Moreover, tear-based wearable devices also have the potential to treat some ocular diseases. This review discusses aspects of tear-based wearable devices and it emphasizes that strict clinical validation is needed before such platforms enter the market. Multifunctional and theranostic strategies would further broaden their clinical use in the future.**

Keywords: Wearable devices, tears, health monitoring, disease management

1. Introduction

Wearable devices utilize sensors to monitor analytes in human biofluids to reflect physiological performance in sports or an aberrant component imbalance in diseases such as diabetes mellitus and cystic fibrosis (*1-3*). Such devices have the potential to revolutionize healthcare and disease management as a surrogate strategy for measuring circulating analyte concentrations in the blood, and especially in a dynamic, noninvasive manner (*4-6*).

Recent advances have led to multiple sensors for mechanoelectrical transduction using nanoparticles, carbon nanotubes, liquid metals, and ionic liquids (*7-10*). The key developments to date have mainly focused on skin interstitial fluid (ISF), tears, saliva, and sweat (*11-16*). Tears have substantial advantage over other biofluids, and tear-based wearable devices have emerged as a promising strategy for monitoring both ocular and common diseases.

The current review summarizes the most recent developments in tear-based wearable devices, and it emphasizes their potential uses in health status management and clinical management.

2. Advantages of tears as a biomarker source

Tears are secreted by the lachrymal gland as a protective film and are part of the anti-fouling mechanism of the eye. Tears are superior to other biological fluids as a target for wearable devices (*17*). Biomarker molecules in tears diffuse directly from the blood and their concentrations in the blood are more closely correlated than those of other biofluids such as sweat (*18*). Unlike sweat that lacks protein biomarkers, tears contain a wide variety of components including proteins, peptides, lipids, metabolites, and electrolytes and are also less complex than blood because of the blood-tear barrier (*18*). Tears possess unique merits in diagnosing specific ocular diseases and also reveal useful information on systemic disorders.

However, *in vitro* diagnoses using reflex tears,

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which are generated during emotional or mechanical stimulation, still have considerable issues due to the small sample volume, ease of evaporation, and variations in production among individuals (*19*). In contrast, basal tears that act as the protective film covering the eye surface at all times have a stable blood-related composition. Therefore, these characteristics make basal tears an attractive target for bloodless diagnosis.

Currently, tear-based sensors have focused primarily on glucose monitoring but show considerable promise for detecting other physiologically important biomarkers. The scope of new tear analytes can be expanded to include additional metabolites and key electrolytes with concentrations in tears that are closely related to those in the blood. For example, direct tear-based noninvasive assays of catecholamines may improve the diagnosis of glaucoma (*20*). Since tear fluid contains thousands of proteins – the most abundant of which are lysozyme, lactoferrin, and albumin – noninvasive tear monitoring could also be used to detect protein biomarkers correlated with various diseases. Proteomic analysis of tear samples may be one approach to identify biomarkers linked to ocular diseases (*21-25*). However, the correlation between the concentration in tears and blood and its relevance to disease progression must be extensively validated before using novel tear analytes.

3. Tear biomarkers associated with health status and diseases

Most diseases-specific biomarkers in tears have been identified using comparative proteomic profiles of diseases-related and healthy tears. Numerous novel protein biomarkers in tear fluid have been found to be correlated with some specific ocular diseases (such as dry eye syndrome, trachoma, glaucoma, keratoconus, and thyroid- associated orbitopathy) and systematic disorders (such as diabetes mellitus, cancer, systemic or multiple sclerosis, cystic fibrosis, Parkinson's disease, and sclerosis) (*17*). Cytokines, growth factors, and mucins are the most frequently validated tear biomarkers. A previous study investigated the endogenous metabolites in the tears of patients with keratoconus, and it found highly abnormal levels of tear metabolites in the urea cycle, tricarboxylic acid (TCA) cycle, and oxidative stress (*26*).

Although various potential biomarkers have been screened, none have been used clinically because their diagnostic power still needs to be validated in large cohorts. Currently, only several long-established tear analytes such as glucose and lactate have been used as targets of wearable devices.

4. Tear-based sensors for continuous analyte monitoring

4.1. *Strip-based sensors*

The earliest tear-based wearable devices were developed using electrochemical sensors on flexible or stretchable strips. Kudo *et al*. (*27*) fabricated a glucose oxidase (GOx)-based glucose sensor on a flexible PDMS substrate coated with a hydrogen peroxide permeable poly membrane to measure tear glucose concentration amperometrically. In a later study, this strategy was further improved by combining film printing technology to develop low-cost and high-fidelity ocular sensors (*28*). Although strip-based ocular sensors have been used in keratoconjunctivitis sicca, transcutaneous oxygen, and glucose monitoring, they lack the integration of sensor and data processing. Moreover, strip-shaped sensors are difficult to keep in place, and hard plastic substrates usually cause eye irritation and the subsequent formation of reflex tears, thus making them less attractive for use as a wearable device.

4.2. *Contact lens-based sensors*

Contact lens systems provide an attractive platform for fabricating tear-based sensors due to the comfort to the wearer, a consistent yield of tear fluid, excellent oxygen permeability, and the ability to provide accurate continuous monitoring.

Currently, two types of contact lens-based sensors, optical and electrochemical biosensing, have been developed (*29*). The earliest optical sensors measured tear glucose by detecting the fluorescence of molecules that competitively bind with glucose such as concanavalin A or phenylboronic acid derivatives (*29*). Several teams developed contact lenses embedded with fluorescent nanoparticles or boronic acid-containing fluorophores to obtain the glucose concentration by measuring the fluorescence intensity and resonance energy transfer. More recently, a colloidal crystal array (CCA) contact lens was able to selectively detect a visible color change reflecting variation in the glucose concentration (*30*).

Another important advance in contact lens-based sensors is electrochemical biosensing. Parviz *et al*. first used an amperometric glucose sensor based on GOx with an in-built wireless readout chip in a contact lens (*31*). Later, they incorporated a dual sensor strategy consisting of activated and deactivated GOx to minimize the interference effect (*32*). Kajisa *et al*. (*33*) developed a highly sensitive hydrogel field effect transistor (FET) glucose sensor electrode suitable for use as a tear-based wearable device. The sensor electrode can significantly suppress the signal noise caused by nonspecific adsorption.

4.3. *Spring-like sensors*

A small spring-like electrochemical sensor coated with a protective polysaccharide-based hydrogel material has been designed by NovioSense (*34*). This sensor can be placed behind the eyelid without irritation. When

coupled with wireless data transmission, this device can measure tear glucose.

4.4. *Eyeglasses-based tear biosensing systems*

Most recently, Sempionatto *et al*. (*35*) integrated a microfluidic electrochemical detector into the nose-bridge pad of eyeglasses to non-invasively monitor tear biomarkers. Unlike other basal tear-based platforms, this system directly collected and measured stimulated tears. Their work was the first to demonstrate that a wearable device could monitor tear analytes outside the eye region, therefore addressing the faults of contact lenses systems, *i.e*. the high risk of infection and vision impairment.

5. Use in healthcare and disease management

5.1. *Glucose monitoring*

Continuous glucose monitoring is particularly important for the successful management of diabetes. The convention finger prick blood test causes frequent pain and inconvenience, so monitoring the glucose level in other body fluids, and particularly in tear fluid, has emerged as a promising strategy. Glucose monitoring is the most widely applicable field of tear-based wearable devices (*36*). Two devices are preparing to enter the commercial market. The concept of a soft contact lens with integrated wireless electrochemistry electronics is now being developed by Google and Novartis, which represents an endeavor to bring contact lens-based wearable devices to the commercial market (*4*). The aforementioned tear glucose monitoring device worn under the lower eye lid developed by NovioSense is now in a phase II clinical trial involving six patients with type 1 diabetes mellitus, and a close correlation between glucose concentrations in tears and blood has been noted in animals and humans (*4,37*).

5.2. *Lactate monitoring*

Lactate is a metabolite associated with an oxygen deficiency, and lactic acidosis characterized by a persistent accumulation of lactate can lead to several life-threatening conditions. Lactate monitoring is of great importance to evaluating an oxygen deficiency in conditions such as cancers, bacterial or fungal infections, cerebral stroke, and trauma (*37-39*). Moreover, continuous lactate monitoring could also expand to sports medicine to indicate the level of an athlete's physical training. Currently, most of the lactate biosensing strategies use two enzymes, lactate oxidase (LOx) and lactate dehydrogenase (LDH) (*37*). Lactate is a main component in basal tears, and it mainly comes from the corneal epithelium. Variation in tear lactate levels is closely associated with many ocular diseases. For example, tear lactate levels decrease in deepithelialized cornea. Thomas *et al*. (*40*) developed a LOx and contact lens-based lactate sensor for real-time monitoring of lactate in tear fluid, and the sensor has a good response time, a high level of sensitivity, and a high level of stability. This device represents a promising step towards the integration of biosensors to measure tear lactate. Unlike efforts at glucose measurement, however, few efforts have been made to fabricate innovative wearable device targeting lactate.

5.3. *Ocular pressure monitoring*

Intraocular pressure is a significant indicator of ocular diseases (*41-43*). For example, increased intraocular pressure is the highest risk factor for glaucoma, which represents the most frequent cause of blindness, so timely monitoring of variations in intraocular pressure is extremely important to early diagnosis and treatment of glaucoma (*44,45*). Kim *et al*. (*46*) developed a multifunctional contact lens sensor to simultaneously monitor tear glucose and intraocular pressure. This system is based on independent different electrical responses, with one element responding to glucose binding and two other elements reflecting structural changes, thus enabling the measurement of intraocular pressure.

5.4. *Alcohol and vitamin detection*

Wearable sensing devices provide a good opportunity for the continuous and real-time monitoring of alcohol intake and intoxication. The first proof of concept of detection of alcohol in tears was performed using a thermal resistivity sensor in vapors above the eyes in the 1980s by Giles *et al*. (*47*). Recently, Sempionatto *et al*. (*35*) indicated that a wearable eyeglasses-based alcohol bioelectronic platform can be used to monitor alcohol in stimulated tears. More importantly, they found a close correlation between alcohol levels in tears and blood. An eyeglasses-based sensing platform has also been used to measure glucose and vitamin concentrations (*35*). Using rapid square wave voltammetry technology, this platform is capable of sensing multi-vitamins, which also represents a major step towards tear-based wearable devices that can monitor personal nutrition (*35*).

5.5. *Theranostic platform for modulation and detection of viral infections*

Mak *et al*. (*48*) proposed and confirmed a new concept of a wearable theranostic device targeting tears. Unlike most other monitoring strategies, this platform is both able to measure interleukin 1 alpha (IL-1α) levels and able to effectively measure herpes simplex virus type 1 (HSV-1) activity in the cornea. This device is based on a contact lens with an anti-viral coating, thus

Table 1. List of tear-targeted wearable devices that have been developed

Year	Analytes & targets	Wearable platform	Monitoring mechanism	Medical use	*Ref.*
2006	glucose	contact lens	optical (fluorescence signal)	glucose monitoring	(*29*)
2006 & 2008	glucose	strip-based sensor	electrochemical	glucose monitoring	(*27,28*)
2011	glucose	contact lens	electrochemical (GOx)	glucose monitoring	(*31*)
2012	glucose	contact lens	electrochemical (dual GOx sensors)	glucose monitoring	(*32*)
2012	lactate	contact lens	electrochemical (LOx)	lactate monitoring	(*40*)
2015	IL-1α (detection), HSV-1 (therapy)	contact lens	surface engineering technique	theranostic	(*48*)
2017	glucose	contact lens	electrochemical (FET)	glucose monitoring	(*33*)
2017	glucose	contact lens	optical (fluorescence signal)	glucose monitoring	(*30*)
2017	glucose, ocular pressure	contact lens	multifunctional electrical response elements	glucose monitoring, and intraocular pressure	(*46*)
2018	glucose	spring-like sensor	electrochemical	glucose monitoring	(*34*)
2019	alcohol, glucose, vitamins	eyeglasses	electrochemical	analyte monitoring	(*35*)

providing a defense against ocular infection. Therefore, the theranostic contact lens holds immense promise as a next-generation wearable device to diagnose ocular diseases.

6. Challenges and perspectives

With the entry of large industrial players like Google, the field of innovative wearable devices targeting tears is expected to grow rapidly, but these products are still in clinical trials and their commercial release is still a ways off. This indicates that some challenges still need to be addressed to successfully achieve a high level of sensitivity, linearity, and accuracy in detecting tear analytes (*49,50*). First, a better understanding of the correlation between analyte concentrations and variations in tear and blood is needed to improve reliability. Second, large, multi-center cohort studies need to validate the performance of tear-based wearable devices before they are used clinically.

Although some obstacles remain, tear-based wearable devices are a potentially cost-effective solution with great prospects for personalized health monitoring. An increasing number of tear biomarkers will presumably soon be used in wearable biosensing platforms. Most of the current systems focus on individual analytes, so the development of multifunctional devices targeting two or more analytes would further enhance the power of diagnosis and monitoring. As miniaturization proceeds and functional improvements are made in electronic interfaces and power sources, such wearable devices systems would represent a great benefit in therapeutic use or monitoring of drug concentrations.

Monitoring harmful chemicals in the human body or from the surrounding environment is crucial to maintaining health (*51*). A panel of organic transistor-based sensors that detect various chemicals such as sulfur dioxide and volatile organics in the human body *via* sweat and urine has been developed (*51*), but analysis of chemicals in tears has yet to be explored. Environmental chemicals might be a key target for tear-based wearable electronic devices in the future.

7. Conclusions

This review has described recent advances in wearable devices targeting tears for continuous personalized health monitoring and management of ocular and systemic diseases (as summarized in Table 1). Currently, such platforms have great prospects in various fields, and this is particularly true for multi-functional and theranostic strategies. Advances in new materials and techniques would greatly benefit the selectivity, stability, reliability, and lifetime of tear-based wearable sensors. However, great challenges still need to be overcome to achieve better performance before validated and reliable data can be obtained. The wearable sensor market is expected to grow rapidly, and particular attention should be paid and considerable efforts should be made to develop tear-based platforms.

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(Received July 15, 2019; Revised August 7, 2019; Accepted August 31, 2019)

Review

DOI: 10.5582/bst.2019.01209

Overview on social security system of rare diseases in China

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Summary **China has initiated the special security project for rare diseases from the national strategic level since 2018. Chinese government has formulated China's First List of Rare Diseases (121 diseases included), established Expert Committee of Diagnosis, Treatment and Security of Rare Diseases and China Alliance of Rare Diseases. The government also encouraged all the provinces to include the security plan and strategies of rare diseases into key work and promoted the establishment of rare diseases clinical research center and the formulation of diagnosis and treatment guidelines. All these actions led to the further improvement of scientific research ability and diagnosis and treatment ability. In terms of the treatment drug of the 121 diseases in China's First List of Rare Diseases, 83 of them are on the market in China and 50 have been included in the national medical insurance accompanied with the tax reduction policy on rare diseases drug and several charity foundations and enterprise donation programs were developed so that drug availability and accessibility for patients are increased. However, there is no clear definition and corresponding coding of rare diseases; the economic burden of most rare diseases could not be calculated accurately because of the difficulty in diagnosis and misclassification. Regarding the service provision system, social security system of rare diseases in China needs further improvement because of the rather few kinds of rare diseases involved in the screening, low level of diagnostic ability, rather few rehabilitation programs and the lack of social caring and knowledge training. It is necessary for China to provide comprehensive and well-rounded health care and social caring for rare diseases patients. Since the formulation and implementation conditions of the policy are different among different regions, the pilot should be carried out first in regions with desired qualifications when China is formulating the rare diseases policy and then a mature national plan should be developed.**

Keywords: Rare diseases, orphan drugs, social security, medical insurance, China

Released online in J-STAGE as advance publication August 20, 2019.

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1. Introduction

With the new round of deepening institutional reform of healthcare in China and the medical security pilot on disastrous diseases of countryside children like leukemia and congenital heart disease (*1*), rare diseases are attracting more and more social concerns. Rare diseases are those with an extremely low incidence rate but there are a variety of them. They have features like complexity, severity and deterioration but are often excluded from the medical insurance system (*2,3*). From the perspective of medicine and risk

regulation,working on the diagnosis and treatment of rare diseases is not only an urgent need to guarantee health rights and the right to life of those patients but also an inevitable requirement for a country to realize solidarity, equity and justice, with great necessity and practical significance (*4*).

In recent years, China has been actively promoting the social security work of rare diseases both on the country level and the regional level. Therefore, this article plans to summarize the current status of the development of China's rare disease security system through literature review, including the definition, list and coding of rare diseases, accessibility of rare disease medication (orphan drugs), policy system of China's rare diseases, financing and payment system of rare diseases, the promotion of scientific research and standardized diagnosis and treatment of rare diseases in China and rare diseases patient organizations and service information platform. It also analyzes the problems and challenges China's rare diseases security system is facing, proposes suggestions to perfect the safeguard work and provides reference to the improvement of security and sustainability of rare diseases prevention and treatment in China.

2. Definition, list and coding of rare diseases in China

2.1. *Definition of rare diseases*

There is no clear official definition of rare diseases in China. On May 17th, 2010, Shanghai, Chinese Society of Genetic Medicine of Chinese Medical Association held an expert seminar about the definition of rare diseases in China. An expert consensuswas reached: Diseases with incidence rate <1/500,000 or new-born incidence rate <1/10,000 could be called a rare disease in China (*5*).

2.2. *List of rare diseases*

Since there are rare epidemiological data of rare diseases in China, it is a more feasible scheme to develop a rare disease list based on the real situation. The exploration of the Chinese rare disease list started from 2016. The former Shanghai Municipal Health and Family Planning Commission (now called Shanghai Municipal Health Commission) published the first provincial main list of rare diseases in 2016 – *Shanghai List of Main Rare Diseases (2016 version)* – which includes 56 rare diseases (*6*). China Organization for Rare Disorders published *China's Reference List of Rare Diseases* with 147 rare diseases on September 23th, 2016 (*7*). National Health Commission of China, Ministry of Science and Technology, Ministry of Industry and Information Technology, National Medical Products Administration and National Administration of Traditional Chinese Medicine formulated and published *China's First List of Rare Diseases* (hereinafter referred to as the Chinese Rare Diseases List, CRDL) on May 22nd, 2018 (*8*). The main principle of this list is to put "rare diseases with a relatively higher incidence rate, heavier disease burden and better treatability" as a priority and the list includes 121 rare diseases like Gaucher disease. 50 diseases in *Shanghai List of Main Rare Diseases (2016 version)* and 88 in *China's Reference List of Rare Diseases* are involved. This list serves as a reference to each department and will help enhance the management of rare diseases in China, improve diagnosis and treatment level for rare diseases and safeguard the health interests of rare diseases patients.

On May 28th, 2018, Bureau of Medical Administration, National Health Commission of China issued *Notification of Printing and Publishing the Working Procedure of the Formulation of Rare Diseases List* (*9*), in which it was proposed that "the formulation of the list should take factors such as economic development, population and social security level into consideration based on the national condition and refer the management experience of countries or regions with similar social development level", "select the list-covered diseases in batch and update the list dynamically with a turnover time not shorter than 2 years in principle". It also points out that diseases involved in the list should meet the following requirements: there is evidence home and abroad that shows the prevalence rate or incidence rate is low; it brings great harm to patients and the family; there are definite diagnosis methods; there are treatment or intervention methods and it is economically affordable or there is no effective treatment or intervention methods but it is included in national special scientific research.

2.3. *Coding of rare diseases*

There is no official definition of rare diseases as well as individual coding system of rare diseases in China. This results in the difficulty in diagnosis and misclassification of rare diseases and makes the disease burden of most rare diseases that could not be calculated accurately. Internationally, with the approval of ICD-11 (*10*), there are more than 5400 kinds of rare disease that have their sole ICD-11 coding (*11*) and China's rare disease coding system will be perfected based on ICD-11 coding.

3. Accessibility of rare disease medication (orphan drugs) in China

Drug accessibility is that people could get the appropriate, highly qualified and culturally acceptable drug with an affordable price and can easily acquire information about reasonable medication, which is, accessibility, affordability and reasonable using of drugs (*12*).

3.1. *Accessibility*

Among the 7000 or more known rare diseases in the whole world, only less than 10% of them have an approved treatment drug or scheme (*13*) and part of them are not in the China market. Taking the treatment drugs of the 121 diseases involved in CRDL as an example, 83 drugs are in the market, among which antineoplastic and immunomodulating agents, alimentary tract and metabolism and nervous system medication are the top 3. The top 3 drugs that are involved in the national medical insurance with the most kinds are antineoplastic and immunomodulationg agents, nervous system and blood and blood forming medications (Table 1).

3.2. *Affordability*

The greatest difference between rare disease drugs and common drugs is the high price. Most rare disease patients could not afford the treatment expense with no support from a medical insurance policy. According to the report of the IQVIA institution, the median annual cost that US rare disease patients spent on single drug treatment in 2017 was 46,000 dollars (without regard to medical insurance payment). For patients of 13 rare diseases that does not have any drugs involved in medical insurance in CRDL, the median annual cost is 204,000 yuan (*14*). Collaborative research between School of Pharmaceutical Sciences of Peking University and China Organization for Rare Disorder (CORD) in 2013 (*15*) found that only less than 1.3% of the 982 patients interviewed can completely cover the medical costs of the whole year with their annual household income. According to a survey, the annual medical cost of rare disease patients in China is 3 times the personal annual income, 1.9 times annual household income, only 17.9% of the interviewees could bear the medical cost and 76.5% mainly depend on family members (*16*). The phenomenon that rare disease patients' family falls into poverty or return to poverty because of illness are serious. Regarding medical insurance involvement (Table 1), by the end of 2018, 50 out of 121 rare diseases in CRDL had been involved in the national medical insurance, among which 17 are in the category A list (the drugs that are formulated uniformly by the government, must go to clinical treatment, widely used and of good efficacy with lower prices compared to drugs of the same category and are 100% reimbursed by medical insurance) and 33 are in category B (the drugs that the basic medical insurance fund will pay part of its cost. Patients need to pay the cost by themselves first and then the basic medical insurance fund will be involved with the cost and pay for it. The detailed reimbursement ratio varies according to different regional policies and the specific drug type) (*17*).

3.3. *Reasonable using*

China has invested many resources to support the development of the discipline of rare diseases in recent years, but the number of treatment drugs is barely enough for the great number of diseases. A survey of rare disease patients registered on the CORD (5810 patients) from 2014 to December of 2018, shows that 42% of the patients (2448) didn't receive any treatment and among the other 58% that received treatment, most could not receive timely enough drug treatment (*18*). Therefore, given that the accessibility of rare diseases drugs is limited, clinical doctors and researchers have to open up new approaches like "off-label" use, "old drug, new use" and "experimental treatment."

4. Policy system of China's rare diseases

Though China hasn't formulated a specific strategy or plan for rare diseases, no individual rare diseases commission (including civil administration, health, medical insurance, medicine administration, charity foundations, scientific research departments, *etc*.) has been established and patients are not involved in the decision making process in the field of rare diseases, work related to rare diseases is getting more attention, especially medication of rare diseases. "Healthy China 2030" Planning outline, a significant strategic plan, clearly points out that national medication security

Table 1. The on-market situation and medical insurance involvement of the treatment drugs for the diseases in CRDL

Anatomical therapeutic chemical classification (First level)	On the market of China (*n*)	Involved in the national medical insurance (*n*)	Ratio of medical insurance involvement (%)
A- Alimentary tract and metabolism	14	5	35.71
B- Blood and blood forming organs	10	6	60.00
C- Cardiovascular system	9	4	44.44
G- Genital urinary system and sex-hormones	7	2	28.57
H-Systemic hormonal preparations, excluding sex hormones and insulins	5	5	100.00
J-Antiinfective for systemic use	4	4	100.00
L-Antineoplastic and immunomodulationg agents	17	10	58.82
M-Muscular-skeletal system	4	4	100.00
N-Nervous system	13	10	76.92
Total	83	50	60.24

Table 2. Introduction to the policy system of rare diseases in China

Items	Special policy theme	Comprehensive policies that mentions
Strategy/Plan	—	"Healthy China 2030" planning outline.
Disease	—	—
Listing	List and its formulation procedure	—
Study	—	Health scientific innovation special subject plan of "13th Five-Year Plan".
Drug	—	"Healthy China" 2030 Planning outline.
R&D	—	Guidance on promoting the healthy development of pharmaceutical industry; Biological industry development plan of "13th Five-Year Plan"; Health plan of 13th Five-Year Plan"; Basic Medical and Healthcare Promotion Law of China (draft; second reviewing draft).
Marketing	—	Drug review and evaluation related policies.
Price	VAT	—
Medical Insurance	—	2019 Adjustment Scheme of National Medical Insurance Drug List.
Diagnosis and treatment service	Diagnosis and treatment collaboration network, diagnosis and treatment guideline	13th Five-Year Plan" Healthscientific innovation Subject Plan.

policies will be perfected (*19*). China has implemented a series of preferential policies for the review and approval of drugs for rare diseases, mainly including priority review and approval, accelerated review and approval, special review and approval (*20*). In recent years, fundamental policies about health scientific innovation and health industry development also put forward that the study of rare diseases and the development of related drug technology should be strengthened and accelerated (*21-23*).

The publishing of CRDL in May, 2018 (*9*) symbolizes that China's rare disease security work has entered into a brand-new phase. Since then, China's rare disease security has had a clear and prioritized disease scope, which laid an important foundation for the publishing of related policies (*24*). At the beginning of 2019, China's rare disease policies were published intensively (Table 2). Rare disease drug VAT, diagnosis and treatment collaboration network, diagnosis and treatment guidelines, etc. were covered. The adjustment scheme of the national medical insurance drug list indicated more clearly that treatment medications of serious diseases like rare diseases should have been taken into priority consideration (*25*). That means, a policy system of rare diseases in China is being perfected and accelerated.

5. Financing and payment system of rare diseases in China

5.1. *National tax reduction and medical insurance funds*

To encourage development of the rare disease pharmaceutical industry and the decrease of medication costs, State Taxation Administration has reduced the VAT of antineoplastic agents in the import process to 3% so that VAT in the domestic process could be calculated by a simplified method for the first list of 21 rare diseases drugs and 4 APIs (*26*). This will result in a further decline of drug costs. In addition, among all diseases in CRDL whose indications have been registered in China, some were involved in the national basic medical insurance, employment injury insurance and maternity insurance drug list or in the pilot of serious illness security (*27,28*). Since June, 2012, the Department of Maternal and Child Health in National Health Commission and National Maternal and Child Monitoring Office initiated the allowance program of specialized milk powder for phenylketonuria children (*16*).

5.2. *Medical insurance and rare diseases funds in some provinces*

Some provinces and cities in China have taken several drugs for rare diseases into local social medical insurance. There are two main security models: *i*) directly include the medication in the drug security list (mainly category B list or formulate for special medication list); *ii*) involve the disease in special disease security scope (co-paid by special financial investment and medical insurance fund) and safeguard related drugs (serious illness medical insurance, outpatient special disease in basic medical insurance or rare diseases special medical insurance). Qingdao is a classic example for the first model (such as hyperphenylalaninemia, idiopathic pulmonary arterial hypertension, Gaucher disease, *etc*.). It established a special drug list in 2012 through serious illness aid system (*29*), and transferred the list into the management of the supplementary medical insurance system in 2017 (*30*). Qingdao's rare diseases medical insurance has an early start, high percentage of payment, a rather complete system. Zhejiang province is typical of the second model. It initiated the rare diseases medical insurance system since 2016, which is

recognized by basic medical insurance, serious illness medical insurance, medical aid and special aid, covering 6 rare diseases (Gaucher disease, amyotrophic lateral sclerosis, and phenylketonuria are included in the rare disease medical insurance, hyperphenylalaninemia, idiopathic pulmonary fibrosis, and idiopathic pulmonary arterial hypertension are only included in serious illness medical insurance) (*31*). Moreover, these two regions give admission to rare diseases drugs on the list through price negotiation and assigning designated hospitals for the diagnosis and treatment of rare diseases patients.

5.3. *Charity funds and enterprise donation programs*

Rare diseases charity funds have been continuously set up such as China-Dolls Fund for Rare Disorders and Rare Diseases Bailout Fund, which provides several forms of help to domestic rare diseases patients. Pharmaceutical companies also actively take social responsibility and initiate assistance programs with China Charity Federation. Cerezyme charity aid program supported by Sanofi-Genzyme has provided the Gaucher-disease-specific drug (Cerezyme) to more than 130 patients with severe Gaucher disease for free and the total amount of assistance until now is over 1,200 million yuan (*32*). The Evolocumab charity aid program supported by Amgen allows eligible patients to have Evolocumab with two years' dosage for free, which is an innovative anticholesteremic agent. There is also an hemophilia-specific Baikeqi Co-pay charity aid program and Advate charity aid program, multiple-sclerosis-specific "China Charity Federation Beteferon Patients Charity Aid Program" , *etc*. that greatly relieve the economic burden and mental stress for patients and improve their life quality (*33*).

6. The promotion of scientific research and standardized diagnosis and treatment of rare diseases in China

6.1. *Expert committee of diagnosis, treatment and security of rare diseases and rare diseases association*

In December 2015, the former National Health and Family Planning Commission (now called National Health Commission) of China formed the Expert Committee of Diagnosis, Treatment and Security of Rare Diseases whose responsibility is to study and put forward the definition and disease type that are consistent with our national circumstances, organize to develop technical specification and clinical pathways of rare diseases prevention and treatment and give suggestions for the prevention, screening, diagnosis and treatment, medication, rehabilitation, and security of rare diseases. Committee members are experts from professional fields like rare diseases screening, diagnosis and treatment, pharmacy, drug supply and security, medical insurance and health economics (*34*). In April, 2017, the committee office was set up in Peking Union Medical College Hospital, responsible for the daily work of the committee (*35*). Since 2010, Shandong, Shanghai, Beijing, Guangdong, and Zhejiang consecutively established rare diseases society or prevention and treatment association and constructed a collaborative platform of rare diseases study, diagnosis and treatment (*36*).

In October 2018, under the permission of Bureau of Medical Administration National Health Commission, China Alliance of Rare Diseases was set up (*37*). The Alliance was initiated by Peking Union Medical College Hospital, China Pharmaceutical Innovation and Research Development Association, Chinese Hospital Association and Chinese Research Hospital Association, consisting of 50 or more medical institutions and institutions of higher learning with rare disease departments, scientific research institutions and enterprises voluntarily. The Alliance is a national, non-profit, cooperative communication platform, aiming to integrate the prevention and treatment resources of China rare diseases and motivate medical institutions, research institutions, social organizations and pharmaceutical enterprises. Tencent, China's internet giant, joined the Alliance in May, 2019 in order to apply artificial intelligence to the field of rare disease (*38*).

6.2. *National rare diseases scientific projects*

The pathogenesis, diagnosis and treatment methods for most rare diseases are unclear and there are few cases with patients scattered in different regions and difficulty in data collection, which makes rare diseases research still have a long way to go (*39*). China is gradually enhancing scientific investment by National Science and Technology Support Program, Rare Diseases Precision Medicine Research, National Major Scientific and Technological Special Project for "Significant New Drugs Development", National Natural Science Foundation of China and Special Scientific Research Fund of Public Welfare Industry of China. Since 2008, China's new drug special project has supported more than 20 R&D projects on rare diseases. The first comprehensive rare diseases prevention and treatment research project "China's Rare Diseases Prevention and Treatment Research and Demonstration" was initiated during the period of "12th Five-Year Plan" and several precision medicine special research projects were set off including rare diseases clinical cohort study, precise diagnosis and treatment technology and Clinical Practice study on important rare diseases in Chinese population during the period of "13th Five-Year Plan" (*40-42*). The amount of funding has been over 120 million yuan. All these studies are based on the actual problem encountered in the field of rare diseases prevention and treatment, and the first national

rare diseases registration system was developed (*43*) as well as a clinical database integrated by multi-omics and multi-center clinical biobank, model rare diseases comprehensive diagnosis and treatment center and imaging expert consultation platform. Several clinical pathways were designed. All play an exemplary role in the research of rare diseases in China and the popularization and promotion of related diagnosis and treatment technologies and give strong data support for rare diseases decision-making (*36,44*).

6.3. *Rare diseases clinical research center*

Clinical research center is a national scientific innovation base that meets the prevention and treatment needs and clinical application orientation, of which medical institutions are the main body and also a national science and technology innovation base supported by a synergetic network. It can speed up the breakthrough of the limitations of current disease diagnosis and treatment technology, explore prevention and control methods that are suitable for China and more economically efficient. The Five-year Development Plan for National Clinical Research Center (2017-2021) points out that 1-3 national clinical research centers will be established for birth defects and rare diseases field, focusing on structural birth defects and inborn errors of metabolism (*45*). After the layout of the national clinical research center for rare diseases, the ability for rare diseases screening, diagnosis and treatment in China will be further promoted.

6.3.1. *Clinical pathway*

To improve the diagnosis and treatment level of rare diseases, promote the standardization of clinical diagnosis and treatment, medical institutions in Beijing and Shanghai have been working actively on the standardization of rare diseases diagnosis and treatment. Regulations and expert consensus on the diagnosis of Gaucher disease, glycogen storage disease type II, hyperphenylalaninemia, Alport syndrome and monographs like *Treatable Rare Disease and Compendium of China's First List of Rare Diseases* were published in order to provide standard diagnosis and treatment guidance on some rare diseases for clinical professionals (*39,46*). In February, 2019, Expert Committee of Diagnosis, Treatment and Security of Rare Diseases, National Health Commission (Chinese Academy of Medical Sciences &Peking Union Medical College) led the formulation and publication of *Rare Disease Diagnosis and Treatment Guide* (2019) which explained in detail the definition, etiology and epidemiology, clinical feature, auxiliary examination, diagnosis, differential diagnosis and treatment and proposed detailed diagnosis and treatment processes, displaying fully that the guide is helpful in the practice of rare diseases diagnosis and treatment in terms of standardization, guidance and practicality (*47*).

6.3.2. *Diagnosis*

The diagnosis and treatment service system of rare diseases in China is still being constructed preliminarily. There is difficulty in diagnosis and misdiagnosis and missed diagnosis. The average length of definite diagnosis is 5-6 years and it is more than 10 years for some patients (*48*). In February, 2019, National Health Commission of China published National Health Commission General Office's Notification on the establishment of national rare diseases diagnosis and treatment collaborative network (Medical Letter of National Health Commission General Office (2019) No.157), and decided to select 324 hospitals in the whole country with stronger diagnosis and treatment capabilities for rare diseases and more cases to form the rare diseases diagnosis and treatment collaborative network. This helps to realize the objective of early detection, early diagnosis, treatability and manageability of rare diseases gradually (*49*). 80% of rare diseases are caused by genetic variation and with development of molecular diagnosis, molecular genetics, and gene sequencing technology (*50*), diagnosis rate of rare diseases increases significantly, length of definite diagnosis shortens and cost declines. In addition, some regions in China are able to choose to use tandem mass spectrometry for the detection of each rare disease (*51*). 13th Five-Year Plan for Development of the Strategic Emerging Industries also points out that high performance medical devices and core components should be developed to support the in vitro rapid and accurate diagnosis and screening of cancer, hereditary diseases and rare diseases (*52*).

6.3.3. *Screening*

Most of the rare diseases are caused by genetic variation, so new-born disease screening could effectively recognize rare diseases that have not emerged. China's new-born diseases screening began from the 1980s. With the publishing of related policies, laws and supporting documents, the preconception and antenatal examination and new-born screening system are continuously improving. Screening has developed quickly with more screening types and a nationwide new-born screening network has developed with a new-born screening coverage rate of 97.5% (*53*). Diseases involved in the nationwide screening are new-born inherited metabolic disorders like congenital hypothyroidism, phenylketonuria and hearing disorders (*54*). What's more, two experts in the field of rare diseases screening from Expert Committee of Diagnosis, Treatment and Security of Rare Diseases are assigned to offer suggestions (*55*).

6.3.4. *Rehabilitation*

Most rare diseases are chronic and serious disease due to gene defects, with a few complications and cannot be cured thoroughly, causing many patients to receive treatment or rehabilitation for their whole life (*56*). According to a survey, the greatest difficulty rare diseases patients meet in the treatment process is over the high treatment expense, followed by the lack of drugs and rehabilitation methods (*57*) that seriously influence life quality. Professional rehabilitation therapists are needed to comprehensively evaluate the physical and psychological status of patients and formulate an individualized rehabilitation scheme. Some rare diseases organizations have initiated programs like rehabilitation training camp/ rehabilitation talents development projects (*58*) and Expert Committee of Diagnosis, Treatment and Security of Rare Diseases should also fully play their role (*55*). Moreover, rare diseases patients are restricted in education or employment, and often isolated and discriminated against in daily life. These bring great psychological pressure to them (*59*) while their family is heavily burdened. Apart from the support of advanced medical technology and finance, patients need certain social support and mental rehabilitation. In China, there are only a few NGOs that could offer help for education, employment and mental health and most can only provide some medical aid or life help services for now (*58*).

7. Rare disease patient organizations and service information platform

7.1. *Rare diseases patient organizations*

Chinese hemophiliacs developed a patient communication and mutual help platform since the beginning of the 21st century. Since then, rare diseases patients organizations in China began to emerge and develop (*60*). The Chongqing Hemophilia Rehabilitation Association was set up in 2006 and is the first rare diseases patients organization registered in China civil administrations (*61*). There are currently about 80 registered rare diseases patients' organizations (*62*). Besides these patient organization for specialized diseases, another patient platform organization (CORD) was established in 2013 and played an important role in promoting the development of Chinese rare diseases patient organizations, including the ability training of patient organizations, activity support and social publicity (*63*). Meanwhile CORD also conducts policy research, patient registration and international collaboration.

Because of the particularity of rare diseases, misdiagnosis or missed diagnoses often happen because of clinical physicians' insufficient understanding. It is of great significance to have a wide-range of rare diseases prevention and control publicity and training of specialist physicians in order to standardize diagnosis and treatment, relieve patients' pain and avoid the waste of medical resources (*64*). National and local health commissions, together with medical institutions, carry out some knowledge training about rare diseases and rare disease organizations also regularly hold ability cultivation training. But the trainings should be enhanced, and a training system still needs to be developed.

7.2. *Rare diseases service information platform*

The main information source for rare disease patients are patient organizations established spontaneously (CORD, Chinese Organization for Albinism, China-Dolls Center for Rare Disorders, *etc.*), websites (Rare Disease in China, National Rare Diseases Registry System of China, Shandong Rare Diseases Prevention and Control Association, *etc*.), exchange meetings (China Rare Diseases Summit, Cross-strait exchange forum of rare diseases, Asian rare diseases patients exchange forum, *etc*.), remote consultation platform of rare diseases/difficult diseases, patient groups, and related books and reports. Patients could acquire rare diseases related policies, scientific and technological knowledge, orphan drug information, activities home and abroad and could get access to rare diseases consulting websites from other countries or regions like EU.

8. Conclusion

Rare disease is a new policy hotspot in the current policy environment of China and the policy of rare disease security system construction is in its window phase. The central government's effort promotes each province to involve the security plan and strategy as the key emphasis in work and also promotes the establishment of rare disease clinical centers and the formulation of diagnosis and treatment guidelines. The Rare diseases security system is developed and implemented on the country level and consists of a series of comprehensive and all-round rare diseases related health policies and social policies. The reasonable allocation of human resources, financial resources and infrastructures plays an important role in the development of the national rare diseases security system. The experience of EU shows that (*65*), the formulation of rare diseases security system should put emphasis on the following two aspects: *i*) Comprehensiveness. Related strategies could complement each other to the greatest extent and not overlap; *ii*) All-roundness. Most rare diseases patients' needs should be fully considered (such as medical services and social services). It is necessary for China to formulate a specific national plan and strategy for rare diseases, provide comprehensive and all-round

health care and social caring for rare disease patients and strengthen the collaboration between regions home and abroad. Since the formulation and implementation conditions of the policy are different among each region, the pilot should be carried out first in regions with desired qualifications when China is formulating the rare disease policy and then a mature national plan should be developed. This is to ensure the accessibility and equity of different regions regarding the diagnosis and treatment services and drugs for rare diseases.

Acknowledgements

This study was supported by grants from the Shanghai Municipal Health Commission for a Special Research Project related to Health Policies entitled "Study on the disease burden of and social security for the vulnerable population in Shanghai (Project number: 19Y04017)" and China Medical Board Collaborating Project "Establishing Health Policy Transformation Network of China (Project number: CMB-CP 14-190)".

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(Received June 12, 2019; Revised August 6, 2019; Accepted August 12, 2019)

Original Article

DOI: 10.5582/bst.2019.01214

Cordycepin induces apoptosis in human bladder cancer T24 cells through ROS-dependent inhibition of the PI3K/Akt signaling pathway

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Summary **Cordycepin, a derivative of nucleoside adenosine, is one of the active ingredients extracted from the fungi of genus *Cordyceps*, which have been used for traditional herbal remedies. In this study, we examined the effect of cordycepin on the proliferation and apoptosis of human bladder cancer T24 cells and its mechanism of action. Cordycepin treatment significantly reduced the cell survival rate of T24 cells in a concentration-dependent manner, which was associated with the induction of apoptosis. Cordycepin activated caspase-8 and -9, which are involved in the initiation of extrinsic and intrinsic apoptosis pathways, respectively, and also increased caspase-3 activity, a typical effect caspase, subsequently leading to poly (ADP-ribose) polymerase cleavage. Additionally, cordycepin increased the Bax/Bcl-2 ratio and truncation of Bid, and destroyed the integrity of mitochondria, which contributed to the cytosolic release of cytochrome c. Moreover, cordycepin effectively inactivated the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, while LY294002, a PI3K/Akt inhibitor, increased the apoptosis-inducing effect of cordycepin. Cordycepin further enhanced the intracellular levels of reactive oxygen species (ROS), while the addition of N-acetyl cysteine (NAC), a ROS inhibitor, significantly diminished cordycepin-induced mitochondrial dysfunction and growth inhibition, and also blocked the inactivation of PI3K/Akt signaling pathway. Furthermore, the presence of NAC significantly attenuated the enhanced apoptotic cell death and reduction of cell viability by treatment with cordycepin and LY294002. Collectively, the data indicate that cordycepin induces apoptosis through the activation of extrinsic and intrinsic apoptosis pathways and the ROS-dependent inactivation of PI3K/Akt signaling in human bladder cancer T24 cells.**

Keywords: Cordycepin, bladder cancer, T24 cells, apoptosis, ROS, PI3K/Akt

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1. Introduction

Apoptosis, a programmed cell death, is one of the most actively studied areas of cancer treatment, and

is largely divided into death receptor (DR)-mediated extrinsic and mitochondria-mediated intrinsic pathways (*1,2*). The extrinsic pathway begins with the activation of caspase-8 by the formation of the death-inducing signaling complex through the binding of death ligand to the cell surface DR. On the other hand, the intrinsic pathway is characterized by the release of pro-apoptotic proteins, such as cytochrome c from the mitochondria to the cytoplasm, with increased mitochondrial permeability and the activation of caspase-9 (*1,3*). Caspase-8 and -9 as initiator caspases ultimately activate downstream effector caspases, including caspase-3, which induce apoptosis through the cleavage of cellular substrates. In addition, these pathways are strictly regulated by a group of proteins that are composed of pro- and anti-apoptotic proteins, such as Bcl-2 protein family proteins (*4,5*). Meanwhile, apoptosis is precisely regulated by a wide variety of cellular signaling pathways. Among them, the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is involved in the inhibition of apoptosis and the promotion of cell growth, thus playing a key role in the pathogenesis of various tumors (*6-8*). Moreover, there is growing interest in reactive oxygen species (ROS), which induce apoptosis of cancer cells through dysregulation of the PI3K/Akt signaling pathway (*9-11*). Therefore, inhibiting the PI3K/Akt signaling pathway, while promoting the generation of ROS, can be an attractive approach to cancer treatment.

Bladder cancer is the sixth most prevalent malignancy in the United States and causes more than 16,000 deaths annually that has a considerable morbidity and mortality impact with particularly poor prognosis (*12,13*). About 90% of affected patients are older than 55 years, and three to four times greater in men than in women to develop the disease (*12*). Established risk factors included male sex, older ages, personal or family history, cigarette smoking and underlying disease such as diabetes mellitus and obesity (*12,13*). The common treatment for bladder cancer focuses on radical cystectomy, but most patients experience relapse after excision (*14*). Because of this, adjuvant chemotherapy is usually performed in an effort to delay recurrence and prolong survival (*14,15*). However, there are reported that adjuvant chemotherapy responded to 50% of muscle-invasive bladder cancer patients (*15,16*). Therefore, there is a need to develop a novel treatment strategies for the overcome these challenges of bladder cancer. In this respect, numerous medical plants and herbal pharmacologically compounds are coming into the spotlight, due to their low cost, low toxicity, and low hostility as dietary supplements (*17*). Recently, many studies into the natural compounds that have been traditionally used in the treatment of various diseases have shown great interest in their use as potential resources for cancer chemoprevention and chemotherapy (*18-20*). Among them, cordycepin is a type of nucleoside analogue that is isolated from the fungi belonging to the Cordyceps genus, such as *Cordyceps militaris* and *C. sinensis* (*21-23*). Although various pharmacological actions of cordycepin have been known, research on the anticancer activity, including the induction of apoptosis of cancer cells, has been conducted most extensively (*21,23-25*). For example, the intrinsic and extrinsic apoptosis pathways may be involved in the induction of apoptosis of human hepatocarcinoma and prostate cancer cells, and mouse Leydig tumor cells by cordycepin (*26-28*). It has also been found that the increase of ROS production in leukemia, gastric and prostate cancer cells plays an important role in the induction of intrinsic apoptosis pathway (*28-30*). In addition, the anticancer effects of cordycepin involve the disturbance of various cell signaling pathways, and in particular, cordycepin-induced apoptosis in human gastric and ovarian cancer cells and leukemia and glioma cells was accompanied by inactivation of the PI3K/Akt signaling pathway (*31-36*). Although the induction of apoptosis by cordycepin in a certain gastric cancer cell line was accompanied by the production of ROS and inactivation of the PI3K/Akt signaling pathway (*24*), the underlying mechanism of ROS involved in the inactivation of PI3K/Akt signaling pathway by cordycepin is still not well known. Therefore, in this study, we investigated the effect of cordycepin on the induction of apoptosis, and evaluated whether its effect was associated with the ROS generation and PI3K/Akt signaling pathway in human urinary bladder transitional cell carcinoma T24 cells.

2. Materials and Methods

2.1. *Cell culture and cordycepin treatment*

T24 cells were purchased from the American Type Culture Collection (Manassas, MD, USA). Cells were cultured at 37°C in 5% CO_2 humidified incubator in complete media consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (WelGENE Inc., Daegu, Republic of Korea). Cordycepin obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich Chemical Co.) to a final concentration of 100 μg/mL, and prior to use, the stock solution was diluted with cell culture medium to the desired concentration.

2.2. *Cell viability*

The viability of the cells was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetra-zolium bromide (MTT) assay, as previously described (37). Briefly, T24 cells were seeded onto 96-well plates at a

density of 1×10^4 cells/well, and incubated overnight. Thereafter, the cells were treated with the desired concentrations of cordycepin for 48 h, and the cells were then incubated with 50 μg/mL MTT solution (Invitrogen, Waltham, MA, USA) for 2 h. Formazan crystals were dissolved in DMSO, and then the absorbance was measured by microplate reader (VERSA Max, Molecular Device Co., Sunnyvale, CA, USA) at 540 nm. The morphological changes of cells were visualized by phase-contrast microscopy (Carl Zeiss, Oberkochen, Germany).

2.3. *Detection of apoptotic morphological changes*

Apoptotic cells containing chromatin condensation and apoptotic body formation in the nuclei were detected by 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) staining. After treatment with cordycepin for 48 h, the cells were harvested, washed with phosphate-buffered saline (PBS), and then fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.) in PBS for 10 min at room temperature (RT). The cells were washed with PBS, and stained with 1 μg/mL DAPI solution for 10 min under light-shielded conditions. The cells were washed with PBS, and the fluorescence intensity was observed using fluorescence microscopy (Carl Zeiss).

2.4. *Determination of apoptosis by flow cytometer*

The magnitude of apoptosis was measured by flow cytometer using propidium iodide (PI) staining. In brief, the cells treated with cordycepin were washed with cold PBS, fixed in ice-cold 70% ethanol, and stored at 4°C. The cells were suspended in cold PBS containing 50 μg/mL PI, 100 μg/mL ribonuclease A, 0.1% (w/v) sodium citrate and 0.1% (v/v) Nonidet-P40 (Sigma-Aldrich Chemical Co.), then incubated on ice for 30 min in the dark at RT. Flow cytometric analysis was carried out using a flow cytometer (BD Biosciences, San Jose, CA, USA), and Cell Quest software was used to determine the relative DNA content. The sub-G1 population was calculated to estimate the apoptotic cell population.

2.5. *Agarose gel electrophoresis for DNA fragmentation assay*

After treatment with cordycepin for 48 h, the cells were lysed in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 0.5% Triton X-100 for 1 h at RT. The lysates were vortexed, and cleared by centrifugation at 10,000× g for 30 min. After extraction of fragmented DNA in the supernatant using phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v, Sigma-Aldrich Chemical Co.), electrophoretic analysis was performed on 1.0% agarose gels containing 0.1 μg/mL ethidium bromide (EtBr, Sigma-Aldrich Chemical Co.).

2.6. *Protein extraction and Western blot analysis*

Total protein was extracted from the cells using the Bradford Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific Inc., Waltham, Utah, USA) were applied for the preparation of mitochondrial and cytosolic extracts of cells, according to the manufacturer's instructions. After quantification of protein concentration using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), an equal amount of protein from the samples was separated by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 1 h, and probed with specific primary antibodies at 4°C overnight. After washing three times with TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at RT. The expression of protein was detected by enhanced chemiluminescence (ECL) kit (GE Healthcare Life Sciences, Little Chalfont, UK), and visualized by Fusion FX Image system (Vilber Lourmat, Torcy, France).

2.7. *Caspase activity*

The activity of caspases was measured using caspase activity assay kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. In brief, cells were harvested, and lysed in the lysis buffer provided in the kit. The supernatants were collected, incubated with the supplied reaction buffer containing dithiothreitol, with or without substrates [Asp-Glu-Val-Asp (DEAD) for caspase-3; Ile-Glu- Thr-Asp (IETD) for caspase-8; and Leu-Glu-His-Asp (LEHD) for caspase-9] labeled with p-nitroaniline (pNA) at 37°C for 2 h in the dark. The optical density of the reaction mixture was determined by absorbance at 405 nm using a microplate reader.

2.8. *Measurement of mitochondrial membrane potential (MMP, Δψm)*

To observe the changes of MMP, 5,5‘,6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1; Sigma-Aldrich Chemical Co.) staining was performed. After treatment with various concentrations of cordycepin, 10 μM JC-1 was added to the cells for 30 min at 37°C. Subsequently, the cells were washed with

PBS to remove unbound dye, and at least 10,000 cells were collected for each sample. The amounts of MMP were detected at 488/575 nm using a flow cytometer (BD Biosciences), by following the manufacturer's protocol.

2.9. *Statistical analysis*

The results of quantitative studies are reported as mean ± standard deviation (SD) using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). All experiments were repeated at least three times. To compare data, One-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test was used, and $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. *Cordycepin inhibits T24 cell viability*

In evaluate the cytotoxicity of cordycepin in T24 cells, the cells were incubated with different concentrations of cordycepin for 48 h, and cell viability was assessed by MTT assay. Figure 1A shows that cordycepin significantly reduced T24 cells viability in a concentration-dependent manner, and fifty percent inhibitory concentration (IC_{50}) values of cordycepin was 41.62 μM. Therfore, we decided that 40 μM (IC_{50}) appropriated as the maximum concentration for investigation of apoptotic effects of cordycepin. Under phase-contrast microscope, the phenotypic characteristics of cordycepin-treated cells showed irregular cell outlines, decrease of cell density, and increase of detached cell (Figure 1B).

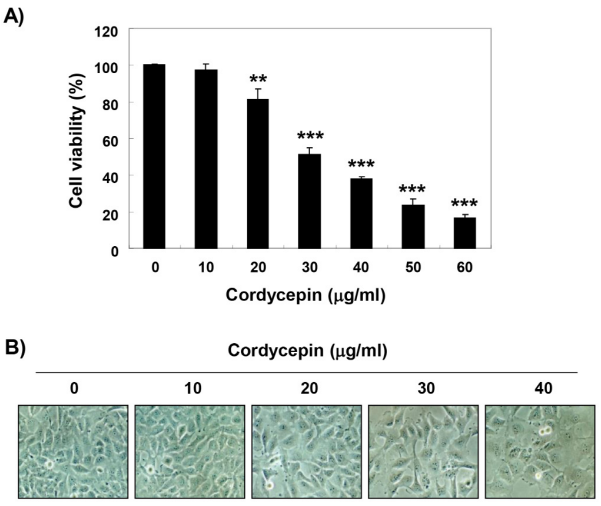

Figure 1. Cordycepin decreases the survival of human bladder cancer T24 cells. (A) T24 cells were treated with the indicated concentrations of cordycepin. The cell viability was assessed after 48 h by MTT assay, as described in the Materials and Methods. Each bar represents the mean ± SD of three independent experiments (**$p < 0.001$ and ***$p < 0.0001$ when compared to control). **(B)** Morphological changes of T24 cells were observed by phase-contrast microscopy. Representative photographs of the morphological changes are presented.

3.2. *Cordycepin induces apoptotic cell death in T24 cells*

We determined whether the growth inhibition of T24 cells by cordycepin was associated with apoptosis induction. Figure 2A shows the results of DAPI staining, which reveal that the morphological changes of nuclei observed in apoptosis-inducing cells, such as nuclear fragmentation, and chromatin condensation, were increased, depending on cordycepin treatment concentration. In addition, the results of flow cytometry analysis and agarose electrophoresis showed that the percentage of sub-G1 cells and fragmentation of DNA were increased in cells treated with cordycepin in a concentration-dependent manner (Figure 2B-D).

3.3. *Cordycepin activates caspases in T24 cells*

We next assessed whether cordycepin activated the caspase signaling pathway in T24 cells, and found that cordycepin reduced the expression of pro-caspase-3, -8, and -9 (Figure 3A) and increased their enzymatic activity in a concentration-dependent manner (Figure 3B). Cordycepin also induced cleavage of poly (ADP-ribose) polymerase (PARP), one of the major substrate

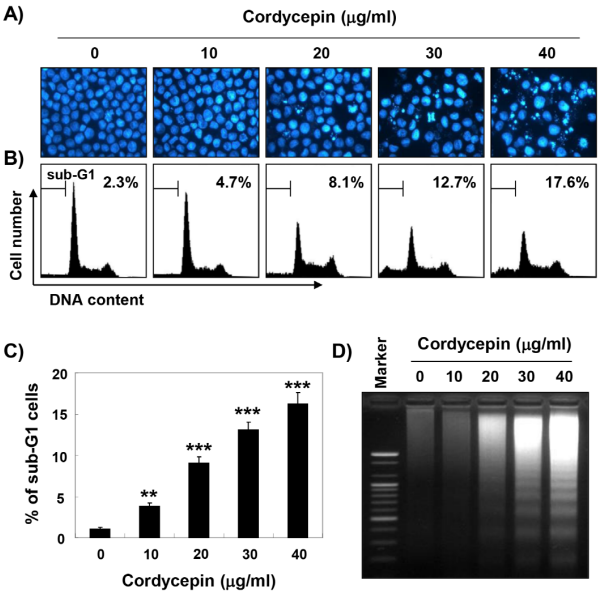

Figure 2. Cordycepin induces apoptosis in T24 cells. (A) After treatment with different concentrations of cordycepin for 48 h, the cells were collected, fixed, and stained with DAPI solution. The stained nuclei were pictured under a fluorescence microscope. **(B** and **C)** The cells cultured under the same conditions were collected, and stained with PI solution for flow cytometry analysis. **(B)** The percentages of apoptotic sub-G1 cells were determined. **(C)** Data were expressed as the mean ± SD of three independent experiments (**$p < 0.001$ and ***$p < 0.0001$ when compared to control). **(D)** DNA fragmentation was analyzed by the extraction of genomic DNA, electrophoresis in agarose gel, and then visualization by EtBr staining.

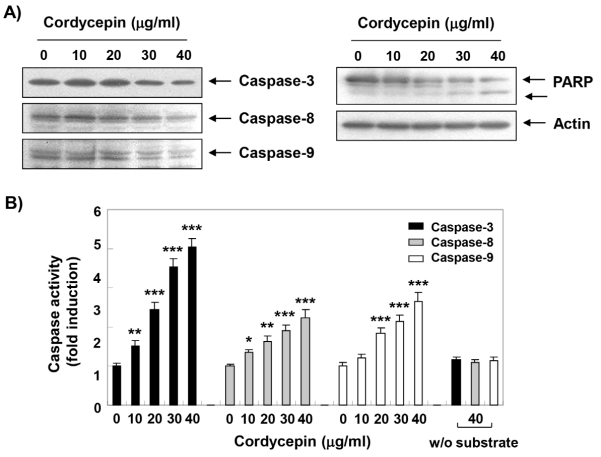

Figure 3. Cordycepin induces the activation of caspases and degradation of PARP in T24 cells. T24 cells were treated with the indicated concentrations of cordycepin for 48 h. **(A)** The cell lysates were prepared, and equal amounts of cellular proteins were separated on SDS-polyacrylamide gels, and transferred to PVDF membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. The equivalent loading of proteins in each well was confirmed by actin. **(B)** The activities of caspases were evaluated using caspases colorimetric assay kits. The data were expressed as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$, when compared to control).

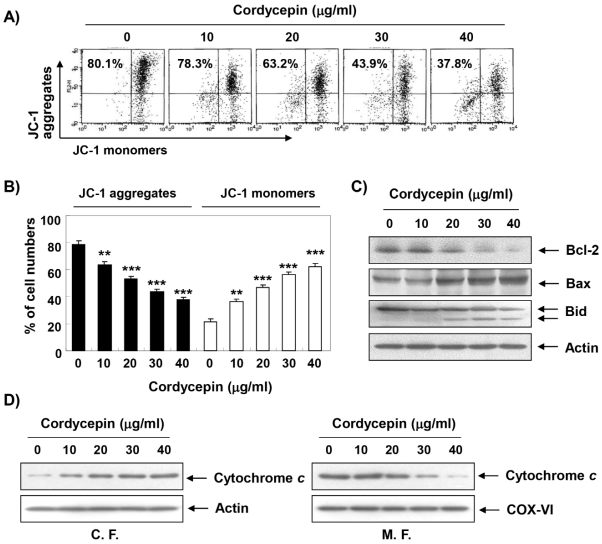

Figure 4. Cordycepin reduces the values of MMP (Δψm), modulates the levels of Bcl-2 family proteins, and induces the cytosolic release of cytochrome c in T24 cells. (A) After 48 h incubation with the indicated concentrations of cordycepin, the cells were stained with JC-1 dye, and were then analyzed by flow cytometry, in order to evaluate the changes in MMP. **(B)** Each bar represents the percentage of cells with JC-1 aggregates and monomers (mean ± SD of triplicate determinations, **$p < 0.001$ and ***p 0.0001, when compared to control). **(C)** The cell lysates were prepared, and the expression of Bcl-2 family proteins (Bc-2, Bax and Bid) was evaluated by Western blot analysis with whole cell lysates. Equal protein loading was confirmed by an analysis of actin. **(D)** Cytosolic and mitochondrial proteins were prepared, and analyzed for cytochrome c expression by Western blot analysis. Equal protein loading was confirmed by the analysis of actin and cytochrome oxidase subunit VI (COX VI) in each protein extract. The results shown are representative of three independent experiments.

proteins of activated caspase-3 (Figure 3A).

3.4. *Cordycepin induces mitochondrial dysfunction in T24 Cells*

We further assessed whether mitochondrial dysfunction was involved in the induction of cordycepin-induced apoptosis, in order to study additional mechanisms involved in inducing apoptosis by cordycepin. As can be seen from the results of JC-1 staining, the MMP-dependent formation of JC-1 aggregates in mitochondria was maintained at a relatively high rate in T24 cells not treated with cordycepin (Figure 4A and B). However, JC-1 aggregates were reduced after treatment with cordycepin in a concentration-dependent manner, indicating a significant depletion of MMP after cordycepin treatment. As indicated in Figure 4C, we also found that cordycepin increased the expression of pro-apoptotic Bax, and decreased the expression of anti-apoptotic Bcl-2. Additionally, the expression of truncated BH3 interacting-domain death agonist (tBid) was increased, and the release of cytochrome c from the mitochondria to the cytoplasm was promoted in cordycepin-treated T24 cells (Figure 4C and D).

3.5. *Cordycepin inactivates PI3K/Akt signaling pathway in T24 cells*

To determine the effect of cordycepin on the PI3K/Akt signaling pathway, we measured the phosphorylation level of PI3K protein and its downstream component Akt. Figure 5A shows that when cells were exposed to cordycepin, the expressions of phosphorylated (p)-PI3K and p-Akt were gradually decreased with increasing time of cordycepin treatment, while total PI3K and Akt protein levels remained constant during cordycepin treatment, which suggests that cordycepin was able to block the activation of the PI3K/Akt pathway in T24 cells. To further confirm the role of the PI3K/Akt pathway in cordycepin-mediated apoptosis, cells were co-treated with LY294002, a specific PI3K inhibitor and cordycepin. The results obtained from DAPI staining, agarose gel electrophoresis, and flow cytometric analysis showed that apoptosis was significantly increased in cells treated with PI3K inhibitor and cordycepin, compared to with cordycepin alone (Figure 5B-E). In addition, after co-treatment with LY294002 and cordycepin, the reduction of cell viability by cordycepin was further reduced (Figure 5F).

3.6. *Cordycepin induces ROS-dependent mitochondrial dysfunction and growth inhibition in T24 cells*

We next investigated whether cordycepin induced the production of ROS, and the effects of increased ROS on cordycepin-induced apoptosis and inhibition of the PI3K/Akt signaling pathway. Examining the

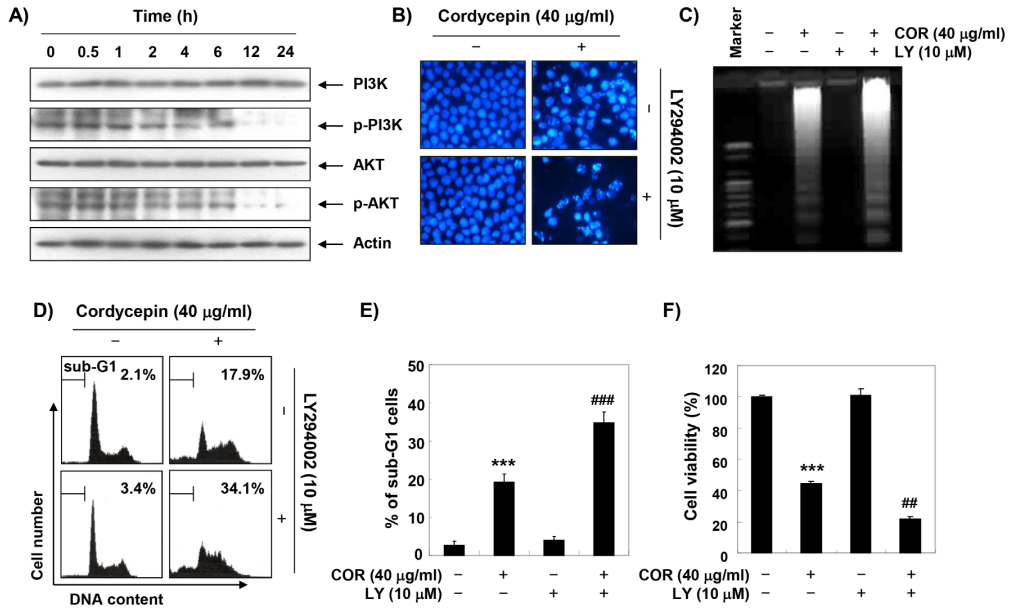

Figure 5. Cordycepin induces the inactivation of PI3K/Akt signaling pathway in T24 cells. The cells were treated with 40 μg/mL cordycepin for the indicated times **(A)**, or pre-treated with 10 μM LY294002 for 1 h, and then treated with 40 μg/mL cordycepin for a further 48 h **(B-F)**. **(A)** The cell lysates were prepared, and the expression of PI3K and Akt proteins was evaluated by Western blot analysis with whole cell lysates. Actin was used as an internal control. **(B)** The DAPI-stained nuclei were then observed by fluorescence microscopy (original magnification, ×400). **(C)** DNA fragmentation was analyzed by the extraction of genomic DNA, electrophoresis in agarose gel, and then visualization by EtBr staining. **(D)** The percentages of apoptotic sub-G1 cells were determined. **(E)** Data were expressed as the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ###$p < 0.001$, when compared to cordycepin-treated cells). **(F)** The cell viability was measured by MTT assay. Data were expressed as the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ##$p < 0.001$, when compared to cordycepin-treated cells). COR, cordycepin; LY, LY294002.

generation of ROS using 2',7'-dichlorofluorescin diacetate (DCF-DA) showed that the accumulation of ROS was highest after 1 h of cordycepin treatment, and then gradually decreased thereafter (data not shown). However, cells co-treated with N-acetyl cysteine (NAC), a potent ROS scavenger, showed significantly reduced ROS levels, compared to those of cordycepin alone treated cells (Figure 6A and B). Consistent with these flow cytometric results, fluorescence microscopy observations confirmed that NAC treatment significantly inhibited cordycepin-induced ROS generation (Figure 6C). In addition, NAC significantly prevented cordycepin-induced loss of MMP (Figure 6D and E), and reduced viability in T24 cells (Figure 6F).

3.7. *Cordycepin-mediated inactivation of PI3K/Akt signaling is ROS-dependent in T24 cells*

We further investigated the role of PI3K/Akt signaling pathway on ROS generation-mediated apoptosis by cordycepin. Figure 7A shows that when the production of ROS was artificially blocked, the reduced phosphorylation levels of PI3K and Akt by cordycepin were maintained at the control level. In addition, NAC treatment significantly protected the apoptosis induced by the co-treatment of cordycepin and LY294002, as observed by the nuclear morphological changes, DNA fragmentation assay, and flow cytometric analysis (Figure 7B-E). Consistent with these results, the reduced cell viability by co-treatment with cordycepin and LY 294002 was also significantly restored by blocking ROS production (Figure 7F).

4. Discussion

Many previous studies have shown that cordycepin induces apoptosis in a variety of cancer cells under conditions that are not toxic to normal cells (*21,23,38*). It has also been reported that several cellular signaling pathways are involved in the induction of apoptosis of cancer cells by cordycepin (*27,32,35,38-40*), and that the accumulation of ROS associated with mitochondrial dysfunction acts as a major signal in this process (*27,29-30,32,39,41*). Although the possibility of involvement of PI3K/Akt signaling pathway was suggested in several previous studies (*32-36,42*), the link between this pathway and the production of ROS is largely unknown.

Our results indicated that cordycepin activated both caspase-8 and -9, initiator caspases for the activation of extrinsic and intrinsic pathways, respectively, and increased the truncation of Bid in T24 cells, consistent with previous studies in breast and prostate cancer cells (*28,42,43*). In addition, mitochondrial dysfunction was induced as confirmed by the loss of MMP in cordycepin-treated T24 cells. The loss of MMP was accompanied by a down-regulation in the Bcl-2/Bax ratio and promotion of cytochrome c release into the cytoplasm from mitochondria, which are typically

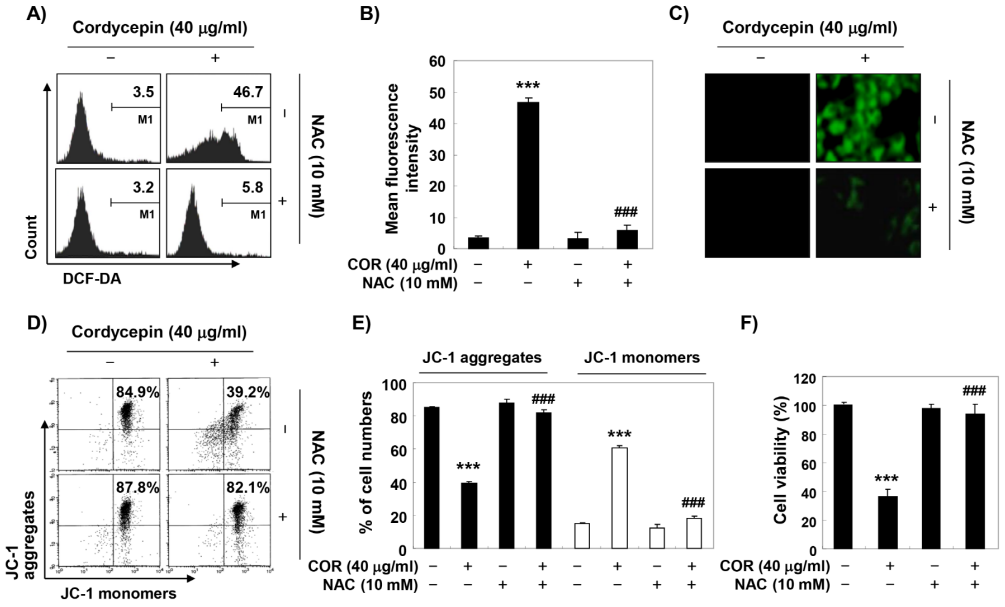

Figure 6. Cordycepin induces ROS generation in T24 cells. (A-C) The cells were either treated with 40 μg/mL cordycepin for 1 h, or pre-treated with 10 mM NAC for 1 h, before cordycepin treatment. **(A)** The medium was discarded, and the cells were incubated at 37°C in the dark for 20 min with new culture medium containing DCF-DA. ROS generation was measured by flow cytometry. **(B)** Each bar represents the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ###$p < 0.0001$, when compared to cordycepin-treated cells). **(C)** Images were obtained by fluorescence microscopy (original magnification: ×200). The images presented here are captured from one experiment, and are representative of at least three independent experiments. **(D-F)** The cells were either treated with 40 μg/mL cordycepin for 48 h, or pre-treated with 10 mM NAC for 1 h, before cordycepin treatment. **(D)** The cells were stained with JC-1 dye, and were then analyzed by flow cytometry, in order to evaluate the changes in MMP. **(E)** Each bar represents the percentage of cells with JC-1 aggregates and monomers (mean ± SD of triplicate determinations, ***$p < 0.0001$, when compared to control; ###$p < 0.0001$, when compared to cordycepin-treated cells). (F) The cell viability was measured by MTT assay. Data were expressed as the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ###$p < 0.0001$, when compared to cordycepin-treated cells). COR, cordycepin.

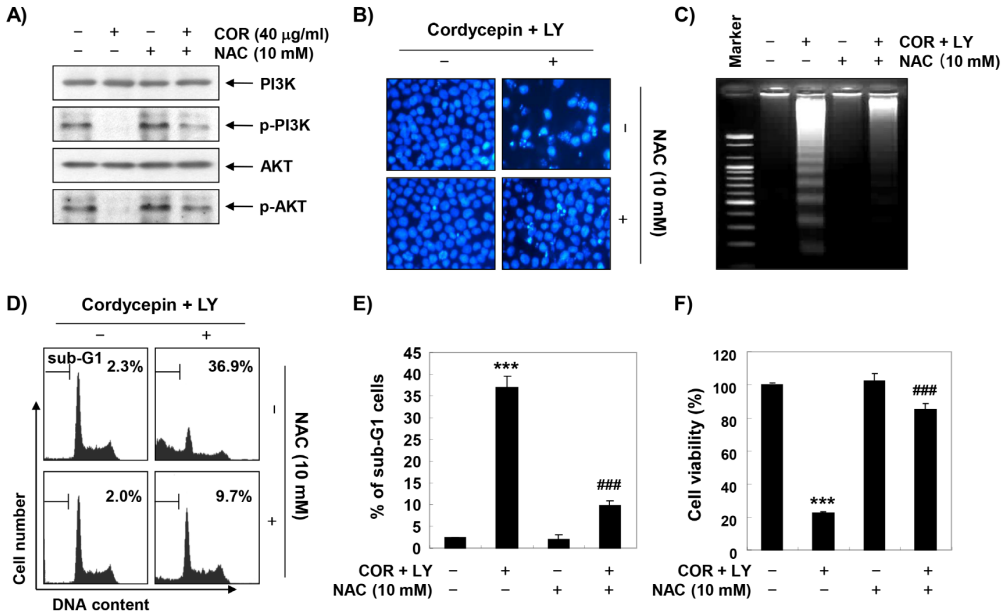

Figure 7. Cordycepin induces the ROS-dependent inactivation of PI3K/Akt pathway in T24 cells. (A) The cells were either treated with 40 μg/mL cordycepin for 48 h, or pre-treated with 10 mM NAC for 1 h before 40 μg/mL cordycepin treatment, and then collected. The cellular proteins were prepared, and the expression of PI3K and Akt proteins was evaluated by Western blot analysis. **(B-F)** The cells were pre-treated with 10 μM LY294002 for 1 h, and then treated with 40 μg/mL cordycepin for a further 48 h, in the presence or absence of 10 mM NAC. **(B)** The DAPI-stained nuclei were then observed by fluorescence microscopy (original magnification, ×400). **(C)** DNA fragmentation was analyzed by the extraction of genomic DNA, electrophoresis in agarose gel, and then visualization by EtBr staining. (D-F) The percentages of apoptotic sub-G1 cells and cell viability were determined by flow cytometry and MTT assay, respectively. (E and F) Data were expressed as the mean ± SD of three independent experiments (***$p <$ 0.0001, when compared to control; ###$p < 0.001$, when compared to cordycepin-treated cells).

observed in the activated intrinsic pathway (*4,5*). Cordycepin treatment also significantly increased the activity of caspase-3, and induced the cleavage of PARP. On the other hand, as is well known in previous studies, caspase-8 activated by the initiation of the extrinsic pathway cleaved and converted Bid, a pro-apoptotic protein belonging to the Bcl-2 family proteins, to tBid (*4,44,45*). tBid in turn translocates to the mitochondria to promote the permeability of the mitochondrial outer membrane, leading to the accumulation of cytochrome c, counteracting the cytoprotective activity of Bcl-2 protein, and amplifying the intrinsic pathway (*45,46*). Therefore, the results indicate that cordycepin induced apoptosis in T24 cells by simultaneously activating the extrinsic and intrinsic pathways through tBid-mediated crosstalk.

Abnormal activation of the PI3K/Akt signaling pathway has recently been shown to be involved in the pathogenesis of multiple human tumors, including bladder cancer (*9-11,47,48*). Activated PI3K initiates the activation of Akt, a downstream kinase of PI3K, which can inhibit apoptosis by protecting caspase cascade through phosphorylation of caspase-9, and promotes the expression of anti-apoptotic proteins of the Bcl-2 family proteins, thereby enhancing cell survival and the proliferation of cancer cells (*49,50*). Because these ultimately contribute to resistance to chemotherapy for inducing apoptosis in cancer cells, PI3K and its regulatory factors are attractive targets for cancer treatment. Therefore, we analyzed whether this signaling pathway was involved in the induction of T24 cell apoptosis by cordycepin, and found cordycepin suppressed the phosphorylated levels of Akt, as well as PI3K. This means that the PI3K/Akt signaling pathway is inactivated by cordycepin treatment, and the results are in good agreement with previous studies performed on several other cancer cell lines (*31-36,42,51*). Furthermore, in line with our previous study using leukemia cells (36), LY294002, a pharmacological inhibitor of PI3K, significantly enhanced the apoptotic effect of cordycepin and further reduced cell viability, supposing that cordycepin-induced apoptosis is mediated by blocking the PI3K/Akt signaling pathway.

Accumulated evidence has shown that the low levels of ROS in the cell act as a secondary messenger in the intracellular signaling pathways, while excessively high levels of ROS induce oxidation of cellular macromolecules, and promote apoptosis through the activation of extrinsic and/or intrinsic pathways (*52-54*). Recent previous studies have reported that several bioactive compounds generated ROS to activate apoptosis signaling in cancer cells, while ROS-dependent suppressing the activity of the PI3K/Akt signaling pathway (*9-11*). These observations suggest that inducing the production of ROS in cancer cells can be used in therapeutic strategies, such as the induction of apoptosis through the inhibition of cell survival signals, such as PI3K/Akt. Therefore, we further assessed whether cordycepin-induced apoptosis in T24 cells was correlated with the production of ROS, and the relationship between ROS production and the PI3K/Akt signaling pathway. Consistent with previous studies (*27-28,30,32,41,43*), our current results showed that cordycepin treatment markedly increased the levels of ROS production; however, as can be predicted, the ROS scavenger, NAC greatly blocked the accumulation of ROS by cordycepin. The quenching of ROS generation also significantly diminished cordycepin-induced disruption of MMP to the control level, followed by significant survival restoration, indicating that ROS act as upstream signaling molecules to enhance cordycepin-induced apoptosis in T24 cells. These results are consistent with our previous findings using prostate cancer cells (*43*), and signified that the cordycepin-induced apoptosis of T24 cells was ROS-dependent. Furthermore, the presence of NAC markedly attenuated cordycepin-induced dephosphorylation of PI3K and Akt proteins, and NAC treatment also significantly blocked the enhanced apoptosis and viability reduction induced by co-treatment of cordycepin and LY294002. Taken together, these results lead us to suggest that the production of ROS by cordycepin plays a critical role in the induction of apoptosis through simultaneous initiation of both extrinsic and intrinsic pathways in T24 cells, and acts as an upstream signal related to the effect of cordycepin on the inactivation of the PI3K/Akt signaling pathway. However, further studies are warranted to determine the direct relationship between cordycepin-mediated inactivation of PI3K/Akt signaling pathway and other cellular signaling pathways, and the identification and role of intracellular organelles involved in ROS generation by cordycepin.

In conclusion, our findings demonstrate that cordycepin exerts an anti-proliferative effect on human bladder cancer T24 cells, through the activation of extrinsic and intrinsic apoptosis pathways. As evidence for this, cordycepin activated caspase-8 and -9, which belong to the initiator caspases of the extrinsic and intrinsic pathways, respectively, followed by the activation of effector caspase 3, resulting in the degradation of PARP. Cordycepin also induced the truncation of Bid and mitochondrial dysfunction, which was associated with an increase in Bax/Bcl-2 expression ratio and cytochrome c release into the cytoplasm. Moreover, the induction of apoptosis by cordycepin was accompanied by inhibition of the PI3K/Akt signaling pathway, and excessive production of ROS. In addition, artificial interception of the PI3K/Akt signal pathway further increased cordycepin-induced apoptosis, and the interruption of ROS generation led T24 cells to escape from apoptosis, while maintaining the activity of PI3K/Akt signaling pathway. Based on these finding, we suggest that cordycepin has chemopreventive potential by inducing apoptosis through ROS-dependent inactivation of the PI3K / Akt signaling pathway in T24 cells.

Acknowledgements

This research was funded by Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korea government (2018R1A2B2005705), Republic of Korea.

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(Received August 12, 2019; Revised August 21, 2019; Accepted August 31, 2019)

Original Article

DOI: 10.5582/bst.2019.01162

Middle-aged female rats lack changes in histone H3 acetylation in the anterior hypothalamus observed in young females on the day of a luteinizing hormone surge

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Summary **Histone acetylation has recently been implicated in gene transcription and estradiol (E2) actions in the hypothalamus. This study aims to determine the involvement of histone acetylation in mediating E2-induced luteinizing hormone (LH) surge to understand the mechanism underlying LH surge dysfunction in female reproductive aging. Young and middle-aged female rats were ovariectomized (OVX) and treated with hormone or oil once per day for two days. At the time of the expected LH surge, blood samples were taken for LH assay. The anterior and posterior hypothalami were dissected, histone H3/H4 acetylation and histone deacetylases (HDACs) 4, -5, -10 and -11 protein expressions were measured using Western blotting. Our results show that in the young females, E2 markedly increased histone H3 acetylation while significantly reducing HDAC10 protein expression in the anterior hypothalamus. Notably, E2-induced alterations of histone H3 acetylation and HDAC10 in the anterior hypothalamus were absent in middle-aged females, associated with a reduced LH release. However, age alters histone H4 acetylation in both the anterior and posterior hypothalamus and significantly increased HDAC 4 and -5 protein expression in the anterior hypothalamus. Taken together, these data suggest that histone H3 acetylation in the anterior hypothalamus may mediate E2 regulation of LH surge and the process possibly through decreasing HDAC10. The missed responsiveness of histone H3 acetylation and HDAC10 expression to E2 in the anterior hypothalamus may contribute to LH surge failure that occurs in female reproductive aging.**

Keywords: Histone acetylation, histone deacetylases, LH, hypothalamus, aging

1. Introduction

A robust and appropriately timed preovulatory luteinizing hormone (LH) surge requires an estradiol (E2) target on estrogen receptor alpha (ERα)-expressing neurons in the hypothalamus to initiate gene transcription and the ensuing coordinated actions of these gene products on gonadotropin-releasing hormone (GnRH) neurons (*1-4*). As female rodents enter middle age, there is a characteristic delayed and attenuated LH surge under an E2 positive feedback condition, which has been considered as the earliest biomarker associated with reproductive aging (*5-7*). The neural and molecular mechanisms that underlie the age-related LH surge impairment remain incompletely characterized (*6-9*), the reduced LH surge is not induced by processes affecting the existence of E2-responsive cells (*e.g.* apoptosis) in the hypothalamus (*8,10*), but rather by mechanisms affecting the transcriptional activity of the network of

Released online in J-STAGE as advance publication August 20, 2019.

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genes related to neurotransmitter/neuropeptide release and recycling (*9,11,12*) in the anterior hypothalamus including the preoptic area (POA) and anteroventral periventricular nucleus (AVPV).

At the time of LH surge, what ultimately causes the decreased transcriptional activity of genes in the middle-aged hypothalamus remains poorly understood. Epigenetic changes such as histone acetylation can be induced by sex steroids (*13,14*) and have recently been implicated in the sexual differentiation of several brain (*15,16*) and behavioral phenotypes (*13,15,17*). Once the amino acid residues on the histone tail are acetylated, the bond between histones and DNA will be relaxed to allow access to transcriptional factors (*18-21*). Of great interest, histone acetylation is required for ERα-mediated gene transcription by affecting ERα to bind estrogen response element (ERE) within the regulatory regions of target genes (*13,22*). In the ventromedial nucleus of hypothalamus (VMH) and POA of female mice, histone H3 and H4 were highly acetylated following E2 administration, accompanied by transcriptional activation of the ERα target gene *Pgr* (*13*) as well as expression of reproductive behavior (*23*). Under E2 positive feedback conditions, histone H3K9/14 acetylation was induced by E2 to recruit ERα binding in the *Kiss1* promoter region in the AVPV and was positively associated with an increase in *Kiss1* gene expression in the nucleus (*24*). These studies indicate that histone acetylation facilitates ERα-mediated gene transcription to coordinate E2 actions in the hypothalamus. Typically, histone acetylation status is regulated by a variety of histone deacetylases (HDACs) (*25-27*). E2 induced histone H3 acetylation at the *Bdnf* promoter pII and pIV enhancing memory consolidation in the hippocampus is coupled with decreased HDAC2 and -3 (*28*), supporting the critical role of HDACs for E2 modulation of histone acetylation.

Neuroendocrine epigenetic modifications induced by aging and hormone cues have increasingly been suggested as good candidates to provide a molecular explanation for aging-related changes of brain function (*29,30*). In middle-aged mice, impaired spatial and contextual memory is associated with deficits in learning-induced H4 acetylation in the hippocampus (*31,32*); when E2 is administered in middle-aged mice, there is a specific increase of histone H3 acetylation at *Bdnf* promoters pI and pIV in the dorsal hippocampus (*28*). These studies implied that interaction of age and hormones could alter histone acetylation level in the brain to induce memory impairment. Thus, it is a perspective that attenuated histone acetylation in the hypothalamus under E2 positive feedback may be the underlying molecular mechanism of reduced transcription of genes in the hypothalamus and contributes to the impaired LH surge in middle-aged females.

The current study was designed to investigate the potential age-related alteration of histone acetylation in the hypothalamus with regard to reduced E2-induced LH surge. We compared young and middle-aged female rats that were treated with a similar exogenous estradiol regimen known to elicit LH surges and determined *i*) whether hypothalamic histone acetylation level exhibits changes coincident with E2-induced LH surge, if so, does this occur similarly in different ages; *ii*) whether age or E2-related hypothalamic HDACs expression exists that may account, in part, for differential histone acetylation in middle-aged females during the LH surge.

2. Materials and Methods

2.1. *Animal care*

All animal procedures were approved by the Institutional Animal Care and Use Committee at Fudan University. Young (3-4 mo) and middle-aged (9-12 mo, retired breeders) female Sprague Dawley rats (Vital River, Beijing, China) were housed in groups and maintained on a 12-hour light, 12-hour dark cycle (lights on at 0700) with free access to chow and water. Rats were handled for 5 min/d for 1 week before monitoring estrous cyclicity by daily vaginal lavage with sterile saline for 2 weeks. Only rats with at least two regular 4- to 5-day estrous cycles were included in the studies (*5,8,9*).

2.2. *Ovariectomy surgery and hormone administration*

Eighteen young and eighteen middle-aged rats were anesthetized with pentobarbital sodium (30mg/kg, *ip*) and ovarioectomized (OVX). To induce LH surges, females received 2μg/0.1ml subcutaneous injections of estradiol benzoate (E2; Hangxiang Inc., China) dissolved in peanut oil. At 0900h on day seven after OVX, rats received the first of two daily injections of 2μg of E2. For the temporal LH surge study, forty-eight hours after the first E2 injection, rats were injected with 500μg of progesterone (P; Steroloids, Inc.). This hormone regimen reliably produces LH surges in female rats (*5,8,9*).

2.3. *Jugular vein catheterization and blood collection*

On postoperative day seven rats were anesthetized and an indwelling catheter placed into the right atrium via the right jugular vein for serial blood sampling (*5*). Catheters were kept patent with daily heparinized saline flushes. Serial blood samples (300μL) were collected from awake, freely moving rats for determination of the LH surge at 1300h, 1500h, 1700h and 1900h. Each sample volume was replaced with warm, sterile, heparinized saline (15 U/mL). Plasma was stored at -80°C until LH determination.

2.4. *Hypothalamic dissection*

At the time of peak LH release, young and middle-aged rats were euthanized by rapid decapitation for Western blotting experiments. As previously described (*5,8,33*), the anterior hypothalamus, which includes the POA, and the posterior hypothalamus, which includes the arcuate and VMH, were flash frozen on dry ice, and stored at -80C until quantitation of proteins.

2.5. *Western blotting*

Samples were resuspended and sonicated in hypotonic lysis buffer (*34*). Total protein content of the lysates was measured using BCA protein assay (Thermo Scientific), sample buffer was added, and samples were boiled for 5 min at 100°C. Samples were loaded onto Tris-HCl polyacrylamide gels (Bio-Rad) for electrophoresis and, after separation, transferred to PVDF membranes (Millipore). Membranes were incubated overnight at 4°C with rabbit polyclonal antibodies recognizing histone H3 (1:1000, Abcam), acetylated histone H3 (1:5000, Millipore), histone H4 (1:1000, Abcam), acetylated histone H4 (1:1000, Millipore), HDAC4 (1:1000, Santa Cruz Bio), HDAC5 (1:1000, Santa Cruz Bio), HDAC10 (1:1000, Santa Cruz Bio), HDAC11 (1:1000, Santa Cruz Bio), or monoclonal β-Actin (1:5000, Sigma). After TTBS wash, membranes were incubated for 1h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) or with goat anti-mouse IgG (Sigma Aldrich) and developed using enhanced chemiluminescence (SuperSignal West Dura, Thermo Scientific). Signal was detected using a Kodak Image Station 440CF and quantified by densitometry using Kodak 1D 3.6 software.

2.6. *LH assay*

Serum LH concentrations were measured using enzyme-linked immunosorbent assay (ELISA) with LH reagents provided by the Beijing Sino-UK Institute of Biological Technology (Chaoyang, Beijing).

2.7. *Data analysis*

GraphPad Prism 6 software was used for statistical analysis. Data are expressed as mean ± SEM. The area under the curve (AUC) for total LH release was calculated using Sigma Plot 10.0; *t*-test was used to determine difference in total LH release. Two-way ANOVA (age × hormone) was utilized to detect differences in histone acetylation and HDACs protein expression in anterior and posterior hypothalamus in young and middle-aged groups. Bonferroni or Turkey's *post-hoc* tests were performed to determine individual group differences following main or interaction ANOVA effects.

3. Results

3.1. *Middle-aged rats exhibit an attenuated LH release under E2 positive feedback*

We first sought to replicate our previous results that E2-induced a reduced LH surge in middle-aged rats under E2 positive feedback. The middle-aged rats exhibited an obvious decreased LH surge after the first E2 injection (Figure 1A). When compared with the middle-aged rats, young rats released 3-fold as much LH ($p < 0.01$) at the time of E2-induced LH surge (Figure 1B). These results are in agreement with our previous studies and others (*5-7*).

3.2. *E2-induced histone H3 acetylation was absent in the anterior hypothalamus of middle-aged rats*

We next determined whether E2-induced LH surge is associated with hypothalamic histone acetylation, and if so, whether histone acetylation in the hypothalamus is altered in middle-aged females. Total extracts of anterior and posterior hypothalamus were isolated

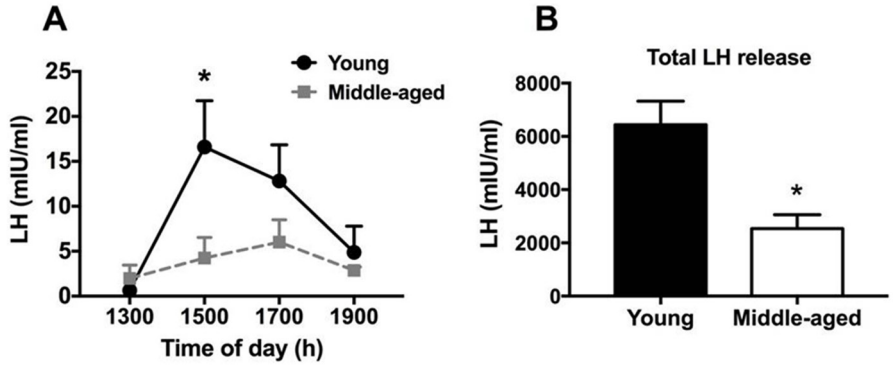

Figure 1. Hormone-induced LH surge was reduced in middle-aged female rats. OVX young and middle-aged rats were primed with two daily doses of E2 benzoate (2 μg) and one injection of P (500μg) on the day of blood collection. **(A)** LH surge and **(B)** Total LH release in young and middle-aged females. Data are shown as means ± SEM, $n = 6$ in both groups, *$p < 0.01$.

from E2 and vehicle-treated young and middle-aged rats during the LH surge, effects of age and hormone treatment on the global acetylation levels of histone H3 and H4 (H3Ac and H4Ac) were analyzed by Western blotting. In the anterior hypothalamus, a significant main effect of E2 ($F = 12.81$, $p < 0.01$) was found for acetylated histone H3, higher in E2 than vehicle-treated young females (Figure 2A). An interaction between age and E2 on H3Ac ($F = 5.14$, $p < 0.05$) was observed as well; E2-induced a higher acetylated histone H3 level in young females, however, this effect was absent in middle-aged rats (Figure 2A). Interestingly, there was no significant main effect of age, or any significant hormone by age interactions, for histone H3 acetylation in the posterior hypothalamus (Figure 2B).

3.3. *Age but not E2 alters histone H4 acetylation in the anterior or posterior hypothalamus*

However, regardless of E2 treatment, a significant main effect of age was found for acetylation of H4 in the anterior hypothalamus ($F = 7.398$, $p < 0.05$) (Figure 3A), with acetyl H4 level higher in middle-aged compared to young females. A significant main effect of age on H4 acetylation was also found in the posterior hypothalamus ($F = 7.398$, $p < 0.01$) (Figure 3B), however, the H4 acetylation level was lower in middle-aged compared to young females. These results implied that the effect of E2 on histone acetylation in the hypothalamus is specific to H3.

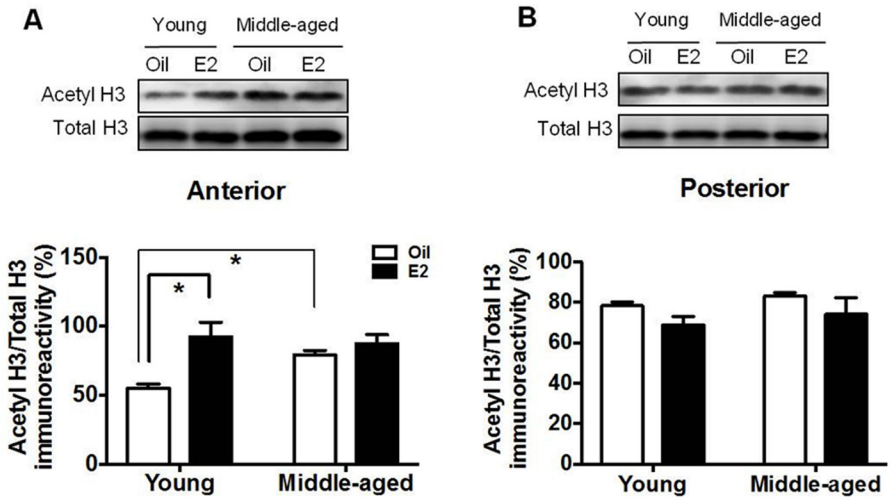

Figure 2. Effect of E2 on histone H3 acetylation in the anterior hypothalamus of young and middle-aged female rats at the time of LH surge. Rats were given two daily doses of E2 benzoate (2 μg) and were killed 52-54 hrs after the first injection of E2. **(A)** Western blot showing E2 increases histone H3 acetylation level in the anterior hypothalamus of young but not middle-aged females. **(B)** E2 does not change histone H3 acetylation in the posterior hypothalamus of young and middle-aged rats. Data are shown as means ± SEM, $n = 6$ in both groups, $*p < 0.01$.

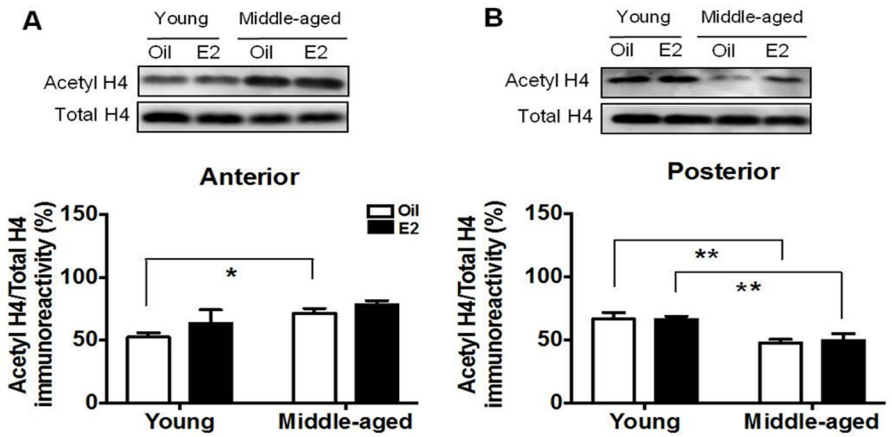

Figure 3. Age alters histone H4 acetylation in the anterior and posterior hypothalamus of young and middle-aged rats. Independent of hormone priming age affects histone H4 acetylation in the anterior and posterior hypothalamus. Rats were given two daily doses of E2 benzoate (2 μg) and were killed 52-54 hrs after the first injection of E2. **(A)** Middle-aged females show an increased histone H4 acetylation in the anterior hypothalamus. **(B)** Acetylated histone H4 decreases in the posterior hypothalamus of middle-aged females. Data are shown as means ± SEM, $n = 6$ in both groups, $*p < 0.05$, $**p < 0.01$.

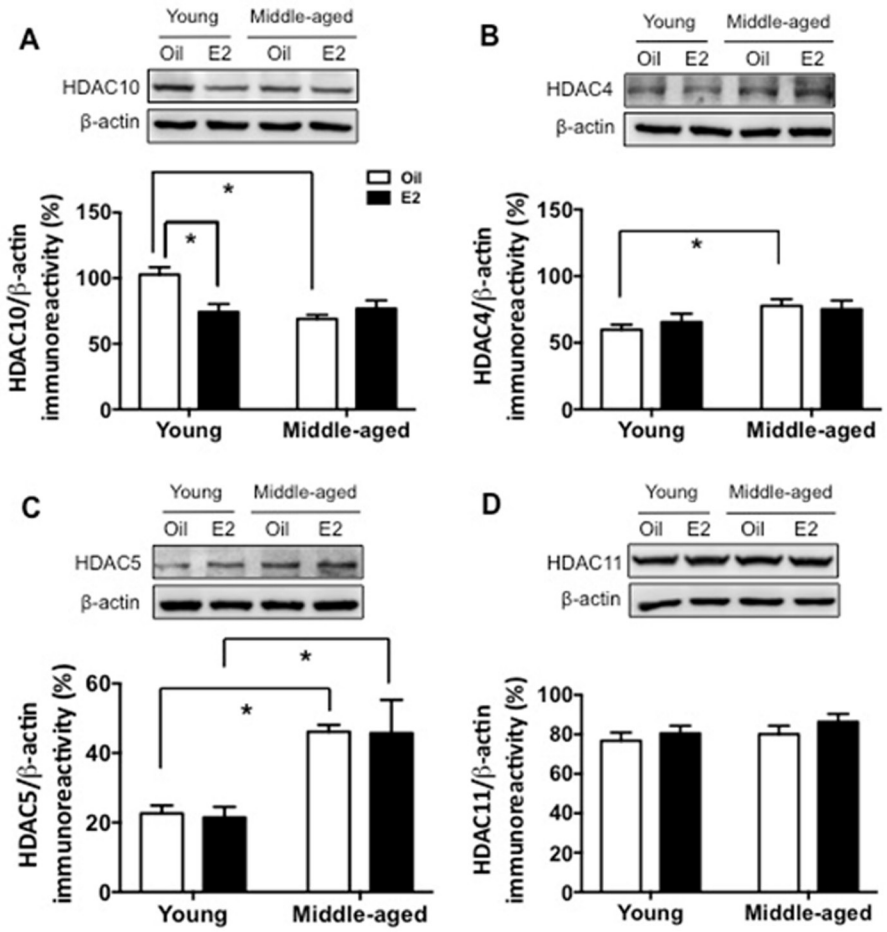

Figure 4. E2 decreases HDAC10 protein expression in the anterior hypothalamus of young but not middle-aged rats at the time of LH surge. Rats were given two daily doses of E2 benzoate (2 μg) and were killed 54-56 hrs after the first injection of E2. Western blotting showing **(A)** E2 priming decreases HDAC10 protein level in the anterior hypothalamus of young but not middle-aged females. **(B)** HDAC4, **(C)** HDAC5 and **(D)** HDAC11 protein expression in the anterior hypothalamus of young and middle-aged females. *$p < 0.05$.

3.4. *E2 decreases level of histone deacetylase 10 in the anterior hypothalamus of young but not middle-aged rats*

HDACs remove acetyl groups from histone tails, thereby condensing the chromatin and decreasing gene transcription (*25,35*). We previously showed that E2 treatment positively decreased mRNA levels of 4 HDACs (*Hdac4*, *-5*, *-10* and *-11*) in the anterior hypothalamus of young females (*36*). However, E2 does not change *Hdac4*, *-5*, and *-11* mRNA expression in middle-aged females except *Hdac10* (*36*). The current Western blotting found that there was an interaction between E2 and age on HDAC10 protein expression in the anterior hypothalamus ($F = 6.063$, $p < 0.05$) (Figure 4A). Although HDAC10 decreased in young rats following E2 treatment, this effect was absent in middle-aged rats. Regardless of E2 treatment, HDAC4 ($F = 5.435$, $p < 0.05$) and HDAC5 ($F = 8.293$, $p < 0.05$) protein was higher in middle-aged compared to young rats (Figure 4B and 4C). There was no significant main effect of age, or any significant hormone by age interactions, for HDAC11 in the anterior hypothalamus (Figure 4D).

4. Discussion

Although sex steroid induction of histone acetylation has been recognized as an important mechanism mediating sexual differentiation of brain (*15,16*) and behavioral phenotypes (*13,15,17*), the possibility that histone acetylation participates in steroid-induced LH surge remains largely unknown. The present experiments demonstrate for the first time that histone H3 acetylation in the anterior hypothalamus is enhanced by E2 at the time of LH surge. Specifically, E2 induced increased histone H3 acetylation may causally be linked to reduced protein expression of HDAC10 in the anterior hypothalamus. Notably, the E2-administration, which led to a significant increase in the level of histone H3 acetylation is absent in the middle-aged anterior hypothalamus, coincident with the typical impaired E2-dependent LH surge. To our knowledge, this is the first evidence demonstrating that E2-induced increased histone acetylation especially H3 in the anterior hypothalamus may be involved in LH surge, which is partially consistent with a recent report indicating that E2 administration led to a significant change in histone H3

acetylation in the *Pgr* gene in the POA of female mice (*13*). Thus, these data suggest that the missed E2-induced histone H3 acetylation in the anterior hypothalamus may be an important mechanistic pathway for the changes of E2 target genes expression and consequently results in age-related impaired LH surge in middle-aged females. However, histone H4 acetylation, unlike histone H3, showed mainly age-related changes in the anterior and posterior hypothalamus.

Acetylation of histone proteins has long been known to promote transcriptional activity (*21,37*) and contribute to E2-induced gene expression in human MCF-7 cells (*14,38,39*) and specific hypothalamic regions (*13,40,41*). Of great interest, histone acetylation is crucial in E2-mediated sexual behavior (*13*) and formation of hippocampus-dependent memory in mice (*28,34,42,43*). We therefore tested the hypothesis that histone acetylation in the hypothalamus would be regulated by E2 to generate a GnRH/LH surge in young females. We observed distinct elevated histone H3 acetylation detected at the level of global chromatin in the anterior hypothalamus in response to E2 at the time of LH surge, suggesting histone hyperacetylation induced by E2 is able to facilitate E2-induced LH surge in female rats. Acetylation of Lys residues on histone H3 and H4 was shown to be critical for an open chromatin structure and gene transcription (*35,38*), especially acetylation of H3K56ac and H3K14ac is associated with expression of genes by regulating genome stability (*35*). However, the current data is limited to reveal the E2-induced specific acetylation marks on histone H3 during LH surge. On the other hand, LH surge requires E2 stimulating appropriate induction of ERα to bind ERE (*14*) within the regulatory regions of target genes in glutamatergic and GABAergic neurons in the POA (*6,7*), histone H3K9/14 acetylation and ERα binding in the AVPV Kiss1 promoter region were induced by E2 and were positively associated with an increase in *Kiss1* gene expression in this nucleus (*24*). Future studies should specifically examine which genes in those specific cell types will be regulated by histone H3 acetylation to gain a better understanding of how E2 regulates gene transcription for a GnRH-LH surge.

HDACs are recognized as important mediators of epigenetic changes via histone deacetylation and chromatin remodeling (*25,27*). Thus, decreased HDACs should alter histone acetylation and open up chromatin, allowing for increased gene expression. Our data show here that female rats with E2-induced LH surge have enhanced histone H3 acetylation in the anterior hypothalamus but a decrease in HDAC10. HDAC10 was found to be expressed in neurons (*44*), fasting decreased HDAC10 in the medial basal hypothalamus (*45*). Our finding that HDAC10 in the anterior hypothalamus decreased at the LH surge time imply that HDAC10 may play a role as a crucial negative regulator of LH surge.

One of the important observations from our study was that E2-regulated histone H3 acetylation is absent in the anterior hypothalamus of middle-aged females at the LH surge time. Age-related changes in the LH surge mechanism are recognized as a reflection of reduced gene transcriptional activity of GnRH neuron excitatory afferent inputs in the POA (*8,9,12*). By analyzing histone acetylation levels in the anterior hypothalamus, we could speculate that a loss of histone H3 acetylation responses to E2 may disrupt the induction of the ERα-mediated gene program in the POA. Nevertheless, it should be noted that we did not use the restricted POA tissues for CHIP experiments, defining the specific targets of the acetylated histone H3 and the putative gene promoters is of paramount importance. Our data showed that middle-aged females lacking E2-induced histone H3 acetylation in the anterior hypothalamus have a consistent unchanged level of HDAC10. The missed response of HDAC10 to E2 could directly link with histone deacetylation and consequently decrease gene transcription by condensing chromatin.

Recent evidence suggests that epigenomic changes can occur extremely early in the aging process and be causative. Hypothalamus-mediated aging may contribute to physiological deterioration and aging-related disease (*29,46*). However, it is largely unknown whether these aging-associated changes are a cause or a consequence of histone acetylation. Interestingly, we found that age changes histone H4 acetylation in both the anterior and posterior hypothalamus and HDAC4 and -5 protein expression in the anterior hypothalamus. Histone H4 modification such as H4K20me3 has been demonstrated to be altered during mammalian aging (*47*), and an increase in H4K20me3 was found in human patients with Hutchinson-Gilford progeria, a premature aging syndrome (*48*). This evidence implies hypothalamic acetylated histone H4 and specific HDACs may be involved in hypothlamus programming aging phenotypes including energy homeostasis or circadian rhythm.

In conclusion, the present study demonstrates histone H3 acetylation in the anterior hypothalamus as a novel mechanism underlying the E2 positive feedback action to induce GnRH-LH surges, and expression of HDAC10 in the anterior hypothalamus is regulated by E2. Our findings provide the first evidence that the middle-aged female anterior hypothalamus loses histone H3 acetylation and HDAC10 expression responsive to E2, and overall suggest an epigenetic mechanism may underlie reduced gene transcriptional activity and the associated LH surge dysfunction in middle-aged females. These findings may provide a key insight into the mechanism of age-related loss of responsiveness of the hypothalamus under E2 positive feedback.

Acknowledgements

This work was supported by the Natural Science Foundation of Shanghai 17ZR1403300 (to Yan Sun), the National Natural Science Foundation of China

31800987 (to Yan Sun). This work was supported by the Science and Technology Commission of Shanghai Municipality 2018 YIXUEYINGDAO project No. 18401902200 (to Ling Wang), the Shanghai Committee of The China Democratic League No. 02054 (to Ling Wang), the National Natural Science Foundation of China No. 31571196 (to Ling Wang), the Science and Technology Commission of Shanghai Municipality 2015 YIXUEYINGDAO project No. 15401932200 (to Ling Wang), the FY2008 JSPS Postdoctoral Fellowship for Foreign Researchers P08471 (to Ling Wang), the National Natural Science Foundation of China No. 30801502 (to Ling Wang), the Shanghai Pujiang Program No. 11PJ1401900 (to Ling Wang), and Development Project of Shanghai Peak Disciplines-Integrative Medicine No.20150407.

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(Received July 4, 2019; Revised July 29, 2019; Accepted August 10, 2019)

Original Article

DOI: 10.5582/bst.2019.01185

GRIM-19 over-expression represses the proliferation and invasion of orthotopically implanted hepatocarcinoma tumors associated with downregulation of Stat3 signaling

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Summary **The retinoid-interferon-induced mortality-19 (GRIM-19) gene has been identified as a negative regulator associated with tumor development. The current study created a model of an orthotopically implanted hepatocarcinoma tumor to verify the inhibitory effect of GRIM-19 *in vivo*. After treatment with GRIM-19 carried by attenuated *Salmonella*, transplanted tumors were measured with an Imaging System. The expression of GRIM-19, Stat3/p-Stat3, cyclinD1, CDK4, PCNA, Bax/Bcl-2, cleaved caspase-9/3, VEGF, and MMP-2/9 was determined using immunohistochemistry and Western blot analysis. The cell cycle was assessed using flow cytometry (FCM). Apoptosis was determined using FCM and a TUNEL assay. Results indicated that GRIM-19 overexpression resulted in inhibition of peritoneal metastasis, induction of cell cycle arrest, and apoptosis *in vivo*. In addition, the expression of Stat3/p-Stat3 was down-regulated by GRIM-19. These results suggest that GRIM-19 overexpression could suppress the growth of orthotopically implanted hepatocarcinoma tumors by reversing the regulation of the Stat3 signaling pathway. This approach could potentially be a powerful treatment for hepatocarcinoma.**

Keywords: GRIM-19, hepatocellular carcinoma, orthotopically implanted tumor, Stat3, apoptosis

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer death worldwide (*1*). Despite the increasing prevalence of HCC and the fact that many patients present with advanced disease, there is a startling lack of treatments (*2*). Fortunately, recent studies suggested that successful targeted therapy could improve outcomes for HCC, even in the late stages of carcinogenesis, and may be the best choice for curative treatment. Sorafenib, a multi-kinase inhibitor, can effectively prolong the median overall survival of patients with advanced HCC from 8 to 11 months (*3*). However, Sorafenib was the sole targeted therapy approved for treating advanced HCC until 2016 (*4*). Many new targeted drugs, such as cabozantinib, regorafenib, ramucirumab, and lenvatinib that can inhibit multi-kinase or angiogenic factors have improved clinical outcomes for patients, but improvements in survival are still modest. Moreover, some of these drugs, like ramucirumab, might fail in subsequent clinical trials when the compound fails to result in effective improvements in overall survival for patients. Hence, identifying more underlying gene targets for initiation and progression of liver cancer is still urgently needed.

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Retinoid-interferon-induced mortality-19 (GRIM-19), an encoded protein localized in the nucleus, mitochondria, and cytoplasm, has been identified as a suppressor in various types of malignancies (*5*). GRIM-19 expression is also reported to be down-regulated in many carcinomas, such as colon cancer, breast cancer, ovarian cancer, and HCC (*6-9*). Over the past 2 decades, an increasing number of studies have indicated that GRIM-19 repressed tumors by inducing apoptosis, arresting the cell cycle, and inhibiting metastasis (*10-12*). In a previous study by the current authors, GRIM-19 was expressed at low levels in 54 HCC samples, and it suppresses liver cancer cell growth by inducing cell cycle arrest and promoting apoptosis in *in vitro* (*13*). Interestingly, the inhibitory effect of GRIM-19 on the signal transducer and activator of transcription 3 (Stat3) has been considered to be the potent mechanism by which GRIM-19 induces antitumor action (*14*).

Stat3, a multifaceted growth mediator in cells, has been recognized as a promoter in many malignancies, including HCC (*15*). In contrast, GRIM-19 was suggested to be a stronger inhibitor of Stat3 activation. Several studies indicated that GRIM-19 could repress the invasion and epithelial mesenchymal transition (EMT) of colorectal cancer *via* inactivation of Stat3 (*8,16*). Other studies reported that a low level of GRIM-19 expression directly induced Stat3 activation in many carcinomas (*6,7,9*). However, the mechanisms of low levels of GRIM-19 expression in carcinomas and the underlying the effects of GRIM-19 on tumorigenesis are still poorly understood. Nowadays, most studies are focusing on targeting carcinogenic molecules that may also be the essential for cell survival in normal organs, and this may cause adverse reactions. The aim here is to develop a strategy that up-regulates GRIM-19 expression using *Salmonella typhimurium* in HCC, a approach that has yet to be tested, as a potential therapy for HCC that causes fewer adverse reactions.

The current study created a mouse model of an orthotopically implanted hepatocarcinoma tumor to observe the effect of GRIM-19 on the growth of the xenografts, and it used an attenuated S. Typhimurium strain to deliver a GRIM-19 expression plasmid to the implanted HCC. In addition, this study also analyzed the expression of Stat3 and its downstream target genes in the xenograft to explore potential mechanisms of GRIM-19 action in tumorigenesis.

2. Materials and Methods

2.1. *Cells and animals*

A murine H22 ascites-derived, hepatocellular carcinoma cell line was donated by the Prostate Disease Research Center, Jilin University (China). Cells were routinely grown in the intraperitoneal space of C57BL/6 mice and then cryopreserved in liquid nitrogen. Inbred male C57BL/6 mice, weighing 18-22 g and ranging from 5-6 weeks of age, were purchased from the animal center of the School of Basic Medicine, Jilin University (China). All mice were housed at a constant temperature and constant humidity in a specific pathogen-free environment with free access to food and water. The study protocol was approved by the local ethics committee.

2.2. *Strains and plasmid*

The eukaryotic expression plasmid pcDNA3.1-GRIM-19 and empty vector pcDNA3.1 were donated by Dr. Hu JD of the University of Maryland (Baltimore, USA). The attenuated *S. Typhimurium* phoP/phoQ mutant strain PQ was stored by the Prostate Disease Research Center, Jilin University (China). Plasmids were electroporated into *Salmonella* to form the recombinant *Salmonella*e, which was then maintained at -80℃ in glycerol.

2.3. *Creation of an orthotopically implanted hepatocarcinoma tumor model*

C57BL/6 mice were subcutaneously inoculated with 0.1 mL (2×10^8 cells per mL) of suspended H22 cells to form a xenograft. When tumors reached 5 mm in diameter under the right axilla of the mice, they were removed and sliced into small pieces of 1 mm3 under sterile conditions. While mice were under anesthesia, the right lobe of the liver was punctured to form a 2-mm-long sinus tract, and a small piece of tumor tissue was inserted into each sinus tract, thus creating a model of orthotopically transplanted HCC tumors.

2.4. *Antitumor activity of GRIM-19 on xenografts*

When the xenografts reached 500 mm^3 in volume in the left lobe of the liver, they were deemed to be the standard model. Standardized mice with HCC xenograph implants were then randomly divided into three groups (n = 20 per group). Before each treatment, all mice were fasted overnight and orally pre-administered 100 μL of 10 g/L $NaHCO_3$ solution. Half an hour later, the Mock group was orogastrically inoculated with 100 μL of PBS, the PQ-Scramble group was similarly inoculated with 1×10^8 cfu of PQ-Scramble, and the PQ-GRIM-19 group was similarly inoculated with 1×10^8 cfu of PQ-GRIM-19. Treatments were administered once every 10 days for a total of two doses. The volume of orthotopic transplantation tumors was monitored using the Vevo770 High-Resolution Imaging System. The mice were sacrificed 21 days after the first treatment, and the tumors were excised, weighed, and photographed. Two hundred mg of fresh tumor tissue was collected to extract total protein and analyze the cell cycle and apoptosis; the remaining tissues were fixed in 4% paraformaldehyde for

morphological assays, immunohistochemical staining, and TUNEL staining.

2.5. *Western blot analysis*

Soluble proteins were extracted from 100 mg of tumor tissues and subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc., Rockford, IL, USA) and immunoblotted with anti-GRIM-19, anti-CyclinD1, anti-β-actin, anti-PCNA, anti-Stat3, anti-p-Stat3(Tyr705), anti-Bax, anti-Bcl-2 or anti-cleaved caspase-9, anti-MMP-2, anti-MMP-9, anti-VEGF, anti-cleaved caspase-3 (C-Caspase-3), anti-CDK4, or anti-β-actin (Cell Signaling Technology, USA) overnight at 4℃ and then incubated with horseradish peroxidase (HRP)-tagged second antibody at room temperature for 1 hour. Finally, the protein level was detected with an ECL plus kit (Millipore, USA) and developed *via* the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA) in accordance with the manufacturer's instructions.

2.6. *Analysis of the cell cycle*

Fifty mg of fresh tumor tissue was homogenized and suspended in RPMI-1640 with 10% FBS (Hyclone, USA). After centrifugation, cells were resuspended in RPMI-1640 with 10% FBS. Cells were collected at a density of 10^6 cells/mL in the presence of propidium iodide (PI) and RNase, both at a final concentration of 50 μg/mL. After storage in the dark at 4℃ for 1 h, DNA content was determined using a flow cytometric analysis with a FACS can flow cytometer (Becton Dickinson, San Jose, CA, USA) as previously described (*9*). Cell cycle analysis was performed using the software Multicycle (Phoenix Flow Systems, Inc., San Diego, CA, USA).

2.7. *Immunohistochemistry assays*

Tumor specimens fixed in 10% formalin were embedded in paraffin and cut into 3mm-thick slides. The slides were soaked in sodium citrate buffer and heated in a microwave oven at 98℃ for 15 min for epitope retrieval. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution at 37℃ for 25 min. Non-specific binding was prevented by normal goat serum at room temperature for 20 min. Immunostaining of target proteins was performed using specific mouse monoclonal antibodies. Goat anti-mouse IgG conjugated with horseradish peroxidase was used as the secondary antibody. The staining procedure was carried out manually at 37℃ for 30 min, using the HRP-avidin complexing method. Reactive products were visualized with 3,3'- diaminobenzidene (DAB) as the chromogen, and the slides were counterstained with hematoxylin. The stained slides were analyzed and photographed with a microscope, and cellular brownish staining was scored as positive.

2.8. *Annexin V/PI apoptosis assay*

Fifty mg of sample was homogenized to detect apoptosis with annexin V/PI. Cells were scraped from the container and stained with annexin V-FITC and PI according to the manufacturer's instructions (Annexin V- FITC Apoptosis Detection Kit, Beyotime Institute of Biotechnology, China). In brief, cells were washed with PBS twice, suspended in 400 μL of binding buffer, and incubated with 5 μL FITC-labeled annexin V for 15 min and 15 μL PI for 5 min, successively, at 4℃ in the dark. Apoptotic cells were measured in the FACScan flow cytometer.

2.9. *TUNEL assay*

The TUNEL assay was performed using the TUNEL System kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, the cells were permeabilized with Proteinase K (20 μg/mL) at 37℃ for 2 min followed by addition of TUNEL in the dark at 37℃ for 1 h. The localized green fluorescence of apoptotic cells was visualized using fluorescence microscopy.

2.10. *Statistical analysis*

Data are expressed as the mean ± standard error. Statistical analyses were performed with SPSS17.0 using an analysis of variance (ANOVA) test. $p < 0.05$ was deemed to be statistically significant.

3. Results

3.1. *GRIM-19 overexpression inhibits the growth of xenografts*

To evaluate the effects of GRIM-19 on tumor growth *in vivo*, an orthotopically implanted xenograft model was created, and xenograft volume and tumor weight were monitored. As shown in Figure 1A, 6 mice survived 21 days after orally receiving PBS and 10 survived after similarly receiving after receiving PQ-Scramble. However, 16 mice in the group treated with PQ-GRIM-19 survived. The xenograft volume was measured with a Vevo770 High-Resolution Imaging System in live mice, and the weight of excised tumors in the PQ-GRIM-19 group decreased significantly ($p < 0.05$) compared to that in the Mock and PQ-Scramble groups (Figure 1B, C and D).

To indicate that GRIM-19 had been transferred into the tumor tissues by the recombinant *S. Typhimurium*, a Western blot assay was used to detect protein expression in xenograft tissues. Importantly, the expression of

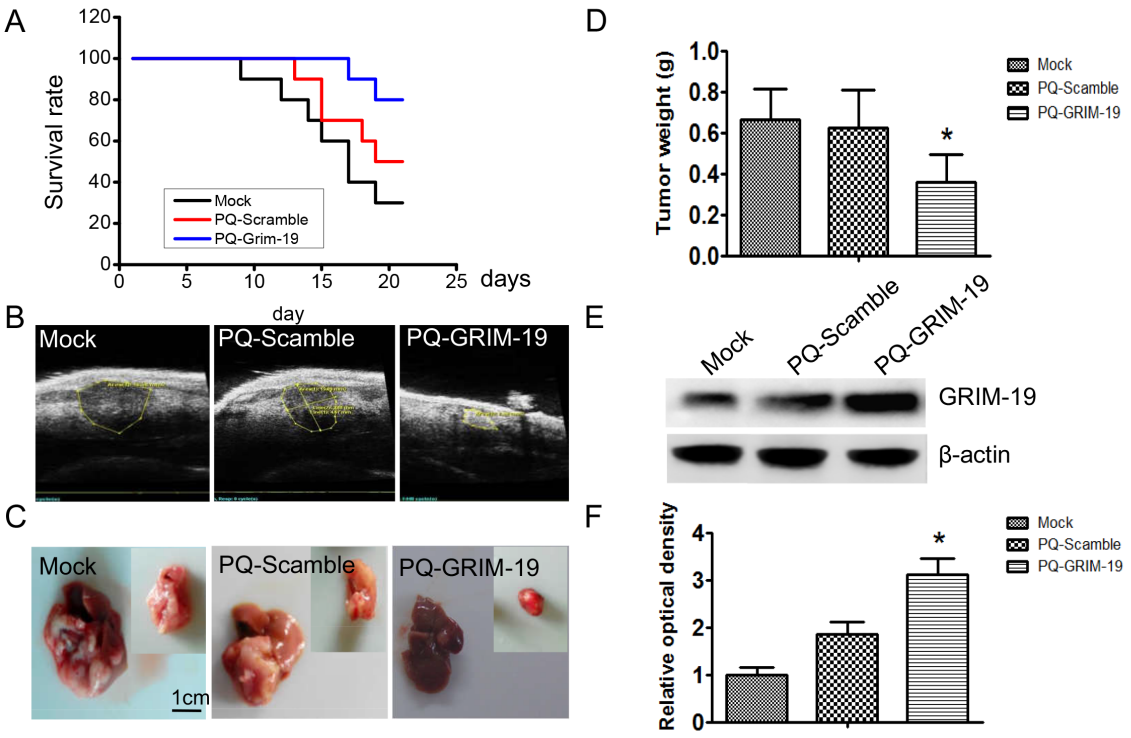

Figure 1. GRIM-19 overexpression inhibited H22 xenograft growth and increased the survival rate of mice with HCC. **(A)** Survival curves of mice bearing an orthotopically implanted hepatocarcinoma tumor following treatment with PQ-GRIM-19. **(B)** Tumor diameters measured with the Vevo770 High-Resolution Imaging System. **(C)** Representative livers and tumors were removed from mice. **(D)** Tumor weight. **(E)** GRIM-19 expression analyzed using Western blot analysis. **(F)** The relative optical density (OD) value for GRIM-19 according to Western blot analysis is shown in part E above.

GRIM-19 in the PQ-GRIM-19 group was significantly higher ($p < 0.05$) than that in the Mock and PQ-Scramble groups (Figure 1E and F). These results indicate that the overexpression of GRIM-19 can inhibit xenograft growth and enhance survival rates of tumor-bearing mice.

3.2. *GRIM-19 inhibits cell cycling, induces apoptosis, and inhibits metastasis of xenografts*

To study the mechanism by which GRIM-19 inhibits HCC growth, cell cycle, apoptosis, and metastasis were examined. Flow cytometric analysis indicated that the cells in PQ-the GRIM-19 group were mostly in the G0-G1 phase, whereas the proportion of cells in S the phase decreased significantly ($p < 0.05$) compared to that in the two control groups. These results suggest that GRIM-19 overexpression may suppress xenograft cell growth by arresting tumor cells in the G0-G1 phase (Figure 2A and B).

The cells were subjected to Annexin V-FITC/PI double staining and TUNEL staining to detect apoptosis. Results revealed that the rate of apoptosis in the PQ-GRIM-19 group was significantly higher ($p < 0.05$) than that in the control groups (Figure 2C, D and E).

Body weight was determined to evaluate ascites formation. There was no significant difference in weight in the Mock and PQ-Scramble groups, but weight was significantly lower in the PQ-GRIM-19 group ($p < 0.05$) compared to that in the two control groups (Figure 2F and G). Remarkably, abdominal tumor metastasis was observed in the Mock and PQ-Scramble groups (Figure 2H).

3.3. *GRIM-19 regulates expression of cell cycle-related proteins in xenografts*

To elucidate the mechanisms by which exogenous GRIM-19 mediated cell cycle arrest in transplantation tumor cells, Western blot analysis was used to examine the expression of cell cycle-related proteins (CDK4, PCNA and CyclinD1). Results indicated no significant difference in the expression of those proteins between the Mock and PQ-Scramble groups., CDK4, PCNA, and CyclinD1 expression were significantly down-regulated in the PQ-GRIM-19 group ($p < 0.05$) compared to levels in control cells (Figure 3A and B). These results were confirmed by immunohistochemical staining (Figure 3C), suggesting that the efficient induction of G0-G1 arrest by exogenous GRIM-19 over-expression was probably associated with the down-regulation of CDK4, PCNA, and CyclinD1 expression.

3.4. *GRIM-19 regulates apoptosis-related protein expression in xenografts*

To further explore the mechanism of cell apoptosis

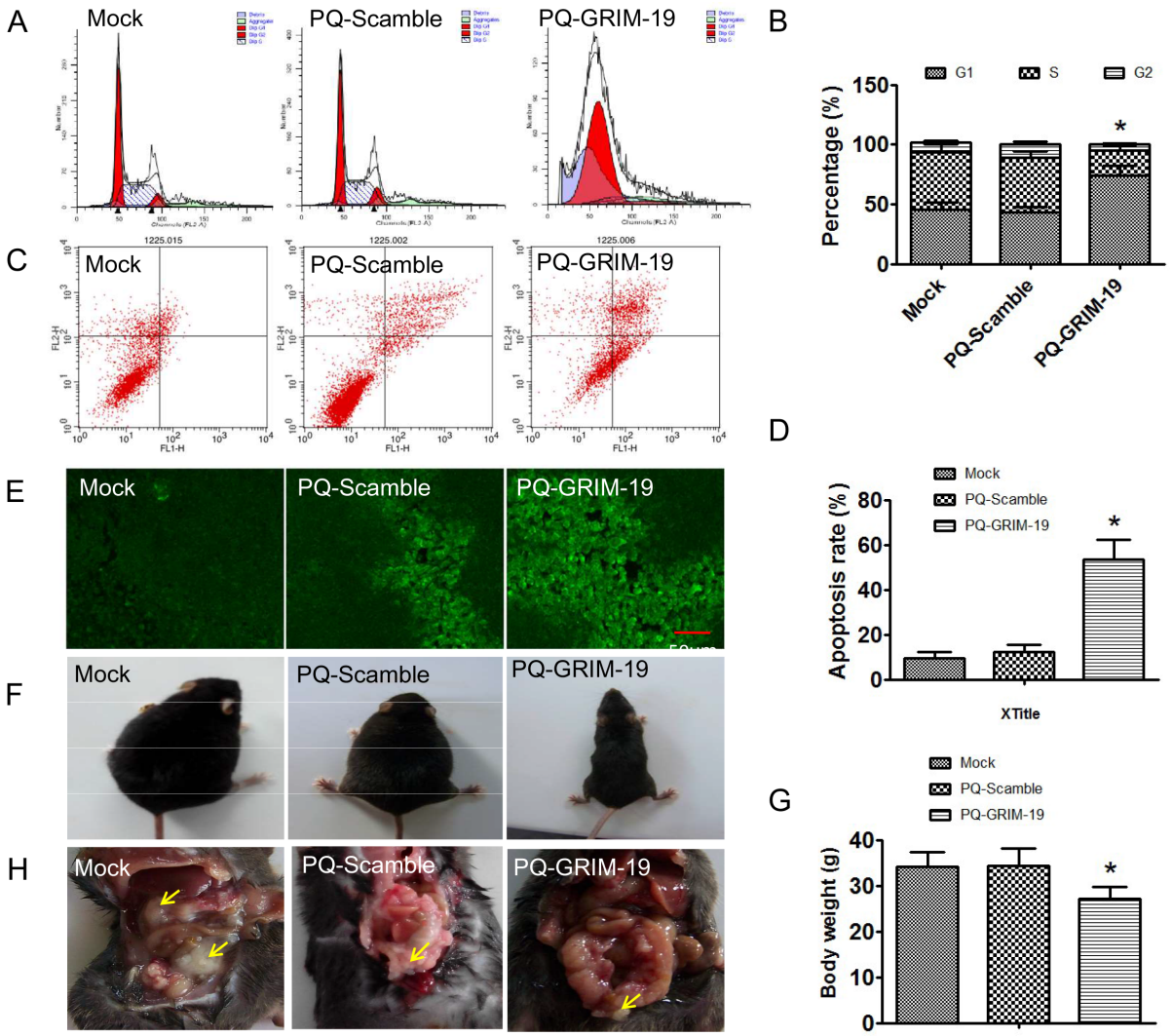

Figure 2. GRIM-19 overexpression induced G1/S arrest, enhanced apoptosis, and suppressed tumor metastasis. (A) FCM data with PI staining for cell cycle arrest. **(B)** Values represent the percentage of cells in each phase of the cell cycle. **(C)** Representative FCM data with Annexin-V-FITC and PI staining for cell apoptosis. **(D)** The average rate of cell apoptosis. **(E)** Analysis of late apoptosis in H22 xenografts with TUNEL fluorescent staining. **(F)** The appearance of mice, showing the extent of ascites formation. **(G)** Average body weight of mice. (H) The formation of abdominal metastases. Arrows indicate the location of tumors.

induced by exogenous GRIM-19, Bcl-2 family proteins Bax and Bcl-2 and caspase family proteins Caspase-3 and Caspase-9 were examined using Western blot analysis. Cells treated with PBS and the empty vector served as negative controls. As shown in Figure 4A and B, exogenous GRIM-19 significantly increased ($p < 0.05$) pro-apoptotic protein Bax expression and significantly down-regulated ($p < 0.05$) anti-apoptotic protein Bcl-2 expression in xenograft cells. Moreover, cleaved Caspase-3 and cleaved Caspase-9 were significantly were upregulated ($p < 0.05$) in the PQ-GRIM-19 group. In accordance with these results, the cells transfected with exogenous GRIM-19 tended to stain more positively for Bax, cleaved caspase-3, and cleaved caspase-9 and weaker for Bcl-2 compared to the negative controls (Figure 4C). These results indicate that tumor cell apoptosis induced by exogenous GRIM-19 was mediated by activation of the mitochondrial apoptosis pathway.

3.5. *GRIM-19 regulates metastasis-related protein expression in xenografts*

To study the molecular mechanisms for inhibition of tumor metastasis by GRIM-19 over-expression, VEGF, MMP9, and MMP2 proteins, which are involved in tumor metastasis, were monitored using Western blot analysis and immunohistochemical assays. As shown in Figure 5, these proteins were significantly down-regulated in the PQ-GRIM-19 group compared to levels in the control groups, revealing that GRIM-19 over-expression could inhibit tumor metastasis into the abdominal cavity by decreasing expression of VEGF, MMP9, and MMP2 proteins.

3.6. *GRIM-19 regulates the Stat3 signaling pathway*

To further elucidate the molecular mechanisms by

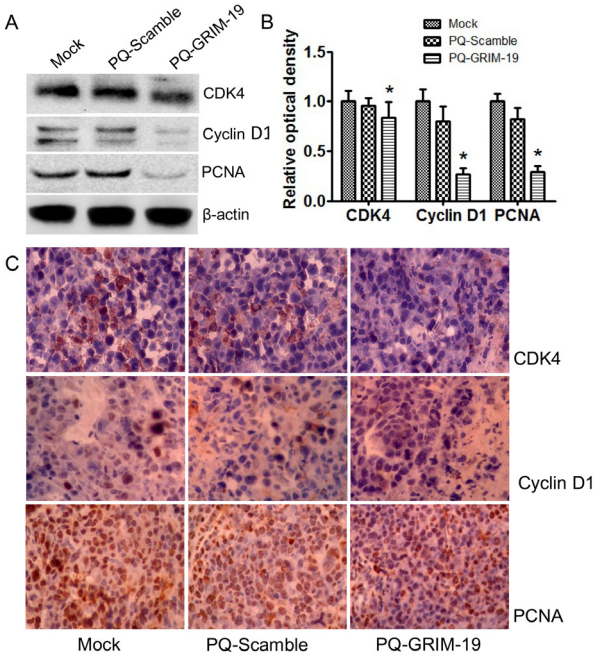

Figure 3. The effects of exogenous GRIM-19 on the expression of CyclinD1, CDK4, and PCNA. (A) Western blot assay for expression of CyclinD1, CDK4, and PCNA proteins. **(B)** Relative optical density (OD) value from Western blot analysis. **(C)** The xenograft tissues were immunostained for CyclinD1, CDK4, and PCNA with corresponding antibodies.

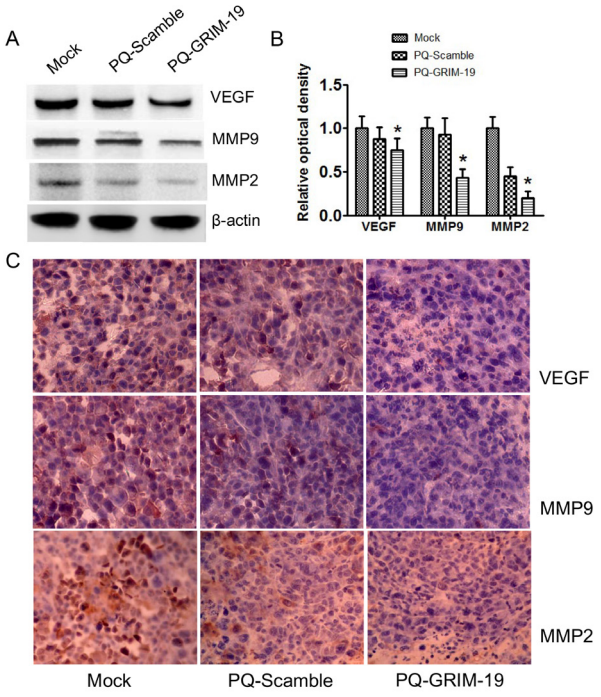

Figure 5. The expression of proteins associated with the inhibition of cell metastasis in xenografts. (A) Western blot assay for expression of VEGF, MMP-9, and MMP-2 proteins in xenografts. **(B)** Relative optical density (OD) value from each group according to Western blot analysis. **(C)** The same treated tumor xenograft tissues were immunostained for VEGF, MMP-9, and MMP-2 with corresponding antibodies.

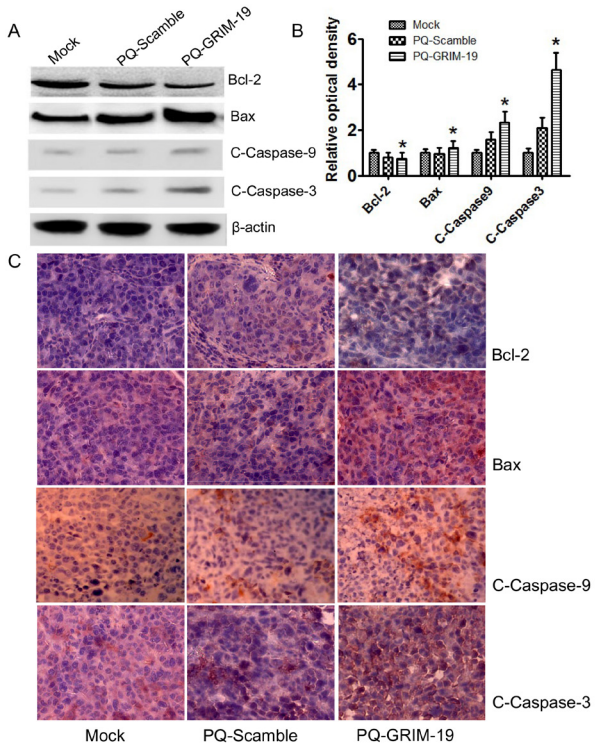

Figure 4. Exogenous GRIM-19 activates the mitochondrial apoptosis pathway. (A) Western blot assay for expression of Bax, Bcl-2, cleaved caspase-3 (C-Caspase-3), and cleaved caspase-9 (C-Caspase-9) proteins in xenografts. **(B)** Relative optical density (OD) values from Western blot analysis. **(C)** The xenograft tissues were immunostained for Bax, Bcl-2, C-Caspase-3, and C-Caspase-9 with corresponding antibodies.

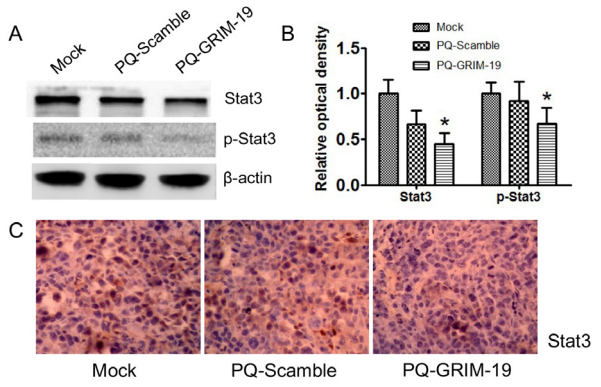

Figure 6. The expression of Stat3/ p-Stat3 proteins in xenografts. (A) Western blot analysis of Stat3/ p-Stat3. **(B)** Relative optical density (OD) value from Western blot analysis. **(C)** The xenograft tissues were immunostained for Stat3 with corresponding antibodies.

which GRIM-19 inhibits metastasis and induces apoptosis in HCC xenograft cells, this study investigated GRIM-19's effects on Stat3 protein expression and phosphorylation, which is a key process in this signaling pathway. As shown in Figure 6A and B, Western blot analyses indicated that exogenous GRIM-19 significantly decreased ($p < 0.05$) Stat3/p-Stat3 expression. Immunohistochemical assays revealed Stat3 down-regulation in cells treated with PQ-GRIM-19 compared to control cells (Figure 6C). These findings

suggest that GRIM-19 may restrict tumor cell growth and metastasis by negatively regulating the JAK/Stat signaling pathway.

4. Discussion

An increasing number of genes essential for normal tissue/organ growth and development have been identified as being involved in HCC (*17,18*). Studies have indicated that malignancies are closely related to genes that affect cellular differentiation, development, and senescence during normal growth. Tumorigenesis of various cancer types is reported to be closely associated with GRIM-19 gene mutation or dysfunction (*8,19,20*). A previous study by the current authors indicated that GRIM-19 expression was down-regulated in most HCC samples in contrast to adjacent non-tumor liver tissues and that over-expression of GRIM-19 suppressed tumor cell growth by inducing cell cycle arrest and promoting apoptosis *in vitro* (*13*). The current study created a mouse model of an orthotopically implanted hepatocarcinoma tumor to verify the effect on tumor suppression of GRIM-19 over-expression *in vivo* and to discuss its mechanism.

Unlike conventional chemotherapeutic drugs, gene-targeted therapies are theoretically more effective in tumor eradication, and less harmful to normal cells, because specific molecular mechanisms are involved (*21*). However, the "Achilles heel" of gene-targeted therapy is vector deficiency. *Salmonella typhimurium*, a type of facultative anaerobic Gram-negative bacteria, can selectively accumulate in solid tumors because of the existence of hypoxic areas within solid tumors (*22*). Attenuated Salmonella phoPphoQ was used in the current study as a GRIM-19-expressing plasmid carrier because of its potent tumor-targeting ability, low toxicity, and ability to efficiently express exogenous gene products (*23*). Several studies have indicated that GRIM-19 expressing plasmids in *phoPphoQ Salmonella* can target solid tumors and be released into implanted tumor cells to successfully over-express GRIM-19 protein.

This study used PQ-GRIM-19, and results indicated that over-expressed GRIM-19 resulted in an increase in the survival rate of mice with HCC and a decrease in tumor volume, ascites, and abdominal metastases, indicating that the growth of orthotopical xenografts was suppressed by over-expressed GRIM-19. These results mirrored those of a previous study by the current authors (*24*).

Although the mechanisms of the anti-tumor actions of GRIM-19 are not completely clear, researchers have often stressed that GRIM-19's function as a tumor inhibitor involves multiple factors. CDK4 and cyclin D1 are important factors during the cell cycle. GRIM-19 can induce G1/S arrest of cancer cells by down-regulating CDK4 and cyclinD1 (*5,10,25*). In the current study, the up-regulation of GRIM-19 causing the decrease in CDK4 and cyclinD1 might have led to a significant arrest of xenograft cells in G1/S, as previously described. PCNA, a nuclear protein localized at active replication sites, is reported to contribute to DNA replication and repair during the S phase (*26,27*). The current results implied that GRIM-19 overexpression efficiently prevents transition from the G1 to S phase by reducing CDK4 and CyclinD1 protein expression, ultimately leading to the decrease in PCNA *in vivo*. Those results were consistent with results when using SMMC-7721 cells *in vitro* (*13*). Accordingly, the hypothesis is that GRIM-19 can suppress the replication and repair of damaged DNA by inducing G1/S arrest, so GRIM-19 could help to limit uncontrolled cancer cell proliferation.

The mitochondria apoptosis pathway reportedly plays a key role in the process of apoptosis and served as a natural barrier to cancer development (*28*). Interestingly, GRIM-19 was originally identified as a nuclear protein affecting mitochondrial apoptosis in interferon/retinoic acid-induced tumor cell death (*11*). The participation of mitochondria in apoptosis was relevant to Bcl-2 family members that triggered apoptosis by regulating the permeability of mitochondrial membranes (*29*). The relative balance of various pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) Bcl-2 family members was a critical cellular homeostasis mechanism. Cleaved-caspase-9 and cleaved-caspase-3 can trigger the caspase cascade and process of apoptosis. The current study revealed that GRIM-19 overexpression promoted apoptosis by increasing the Bax/Bcl-2 ratio and cleaved caspase-3 and cleaved caspase-9. Analogous results have been observed in lung cancer and glioma (*12,30*), suggesting that GRIM-19 induces apoptosis *via* activation of the mitochondria apoptosis pathway.

In addition to suppressing growth, GRIM-19 inhibits the metastasis of hepatocarcinoma tumor cells. Several studies have claimed that down-regulation of GRIM-19 correlates with invasive features of breast cancer, glioma, and HCC (*7,12,31*). A study by the current authors indicated that treatment of tumor-bearing mice with PQ-GRIM-19 for 10 days led to an appreciable decrease in vascular endothelial growth factor (VEGF) and matrix-degrading enzyme metalloproteinase-2 (MMP-2) and MMP-9 activity, findings which were similar to those of a study of gastric cancer (*32*). MMP-9 and MMP-2 play crucial roles in regulating cancer cells invasion and metastasis (*33*). VEGF reportedly contributes to an increase in the invasive potential in colorectal cancer (34). A reduction in MMP-2 and VEGF could inhibit angiogenesis-mediated human hepatocellular metastasis (*35*). Thus, preventing upregulation of VEGF, MMP-2, and MMP-9 by PQ-GRIM-19 treatment might be a potential strategy to render HCC less invasive.

Several studies have indicated that signal transducers and activators of transcription 3 (Stat3), a key controller of the JAK/Stat signaling pathway, are involved in carcinogenesis by promoting cell proliferation, differentiation, and cell cycle progression, as well as by inhibiting apoptosis. Indeed, constitutive activation of Stat3 705 tyrosine phosphorylation and its up-regulation have been observed in diverse human tumors, including gliomas, as well as in prostate and thyroid cancers (*12,36*). Recent studies have indicated that Stat3 activation can accelerate tumor progression and metastasis, prolong cell survival, and inhibit cell apoptosis by incessantly regulating its downstream target genes, such as Bcl-2, Bax, Cyclin D1, MMP-2, and MMP-9 (*36*). Importantly, a previous study indicated that GRIM-19 was suppressed in HCC tissues, with a corresponding increase in p-Tyr(705)-Stat3 (p-Stat3) activity (*6*). These results might indicate a delicate relationship between GRIM-19 deactivation and Stat3 activation. GRIM-19 might regulate Stat3 and p-Stat3 expression and then mediate cell cycle progression, cell apoptosis, and cell migration. To verify this contention and to further investigate the possible mechanisms by which GRIM-19 inhibits growth, GRIM-19's effects on Stat3/p-Stat3 protein in xenograft tissues were examined. The data in this study suggest that exogenous GRIM-19 down-regulated Stat3 expression and phosphorylation, implying that GRIM-19 might inversely regulate Stat3 signaling in HCC.

In summary, a mouse model of orthotopically implanted HCC tumors has demonstrated that GRIM-19 acts as a regulator that induces G1/S arrest, suppresses tumor cell migration, and promotes tumor apoptosis *in vivo*. Furthermore, evidence indicates that GRIM-19 inhibits Stat3 expression and phosphorylation, which apparently deactivate the Stat3 signaling pathway and then inhibit tumor growth. The mechanism by which GRIM-19 affects Stat3 expression and phosphorylation remains undetermined. GRIM-19 apparently affects hepatocarcinogenesis at multiple levels and could be a potential therapy for HCC. Administration of GRIM-19 carried by attenuated *S. Typhimurium* could be a novel and targeted strategy with which to treat hepatocellular cancer.

Acknowledgements

This study was support by the Zhejiang Medical and Health Science and Technology Project (grant no. 2018254697).

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(Received July 21, 2019; Revised August 30, 2019; Accepted August 31, 2019)

Original Article

DOI: 10.5582/bst.2019.01212

The combination of the preoperative albumin-bilirubin grade and the fibrosis-4 index predicts the prognosis of patients with hepatocellular carcinoma after liver resection

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Summary **There is little information regarding the use of a combination of the albumin-bilirubin (ALBI) grade and the fibrosis-4 index (FIB-4) in predicting hepatocellular carcinoma (HCC) patient outcomes after liver resection. In this study, we aimed to analyze the predictive ability of a combination of the ALBI grade and the FIB-4 score (ALBI-FIB-4) for HCC patients within the Milan criteria after liver resection. The data of HCC patients within the Milan criteria who underwent liver resection between 2011 and 2019 at our center were reviewed (n = 544). Patients with an FIB-4 index > 3.25 were considered to have a high FIB-4 index and were given a score of 1, whereas patients with an FIB-4 index ≤ 3.25 were considered to have a low FIB-4 index and were given a score of 0. The ALBI-FIB-4 score was a summary score that combined the ALBI grade and the score based on the FIB-4 index. During the follow-up period, 279 patients experienced recurrence, and 175 patients died. Multivariate analysis showed that tumor size, the presence of multiple tumors, the presence of microvascular invasion and the ALBI-FIB-4 score were four independent risk factors for both postoperative recurrence-free survival (RFS) and overall survival (OS). The 5-year RFS of patients with high ALBI-FIB-4 scores of 1, 2, and 3 were 55.0%, 44.2% and 35.3%, respectively (p = 0.004). The 5-year OS rates of patients with high ALBI-FIB-4 scores of 1, 2, and 3 were 72.9%, 66.4% and 54.8%, respectively (p = 0.011). The ALBI-FIB-4 score may be a surrogate marker for predicting the prognosis of patients with HCC after liver resection. A high ALBI-FIB-4 score was associated with a high incidence of postoperative recurrence and mortality.**

Keywords: Hepatocellular carcinoma, albumin-bilirubin grade, fibrosis-4 index

1. Introduction

Because of the rapidly increasing incidence of hepatocellular carcinoma (HCC), HCC has become the second leading cause of cancer-related death worldwide (*1*). Due to the high prevalence of hepatitis B virus (HBV) infection, more than half of new cases and deaths occurred in China (*2*). Liver resection is one of the methods of curative management for patients with early stage HCC and compensated liver function. However, a high incidence of postoperative recurrence has greatly limited the long-term survival of patients with HCC after liver resection (*3*).

HCC patients are often affected by various degrees of liver fibrosis (*4*). Previous studies have confirmed that severe liver fibrosis or cirrhosis could result in serious postoperative complications, such as postoperative liver failure and poor long-term survival for patients with HCC (*5,6*). Liver biopsy is the standard method to evaluate the degree of liver fibrosis. However, liver biopsy is an invasive management technique that can cause severe complications, such as intra-abdominal bleeding (*7*). Some noninvasive methods based on laboratory measurements have been

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proposed to assess the severity of liver fibrosis (*8,9*). The fibrosis-4 index (FIB-4) is a noninvasive method used to assess the severity of fibrosis in patients that considers four parameters: age, platelet counts, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (*10*). Some investigators have suggested that FIB-4 can predict the outcomes of liver resection (*5*).

In addition to liver fibrosis, liver function also greatly impacts HCC patient outcomes after liver resection. Poor liver function can cause high postoperative morbidity and shorter long-term survival (*11*). Although FIB-4 was recognized for assessing liver fibrosis, it was not able to assess liver function in patients. Recently, the albumin-bilirubin (ALBI) grade was proposed for assessing liver function in patients instead of the conventional Child-Pugh score (*12*). Unlike the Child-Pugh score, the ALBI grade only includes two objective parameters, serum albumin and total bilirubin (*12*). Whether a combination of the FIB-4 score and the ALBI grade could better predict the prognosis of HCC patients after liver resection is not well established. In this study, we tried to clarify this issue.

2. Materials and Methods

We retrospectively reviewed data from patients with HCC within the Milan criteria who underwent liver resection between 2011 and 2019 at our center. The exclusion criteria included reresection, ruptured HCC, receipt of preoperative antitumor treatment, a positive surgical margin, and the presence of other types of tumors. The diagnosis of HCC was confirmed based on the histological examination of resected specimens. This study was approved by the ethics committee of West China Hospital.

2.1. *Follow-up*

All preoperative blood tests were performed one week before the operation. After discharge, patients were followed up regularly every 3 months. Before and after surgery, antiviral drugs (entecavir, lamivudine or tenofovir) were conventionally administered to patients with a positive hepatitis B virus-DNA (HBV-DNA) load. Follow-up assessments included blood cell tests, hepatic function tests, serum measurements of alpha-fetoprotein (AFP), and HBV-DNA tests. Visceral ultrasonography, as well as computed tomography or magnetic resonance imaging and chest radiography, were performed for all patients during each follow-up. Bone scintigraphy was performed whenever HCC recurrence was suspected. Postoperative recurrence was defined as positive imaging findings compared to those obtained during the preoperative examination or was confirmed by biopsy or resection.

2.2. *Definitions*

The FIB-4 index was calculated as the AST (IU/L) × age (years)/[platelet count (10^9/L) × ALT (IU/L)$^{1/2}$]. The ALBI score = (log10 bilirubin ×0.66) + (albumin × −0.085). ALBI grades were defined as grade 1 (score ≤ –2.60), grade 2 (score > –2.60 and ≤ –1.39), and grade 3 (score > –1.39). Preoperative AFP greater than 400 ng/mL was considered a high preoperative AFP level (*3*). An FIB-4 index > 3.25 (cirrhosis) was considered a high FIB-4 index, which was defined as a score of 1 (*8*). An FIB-4 index ≤ 3.25 (noncirrhosis) was considered a low FIB-4 index, which was defined as a score of 0 (*8*). The combined ALBI and FIB-4 score (ALBI-FIB-4) was defined as the combination of the scores of the ALBI grade and the FIB-4 score and ranged from 1 to 4. The neutrophil-to-lymphocyte ratio (NLR) was defined as the absolute neutrophil count divided by the lymphocyte count (*13*). The platelet-to-lymphocyte ratio (PLR) was defined as the platelet count divided by the lymphocyte count (*13*).

2.3. *Statistical analysis*

All statistical analyses were performed using SPSS 21.0 (SPSS Company, Chicago, IL) for Windows. All continuous variables were compared using one-way analysis of variance. Categorical variables were compared using the χ^2 test or Fisher's exact test. Recurrence-free survival (RFS) and overall survival (OS) were determined using the Kaplan-Meier method, and comparisons were made using the log rank test. A Cox proportional hazard regression model was generated to evaluate hazard ratios for risk factors OS and RFS. All variables found to be significant ($p < 0.05$) by univariate analysis were included in the multivariate analysis. A p-value less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. *The clinicopathological characteristics*

The clinicopathological data of the 544 patients are shown in Table 1. The mean age was 52.0 ± 11.7 years in the current study, and the patients were predominantly male. Multiple tumors were observed in 43 patients. Microvascular invasion was detected in 74 patients. High preoperative AFP levels were observed in 154 patients. Positive HBV-DNA was detected in 262 patients. In the current study, no patients with an ALBI grade of 3 were observed. A total of 411 patients had an ALBI grade of 1, whereas 133 patients had an ALBI grade of 2. According to the FIB-4 index, 233 patients had a high FIB-4 index, whereas 311 patients had a low FIB-4 index. In the present study, the number of patients who had ALBI-FIB-4 scores of 1, 2, and 3 were

Table 1. Clinicopathological characteristics of present study

Variables	No./mean ± SD
Age (years)	52.0 ± 11.7
Female/male	81/463
Multiple tumors (yea/no)	43/501
Presence of MVI (yea/no)	74/470
Differentiation (well/moderate/poor)	18/373/153
Tumor size (cm)	3.4 ± 1.1
Positive HBV-DNA load (yea/no)	262/282
Preoperative AFP > 400ng/mL (yea/no)	154/390
Preoperative platelet counts (109/L)	116 ± 50
NLR	2.2 ± 1.4
PLR	81.6 ± 37.5
FIB-4 index > 3.25 / ≤ 3.25	233/311
ALBI grade 1/2	411/133
ALBI-FIB-4 score 1/2/3	261/199/84

ALBI, albumin-bilirubin; AFP, alpha-fetoprotein; FIB-4, fibrosis index 4; HBV, hepatitis B virus; MVI, microvascular invasion; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio, SD, standard deviation.

261, 199, and 84, respectively. There were no patients with an ALBI-FIB-4 score of 4. Based on a median follow-up of 52.5 months, 279 patients experienced recurrence, and 175 patients died. As shown in Figure 1, the 1-, 3-, and 5-year RFS rates (Figure 1A) were 83.1%, 61.8% and 47.9%, respectively, whereas the 1-, 3-, and 5-year OS rates (Figure 1B) were 97.4%, 83.4% and 67.6%, respectively.

3.2. *Univariate and multivariate analyses for RFS*

Table 2 lists the results of the univariate and multivariate analyses of the predictors of postoperative RFS. Univariate analysis revealed that tumor size, preoperative platelet counts, ALBI-FIB-4 score, presence of MVI, multiple tumors, FIB-4 score, and ALBI grade were significant. However, in the multivariate analysis, only four independent risk factors were identified: tumor size

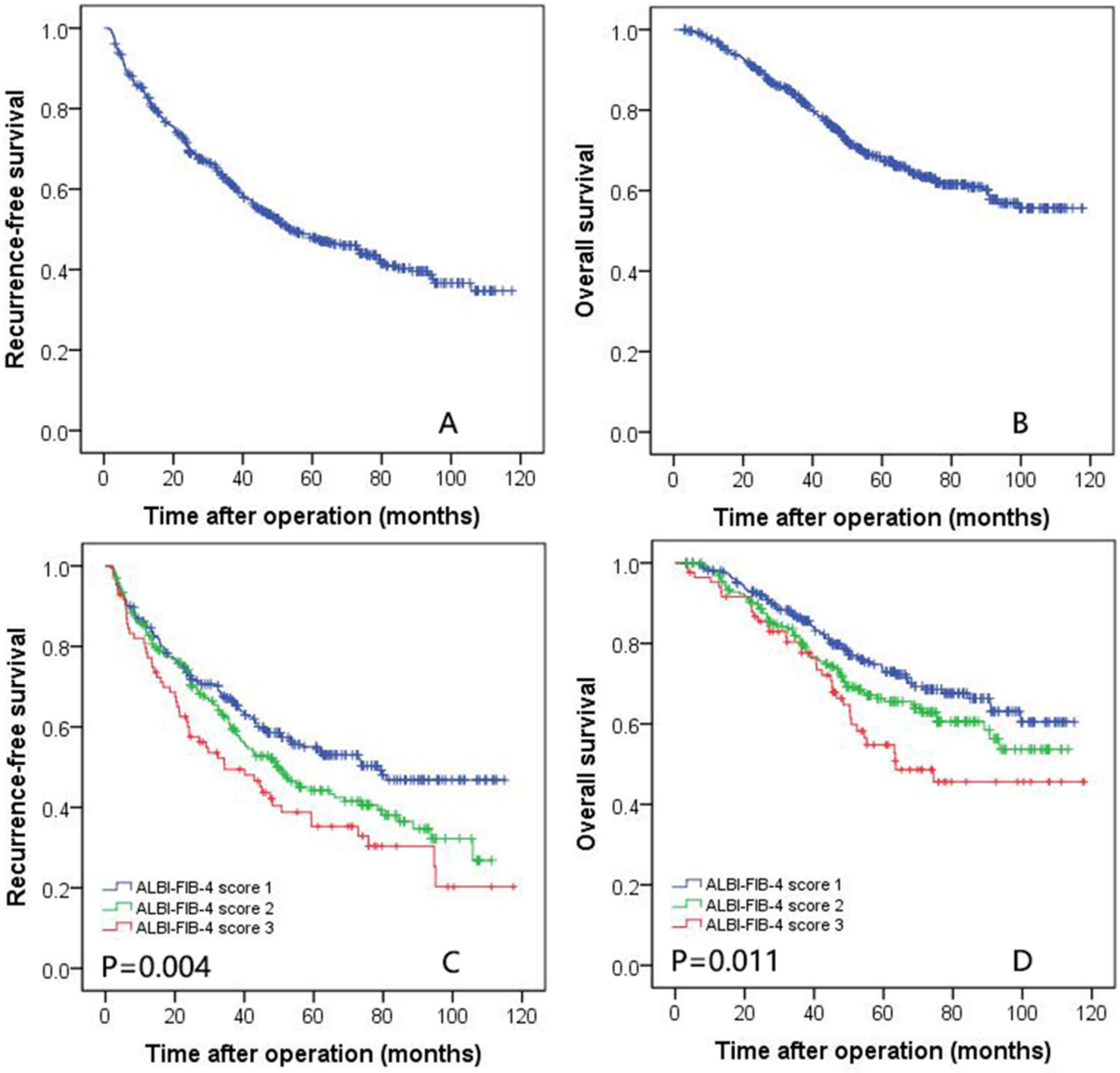

Figure 1. The recurrence-free survival (A) and overall survival (B) curves of the current study. Comparison of the recurrence-free survival (C) and overall survival (D) in patients with different ALBI-FIB-4 scores.

(HR = 1.296, 95% CI = 1.154-1.457, $p < 0.001$), multiple tumors (HR = 2.673, 95% CI = 1.801-3.976, $p < 0.001$), ALBI-FIB-4 score (HR = 1.278, 95% CI = 1.090-1.498, $p = 0.003$), and the presence of MVI (HR = 1.681, 95% CI = 1.225-2.307, $p = 0.001$).

As displayed in Table 3, univariate analysis identified several factors associated with poor OS: tumor size, preoperative platelet counts, ALBI-FIB-4 score, FIB-4 score, positive HBV-DNA, and the presence of MVI. However, the multivariate analysis suggested that only tumor size (HR = 1.386, 95% CI = 1.196-1.607, $p < 0.001$), presence of MVI (HR = 2.090, 95% CI = 1.449-3.015, $p < 0.001$), multiple tumors (HR = 2.436, 95% CI = 1.505-3.943) and ALBI-FIB-4 score (HR = 1.335, 95% CI = 1.091-1.633, $p = 0.005$) were independent risk factors associated with postoperative mortality.

3.3. *Comparison of postoperative RFS and OS in patients with different ALBI-FIB-4 scores*

As shown in Figure 1C, the 1-, 3-, and 5-year RFS rates were 85.0%, 67.1%, and 55.0% for patients with an ALBI-FIB-4 score of 1, 83.2%, 60.1%, and 44.2% for patients with an ALBI-FIB-4 score of 2 and 77.2%, 49.4%, and 35.3% for patients with an ALBI-FIB-4 score of 3, respectively. A significant difference was observed (Figure 1C; $p = 0.004$). As shown in Figure 1D, the 1-, 3-, and 5-year OS rates were 98.1%, 86.0%, and 72.9% for patients with an ALBI-FIB-4 score of 1, 97.4%, 81.4%, and 66.4% for patients with an ALBI-FIB-4 score of 2 and 95.2%, 80.3%, and 54.8% for patients with an ALBI-FIB-4 score of 3, respectively (Figure 1D; $p = 0.011$).

4. Discussion

Previous studies suggested that the severity of fibrosis in patients was associated with both postoperative morbidity and mortality (*6,14*). The FIB-4 index, as a

Table 2. Factors associated with postoperative recurrence-free survival

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*	HR	95% CI	*p*
Age (years)	1.005	0.995-1.016	0.319			
Tumor size (cm)	1.257	1.122-1.407	< 0.001	1.296	1.154-1.457	< 0.001
Multiple tumors (yes/no)	2.131	1.457-3.117	< 0.001	2.673	1.801-3.967	< 0.001
Female/male	1.390	0.975-1.981	0.069			
AFP > 400ng/mL (yes/no)	1.096	0.847-1.416	0.486			
Positive HBV-DNA load (yes/no)	1.203	0.951-1.521	0.123			
Differentiation (poor/moerate/well)	0.926	0.735-1.165	0.511			
MVI (yes/no)	1.979	1.313-2.461	< 0.001	1.681	1.225-2.307	0.001
Platelet counts (10^9/L)	0.997	0.995-1.000	0.032			
PLR	0.999	0.996-1.002	0.624			
NLR	1.016	0.942-1.096	0.675			
ALBI grade	1.359	1.049-1.761	0.020			
FIB-4 index	1.387	1.097-1.755	0.006			
ALBI-FIB-4 score	1.304	1.114-1.525	0.001	1.278	1.090-1.498	0.003

ALBI, albumin-bilirubin; AFP, alpha-fetoprotein; FIB-4, fibrosis index 4; HBV, hepatitis B virus; HR, hazard ratio; MVI, microvascular invasion; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio.

Table 3. Factors associated with postoperative overall survival

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*	HR	95% CI	*p*
Age (years)	1.004	0.991-1.017	0.557			
Tumor size (cm)	1.379	1.194-1.594	< 0.001	1.386	1.196-1.607	< 0.001
Multiple tumors (yes/no)	1.856	1.164-2.959	0.009	2.436	1.505-3.943	< 0.001
Female/male	1.356	0.859-2.139	0.191			
AFP > 400ng/mL (yes/no)	1.253	0.916-1.715	0.158			
Positive HBV-DNA load (yes/no)	1.197	0.889-1.612	0.236			
Differentiation (poor/moerate/well)	0.777	0.581-1.040	0.090			
MVI (yes/no)	2.332	1.625-3.346	< 0.001	2.090	1.449-3.015	< 0.001
Platelet counts (10^9/L)	0.997	0.994-1.000	0.057			
PLR	1.001	0.997-1.005	0.534			
NLR	1.043	0.964-1.127	0.295			
ALBI grade	1.268	0.913-1.761	0.156			
FIB-4 index	1.563	1.162-2.103	0.003			
ALBI-FIB-4 score	1.345	1.105-1.637	0.003	1.335	1.091-1.633	0.005

ALBI, albumin-bilirubin; AFP, alpha-fetoprotein; FIB-4, fibrosis index 4; HBV, hepatitis B virus; HR, hazard ratio; MVI, microvascular invasion; NLR, neutrophil to lymphocyte ratio; PLR, platelet to lymphocyte ratio.

noninvasive marker that could assess the severity of fibrosis, was proven to predict the prognosis of HCC patients after liver resection (*5*). However, the FIB-4 index cannot predict the liver function of patients with HCC. Our study confirmed that the combination of the FIB-4 index and the ALBI score could better predict the outcomes of patients with HCC after liver resection.

In 2015, Johnson *et al*. (*12*) proposed the use of the ALBI grade to assess patient liver function. Compared to the conventional liver function method, the Child-Pugh score, ALBI only included total bilirubin and serum albumin as the two objective factors. However, the Child-Pugh score includes ascites and hepatic encephalopathy, which are two subjective parameters. Subsequently, many investigations confirmed that the ALBI grade may be better than the Child-Pugh score for evaluating patient liver function (*15,16*). A number of studies also revealed that the ALBI grade could predict the prognosis of patients with HCC after liver resection, radiofrequency ablation, liver transplantation, transarterial chemoembolization and other treatments (*17-21*). A large, multicenter study performed by Pinato *et al*. (*22*) confirmed that the ALBI grade showed predictive value for patients with HCC in several Barcelona Clinic Liver Cancer system stages. Studies have even suggested that when the ALBI grade was used instead of the Child-Pugh score, the ALBI-based Barcelona Clinic Liver Cancer staging system and the ALBI-based Japanese Integrated Staging system showed similar or better prognostic prediction power than the Child-Pugh-based Barcelona Clinic Liver Cancer staging system and the Child-Pugh-based Japanese Integrated Staging system (*16,23*). Moreover, poor liver function contributed to a high incidence of postoperative complications, and shorter long-term survival was also found in many studies (*11,24*).

Another explanation for the relationship between a high ALBI grade and a high postoperative recurrence rate is the anti-cancer effect of albumin. Low serum albumin levels may cause a high ALBI grade. Carr *et al*. (*25*) suggested that patients with low serum albumin had larger tumor sizes, higher AFP levels, more tumors and increased portal vein tumor thrombosis. Carr *et al*. (*25*) suggested that decreased serum albumin levels may contribute to the aggressiveness of HCC. A basic research study suggested that albumin plays a direct role in inhibiting the growth of HCC (*26*).

Previous studies have suggested that liver fibrosis is associated with HCC patient outcomes after liver resection. As a noninvasive marker of liver fibrosis, the preoperative FIB-4 index was shown to predict postoperative complications and recurrence in patients with HCC after liver resection (*5*). Previous studies have confirmed a good correlation between FIB-4 index and degree of fibrosis using specimens from liver biopsies (*27*). The FIB-4 index included four parameters: age, ALT, AST and platelet count. These four parameters are also associated with HCC patient outcomes. For example, a national survey confirmed that elderly patients suffered from significantly worse long-term survival than middle-age and young patients (*28*). Kaneko *et al*. (*29*) confirmed that a low preoperative platelet count was associated with high mortality after liver resection. AST and ALT are sensitive and reliable biochemical markers of liver inflammation. A study performed by Zhou *et al* revealed that both AST and ALT could predict early recurrence and OS in patients with HCC following liver resection (*30*).

Our study confirmed that the ALBI-FIB-4 score was better than both the ALBI grade and the FIB-4 index alone in predicting postoperative recurrence and mortality in HCC patients after liver resection. We acknowledge that the ALBI grade can accurately reflect liver function. However, some other situations may also result in a high ALBI grade, such as malnutrition. In this situation, patients may not have severe liver fibrosis. Similarly, some patients with a high FIB-4 score may have good liver function. Accordingly, the ALBI-FIB-4 score may more accurately reflect the patient's status than ALBI grade or FIB-4 index alone. Liao *et al*. (*31*) also suggested the combination of ALBI and FIB-4 could predict postoperative recurrence of patients with HCC after liver resection. Compared with Liao *et al*'s study, our study only included HCC patients within Milan criteria (*31*). Like other centers, patients with HCC beyond Milan criteria may receive some treatments after liver resection. We believe, these treatments may also impact the outcomes of HCC patients after liver resection. Moreover, our study confirmed a combination of ALBI and FIB-4 may not only predict the RFS but also the OS of patients with HCC after liver resection.

There are also some limitations in this study. This is a single center respective study. Additional studies are needed to validate its conclusions. Moreover, in China, more than 90% of HCC cases are HBV-related HCC (*32*). Whether our conclusions pertain to other causes of HCC requires further study.

In conclusion, our study suggested that a combination of ALBI grade and FIB-4 index may better predict the prognosis of patients with HCC after liver resection. Patients with a high ALBI-FIB-4 score may have a higher incidence of postoperative recurrence and poorer long-term survival.

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(Received August 10, 2019; Revised August 21, 2019; Accepted August 31, 2019)

Communication

DOI: 10.5582/bst.2019.01240

China should take more measures to raise its breastfeeding rate

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Summary **A growing number of studies show that breastfeeding is the best source of nutrition for infants and young children and one of the most effective measures to ensure the healthy growth and development of children. However, a study from the China Development Research Fund found that the rate of exclusive breastfeeding among Chinese infants within six months of birth is only 29%, a rate that is lower than that in other countries and regions of the world. Other data provided by the World Bank shows that the rate of exclusive breastfeeding in China declined from 1998 to 2013 and that it increased slightly by 2018, but it was still lower than the global average. Compared to other Asian countries, the exclusive breastfeeding rate in China is also low. Establishing a sound breastfeeding support system and creating a breastfeeding environment with full support for mothers of infants is a way to effectively improve the state of breastfeeding in China, to ensure the healthy growth of children, and to achieve the goals of Healthy China 2030.**

Keywords: Exclusive breastfeeding, infants within six months of birth, rate, China

Children's nutrition and health problems are an increasing concern for the entire world. Improving the health status and nutrition of children has become an important basis for the promotion of all-round human development, the improvement of quality human capital, and sustainable economic and social development. A growing body of research shows that the period from birth to age 2 is critical for determining a child's lifetime nutrition and health status (*1*). Breastfeeding is the best source of nutrition for infants and young children during this period and is one of the most effective measures to ensure the healthy growth and development of children (*2*).

The benefits of breastfeeding are self-evident. It is widely recognized as the most effective intervention to improve health status by preventing diseases for both infants and mothers (*3-5*). A study has pointed out that breastfeeding has positive effects not only for the growth and development of infants but also for nursing women (*6*) . Breastfeeding can prevent breast cancer, shorten the birth interval, and possibly prevent ovarian cancer and type 2 diabetes. Because of its benefits, the World Health Organization (WHO) advocates and recommends exclusive breastfeeding for the first six months of life (*7*). According to the WHO, exclusive breastfeeding means that the baby is not fed water, liquid, or food other than breast milk for 6 months after birth. After 6 months, breast milk can be supplemented with solid food, and breastfeeding can be continued until the age of 2 years or until desired. In 2012, the World Health Assembly proposed that 50% of babies aged 0 to 6 months should be exclusively breastfed by 2025. In 2017, China (*8*) proposed reaching the same goal by 2020.

However, the exclusive breastfeeding in China still remains rather low (*9,10*). According to a survey on factors affecting breastfeeding in China (*11*) by the China Development Research Foundation, the rate of exclusive breastfeeding among Chinese infants within six months of birth is 29%, which is lower than in other countries around the world (Figure 1). Other data provided by the World Bank shows that the rate of exclusive breastfeeding in China declined from 1998 to 2013 and increased slightly by 2018 (Figure 2), but it was still lower than the global average. Compared to other Asian countries, the exclusive breastfeeding rate in China is also low. South Asia had seen the fastest rise in the exclusive breastfeeding rate globally since 2000. Between 2000 and 2015, the exclusive breastfeeding rates in South Asia increased by 17% from 47% to 64%

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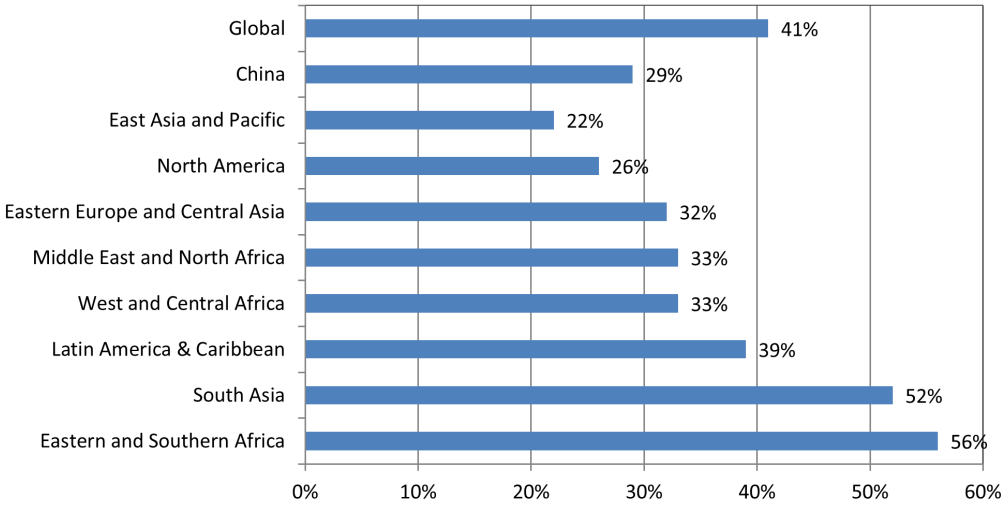

Figure 1. Exclusive breastfeeding rates worldwide in 2018. Data source: United Nations Children's Fund, *https://data.unicef.org/topic/nutrition/infant-and-young-child-feeding/*

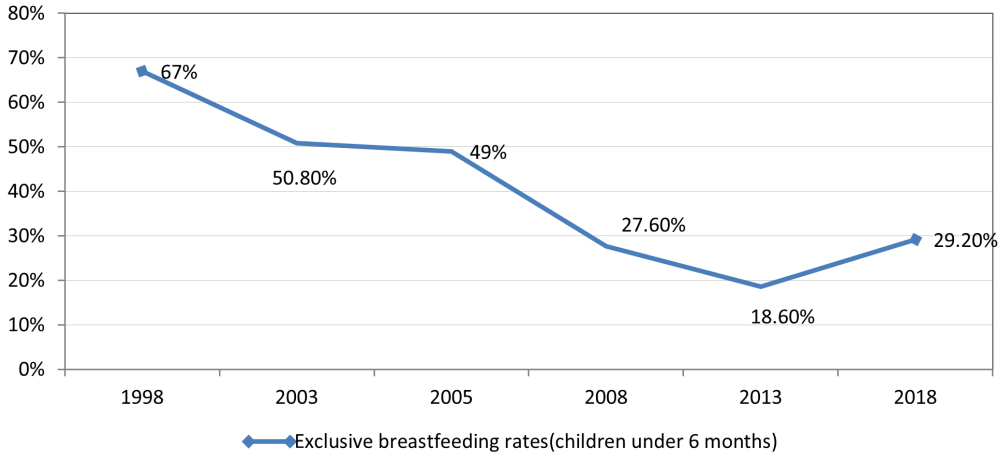

Figure 2. Exclusive breastfeeding rates in China from 1998 to 2018. Data source: The World Bank, *https://data.worldbank.org/indicator/SH.STA.BFED.ZS?end=2018&most_recent_year_desc=true&start=1986&view=map*

(*1*). The exclusive breastfeeding rate in India reached 64.5% in 2013, up 18.5% from 2005. Bangladesh's exclusive breastfeeding rate increased from 37.4% in 2006 to 55.3% in 2014 (*12*).

According to the literature, there are several possible reasons for the low rate of exclusive breastfeeding in China. First, Zhang *et al.* (*13*) conducted a survey in China and found that the maternal perception that one has an "insufficient supply of breast milk" may be the main reason for introducing infant formula before 6 months postpartum. Second, some researchers have pointed out that many mothers have limited awareness of the importance of breastfeeding, especially in their understanding of the benefits of the exclusive breastfeeding in the first 6 months after birth (*14*).

To resolve this situation, relevant laws, regulations, and policies in China need to be drafted and implemented. Providing breastfeeding-related health education to fertile women is vital to raising the breastfeeding rate in China. In addition, the resources and backing of the government, medical and health care facilities, companies, communities, and families need to be mobilized to establish a multi-faceted breastfeeding support system with the participation of the entire society, and a breast-feeding environment and support system with full support for mothers of infants needs to be provided to promote and support breastfeeding.

All in all, improving the state of breastfeeding in China is not just the responsibility of parents and families but also of the country and the entire society. Establishing a sound breastfeeding support system and creating a breastfeeding environment with full support for mothers of infants isa way to effectively improve the state of breastfeeding in China, to ensure the healthy growth of children, and to achieve the goals of Healthy China 2030.

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(Received August 15, 2019; Revised August 27, 2019; Accepted August 31, 2019)

Letter

DOI: 10.5582/bst.2019.01221

Difference in distribution of malignant melanoma and melanocytic nevus in the palm and finger

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Summary **We conducted a study to try to plot the lesions of melanocytic nevus and malignant melanoma on the palm and fingers, and compared them to identify the different distribution pattern of both lesions. Data on 8 patients with melanomas (4 male and 4 female) and 26 patients with melanocytic nevus (6 male and 20 female) of palm and finger pulp who visited Wakayama Medical University Hospital between 1986 and 2018 was retrospectively collected. We found that all of the 8 lesions of melanoma were located on the finger pulps and distal to the 'distal transverse crease' of the palm, and that melanomas were not present proximal to the transverse crease. On the other hand, melanocytic nevus was present in the proximal area to the distal transverse crease of the palm more frequently than melanomas (50.0% *vs*. 0%), and there was statistically significant difference (p = 0.011 by Fisher's exact probability test). From these observations, our findings may reveal the contribution of mechanical stress to the cause of palmar melanoma, and may facilitate clinical differentiation between malignant melanoma and melanocytic nevus by the localization. Further studies with increased number of patients are needed to validate the finding.**

Keywords: Acral lentiginous melanoma, palmar melanoma, nevus cell nevus

It is important to investigate the causes, risk factors, or mechanism of tumorigenesis in malignant melanoma. Ultraviolet rays are clearly involved in the development of melanoma via the mutagenesis of BRAF or NRAS gene. However, melanoma also occurs in the unexposed area to ultraviolet rays including the palm and sole.

Acral lentiginous melanoma (ALM), lesions occurring on the extremities, was firstly defined by Reed (*1*). ALM is the most common form of melanoma in Japanese patients, and accounts for approximately 50% of all melanoma cases (*2*). Among ALM, the lesions on foot sole is most frequent, followed by the lower nail lesions (*3*).

On the other hand, the occurrence frequency of malignant melanoma in the palm is relatively low (10%) (*4*), thus suggesting that cases with palmer ALM are rare, and that there may be different etiology between palmar melanoma and planter melanoma. Although characteristic genetic alterations are detected in melanomas according to their involved areas, BRAF or NRAS are not frequently found in ALM, and no previous studies have reported characteristic genetic alteration of palmar melanoma. Accordingly, we supposed that factors other than genetic abnormality were involved in the etiology of palmar ALM.

In addition, as far as we searched, there has been no report describing the predilection sites of melanocytic nevus. In general, Unna melanocytic nevus tends to occur in the trunk whereas Miescher nevus is known to be found in the face. In this study, we tried to plot the lesions of melanocytic nevus and malignant melanoma on the palm and fingers, and compared them to identify the different distribution pattern.

We retrospectivity collected data on 8 patients with melanomas (4 male and 4 female; 2 patients in the palm and 6 patients in the finger pulp) and 26 patients with melanocytic nevus (6 male and 20 female; 16

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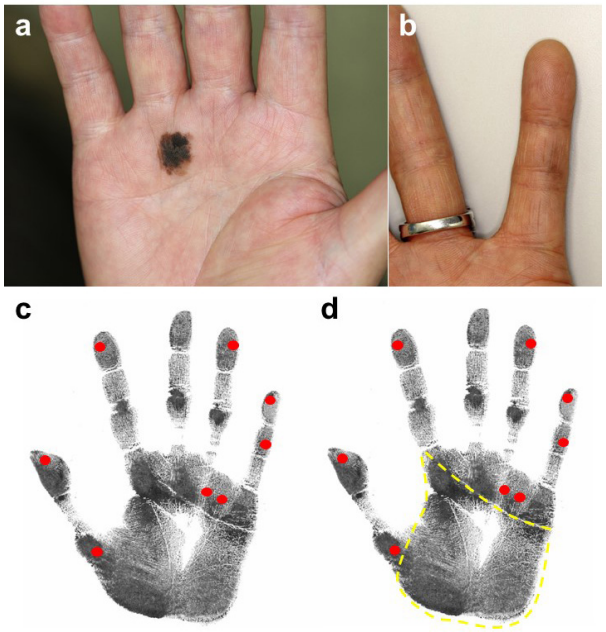

Figure 1. Distribution of melanoma on the palm and fingers. (**a** and **b**) Representative clinical images of patients with melanoma on the palm (**a**: 64 year-old male) and finger pulp (**b**: 59-year-old female). (**c** and **d**) The center of each lesion of 8 melanoma is plotted in red. In (**d**), proximal to the distal transverse crease of the palm was surrounded by yellow dotted line.

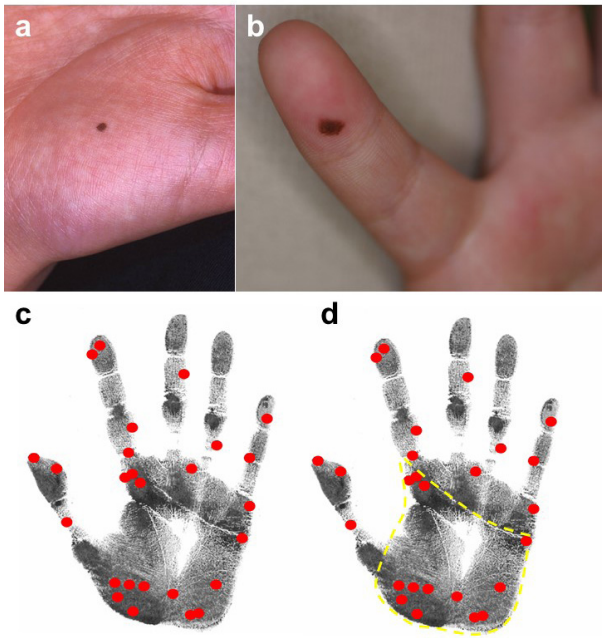

Figure 2. Distribution of nevus on the palm and fingers. (**a** and **b**) Representative clinical images of patients with nevus on the palm (**a**: 9 year-old male) and finger pulp (**b**: 5 year-old female). (**c** and **d**) The center of each lesion of 26 nevus is plotted in red. In (**d**), proximal to the distal transverse crease of the palm was surrounded by yellow dotted line.

patients in the palm and 10 patients in the finger pulp) of palm and finger pulp who visited Wakayama Medical University Hospital between 1986 and 2018. Lesions that developed primarily in the subungual, periungual and dorsum areas of hand were excluded from the analysis. All patients were diagnosed based on clinical manifestation, clinical course, and/or dermoscopic findings. Furthermore, the diagnosis was histopathologically confirmed in all patients with melanomas and 6 of 26 patients with melanocytic nevus. We then adjusted the size of the palm and fingers in each clinical image by means of digital magnification according to the previous study (*5*), and plotted the center of each lesion.

Among the six melanoma lesions of fingers, two were present on 1st fingers, one on 2nd fingers, one on 4th fingers, and two on 5th fingers. Representative clinical images of patients with melanoma of palm and finger pulp were shown in Figure 1a and 1b, respectively, and there were no significant differences in sex, gender and histopathological features between them. However, we found that all of the 8 lesions of melanoma were located on the finger pulps and distal to the 'distal transverse crease' of the palm (Figure 1c), and that melanomas were not present proximal to the distal transverse crease (surrounded by yellow dotted line in Figure 1d).

On the other hand, there were three nevus lesions on 1st fingers, three on 2nd fingers, and one on 3th fingers, one on 4th fingers, and two on 5th fingers, thus indicating that both melanoma and melanocytic nevus showed a tendency to be present on 1st and 5st finger. As shown in Figure 2a and 2b as representative clinical images of patient with nevus of palm and finger pulp, respectively, there were no significant differences in sex, gender and histopathological features between them. Unlike melanomas, however, we found that 13 melanocytic nevus were present (Figure 2c) proximal to the distal transverse crease of the palm (surrounded by yellow dotted line in Figure 2d).

We then compared the frequencies of melanoma and melanocytic nevus between in the proximal area of the distal transverse crease of the palm and in remaining parts of palm and fingers. As a result, we found that melanocytic nevus were present in the proximal area to the distal transverse crease of the palm more frequently than melanomas (50.0% *vs*. 0%), and there was statistically significant difference (p = 0.011 by Fisher's exact probability test). Therefore, our results suggest that the distribution of melanoma is different from that of melanocytic nevus in the palm and finger pulp.

ALM in the sole is known to be mostly found in the heel, followed by in the big toe. On the other hand, Minagawa et al. reported that the frequency of melanoma in the rear and front of the foot was extremely high compared to the arch area (*5*). The authors concluded that there may be more mechanical

stress such as plantar pressure and shear stress in the rear and front of the foot, which results in the increased susceptibility for melanomas.

Our finding that ALM tends to occur in the finger pulp and distal area of the distal transverse crease of the palm may indicate that mechanical stress also induces the formation of palmar melanoma: Blisters and calluses are often induced by mechanical stress on the hand: Actually, they are usually seen in the finger pulps and palm distal to the 'distal transverse crease', but rarely in the proximal area of the palm. Consistently, lower occurrence frequency of ALM in the palm can be explained by the minor mechanical stress compared to that in the sole.

Taken together, our findings may reveal the contribution of mechanical stress to the cause of palmar ALM, and may facilitate clinical differentiation between malignant melanoma and melanocytic nevus by the localization. Further studies with increased number of patients are needed to validate the finding.

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(Received August 4, 2019; Revised August 22, 2019; Accepted August 26, 2019)

Letter

DOI: 10.5582/bst.2019.01237

Accidental awareness while under general anaesthesia

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Summary **Accidental awareness during general anaesthesia may cause many intraoperative discomforts and bring further moderate to severe long-term symptoms including flashbacks, nightmares, hyperarousal or post-traumatic stress disorder. The incidence of awareness varied from 0.017% to 4% among studies. The relatively reliable incidence of intraoperative awareness with postoperative recall is 0.02%. The reason causing awareness was unclear. Insufficient anaesthetic dosing was thought as the principal cause. Even awareness was not comprehensively understood, some endeavors have been raised to prevent or reduce it, including *i*) Reducing the insufficient anaesthetic dosing induced by negligence; *ii*) Providing close clinical observation and clinical parameters from the monitor such as bispectral index or electroencephalogram, as well as isolated forearm technique and passive brain-computer interface may bring some effects sometimes. Because current studies still have some flaws, further trials with new detecting approach, superior methodology and underlying aetiology are needed to unfasten the possible factors causing awareness.**

Keywords: Accidental awareness, general anaesthesia

Unintended or accidental awareness during general anaesthesia (AAGA) was regarded as a failure in the general anaesthesia process. It was difficult to define awareness or AAGA accurately since consciousness and anaesthesia were also difficult to define. Unintentional or accidental consciousness during general anaesthesia (GA), without emphasizing recall, has been referred to as awareness earlier, while AAGA, defined as explicit recall of GA and could be spontaneously reported by the patient or detected by direct questioning or promoting, has been mentioned more (*1,2*). Although AAGA was relatively uncommon, it brought negative experiences including but not limited to intraoperative hearing voices or equipment noises, sensation of paralysis or pain, awareness of tracheal intubation and inability to breathe, etc. Furthermore, moderate to severe symptoms including flashbacks or nightmares, avoidance of situations relating to the experience, hyperarousal and post-traumatic stress disorder (PTSD) may suffer in 79% of patients who experienced AAGA long-term (*3*). However, no studies have provided any strong evidence that awareness without recall has important negative consequences to date (*2*).

The incidence of awareness is difficult to be accurately determined. The identification of awareness in most researches based on explicit postoperative recall ranges, spontaneous patient reports or structured postoperative interviews, such as the Brice Questionaire (*6-12*). The relatively reliable incidence of intraoperative awareness with postoperative recall is 0.02%, while the incidence without explicit recall may be much higher (*3,7,13,14*) (Table 1). Variation among studies in the proportion of patients with a possible awareness event may be up to 200-fold (4% *vs*. 0.017%) (Table 1). It was unclear whether the differences in incidence resulted from disparities in patient population, sample size, time of investigation, anaesthetic technique, study design, clinical severity, method of identification or definition of awareness. It was found that different definition of awareness, for instance, explicit recall

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Table 1. The incidence of accidental awareness during general anaesthesia (2000-2017) (*4,5*)

Study and year	Sample size, N	Incidence	Method of identification	Study design	Explicit postoperative recalls or not
Sandin *et al*, 2000	11,785	18 (0.15%)	Modified Brice questionnaire: PACU, 1–3 days and 7–14 days postoperatively	Observational prospective case study	Explicit recalls
Myles *et al*, 2000	11,811	12 (0.11%)	Question and answer survey within 24 h of surgery	Observational prospective case study	Explicit recalls
Myles *et al*, 2004 (*11*)	Total: 2,463 BIS: 1,225 Routine care: 1,238	Total: 13 (0.52%) BIS: 2 (0.16%) Routine care: 11 (0.89%)	Modified Brice questionnaire: 2–6 h, 24–36 h, and 30 days postoperatively	Randomised controlled trial	Explicit recalls
Andrade *et al*, 2011	Total: 5,731 BIS: 2,861 ETAC: 2,852	Total: 9 (0.15%) BIS: 7 (0.24%) ETAC: 2 (0.07%)	Modified Brice questionnaire: 72 h and 30 days postoperatively	Randomised controlled trial	Explicit recalls
Andrade *et al*, 2011	Total: 18,832 BIS: 9,460 ETAC: 9,376	Total: 20(0.11%) BIS: 8 (0.08%) ETAC: 11 (0.12%)	Brice questionnaire: 28-30 days postoperatively	Randomised controlled trial	Explicit recalls
Pandit *et al*, 2014	2,766,600	471 (0.017%)	Spontaneous complaints/reports of awareness	Cross-sectional observational study	Explicit recalls
Andrade *et al*, 2008	184	2 (1.1%)	IFT and postoperative structured interview	Observational prospective case study	No cases of explicit postoperative recall
Sanders *et al*, 2017	260	12 (4%)	IFT followed by modified Brice questionnaire	Observational prospective case study	No cases of explicit postoperative recall
Total	2,817,666	557 (0.020%)			

IFT, isolated forearm technique; BIS, bispectral index; ETAC, end-tidal anaesthetic gas concentration.

was regard as necessary or not may differentiate the results mostly (*15,16*). However, what was certain was that with upwards of millions admissions leading to surgical intervention annually, patients suffered from the accidental awareness could be an enormous number of cases.

What incentives awareness was not clear totally. Insufficient anaesthetic dosing, sometimes caused by administration equipment failure or the negligence of an anesthetist, was thought as the pivotal cause of awareness. However, some occult factors might cause consciousness and memory despite clinicians may consider adequate anaesthesia. It is unclear whether the reduced potency of anaesthetic was induced by a genetic contribution (*17-20*).

Even awareness was not comprehensively understood, some endeavors to prevent awareness have been advocated. Reducing the insufficient anaesthetic dosing induced by negligence should be the first step. Anaesthesia equipment before each use, especially vaporiser, circuit and drug-infusion pump, must be checked carefully. Drug error should be avoided by double-checking and labelling all drug syringes. Clinical parameters from the monitor or clinical signs being directly observed might work sometimes. The isolated forearm technique (IFT) was thought as the current gold standard for connected consciousness monitoring and was used to remind the anesthetist (*21*). Electroencephalogram derived bispectral index (BIS) or electroencephalogram (EEG) was once proved to be critical in preventing intraoperative awareness with explicit recall compared with clinical signs in most clinical researches (*8-10,12*). However, a study has shown that the BIS protocol was less useful in preventing awareness than end-tidal anesthetic-agent concentration (*9*). Moreover, innovative techniques, such as passive brain-computer interface (BCI) based on an intention of movement may provide a foundation that would allow to detect awareness (*22*).

Although substantial progress has been made in understanding awareness about the incidence, consequences, and prevention, lack of gold standard of the definition, detection and prevention still prevent us from minimizing it. Thus, further trials about AAGA, especially with new detecting approach, superior methodology, underlying aetiology and novel results compared with the existing literature are still precious in the future.

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(Received August 16, Accepted August 30, 2019)

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