Effects of Chitosan on Xenograft Models of Melanoma in C57BL/6 Mice and Hepatoma Formation in SCID Mice

MING-YANG YEH[1,#], MING-FANG WU[4,#], HUNG-SHENG SHANG[5], JIN-BIOU CHANG[5,6], YUNG-LUEN SHIH[7,8,9], YUNG-LIANG CHEN[6], HSIAO-FANG HUNG[10], HSU-FENG LU[2], CHUN YEH[3], W. GIBSON WOOD[11], FANG-MING HUNG[12*] and JING-GUNG CHUNG[13*]

Departments of [1]Office of Director, [2]Clinical Pathology, [3]Division of Gastroenterology, Cheng Hsin General Hospital, Taipei, Taiwan, R.O.C.;
[4]Animal Medicine Center, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C.;
[5]Department of Pathology, National Defense Medical Center, Division of Clinical Pathology, Tri-Service General Hospital, Taipei, Taiwan; R.O.C;
[6]Department of Medical Laboratory Science and Biotechnology, Yuanpei University, Hsinchu, Taiwan, R.O.C.;
[7]Department of Pathology and Laboratory Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, R.O.C.;
[8]School of Medical Laboratory Science and Biotechnology, Taipei Medical University, Taipei, Taiwan, R.O.C.;
[9]School of Medicine, Fu-Jen Catholic University, New Taipei City, Taiwan, R.O.C.;
[10]Jen-Teh Junior College of Medicine, Nursing and Management, Houlong Township, Taiwan, R.O.C.;
[11]Department of Pharmacology, University of Minnesota School of Medicine, and GRECC, VAMC, Minneapolis, MN, U.S.A.;
[12]Department of Surgical Intensive Care Unit, Far Eastern Memorial Hospital, Taipei, Taiwan, R.O.C.;
[13]Department of Biological Science and Technology, China Medical University, Taichung, Taiwan, R.O.C.

Abstract. *According to the World Health Organization, Complementary and alternative medicine (CAM) is a comprehensive term referring to traditional medical treatments and various forms of indigenous medicines, also known as indigenous or folk medicine. Cancer patients often use CAM in the form of nutritional supplements, psychological techniques and natural medical approaches in the place of or in parallel to conventional medicine. The present study aimed to determine if Chitosan can inhibit lung metastasis and hepatoma formation, by studying xenograft of B16F10 melanoma cells in C57BL/6 mice and of Smmu 7721 cells in SCID mice, respectively. For the lung metastasis model, after a five-week treatment, the survival rates of B6 mice were 15% for the control group and 35%, 20%, 45% and 40% for the 320,000 kDa, 173,000 kDa, 86,000 kDa and 8,000 kDa molecular-weight treatment groups, respectively. Chitosan treatment dramatically increased lifespan and inhibited tumor metastasis especially in treatment groups of the low-molecular weight compound. For the hepatoma growth model, the size of the liver tumor mass was approximately >14 mm in the control group. In comparison to the control group, the tumor mass grew slowly with Chitosan treatment, especially at the low-molecular weight treatment group. Chitosan slowed-down the rate of tumor growth but did not inhibit tumor formation. Data presented herein demonstrate that Chitosan has anticancer effects and thus further study of the substance is warranted to examine for mechanisms of action and optimal dosage.*

In 2008 there were approximately 12.7 million cancer cases worldwide, with 6.6 million cases affecting men and 6.0 million women. The total number is expected to increase to 21 million by year 2030. Lung cancer is the most common cancer worldwide, contributing 1.61 million (nearly 13%) of the total number of new cases diagnosed in 2008 (1). Liver cancer is the sixth most common cancer in the world, with 750,000 new cases diagnosed in 2008. This accounted for

#*These Authors contributed equally to this work.

Correspondence to: Jing-Gung Chung, Ph.D., Department of Biological Science and Technology, China Medical University. No 91, Hsueh-Shih Road, Taichung 40402, Taiwan. Tel: +886 422053366 ext. 2161, Fax: +886 422053764, e-mail: jgchung@mail.cmu.edu.tw and Dr. Fang-Ming Hung, Department of Surgical Intensive Care Unit, Far Eastern Memorial hospital, No. 21, Sec.2, Nan-Ya south Rd. Banciao 220, Taipei, Taiwan, R.O.C. e-mail: philip@mail.femh.org.tw

Key Words: Chitosan, lung metastasis, hepatoma formation, xenograft models.

about 6% of the total number of cases of cancer in 2008. Mongolia has the highest rate of liver cancer, followed by Gambia and Taiwan (1).

Surgery, chemotherapy, and radiotherapy are the major conventional cancer therapies. However, these therapies have numerous limitations and drawbacks: i) most cancer patients are diagnosed too late to undergo surgery; ii) most cancers have a postoperative survival rate of less than 5 years and recurrence is quite common in patients who have had a resection; iii) although chemotherapy and radiotherapy are effective against cancer, they also have serious side-effects and complications *e.g.* fatigue, pain, diarrhea, nausea, vomiting, and hair loss; and iv) certain cancers are relatively resistant to chemo- or radiotherapy (2, 3). Therefore an urgent need for effective therapies or combination therapies to treat cancer is noted. Over the past few years, use of complementary and alternative medicine (CAM) has become increasingly popular among cancer patients in Western countries, with prevalence as high as 80%, for patients using some form of CAM (4, 5). Traditional medicine and herbal medicines in particular, have been used in cancer treatment for thousands of years in China, Japan, and other countries. These medicines are widely accepted as current forms of CAM in cancer treatment in the United States and Europe (6, 7). As recent pre-clinical and clinical studies have shown, Traditional Chinese medicine (TCM) combined with conventional Western medicine (chemotherapy and radiotherapy) can provide effective supportive care for cancer patients. TCM adds great advantages in terms of increasing the sensitivity of chemo- and radio-therapeutics, reducing the side-effects and complications associated with chemotherapy and radiotherapy, and improving patient quality of life and survival (8). Therefore, an understanding of Chinese herbal medicines is needed by physicians and other health care providers.

Chitosan was first described by Rouget in 1859 and in 1894; and was formally named by Hoppe-Seyler (9-12). Chitosan, largely available in the exoskeletons of shellfish and insects, is a collective name for a group of partially and fully de-acetylated chitins. Chitin is a linear polysaccharide consisting of β(1→4) linked N-acetyl–*D*-glucosamine (GlcNAc; A) residues (13). De-acetylation of chitin is established by boiling chitin from crab and shrimp shells in sodium hydroxide after decolorization with potassium permanganate (9-12). When the number of N-acetylglucosamine units exceeds 50%, the biopolymer is termed chitin, whereas the term "chitosan" is used to describe an N-acetyl-glucosamine unit content less than 50% (9). The unique structural feature of chitosans is the presence of the primary amine at the C-2 position of the glucosamine residues. This polysaccharide is non-toxic, biocompatible and biodegradable. Chitosan has many uses either alone or in blends with other natural polymers (starch, gelatin, alginates) in agriculture, cosmetics, water treatment, medicine, environmental protection, biotechnology, functional food and the pharmaceutical industry, mainly due to its high biodegradability and anti-microbial properties (11-15). Chitosan exhibits a variety of interesting physicochemical and biological properties. Chitin is insoluble in water but chitosan is soluble in dilute aqueous acid solutions. Chitosan is digested by chitinases after oral administration. Chitinases are secreted by intestinal microorganisms and are also present in plant ingredients of food, or by lysozymes (16, 17). The US Food and Drug Administration approved chitosan as a food additive in 1983. Because of its low production costs, biodegradability, biocompatibility and recent FDA approval, the pharmaceutical and food applications of chitosan have increased remarkably over recent years (18, 19).

There is evidence that chitosan has numerous medicinal uses, *e.g.* against asthma (20-23), cholesterol-lowering effects (17, 24), as an anti-bacterial agent (18), ingredient in wound-dressings (25, 26), as vector in gene-therapy (27, 28), anti-fungal activity (29, 30) and in reducing serum glucose levels in diabetics (31). Furthermore, chitosan may increase bone-strength in osteoporosis (32, 33) and it may inhibit the malaria *Plasmodium* parasites (34). The biological activity of chitosan depends on its structural properties including its molecular weight. The purpose of the present study was to determine if low molecular weights of chitosan were more effective in reducing tumor size compared to high molecular weights. This hypothesis was tested in xenograft mouse models of melanoma and hepatoma.

Materials and Methods

Experimental animals and housing conditions. The studies involving mice were approved by the Institutional Animal Care and Use Committee of Chen Hsin General Hospital (Taipei, Taiwan, ROC). C57BL/6 male mice and SCID male mice, specific pathogen-free and 6 weeks old, were obtained from the National Taiwan University College of Medicine Animal Medicine Center. Animals were kept in polypropylene cages (5 animals/cage) covered with metallic grids in a room maintained under constant environmental conditions, with air filter tops in a filtered laminar air flow, with an ambient temperature of 20±2˚C, relative humidity 75±15%, and a 12-h light-dark cycle. Mice were given autoclaved water and fed laboratory pellet chow *ad libitum* following the animal procedures approved by the National Science Council of the Republic of China. Experiments were performed according to law, regulations and guidelines for animal experiments in Taiwan, which are in agreement with the declaration of Helsinki.

Preparation of Chitosan with four different molecular weights. Chitosan powder with molecular weights of approximately 320,000 kDa, 173,000 kDa, 86,000 kDa and 8,000 kDa (Koyo Chemical Co., Ltd, Sakaiminato, Tottori, Japan) was obtained from the National Taiwan University College of Medicine Animal Medicine Center (Taipei, Taiwan, R.O.C.). The dose of 0.125 mg/day/mouse was separately suspended in 0.2 ml distilled water at 50˚C for 10 min, then cooled to room temperature and stirred for 1 h.

Table I. *In the lung metastasis model, after 5 weeks' treatment, survival rates were 15% for the control group and 35%, 20%, 45% and 40%, for the 320,000 kDa, 173,000 kDa, 86,000 kDa, 8,000 kDa treatment groups, respectively.*

	Control		320,000 kDa		173,000 kDa		86,000 kDa		8,000 kDa	
	Dupli 1	Dupli 2	Dupli 1	Dupli 2	Dupli 1	Dupli 2	Dupli 1	Dupli 2	Dupli 1	Dupli 2
1w	0	0	0	0	0	0	0	0	0	0
2w	0	0	0	0	1	0	0	0	0	0
3w	1	1	1	1	1	2	1	0	0	0
4w	2	3	2	3	3	2	2	3	3	3
5w	5	5	3	3	4	3	2	3	3	3
Survivors	2	1	4	3	1	3	5	4	4	4

Table II. *Data showing the tumor status score of all mice. Significant differences between the control group and the experimental groups are apparent.*

	Control		320,000 kDa		173,000 kDa		86,000 kDa		8,000 kDa	
	Dupli 1	Dupli 2	Dupli 1	Dupli 2	Dupli 1	Dupli 2	Dupli 1	Dupli 2	Dupli 1	Dupli 2
+	0	0	0	0	0	0	4	3	4	3
++	1	2	1	2	2	2	4	4	3	3
+++	3	3	3	3	2	3	1	2	2	2
++++	6	5	6	5	6	5	1	1	1	2

Numbers for tumor status was categorized into 4 scales: +, 1-12 tumor masses; ++, 13-24; +++, 25-36; ++++, over 36 tumor masses.

Experimental design and treatment for metastasis formation. B16-F10 mouse melanoma cell lines were purchased from the American Type Culture Collection (ATCC, Rockville MD, USA) and preserved by the National Taiwan University College of Medicine Animal Medicine Center. Cells were cultured in RPMI1640 medium (Gibico BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) plus 100 μg/ml amikacin in a 37˚C humidified chamber containing 5% CO_2.

Fifty-five 8-week-old C57BL/6 mice were inoculated with 5×10^4 B16F10 cells suspended in 0.1 ml PBS into the tail vein and then divided into 5 groups (each group consisted of 10 mice except for the positive control group). The positive control group (5 mice) was sacrificed between the 8th to 10th day to observe whether metastasis was present or not; from past experience, 10 days were needed for mice to show lung metastasis. All mice were fed with the regular diet and double distilled water. After 10-day inoculation, the positive control group was assessed for black points of needle size on the lung surface by visual inspection which was observed in the positive control group. Chitosan (molecular weights of 320000 kDa, 173000 kDa, 86000 kDa and 8000 kDa) was administered daily by oral gavage (0.125mg/ day/ mouse) for 5 weeks. Lung tissue was collected and tumor lesions were scored immediately after the animals were sacrificed. The survival rate was assessed by counting the surviving mice at the end of the 5th week of treatment. Survivors were sacrificed under anesthesia by CO_2. We duplicated the experiment by the above procedures to the other 55 animals. The number of melanotic nodules on each lung was counted and scored under gross examination. Tumor status was categorized into 4 scales: +, 1-12 tumor masses; ++, 13-24 tumor masses; +++, 25-36 tumor masses; ++++, more over 36 tumor masses.

Experimental design and treatment for hepatoma formation by Smmu 7721 cells in SCID mice. Mice were injected subcutaneously (*s.c.*) with Smmu 7721 cells (3×10^7 cells/mouse) in the dorsal area, while growing to 8 weeks old. Following 2-3 weeks (week 0) after the injection, mice with tumors of 1-3 mm in diameter were divided into five groups with 10 mice per group. Mice were fed with the regular diet and double-distilled water. Chitosan at the different molecular weights was administered orally (0.125 mg/day) for 6 weeks and then surviving mice were sacrificed under anesthesia by CO_2. Liver tumor status was defined by 4 sizes: +, <7 mm; ++, 7-<14 mm; +++, 14 - <21 mm; ++++, >21 mm.

Results

Chitosan reduces metastasis induced by B16F10 melanoma cells in C57BL/6 mice. Survivor rates improved after 5 weeks of treatment with different molecular weights of Chitosan. For the tumor metastasis model, all five mice of the indicator group formed metastases at around 10 days. Injection of B16F10 melanoma tumor cells into C57BL/6 mice induced metastasis. Chitosan treatment increased survival rates which were 15% for mice of the control group and for the different Chitosan molecular weight groups: 35% for the 320,000 kDa, 20% for the 173,000 kDa, 45% for the 86,000 kDa and 40% for the 8,000 kDa (Table I). Following 5 weeks of Chitosan administration, surviving mice were sacrificed to collect lung tissues (Figure 1). Treatment effects were

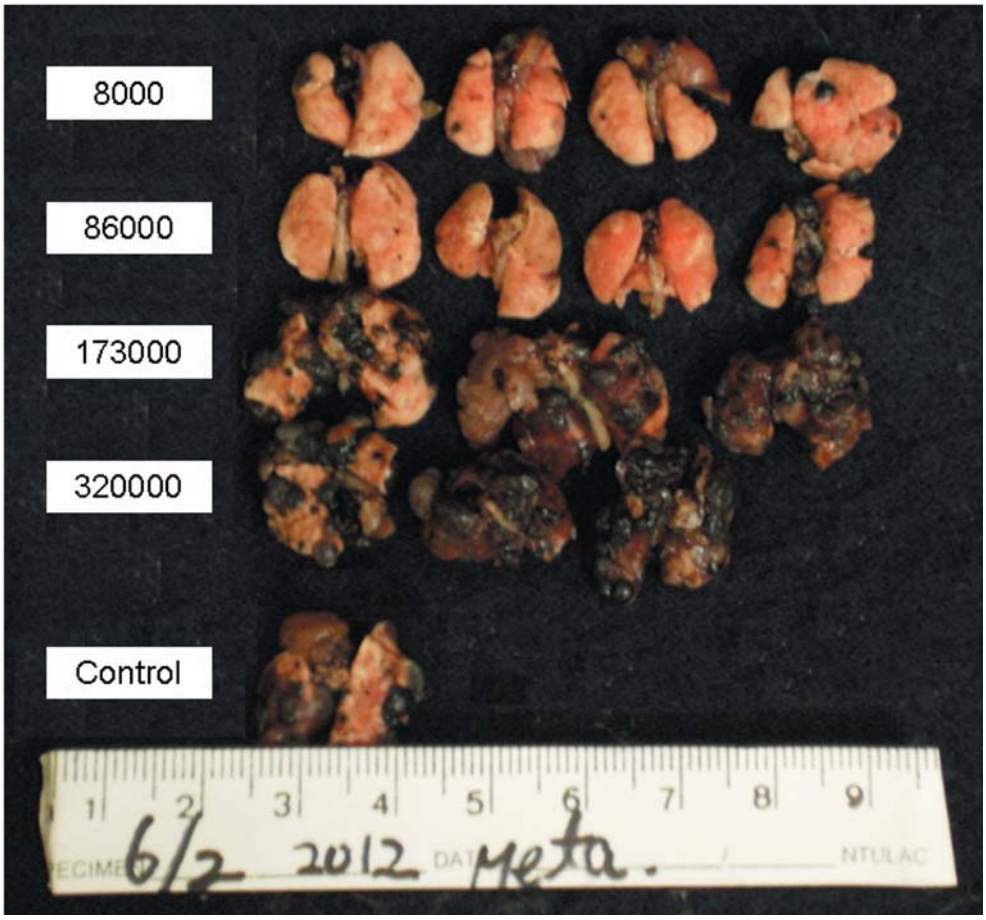

Figure 1. *The tumor mass in control and treatment mice were induced by inoculation with B16F10 cells. Chitosan was administered orally daily for 5 weeks to four experimental groups of mice. At the end of the experiments, all survivors were sacrificed and the number of melanotic nodules on lungs was counted and scored under gross examination.*

dependent on the molecular weight of Chitosan. Table II lists data showing the manner in which Chitosan reduced tumor mass.

Chitosan alters hepatoma formation in SCID mice. Injection of Smmu 7721 tumor cells into SCID mice induced tumors of 1-3 mm in diameter at 2-3 weeks post-injection. Hepatoma was successfully induced in 40 mice in this study. After 6 weeks of treatment, the survival rates were 50% for the control group and for the different Chitosan molecular weights: 320,000 kDa 40%, 173,000 kDa 40%, 86,000 kDa 70% and 8,000 kDa 90% (Table III). The 8,000 kDa and 86,000 kDa treatment groups had greater survivor rates than the higher Chitosan molecular weight groups.

Observation clearly revealed a tumor mass in the liver of the positive control group (Figure 2). Surviving mice in control group, all scored the highest in tumor mass (Table IV). The size of the tumor mass grew rapidly and was more than 21 mm at the end of 6th week post-injection. Chitosan did not inhibit tumor growth but compared with the positive control group, tumor mass seemed to grow more slowly among the different Chitosan molecular weight groups. Data

Table III. *In the hepatoma formation model, after 6 weeks' treatment, the survival rates were 50% for the control group and 40%, 40%, 70% and 90%, for the 320,000 kDa, 173,000 kDa, 86,000 kDa and 8000 kDa treatment groups, respectively.*

	Control	320000	173000	86000	8000
1w	0	0	0	0	0
2w	0	0	0	0	0
3w	0	0	0	0	0
4w	1	0	0	0	0
5w	2	2	1	0	0
6w	2	4	5	3	1
Survivors	5	4	4	7	9

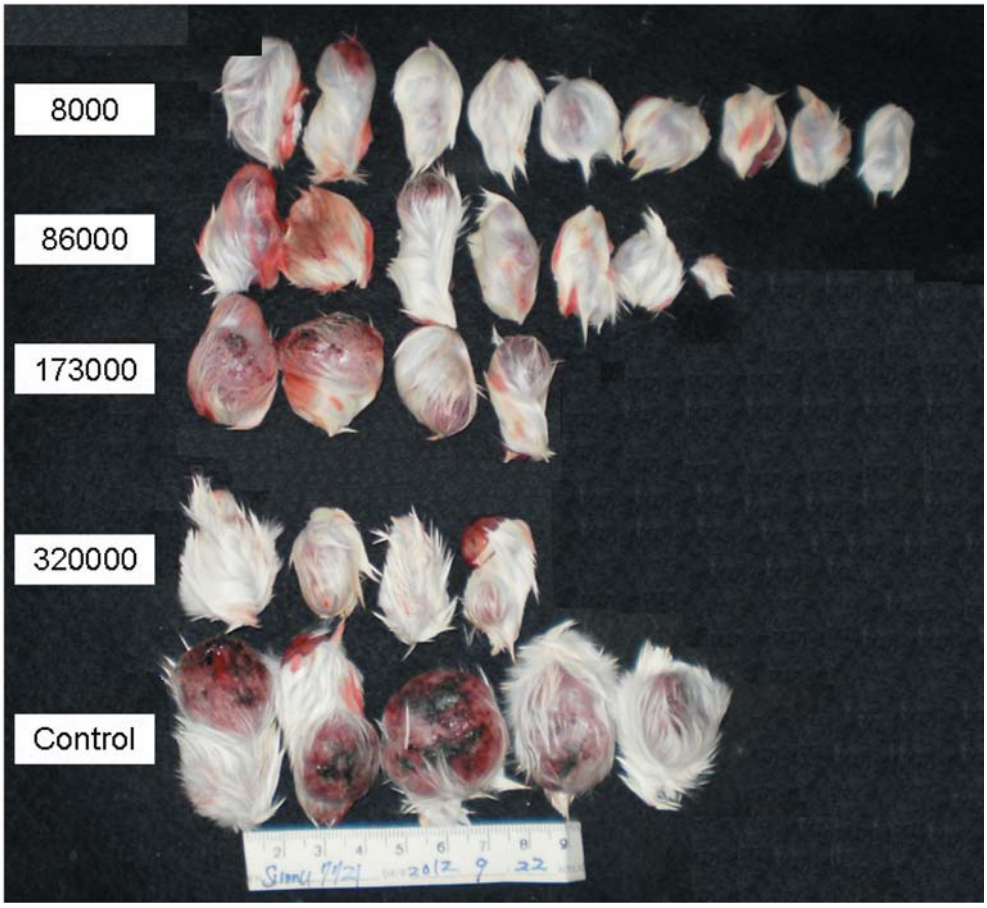

Figure 2. *Following Smmu 7721 inoculation to initialize hepatoma, mice of experimental groups were orally administered Chitosan of different molecular weights. After 6 weeks' treatment, all survivors were sacrificed, and the sizes of liver tumors were assessed. Low-molecular weight Chitosan treatment can evidently reduce tumor growth compared to control groups.*

in Figure 2 show that all four molecular weights were effective in inhibiting tumor growth especially the 8,000 kDa and 86,000 kDa groups. Surviving mice treated with 8,000 kDa and 86,000 kDa Chitosan scored the lowests level for tumor growth. Tumor growth may be dependent upon the molecular weight of administered Chitosan.

Disscussion

Recently, low-molecular weight (LMW) chitosan has been shown to present advantages as a colloidal drug carrier due to its high water solubility, non-toxicity, bio-compatibility, bio-degradability, bio-adhesiveness and absorption-enhancing properties. Moreover, the potential biological activities of LMW chitosan, such as anti-oxidant and antitumor activities make it an ideal candidate for biomedical applications (35, 36). The mechanisms of its bioactivities are poorly-understood. Its activities have been reported only once or twice, providing insufficient basis for general conclusions about the applicability of chitosan. Although many studies are available on the aforementioned biological activities, the relationships of these activities with molecular weight and water-solubility of chitosan deserve to be investigated. It can be easily hypothesized that the biological properties of chitosan may be closely-related to its molecular weight and water-solubility.

Table IV. *Tumor size score after 6-week treatment of SCID mice with different molecular weight of Chitosan. Tumor status categorized as: +: up to 7 mm; ++: up to 14 mm, +++: up to 21 mm; ++++: over 21 mm.*

	Control	320,000	173,000	86,000	8,000
+	0	0	0	0	0
++	0	0	0	1	3
+++	2	6	7	7	7
++++	8	4	3	2	0

Chitosan has an antitumor activity (37) yet there are only few studies on lung metastasis induced by B16F10 melanoma cells in C57BL/6 mice, and hepatoma induced by smmu 7721 cells in SCID mice. The aim of the present study was to examine whether different molecular weights of Chitosan were effective against tumor-bearing mice. Chitosan has a wide spectrum of possible bioactivities (38).

There are some studies which have reported on antitumor effects of Chitosan (39). The present study is the first to evaluate effects of Chitosan on inhibition of metastasis induced by B16F10 melanoma cells in C57BL/6 mice and hepatoma formation by Smmu 7721 cells in SCID mice. Chitosan reduced metastasis and tumor growth with the most potent effect seen at the lower molecular weights tested. Chitosan had antitumor effects in mouse models, and our results provide the rationale for further studies on potential mechanisms of this promising anti cancer drug.

Acknowledgements

This study was supported by the grant CH101-02 from Cheng Hsin General Hospital.

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Received September 11, 2013
Revised October 4, 2013
Accepted October 7, 2013