ISSN 1881-7815 Online ISSN 1881-7823

BST
BioScience Trends

Volume 6, Number 1
February, 2012

IRCA
BSSA

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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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(This journal was partially supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.)

Brief Report

DOI: 10.5582/bst.2012.v6.1.1

Can health systems be enhanced for optimal health services through disease-specific programs? – results of field studies in Viet Nam and Cambodia

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Summary **Developing better health systems is the key to delivering optimal health services, although more evidence of effective strategies to do so is needed. Field surveys were conducted in Viet Nam and Cambodia to identify best practices in addressing health system bottlenecks to scale up disease control programs. The two countries were compared over time using a framework for analysis developed by the authors. In Viet Nam, a health system was in place for decades at the central to municipal levels, although it was fragile until the 1990s, when the government started taking measures. In Cambodia, the previous health system had been destroyed during previous internal conflict. In the post-conflict period, the health system was rebuilt with support for programs followed by centralization of health services. In different settings, different measures were taken to deal with similar bottlenecks. In Cambodia, vertical programs were dominant, so the government sought to centralize drug management to deal with shortages of essential drugs, while Viet Nam sought to mobilize resources to ensure drug distribution at all levels. This study shows there is no single successful approach to health systems, and a systemic approach needs to be taken because elimination of one bottleneck may reveal another. Efforts to enhance disease-specific programs may not always contribute to overall enhancement of the health system, and the best possible approach may not be the same in different countries. Further study is needed to explore common issues and principles for effective strategies to enhance health systems in different contexts.**

Keywords: Health system enhancement, disease-specific program, global health initiatives, Cambodia, Viet Nam

1. Introduction

In the past decades, several strategies such as the Directly Observed Treatment, Short-course (DOTS) strategy for tuberculosis control and the Expanded Program for Immunization (EPI) were developed to effectively implement disease-specific programs through simple and universally adopted interventions.

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The DOTS strategy is a simple package for tuberculosis (TB) control developed as a cost-effective health intervention that can be easily implemented in various settings. The strategy highlights identification and monitoring of patients through a sputum smear exam, direct observation to prevent drop-outs, and case management and evaluation by cohort analysis through recording and reporting. The DOTS strategy has been adopted by most countries with obvious results, although the world is not yet on track to decrease mortality and morbidity of TB due to health system bottlenecks (*1*). EPI aims to promote basic vaccination in order to protect children from vaccine-preventable diseases. EPI features strong logistic management including cold chain logistics and has markedly

increased vaccination coverage, but its constraints are that vaccine coverage depends on social status as well as sources of financing (*2*). Although these strategies have achieved marked results through their respective programs, the scaling up of these programs is constrained by weak health systems (*3*). The global health community and the Global Health Initiatives (GHIs) are reaching the consensus that enhancing health systems is essential to improving delivery of these health services, although a strategy to achieve that goal is not clearly defined and is still under discussion (*4*).

The current authors conducted a study to identify the best practices that are addressing health system bottlenecks and common strategies of health system strengthening (HSS) to scale up the disease programs. This study focuses on two countries with different health system settings, *i.e.,* Viet Nam and Cambodia.

In Viet Nam (*5*), the health system has been in place since the 1980s with central, provincial, district, and municipal levels at which services have been provided. The economic reforms initiated in 1986 for a "socialist-oriented market economy" have had a great impact on the health sector as well, and the government has acted with partners to implement health system reform and enhancing the health system since the 1990s. Under the Ministry of Health (MOH), national steering committees are organized to manage national health programs with strong leadership, efficiency, and inter-sectoral collaboration. At the district level, structural reforms were carried out in 2006 and in 2009, and district health offices manage health services and supervise family planning centers (*6*). Municipal health stations provide health care services to locals through health workers (volunteers receiving a small allowance). Village health workers are under the direct management of the municipal health station and village leaders, performing various tasks such as providing primary health care (PHC) and implementing national health programs.

In contrast, the health system in Cambodia (*7*) was previously destroyed through internal conflict. In the post-conflict period, the system is being rebuilt. Efforts to support disease-specific programs were implemented in the 1980s, followed by centralization of some health services in the 1990s. The current system consists of strong and vertical disease-specific programs with relatively weak horizontal links supported by an overwhelming numbers of development partners and non-governmental organizations (NGOs). The health system consists of the MOH, provincial or municipality health departments (PHDs), and operational district health offices (ODs). According to the health coverage plan (*8*), Cambodia has 75 referral hospitals and 967 health centers (HCs), 7% of which only provide outreach services with no fixed site. In remote areas, health posts provide basic health services to locals in addition to HCs. All health facilities down to health posts have professional staff with a government salary. In communities, each village is supposed to have a village health support group (VHSG, with 2 volunteers per village) that performs numerous tasks such as connecting people to HCs through immunization outreach, implementing community DOTS, and distributing mosquito nets. National programs have a central program manager team, provincial supervisors at PHDs, and OD supervisors.

2. Methods

This study was designed in collaboration with Western Pacific Regional Office of the World Health Organization (WHO-WPRO) in preparation for the Workshop on Maximizing Synergies between GHIs and Health System held in Lipa City, the Philippines, November 25-28, 2009 (*http://www.wpro.who.int/sites/hsd/documents/ Workshop+on+Maximizing+Synergies+between+Global+Health+Initiatives+and+Health+Systems.htm*).

This study was conducted using qualitative methods, *i.e.,* interviews with key informants *via* a semi-structured questionnaire, and a review of relevant documents. The authors first determined the core functions of programs, identified the key informants, and agreed on items on the questionnaire. The document review was carried out to identify items from the questionnaire in documents from the two countries. The same methods were used in both countries. Results of analyses were determined through agreement by the authors.

2.1. *Key informant interviews*

Key informant interviews were conducted with health staff at each level of health facility, health managers at the district and provincial levels, and program managers of the MOH, NGOs, and other partner agencies. Consent to the interview was obtained verbally through communication with the authors before the interview. At the central level, information on management of national programs was collected through interviews in accordance with the 6 blocks of a health system (leadership and governance, service delivery, financing, human resources, logistics, and information) (*9*). A semi-structured questionnaire asked about financing and funding streams; overall planning, human resource strategy, and lines of authorities for supervision and quality control; and supply chains and delivery services. Key informants at the central level were managers of EPI and malaria programs in Viet Nam and the national immunization program (NIP, in Cambodia) and the national tuberculosis control program (NTP) in Cambodia. Information on the Global Fund against AIDS, Tuberculosis and Malaria (GFATM) and the Global Alliance for Vaccines and Immunization (GAVI) management and

partner coordination was collected from managers and from related departments of the MOH, departments of planning and health information, departments of budgeting and finance, and partners. At the provincial and district levels, interviews were conducted on bottlenecks in the health system, integration of service delivery and program management, and HC service delivery, including outreach at HCs. In Viet Nam, Thanh Hoa Province (Lang Chanh District) and Dien Bien Province were studied. In Cambodia, the authors joined the NIP supervisory team and visited three ODs in Seam Reap Province (Angkor Chum OD with GAVI support, Seam Reap, and Sothnikum OD) and 4 HCs. The authors interviewed PHD/OD chiefs and program supervisors. During the visit to HCs, interviews were conducted with the HC chief (if available) and staff. In total, 62 key informants were interviewed: 29 in Viet Nam (4 at the central level and 25 in the field) and 33 in Cambodia (14 at the central level and 19 in the field).

2.2. *Document review*

Related documents from the 1990s and 2000s were also reviewed to corroborate the interviews, *i.e.,* documents on GHI or GFATM support in each country, changes in the health system, activities and achievements of respective programs in each country, and field reports from the Japan International Cooperation Agency (JICA) and other projects.

2.3. *Analysis*

Information and findings obtained from key informant interviews at each level were compared with quantitative data such as the amount of assistance and program coverage to verify this study's results. The authors developed a framework for analysis. In it, bottlenecks, solutions to bottlenecks in terms of basic health service delivery at each level, and good practices that subsequently resulted was categorized based on the six building blocks of a health system, *i.e.,* service delivery, workforce, information, medical products, health financing, and leadership and governance. The positive and negative effects of each bottleneck and practice on health systems and on PHC principles (*10*) were described. The outcomes of solutions to common bottlenecks in the two countries were compared using the tables. A framework of analysis was thus developed and the cause-and-effect relationship between common bottlenecks and their solutions was determining through consensus.

3. Results and Discussion

3.1. *Results*

Bottlenecks and solutions were identified in the two health system blocks of logistics and service quality. Figure 1 summarizes the health system bottlenecks and good practices in both countries from the 1980s to the 2000s, showing the cause-and-effect relationship between bottlenecks and the solutions implemented to resolve them.

Described here are two examples of common bottlenecks and how they were resolved in the two countries. One common bottleneck was the shortage of drugs due to poor drug distribution at the local level. In Cambodia, rebuilding of the health system started in the 1980s through establishment of basic health services. The government sought to manage its limited budget effectively and make the system functional by implementing disease-specific programs. Experiences with NIP and NTP were positive until the 1990s as programs were started and successfully expanded despite a health system with a very limited capacity. Drug distribution to the local level became more efficient and faster with both NIP and NTP, but a shortage of essential drugs and lack of other programs remained particularly at the district level and local health centers. To resolve this problem, an attempt was made to integrate and centralize drug procurement and distribution. At this stage, this integration contributed to HSE in the areas of drug supply management and HIS (GP-C2 in Figure 1). However, further drug shortage bottlenecks (BN-C2) appeared due in part to the delay in reporting by the national HIS. To compensate for the deficiencies in the centralized system (GP-C4), disease programs kept parallel systems for emergency drug distribution as well as vertical reporting and supervision (*11,12*). Under a communist government, Viet Nam has experienced a drug shortage despite its extensive health network from the central to the municipal level (BN-V1 in Figure 1). To resolve this bottleneck, Viet Nam mobilized domestic financial and human resources and made some improvements in its drug supply system (GP-V1). However, certain bottlenecks still remain in hard-to-reach areas and in relation to the migrant population who were not reached by these efforts (BN-V2).

Another example is the low quality of service delivery due to the lack of qualified health personnel at the local level. Table 1 depicts service delivery in Cambodia using the framework for analysis. In Cambodia, as shown in Table 1, one reason for the low quality of services was low motivation of personnel caused by a low salary, delay in salary payment, and insufficient mechanisms of support for health staff (BN-C2 in Figure 1). To resolve these bottlenecks, performance-based incentives (PBIs) were introduced through contracting or through the support of the GHI, *i.e.*, the Global Alliance for Vaccines and Immunization (GAVI)-HSS project since 2002 (GP-C3). Contracting services or PBIs seemed to have positive impacts on staff motivation and improved the number of

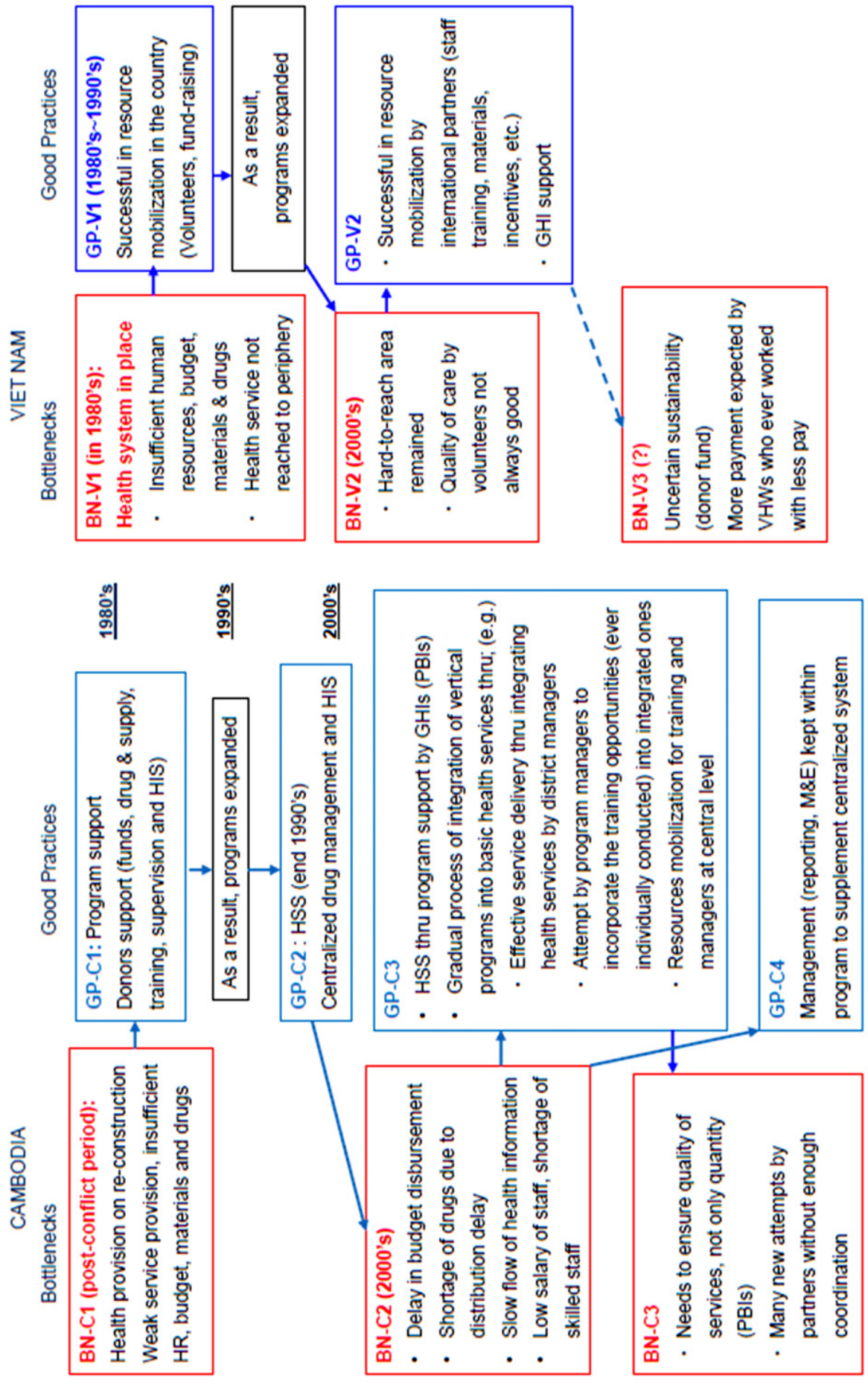

Figure 1. Summary of the health system bottlenecks and good practices observed since the 1980s in both countries.

Table 1. Cambodian workforce

Bottlenecks	Good practices	Effects (+/-) on HS	
		Effects on HS	Effects on PHC principles
(1) Shortage of HR at HCs and referral hospitals in rural areas. (2) Low salary and delayed payment. (3) Workforce misallocation to better funded programs. (4) Training budget decreasing/ unreliable (MOH, UNICEF, WHO). Training for new staff/techniques always essential.	PBI for service providers	(1) PBI motivates staff to work, HC is staffed 24 h a day. (2) No change of government system of basic salary. No guarantee of funding for PBI in the future.	(1) Universal coverage: HCs now staffed 24 h a day. (2) Quality of care: PBI focused only on quantity of services delivered, not quality (*i.e.*, ANC coverage). (3) People-centered care: Moral issues might arise.

services delivered (Unpublished report by JICA: Performance-based financing of maternal and child health services: Financial and behavioral impacts at the field level in Kompong Cham Province. 2009). That said, the need to ensure the quality of services and not merely their quantity was noted, as indicated by TBC challenges (Unpublished report by BTC: Assessment of performance contracting in Kampong Cham, Seam Reap, and Odoymeanchey provinces, Cambodia. 2008). Too many different initiatives have been implemented *via* partners without appropriate coordination, creating a bypass and hampering coordination at any level (BN-C3) (*14,15*). In Viet Nam, support from the GHIs helped with training and motivating staff and village health volunteers (VHVs, GP-V2). This successful resource mobilization through GHIs resulted in an increasing need for monetary incentives to maintain quality services by VHVs, who have historically worked without such incentives. The success and sustainability of the current approach is also affected by uncertain continuity of funding from the GHIs (BN-V3 (?)).

3.2. *Discussion*

The aforementioned findings led to several conclusions. First, efforts to enhance disease-specific programs may not always contribute to overall health system enhancement. Several vertical approaches were needed to promptly improve service delivery but were found to have a negative effect on the health system. That said, some good practices for disease-specific programs were also observed. This study showed that "vertical" program management can complement weak health systems when starting or continuing to provide services. Second, there is no single approach to HSS. Effective health system approaches have to be identified according to the country, and approaches may differ in different countries and different contexts. This study shows that different solutions to common bottlenecks were historically adopted in the two countries. Likewise, a good practice in one country may not always produce the same positive outcome in another country. Third, this study also reveals the historical fact that a solution to a bottleneck can be beneficial but it may also lead to another bottleneck. Decisions on measures to take must be made in light of what currently scheduled efforts may lead to. The current case studies provide hints and tips to possible outcomes that may result from later efforts, so "history is the teacher of life" (*16*). This study suggests that a systemic perspectives needs to be taken because resolving one bottleneck may reveal another.

Interviews with key informants revealed several vertical approaches that may or may not positively affect health systems, even though these approaches were needed to promptly improve service delivery. PBIs may be an example of a good practice to enhance program implementation although they do not always help to enhance health systems. That said, other good practices for disease-specific programs were identified in Cambodia (GP-C3). Many good practices may not be sustainable or may cause other bottlenecks. The gradual process of integration of vertical programs (NIP and NTP) into basic health services in Cambodia is a good practice to enhance the health system, which is sustainable despite limited resources. One example of a pioneering effort is an attempt by program managers at the central level to incorporate individual training opportunities into integrated training and mobilize resources using governmental and partner funds. These innovative approaches were the idea of central and district managers who faced constraints in their own districts.

The current study had several limitations. This study did not cover all national programs since programs were selected through discussion with WHO offices in each country based on feasibility. In Cambodia, the study did not cover HIV and malaria programs that receive significant amounts of support from GFATM. Second, the selection of the provinces studied was not random but was dictated by accessibility, the possibility of field visits (in Cambodia), and the availability and quality of reports for document review. Third and most important, time constraints and budget limitations meant that this study did not include extensive quantitative analysis in terms of finances, human resources, drug procurement

and management, and health information systems. As it stood, the topic of the current study was already rather complex and extensive, *i.e.,* health systems, health programs, and their relationships. However, personal networks cultivated through long-standing collaboration did help to facilitate interviews with key informants. These interviews depicted cause-and-effect relationships between bottlenecks and their solutions that were corroborated by document reviews.

3.3. *Conclusion*

The current study indicates that there is no single successful approach to health systems as such, but there are common issues faced by and principles to be learned from experiences of different countries. Since this study was conducted in only two countries, more studies need to be conducted in other countries to further explore these common issues and principles of health system enhancement, including the possibility of identifying issues by clustering several countries in similar stages and with similar characteristics of health system development.

Acknowledgements

This study was funded by the WHO-WPRO and the research fund of the National Center for Global Health and Medicine, Japan.

The authors would like to thank Dr. Dean Shuey, Regional Advisor for Health Services Development, WHO-WPRO for his review of this manuscript and valuable comments. The authors would also like to express thanks to Dr. Benjamin Lane of the WHO Representative Office in Cambodia, Dr. Lokky Wai of the WHO Representative Office in Viet Nam, and the health ministries of Cambodia and Viet Nam for their full support during this study.

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(*Received August 15, 2011; Revised January 10, 2012; Accepted January 22, 2012*)

Brief Report

DOI: 10.5582/bst.2012.v6.1.7

A study on indoor environment contaminants related to dust mite in dwellings of allergic asthma patients and of healthy subjects

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Summary **This study investigated the pollution of dust mite allergens in the houses of 30 families and their infection to young allergic asthma patients in Shanghai. Medical records, family information, and dust samples were collected from the dwellings of 15 young allergic asthma patients and 15 healthy subjects. Der 1 allergen, which is a common allergen causing allergic asthma, was measured in collected dust samples using the Pharmacia Uni-CAP System. A significant correlation was found between the number of Der 1 allergens collected from floor surfaces and the number of Der 1 allergens collected from bed surfaces. Some factors influencing Der 1 allergen levels were found in this study. Relative humidity in dwellings was found to be most influential to the allergen levels. The findings suggested that traditional reduction methods for coarse particles, such as opening windows and periodic cleaning of beddings, may be effective in removing dust mite allergens.**

Keywords: Dust mite, dust mite allergen, allergic asthma, Der 1, indoor, dwelling

1. Introduction

Allergic diseases, especially asthma, are public health problems worldwide. In most countries, the spread and mortality cases of asthma are continuously increasing. The World Health Organization (WHO) estimated 300 million asthmatic patients around the world, with incidence ranging from 1% to 18%. More than 50% of adult patients and 80% of young patients are sensitive to allergic factors (*1,2*).

Dust mite allergen is not only a familiar indoor allergen, but is also one of the most important pathogens causing allergic asthma (*3-6*). Dust mites belong to Acariformes, Acaroidea, Pyroglyphidae, and Pyroglyphidae, including Pyroglyphinae and Dermatophagoidinae. More than 10 genera and 40 species of dust mites exist all over the world. Two of the most important strains are *Dermatophagoides pteronyssinus* (D.p) and *Dermatophagoides farinae* (D.f). D. *pteronyssinus* is one of the first advantaged dust mites found in Eurasia, whereas D. *farinae* is the advantaged dust mite common in America (*7,8*).

Data reveal that China's cities are seriously polluted by dust mite allergens (*9*). A research on dust mites in 15 cities in China showed that house dust mites thrive in over 40% of houses in cities, except in high latitude areas, such as Zhangjiakou and Akesu. House dust mites were also found in 67% of houses in Shanghai (*10*).

Allergic asthma is a disease threatening human health which involves long-term treatment and high costs. Dust mite allergen is one of the most important pathogens causing allergic asthma, which exist in every part of the house. Thus, effectively decreasing indoor dust mite allergen levels can actively contribute to the prevention and treatment of allergic asthma. The objectives of this study are to determine the pollution caused by dust mite allergens in dwellings in Shanghai and to formulate a control method which will reduce indoor air pollution related to dust mite allergens.

2. Materials and Methods

This observational testing and analysis study evaluated indoor air contaminants related to dust mite from 2006

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to 2007 in dwellings located in Shanghai. Similar with a related research (*11*), the present study gathered 15 young allergic asthma patients hospitalized in public pediatric clinics in Shanghai. The parents or legal guardians of eligible child subjects received detailed orientation on the aim of the study. Subjects were selected according to the following criteria: aged 1-16 years old, resident of Shanghai, reported with allergic asthma, income, and house types. Another set of 15 healthy subjects was chosen to participate in the study as control. The healthy subjects were selected based on the same criteria as the allergic asthma patients. Measurements were taken in the living room of each of the subject's dwellings.

A questionnaire survey was also carried out, which focused on medical records, house conditions, and lifestyles. Necessary information was inquired and documented in detail.

The indoor air temperature and relative humidity in each dwelling were measured by a hygrothermograph (THERMO RECORDER RS-11) positioned at the center of the living room. After a waiting time of two minutes, the data were read and recorded.

After measuring air temperature and relative humidity, house dust was collected from the surfaces of living room beds and floors using a paper filter equipped with a hand cleaner (HC-V15, National). The hand cleaner instantly absorbed house dust from the beds and floors. Subsequently, the collected samples of house dust were placed in a paper filter every minute. The samples were labeled and used for the analysis of dust mite allergen levels using the Pharmacia Uni-CAP System. The Pharmacia Uni-CAP System identified the dust mite allergen levels of the dust samples and automatically printed the results.

Results were tabulated and analyzed as μg/g fine dust for the dust mite allergen, Der 1. The levels of the allergen were compared based on geographical location, sampling locations, air temperature, and relative humidity.

The different parameters did not approach a normal distribution, even after log transformation. Non-parametric Mann-Whitney *U* test was used to evaluate the correlation between the dwellings of allergic asthma patients and those of healthy subjects in different sampling locations.

Correlations between Der 1 levels and temperature and relative humidity were assessed by means of the Spearman correlation coefficient (rho). The limit of significance was 0.05. Statistical analysis was performed with SPSS for Windows.

3. Results and Discussion

A total of 30 dwellings were assessed in this study: 15 dwellings of young allergic asthma patients and 15 dwellings of healthy subjects. Investigations conducted in each dwelling were successfully completed, and results were collected.

Der 1 mite allergens were detected in 100% of the analyzed dust samples (n = 60) collected from the dwellings. In dwellings of young allergic asthma patients, the level of Der 1 on the surfaces of living room beds varied widely from 0.06 μg/g to 21.30 μg/g (geometric mean: 1.74 μg/g). The Der 1 level on bed surfaces was higher than that on the surfaces of living room floors ($p < 0.001$), which ranged from 0.03 μg/g to 2.09 μg/g (geometric mean: 0.34 μg/g) (Figure 1). In the dwellings of healthy subjects, the level of Der 1 collected from the surfaces of living room beds varied widely from 0.65 μg/g to 69.33 μg/g (geometric mean: 7.14 μg/g), which was higher than the Der 1 level on the surfaces of living room floors ($p = 0.045$) that ranged from 0.01 μg/g to 22.22 μg/g (geometric mean: 2.05 μg/g) (Figure 1). In comparison, significantly higher levels of Der 1 were found in dwellings of healthy subjects than in dwellings of allergic asthma patients ($p < 0.001$).

Factors such as medical records of young allergic asthma patients, building structures, and family lifestyles were examined to determine their effects on Der 1 allergen levels; data came from the questionnaire survey. No association was found between the concentration of mite allergens and environmental characteristics, such as type of ventilation, lifestyles, and building structures.

Air temperature in the dwellings of allergic asthma patients ranged from 18.1°C to 21.6°C (mean: 19.9° C), whereas relative humidity ranged from 60% to 75% (mean: 69%). Air temperature in the dwellings of healthy subjects ranged from 19.7°C to 24.2°C (mean: 21.6°C), whereas relative humidity ranged from 41% to 62% (mean: 49.4%). The average air temperature in the patients' dwellings was lower than that of the healthy subjects' dwellings. A significant relationship was found between relative humidity and Der 1 levels on bed surfaces in patients' dwellings ($r = 0.577$; $p = 0.024$) (Figure 2), whereas no obvious correlation was found between relative humidity and Der 1 levels on bed surfaces in the dwellings of healthy subjects.

The dwellings of six families who use cleaners reflected higher Der 1 levels compared with those of the nine families who do not use cleaners. A significant relationship between the usage of cleaners and Der 1 levels on bed surfaces was determined. Der 1 levels were evidently high in dwellings of families using cleaners. On the other hand, statistical analysis indicated a high Der 1 level when relative humidity was high. Thus, relative humidity may be the most important factor affecting Der 1 level and also decreases the influence of other factors.

The significantly higher levels of Der 1 in the dwellings of healthy subjects compared with those of allergic asthma patients could be attributed to the attention given to the conditions of their houses. Families of allergic asthma patients apparently spent more time in house cleaning to reduce further exposure of their children to dangerous allergens. Another possible reason

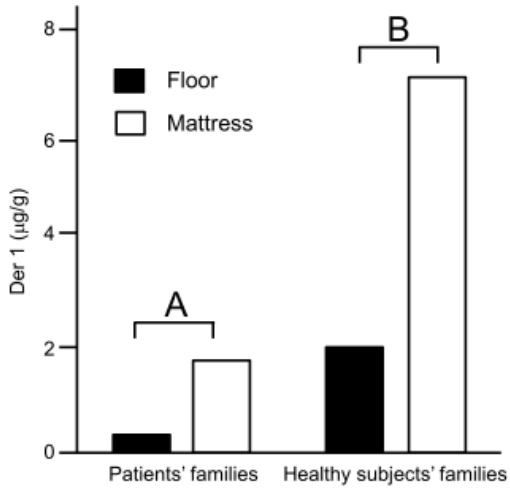

Figure 1. The geometric mean of Der 1 concentrations in dust samples. Black pillar indicates dust samples collected from floor and white pillar indicates dust samples collected from mattress. **(A)** Dust samples collected from the dwellings of allergic asthma patients (mattress *vs.* floor surfaces: $p < 0.001$). **(B)** Dust samples collected from the dwellings of healthy subjects (mattress *vs.* floor surfaces: $p < 0.001$).

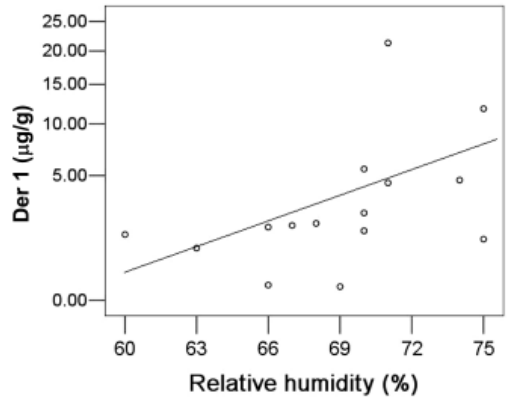

Figure 2. Correlation between Der 1 levels of dust samples collected from the dwellings' mattress of allergic asthma patients and indoor relative humidity ($r = 0.577$; $p = 0.024$).

could be the occurrence of allergic symptoms or allergic asthma among patients even from only a few dust mite allergens.

Der 1 levels, as well as relative humidity, in the dwellings of two healthy subjects were especially higher than those in others. The higher Der 1 levels may be erroneously caused by a technician feeding dust mites as their jobs in each of the two families. However, the result may also be attributed to high relative humidity. Further study is necessary to verify this.

The geometric mean level of Der 1 collected from the surfaces of living room beds was higher than that collected from the surfaces of living room floors. This finding implied that maintaining an orderly bed and periodic cleaning of beddings were of more concern for families with members who had allergic asthma.

From the present study, relative humidity was found to be the most significant factor affecting indoor dust mite levels. Yanagi *et al.* (*11*) reported the relationship between ventilation, purification, and relative humidity. Relative humidity decreased sharply after opening windows and normalized quickly as time elapsed. Therefore, opening windows (*e.g.* once per hour) is effective in reducing indoor relative humidity and dust mite levels.

Acknowledgements

This work was supported by grants from the National Science Foundation for Fostering Talents in Basic Research of the National Natural Science Foundation of China (Grant No. JO730860), and the Hi-Tech Research and Development Program of China (Grant No. 2007AA02Z472), and the Special Research Foundation of the Health Industry (Grant No. 200802001).

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(*Received November 30, 2011; Revised January 30, 2012; Accepted February 12, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.1.10

Characteristics of family caregivers with sleep dissatisfaction in Japan: Identification using CHAID dendrograms

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Summary **The rapid increase in the population of the elderly has raised several social issues. The current study focused on sleep dissatisfaction in family caregivers to identify family caregivers with a heavy care burden. This study aimed to detect the characteristics of caregivers who are most likely to have sleep dissatisfaction. A chi-squared automatic interaction detection technique was used to analyze data collected from 92 research care managers who collected demographic and sleep dissatisfaction information from 280 caregivers and their care recipients. Caregivers whose care recipients were unstable and bedridden were most likely to have sleep dissatisfaction. When care recipients were not stable or non-bedridden, had severe dementia symptoms, and were physically independent, their caregivers were the second most likely to have sleep dissatisfaction. When care recipients were not stable or non-bedridden, had moderate dementia symptoms, and did not need help in transferring, their caregivers had the lowest risk of sleep dissatisfaction. Although many recent studies have found a high prevalence of insomnia among the elderly, describing the characteristics of caregivers who are most likely to have sleep dissatisfaction is a significant challenge. When care recipients are physically independent, the severity of the recipient's dementia symptoms relates to the caregiver's dissatisfaction with his/her sleep. In physically dependent care recipients, the severity of the recipient's dementia did not contribute to the caregiver's dissatisfaction with his/her sleep.**

Keywords: Sleep dissatisfaction, long-term care, caregiver burden, community nursing

1. Introduction

Increasing health care costs for the disabled elderly have become an important issue in developed countries. Prolonging home care has been effective at reducing health care costs (*1*), but the disabled elderly must then remain independent and stay at home. In Asian countries, family members are often expected to play a caregiving role at home. Family members often experience a conflict between the caregiving role and their other roles (*2*) or are subjected to a heavy burden of providing care. When looking at prolonged home care, family caregivers with a heavy care burden must be identified and their load must be reduced.

Some studies have reported that sleep problems, which include insomnia, are an important component of the caregiving load that causes mental disorders among caregivers (*3-5*). In a recent review, McCurry, Logsdon, Teri, and Vitiello (*6*) noted that changes in caregiver sleep patterns are caused by multiple factors, including factors on the part of both the caregiver and care recipient. The most common factors in caregivers' sleep disorders are gender and age. Older females have a higher risk of increased sleep latency, decreased sleep maintenance, decreased slow-wave and rapid eye movement (REM) sleep, nocturnal temperature dysregulation, more frequent shifts between sleep stages, and circadian rhythm disturbances, particularly in the advanced sleep phase (*7*). These age-related changes contribute to increased nighttime wakefulness and fragmentation of sleep.

That said, factors related to care recipients also

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cause changes in caregivers' sleep patterns. When care recipients need frequent assistance during the night, caregivers experience events that precipitate nighttime wakefulness. In particular, nighttime behavior by care recipients affects sleep patterns of their caregivers. Research shows that nighttime activity, as well as waking at night to urinate or taking a walk around the house, is one of the most common reasons cited for moving a family member with dementia into an institutional setting (*8*).

With society aging, more care recipients will need personal and medical care during the night, resulting in more caregivers suffering from sleep disorders. However, no studies have described caregivers who are at-risk for a sleep disorder. Data on the characteristics of caregivers who are the most likely to have a sleep disorder may help health care professionals to assess nocturnal care needs in various clinical settings and develop strategies to decrease a family caregiver's burden.

Data mining has been found to help understand how subjects' patterns relate to their own special situations. One commonly used data mining technique includes the chi-squared automatic interaction detector (CHAID), which has been used to segment the population into homogeneous subgroups. Forthofer and Bryant (*9*) used CHAID to compare various approaches to developing strategies to change health behaviors. Their results not only provide insight into the determinants of breast cancer screening among women but also enable researchers to identify unique characteristics of population segments with greater needs and to focus intervention resources in a manner that is likely to maximize intervention impact. Naruse *et al*. used CHAID to develop a service need pattern model for the elderly. They identified service needs among the elderly based on patterns (*10*).

Sleep dissatisfaction is one of the main factors that cause insomnia (*11*). Insomnia, which is characterized by inadequate or poor quality of sleep (*12*), may be a primary sleep disorder or might manifest itself as a co-occurring condition with a psychiatric, medical, or other sleep disorder. Although sleep dissatisfaction is not the same as sleep disturbance, it may be a reasonable index of insomnia. Therefore, the purpose of the current study is to use data mining – CHAID – in order to segment caregivers into homogeneous subgroups on the basis of their sleep dissatisfaction rates. This approach aims to develop a strategy that detects and helps caregivers who are at-risk for sleep dissatisfaction.

2. Materials and Methods

2.1. *Individuals cared for under the long-term care insurance system in Japan*

Long-term care insurance (LTCI) was established in April 2000 (*13*). The Japanese government has recommended that the elderly continue to live in their own homes and use home-based services provided by this insurance system for support. Under the system, two groups of people can use the LTCI services: people aged 65 years or older who are in need of daily care, including those who are bedridden, suffering from dementia, or housebound, and people aged 40 to 64 years who need daily care because of illnesses such as dementia and cerebrovascular disease. Levels of care range from support and care level 1 (requires help with activities of daily living (ADL)) to level 5 (requires maximum care). To ensure their independence, users of LTCI can tailor their services to conform to their own care plan. Usually, such plans are developed by care managers who are professionals in managing the care of the elderly.

2.2. *Study design and participants*

This study analyzed data that were obtained from individuals who participated in an earlier study investigating the characteristics of the elderly; these elderly individuals were most likely to need home help and home nursing (*10*). Ninety-two care managers, who are professional care planners in the LTCI system (*13*), from 32 care management offices in the southern district of Shiga Prefecture participated in this study as research care managers (RCMs). The RCMs chose primary caregivers for care recipients (from care levels 2, 3, 4, and 5) as study participants. A primary caregiver was defined as the person who took the most care of the care recipient among his/her family members. To avoid selection bias, caregivers whose care recipient's date of birth was closest to August 1 were selected at each level of care. A total of 320 elderly individuals were selected.

Between July and September 2008, the researchers visited the primary caregivers of those individuals and explained the purpose of the survey. If they agreed to participate, the RCMs gathered data, including demographic information and assessments of the status of the elderly individual. Participants were then interviewed by the RCMs using a structured questionnaire. This study was approved by the Ethics Committee of the Graduate School of Medicine at the University of Tokyo.

2.3. *Conceptual guide*

Independent variables that explain sleep dissatisfaction were selected by referring to a disease model that explained the causes of insomnia. Insomnia, which is characterized by inadequate or poor quality of sleep, may be a primary sleep disorder or manifest itself as a co-occurring condition with a psychiatric, medical, or different sleep disorder. Spielman's 3P model shows that three types of factors are involved at different

points during the course of insomnia: predisposing, precipitating, and perpetuating factors (*14*). Predisposing factors are not a direct cause of insomnia, but they increase an individual's risk of developing sleep difficulties. Precipitating factors are the life events and the medical, environmental, or psychological factors that trigger insomnia, and perpetuating factors maintain or exacerbate sleep difficulties. Caregivers' demographic variables were selected as predisposing factors, and care recipients' demographic variables were selected as precipitating and perpetuating factors.

2.4. *Variables*

To formulate a questionnaire for the RCMs and family caregivers, five care managers were recruited for a pilot test. All variables were selected and modified in order to create a clear and feasible instrument for RCMs. According to the care managers, lifestyles of care recipients and caregivers were so irregular that they could not answer questions about them over the long-term. Taking these constantly changing conditions into consideration, all questions concerned the lives of family caregivers over the past week.

Caregivers' sleep dissatisfaction was examined as a dependent variable. Here, the sleep dissatisfaction index from the life habit inventory developed by the Tokyo Metropolitan Institute for Neuroscience (*15*) was adopted. As in Hayashi and Hori (*16*), participants were divided into 2 categories: those who were satisfied with their sleep and those who were dissatisfied with their sleep. On this scale, sleep dissatisfaction was measured using three items: mood (good, bad), amount of sleep (sufficient, insufficient), and quality of sleep (good, bad). If a person answered "good" or "sufficient" to all of the items, he/she was considered to be satisfied with his/her sleep. If a person answered otherwise, he/she was considered to be dissatisfied with his/her sleep.

Demographic variables for caregivers included age, gender, family relationship, living situation, duration of caregiving, and frequency of care by a second caregiver. Demographic variables for care recipients included age, gender, daily functioning, severity of dementia symptoms, severity of illness and presence of conditions or impairments, level of care, and living arrangements.

Using the Katz index of independence, the difficulties experienced by care recipients' in ADL (bathing, dressing, toileting, transferring, incontinence, and feeding) (*17*) were measured. In addition, the degree of independent living for the elderly was used (*18*). This scale was established by the Japanese Ministry of Health and Welfare and is commonly used among Japanese community health agencies to assess daily conditions among the elderly. Using this index, RCMs judged whether the elderly person was independent (did not need help to perform daily activities at home) or dependent (needed help to perform daily activities at home). The severity of dementia symptoms was assessed using the Japanese index of independence/ dementia symptoms (*19*). This index was established by the Japanese Ministry of Health and Welfare. Using this index, care managers judged whether the elderly had moderate dementia symptoms (did not need help in daily life because of dementia symptoms) or severe dementia symptoms (needed help in daily life because of dementia symptoms). The presence of conditions or impairments in each care recipient was also measured using the two most important medical diagnoses considered by RCMs in devising a care plan. Further, the RCMs indicated whether or not the care recipients were terminal.

To compare the care needs of the elderly, which were categorized based on dendrograms, the RCMs asked caregivers about the time spent caregiving and considered care during the night before the interview. In all, four care items were examined: suction, postural change, toileting support, and diaper change. Toileting support included transferring the elderly person to the toilet, helping them remove clothing, and wiping the diaper area clean. These factors were selected as they were considered necessary throughout the day.

2.5. *Statistical analysis*

Before data mining for the research question, the relationships between the demographic variables and participants' sleep dissatisfaction were examined by using an unpaired *t* test, chi-squared test, and Fisher's exact test.

Next, the research question was addressed using CHAID analysis, a nonparametric analysis based on statistically recursive partitioning algorithms. The CHAID technique determines the relative importance of each of the independent (predictor) variables in explaining group membership in a categorical dependent (outcome) variable. The technique involves two steps. In the first, the independent variables are stratified into alternative ordinal groupings to ensure similar percentage distributions of the dependent variable among these categories. Groups may be formed by any possible combination of the levels of an independent variable or by placing cut points at any value of a continuous predictor. In the second step, the technique uses χ^2 significance levels to determine which independent variable explains the most variance in the dependent variable. The process is repeated for all significant predictor variables until no further significant χ^2 values are obtained.

Dendrograms are used to display the relative importance of significant independent variables for the dependent variable. The hierarchical nature of the CHAID dendrograms provides a visual depiction of criterion and predictor variable interactions that might

not be detected in traditional analytical procedures. The variable at the highest level of the tree is considered to have the closest statistical association with the dependent variable. The CHAID technique has traditionally been used in business and marketing research (*20*), and its use is increasing in rehabilitation and mental health studies (*21,22*).

In the current analysis, groups were split until the following criteria were reached. The tree depth was limited to three levels – no group smaller than 40 was split, and no group smaller than 20 was formed. The alpha level for all statistical tests was 0.05, corrected for the number of statistical tests within each predictor using a correction factor analogous to the Bonferroni technique. SPSS ver.17 and Decision tree ver.17 were used for the CHAID analysis.

3. Results

3.1. *Response rates and demographic characteristics*

Of the 320 caregivers selected for this study, 300 (93.8%) agreed to participate. Two hundred and eighty (87.5%) participants were included in the analysis, and 20 were excluded because of incomplete data. Table 1 shows the demographic characteristics of caregivers. Their average age was 64 years. Of the caregivers, 50% were under 64 years, 80% were female, 40% were daughters (including in-laws), and 30% were wives. Almost all the caregivers lived with the elderly individual, and half of them spent more than half a day providing care. Seventy percent were supported by second caregivers.

One hundred eighty-nine participants (67.5%) were dissatisfied with their sleep. Time spent caregiving (χ^2 = 7.85, p = 0.049) and the frequency of care by a second caregiver (χ^2 = 12.5, p = 0.014) were significantly related to participants' sleep dissatisfaction.

3.2. *Demographic characteristics of care recipients*

Table 2 shows the demographic characteristics of care recipients. Their average age was 80 years. Of the care recipients, 50% were female. There were 81 (28.9%) care recipients at care level 2, 76 (27.1%) at level 3, 68 (24.3%) at level 4, and 55 (19.6%) at level 5. Over 60% had ADL difficulties in bathing (84.3%), dressing (81.8%), toileting (80.7%), and transferring (62.9%), as well as incontinence (78.6%). Almost 30% had difficulty eating. Around half the recipients needed help in daily activities as they were physically dependent (59.6%) and had severe dementia (47.5%).

A significant number of participants caring for care recipients who had difficulty with incontinence and

Table 1. Characteristics of participants (n = 280)

Variables	Total		Sleep satisfaction				p
			Satisfied (n = 91)		Dissatisfied (n = 189)		
	n	(%)	n	(%)	n	(%)	
Age (years)							
< 65	137	(48.9)	42	(46.2)	95	(50.3)	0.527[a]
≥ 65	143	(51.1)	49	(53.8)	94	(49.7)	
Gender							
Female	216	(77.1)	65	(71.4)	151	(79.9)	0.129[a]
Family relationship							
Wife	92	(32.9)	26	(28.6)	66	(34.9)	0.512[a]
Husband	37	(13.2)	16	(17.6)	21	(11.1)	
Daughter	116	(41.4)	36	(39.6)	80	(42.3)	
Son	23	(8.2)	9	(9.9)	14	(7.4)	
Other	12	(4.3)	4	(4.4)	8	(4.2)	
Duration of caregiving (years)							
< 1	39	(13.9)	9	(10.0)	30	(16.2)	0.180[a]
1-5	109	(38.9)	33	(36.7)	76	(41.1)	
> 5	127	(45.4)	48	(53.3)	79	(42.7)	
Living situation							
Living with the elderly person	266	(95.0)	87	(95.6)	179	(94.7)	1.000[a]
Living apart	14	(5.0)	4	(4.4)	10	(5.3)	
Time spent caregiving per day							
All day	71	(25.4)	15	(17.0)	56	(30.3)	0.049[a]
Half a day	72	(25.7)	24	(27.3)	48	(25.9)	
Two or three hours	65	(23.2)	22	(25.0)	43	(23.2)	
Often	59	(21.1)	26	(29.5)	33	(17.8)	
Frequency of care by a second caregiver							
No second caregiver	63	(22.5)	15	(16.9)	48	(25.4)	0.014[a]
One-two days/week or less	109	(37.9)	47	(52.8)	62	(32.8)	
Three-four days/week	40	(14.3)	13	(14.6)	27	(14.3)	
More than five days/week	66	(23.6)	14	(15.7)	52	(27.5)	

Numbers are n (%), [a] Chi-squared test.

Table 2. Characteristics of care recipients (*n* = 280)

Variables	Total		Sleep satisfaction				*p*
			Satisfied (*n* = 91)		Dissatisfied (*n* = 189)		
	n	(%)	*n*	(%)	*n*	(%)	
Age (years)							
< 65	18	(6.4)	7	(7.7)	11	(5.8)	0.605[e]
≥ 65	262	(93.6)	84	(92.3)	178	(94.2)	
Gender							
Female	153	(54.6)	52	(57.1)	101	(53.4)	0.560[e]
Care level[a]							
Level 2	81	(28.9)	29	(31.9)	52	(27.5)	0.271[e]
Level 3	76	(27.1)	28	(30.8)	48	(25.4)	
Level 4	68	(24.3)	22	(24.2)	46	(24.3)	
Level 5	55	(19.6)	12	(13.2)	43	(22.8)	
Functional status (ADL[b] difficulties)							
Bathing	236	(84.3)	72	(79.1)	164	(86.8)	0.099[e]
Dressing	229	(81.8)	71	(78.0)	158	(83.6)	0.258[e]
Toileting	226	(80.7)	68	(74.7)	158	(83.6)	0.078[e]
Transferring	176	(62.9)	51	(56.0)	125	(66.5)	0.090[e]
Incontinence	220	(78.6)	63	(69.2)	157	(83.1)	0.008[e]
Eating	80	(28.6)	19	(20.9)	61	(32.3)	0.048[e]
Degree of independent living[c]							
Independent	113	(40.4)	35	(38.5)	78	(41.3)	0.654[e]
Dependent	167	(59.6)	56	(61.5)	111	(58.7)	
Severity of dementia[d]							
Moderate	147	(52.5)	58	(63.7)	89	(47.1)	0.009[e]
Severe	133	(47.5)	33	(36.3)	100	(52.9)	
Presence of conditions or impairments							
Dementia	117	(41.8)	35	(38.5)	82	(43.4)	0.434[e]
Cerebrovascular disorder	99	(35.4)	39	(42.9)	60	(31.7)	0.069[e]
Heart disease	32	(11.4)	7	(7.7)	25	(13.2)	0.173[e]
Neurological disorder	28	(10.0)	10	(11.0)	18	(9.5)	0.702[e]
After fracture	27	(9.6)	7	(7.7)	20	(10.6)	0.443[e]
Severity of illness index							
Stable	226	(80.7)	78	(85.7)	148	(79.1)	0.016[e]
Unstable and not bedridden	31	(11.1)	12	(13.2)	19	(10.2)	
Unstable and bedridden	21	(7.5)	1	(1.1)	20	(10.7)	
Terminal status	4	(1.4)	2	(2.2)	2	(1.1)	0.597[f]
Living arrangements							
Alone	8	(2.9)	2	(2.2)	6	(3.2)	0.682[e]
With one person	69	(24.6)	25	(27.8)	44	(23.4)	
With two or more persons	201	(71.8)	63	(70.0)	138	(73.4)	

Numbers are *n* (%). [a] Care level = individual's level of care needed: "Level 2" = moderate care needed, "Level 3" = considerable care needed, "Level 4" = critical care needed, "Level 5" = maximum care needed. [b] ADL = activity of daily living. [c] Degree of independent living: "Independent" = did not need help to perform daily activities at home, "Dependent" = needed help to perform daily activities at home. [d] Severity of dementia: "Moderate" = did not need help in daily life because of dementia symptoms, "Severe" = needed help in daily life because of dementia symptoms. [e] Chi-squared test. [f] Fisher's exact test.

eating (χ^2 = 7.0, p = 0.008; χ^2 = 3.9, p = 0.048) and who had severe dementia (χ^2 = 6.83, p = 0.009) tended to be dissatisfied with their sleep. In addition, the care recipients' severity of illness index was significantly associated with participants' sleep dissatisfaction (χ^2 = 8.3, p = 0.016).

3.3. *CHAID dendrograms describing the characteristics of those who are most likely to be dissatisfied with their sleep*

CHAID dendrograms were used to explore the demographics of the participants who were dissatisfied with their sleep. Figure 1 illustrates the relative importance of the significant independent variables in determining sleep dissatisfaction. The independent variables used in the model were the demographic characteristics of the participants and care recipients. Among the factors most closely associated with a participant's sleep dissatisfaction, the care recipient's severity of illness index was the most significant (χ^2 = 12.1, df = 1, p = 0.008) in two categories: (a) unstable and bedridden and (b) stable or non-bedridden. Participants caring for care recipients who were unstable and bedridden tended to be dissatisfied with their sleep (95.7%) (Group A, Node 2) more than those caring for other groups of care recipients.

Participants caring for care recipients who were stable or non-bedridden tended not to be dissatisfied with their sleep (65.0%) (Node 1) compared to those caring for care recipients in Group A. For this group, care recipients' severity of dementia was the next most

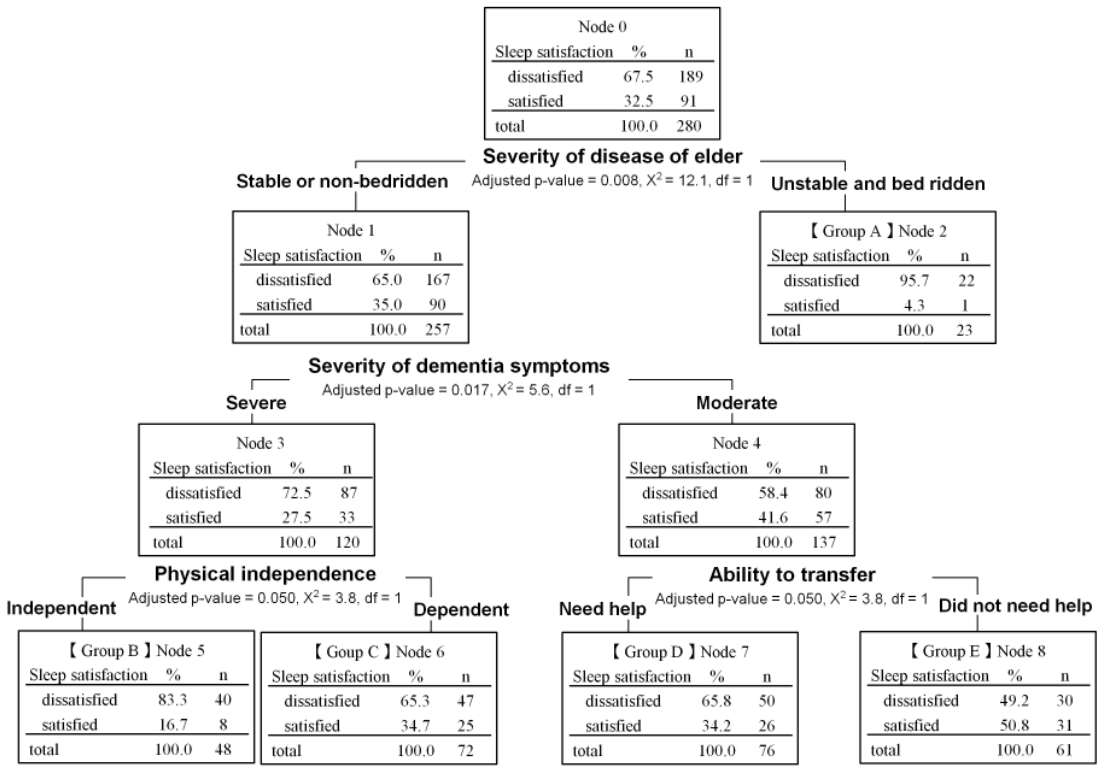

Figure 1. Dendrogram of Chi-squared Automatic Interaction Detection for sleep dissatisfaction. Care recipients cared for by a total of 280 participants were divided into 5 groups: Groups A, B, C, D, and E. The prevalence of participants who were dissatisfied with their sleep was highest among those caring for care recipients in Group A (95.7%) and lowest among those caring for care recipients in Group E (49.2%).

significant variable in determining participants' sleep dissatisfaction ($\chi^2 = 5.6$, df = 1, $p = 0.017$). Participants caring for care recipients who were stable or non-bedridden and had severe dementia symptoms tended to be dissatisfied with their sleep more (72.5%) (Node 3). In this group, participants caring for care recipients who were physically independent tended to be more dissatisfied (83.3%) (Group B, Node 5) than those caring for the other group of care recipients (65.3%) (Group C, Node 6).

Participants caring for care recipients who were stable or non-bedridden and had moderate dementia symptoms tended to be not dissatisfied with their sleep (58.4%) (Node 4). Unlike the previous group, transferring was the next most significant predictor of participants' sleep dissatisfaction ($\chi^2 = 3.8$, df = 1, $p = 0.050$); participants whose care recipients did not need help with transferring (49.2%) (Group E, Node 8) tended to be dissatisfied with their sleep significantly less than those caring for care recipients who needed help (65.8%) (Group D, Node 7). Caregivers who were dissatisfied with their sleep were least prevalent among participants caring for care recipients in Group E.

3.4. *Care needs of groups of care recipients in CHAID dendrograms*

Table 3 shows the care recipients' care needs at night. In Group A, more recipients had suction treatment needs (26.1%) and postural change needs (47.8%) than the other groups of care recipients. Care recipients in Groups A and C received care for more than two hours at night and needed more diaper changes (56.5%, 59.7%) than the other groups. Elderly individuals in Group E received care for a shorter duration than the other groups and had fewer care needs.

4. Discussion

Most developed nations emphasize preventing the deterioration associated with chronic conditions because of the increasing burden on caregivers for the elderly. To this end, clinical investigators and health care organizations have been using care services to address caregivers' sleep disturbances. Moreover, health care professionals need to develop strategies for efficient and effective care delivery.

The current study examined the characteristics of caregivers who are dissatisfied with their sleep. The independent variables used in the CHAID models are well-known risk factors associated with a sleep disturbance (*23,24*). Therefore, the primary contribution of this study is to identify caregivers who are most likely to have sleep dissatisfaction. An elderly person who needs more or greater care has a greater impact on the caregiver's sleep dissatisfaction than dose the status

Table 3. The care needs of elderly during the night

Variables	Group A[a] (*n* = 23) M (S.D.)	Group B[b] (*n* = 48) M (S.D.)	Group C[c] (*n* = 72) M (S.D.)	Group D[d] (*n* = 76) M (S.D.)	Group E[e] (*n* = 61) M (S.D.)
Length of care (min/day)	130.4 (72.8)	97.5 (87.7)	145.0 (120.3)	100.6 (71.9)	59.2 (70.8)
Care provided (*n* (%))					
Suction	6 (26.1)	0 (0.0)	1 (1.4)	3 (3.9)	0 (0.0)
Postural change	11 (47.8)	0 (0.0)	19 (26.4)	10 (13.2)	0 (0.0)
Toileting support[f]	0 (0.0)	11 (22.9)	16 (22.2)	27 (35.5)	11 (18.0)
Diaper change	13 (56.5)	18 (37.5)	43 (59.7)	25 (32.9)	4 (6.6)

Numbers are *n* (%). [a] Group A: Care recipients were unstable and bedridden. [b] Group B: Care recipients were stable or non-bedridden, had severe dementia, and could perform daily activities independently. [c] Group C: Care recipients were stable or non-bedridden, had severe dementia, and were physically dependent on caregivers to perform daily activities. [d] Group D: Care recipients were stable or non-bedridden, had moderate/no dementia, and needed help transferring. [e] Group E: Care recipients were stable or non-bedridden, had moderate/no dementia, and did not need help with transferring. [f] Toileting support includes transferring to the toilet, helping the elderly person to remove clothing, and wiping the diaper area clean.

or condition of the caregiver.

In accordance with Spielman's 3P model, sleep disturbances will occur when a person who is predisposed to poor sleep experiences an event that precipitates nighttime wakefulness. For caregivers, the precipitating event may often be providing care at night.

Care recipients in Group A were unstable and bedridden, so their caregivers had the highest risk of sleep dissatisfaction. The care recipients in this group needed more medical treatment and personal care than those in the other groups. Conversely, care recipients in Group E were stable or non-bedridden and were capable of performing ADL independently, so their caregivers had the lowest risk of sleep dissatisfaction; the prevalence of sleep dissatisfaction among these caregivers was as low as that among the general Japanese population (*25*). These results indicate that caring for an elderly individual who needs more care at night will impact the sleep of the person caring for that individual.

Care recipients in Group B needed daily help because of severe dementia but their physical abilities were unaffected, and caregivers caring for this group had the second highest risk of sleep disorders. Over 80% of caregivers caring for care recipients in Group B were dissatisfied with their sleep, which is about 20% higher than those caring for care recipients in Groups C and D. The severity of dementia among the care recipients in Group C was similar to that among care recipients in Group B, and care recipients in Group C also needed physical support. Indeed, more care recipients in Group C needed a postural change and elimination assistance than did those in Group B. Furthermore, dissatisfaction among caregivers caring for care recipients in Group C was on par with that among caregivers caring for care recipients in Group D, who did not have severe dementia but needed physical support. Clearly, if a person with dementia is awake and moving around the house at night, this behavior impacts the sleep of his/her caregiver. In other words, a caregiver's dissatisfaction with his/her sleep was not explained only by caring for an individual who had severe dementia and was physically dependent. This result shows that when care recipients were physically independent, the severity of the recipient's dementia was associated with the caregiver's dissatisfaction with his/her sleep. When care recipients were physically dependent, however, the severity of the recipient's dementia did not contribute to the caregiver's dissatisfaction with his/her sleep.

Over 80% of caregivers caring for care recipients in Groups A and B were dissatisfied with their sleep, so these caregivers are considered to have a higher risk of sleep dissatisfaction. For Group A, formal visiting nurses, rather than informal caregivers, are needed to provide medical or personal care in order to resolve the caregiver's dissatisfaction with his/her sleep. However, in Japan there is a particularly notable discrepancy between need for home-visiting nursing services at night and their actual use (*10*). More home-visiting nurses should provide care at night so that individuals caring for care recipients in Group A will be more satisfied with their sleep.

Care recipients in group B have dementia and are thus awake and moving about at night, so time-specific home care services would have difficulty resolving the dissatisfaction of persons caring for such individuals. Because a home care service provider can stay by the individual's side for only a short time of about 30 to 60 min, these services do not allow caregivers respite when the individuals they care for stay awake throughout the night.

Lessen the caregiving load of elimination assistance is crucial to resolving sleep dissatisfaction among caregivers. About 35% or more of the care recipients in all groups other than Group E needed elimination assistance, including toileting support and diaper change at night. A previous study reported that elimination assistance for community-dwelling elderly at night was usually provided by family caregivers rather than formal services (*26*).

This study had several limitations. Examining sleep dissatisfaction cannot be equated with the study of sleep disturbance. More objective examination is needed to understand sleep disturbance among high-

risk groups. Since this study was conducted in only one prefecture, it does not allow generalizations. Furthermore, the sleep hygiene and use of psychological sleep therapy, employment status, and other daily activities of caregivers were not considered. Moreover, psychological factors such as caregivers' anxiety and distress, which are considered to be related to sleep disturbance (*27*), were not included as predisposing factors. This was because this study's aim was to include only objective factors in dendrograms to facilitate use in clinical settings. However, a more multidimensional examination may better explain the sleep problems of caregivers.

Despite its limitations, this study has clarified the characteristics of caregivers who are dissatisfied with their sleep and the prevalence of sleep dissatisfaction among those individuals. In order to plan appropriate care, health care professionals need to understand the care recipient's needs and address the gap between the care provided and care recipient's needs. Caregivers providing care to care recipients in Group A were the most dissatisfied with their sleep, so more home-visiting nurse services should provide care at night. Caregivers providing care to care recipients in Group B were also dissatisfied with their sleep, but time-specific home care services would have difficulty resolving their sleep dissatisfaction. However, long-term-stay health care professionals or respite services in the community could help those caregivers.

Many studies have found a greater prevalence of insomnia among older people (*28,29*). In the near future, caregivers will also grow older. An effective and efficient health care system that addresses caregivers' sleep problems will thus be increasingly important. The current results can help health care professionals assess community needs and improve, manage, and continue the provision of care at home.

5. Conclusion

Caregivers caring for care recipients who were unstable and bedridden had the highest risk of sleep dissatisfaction. Caregivers caring for care recipients who needed daily help because of severe dementia but whose physical abilities were unaffected had the second highest risk of sleep dissatisfaction. When care recipients could not leave their bed without help, their caregivers had a slight risk of sleep dissatisfaction, regardless of the recipient's dementia and need for care. Although many recent studies have found a greater prevalence of insomnia among older people, describing the characteristics of caregivers who are most likely to experience sleep dissatisfaction is a significant challenge. When care recipients were physically independent, the severity of the recipient's dementia symptoms was related to the caregiver's dissatisfaction with his/her sleep. Among physically dependent care recipients, the severity of the recipient's dementia did not contribute to the caregiver's dissatisfaction with his/her sleep.

Acknowledgements

Financial support for this study was provided by the Health Labor Sciences Research program in 2009 in Japan. The authors wish to thank the chief care managers at the Comprehensive Community Support Center in the Konan area for their assistance in carrying out this research project. Last, but not least, the authors wish to thank the care managers and caregivers who participated in this survey.

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(*Received June 10, 2011; Revised October 16, 2011; Re-revised October 31, 2011; Accepted January 15, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.1.19

Protective effect of naringenin-7-*O*-glucoside against oxidative stress induced by doxorubicin in H9c2 cardiomyocytes

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Summary **Doxorubicin (DOX) is one of the most effective chemotherapeutic agents, but cardiotoxicity limits its clinical use. Although the mechanisms are not entirely understood, reactive oxygen species (ROS) and cardiomyocyte apoptosis appear to be involved in DOX cardiotoxicity. Protection or alleviation of DOX cardiotoxicity can be achieved by administration of natural phenolic compounds *via* activating endogenous defense systems and antiapoptosis. Naringenin-7-*O*-glucoside (NARG), isolated from *Dracocephalum rupestre* Hance, could protect from cardiomyocyte apoptosis and induce endogenous antioxidant enzymes against DOX toxicity, but the effects on intracellular ROS generation and cell membrane stability were not demonstrated. In the present study, we investigated the effects of NARG on H9c2 cell morphology, viability, lactate dehydrogenase (LDH) and creatine kinase (CK) leakage, glutathine peroxidase (GSH-Px) activity, intracellular Ca^{2+} concentration, and ROS generation. Compared with DOX alone treatment group, the morphological injury of the cells in groups treated by DOX plus NARG was alleviated, cell viability was increased, the amount of released LDH and CK was significantly decreased, the activity of GSH-Px was increased, the content of intracellular Ca^{2+} and ROS generation was lowered remarkably. These results suggest that NARG could prevent cardiomyocytes from DOX-induced toxicity by their property of stabilizing the cell membrane and reducing ROS generation.**

Keywords: Naringenin-7-*O*-glucoside (NARG), doxorubicin (DOX), reactive oxygen species (ROS)

1. Introduction

Doxorubicin (DOX) is an anthracycline antibiotic with a broad spectrum of activity and high potency against human malignant neoplasms. However, its clinical use is limited by its severe cumulative dose-related cardiotoxicity (*1*). It has been suggested that one of the molecular mechanisms responsible for DOX cardiotoxicity is the formation of reactive oxygen species (ROS) (*2*). The quinone moiety of DOX undergoes a one-electron reduction that is catalyzed by NAD(P)H reductases to yield a semi-quinone free-radical intermediate, which regenerates the parent quinone by reacting with O_2 to form O_2^- and H_2O_2 (*3,4*). These ROS can then be transformed into the more potent hydroxyl radical HO• which is capable of damaging DNA and proteins, and initiating membrane lipid peroxidation, thus eventually leading to intracellular calcium overload (*5*), cell death, and cardiac damage. Oxidative stress is now considered a major contributor as a trigger for cardiomyocyte death by apoptosis or cell necrosis (*1,6,7*). Treatment with antioxidants or natural phenolic compounds has been found to protect against DOX-induced cardiotoxicity (*8*).

The Chinese traditional medicine *Dracocephalum rupestre* Hance, a wild perennial herb found throughout western China, contains a high content of flavonoids (*9*), and has offered therapeutic potential for

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cardiovascular diseases. In our continuous search for cardioprotective substances from these natural products (*10-12*), naringenin-7-*O*-glucoside (NARG), a major active flavonoid isolated from *D. rupestre*, has been demonstrated that NARG was able to up-regulate the expression of heme oxygenase-1 (HO-1) and attenuate DOX-induced H9c2 cell apoptosis (*13*). NARG could prevent cardiomyocytes from DOX-induced toxicity by induction of endogenous antioxidant enzymes *via* phosphorylation of ERK1/2 and nuclear translocation of Nrf2 (*14*). In the present study, we investigated the effects of NARG on cell membrane stability and ROS generation in H9c2 cardiomyocytes treated with DOX. These results suggest that NARG could prevent cardiomyocytes from DOX-induced injury by their property of stabilizing the cell membrane and reducing ROS generation.

2. Materials and Methods

2.1. *Chemicals and materials*

NARG was isolated from *D. rupestre* in our laboratory (*15,16*) and dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* bioassay. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was bought from Tianjin TBD Biotechnology Development Center (Tianjin, China). 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Sigma. Lactate dehydrogenase (LDH), glutathione peroxidase (GSH-Px) and creatine kinase (CK) assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); the ROS assay kit was from Applygen Technologies Inc. (Beijing, China). Fura-2/AM Ester was provided by Biotium (Hayward, CA, USA).

2.2. *Cell culture*

Rat cardiac H9c2 cells (ATCC Rockville, MD, USA) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 5% CO_2 at 37°C. The cells were fed every 2-3 days and subcultured once they reached 70-80% confluence. Cells were plated at an appropriate density according to each experimental design.

2.3. *Cell treatment with NARG*

H9c2 cells were incubated with NARG for 24 h followed by incubation with DOX (10 μM) for another 24 h. After this incubation, cells were harvested after trypsin digestion by centrifugation (1,000 rpm × 5 min) and parameters were measured as described in materials and methods.

2.4. *In vitro cell proliferation*

H9c2 cells were seeded in 96-well plates at a density of 5,000 cells/well. Following overnight adherence, cells were incubated with NARG (5, 10, 20, 40, and 80 μM) in DMEM supplemented with 0.5% fetal bovine serum at 37°C for 24 h followed by incubation with/without DOX (10 μM) for another 24 h, and then cell proliferation was determined by MTT assay. Cells were treated with MTT solution (final concentration, 0.5 mg/mL) for 4 h. The supernatants were removed carefully, followed by addition of 100 μL DMSO to each well to dissolve the precipitate. The absorbance was measured at 570 nm in a microplate reader (Synergy HT).

2.5. *Analysis for generation of ROS*

The production of ROS was measured by detecting the fluorescent intensity of an oxidant-sensitive probe CM-H2DCFDA, which is a stable nonfluorescent molecule that passively diffuses into cells, where the acetate can be cleaved by intracellular esterases to produce a polar diol that is well retained within the cells. H9c2 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and 1×10^6 cells/well in a 6-well plate. The next day, cells were pretreated with/without NARG (5, 10, and 20 μM) for 24 h followed by incubation with DOX (10 μM) for another 24 h. Then cells were loaded with CM-H2DCFDA (10 μM) as per the manufacturer's protocol for 3 h. Fluorescent intensity was recorded by excitation at 485 nm and emission at 535 nm using a Wallac 1420 Multilabel Counter (Wallac, Turku, Finland). Cells in the 6-well plate were observed under a fluorescence microscope (IX-7, Olympus, Tokyo, Japan) (×200).

2.6. *Cell morphological analysis*

H9c2 cells were seeded at a density of 1×10^6 cells/well in a 6-well plate, and the cells were grown overnight at 37°C in a humidified incubator with 5% CO_2. The next day, cells were pretreated with/without NARG (10 and 20 μM) for 24 h, and then exposed to DOX (10 μM) for another 24 h. After this incubation, cell morphology was examined without/with Wright's Giemsa staining under an inversion microscope (×200).

2.7. *Measurement of excellular LDH content, CK levels and cellular GSH-Px activity*

H9c2 cells were treated with NARG for 24 h, followed by incubation with 10 μM DOX for another 24 h. After collecting cell culture supernatants extracellular LDH content and CK levels were measured using commercially available colorimetric assay kits respectively. Treated cells were harvested and resuspended in cell lysis buffer (*13*), supernatants

separated were used for measurement of GSH-Px activity according to the commercially available colorimetric assay kits.

2.8. *Measurement of intracellular Ca^{2+} concentration*

Intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was measured by incubating H9c2 cells with the fluorescent Ca^{2+} indicator Fura-2/AM using the Multilabel Counter Victor-1420. Briefly, H9c2 cells were seeded at a density of 1×10^6 cells/well in a 6-well plate, and the cells were grown overnight at 37°C in a humidified incubator with 5% CO_2. The next day, cells were pretreated with/without NARG (5, 10 and 20 μM) for 24 h, and exposed to DOX (10 μM) for another 24 h. After this incubation, cells were resuspended in Hanks' solution at a density of 10^6-10^7 cells/mL. Trypan blue staining showed a 90%-95% cellular viability rate. A final concentration of 5 μM Fura-2/AM was added to the above isolated cell suspensions incubated at 37°C for 35 min, and then were washed with Hank's solution containing 0.1% bovine serum albumin to wash out the residual Fura-2/AM. The cells were resuspended in Hank's solution and incubated for five minutes at 37°C prior to measurements. The basal emission was measured by stimulating the cells with 340 to 380 nm light and recording the emitted fluorescence intensity at 510 nm. In order to calculate $[Ca^{2+}]i$, calibration at high and low $[Ca^{2+}]i$ was made after the cells were treated with 0.1% Triton X-100 in PBS, followed by exchanging with calcium-free medium containing 5 mM EGTA in PBS. This produced calcium signals equivalent to saturated calcium and to zero calcium. Calculation of intracellular calcium was made using the following equation (*17,18*): $[Ca^{2+}]i = K_d(R\text{-}R_{min})/(R_{max}\text{-}R)F_{min}/F_{max}$ where K_d is the dissociation constant of Fura-2 for Ca^{2+} and is assumed to be 224 nM at 37° C. R is the ratio of corrected fluorescence at 340 and 380 nm. Rmax is the ratio obtained after 0.1% Triton X-100 treatment. Rmin is the ratio obtained after EGTA treatment. Fmin and Fmax are fluorescent intensity at 380 nm after EGTA and 0.1% Triton X-100 treatment respectively.

2.9. *Statistical analysis*

All data are expressed as mean ± S.D. from at least four independent experiments. Differences between mean values of multiple groups were analyzed by Student's *t*-test. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. *NARG protects H9c2 cells from DOX-induced cytotoxicity*

H9c2 cells were treated with NARG (5, 10, 20, 40, and 80 μM) in the absence of DOX for 24 h and then the rates of cell growth inhibition were evaluated. As shown in Figure 1A, NARG at each of these concentrations alone did not cause any apparent cytotoxicity. To analyze the effects of NARG on DOX-induced cytotoxicity in H9c2 cells, cell proliferation was examined after incubation with NARG in the presence of DOX (10 μM). As shown in Figure 1B, NARG (5, 10, 20, 40, and 80 μM) pretreatment provided significant protective effects on DOX-mediated cytotoxicity in a dose-dependent manner at low doses.

To determine whether NARG could protect H9c2 cells from DOX-induced injury, cell morphology was examined without/with Wright's Giemsa staining under an inverted microscope. As shown in Figure 2A, normal cells were seen as spindle-shaped with integral and clear structures (Aa). NARG (10 and 20 μM) alone had no apparent effects on H9c2 cells (Ab and Ac). Treatment with DOX (10 μM) for 24 h leads to alterations in cell shape, which include cell shrinkage starting from the periphery and accompanied by membrane blebbing. DOX also induced rapid changes in the nuclear morphology of H9c2 cells with increased nucleus size and chromatin condensation (Ad). Pretreatment with 10 and 20 μM NARG significantly protected the cells from the morphological changes induced by DOX as shown in Figure 2A (Ae and Af). This result is consistent with the morphological analysis using Wright's Giemsa

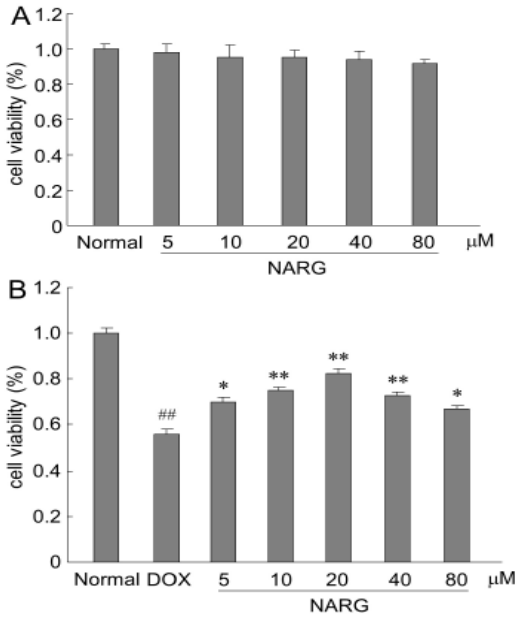

Figure 1. Effects of NARG on DOX-induced injury in H9c2 cells. Proliferation of H9c2 cells exposed to NARG in the absence **(A)** or presence **(B)** of DOX *in vitro*. Cells were incubated without or with NARG (5, 10, 20, 40, and 80 μM) for 24 h, followed by incubation with (10 μM) DOX for another 24 h. After this incubation, cell viability was determined using the MTT assay. Values represented are means ± S.D. ($n = 6$). $^{\#\#}$ $p < 0.01$ compared to the normal group; * $p < 0.05$, ** $p < 0.01$ compared to DOX group.

staining (Figure 2B).

3.2. *Determination of cellular ROS*

One potential mechanism for DOX-induced cell damage seems to be linked to an increased production of ROS (*2,19*). Oxidative stress precedes the development of irreversible cell injury after DOX exposure in H9c2 cells. To determine whether pretreatment with NARG mitigated DOX-induced early oxidative stress, cellular ROS contents were measured by incubating the control or drug-treated cells with 10 μM CM-H2DCFDA. As shown in Figures 3A and 3B, exposure to DOX without NARG significantly increased fluorescence, indicating that DOX generated oxidative stress. Pretreatment with NARG (5, 10, and 20 μM) significantly inhibited the elevated intracellular concentration of ROS compared with DOX treated cells.

3.3. *Measurement of excellular LDH content and CK levels and GSH-Px activity*

The integrity of plasma membranes was determined by monitoring the activity of cytoplasmic enzyme LDH and CK in the extracellular incubation medium, which represents a common procedure to determine membrane leakage and cellular damage. As shown in Table 1, compared with normal cells, exposure to DOX without NARG, the released amount of LDH and CK was increased for both; the activity of GSH-Px was decreased. Compared with DOX, NARG significantly decreased the amount of released LDH and the content of CK, but the activity of GSH-Px was increased; these results suggest that NARG could prevent cardiomyocytes from DOX-induced toxicity partly by their property of stabilizing cell membranes.

3.4. *The measurement of the intracellular Ca^{2+} concentration*

DOX-mediated alteration of Ca^{2+} homeostasis is another possible mechanism of cardiotoxicity. Intracellular Ca^{2+} accumulation is thought to initiate myocardial injury and contractile impairment. In the present study, intracellular Ca^{2+} levels were determined using the intracellular Ca^{2+} probe Fura-2/AM. As depicted in Figure 4, $[Ca^{2+}]i$ level was quite low in normal cells. Cardiomyocytes exposed to DOX without NARG had

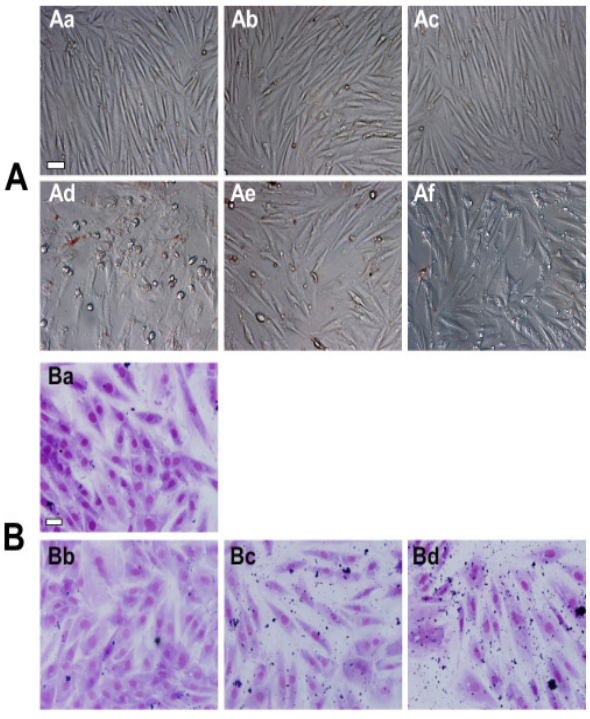

Figure 2. Morphology of H9c2 cells treated with NARG and DOX. H9c2 cells were treated without or with (10 and 20 μM) NARG for 24 h, followed by incubation with 10 μM DOX for another 24 h. Morphology of H9c2 cells was assessed using an Olympus inverted phase contrast microscope (×200) equipped with a quick imaging system. All images are the same magnification; scale bar = 20 μm. **(A)**: images without Wright's Giemsa staining. **Aa**, contol; **Ab**, 10 μM NARG; **Ac**, 20 μM NARG; **Ad**, 10 μM DOX; **Ae**, 10 μM NARG plus 10 μM DOX; **Af**, 20 μM NARG plus 10 μM DOX. **(2B)**: images with Wright's Giemsa staining. **Ba**, contol; **Bb**, 10 μM DOX; **Bc**, 10 μM NARG plus 10 μM DOX; **Bd**, 20 μM NARG plus 10 μM DOX.

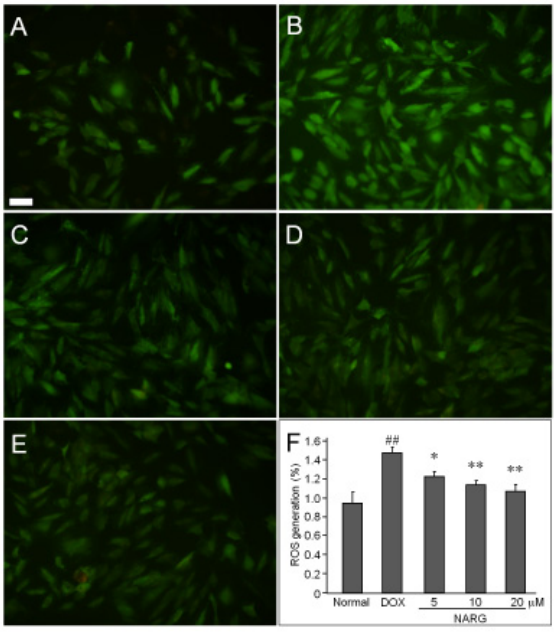

Figure 3. Effect of NARG on intracellular ROS concentration in H9c2 cells. H9c2 cells were treated without or with (5, 10 and 20 μM) NARG for 24 h, followed by incubation with 10 μM DOX for another 24 h. ROS generation was assayed by CM-H2DCFDA (10 μM) oxidation-based fluorescence using a fluorescence microscope (magnification, ×200). All images are the same magnification; scale bar = 20 μm. **(A)**, contol; **(B)**, 10 μM DOX; **(C)**, 5 μM NARG plus 10 μM DOX; **(D)**, 10 μM NARG plus 10 μM DOX; **(E)**, 20 μM NARG plus 10 μM DOX. Fluorescent intensity was measured using a microplate reader **(F)**. Signal intensity from six independent experiments was averaged for each condition. $^{\#\#}p < 0.01$ compared to the normal group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to DOX group.

Table 1. Effects of NARG on excellular LDH content, CK level, and activities of cellular GSH-Px (mean ± S.D., $n = 5$)

Group	Final concentration (μM)	LDH (U/mL)	TCK (U/mL)	GSH-Px (U/mL)
Normal	Equal volume	0.42 ± 0.02	0.91 ± 0.01	167.0 ± 6.6
DOX	10	0.78 ± 0.10##	1.83 ± 0.15##	76.6 ± 9.3##
NARG	5	0.73 ± 0.06	1.29 ± 0.14*	98.5 ± 8.4*
	10	0.60 ± 0.04*	1.13 ± 0.02**	114.7 ± 7.7**
	20	0.59 ± 0.08*	1.02 ± 0.02**	127.1 ± 8.1**

$p < 0.01$ *vs.* normal and * $p < 0.05$, ** $p < 0.01$ *vs.* DOX.

significantly increased concentrations of intracellular Ca^{2+} ($p < 0.01$). However, pretreatment with NARG (5, 10, and 20 μM) inhibited DOX-induced $[Ca^{2+}]i$ rise in a dose-dependent manner.

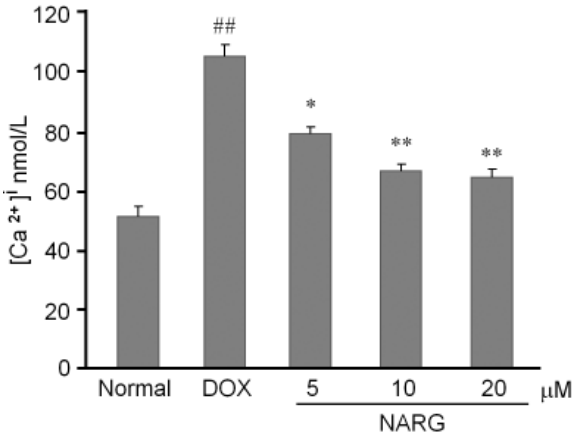

Figure 4. Effect of NARG on intracellular Ca^{2+} concentration in H9c2 cells. H9c2 cells were treated without or with NARG for 24 h, followed by incubation with 10 μM DOX for another 24 h. The intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was measured by incubating H9c2 cells with Fura-2/AM. ## $p < 0.01$ compared to the normal group; * $p < 0.05$, ** $p < 0.01$ compared to DOX group.

4. Discussion

DOX is a widely used chemotherapeutic agent for treatment of various cancers. However, its clinical use is limited by its severe cumulative dose-related cardiotoxicity (*20,21*). Although DOX-induced injury appears to be multifactorial, one of the possible mechanisms is cellular damage mediated by generation of ROS (*22*). DOX-induced cardiotoxicity is associated with the accumulation of DOX in the mitochondria and ROS production (*19,23*). The heart is especially susceptible to oxidative damage because cardiomyocytes are rich in mitochondria, the site of basal ROS generation, and are exposed to relatively high oxygen tension compared to other tissues. In normal healthy cells, endogenous antioxidants, such as manganese superoxide dismutase (MnSOD), are able to detoxify basal ROS generation in mitochondria. However, as DOX accumulates and generates ROS, the system becomes easily overwhelmed, and thus, oxidative damage occurs. Therefore, an effective cardioprotective agent perhaps acts partly by preventing DOX-induced ROS production.

Furthermore, ROS may lead to cardiomyocyte apoptosis, endogenous antioxidant system destruction and Ca^{2+} overload. Ca^{2+} overload can further increase ROS synthesis and is considered an initiating agent of cell apoptosis. Therefore, protection or alleviation of DOX cardiotoxicity can be achieved by reducing oxidative damage, decreasing Ca^{2+} overload, and activating endogenous defense systems.

Due to the successful action of DOX as a chemotherapic agent, several strategies have been tried to prevent/attenuate the side effects of DOX. Treatment with antioxidants or natural phenolic compounds such as flavonoids has been found to protect against DOX-induced cardiotoxicity *via* radical-scavenging (*24*), iron-chelating (*25*), activating endogenous defense systems (*26,27*) and regulating intracellular Ca^{2+} homeostasis (*5*).

It has been demonstrated that NARG, a major active flavonoid isolated from *D. rupestre* Hance, did not cause apparent cytotoxicity at each investigated low dose alone and could prevent H9c2 cardiomyocytes from DOX-induced toxicity by induction of endogenous antioxidant enzymes *via* phosphorylation of extracellular signal-regulated kinase1/2 (Erk1/2) and nuclear translocation of nuclear factor E2 P45-related factor 2 (Nrf2) (*13*). NARG could protect against cardiomyocyte apoptosis by modulation of apoptosis-related genes (Bcl-2, caspase-3, and caspase-9) and HO-1 expression (*14*). Pretreatment with NARG could elevate not only the activities of SOD and catalase (CAT) but also phase 2 metabolizing enzymes such as HO-1 and NAD(P)H: quinine oxidoreductase 1 (NQO1). NARG also could decrease the level of malonaldehyde (MDA) and increase the intracellular reduced glutathione (GSH) level by up-regulating the expression of glutamate-cysteine ligase modifier subunit (GCLM) and glutamate-cysteine ligase catalytic subunit (GCLC) mRNA (*13*). In this study, we found that pretreatment with NARG increased the activity of GSH-Px.

Efficient detoxification of ROS requires the coordinated actions of various cellular antioxidant enzymes. Accordingly, simultaneous induction of key cellular antioxidant enzymes by NARG in cardiomyocytes appears to be a promising strategy for protecting against oxidative injury and may be an

important mechanism underlying the protective effects of NARG observed in DOX-induced cardiotoxicity. Although the direct antioxidant effects of NARG were not studied. In the present study, we found that compared with DOX, the morphological injury of cells treated with NARG was alleviated, cell viability was increased; the amount of released LDH and CK was significantly decreased; and the content of intracellular Ca^{2+} and ROS generation was lowered remarkably. These results suggest that NARG could prevent cardiomyocytes from DOX-induced toxicity by its property of stabilizing cell membranes and reducing ROS generation.

In conclusion, the protection by NARG against DOX-induced oxidative damage and cardiomyocyte death occurs through inhibition of cell death, increased antioxidant activity, decreased cellular calcium overload, and increased mitochondrial function. The findings in our paper of NARG protection against DOX-induced cardiotoxicity conclude that in H9c2 cardiomyocytes NARG is able to prevent DOX-induced oxidative damage and cell death, which is very promising; however, whole animal studies are necessary as the next step to evaluate the ability of NARG to protect the heart from DOX *in vivo*.

Acknowledgements

This work was supported by grants from the Natural Science Foundation of Shandong Province (No. Y2007C098) and the Technology Development Planning of Shandong Province (No. 2009GG10002083) of China.

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(*Received November 19, 2011; Revised February 8, 2012; Accepted February 9, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.1.26

Experimental study on inhibition of rat ventricular I_{k1} by RNA interference targeting the *KCNJ2* gene

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Summary **The dominant-negative inhibition of *KCNJ2*-encoded inward rectifier potassium channels (Kir2) is currently considered the best approach to biological pacemakers. We hypothesized that inhibition of the inward rectifier potassium current (I_{K1}) in ventricular myocytes by RNA interference (RNAi) would convert ventricular myocytes into pacemaker cells. Five pieces of short hairpin RNA (shRNA) were designed to target the *KCNJ2* gene and then plasmids incorporating shRNA and green fluorescent protein (GFP) as a marker were constructed for transfection into rat ventricular myocytes. The levels of *KCNJ2* mRNA were analyzed with real-time quantitative RT-PCR to screen for pieces of shRNA that were effective at inhibiting the expression of the *KCNJ2* gene. The activity of potassium ionic channels was then studied in the transfected ventricular myocytes. In the recombinant plasmids, LYS2 transfection significantly inhibited the mRNA of the *KCNJ2* gene in comparison to other groups ($p < 0.05$), and the beating frequency of ventricular myocytes increased after LYS2 transfection. The open probability of I_{K1} potassium ion channels of cardiac myocytes transfected with the LYS2 plasmid was significantly down-regulated ($p < 0.05$) and the I_{K1} of ventricular myocytes was also significantly suppressed compared to the negative group ($p < 0.05$). Our study demonstrated that I_{K1} was clearly inhibited after the inhibition of *KCNJ2* gene expression by RNAi, and this may represent a new approach to the study of biological pacemakers.**

Keywords: RNA interference, *KCNJ2*, plasmid, biological pacemaker

1. Introduction

Since Swedish cardiac surgeon Ake Senning implanted the first built-in cardiac pacemaker in 1958 (*1*), the electrical cardiac pacemaker has become the gold standard for treatment of sinus bradycardia caused by sick sinus syndrome and a high-degree atrioventricular block. However, there are some complications that may be difficult to overcome with a built-in pacemaker, and such pacemakers are not suitable for patients with a high risk of infection or who are too young (*2*). Given cardiac physiological function and body adaptability, the biological pacemaker is expected to be the ideal pacemaker (*3*). At present, the study of biological pacemakers is focused mainly on three gene therapy strategies: i) Upregulation of β2-adrenergic receptors, which was found to increase the atrial rate in porcine hearts (*4*), ii) Overexpression of inward depolarizing current, which is coded for by hyperpolarization-activated, cyclic-nucleotide-gated (HCN) and is the primary pacemaker current in the sinoatrial node (*5*), and iii) Dominant-negative therapy to inhibit the inward rectifier potassium current (I_{K1}) (*6*). In the latter two strategies, the resting membrane potential (RMP) is disturbed to generate spontaneous slow diastolic depolarization. In addition to these gene therapy strategies, various cell therapy approaches such as stem cell therapy and an adult somatic cell-fusion approach have yielded some results (*7*).

The use of dominant-negative therapy to inhibit the inward rectifier I_{K1} is currently the most promising approach to biological pacemakers (*8*). I_{K1} is considered to serve as the primary conductance controlling the RMP and contributes significantly to repolarizing

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current during the terminal phase of the action potential (AP) in ventricular myocytes. Kir2.1 subunits, encoded by the *KCNJ2* gene, assemble to form tetrameric inward rectifier potassium channels in many cell types, including cardiac myocytes (*9,10*). Thus, Kir2.1 is essential to the generation of I_{K1}. The possibility that pacemaker activity is latent and is normally repressed by I_{K1} was investigated in ventricular myocytes. Ventricular myocytes will presumably be converted to pacemaker cells when I_{K1} is inhibited (*11*). Miake *et al.* (*6*) built a dominant negative construct by replacing three amino acid residues in the pore of Kir2.1, resulting in idioventricular pacemaker function in the guinea pig ventricle.

RNA interference (RNAi) is a type of effective experimental technique in molecular biology that was recently developed (*12,13*). The current study constructed and screened out short hairpin RNA (shRNA) in response to the *KCNJ2* targeting gene and then transfected that shRNA into rat ventricular myocytes. The open probability (Po) of I_{K1} potassium ion channels in transfected recombinant plasmids should be significantly down-regulated and the I_{K1} of the ventricular myocytes should be significantly suppressed, providing a new idea for and approach to the study of biological pacemakers.

2. Materials and Methods

2.1. *Design and synthesis of coded shRNA oligonucleotide DNA segments*

In accordance with the mRNA nucleotide sequence of the rat gene *KCNJ2* (NM-017296), five pairs of shRNA sequences were designed depending on bases in the coding area: +109 ~ +117 AACGCAATGCCGGAGTTCATA, +361 ~ +379 AAGCGTGTGTGTCTGAGGTCA, +926 ~ +934 AAGTCCATACCCGACAACAGT, +1156 ~ +1174 AAGAGGAAGAGGACAGTGAGA, +1176 ~ +1194 AACGGAGTTCCAGAGAGCACA. The structure of the DNA primer that transcribes the shRNA is as follows: BamHI + Sense + Loop + Antisense + Terminal Signal + EcoRI/SacI/SalI/SacI/XbaI + HindIII (the enzyme digestion sites of EcoRI, SacI, SalI, SacI and XbaI were designed respectively in the inserted segments targeting the gene in question, and segments were inserted between the two enzyme digestion sites of BamHI and HindIII).

2.2. *Plasmid construction*

Double enzyme digestion of BamHI + HindIII was carried out on the plasmid Pgenesil-1 (Figure 1A) and then longer segments were collected on a 1% agarose gel. Synthesized single-chain targeting gene segments were synthesized with 50 μL of annealing buffer and then cooled to room temperature after a water bath at 94ºC. Annealed segments were ligated with linear Pgenesil-1 plasmid-expressing vectors. After cloning into *Escherichia coli* DH5a, clones were selected on LB plates. Plates were left overnight in a warm box at 37ºC, and then the plasmid was extracted. The recombinant plasmids were termed LYS1, LYS2, LYS3, LYS4, LYS5, and HE (negative control). Enzyme digestion and sequencing of the recombinant plasmids were done by Wuhan Genesil Biotechnology Co., Ltd. The DNA sequence of transcribed shRNA includes 47 bps, with two 19-bp reverse repetitive sequences on both ends and a 9-bp ringlike structure in the middle. The hairpin DNA sequence with the U6 promoter was transcribed into shRNA by internal RNA polymerase III.

2.3. *Separation and identification of ventricular myocytes in newborn rats*

Fifteen newborn Wistar rats (provided by the laboratory animal center of Shandong University) 1-2 days of age were sacrificed by decapitation and their cardiac ventricles were removed. Ventricular myocytes were purified twice through enzyme digestion and differential attachment in culture dishes. Myocytes were cultured in six-well tissue culture plates, and 5×10^5 ventricular myocytes that were cultured at 37ºC in 5% CO_2 were added to each well. Ventricular myocytes were identified by immunohistochemical analysis with anti-α-actin antibody and anti-myosin antibody.

2.4. *Plasmid transfection in ventricular myocytes*

Liposomes were used to facilitate transfection. Metafectene liposomes and plasmid were mixed at a ratio of 3:1 (*14*). The culture medium with serum and antibiotic was removed before transfection and washed with PBS three times, and then culture medium without serum and antibiotic was added. One hour later, the ventricular myocytes were transfected when the myocytes reached 50-60%. The mixture was placed in six-well culture plates and 0.8 mL of culture medium without serum and culture was added; plates were kept at 37ºC for 6-8 h. Then, 1 mL of culture medium with 20% fetal bovine serum was added in 5% CO_2 and cultured for another 48 h at 37ºC. The transfection rate was measured using flow cytometry.

2.5. *Establishment of a method of real-time PCR detection of KCNJ2 mRNA*

Total RNA from rat ventricular myocytes was extracted, reverse transcription was used to construct cDNA, and *KCNJ2* and β-actin segments were amplified. Specific primers were designed in accordance with the mRNA sequence of the *KCNJ2* gene and the *KCNJ2* and β-actin segments were amplified. The primers were provided by

Shanghai Bioengineering Co., Ltd. Primer for the *KCNJ2* gene: upstream 5'- TGCCCGATTGCTGTTTTC-3', downstream 5'- GGCTGTCTTCGTCTATTT-3'(amplified segment 373 bp; Primer for β-actin: Upstream 5'-AACCCTAAGGCCAACCGTGAAA-3', downstream 5'- TCATGAGGTAGTCTGTCAGGTC-3'(amplified segment 241 bp). The RT-PCR reaction system for the *KCNJ2* gene and β-actin was as follows: reaction conditions: 94ºC for 10 min, 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 30 sec, 40 cycles; 72ºC for 2 min. The segments were cloned into plasmids as usual. Conditions for real-time quantitative RT-PCR were varied. The results were analyzed by calculating the Ct values for *KCNJ2* and β-actin in samples. PCR products were quantitatively analyzed given a standard quantitative curve.

Cultured ventricular myocytes from newborn Wistar rats were divided into three groups: (1) Experimental group (positive group): transfected with vector plasmid. (2) Control group (negative group): transfected with empty vector plasmid, and (3) Blank group: No treatment. The relative rate of *KCNJ2* mRNA expression in the three groups was analyzed *via* real-time quantitative RT-PCR, and the rate of suppression of mRNA was calculated.

2.6. *Western blot analysis*

KCNJ2 expression was examined in ventricular myocytes transfected with plasmid vectors using Western blotting. Cells were harvested in a lysis buffer (2% SDS, 50 mM Tris, pH 7.4/1 mM EDTA/ protease inhibitor mixture) 96 h after transfection and homogenized by sonification. Equal amounts of protein (40 μg) were separated by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels, blotted on nitrocellulose, probed with anti-Kir2.1 goat polyclonal IgG (Santa Cruz Biotechnology) and subsequently with rabbit-anti-goat (HRP) (Beijing Zhong Shan Golden Bridge Biological Technology), and detected by chemiluminescence (Roche). Antibodies against β-actin (Sigma-Aldrich) were used to measure protein loading.

2.7. *Recording of single-channel current*

The activity of inward potassium channels was recorded using the whole-cell patch-clamp technique at 37°C with an Axopatch 200B amplifier while sampling at 10 kHz (for currents) or 2 kHz (for voltage recordings) and filtering at 2 kHz. Pipettes had a tip resistance of 2-4 MΩ when filled with the internal recording solution.

Cells were superfused with a physiological saline solution containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 1 mM $MgCl_2$, and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. The pipette solution consisted of 130 mM K-glutamate, 19 mM KCl, 10 mM Na-HEPES, 2 mM EGTA, 5 mM Mg-ATP, and 1 mM $MgCl_2$; pH was adjusted to 7.2 with KOH. For I_{K1} recording, $CaCl_2$ was reduced to 100 μM, $CdCl_2$ (200 μM) was added to block $I_{Ca,L}$, and I_{Na} was steady-state inactivated by using a holding potential of –40 mV. To obtain I_{K1} as a Ba^{2+}-sensitive current, currents recorded before and after the addition of Ba^{2+} (500 μM) were subtracted. Data were recorded with pClamp9.0 software.

2.8. *Analysis of single-channel current*

Single-channel data were analyzed with QUB single-channel activity analyzers in order to obtain the current of single channels, channel close probability (Pc), and the number of channels in the membrane. The normalized value of 5 sec was chosen for each channel analyzed. The following formula was used to calculate the open probability (Po) of a certain channel: $Po = 1 - Pc^{1/N}$ (N refers to the number of channels in the membrane, and Pc refers to the probability that N ionic channels were closed at the same time) (*10*).

2.9. *Statistical analysis*

The SPSS 11.0 for WINDOWS statistical software package was used to analyze one-way variance in basic data that were expressed as the mean ± S.E.M. A *t*-test was used for comparison among the groups, and $p < 0.05$ represented a significant difference. One-way ANOVA was used to compare the effects stretch-induced channel activity. $p < 0.05$ indicated a significant difference, and $p < 0.01$ indicated a highly significant difference.

3. Results

3.1. *Identification of plasmid Pgenesil-1 and enzyme digestion of recombinant plasmids*

BamHI and HindIII enzyme digestion was carried out on the plasmid Pgenesil-1. After enzyme digestion, ringlike plasmids were converted into linear plasmids (Figure 1B). The multiple cloning sites (MCS) of the plasmid Pgenesil-1 were as follows: –HindIII–insertDNA–BamHI–U6 Promoter–EcoRI–SalI–XbaI–DraIII–. Analysis of enzyme digestion indicated that the plasmids LYS1-1, LYS2-4, LYS3-8, LYS4-22, and LYS5-9 all met the design requirements (Figures 1C and 1D).

3.2. *Sequencing of recombinant plasmids*

The transfected plasmid bacterium solution was used to sequence the plasmid. The shRNA-encoding sequences of six recombinant plasmids were identical to those of the designed segments, indicating that the recombinant plasmids were correctly constructed.

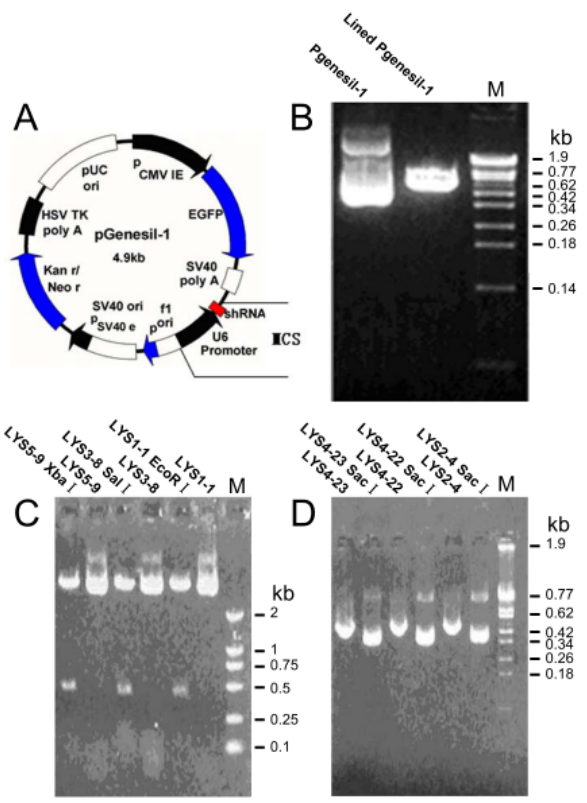

Figure 1. Restriction map of the Pgenesil-1 plasmid and recombinant plasmids.

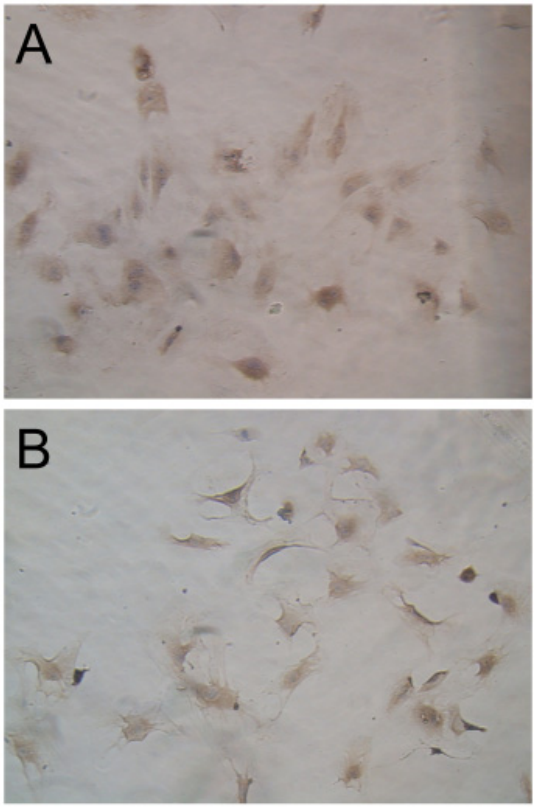

Figure 2. Identification of ventricular myocytes. Anti-actin **(A)** and anti-myosin **(B)** immunohistochemistry(inverted microscope, 200×): the cytoplasm of ventricular myocytes was stained brownish-yellow.

3.3. *Identification of ventricular myocytes*

The purity of the ventricular myocytes was 95.1% (according to positivity for anti-α-actin) and 94.8% (according to positivity for anti-myosin) (Figure 2).

3.4. *Inhibition of mRNA of the KCNJ2 gene after LYS2 transfection*

A logarithmic chart of the corresponding concentrations according to the cycle threshold value (Ct) of β-actin and *KCNJ2* resulted in two straight lines. The relevant coefficients of β-actin and *KCNJ2*, *i.e.*, straight lines indicated by real-time quantitative measurement, were –0.993 and –0.999, respectively, and the gradients were –3.24. According to the formula E = 10-1/S-1, the amplification efficiency of the two genes was 100%. The Ct value for plasmid LYS2 was higher in the intervention group than in the blank group, and the rate of inhibition after correction was 86%, which was significantly greater than other suppression rates ($p < 0.05$). Plasmid LYS2 was thus used to transfect cells.

3.5. *Inhibition of Kir2.1 protein expression after LYS2 transfection*

Western blot analysis indicated that the experimental group had low levels of expression of the Kir2.1 protein

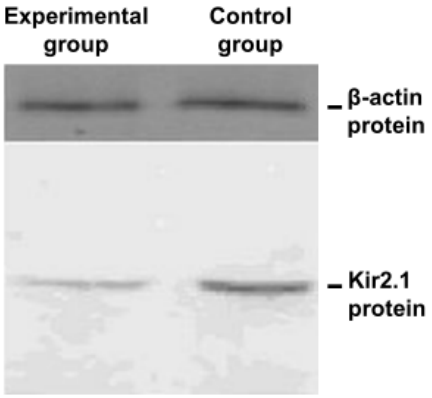

Figure 3. Western blotting analysis of LYS2-transfected ventricular myocytes and non-transfected ventricular myocytes (left-to-right). The experimental group had a lighter Kir2.1 protein blot than the control group, indicating that Kir2.1 protein expression was suppressed by RNAi in the experimental group.

while the control group had high levels of expression of that protein. There were no significant differences in β-actin protein expression in the two groups (Figure 3).

3.6. *Increase in the beating frequency of ventricular myocytes after RNAi*

Observed under a microscope, adjacent ventricular myocytes extended pseudopods to interlace and connect

as a syncytium with the same beating frequency, which is designated here as a cell cluster. After 24 h (transfected LYS2), the experimental group and negative plasmid control group had significantly fewer beating cell clusters than the blank group, and their beating frequency was lower than that in the blank group ($p < 0.01$). After 48 h, the number of beating cell clusters in the experimental group and the negative plasmid control group increased, and the beating frequency increased compared to the beating frequency at 24 h. The increase in clusters was more obvious in the experimental group, and the beating frequency was more obvious in the experimental group, and the beating frequency was greater than that in the two control groups ($p < 0.01$). There were no significant differences between the two control groups. After 72 h, the beating frequency increased in the experimental group compared to the beating frequency at 48 h. There were no significant changes in the two control groups. The experimental group had a significant faster beating frequency than did the two control groups ($p < 0.01$). There were no significant changes in the beating frequency from 72 h to 96 h for any of the groups, indicating that the RNAi effect was stable (Figure 4A).

3.7. *Identification of I_{K1} potassium ionic channels in rat ventricular myocytes*

Ca^{2+} current was blocked by adding 0.3 mM of CdCl to Tyrode's solution. The membrane voltage was hyperpolarized to –120 mV and then depolarized to +30 mV in steps. I_{K1} current was induced for 300 msec with a stimulation frequency of 0.5 Hz and command voltage of 10 mV at each step (Figures 4B-4D).

3.8. *Suppression of the I_{K1} of ventricular myocytes by RNAi*

Five membranes with little background channel activity were analyzed. At a voltage of +10 mV, the normal group had a significantly higher Po than did the transfected plasmid group ($p < 0.05$). At a voltage of −40 mV, the normal group had a higher Po than did the transfected plasmid group ($p < 0.01$) (Table 1). At different voltages, the transfected plasmid group had a significantly lower I_{K1} current in ventricular myocytes than did the control group ($p < 0.05$) (Figure 5).

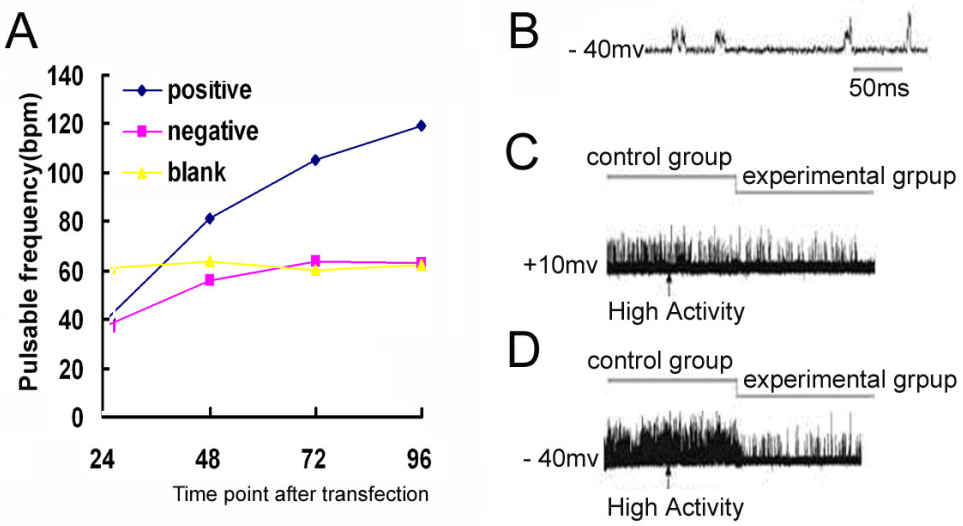

Figure 4. The I_{k1} activity of ventricular myocytes decreased and their beating frequency increased after RNAi. (A) Changes in the beating frequency of cardiomyocytes after transfection; **(B)** Activity of the normal inward rectifier K^+ channel current in rat ventricular myocytes (in a cell-attached state, −40 mV membrane potential control. Sustained recording for 1 min); **(C)** At a voltage of +10 mV, the LYS2-transfected group had less I_{k1} activity than the control group; **(D)** At a voltage of −40 mV, the LYS2-transfected group had much less I_{k1} activity than the control group.

Table 1. Real-time quantitative detection of *KCNJ2* mRNA expression after 72 h of RNAi

Group	*LYS1*	*LYS2*	*LYS3*	*LYS4*	*LYS5*	*HE*	Blank
ΔCt	−10.23	−10.17	−10.24	−10.28	−10.26	−10.26	−10.21
ΔΔCt	−0.02	−0.5	−0.03	−0.07	−0.05	−0.05	
Suppression rate	--	0.29	--	--	--	--	
Corrected suppression rate	--	0.86	--	--	--	--	

Note: ΔCt: The difference between β-actin Ct and *KCNJ2* Ct; ΔΔCt =ΔCtb − ΔCtf (ΔCtb: The difference between β-actin Ct and *KCNJ2* Ct in the transfected groups and negative group; ΔCtf: The difference between β-actin Ct and *KCNJ2* Ct in the blank control group); Suppression rate =$1-2^{-\Delta\Delta Ct}$; Corrected suppression rate = Suppression rate/transfection rate.

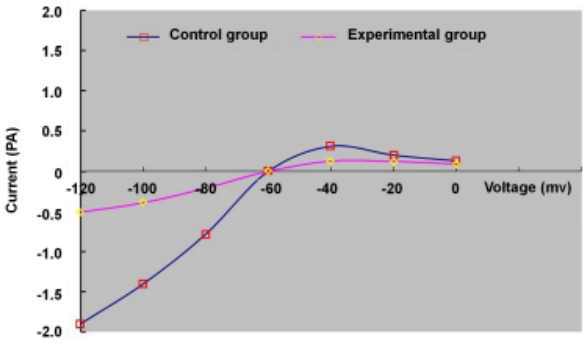

Figure 5. I-V line graph of the experimental group and control group. At different voltages, the ventricular myocytes in the transfected plasmid group (experimental group) had a significantly lower I_{K1} current than the control group ($p < 0.05$).

4. Discussion

The inward rectification of I_{K1} plays an important role in the ventricular myocardial activity (*15,16*). When the membrane potential is higher than the potential for K^+ equilibrium, an outward current is generated, speeding up the complex polarization of the AP. When the membrane potential is lower than the potential for K^+ equilibrium, an inward current is generated. This plays a leading role in maintaining the resting potential and in the potassium sensitivity of cells (*17*). At the end of complex polarization, most channels are inactivated and I_{K1} is activated, facilitating rapid complex polarization and inhibiting early after-depolarization (*18*). Adult myocardial cells have latent pacing ability but are inhibited by an inward rectifier current (I_{K1}) that stabilizes the resting potential at a negative level. I_{K1} is highly expressed in ventricular and atrial myocytes (*19*), so it inhibits automatic rhythmicity of the ventricle and atrium. Miake (*20*) provided direct proof-of-concept for biological pacemaking. Thus, the current study used an RNAi technique to suppress the *KCNJ2* gene in order to provide an approach for the development of biological pacemakers. RNAi has been widely used as a tool for targeted gene silencing (*21,22*). In the current study, five groups of shRNA recombinant plasmids specific to the *KCNJ2* gene were constructed based on the concept of RNAi. Analysis of enzyme digestion indicated that the constructed recombinant plasmid had the same size as expected. There were no base mutations according to sequencing analysis, indicating that construction was successful. For the five plasmids expressing shRNA in the current experiment, a LYS2 vector provided the optimal level of mRNA inhibition based on the results of quantitative mRNA detection and the corrected inhibition rate of 86%, which is significantly higher than that of other vectors.

Based on this experiment, I_{K1} was studied in rat ventricular myocytes using the membrane clamp technique. Membranes were attached to cells. When the K^+ ion concentration in the internal solution is 140 mM, the equilibrium potential (Ek) of K^+ ions approaches 0 mV (*23*). When the potential of the membrane is below 0 mV (hyperpolarization), a type of inward pulse of current is recorded in most membranes. Channels are randomly opened temporarily, and the most common pattern is clustered opening (*24*). The current increases with the increasing polarization of the membrane. When the potential of the membrane exceeds 0 mV, channel opening is difficult to record. Even if opening is recorded accidentally, the channel amplitude is smaller, *i.e.,* the channel has intense inward rectification. Its reverse potential is –60 mV, so when the potential is less than –60 mV the channel current reflects inward rectification. When the potential is more than –60 mV, the channel current reflects outward rectification. Potential was kept at –40 mV and electrical stimulation was supplied with 500-ms square-wave pulses. The potential was depolarized from –120 mV to +30 mV, and a step voltage of 10 mV was used. I_{K1} in ventricular myocytes is a stable current that does not change over time. It is part of the inward current during hyperpolarization and has a larger amplitude. It becomes part of the outward current during depolarization, indicating inward rectification.

Results indicated significant differences ($p < 0.05$) in the Po of channels in the control group and the LYS2-transfected group at voltages of +10, 0, and –40 mV. There were also significant differences ($p < 0.01$) in the Po of channels in the control group and LYS2-transfected group at voltages of –80, –100, and –120 mV. This experiment measured current at voltages of –120, –100, –80, –40, –20, and 0 mV, which are more sensitive to current activity. After transfection of LYS2, the channel current decreased but was not blocked completely, indicating that LYS2 transfection had interfered with I_{K1} potassium channels and significantly inhibited the I_{KI} in rat ventricular myocytes.

In conclusion, RNAi affected I_{K1} in rat ventricular myocytes, and the current amplitude of I_{K1} potassium channels clearly decreased after transfection of the plasmid LYS2. This RNAi technique has provided a new method for the study of cardiac pacemakers and has laid the foundation for clinical cardiac pacemaking.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (Grant No. 30471711).

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(Received September 15, 2011; Revised January 25, 2012; Accepted February 16, 2012)

Original Article

DOI: 10.5582/bst.2012.v6.1.33

Ageing in Werner syndrome

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Summary **Oxidative stress markers including pentosidine and homocysteine were examined comparing them with inflammation markers including highly sensitive C-reactive protein (hsCRP) and matrix metalloproteinase-9 (MMP-9) in serum from patients with Werner syndrome (WS) and healthy individuals. Elevation of serum pentosidine correlated significantly with normal aging in healthy individuals ($p < 0.0004$). Serum pentosidine in WS increased significantly compared with age-matched healthy individuals ($p < 0.05$). Serum homocysteine levels increased insignificantly with normal aging in healthy individuals and in WS compared with age-matched healthy individuals. As both pentosidine and homocysteine levels did not correlate with hsCRP nor MMP-9, both oxidative stress markers may be differentially regulated by inflammation.**

Keywords: Aging, homocysteine, inflammation, oxidative stress, pentosidine, C-reactive protein (CRP), matrix metalloproteinase-9 (MMP-9)

1. Introduction

Human ageing has been believed to be an irreversible and detrimental process counted by the advance of calendar years leading finally to death, though the fundamental mechanism(s) remains unclear. We still do not know whether ageing is a physiological one-way process following development and maturation that may be genetically tuned, or is just the result of a stochastic accumulation of damage resulting from daily metabolic/catabolic activities leading to systemic destruction that may accelerate a chance of death along with the passage of time. Also, we cannot discriminate definitely natural (physiological) ageing and age-related diseases (pathological ageing) (*1*).

Dyslipidemia (DL) and other risk factors for atherosclerosis include pentosidine and homocysteine (HC). Pentosidines are a family of advanced glycation endproducts (AGEs) generated under oxidative stress and ageing (*2-8*). AGEs are a group of reactive intermediates resulting from a series of non-enzymatic chemical reactions after an initial glycosylation such as rearrangement, dehydration, oxidation, and fragmentation reactions of glucose or its adducts to protein (*9,10*). AGEs tend to accumulate with age in long-lived tissue proteins such as collagen, lens crystalline, immunoglobulin chains, amyloid β-peptide and nucleic acids, partly under the influence of oxidative stress (*10,11*). The chronic, systemic inflammation tightly associated with ageing such as diabetes mellitus (DM) (*12,13*), atherosclerosis (*14*), arthritis (*15,16*) and hemodialysis (*17,18*) may accelerate AGEs formation through oxidative reactions. Collectively, the term: inflammaging has been proposed to explain pathophysiology of human ageing and ageing-related diseases (*1,19*).

HC, metabolized into cysteine or recycled into methionine, is a sulfhydryl-containing amino acid exclusively derived from demethylation of dietary methionine in our body (*20,21*). About 80% of the HC is protein bound and HC promotes oxidative stress of protein *via* reactive oxygen species (ROS) generation upon disulfide bond formation (*22*). Elevated serum HC enhanced the expression of acute stress-related genes and pro-inflammatory transcription factor, NF-κB, leading to AGEs formation, vascular inflammation and

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accelerated atherosclerosis in mice (*23,24*). Hyper HC serum is also associated with cardiovascular mortality, osteoporosis, Alzheimers disease, renal failure, and physical dysfunction in elderly people (*25-28*).

Although plasma HC level has been believed to be a biomarker for atherothrombotic events and folate supplementation has been recommended (*29*), so-far no convincing preventive data of recurrent vascular events have been reported, even if folate supplementation successfully reduced plasma HC levels (*30-32*). Even though the magnitude of predictive values of HC has recently decreased after fortification of folate in the United States (*33,34*) and a variety of Japanese foods has been revealed to contain enough folate (*35*), the epidemiological, clinical and basic findings addressed an important new link between serum HC level, cardiovascular diseases and oxidative stress (*22,36*).

Werner syndrome (WS; MIM#27770), the representative progeroid syndrome, has been extensively studied as the natural model of human ageing (*37*). Patients with WS show a wide variety of ageing-associated clinical manifestations such as gray hair/alopecia, hoarseness, cataracts, skin atrophy, skin hyper-/hypo-pigmentation, skin ulcers (SU), sarcopenia, DM, hypogonadism, DL, atherosclerosis, osteoporosis, and malignancy at a relatively early stage of their life followed by death at around 50 y.o. due to atherosclerosis-related diseases or malignancy. Interestingly, the patients with WS usually manifest a low grade inflammation represented by high sensitivity CRP (hsCRP) and matrix metalloproteinase-9 (MMP-9) despite apparent inflammation such as infection and malignancy (manuscript submitted). Because investigation of oxidative stress monitored by AGEs and HC in WS have never been reported, we conducted a study of serum levels of pentosidine and HC in Japanese patients with WS and healthy Japanese individuals.

2. Materials and Methods

2.1. *Subjects*

A total of 55 serum samples [35 healthy individuals (M = 14, F = 21) ages from 33 to 83 y.o. and 20 patients (M = 12, F = 8) with mutation-proven WS ages from 35 to 70 y.o. (a part of "Goto collection of Werner syndrome": *http://www.brc.riken.jp/lab/cell/gmc/*)] was selected for the study as shown in Table 1 (*37*). Diagnosis of DL was made if one of the following criteria was met: total cholesterol (TC) ≥ 220 mg/dL, low-density lipoprotein-cholesterol (LDL-C) ≥ 140 mg/dL, high-density lipoprotein-cholesterol (HDL-C) < 40 mg/dL or triglycerides (TG) ≥ 150 mg/dL, as previously described (*38*). The definition of healthy is based on the following criteria: healthy individuals, enjoying a usual daily life by themselves at home without any specifictreatment, had no apparent inflammatory diseases or infection at the time of serum sampling.

2.2. *Measurements*

Human hsCRP in the sera was measured by using a CircuLex high-sensitivity CRP ELISA kit (CycLex Co., Nagano, Japan) according to the users manual. HC (nmol/mL) was examined by HPLC (*39*) and pentosidine (μg/mL) was assayed by ELISA as previously described (*40*). The concentration of MMP-9

Table 1. Clinical characteristics of Werner syndrome patients

ID	Sex	Age	SU	DM	DL	Pentosidine (μg/mL)	HC (nmol/mL)	hsCRP (μg/mL)	MMP-9 (ng/mL)
WS0101	M	46	+	+	+	0.0806	25.1	15	49.3
WS4705	F	67	+	+	+	0.1131	23.1	11.8	224.3
WS6301	M	46	+	+	+	0.075	15.3	27.2	184
WS12201	M	39	+	+	+	0.1106	11.6	0.79	106
WS51601	F	40	+	+	+	0.0472	7.2	0.98	222
WS57801	M	41	+	+	+	0.3982	10.6	1.04	122
WS58301	M	53	+	+	+	0.0467	16.1	3.21	32.1
WS12901	F	48	+	+	+	0.0534	8	0.88	134
WS19201	M	44	+	+	+	0.071	9.5	18.7	82.2
WS53101	F	39	+	-	+	0.045	12.5	22.9	294
WS54801	M	57	+	+	−	0.0472	6	5.88	85.8
WS56201	M	70	+	+	−	0.0461	10.6	10.3	85.8
WS56301	M	39	+	+	−	0.0444	6.5	0.79	284
WS58501	M	51	+	+	−	0.0421	8.6	4.02	357
WS53801	F	46	+	−	−	0.0445	9.9	0.98	103
WS54001	F	57	+	−	−	0.0721	7.7	7.04	70.3
WS59501	F	37	+	−	−	0.0512	5.5	0.98	222
WS57701	F	38	−	+	+	0.0394	5.2	2.07	197
WS58701	M	35	−	+	+	0.0604	8	2.93	108
WS57401	M	41	−	+	−	0.0525	7	26.9	0

Abbreviations: SU, skin ulcers; DM, diabetes mellitus; DL, dyslipidemia; HC, homocysteine; hsCRP, highly sensitive C-reactive protein; MMP-9, matrix metalloproteinase-9.

in the sera was determined by specific sandwich ELISA using a Human MMP-9 ELISA kit (GE Healthcare, Buckinghamshire, UK) as described previously (*41*).

2.3. *Statistical analysis*

Statistical analysis was performed using SAS software version 9.2. Mean values between groups and within each group of patients were compared using Student's *t* tests after confirming that the data followed a normal distribution, and nonparametric methods (Mann-Whitney *U* test). Correlations were determined using Pearson's test. *p* values less than 0.05 were considered significant.

3. Results

The serum level of pentosidine increased significantly with ageing in the healthy population ($r = 0.475$; $p < 0.0004$), but not in WS patients as shown in Figure 1. The serum pentosidine level (Mean ± S.D.) was significantly elevated in the WS patients (0.077 ± 0.079 μg/mL, $p < 0.05$) compared with age-matched healthy controls (0.039 ± 0.015 μg/mL) as indicated in Figure 2. Although pentosidine was usually associated with inflammation, serum levels of pentosidine in the healthy population and WS patients did not correlate significantly with inflammation assessed by hsCRP or MMP-9 in the present study (data not shown). If WS patients were grouped into SU(+)(-), DM(+)(-), and DL(+)(-), respectively, and compared with (+) and (-) in each group, the differences of both serum pentosidine and hsCRP or MMP-9 levels were insignificant between all groups.

The serum level of HC increased insignificantly with ageing in the healthy population ($r = 0.316$; $p = 0.065$) and in WS patients as shown in Figure 3. Although HC was also usually associated with inflammation, serum levels of HC in healthy individuals and WS patients did not correlate with inflammation assessed by hsCRP or MMP-9 in the present study (data not shown). The serum HC level was comparable between WS patients (10.7 ± 5.4 nmol/mL) and age-matched healthy individuals (9.4 ± 2.8 nmol/mL) (Figure 4). However, serum HC level in the DL(+) group in WS (12.7 ± 6.23 nmol/mL; $p < 0.05$) was significantly elevated compared with the DL(-) group (7.8 ± 1.84 nmol/mL).

4. Discussion

The significantly elevated pentosidine level in WS serum may suggest increased AGE production associated with accelerated ageing in WS. However, the other oxidative reaction marker in serum, HC, was not significantly elevated in the same WS serum compared with the age-matched healthy population. Although HC induced oxidative stress may contribute

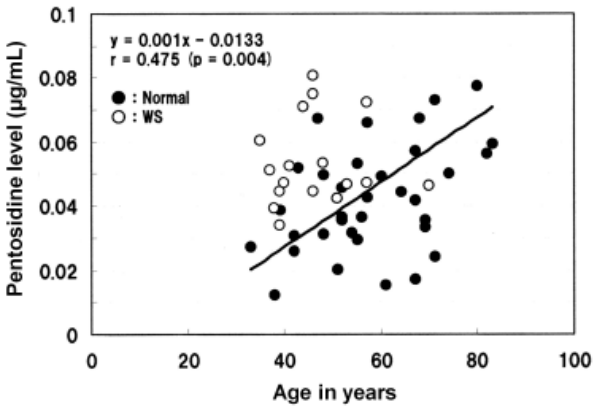

Figure 1. Age-associated change of serum pentosidine in healthy individuals.

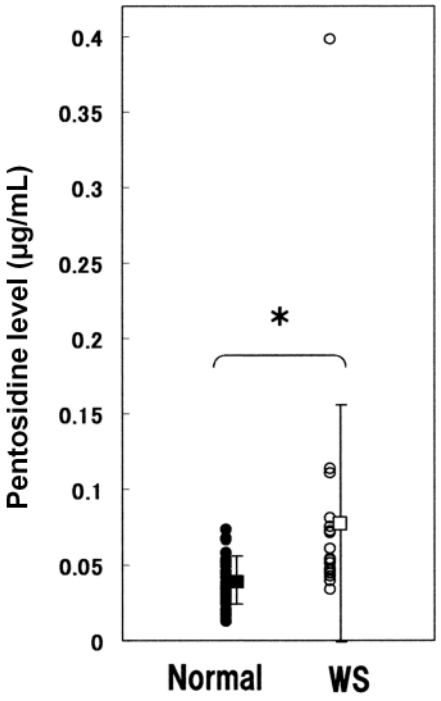

Figure 2. Pentosidine in Werner syndrome. * $p < 0.05$.

to vascular damage by activating MMP-9 (*42*), and both HC and pentosidine were in general increased with inflammation monitored by hsCRP, both oxidative markers were neither correlated with each other, nor serum levels of hsCRP and MMP-9 in WS. Serum pentosidine and HC may be differentially regulated by inflammation as was reported (*4,6,14,17,23,24,43*). Although serum HC in WS did not correlate with TC, HDL-C, LDL-C, and TG (data not shown), the serum HC level in the DL(+) group in WS was significantly elevated irrespective of age compared with the DL(-) group. In animal studies, elevated HC may activate oxidative enzymes leading to podocyte damage resulting in glomerulosclerosis (*44*). The oxidative damage of HC on the vascular system has been reported in most studies using supraphysiological concentrations of HC or genetic diseases like homocystinuria (*45*).

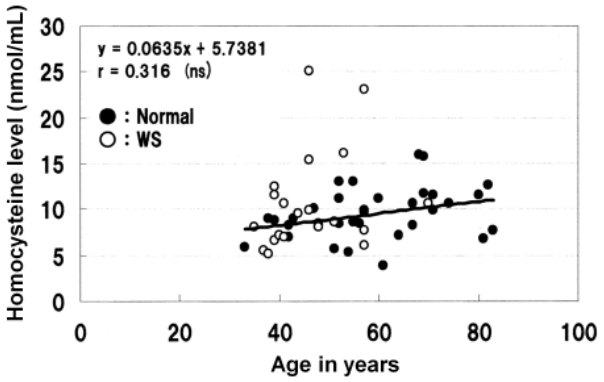

Figure 3. Age-associated change of serum homocysteine in healthy aging.

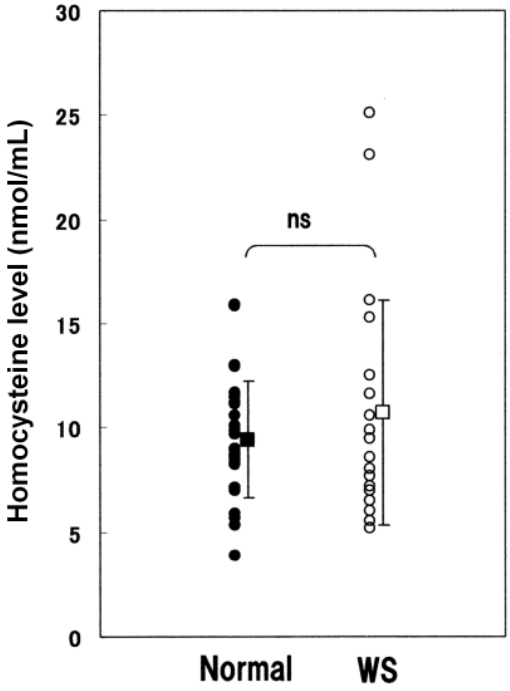

Figure 4. Homocysteine in Werner syndrome. ns, not significant.

In any case, Pagano *et al*. reviewed that the multiple involvement of oxidative stress in WS (*46*) and the concomitant association of moderate hyperhomocysteinemia and DL, in addition to elevated AGE production may accelerate oxidative injury in cardiovascular diseases and other ageing associated phenotypes in WS.

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(*Received December 9, 2011; Revised January 16, 2012; Accepted January 22, 2012*)

Original Article

DOI: 10.5582/bst.2012.v6.1.38

Stroke volume variation and pleth variability index to predict fluid responsiveness during resection of primary retroperitoneal tumors in Hans Chinese

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Summary **Respiration variation in arterial pulse pressure (ΔPP) and pulse oximetry plethysmographic waveform amplitude (ΔPOP) are accurate predictors of fluid responsiveness in mechanically ventilated patients. We hypothesized that stroke volume variation (SVV) and pleth variability index (PVI) can predict fluid responsiveness in mechanically ventilated patients during major surgical procedures in Hans Chinese. This prospective study consisted of fifty-five Hans Chinese patients undergoing resection of primary retroperitoneal tumors (PRPT). During the surgical procedures, hemodynamic data [central venous pressure (CVP), cardiac index (CI), stroke volume index (SVI), SVV, and PVI] were recorded before and after volume expansion (VE) (8 ml•kg^{-1} of 6% hydroxyethyl starch 130/0.4). Fluid responsiveness was defined as an increase in SVI ≥ 10% after VE. Four patients were excluded from analysis for arrhythmia or obvious hemorrhage during VE. Baseline SVV correlated well with baseline PVI, and the changes in SVV was correlated with the changes in PVI ($p < 0.01$) after VE. There were significant increases of CI, SVI and decreases of SVV, PVI in responder (Rs) after VE. ROC results showed that the areas for SVV, PVI were significantly higher than the areas for CI, MAP, CVP, PI ($p < 0.05$). The best threshold values to predict fluid responsiveness were more than 12.5% for SVV and more than 13.5% for PVI in the real surgical setting. The baseline value of SVV, and PVI correlated significantly with volume-induced changes in SVI ($p < 0.01$). Both SVV and PVI could be used to predict intraoperative fluid responsiveness during resection of PRPT in Hans Chinese.**

Keywords: Pulse oximeter, *i.v.* fluids, intraoperative monitoring

1. Introduction

Goal-directed intraoperative fluid administration has been shown to reduce postoperative morbidity and shorten hospital stay following abdominal surgery (*1*). Primary retroperitoneal tumors (PRPT) are a rare but diverse group of neoplasms that arise within the retroperitoneal space. Surgical resection is difficult because of close proximity of vital organs and adjacent vascularities. Sufficient blood and intravascular fluids should be prepared according to the size and localization of tumor, so intraoperative transfusion management is particularly important. During resection of PRPT, patients with preexistent cardiac disease or poor myocardial function are exposed to the risk of pulmonary and peripheral edema. Preload assessment is therefore crucial to guide fluid therapy and to prevent excessive fluid loading during resection of PRPT. Static indicators, such as central venous pressure (CVP), and pulmonary capillary wedge pressure (PCWP) have been shown to be poor predictors of fluid responsiveness (*2*). Dynamic indicators of cardiac preload, based on respiratory variations of arterial pulse pressure (ΔPP) and pulse oximetry plethysmographic (ΔPOP) changes have been shown to be sensitive to changes in preload and can predict hemodynamic response to volume expansion in mechanically ventilated patients during perioperative periods (*3-7*).

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A recent Vigileo™/FloTrac™ system (Edwards Lifescience, Irvine, CA, USA) allows for continuous monitoring of cardiac output (CO) based on pulse contour analysis and of respiratory variations in stroke volume (SVV) based on analysis of systemic arterial pressure wave. A few studies performed in the post-anaesthesia, pre-surgery time window (*8*,*9*) or postoperatively (*10*,*11*) have shown controversial results. Another new device (Masimo Radical™ 7 system, Masimo Co., Irvine, CA, USA) can automatically display perfusion index (PI) and calculate the pleth variability index (PVI), which is a new algorithm that automatically calculates ΔPOP. But the ability of PVI to predict fluid responsiveness was evaluated in mechanically ventilated patients preoperatively (*12-14*) or after passive leg rising in spontaneously breathing volunteers (*15*). Whether these two indices can be used for intraoperative fluid responsiveness predictions and fluid optimization in patients undergoing major surgical procedures still has to be demonstrated, and the optimal threshold value of these indices in the surgical setting still has to be determined.

The aim of this study was to test the ability of SVV and PVI to predict intraoperative fluid responsiveness in mechanically ventilated patients during resection of PRPT in Hans Chinese and to compare them with other indicators in this surgical setting.

2. Materials and Methods

2.1. *Patients characteristics*

This prospective study was approved by institutional review board of General Hospital of PLA. All patients gave informed consent. Between September 2009 and May 2011, there were a total of 55 patients undergoing resection of PRPT that received intraoperative infusion with colloids. All patients were diagnosed preoperatively by ultrasonography, computerized tomograph (CT) and/or magnetic resonance imaging (MRI), or digital subtraction angiography (DSA). Exclusion criteria were: patients younger than 18 years, arrhythmias and intracardiac shunts. Among the final 51 eligible patients, 9 had hypertension and 4 had diabetes, which were treated medically to keep preoperative blood pressure below 140/90 mmHg and fasting plasma glucose under 8 mM. All patients were kept supine during the operation. Histologic types of the 51 cases of retroperitoneal tumors consisted of 18 liposarcoma, 6 lipoma, 11 leiomyosarcoma, 9 nuerofibroma, 4 neurilemmoma, and 3 teratoma cases. The tumors ranged in size from 7 cm to 34 cm in their long axis, and the average diameter was 16.8 cm.

2.2. *Anaesthesia methods*

Anaesthesia was induced with *i.v.* bolus administration of fentanil (3 μg/kg), and 2 min later propofol (1.5-2 mg/kg). Orotracheal intubation was facilitated with rocuronium (0.6-0.9 mg/kg). After induction of anaesthesia, a two lumen, 7.0-French central venous catheter (Arrow International Inc.) was inserted in the right internal jugular vein. A radial artery catheter (REFRA-04220, Arrow international Inc.,USA) was inserted in the radial artery. Pressure transducers were placed on the midaxillary line and fixed to the operation table in order to keep the transducer at atrial level during the study protocol. All transducers were zeroed to atmospheric pressure. Anaesthesia was maintained with target controlled infusion (TCI) of propofol (2-4 μg/mL) and continuous infusion of remifentanil (0.3-0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) with bispectral index (BIS, Aspect 1000™, Aspect Medical Systems Inc., Natick, MA, USA) kept between 40 and 50. All patients were ventilated in a volume-controlled mode with a tidal volume of 8-10 mL/kg body weight and an inspiratory/expiratory ratio of 0.5. The ventilatory frequency (10-12 cycles) was set to maintain an end-tidal P_{CO2} range of 3.8-4.7 kPa. Positive end-expiratory pressure was set at 0 cm H_2O.

2.3. *Data recording and analysis*

A dedicated transducer (FloTrac™, Edwards Lifesciences) was connected to the radial arterial line on one side and to the Vigileo™ System (Edwards Lifesciences) on the other side. The system enables the continuous monitoring of SV, SVI, CO, CI and SVV without calibration. The Vigileo (Software version 1.14) analyzes the pressure waveform 100 times per second (100 Hz), and performs its calculations on the most recent 20 s data (*10*,*16*). SVI obtained with this device was recorded and used to discriminate responder and non-responder patients after VE. SVV was calculated as the variation of beat-to-beat SV from the mean value during the most recent 20 sec data and was displayed continuously.

Masimo Radical™ 7 monitor: A pulse oximeter probe (LNOP® Adt, Masimo Co., Irvine, Canada) was placed on the index finger and wrapped with black paper to minimize light interference. The probe was connected to a Masimo Radical 7 monitor with PVI software (version 7.0.3.3). PVI is an automatic measure of the dynamic change in PI that occurs during a complete respiratory cycle. PVI calculation measures changes in PI over a time interval sufficient to include one or more complete respiratory cycles and was displayed continuously (*14*). At each step of the study protocol, the following were recorded simultaneously: heart rate (HR), systolic arterial pressure, mean arterial pressure (MAP), diastolic arterial pressure, and end-expiratory CVP.

2.4. *Experiment protocol*

Intraoperative infusion with 8 $mL \cdot kg^{-1}$ of 6% hydroxyethyl starch were started when MAP dropped

more than 20% from preoperative values, and completed between 20 to 30 min. Hemodynamic measurements were performed before, and within 30 sec after volume expansion without stimulation, in order to limit changes in vasomotor tone that may have affected PVI value (*14*). During the volume expansion, ventilator settings were kept consistent. If obvious hemorrhage (volume > 100 mL) or arrhythmias happened, the infusion protocol would be terminated and patient would be treated accordingly.

2.5. *Statistical analysis*

All data are presented as mean ± S.D. Distribution normality was assessed using the Kolmogorov-Smirnov test. Changes in hemodynamic measures induced by volume expansion were assessed using one-way analysis of variance. Patients were divided into two groups according to the percent increase in SVI after intravascular volume expansion: responders (Rs) were defined as patients demonstrating an increase in SVI ≥ 10% after intravascular volume expansion and non-responders (NRs) as patients whose SVI changed < 10%. Receiver operating characteristic (ROC) curves were generated for SVV, PVI, SVI, CI, CVP, MAP, and PI varying the discriminating threshold of each and areas under the ROC curves were calculated. The areas of ROC curves were compared according to the method described by Hanley and McNeil (*17*). Threshold values for each parameter were determined by considering values that yielded the greatest sensitivity and specificity. Pearson's test was used to test correlation. A *p*-value less than 0.05 was considered as statistically significant. All statistical analysis was performed using statistical software (Statview 5.01, SAS Institute, Cary, NC, and SPSS 15.0, SPSS, Chicago, IL, USA).

3. Results

3.1. *Patients selection*

Fifty-five patients were initially included, four patients were excluded from analysis for arrhythmia (three patients: two had ventricular premature contraction, one had atrial fibrillation) or obvious hemorrhage during the protocol (one patient; bleeding > 100 mL during volume loads). Fifty-one patients consisted of 26 males and 25 females between 19 and 69-year-old (mean age, 48.7 ± 13.4 year).

3.2. *Changes in hemodynamic variables after volume expansion*

Hemodynamic measurements in Rs and NRs at baseline and after VE are given in Table 1. After VE, no significant changes were found in NRs, while in Rs, there were significant changes of CI (from 2.9 ± 0.5 to 3.3 ± 0.7 $L \cdot min^{-1} \cdot m^{-2}$; $p = 0.009$), SVI (39.1 ± 8.1 to 46.8 ± 10.3 mL/m^2; $p = 0.008$). At the same time we observed significant decreases in both SVV (from 18.4 ± 5.8% to 8.7 ± 3.7%; $p = 0.004$) and PVI (from 19.5 ± 6.6% to 12.0 ± 5.0%; $p = 0.002$) in Rs. Before VE, SVV and PVI were significantly higher in Rs than in NRs, but there was no difference in CI, SVI, CVP, MAP and PI at baseline (Table 1). We found a significant correlation between SVV and PVI at baseline ($r = 0.727$, $p = 0.0001$).The change in SVV after VE was correlated with the change in PVI after VE ($r = 0.693$, $p = 0.002$) (Figure 1).

3.3. *Dynamic indices and static indices to predict fluid responsiveness*

Thirty-one patients were Rs (ΔSVI ≥ 10%) and 20 were NRs. The areas under the ROC curve, showing the ability of the hemodynamic parameters to discriminate between Rs and NRs, are shown in Table 2. The areas for SVV, PVI were significantly higher than the areas for SVI, CI, MAP, CVP and PI ($p < 0.05$). An SVV threshold of > 12.5% discriminated Rs with a sensitivity of 87.9% and a specificity of 83.3%. A PVI threshold of > 13.5% discriminated Rs with a sensitivity of 77.4% and a specificity of 80.0%. There was no significant difference between the areas under the ROC curve for SVV and PVI.

3.4. *Dynamic indices and static indices to quantify response to intravascular volume expansion*

There were no significant correlations between baseline values of MAP, CVP and CI and the percent change in SVI (ΔSVI) after fluid expansion (respectively, $r = -0.284$, $p = 0.054$; $r = -0.220$, $p = 0.121$; $r = -0.241$, $p=0.090$). In contrast, the baseline value of SVV and PVI correlated significantly with the change in SVI induced by fluid expansion (respectively, $r = 0.446$, $p = 0.001$; $r = 0.362$, $p = 0.009$), which indicated that the higher SVV and PVI at baseline, the higher ΔSVI (Figure 2).

4. Discussion

This study demonstrates that SVV measured by the Vigileo™ System and PVI measured by the Masimo™ Radical 7 monitor can be used to predict the effects of volume expansion during the resection of RPRT in Hans Chinese.

The results showed that 31 patients were Rs (ΔSVI ≥ 10%) and 20 were NRs. This may be related to inclusion criteria of patients who needed colloid infusions. This study compared the relationship between SVV and PVI intraoperatively, which showed that SVV has a good agreement with PVI. This finding is in agreement with previous studies (*7*,*18*), which compared ΔPOP and ΔPP in mechanically ventilated patients under general

Table 1. Hemodynamic variables before and after volume expansion in fluid responders and fluid non-responders

	Fluid non-responders (n = 20)			Fluid responders (n = 31)			
	Baseline	Volume expansion	P1	Baseline	P2	Volume expansion	P3
HR (beats min^{-1})	69.7 (17.7)	71.2 (15.7)	0.752	68.9 (14.6)	0.520	72.7 (14.5)	0.797
MAP (mmHg)	73.1 (12.7)	81.5 (18.9)	0.649	66.0 (10.5)	0.060	76.1 (15.2)	0.185
CVP (mmHg)	9.4 (5.0)	11.3 (5.3)	0.680	7.8 (3.4)	0.126	9.8 (3.6)	0.717
CI (liter min^{-1}• m^{-2})	3.3 (0.8)	3.5 (0.9)	0.809	2.9 (0.5)	0.190	3.3 (0.7)	0.009
SVI (mL•m^{-2})	46.7 (7.8)	50.2 (8.6)	0.837	39.1 (8.1)	0.091	46.8 (10.3)	0.008
SVV (%)	11.2 (3.7)	6.3 (3.2)	0.126	18.4 (5.8)	0.001	8.7 (3.7)	0.004
PVI (%)	11.3 (6.2)	8.7 (3.8)	0.618	19.5 (6.6)	0.001	12.0 (5.0)	0.002
PI (%)	3.9 (2.5)	3.6 (2.4)	0.922	3.3 (1.8)	0.166	3.3 (1.6)	0.643

Data are mean (S.D.). HR, heart rate; MAP, mean arterial pressure; CVP, central venous pressure; CI, cardiac index; SVI, stroke volume index; SVV, stroke volume variation; PVI, pleth variability index; PI, perfusion index. P1, volume expansion value *vs.* baseline value in non-responders; P2, baseline value in responders *vs.* baseline value in non-responders; P3, volume expansion value *vs.* baseline value in responders.

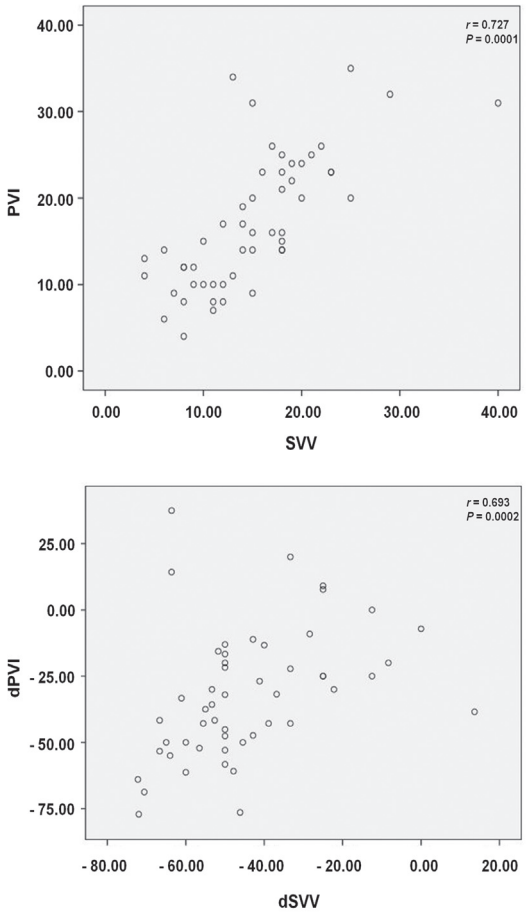

Figure 1. Relationships between baseline values of SVV and PVI (*upper*), and between percent changes in SVV and PVI after volume expansion (*lower*).

anaesthesia preoperatively. All of these studies proved that ΔPOP is closely related to ΔPP perioperatively, and is sensitive to changes in ventricular preload (*19*).

SVV obtained with the Vigileo™ system showed its efficacy of predicting responsiveness to fluid loading under general anesthesia in stable conditions (*8-10*). Conversely, one study found that SVV was unable to predict fluid responsiveness after cardiac surgery (*11*). Thus, further studies are required to address this controversy. In this study, the ability of SVV-Vigileo™ to predict responsiveness to fluid loading was assessed during surgical procedures. ROC curves demonstrated that SVV, PVI could predict fluid responsiveness to colloids, and more efficiently than CI, CVP, and MAP, which is in agreement with increasing evidence that static preload indicators are not suited for functional hemodynamic monitoring (*20*). The results showed that SVV > 12.5% discriminated Rs with a sensitivity of 87.9% and a specificity of 83.3% during major surgery. Monitoring of hemodynamic variables such as SV/SVI and CO/CI are regarded to be more reliable measures for assessing the adequacy of volume replacement therapy than simple pressure monitoring (*21*). If SVI were low and SVV, and PVI were high, it may be more accurate to make a diagnosis of hypovolemia, and may help for fluid optimization in complicated surgery procedure settings.

Respiratory variation in the ΔPOP waveform amplitude has been studied in mechanically ventilated patients (*18*,*22*). Pleth variability index (PVI) (Masimo Co., Irvine, Canada) is a novel algorithm allowing for automated and continuous monitoring of ΔPOP (*7*). Some studies had extended PVI assessment to the perioperative period, and their results showed that there was a significant correlation between PVI before volume expansion and change in CI/SVI after volume expansion (*9*,*14*). But Broch *et al.* showed that PVI was not able to predict fluid responsiveness with sufficient accuracy, and the accuracy of PVI to predict fluid responsiveness was improved on analyzing patients with higher PI values (*25*). These studies were performed under stable hemodynamic conditions, right after induction of general anaesthesia and before main surgical procedures. Whether this index can be used for intraoperative fluid responsiveness predictions still has to be demonstrated. To our knowledge, this is the first paper to observe the relationships between PVI and fluid response during surgical procedures. Our results showed a significant positive linear correlation between PVI at baseline and percent changes in SVI (ΔSVI) induced by intravascular volume expansion. ROC curves results also showed that PVI has predictive

Table 2. Areas under the ROC curves and cutoff values of various hemodynamic parameters for prediction of fluid responsiveness

	Optimal threshold value	Sensitivity (%)	Specificity (%)	AUC (95% CI)	*p*-value
SVV	12.5%	87.9	83.3	0.862 (0.761-0.963)	0.001
PVI	13.5%	77.4	80.0	0.785 (0.651-0.920)	0.002
SVI	43.5 mL•m^{-2}	83.3	91.0	0.726 (0.577-0.875)	0.057
CI	2.85 liter min^{-1}• m^{-2}	72.2	75.8	0.651 (0.488-0.813)	0.071
CVP	7.5 mmHg	61.1	63.6	0.606 (0.447-0.779)	0.203
MAP	67.5 mmHg	66.7	69.7	0.686 (0.517-0.776)	0.059
PI	3.49%	61.1	66.7	0.647 (0.485-0.808)	0.079

AUC (95% CI), area under ROC curve (95% CI); SVV, stroke volume variations; PVI, pleth variability index; SVI, stroke volume index; CI, cardiac index; CVP, central venous pressure; MAP, mean arterial pressure; PI, perfusion index. *p*-value, comparison with AUC = 0.5.

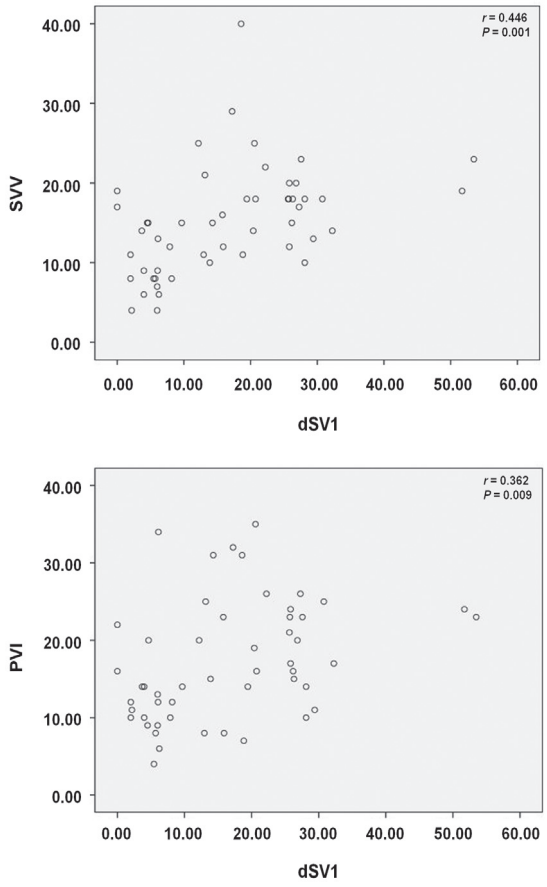

Figure 2. Relatiships between SVV (*upper*) and PVI (*lower*) before volume expansion and percent increase in stroke volume index after VE.

value for fluid responsiveness intraoperatively. So, monitoring fluid responsiveness using a non-invasive device may help for fluid optimization in the operating room, especially in some patients who do not need invasive artery monitoring.

In the surgical setting, whether some surgical stress factors, such as nociceptive stimulation, intraoperative bleeding and fluid loss have influenced PVI are still unknown. PI depends on vasomotor tone and sympathetic tone, which may affect the pulsatile absorption component (*23*). Keller *et al.* found that PVI was a weak predictor of fluid responsiveness in a spontaneously breathing volunteer (*15*). It appears that PVI is not yet able to distinguish between changes in PI induced by respiration from changes induced by any other phenomenon. Cannesson *et al.* proved that PVI was more stable in mechanical ventilated patients under general anaesthesia preoperatively (*14*). This may be related to a decrease in sympathetic tone related to general anaesthesia and vasomotor tone does not impact PVI (*12*,*13*). The accuracy of PVI to predict fluid responsiveness was improved on analyzing patients with higher PI values (*25*). If a patient's finger is inaccessible for monitoring purposes, or during states of low peripheral perfusion, the plethysmographic dynamic index can be used in the forehead or ear (*26*). Our results showed that SVV and PVI could predict fluid responsiveness during major abdominal surgery in complicated dynamic conditions, which proved that surgical stress factors did not affect SVV and PVI's clinical value as a predictor of fluid responsiveness. In this study, the areas under the ROC curve for SVV (0.862) and PVI (0.785) are less than Zimmermann's results (*9*), in which the areas of ROC curves for SVV and PVI were 0.993 and 0.973 respectively. But both SVV and PVI achieved statistical significance in this real surgical setting. Our results of the best threshold values to predict fluid responsiveness were more than 12.5% for SVV and more than 13.5% for PVI during the surgical setting, compared with Zimmermann's results of 11% for SVV and 9.5% for PVI. So, during the surgical procedure, many intraoperative factors such as nociceptive stimulation, intraoperative occult bleeding and fluid loss can easily affect SVV and PVI's readings, but our results showed that both of them were still good indicators of intraoperative fluid responsiveness, and the threshold values derived from this study maybe more instructional in guiding fluid expansion during major surgical procedures.

In conclusion, the baseline value of SVV, PVI correlated significantly with volume-induced changes in SVI, SVV have good agreement with PVI during resection of PRPT in Hans Chinese. Both of them could predict fluid responsiveness in a complicated surgical setting.

Acknowledgements

Dr. Qiang Fu was supported by a Japan-China Sasagawa

medical research fellowship. Thanks to Dr. Junko Kouhei and Prof. Makoto Ozaki (Tokyo Women's Medical University) for their kind guidance and help.

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(Received October 10, 2011; Revised December 15, 2011; Accepted January 25, 2012)

Case Report

DOI: 10.5582/bst.2012.v6.1.44

Resection of the second portion of the duodenum sacrificing the minor papilla but preserving the pancreas for a recurrent duodenal adenocarcinoma: Report of a case

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Summary **Duodenal adenocarcinoma is a relatively rare malignancy and pancreaticoduodenectomy would be a standard procedure to achieve curative resection. We report a case of resection of the 2nd portion of the duodenum with nodal dissection preserving the pancreas. The patient was a 75-year-old man with right-sided paresis suffering from early cancer in the 2nd portion of the duodenum. Despite 3 times of endoscopic mucosal resections, mucosal local recurrence was found. The depth of the tumour involvement continued to be limited within the mucosal layer. We performed segmental duodenal resection with nodal dissection sacrificing the minor papilla, while preserving the pancreas and the major papilla. The pathological diagnosis was primary intramucosal adenocarcinoma; the surgical margin was negative for cancer and there was no nodal metastasis. This procedure can be an alternative to pancreaticoduodenectomy in patients with early-stage adenocarcinoma in the 2nd portion of the duodenum when the major papilla can be spared, especially in high-risk patients.**

Keywords: Duodenal resection, adenocarcinoma, major papilla, pancreaticoduodenectomy, pancreas-sparing duodenectomy

1. Introduction

Duodenal adenocarcinoma is a relatively rare digestive malignancy and pancreaticoduodenectomy (PD) would be a standard curative procedure (*1*). Recent insights into the surgical anatomy of the pancreaticoduodenal region (*2*) have permitted pancreas-sparing duodenectomy (PSD) for low grade malignancies (*3-7*). Some authors suggested that surgical indications of PSD can be divided into 3 categories: early-stage neoplasms, isolated duodenal neoplasms in high-risk patients, and duodenal involvement from adjacent organ malignancies (*5*). Herein, we report a case of resection of the 2nd portion of the duodenum with nodal dissection for a recurrent intramucosal adenocarcinoma in the duodenum for a high-risk patient.

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2. Case report

2.1. *Patient and present illness*

An asymptomatic 75-year-old man was admitted to a local hospital for the endoscopic treatment of a papillary tumour, measuring 3 cm in diameter, in the second portion of the duodenum. He underwent endoscopic piecemeal mucosal resection 3 times, however a local recurrence was found on the primary site. The pathological finding of the piecemeal specimen was adenocarcinoma with a tubulovillous adenoma component. He was referred to our hospital for radical resection of the recurrent lesion. He had been suffering from lumbar canal stenosis and cerebral infarction since he was 60 and 70 years old, respectively. Because he had right-sided paresis, he used a wheelchair in his

daily life. He also had moderate dementia associated with cerebral vascular disease. The patient's laboratory data showed slight anemia and hypo-albuminemia; serum albumin, 3.6 g/dL and hemoglobin, 12.7 g/dL. Serum level of carbohydrate antigen 19-9 (CA19-9) was 65 U/mL (normal range, < 37 U/mL).

Upper gastrointestinal fiberscope revealed a papillary tumour, 3 cm in diameter, originating from the 2nd portion of the duodenum. Endoscopic ultrasonography showed no extraduodenal involvement, and the tumour appeared to be limited within the mucosal layer (Figure 1A). On enhanced abdominal CT, no apparent nodal metastasis or distant metastasis was found. Coronal Magnetic Resonance Images revealed that the tumour was located 1.5 cm proximal to the minor papilla (Figure 1B). The existence of pancreatic divisum was denied by Magnetic Resonance Cholangio pancreatography. Thus, the preoperative diagnosis was recurrent duodenal cancer involving the mucosal layer without nodal or distant metastasis (T1N0M0).

2.2. *Surgical procedure*

Considering the poor-risk of the patient and the limited extent of tumour spread, we conducted duodenal resection concomitant with antrectomy of the stomach and nodal dissection, as a substitute procedure for PD. After complete mobilization of the duodenum, cholecystectomy was performed, and a 6Fr feeding tube was inserted from the cystic duct to find out the position of the major papilla by manipulation, which should be preserved. The lymph nodes along the right gastroepiploic arteries (#No. 6 and No. 4) and some of the nodes along the right gastric artery (#No. 5 and No. 3) were dissected. After antrectomy using a linear stapler, the duodenum was dissected from the pancreatic head preserving the pancreatic parenchyma. The root of the minor papilla, connecting to the duct of Santorini, was identified along the dissecting plane, which was situated about 2 cm ventroproximal to the major papilla. It was encircled by vessel loop (Figure 2A) and was clipped. To confirm the preservation of the major papilla, intraoperative cholangiography was performed by using above-mentioned 6Fr feeding tube. And the root of the minor papilla was ligated and divided. The anal side of the duodenum was divided using another linear stapler, preserving the orifice of the major papilla. An ante-colic Roux-en-Y loop was then made, and an end-to-side gastro-jejunostomy was performed using a circular stapler (Figure 2B) (*8*). The operative time and intraoperative blood loss were 355 min and 100 mL, respectively.

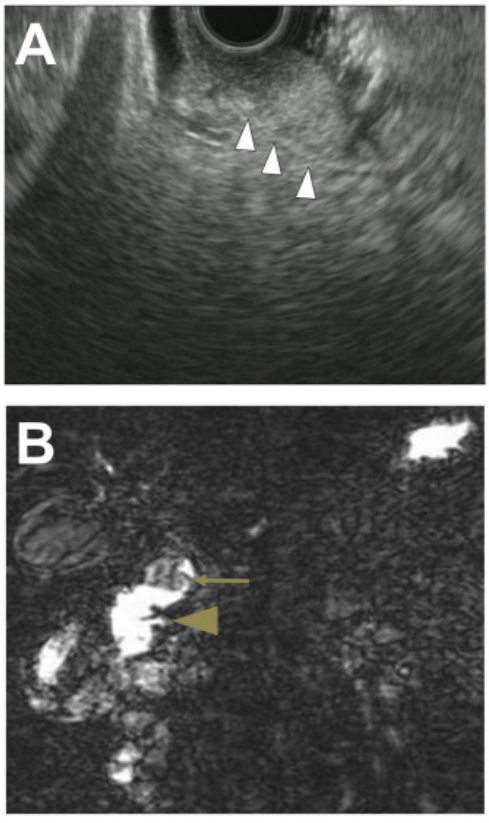

Figure 1. Endoscopic ultrasonography and coronal-view MR diagnosis. (A) Endoscopic ultrasonography revealed that the tumour had not invaded the proper muscular layer of the duodenum. The hypoechoic line (arrowhead), which suggested the proper muscular layer, was intact. **(B)** Coronal-view MR showed that the tumour (arrow) existed in the second portion of the duodenum, 1.5 cm proximal to the minor papilla (arrowhead).

2.3. *Postoperative outcome immunohistochemistry*

The postoperative course was uneventful, except for delayed gastric emptying. Neither pancreatitis nor pancreatic leakage was documented. The patient was transferred to a local hospital on day 31 for further rehabilitation. The pathological diagnosis was primary duodenal intramucosal adenocarcinoma with tubulovillous adenomas. There was no nodal metastasis (= 0/6). The proximal and distal resection margins were negative for cancer (Figure 2C). There was neither perineural nor microvascular invasion.

3. Discussion

We successfully performed resection of the 2nd portion of the duodenum with nodal dissection sacrificing the minor papilla, while preserving the pancreas and the major papilla for a recurrent duodenal cancer limited within the mucosal layer. The patient had poor surgical risks and was not a candidate for an extensive resection. Surgical margins were negative for cancer and there was no nodal involvement. The postoperative course was uneventful. The present PSD with nodal dissection would be a less invasive surgery feasible in a poor risk patient and could be an alternative procedure for adenocarcinoma in the 2nd portion of the duodenum.

Although several authors have reported PSDs for

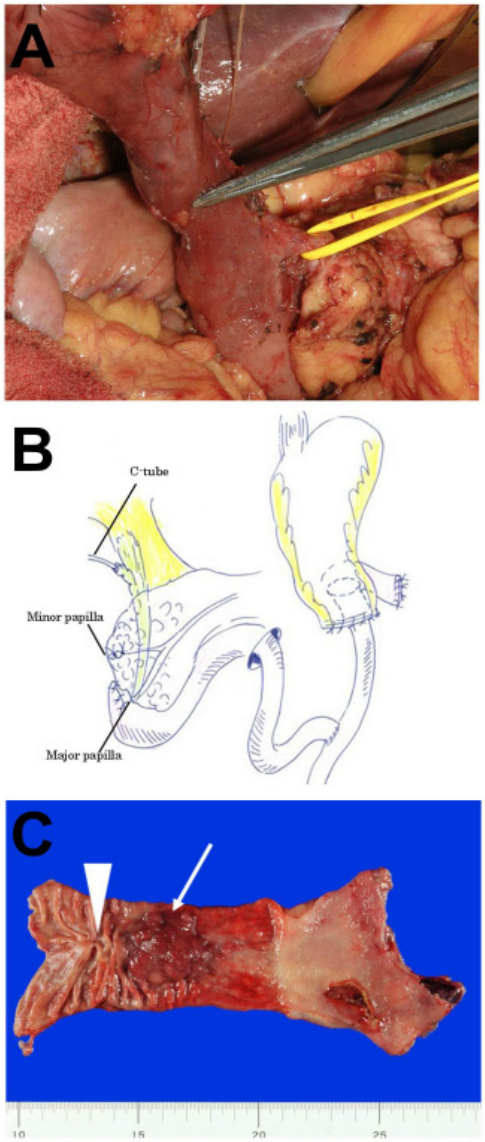

Figure 2. Intraoperative surgical findings. (A) Intraoperative findings showed the anal margin of the tumour, indicated by the forceps, and encirclement of the duct of Santorini by vessel loop. **(B)** Roux-en-Y reconstruction was performed to separate the alimentary root from the stump of the duodenum. **(C)** Macroscopic findings showed that the papillary elevated tumour (arrow) was located 1 cm proximal to the minor papilla (arrowhead) (Right side: oral side).

various duodenal malignancies, reports on resection of the 2nd portion of the duodenum for a duodenal adenocarcinoma are very limited. Only 1 report of segmental resection of the 2nd portion of the duodenum has been found in 2 patients in the literature to our knowledge (*5*). However, techniques for dividing the minor papilla have not been clearly described in the previous report. The key of the present surgical procedure was safely sacrificing the minor papilla and sparing the major papilla. In the present case, minor papilla had to be resected to secure adequate surgical margins. To safely sacrifice the minor papilla, it is very important to confirm the position of the major papilla, using the tube in the duodenum through the cystic duct and the major papilla. Adequate nodal dissection would be necessary in case of adenocarcinoma, not likely in case of duodenal gastrointestinal stromal tumour (GIST) (*7*).

The definition of the term PSD is variable. Chung *et al*. first used PSD in 1995 and described the procedure as near total duodenectomy (*9*). Tsiotos *et al*. used the term PSD to mean almost complete resection of the duodenum (*10*). Meanwhile, Maher *et al*. and we used PSD to describe resection of only the third and fourth portions of the duodenum (*7,11*). This procedure has also been named segmental duodenectomy (*12-14*). Cho *et al*. reported proximal segmental duodenectomy under the term PSD (*15*). In addition, the reported PSD involves operations with and without reconstruction of the major papilla. Papilloplasty and implantation of the major papilla would be technically too complicated, and severe surgical complications have been reported, such as bile leakage, pancreatic fistula, acute pancreatitis, and passage disturbance at the anastomotic site (*9,12,16-18*). Therefore, we consider that not PSD, but PD should be indicated when the major papilla cannot be preserved. As for the alimentary reconstruction in the present method, Asakawa *et al*. described the possible advantage of Roux-en-Y reconstruction to separate the alimentary root from the stump of the duodenum (*6*).

Surgical indication of PSD for primary early adenocarcinoma arising from the duodenum remains controversial. PD would be necessary in patients with duodenal cancer invading the pancreas or with possible nodal metastases. Ryu *et al.* observed no nodal metastasis in 122 patients with duodenal cancer limited in the mucosal layer (*19*). Just when the intramucosal duodenal cancer is confirmed preoperatively, we might be able to omit the nodal dissection. However when the submucosal minimal invasion couldn't be denied completely, perilesional nodal dissection would be efficient. In these patients, less invasive surgery by use of PSD offers the good chance for a cure and good postoperative quality of life.

In conclusion, we successfully performed a resection of the 2nd portion of the duodenum with nodal dissection sacrificing the minor papilla, while preserving the pancreas and major papilla, for a recurrent duodenal cancer limited within the mucosal layer. This procedure can also be an alternative to PD in patients with low grade-malignancy in the 2nd portion of the duodenum when the major papilla can be spared.

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(Received February 10, 2012; Revised February 16, 2012; Accepted February 22, 2012)

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(*Revised October 2011*)

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