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Editorial Board

Editorial Office

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan
Tel: +81-3-5840-8764 Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com

BioScience Trends

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: +81-3-5840-8764, Fax: +81-3-5840-8765
E-mail: office@biosciencetrends.com
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Traditional, complementary, and alternative medicine: Focusing on research into traditional Tibetan medicine in China

Peipei Song[1], Jufeng Xia[2], Caidan Rezeng[3], Li Tong[3,*], Wei Tang[2]

[1] *Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa-shi, Chiba, Japan;*
[2] *Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[3] *The Research Center of Chinese and Tibetan Medicine, Medicine College, Qinghai University, Xining, Qinghai, China.*

Summary **As a form of traditional, complementary, and alternative medicine (TCAM), traditional Tibetan medicine has developed into a mainstay of medical care in Tibet and has spread from there to China and then to the rest of the world. Thus far, research on traditional Tibetan medicine has focused on the study of the plant and animal sources of traditional medicines, study of the histology of those plants and animals, chemical analysis of traditional medicines, pharmacological study of those medicines, and evaluation of the clinical efficacy of those medicines. A number of papers on traditional Tibetan medicines have been published, providing some evidence of the efficacy of traditional Tibetan medicine. However, many traditional Tibetan medicines have unknown active ingredients, hampering the establishment of drug quality standards, the development of new medicines, commercial production of medicines, and market availability of those medicines. Traditional Tibetan medicine must take several steps to modernize and spread to the rest of the world: the pharmacodynamics of traditional Tibetan medicines need to be determined, the clinical efficacy of those medicines needs to be verified, criteria to evaluate the efficacy of those medicines need to be established in order to guide their clinical use, and efficacious medicines need to be acknowledged by the pharmaceutical market. The components of traditional Tibetan medicine should be studied, traditional Tibetan medicines should be screened for their active ingredients, and techniques should be devised to prepare and manufacture those medicines.**

Keywords: Minority medicine, traditional medicine, evidence-based medicine, components of traditional Tibetan medicines, active ingredient, quality standards

1. Introduction

The increasing prevalence of complex multi-factorial chronic diseases and multi-morbidity has indicated the need for greater therapeutic options. In medical practice, the traditional, complementary, and alternative medicine (TCAM) is an important part of health care along with conventional medicine, and growing numbers of patients are relying on TCAM for preventive or palliative care worldwide. Many studies have indicated that TCAM users were more likely to suffer from one or more chronic conditions, and especially mental, musculoskeletal, and metabolic disorders (*1-3*). The prevalence of TCAM use is reported to be 22.7-66.7% (*4,5*). A meta-analysis indicated an increase in TCAM use for patients with cancer from an estimated 25% in the 1970s and 1980s to more than 32% in the 1990s and to 49% after 2000 (*6*). Data from recent studies have indicated that the rate of TCAM use is 77% or even higher (*7-9*).

Medicines used in TCAM include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants, other plant materials, or combinations thereof, as active ingredients. TCAM practices involving medication and procedure-based health care have been implemented; as an example, acupuncture is now used worldwide. According to reports supplied by 129 countries, 80% (103 countries)

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**Address correspondence to:*
Dr. Li Tong, The Research Center of Chinese and Tibetan Medicine, Medicine College, Qinghai University, No.16, Kunlun Road, Xining 810001, Qinghai, China.
E-mail: qhdx2011@126.com

recognized the use of acupuncture as of 2012 (*10*).

The World Health Organization (WHO) stresses that TCAM can play an important role in achieving the goal of "Health for All" and the WHO is dedicated to facilitating the integration of TCAM and modern medicine worldwide (*11*). With the implementation of the "WHO Traditional Medicine Strategy 2002-2005," great efforts were made to advance TCAM by developing national and regional policies and regulations. Furthermore, the "WHO Traditional Medicine Strategy 2014-2023" has also been formulated to promote the safe and effective use of TCAM (*10*). Current data indicate that over 100 million Europeans are currently TCAM users, with one-fifth regularly using TCAM and the same number preferring health care that includes TCAM; there are many more TCAM users in Africa, Asia, Australia, and North America (*10*).

In China, TCAM includes traditional Chinese medicine (TCM) as well as minority medicines such as traditional Tibetan medicine, Mongolian medicine, Uygur medicine, and Dai medicine. Among the minority medicines that originated more than 2,300 years ago, traditional Tibetan medicine has developed into a mainstay of medical care in Tibet and it has spread from there to China and then the rest of the world (*12*). In China today, traditional Tibetan medicine plays an important role in the health care system in the Tibet Autonomous Region and other Tibetan regions including Qinghai Province, Tibet Autonomous Region, Gannan Prefecture of Gansu Province, Ganzi Prefecture and Aba Prefecture of Gansu Province, and Diqing Prefecture of Yunnan Province. However, the development of traditional Tibetan medicine faces several challenges, particularly with regard to evaluation of its safety and efficacy, control of its quality, and standardization. Traditional Tibetan medicine must take several steps to modernize and spread to the rest of the world: the pharmacodynamics of traditional Tibetan medicines need to be determined, the clinical efficacy of those medicines needs to be verified, criteria to evaluate the efficacy of those medicines need to be established in order to guide their clinical use, and efficacious medicines need to be acknowledged by the pharmaceutical market.

2. Traditional Tibetan medicine: Types and characteristics

Traditional Tibetan medicine is a form of medicine guided by a traditional system of practices and theories. Traditional Tibetan medicine is an important part of minority medicines and an integral part of herbal medicine and natural medicine. Plants used to prepare traditional Tibetan medicines are typically cold-resistant, drought-resistant, and perform intensive photosynthesis. Traditional Tibetan medicines include a large number of biologically active substances, trace elements, and amino acids. The efficacy of traditional Tibetan medicines is greater than that of similar traditional medicines from regions at lower altitudes.

There are about 2,000 to 3,000 traditional Tibetan medicines available in China. Based on long-term collection of specimens and statistical data (*13*), plants used in traditional Tibetan medicine come from 191 families, 692 genera, and 2,085 species. Animals used in traditional medicines come from 57 families, 111 genera, and 159 species. Minerals used in traditional Tibetan medicine include 80 types of compounds. The abundant sources of traditional Tibetan medicines suggests that some of these medicines may offer promise as clinical treatments.

In 2012, a study analyzed 439 traditional Tibetan medicines in the Chinese Pharmacopoeia, the Drug Standards of the Ministry of Health of the People's Republic of China (Volume: Traditional Tibetan Medicine), the Tibetan Drug Standards, and the related literatures. It also investigated 711 traditional Tibetan medicines formulations from 40 medical facilities and pharmaceutical companies (*14*). The study found that 502 raw ingredients were used in hospital formulations and formulations described in the literature; 416 of those raw materials had been documented, including 287 herbs, 78 animals, and 51 minerals.

Currently, over 200 traditional Tibetan medicines have been incorporated into the Chinese Pharmacopoeia. Rannasangpei, Shiwuweiheiyao pills, Jiuweiniuhuang pills, and 40 other medicines have been approved, and 78 medicines have been incorporated into the Catalogue of Medicines Covered by National Basic Medical Insurance (*15*).

3. Pharmacological research and its clinical application: Status and challenges

A number of published papers have described studies of traditional Tibetan medicine. In 2003, a study classified and statistically analyzed 14 relevant books and 543 relevant papers published from 1953 to 2002 (*16*). That study suggested that the focus of research into traditional Tibetan medicine has shifted from study of the plant and animal sources of traditional medicines to the study of the histology of those plants and animals, chemical analysis of traditional medicines, pharmacological study of those medicines, and determination of their efficacy in clinical settings.

Research in the 1950s consisted primarily of an introduction to traditional Tibetan medicine and study of the plant and animal sources of traditional medicines. In the early 1970s, research consisted primarily of the study of the plant and animal sources of traditional medicines and surveys of the literature. In the late 1970s, research consisted primarily of the ethnobotanical study of traditional medical texts, chemical analysis of traditional medicines, and pharmacological study of those medicines. Starting

in the 1980s, studies on traditional Tibetan medicine diversified, including study of the plant and animal sources of traditional medicines, study of the histology of those plants and animals, chemical analysis of traditional medicines, pharmacological study of those medicines, and clinical evaluation of the efficacy of those medicines. In recent years, pharmacological research and its clinical application have become the focus of research on traditional Tibetan medicine.

3.1. *Pharmacological research on traditional herbal Tibetan medicine*

Basic research on traditional Tibetan medicine started late, but several studies have made great progress. A number of pharmacological studies of the active compounds of traditional herbal Tibetan medicines have been published (Table 1). For instance, studies have suggested that salidroside, an active compound extracted from *Rhodiola eoccinea*, has anti-aging action, it alleviates fatigue, it has antioxidant action, and it improves memory (*17-19*). Studies have indicated that salidroside alleviates hydrogen peroxide-induced endothelial injury by improving mitochondrial activity (*20*) and it improves microcirculation (*21*). Thus, salidroside has been used to treat high-altitude erythrocytosis. *Crocus sativus L.*, which is rich in crocin, crocetin, and safranal, is used as an anti-tumor treatment (*22*). *C. sativus* protects the heart from subchronic diazinon toxicity by decreasing lipid peroxidation and apoptosis (*23*).

3.2. *Pharmacological research on patent medicines of traditional Tibetan medicine*

Following the development of modern pharmacology, many pharmacological studies on patent medicines of traditional Tibetan medicine have been published. For instance, sea buckthorn cream (Shaji Gao) was widely used to "clear heat" (antipyretic action), relieve a cough, promote blood circulation to dispel blood stasis, and treat an ulcer. Flavones, the main ingredients

Table 1. Active ingredients and pharmacological analysis of traditional herbal Tibetan medicines

Raw material (*Ref.*)	Active ingredients	Pharmacological analysis
Rhodiola rosea L. (*17-21*)	Salidroside	anti-aging action, alleviating fatigue, antioxidant action, improving memory; increasing mitochondrial activity to protect from H_2O_2-induced endothelial injury; improving microcirculation.
Crocus sativus L. (*22,23*)	Crocin, crocetin, safranal	anti-tumor activity; protecting the heart from subchronic diazinon toxicity by decreasing lipid peroxidation and apoptosis.
Swertia (*24-27*)	Triterpenes, flavonoids, chimonin, gentiamarin, swertiamarin	inhibiting hepatitis B, cirrhosis, and *E. coli*; anti-tumor activity; scavenging NO_2^-; slightly inhibiting growth of the S180 mouse cancer cell line and moderately inhibiting growth of the RS321 tumor cell line.
Phyllanthus emblica (*28-31*)	ellagic acid, gallic acid, sesquiterpenoids, volatile oils	antioxidant action; anti-tumor activity; antibiosis; anti-inflammatory action; protecting the cardiovascular system.
Hypecoum erectum L. (*32-35*)	Alkaloids (hypecorin), cis-anethole, cardiac glycoside	anti-inflammatory action; antibiosis; anti-viral action; liver protection; analgesic action.
Aconitum carmichaelii Debx. (*36-39*)	Alkaloids (bulleyaconitine A, lappaconitine), flavonoids, steroids	analgesic action; anti-inflammatory action; cardiotonic activity; anti-tumor activity; antiepileptic action; antiparasitic action.
Terminalia chebula Retz. (*40-43*)	Tannins, phenolic acids, triterpenes, flavonoids, volatile oils	antioxidant action; preventing diabetes mellitus; antibiosis; anti-inflammatory action; analgesic action.
Meconopsis (*44-47*)	Flavonoids, alkaloids	treating fractures; increasing blood circulation to dissipate blood stasis; analgesic action.
Herpetospermum pedunculosum (*48-51*)	Herpetone, dehydrodiconiferyl alcohol, Herpetolide C	treating hepatitis, cholecystitis, or indigestion.
Corydalis (*52-55*)	Isoquinoline alkaloid	treating a cold, fever, hepatitis, edema, gastritis, cholecystitis, or hypertension; antibiosis, analgesic action, anti-inflammatory action, anti-arrhythmia, liver protection; pharmacological action on the cardiovascular system, central nervous system, and smooth muscle.
Gentiana macrophylla Pall. (*56-58*)	Iridoid glycoside, secoiridoid glycoside	improving the flow of interstitial fluid and removing algogenic substances, treating arthritis, rheumatism, or muscular constriction.

in sea buckthorn cream, have been found to treat arrhythmia, improve the function of cardiomyocytes, treat a thrombus, counteract hypoglycemia, scavenge oxygen radicals, inhibit tumor growth, and facilitate bacteriostasis (*59-61*). RanNaSangPei (RNSP) decreases blood viscosity, it reduces blood pressure, it alleviates angina pectoris, and it is effective at treating cardiovascular diseases (*62,63*). Ershiwuwei Donkey Blood Pills are used to treat rheumatoid arthritis, ankylosing spondylitis, and rheumatoid arthritis (*62*).

3.3. *Challenges in the pharmacological study of traditional Tibetan medicine*

In recent years, the pharmacological study of traditional Tibetan medicine has made some progress, but the methods used in research are mostly traditional. The lack of in-depth and extensive pharmacological study of traditional Tibetan medicines means that traditional Tibetan medicine is still far from modern. Methods of modern pharmacology, such as chronopharmacology, *in vitro* pharmacology, component analysis, and pharmacokinetics, should be capitalized upon in order to improve the quality of research, determine the mechanisms of action of traditional Tibetan medicines, and obtain international recognition of the efficacy of those medicines.

3.4. *Clinical application: Traditional Tibetan medicine preparations*

Traditional Tibetan medicine has been used clinically to treat common ailments, frequently encountered diseases, altitude sickness, and endemia, and traditional Tibetan medicines have been developed to treat 5 types of diseases including cardiovascular diseases, hepatobiliary diseases, gastrointestinal diseases, rheumatism, and gynecological diseases.

There are more than 20 dosage forms for traditional Tibetan medicine, such as decoctions, powders, pills, ointments, lotions, and medicinal liquors. Although there are many traditional Tibetan medicine dosage forms, the types of preparations, the diversity of dosage forms, and their clinical are expected to be improved. Modern techniques to prepare and manufacture drugs should be used to accelerate the development of novel drugs while retaining the characteristics of traditional Tibetan medicine.

4. Study of the chemical constituents of traditional Tibetan medicines and quantitative analysis of those traditional medicines

4.1. *Promoting a quantitative analysis of traditional Tibetan medicines*

Chemical constituents are the reason for a medicine's effect. From 2003 to 2013, studies of traditional Tibetan medicine focused on extraction, identification, and quantitative determination of the chemical constituents of traditional medicines. Chemical constituents of about 100 traditional Tibetan medicines, such as Tibetan *Artemesia capillaris*, *Lamiophlomis rotata* Kudo, and *Arenaria serpyllifolia* L., were extensively studied.

High performance liquid chromatography (HPLC) was widely used to establish quality control standards by quantitatively analyzing a traditional medicine's chemical constituents. Zhong *et al*. used HPLC to analyze gallic acid in the traditional Tibetan medicine Sanguo Tang Lozenges (*64*). De *et al*. used HPLC to prolife Sanguo Tang San (*65*). Luo *et al*. used HPLC to determine the flavonoid content of Shibawei Dujuan Pills (*66*). Xie *et al*. used HPLC to quantitatively detect shikimic acid and gallic acid in *Geranium pretense* L. and they established quality control standards for quantitative analysis of *Geranium pratense* (*67*).

Besides, Li *et al*. used Fourier transform near-infrared (FT-NIR) spectroscopy to quantitatively analyze salidroside and p-Tyrosol in the traditional Tibetan medicine *R. crenulata*. They found that FT-NIR spectroscopy provided a precise and rapid method for quantitative analysis of the major active ingredients in *R. crenulata* and that it could be used to control the quality of *R. crenulata* (*68*).

4.2. *Examining the active compounds in traditional Tibetan medicines to provide indicators for quality control*

About 500 papers have been published on the chemical constituents of traditional Tibetan medicines, but these papers have only scratched the surface of traditional Tibetan medicine. Unknown active compounds hamper the establishment of drug quality standards and the development of novel drugs. Thus, new techniques such as droplet countercurrent chromatography, supercritical extraction, infrared absorption spectroscopy, thin slice scanning, gas chromatography mass spectrometry, liquid chromatography mass spectrometry, and nuclear magnetic resonance could be used. These techniques could improve studies of the active compounds in traditional Tibetan medicines, examine various indicators to direct production and ensure the consistency, efficacy, stability, and reliability of traditional medicines, promote the modernization of quality standards, and encourage the development and utilization of traditional Tibetan medicines.

5. Evidence-based medicine, quality control, and the modernization and international recognition of traditional Tibetan medicine

Numerous studies have focused on the study of the plant and animal sources of traditional Tibetan

medicines, the study of the histology of those plants and animals, analysis of the chemical composition of traditional medicines, pharmacological study of those medicines, and evaluation of the clinical efficacy of those medicines in order to provide evidence for development of traditional Tibetan medicine. However, the traditional Tibetan medicines studied thus far only account for 10% of all traditional Tibetan medicines. Many traditional Tibetan medicines have unknown active ingredients, hampering the establishment of drug quality standards, the development of new medicines, commercial production of medicines, and market availability of those medicines. Traditional Tibetan medicine must take several steps to modernize and spread to the rest of the world: the pharmacodynamics of traditional Tibetan medicines need to be determined, the clinical efficacy of those medicines needs to be verified, criteria to evaluate the efficacy of those medicines need to be established in order to guide their clinical use, and efficacious medicines need to be acknowledged by the pharmaceutical market.

5.1. *Evidence-based medicine and component analysis on traditional Tibetan medicine*

Development of traditional medicines involves many obstacles such as safety, efficacy, and evaluation of quality. Component analysis has been used to meet this challenge and this approach has been used to study the functions and active compounds of traditional medicines (*69-71*). In recent years, component analysis of traditional Tibetan medicines has developed rapidly and has been used to standardize traditional Tibetan medicines (*72,73*). The crux of research into traditional Tibetan medicine is the study of active ingredients based on their mechanism of action. Several studies have examined the compatibility of active ingredients, they have determined the quality of the active ingredients in traditional Tibetan medicines, they have studied the relationship between efficacious ingredients and active ingredients, and they have examined the aspects and targets of the efficacious ingredients of traditional Tibetan medicines.

Studies have suggested that traditional Tibetan medicines developed using modern pharmaceutical manufacturing techniques and animal models of pharmacodynamics could contain components that offer the clinical benefits of traditional Tibetan medicines (*74,75*). Thus, suitable quality standards for traditional Tibetan medicines need to be established to ensure preparations have a specific chemical composition, an identified mechanism of action, and controlled quality. In addition, component analysis of traditional Tibetan medicines can be used to rule out ingredients that are inactive or deleterious, leaving only efficacious ingredients. Thus, component analysis of traditional Tibetan medicine can lead to better symptomatic treatment, it can clarify an ingredient's mechanisms of action, and it can facilitate international acceptance (*72,73*). Component analysis of traditional Tibetan medicines should be performed further in order to provide evidence for the development of traditional Tibetan medicine.

5.2. *Compound screening and new drug development*

Compound screening and new drug development are being used to develop traditional Tibetan medicines. In 2015, a study examined 100 traditional Tibetan medicines in order to discover new anticancer drugs (*76*). The study obtained cyclohexane extracts, acetic ether extracts, and methanol extracts of traditional Tibetan medicines through rapid solvent extraction. The same study used an MTT assay to detect the anti-tumor activity of those medicines in 2 human liver cancer cell lines (HepG2 and SMMC-7721) and it evaluated toxicity in the L02 human liver cell line. Results suggested that 15 traditional Tibetan medicines had anti-tumor activity, with an IC_{50} of less than 150 μg/mL. The identified substances included acetic ether extracts of *Chenopodium album* L., *Carpesium abrotanoides*, and *Aster ageratoides* Turcz. Those extracts had significant anti-tumor activity, with an IC_{50} less than 50 μg/mL, and none were toxic to L02 cells. Those findings offered a glimpse at the development of new anti-tumor drugs from traditional Tibetan medicines.

5.3. *Using modern scientific techniques and modern preparation and manufacturing techniques to promote the research and development of traditional Tibetan medicine*

Research and development of traditional Tibetan medicine should be promoted in order to yield different preparations and to facilitate the clinical use of traditional medicines. However, the characteristics of traditional Tibetan medicine need to be retained while using modern scientific techniques and preparation and manufacturing techniques: *i*) increasing the use of traditional Tibetan medicines by developing new techniques based on the medicine's characteristics; a medicine in powder form, for example, could be improved by superfine grinding and other preparations could be improved by supercritical fluid extraction and separation and purification using macroporous adsorbent resin; *ii*) compounds of raw materials could be improved through screening with pharmacological indicators; *iii*) an appropriate preparation could be chosen based on treatment goals; an injection, for example, is the preparation of choice for acute conditions such as angina pectoris and asthma while slow-release preparations are suitable for chronic diseases; *iv*) selecting a preparation based on a medicine's properties; an oral preparation, for example,

is suitable for a drug that is poorly soluble and this preparation could be used to improve drug absorption; *v*) development goals: efficiency, quick results, controlled release, a low dose, a low level of toxicity, and few adverse reactions, consistent timing and site of action, and a fixed rate of action.

6. Conclusion

Traditional Tibetan medicine is gradually becoming more scientifically based and the clinical efficacy of traditional medicines is gradually being determined. Nevertheless, traditional Tibetan medicine still faces several challenges. The consistent quality of traditional Tibetan medicines is directly related to their safety, efficacy, and control of their quality. A number of steps need to be taken in that direction. The sources of traditional medicines need to be explored, basic research needs to be conducted on those medicines, their active ingredients and their mechanisms of action need to be determined, quality standards need to be studied and implemented, traditional preparations need to be screened and more types of preparations need to be developed, and the efficacy of traditional medicines needs to be ensured while limiting their toxicity. These steps will modernize traditional Tibetan medicine and make those medicines more consistent, significantly facilitating the international recognition of traditional Tibetan medicine. Ingredients in traditional Tibetan medicines should be studied, those medicines should be screened for their active ingredients, and techniques should be devised to prepare and manufacture those medicines.

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Review

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Predictive biomarkers for targeted and cytotoxic agents in gastric cancer for personalized medicine

Shalong Wang, Lianwen Yuan*

Geriatric Surgery Department, Second Xiangya Hospital Affiliated with Central South University, Changsha, China.

Summary **Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer. The treatment of GC remains challenging as the outcomes achieved with surgery alone or adjuvant or neoadjuvant chemotherapy and radiotherapy are relatively poor. New treatment strategies are emerging and are being tested in solid tumors including GC. Over the past few years, the treatment of metastatic colorectal cancer (CRC) has made great advances, but strategies to manage GC have improved little. Multiple drug resistance is common in GC chemotherapy and targeted therapy; some patients appear to receive treatment that is suboptimal or even inefficacious. Unfortunately, there are few validated predictive biomarkers to guide the tailored treatment of GC. ToGA and AVAGAST are two phase III trials that tested the efficacy and safety of targeted agents in advanced gastric cancer (AGC), and results clearly indicated that patients need to be selected and that targeted agents are the best hope for better results. This review aims to provide an overview of potential predictive biomarkers for cytotoxic and targeted agents in GC.**

Keywords: Gastric cancer, biomarkers, chemotherapy, targeted therapy, personalized medicine, predictive marker

1. Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer related death worldwide, with an estimated 800,000 deaths caused by the disease (*1*). The incidence of gastric cancer varies widely by geographic region and is particularly common in East Asia (*2*). GC is primarily adenocarcinoma (approximately 95%), and GC can be further categorized into an intestinal form and a diffuse form based on its clinicopathologic features. The intestinal form develops amidst chronic atrophic gastritis, which is usually related to an *H. pylori* infection. In contrast, the diffuse form of gastric cancer is found in the proximal stomach and gastroesophageal junction (GEJ) and is common in populations suffering from chronic reflux disease (*3*).

Because of the early detection of GC and advances in chemotherapy and targeted therapy, the mortality rate of GC has decreased in most parts of the world. Surgical resection offers the best chance for curative therapy, but most newly diagnosed patients present with advanced and unresectable GC, and use of surgery alone or chemotherapy and radiotherapy to treat advanced gastric cancer (AGC) results in poor outcomes. The 5-year survival rate drops from 50-70% in early-stage GC to 4-10% in AGC. For these patients, chemotherapy is the primary treatment option (*4,5*). New treatment strategies for AGC, including targeted therapies, are emerging and being tested, but their efficacy is limited due to development of chemo-resistance.

Mounting evidence indicates that prognosis and treatment responses of a variety of cancers depend on the stage of the tumor as well as the phenotypic and genotypic characteristics of the tumor. Some patients appear to receive treatment that is suboptimal or even inefficacious. In personalized medicine, predictive biomarkers can be used to exclude therapies that the tumor will not respond to or to select therapies that the tumor is likely to respond to. Advances in the

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**Address correspondence to:*
Dr. Lianwen Yuan, Geriatric Surgery Department, Second Xiangya Hospital affiliated with Central South University, No. 139 Renming Road, Changsha, 410011, China.
E-mail: yuanlianwen1971@aliyun.com

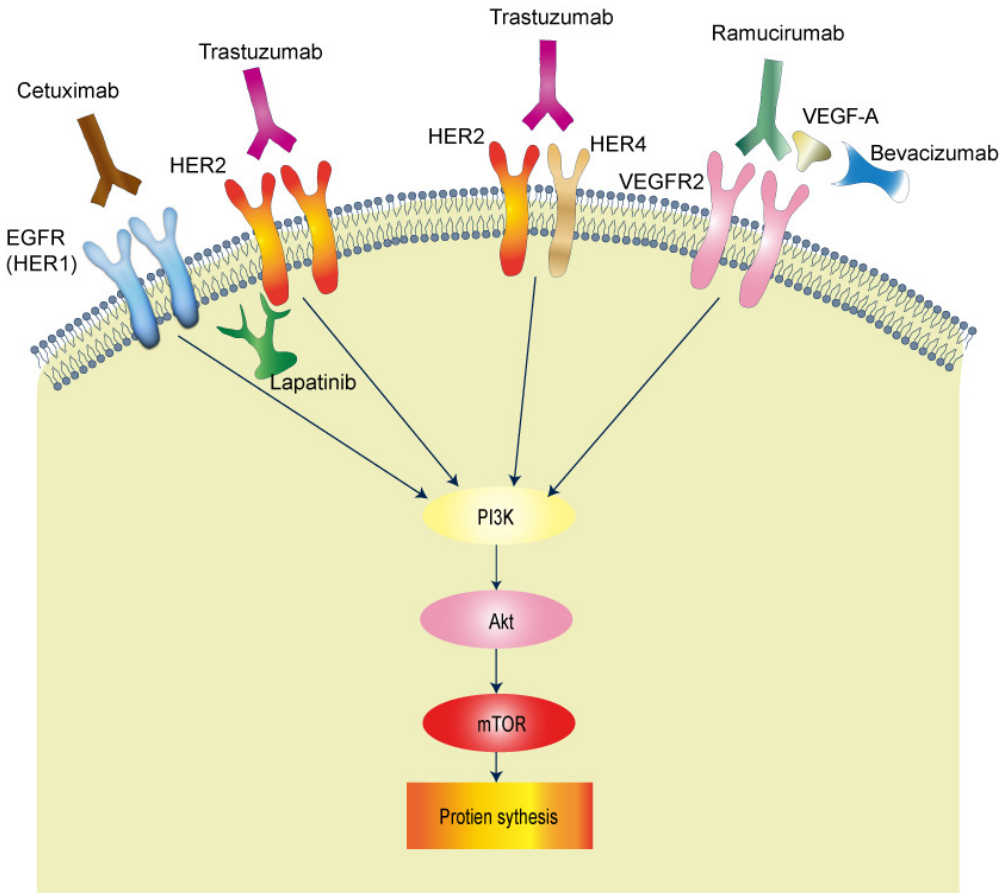

Figure 1. Molecular mechanisms of targeted therapy for GC. Targeted therapy with antibodies or specific small molecule inhibitors to treat GC involves overexpressed or amplified receptors, specific ligands, or receptor tyrosine kinases (RTKs). The targeted RTKs include EGFR (HER1), HER2, HER4, and VEGFR. VEGFR-A is the ligand targeted by bevacizumab. The activation of (RTK) signals via PI3K leads to the activation of mTOR.

identification and verification of prognostic biomarkers aids in early detection of GC and monitoring its recurrence, but the current understanding of predictive biomarkers is relatively limited. Personalized medicine based on predictive biomarkers is urgently needed to optimize patient selection and maximize treatment efficacy.

The aim of this review is to provide up-to-date information about predictive biomarkers for GC. Over the past few years, considerable effort has been devoted to identifying predictive biomarkers. Those biomarkers may include DNA repair enzymes, circulating tumor cells (CTCs), and microRNAs (miRNAs).

2. Predictive biomarkers for GC

2.1. *Human epidermal growth factor 2 (HER2)*

HER2 (encoded by *ERBB2*) is one of the four members of the human epidermal growth factor receptor family (EGFR or HER1, HER2, HER3 and HER4) in the receptor tyrosine kinase superfamily (Figure 1). *ERBB2* amplification and HER2 overexpression have been studied most often in breast cancer, and *ERBB2* amplification and HER2 overexpression are two of the most common biomarkers for GC. In breast cancer, amplification and overexpression of the *HER2* gene is associated with poor outcomes, higher mortality, and higher recurrence (*6*). *ERBB2* amplification or HER2 overexpression has been reported in 7-34% of patients with GC (*7-9*).

Results regarding the prognostic value of HER2 in GC are controversial. Some studies have reported that *ERBB2* amplification is associated with a poor prognosis and aggressive disease (*7,8,10*). However, other studies have reported finding no difference in prognosis between HER2-positive and HER2-negative tumors (*11,12*). Inhibition of HER2 has been induced as a potential targeted therapy for GC. Trastuzumab, a monoclonal antibody that targets HER2, inhibits HER2-mediated signaling and prevents cleavage of its extracellular domain (ECD).

The ToGA trial (NCT01041404) is a phase III international study that assessed the efficacy of a combination of trastuzumab with cisplatin plus 5-fluorouracil (5-FU) or capecitabine in patients with GC. The trial recruited 584 gastric/GEJ cancer patients with either HER2 overexpression (immunohistochemistry (IHC) 3+) or *ERBB2* gene amplification. The addition of trastuzumab to chemotherapy significantly increased the

response rate (47% *vs*. 35%), progression-free survival (PFS; 6.7 *vs*. 5.5 months), and overall survival (OS; 13.8 *vs*. 11.1 months). Moreover, the trial verified the predictive value of positivity for HER2. The median OS within the whole trastuzumab arm was 13.8 months, whereas it was 17.9 months in the "Very High HER2" group and 16 months in the "High HER2" group. Patients with FISH-positive and IHC 0/1+ GC did not benefit from trastuzumab treatment (*13*).

Results of several recent prospective studies also indicated that the level of *HER2* gene amplification significantly predicts sensitivity to therapy and OS in AGC treated with trastuzumab-based chemotherapy. CGOG1001 (NCT01364493) is a multicenter, prospective phase II study that evaluated the addition of trastuzumab to oxaliplatin/capecitabine in patients with chemotherapy-naive HER2-positive AGC. Patients with a HER2/CEP17 ratio of greater than five had an improved OS (20.9 *vs*. 19.5 months, $p = 0.001$) (*14*). Another prospective study in 90 patients with metastatic GC yielded similar results. In that study, a mean HER2/CEP17 ratio of 4.7 was identified as the optimal cutoff value distinguishing sensitive and refractory patients, and the optimal cutoff for predicting a survival longer than 12 months was 4.45 *(15*) . A cohort study of 126 patients with HER2-positive AGC treated with trastuzumab plus chemotherapy indicated that patients with HER2 IHC 3+ had a significantly longer OS than patients with IHC ≤ 2+. An HER2/CEP17 ratio of 4.48 was the optimal cutoff for predicting a longer OS (26.9 *vs*. 14.7 months; $p = 0.027$). In patients with IHC ≤ 2+, however, an HER2/CEP17 ratio of more than 3.69 and an *HER2* gene copy number (GCN) higher than 7.75 were positive predictive factors for better outcomes (*16*). Hence, HER2 and the HER2/CEP17 ratio can serve as predictive biomarkers for trastuzumab-targeted therapy in AGC.

Zhou *et al*. sought to identify blood-based predictive biomarkers for trastuzumab-treated AGC, and they found that the levels of HER2 ECD in serum were closely correlated with the HER2 status of tissue in AGC. There was a significantly better overall response rate and PFS for patients with abnormal baseline serum HER2 ECD than for patients with normal serum HER2 ECD. A change in serum HER2 ECD during chemotherapy was significantly correlated with a response to chemotherapy and PFS in patients with HER2-positive tumor tissue. These results substantiate the clinical utility of measuring serum HER2 ECD levels in patients with AGC. Baseline and early changes in serum HER2 ECD could be useful for monitoring clinical outcomes in patients with HER2-positive AGC receiving trastuzumab-combined chemotherapy (*17*).

A study investigated the role of the phosphoinositide 3-kinase (PI3K) pathway activation in HER2-targeted therapy in 48 patients receiving trastuzumab or lapatinib combination chemotherapy (*18*). Among the patients with responsive disease, the time to best response did not differ by phosphatase and tensin homolog (*PTEN*) status, but the duration of response was significantly shorter for patients with *PTEN* loss (median 4.2 *vs*. 6.1 months, $p = 0.04$). In addition, patients with *PTEN* loss had a significantly shorter PFS (median 4.9 *vs*. 7.3 months, $p = 0.047$). These findings suggest that a *PTEN* deficiency is an important predictive marker for early resistance to HER2 inhibitor treatment in patients with GC.

Lapatinib is a dual inhibitor of HER2 and EGFR tyrosine kinases (Figure 1). A Phase III trial (TyTAN) of lapatinib in combination with weekly paclitaxel versus weekly paclitaxel alone was conducted in patients with HER2-positive GC. The trial failed to find any improvement in OS. However, patients in the HER2 IHC 3+ subgroup who received lapatinib had a significantly prolonged survival (14 *vs*. 6.4 months; HR = 0.59; $p = 0.018$) and higher response rate (27% *vs*. 9%) (*19*).

2.2. *EGFR (HER1)*

EGFR is a member of the ERBB family of transmembrane RTKs. EGFR activation results in proliferation, angiogenesis, and migration *via* the MAP kinase and PI3K/AKT pathways (*20*). Cetuximab, a monoclonal antibody, attenuates the malignancy signal mediated by EGFR (Figure 1). A Phase III trial (EXPAND) involving 904 patients with GC indicated that addition of cetuximab to capecitabine/cisplatin as first-line treatment provided no additional benefit (*21*). A phase II trial (NCT00477711) indicated that EGFR overexpression predicted the efficacy of cetuximab combined with cisplatin and capecitabine in AGC or GEJ adenocarcinoma (*22*). Conversely, several phase II trials failed to verify the ability of EGFR IHC to predict the clinical response to cetuximab (*23,24*) or EGFR tyrosine kinase inhibitors (*25*) in GC.

Patients with metastatic colorectal cancer (mCRC) who responded to anti-EGFR treatment (cetuximab or panitumumab) had a significantly increased tumor *EGFR* GCN according to FISH (*26*). A study investigating the predictive role of *EGFR* gene amplification in patients with GC yielded similar results (*27*). The study used dual *in situ* hybridization to determine *EGFR* GCN gain (≥ 2.5 EGFR signals per cell), which it detected in 194 patients (22.7 %); *EGFR* GCN gain also predicted a poor prognosis. *EGFR* GCN gain is a more accurate prognostic biomarker than EGFR overexpression in patients with GC. Continued EGFR signaling might play a more important role in survival of *EGFR*-amplified GC cells than EGFR overexpression. Various oncogenic signals, such as *c-Jun* activation (*28*), may be involved in EGFR protein overexpression without gene amplification. A prospective study investigated EGFR expression and ligand levels in patients with GC and found that patients with EGFR expression and low ligand levels may have better outcomes with cetuximab/mFOLFOX6

treatment (*29*). Moreover, the study found that ligand levels increased when disease progressed in 7 of 8 patients with EGFR expression and low baseline ligand levels. Drawing any conclusions from that study is difficult due to the small number of evaluable patients (*n* = 38), but evaluation of *EGFR* amplification in large-scale trials might yield promising results.

Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mediates the transduction of signals between *EGFR* and the nucleus, and *KRAS* mutations may serve as a negative predictor of the response of CRC to cetuximab *(30*). However, *KRAS* mutations have not been fully evaluated as predictive markers of EGFR-targeted therapy in patients with GC due to their low frequencies in patients with GC. Results of *in vitro* experiments indicated that *KRAS* mutations were associated with cetuximab resistance in 5 GC cell lines (*31*). The growth of GC cells with wild-type *KRAS* and xenografted GC cells with a *KRAS* (G→A) mutation was significantly inhibited by cetuximab. However, apoptosis was induced in GC cells with wild-type *KRAS* but not in xenografted GC cells with a *KRAS* (G→A) mutation after cetuximab treatment. A *KRAS* (G→A) mutation does not affect the anti-cancer efficacy of cetuximab in GC cell lines (*32*). These findings imply that *KRAS* point mutations might predict the response of GC to cetuximab.

2.3. *Vascular endothelial growth factor (VEGF)*

The role of VEGF has been extensively studied in several cancers, including GC. Vascular endothelial growth factor A (VEGF-A) and its receptors (VEGFRs) play an important role in angiogenesis leading to tumourigenesis and metastasis. Expression of VEGF-A was reported in 40% of patients with GC and expression of VEGFR was reported in 36% (*33*). VEGF-A expression in a tumor and serum correlates with a lack of a response to chemotherapy, as well as with more aggressive behavior of the tumor, both in resectable GC and AGC.

Bevacizumab, a monoclonal antibody against VEGF-A, has been the anti-angiogenesis agent most often used to decrease the vascular supply to a tumor and slow metastasis (Figure 1). Bevacizumab has successfully improved OS in advanced CRC (*34*). A phase III randomized, double-blind, contrast study (AVAGAST, NCT00548548) tested the efficacy of first-line bevacizumab in 774 unselected patients with GC (*35*). Patients were treated with capecitabine and cisplatin in combination with either bevacizumab or a placebo. The median rate of OS was 10.1 months for the placebo group and 12.1 months for the bevacizumab group (HR = 0.87; $p = 0.1002$), failing to meet the primary endpoint. Nevertheless, the addition of bevacizumab to chemotherapy (capecitabine/cisplatin) improved PFS and the response rate compared to chemotherapy plus a placebo. Moreover, high levels of plasma VEGF-A predicted an improvement in OS (HR = 0.72; 95% CI, 0.57 to 0.93), and low expression of tumor neuropilin-1 also predicted an improvement in OS (HR = 0.75; 95% CI, 0.59 to 0.97) in the bevacizumab group. In contrast, the predictive value of neuropilin-1 was not noted in another phase II trial (*36*). According to an analysis of AVAGAST subgroups, VEGF-A and neuropilin-1 had the ability to predict OS only in non-Asian patients (*37*). Another trial found a similar ethnic difference since VEGR-A levels were independent predictors of OS in Caucasians with GC but not in Asians with GC (*38*). Therefore, the predictive role of VEGF-A might depend on ethnicity and the type of tumor.

2.4. *Thymidylate synthase (TS)*

TS provides an effective predictor of the response to chemotherapy with 5-FU. One mechanism by which 5-FU displays anticancer action is through the inhibition of TS, which is a key enzyme in the process of DNA replication and repair. Most GC chemotherapy regimens include 5-FU, so responsiveness to chemotherapy may, in theory, be affected by TS status.

Patients with GC have significantly higher levels of TS mRNA in plasma than do healthy controls, and levels of TS mRNA in plasma were significantly correlated with levels of TS mRNA in tumor tissues *(39*). Low levels of expression of TS mRNA in a tumor and plasma were significantly correlated with raltitrexed sensitivity. When expression of TS mRNA in a tumor and plasma was used to predict the response to chemotherapy, raltitrexed sensitivity predicted on the basis of levels of TS mRNA in plasma had a sensitivity of 82% and an accuracy of 60% while sensitivity predicted on the basis of levels of TS mRNA in a tumor had a sensitivity of 70% and an accuracy of 68% (*39*). Endoscopic biopsies in patients with AGC have also indicated that levels of expression of TS were significantly higher in a tumor than in normal tissue and significantly lower in S-1/cisplatin responders than in S-1/cisplatin non-responders. Interestingly, a significant increase in TS expression was detected in several patients, who changed from "responders" to "non-responders" after chemotherapy (*40*). An *in vitro* study has found that levels of TS in plasma and tissue are negatively associated with pemetrexed sensitivity (*41*).

2.5. *DNA repair enzymes*

X-ray repair cross complement group 1 (XRCC1), excision repair cross-complementing 1 (ERCC1), and BRCA1, known as DNA repair enzymes, have recently garnered attention because of their role in predicting the response to chemotherapy in patients with GC. XRCC1 and ERCC1 expression was significantly

Table 1. MiRNAs as predictive biomarkers in GC*

MiRNA	Year	Specimens (up or down-regulated in chemo-resistant specimens)	Target	Function	*Ref.*
MiR-125a-5p	2011	Tissues and cell lines	*ERBB2*	Enhances antitumor efficacy in combination with trastuzumab	(*60*)
MiR-27a	2011;2014	Cell lines and mouse model; Plasma	-	Modulates MDR; Predicts resistance to fluoropyrimidine-based chemotherapy	(*61,62*)
MiR-497	2012	Cell lines (down-regulated)	*Bcl-2*	Modulates MDR	(*63*)
MiR-200bc/429 cluster	2012	Cell lines (down-regulated)	Bcl-2,XIAP	Modulates MDR	(*64*)
MiR-17-5p,MiR-20a	2012	Plasma and mouse model	-	Modulates chemotherapeutic effects	(*65*)
miR-21	2013	Cell lines (up-regulated)	*PTEN*	Modulates MDR	(*66*)
MiR-106a	2013	Cell lines (up-regulated)	*PTEN*	Modulates MDR	(*67*)
MiR-1271	2014	Cell lines (down-regulated)	IGF1R, IRS1, mTOR, and BCL-2	Modulates MDR	(*68*)
MiR-429	2015	Tissues and cell lines	Bcl-2	Modulates chemotherapeutic effects	(*69*)
MiR-218	2015	Cell lines (down-regulated)	SMO	Inhibits MDR	(*70*)
MiR-143	2015	Cell lines (down-regulated)	IGF1R and BCL-2	Modulates MDR	(*71*)
MiR-103/107	2015	Cell lines (down-regulated)	caveolin-1	Modulates MDR	(*72*)
MiR-223	2015	Cell lines	FBXW7	Modulates trastuzumab-induced apoptosis	(*73*)
MiR-26a	2015	Cell lines (down-regulated)	NRAS and E2F2	Modulates MDR	(*74*)
MiR-23b-3p	2015	Cell lines and mouse model (down-regulated)	ATG12 and HMGB2	Modulates MDR	(*75*)
MiR-1284	2016	Tissues and cell lines (down-regulated)	EIF4A1	Modulates MDR	(*76*)
MiR-375	2016	Cell lines (down-regulated)	ERBB2	Modulates MDR	(*77*)
MiR-181	2016	Cell lines and mouse model (down-regulated)	ATG5	Modulates autophagy and chemo-resistance	(*78*)
MiR-27b	2016	Cell lines and mouse model	CCNG1	Modulates MDR	(*79*)

*XIAP: X-linked inhibitor of apoptosis protein; IGF1R, type 1 insulin-like growth factor receptor; IRS1, insulin receptor substrate 1; BCL-2, B cell leukemia/lymphoma 2; SMO, smoothened, frizzled class receptor; FBXW7, F-box/WD repeat-containing protein 7; NRAS, neuroblastoma RAS viral (v-ras) oncogene homolog; E2F2, E2F transcription factor 2; ATG12, autophagy related 12; HMGB2, high mobility group box 2; EIF4A1, eukaryotic translation initiation factor 4A1; ATG5, autophagy related 5; CCNG1, cyclin G1.

downregulated in GC tissue and XRCC1 and ERCC1 have been identified as negative markers of OS in most studies (*42-45*). ERCC1 mRNA overexpression is associated with an unfavorable response to regimens with platinum agents (*46-48*). In contrast, the *XRCC1* and *ERCC1* genes are not able to predict the disease control rate (*49,50*). Two studies failed to verify the ability of tumor ERCC1 expression to predict the clinical response or survival of patients with AGC (*51,52*). Due to these conflicting results, the predictive role of XRCC1 and ERCC1 needs to be verified in large-scale prospective clinical trials.

Studies of *XRCC1* and *ERCC1* gene polymorphisms as a predictor of the response to chemotherapy have yielded encouraging results. In GC, the specific presence of an A/G polymorphism in *XRCC1* at codon 399 and the combination of the A/G polymorphism in *XRCC1* at codon 399 and a C/T polymorphism in ERCC1 at codon 118 is a predictor of median OS for patients receiving oxaliplatin/5-FU-based chemotherapy (*49,50*). A polymorphism in *XRCC1* at codon 194 (Arg>Trp) was correlated with a better response to chemotherapy (*53*). The *XRCC1* 194 C/T genotype could be a modest predictor of AGC response in patients treated with taxane and cisplatin (*54*). Retrospective studies found that the *ERCC1* rs3212986 and rs11615 polymorphisms influenced the response to chemotherapy and the OS of patients with GC (*55-58*).

Germline mutations in *BRCA1* are associated with an increased risk of developing breast cancer, ovarian cancer, gastric cancer, and other types of cancers. *BRCA1* heterozygosity has been found to cause a predisposition to GC (*59*). A low level of *BRCA1* mRNA in a tumor was associated with an increased response rate (*59*). However, conflicting results have been reported since BRCA1 levels in plasma and a tumor were positively associated with docetaxel sensitivity. The *BRCA1* TT genotype could be a modest predictor of AGC response in patients treated with taxane and cisplatin (*54*). *BRCA1* mRNA and *BRCA1* polymorphisms may be potential predictive biomarkers for chemotherapy.

2.6. *miRNAs*

miRNAs are a relatively novel class of regulatory molecules that control the translation and stability of mRNAs on a post-transcriptional level *via* interaction with the 3'-untranslated region (UTRs) of target mRNAs, eventually leading to destabilization and/or inhibition of their translation. Multidrug resistance (MDR) correlates with treatment failure and a poor

prognosis among patients with GC. Aberrant patterns of miRNA expression have been implicated in MDR in GC cells. miRNAs could potentially be used to predict the response to chemotherapy. The current literature describing the impact of miRNAs on the prediction of and changes in sensitivity to anticancer treatment is summarized in Table 1.

An association between aberrant patterns of miRNA expression in GC and MDR has been noted *in vivo* and *in vitro*. Expression of 12 miRNAs (miR-497 (*63*), miR-200bc/429 cluster (*64*), miR-1271 (*68*), miR-218 (*70*), miR-143 (*71*), miR-103 (*72*), miR-107 (*72*), miR-26a (*74*), miR-23b-3p (*75*), miR-1284 (*76*), miR-375 (*77*), and mi-181 (*78*)) was downregulated in GC cells. Overexpression of these miRNAs sensitized tumors to anticancer drugs. miR-21 (*66*) and miR-106a (*67*) were found to be up-regulated in chemo-resistant GC cell lines. Overexpression of miR-21 and miR-106a significantly decreased the antiproliferative effects of anti-cancer drugs and the apoptosis they induced, while knockdown or suppression of miR-21 and miR-106a dramatically increased the cytotoxicity of anti-cancer drugs. Down-regulation of miR-27a conferred sensitivity to chemotherapy in GC cells (*61*). Moreover, patients with up-regulated levels of miR-27a expression had a significantly worse OS than patients with lower levels of miR-27a expression ($p = 0.024$). miR-27a is a potential biomarker to predict resistance to fluoropyrimidine-based chemotherapy in patients with metastatic or recurrent GC (*62*). Ectopic miR-27b in GC tumors led to increased sensitivity to chemotherapy *in vitro* and *in vivo* (*79*). The levels of miR-17-5p/20a in serum decreased markedly in treated mice with a decreased tumor volume (*65*). Suppression of miR-429 in GC cells promotes Bcl-2-mediated cancer cell survival in response to chemotherapy-induced cell death. Restored levels of miR-429 expression may enhance cancer apoptosis in GC cells during chemotherapy (*69*). These findings suggest that these miRNAs have the potential to be molecular markers of pathological progression, to predict prognosis, and to monitor the response of GC to chemotherapy.

miRNAs have also been found to be potential biomarkers for targeted therapy. *In vitro* assays indicated that *ERBB2* is a direct target of miR-125a-5p; miR-125a-5p potently suppresses the proliferation of GC cells and it suppresses that proliferation even more so in combination with trastuzumab (*60*). Overexpression of miR-223 decreased the sensitivity of GC cells to trastuzumab while suppression of miR-223 restored the sensitivity of GC cells to trastuzumab. Moreover, overexpression of miR-223 significantly suppressed trastuzumab-induced apoptosis (*73*).

Despite the promising results described here, research on miRNAs is still in its infancy. Studies of miRNAs in GC are limited and thus far only describe experiments and clinical observations. Unfortunately, few clinical trials have involved patients with GC. From a clinical point of view, there are no reliable biomarkers available that allow the prediction of the response to chemotherapy (*80*).

2.7. *CTCs*

Advances in techniques have allowed the detection and characterization of CTCs (and even rare types of those cells) in peripheral blood. Metastasis of a solid tumor requires tumor cells to enter the circulation and travel to distant sites to establish a metastatic focus. Studies have focused on the potential role of CTCs in metastasis. Different methods have been used to detect and isolate CTCs. These include RT-PCR of whole blood, plasma, and sera, flow cytometry, and the related technique of immunomagnetic separation.

Analysis of CTCs has been used to predict prognosis, monitor the response to treatment, and monitor a relapse in breast cancer, mCRC, and melanoma (*81-83*). The role of CTCs in GC was evaluated in a phase II study involving patients with advanced HER2-negative GC or GEJ adenocarcinoma. The presence of CTCs at the baseline was found to be strongly predictive of PFS (HR = 3.8; $p = 0.007$) and OS (HR = 3.4; $p = 0.014$) (*84*). Patients who were CTC-positive at the baseline had a significantly shorter median PFS and OS (*85*). These findings suggest that a favorable clinical response depends significantly on negativity for CTCs.

Measuring the CTC count to monitor the response to treatment is an attractive area of research. The CTC count was found to decrease on day 16 following chemotherapy and then increase again during the chemo-resistant phase in AGC (*86*). Matsusaka *et al.* used immunomagnetic separation to isolate CTCs and they measured the CTC count in whole blood at the baseline and 2 and 4 weeks after initiation of chemotherapy (*87*). Patients with AGC receiving S-1-based or paclitaxel chemotherapy with ≥ 4 CTCs at 2 weeks and 4 weeks had a shorter median PFS and OS. An epithelial-to-mesenchymal transition (EMT) was also evident in a few cells of primary tumors and more so in CTCs from the blood of patients with GC, so this phenomenon might be used to monitor the response to treatment (*88*). HER2-positive CTCs were effectively eliminated by HER2-targeted therapy in patients with HER2-positive AGC. Determining the number of copies of chromosome 8 in CTCs provides a potential approach to predicting chemotherapeutic efficacy and monitoring chemo-resistance (*89*). Thus, the CTC count may serve as an early biomarker that allows the evaluation of therapeutic efficacy.

3. Conclusion and prospects for the future

This review has described most of the predictive markers that guide the choice of the most suitable

therapy for individual patients with GC. Compared to major developments in targeted treatment of mCRC in recent years, strategies to manage GC have improved little. Anti-HER2 therapy is the only targeted therapy that is currently accepted as standard treatment for GC, and very high levels of HER2 expression predict which patients will benefit from this therapy.

TNM staging has been a vital tool to assess prognosis and predict the need for systemic treatment of resectable GC. However, several studies have highlighted the importance and necessity of genotypic and phenotypic classification of GC to facilitate patient treatment. The success of the ToGA trial and, more recently, the failure of bevacizumab in a large phase III study (*37*) in unselected patients with GC clearly show that patients need to be selected and that patients selection is the best hope for better results of targeted agents. Potential tumor and blood-based predictive biomarkers need to be further investigated for appropriate patient stratification and personalized oncologic treatment.

Since MDR is a common and complex phenomenon attributed to crosstalk and feedback between multiple signaling pathways, a single biomarker may have limited power to predict a clinical response. Rapid advancements in sequencing and mass spectrometry techniques have allowed simultaneous evaluation of multiple signaling pathways in specimens. An evaluation of multiple signaling pathways may help with efforts to improve personalized treatment with targeted agents and, possibly, cytotoxic agents (*90,91*).

Most clinically actionable targets are relatively infrequent in GC. In order to evaluate the predictive value of potential markers, a combined effort is needed to procure an adequate number of pretreatment tumor specimens to ensure that projects to identify biomarkers have robust statistical power. Novel techniques may help in the early evaluation of tumor response after anti-cancer treatment. *In vivo* apoptosis imaging using Apopep-1 (*92*) has been found to be a sensitive and predictive tool for early determining of the response of GC after anti-cancer treatment.

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Original Article

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Cloning, expression and cytotoxicity of granulin A, a novel polypeptide contained in human progranulin

Xuan Wang[1,2], Huanli Xu[3], Xiaoliang Chen[3], Ye Tian[3], Fengshan Wang[1,2,*], Xiukun Lin[2,3,*]

[1] *Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong, China;*
[2] *SDU-ZB Biomedical R&D Institute, Zibo, Shandong, China;*
[3] *Department of Pharmacology, Capital Medical University, Beijing, China.*

Summary **In our previous study, we isolated an antitumor polypeptide, CS5931, from the sea squirt *Ciona savignyi*; it shares high homology with *Ciona intestinalis* Granulin A (GRN A). However, little is known about the anticancer effect of GRN A. In the present study, GRN A was cloned and expressed in the yeast *Pichia pastoris*. The polypeptide was purified to almost homogeneity using a Ni-NTA column. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] assay reveals that GRN A displays potent cytotoxicity to several human cancer cells. The polypeptide induces cell apoptosis as analyzed by morphological observation and flow cytometry assays. This study provides evidence that GRN A possesses potential to be developed as a novel anticancer agent.**

Keywords: Granulin A, cloning, expression, anticancer activity

1. Introduction

Granulins (GRN), also known as epithelins, consists of a family of cysteine-rich peptides with diverse functions. All members of the GRN family contain 12 cysteine residues, arranged in highly conserved positions, and the cysteine residues form intramolecular disulfide bridges, resulting in characteristic tightly packed structures (*1*). The GRN family includes GRN A, B, C, D, E, F and G (*2*). GRN A is an acid- and heat-stable polypeptide with low molecular mass. The polypeptide contains 12 cysteine residues, and peptides bearing this domain are found in a wide range of organisms, from eubacteria to mammals (*3,4*). Studies have shown that GRN A plays a critical role in epithelial homeostasis, tumorigenesis, and in reproductive, immunological, and neuronal functions (*5,6*).

In our previous study, we isolated a novel antitumor polypeptide termed CS5931 from the sea squirt *Ciona savignyi*, CS5931 shares high homology with *Ciona intestinalis* GRN and is conserved during evolution (*7*). The polypeptide shows a specific inhibition effect on the growth of several tumor cells *in vitro* (*8*). Prediction of 3D structure of the polypeptide revealed that CS5931 consisted of six disulfide bonds and two beta-hairpins, similar to human GRN A (*9,10*). Therefore, there is potential that GRN A may also display anticancer activity. However, little is known about the antitumor effect of GRN A as well its mode of action. Additionally, since the content of GRN A in tissue is very low, developing a novel approach for obtaining a sufficient amount of the polypeptide is promising.

Pancreatic cancer, one of the most common malignant tumors in man, is the fourth most common cause of cancer-related death in industrialized countries (*11*). Despite advances in surgical and nonsurgical treatments, it remains a tumor with poor prognosis; the overall 5-year survival rate is only 3-5% owing to the lack of symptoms and screening techniques for early detection, aggressive metastatic behavior, and resistance to conventional chemotherapy and radiotherapy regimens (*12*). Therefore, there is an urgent need to develop novel agents and alternative strategies for

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*Address correspondence to:
Dr. Fengshan Wang, Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Shandong University, Ji'nan, Shandong 250012, China.
E-mail: fswang@sdu.edu.cn

Dr. Xiukun Lin, Department of Pharmacology, Capital Medical University, 10 Xitoutiao, Fengtai, Beijing 100069, China
E-mail: linxiukun@yahoo.com

treatment of pancreatic cancer (*13*).

In the present study, we developed a highly effective approach for expressing the polypeptide in the yeast *Pichia pastoris*; the fermentation conditions were optimized, and the cytotoxicity as well as the pro-apoptotic activity of the polypeptide are also presented.

2. Materials and Methods

2.1. *Materials*

Gel Extraction Kit and Plasmid Mini Kit I were purchased from Omega (Norcross, GA, USA), and plasmid pGAPZαA and *P. pastoris* SMD1168H were a gift from the Academy of Pharmaceutical Science of Shandong Province (Jinan, China). Protein markers and PCR Mix were products of KeyGEN (Nanjing, China), while T4 DNA ligase, restriction enzymes, DNA molecular markers and Zeocin were obtained from Themo (Waltham, MA, USA).

2.2. *Cell lines and culture*

PANC-28 cells, BEL-7402 cells, and HCT-116 cells were purchased from ATCC (Manassas, VA, USA). Cell culture medium DMEM (Dulbecco's modified eagle medium) and RPMI (Roswell Park Memorial Institute) -1640 were purchased from Hyclone (Logan, Utah, USA).

2.3. *Cloning of GRN A*

Oligonucleotide primers for the amplification of the GRN A were designed based on the genomic sequence, and their sequences are as follows: Forward primer: 5'-CCGGAATTC GATGTGAAATG TGACATGAG-3'; Reverse primer: 5'-CTAGTCGATA CCCTGTTCACAG GTACCCTTCT-3'. The restriction sites for *Eco* RI and *Xba* I are indicated by underlines. The PCR (Polymerase Chain Reaction) amplification conditions were initiated at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 30 s, and a final extension at 72°C for 10 min (*14*). The amplified DNA fragment was purified using Gel Extraction Kit, and digested with *Eco* RI and *Xba* I. The fragment was ligated into the pGAPZαA plasmid pretreated with *Eco* RI and *Xba* I. The synthesized vector, pGAPZαA/GRN A was transformed into *E. coli* DH-5α, and the transformants were selected on low salt LB medium supplemented with 25 μg/mL zeocin (*15*).

2.4. *Expression of recombinant GRN A*

The expression plasmid pGAPZαA/GRN A was linearized with *Bsp* HI and transformed into the genome of the yeast *P. pastoris* SMD1168H by electroporation using the Gene pulser Xcell system (Bio-Rad, California, USA) at 1.5 kV, with a 0.2 cm cuvette (*16*). Transformants were selected on YPDs (yeast extract peptone dextrose solid medium) plates (1% yeast extract, 2% peptone, 2% dextrose) containing 100 μg/mL zeocin (*17*). Protein expression was examined by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electropheresis). Western blotting analysis was also used to check the expressed protein. Briefly, the purified GRN A polypeptide was resolved on 15% SDS-PAGE. The gel was then semi-dry electroblotted onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA) at 2.5 mA/cm^2 for 20 min. The membrane then was then incubated in blocking buffer (5% fat milk in PBS) for 1 h at room temperature, washed with PBST three times for 10 min each, and incubated in PBST overnight at 4°C with anti-his monoclonal antibody (anti-His 1:1,000, Santa Cruz Biotechnology, Delaware, CA, USA). After washing the membrane with PBS, an HRP-conjugated antibody (goat anti-rabbit) was used as the secondary antibody and incubated for 1 h. The membrane was processed using the enhanced chemiluminescence method (Thermo, Waltham, USA), and the protein band was visualized by Gel imaging (Bio-Rad, Gel Doc XR+, Hercules, CA, USA).

2.5. *Optimization of the expression conditions*

2.5.1. *Inoculation amount*

The expression of GRN A in *P. pastoris* was determined by SDS-PAGE analysis, and the transformant with highest expression activity of GRN A was inoculated in YPD medium and cultured overnight at 30°C, 200 rpm. Inoculated culture was added to the YPD medium according to the proportions of 3, 5, 7, and 10%. After incubation for 96 h at 30°C, the culture medium was collected by centrifugation at 10,000× g for 20 min, and analyzed using SDS-PAGE.

2.5.2. *Effect of temperature and culture time on the expression of GRN A*

The temperature of the expression conditions of *P. pastoris* was set to 25 and 30°C, while the culture time was 48, 72, 96, 120 h respectively. SDS-PAGE analysis and MTT assay were performed to determine the amount and cytotoxicity of GRN A.

2.6. *Expression and purification of recombinant GRN A*

Transformant was inoculated in YPD medium and cultured at 25°C, 200 rpm overnight. After culture for 96 h, the supernatant was collected by centrifugation (10000× g, 10 min, 4°C) and purified using a Ni^{2+} chelating Sepharose column (GE Healthcare, Marlborough, MA, USA). After washing with 50 mM imidazole 2 times, the polypeptide was eluted using

imidazole at a concentration of 250 mM (*18*). The eluted polypeptide was dialyzed in dialysis buffer TE (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA) at 4°C. The concentration of purified recombinant GRN A polypeptide was quantified by BCA (bicinchoninic acid) protein assay Kit (KeyGen, Nanjing, China) and further analyzed using SDS-PAGE on 15% polyacrylamide gel, and stained with Coomassie brilliant blue R250.

2.7. *MTT assay*

MTT assay was performed to evaluate the anti-proliferative effects of recombinant GRN A against human liver cancer cells BEL-7402, human colon carcinoma HCT116 and human pancreatic cancer cells PANC-28. Briefly, cells were plated on to 96-well-plates and incubated at 37°C in humidified air atmosphere with 5% CO_2. After incubation for 24 h, cells were treated with various concentrations of recombinant GRN A. After culture for another 48 h, 20 μL of MTT [5 mg/mL MTT in PBS (phosphate buffered saline)] (Sigma, St. Louis, MO, USA) was added to each well and the cells were incubated for an additional 4 h (*19*). DMSO (dimethyl sulphoxide) 150 μL was added to each well to dissolve the reduced MTT crystals. The MTT-formazan product dissolved in DMSO was estimated by measuring the absorbance at 570 nm with a micro plate reader (Biotech, power wave, USA). The cell proliferation rate was calculated using the following formula: Relative inhibition rate (%) = $(OD_{control} - OD_{treated})/OD_{control} \times 100\%$ (*20*).

2.8. *Cell morphological observation*

Human pancreatic cancer cells PANC-28 were seeded into 6-well culture plates with or without recombinant GRN A. After incubation for 24 h, the cells were fixed with 4% paraformaldehyde for 1 h at 4°C, washed with PBS, and incubated with 5 μg/mL of DAPI (*21*) (4',6-diamidino-2-phenylindole) (Beyotime Institute of Biotechnology, Shanghai, China) for 10 min in the dark. Cell morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan). Apoptosis was defined by the appearance of chromatin condensation.

2.9. *Flow cytometry analysis*

Annexin V-FITC (fluorescein isothiocyanate) and PI (propidium iodide) double-staining assay was performed to distinguish apoptotic from necrotic cells. Cells (2.5×10^5) were seeded in 6-well tissue culture plates and incubated for 24 h at 37°C. GRN A at several concentrations was directly added into 6-well plates and incubated for an additional 48 h. The cells were harvested and resuspended in PBS buffer. Apoptotic cells were identified using Annexin V-FITC Apoptosis Detection kit (KeyGen, Nanjing, China) according to the manufacturer's instructions. Then the cells were examined by Cytomics FC 500 flow cytometer (Beckman Coulter, CA, USA).

3. Results

3.1. *Expression of recombinant GRN A*

In order to screen out the multiple copy colonies, the effect of zeocin concentration on GRN A production was studied. Zeocin was added at concentrations of 100, 300, 500, and 1,000 μg/mL respectively. Our results showed that a high yield of GRN A was found in 100 μg/mL of zeocin (data not shown). Random selection of positive clones from different batches of electroporation was beneficial to screen out the multiple copy colonies. SDS-PAGE revealed that the recombinant polypeptide was found in the supernatant of culture medium with a molecular weight around 15 kD (Figure 1A). Of note, the theoretical molecular weight of GRN A was 6.9 kD; it is conceivable that the six paired disulfides of GRN A influenced the electrophoretic mobility and the SDS-PAGE molecular weight (*9*). To further confirm the expression of GRN A, Western blotting analysis was performed (Figure 1B). The results showed that a clear band appeared at the same position corresponding to SDS-PAGE analysis (Figure 1B). The results indicated that the polypeptide GRN A was successfully expressed in the yeast *P. pastoris*.

3.2. *Optimization of expression conditions*

We first studied the effect of inoculated amount on the

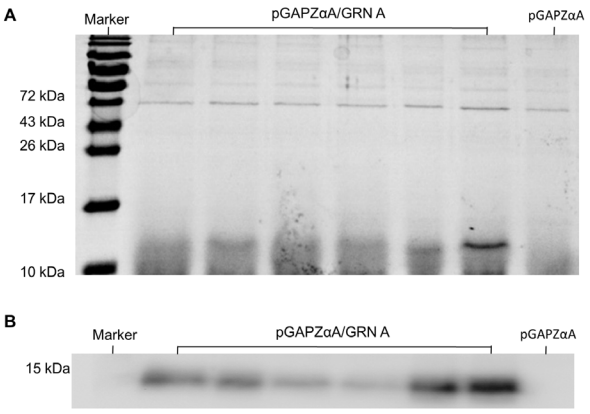

Figure 1. Expression of recombinant GRN A. (A) SDS-PAGE analysis of GRN A expression. pGAPZαA/GRN A and pGAPZαA were transformed in the yeast *P. pastoris*, and induced by zeocin. After culture for 96 h, the culture medium was collected using centrifugation. **Lane 1~6** represents the expression of GRN A in different clones. **Lane 7** represents the result in the strain transfected with the parent plasmid pGAPZαA. **(B)** Western blotting analysis of GRN A expression. **Lane 1~6** represents the expression of GRN A in different clones. **Lane 7** represents the result in the strain transfected with the parent plasmid pGAPZαA.

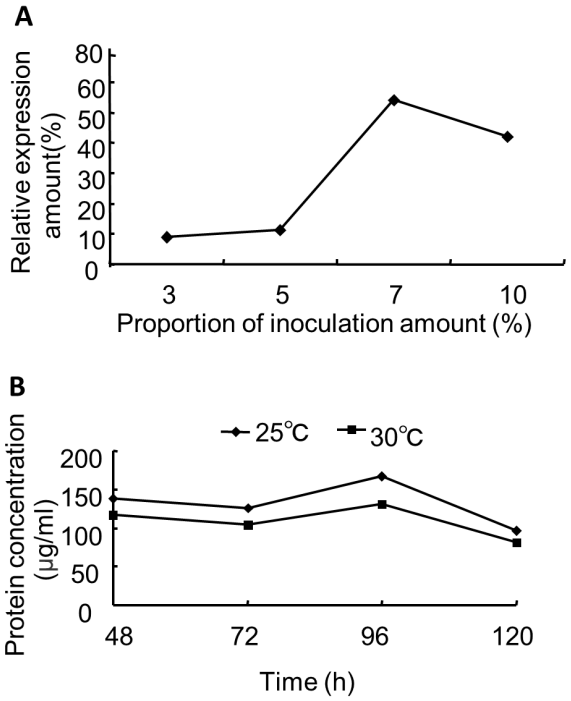

Figure 2. Optimization of expression conditions of the yeast *P. pastoris*. (A) Effect of inoculation amount on GRN A expression. The proportion of inoculation amount was 3, 5, 7, 10% respectively, and the expression of GRN A was determined by SDS-PAGE analysis. **(B)** Effect of temperature and fermentation period on GRN A production. The inoculated medium was cultured at 25 and 30°C for 48, 72, 96, 120 h respectively. The expression amount was analyzed using SDS-PAGE analysis.

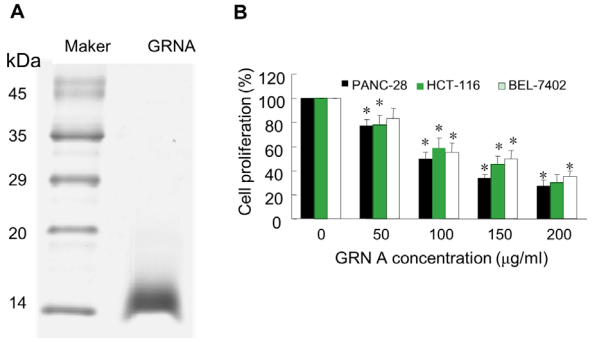

Figure 3. Purification of GRN A and its cytotoxicity on human cancer cells. (A) SDS-PAGE of purified recombinant GRN A. **(B)** Recombinant GRN A inhibits the growth of tumor cells. Human liver cancer cells BEL-7402, human colon carcinoma HCT116 and human pancreatic cancer cells PANC-28 were treated with certain concentrations of recombinant GRN A for 48 h. The cell inhibitory rate was determined by MTT assay as described in Materials and methods section. The experiments were repeated three times and the results are presented as mean ± SD; * $p < 0.01$.

expression of GRN A. Inoculated culture was added with proportions of 3, 5, 7, 10% respectively to the YPD medium. After culture for 96 h, the expression of GRN A was analyzed by 15% SDS-PAGE. Maximum expression of the polypeptide was obtained when the proportion of inoculation culture was 7% (Figure 2A).

Next, the effect of temperature on GRN A expression was investigated at 25 or 30°C respectively. The expression amount of GRN A was higher when the strains were cultured at 25°C than at the high temperature, 30°C (Figure 2B). Additionally, the expression of GRN A was also time-dependent and the highest production was obtained when incubated at 25°C for 96 h (Figure 2B).

3.3. *Recombinant GRN A displays potent cytotoxicity to several cancer cells*

GRN A was expressed at the optimized conditions, and purified using Ni-NTA column. The polypeptide was purified to almost homogeneity with a band at molecular weight of 15 kD (Figure 3A). Using the optimized conditions, 5 mg of GRN A was obtained in one liter of medium. MTT assay was conducted to check the cytotoxicity of the purified polypeptide (Figure 3B). GRN A displayed potent cytotoxicity to several human cancer cells. The IC_{50} of GRN A for PANC-28, HCT-116 and BEL-7402 cancer cells is 85.6, 100.4, and 90.9 μg/mL respectively (Figure 3B). The results revealed that the recombinant polypeptide possesses potential to be developed as a novel anticancer agent for treatment of human cancer. Compared with other cancer cells, GRN A displayed higher cytotoxicity on PANC-28 cancer cells (Figure 3B). Therefore in our next experiments the pancreatic cancer cells were used for further study.

3.4. *GRN A induces cell apoptosis*

PANC-28 cells were treated with GRN A, and stained with DAPI. The cell morphology was observed by fluorescence microscope. As shown, treatment of the cancer cells with GRN A resulted in the production of nucleus pyknosis, condensation (Figure 4A, *arrow*). To further confirm the apoptosis induction effect of GRN A, Annexin V-FITC/PI double staining was performed (Figure 4B), a dose-dependent increase in the percentage of total apoptotic cells was observed in cells treated with GRN A; the percentage of total apoptotic cells was 9.64% in untreated cells (Figure 4C), whereas the percentages of total apoptotic cells increased to 18.52, 44.13, and 69.5 % in PANC-28 cells treated with 50, 100, and 150 μg/mL of GRN A respectively (Figure 4C). These results suggest that the recombinant polypeptide is able to inhibit tumor cell growth *via* the apoptotic pathway.

4. Discussion

Peptides play crucial roles in many physiological processes and a lot of peptides have been used clinically to treat human disorders. For many years, researchers have been searching for anticancer agents from the human body, since peptides from humans display little immunogenicity. Several peptides from human sources

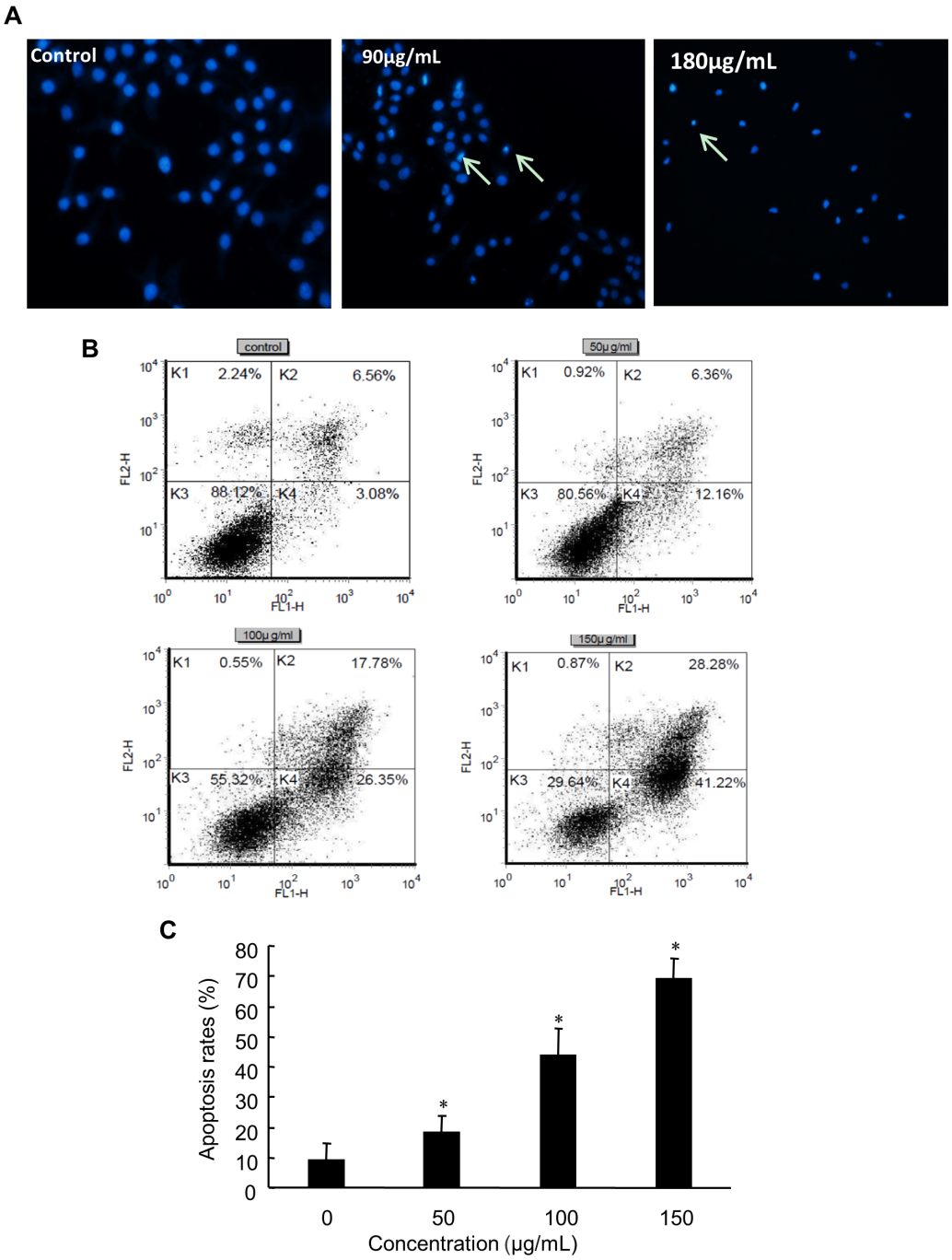

Figure 4. GRN A induces cell death via apoptotic pathway. (A) Morphological observation. PANC-28 cells were treated without (control) or with 90 μg/mL and 180 μg/mL GRN A for 24 h respectively. After staining with DAPI, the cell morphology was observed using a fluorescence microscope. The arrow indicates nuclei condensation. **(B)** Flow cytometry analysis. PANC-28 cells were treated without (control) or with certain concentrations of GRN A (50, 100, 150 μg/mL) and the cells were stained using Annexin V/PI double staining and analyzed by flow cytometry. The proportion of cell number is shown in each quadrant. The proportion of viable cells is shown in K3 quadrant (Annexin V-FITC$^-$, PI$^-$), while the early apoptotic cells is shown in K4 quadrant (Annexin V-FITC$^+$, PI$^-$). The K2 quadrant (Annexin V-FITC$^+$/PI$^+$) represents the late apoptotic/necrotic cells, while the necrotic cells are shown in K1 quadrant (Annexin V-FITC$^-$, PI$^+$). (C) represents the quantity of apoptotic ratio in cells treated as above. The percentage of total apoptotic cells was calculated from at least three separate experiments. All statistical significance was determined by a paired *t*-test; * $p < 0.01$ versus control.

have been successfully used clinically for treatment of human cancer, such as interferons and interleukins (*22-24*). However, these kinds of peptides usually do not inhibit cancer cell growth directly; they affect the growth of tumors *via* an immune effect. In the present study, we found that the GRN A possesses the ability to inhibit proliferation of cancer cells directly. This result provides primary evidence that GRN A has the potential to be developed as a novel kind of anticancer agent. Study is on-going in our laboratory to confirm the antitumor activity of GRN A in nude mice bearing human tumors.

Since the structure of GRN A contains multiple disulfide bonds, the correct folding of the polypeptide is important for maintaining the activity of GRN A. In the beginning, we tried to use a prokaryotic system to express GRN A, but the expressed polypeptide did not display any activity on human cancer cells. The results suggested that the right folding of GRN A is needed for obtaining an active polypeptide. It is well established that the apoptotic pathway is a main target for chemotherapeutic anticancer agents, and most anticancer drugs used clinically display tumor inhibition effect *via* the apoptotic pathway. The results of the present study confirmed that GRN A displays cytotoxicity to several human cancer cells (Figure 3B), and induced cell death *via* the apoptotic pathway in human pancreatic cells (Figures 4A-4C). It is well known that pancreatic cancer is very difficult to cure; the overall 5-year survival rate is only 3-5%. This study provides primary evidence that GRN A possesses the possibility to be a novel anticancer agent, especially on human pancreatic cancer. The study also provides a basis for further study of the function of GRN A both *in vitro* and *in vivo*.

In conclusion, GRN A was cloned and expressed in *P. pastoris* (Figures 1A and 1B). The polypeptide was purified to homogeneity using Ni-NTA column (Figure 3A). MTT assay confirmed that GRN A displays potent cytotoxicity to several human cancer cells (Figure 3B). Morphological observation as well as cytometry analysis reveals that the polypeptide is able to induce cell death *via* the apoptotic pathway (Figures 4A-4C). In recent years, peptides as drugs have attracted great attention due to their high efficiency and low resistance. However, up until now, there are no peptides inhibiting cancer cell growth directly, found from the human body. This study provides a novel strategy to search for anticancer agents.

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Original Article

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Protection against vascular endothelial dysfunction by polyphenols in sea buckthorn berries in rats with hyperlipidemia

Fang Yang[1,2,3], Yourui Suo[1,4,*], Dongli Chen[5], Li Tong[2]

[1] *Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, Qinghai, China;*
[2] *Medical College , Qinghai University, Xining, Qinghai, China;*
[3] *University of Chinese Academy of Sciences, Beijing, China;*
[4] *Qinghai Academy of Agriculture and Forestry Science, Qinghai University, Xining, Qinghai, China;*
[5] *AA Pharma, Inc., Massachusetts, USA.*

Summary **Chronic hyperlipemia increases the incidence of vascular endothelial dysfunction and can even induce cardiovascular disease. Sea buckthorn contains a host of bioactives such as flavonoids and polyphenols that can prevent the development of cardiovascular disease. The current study isolated active ingredients, polyphenols, from sea buckthorn berries (SVP) and orally administered SVP at a dose of 7-28 mg/kg. This treatment significantly reduced serum lipids, it enhanced the activity of antioxidant enzymes, and it decreased the level of serum TNF-α and IL-6. SVP also alleviate vascular impairment by decreasing the expression of eNOS, ICAM-1, and LOX-1 mRNA and proteins in aortas of rats with hyperlipidemia. Based on these findings, SVP has antioxidant action and it protects endothelium.**

Keywords: Vascular endothelial dysfunction, polyphenol, sea buckthorn berries, hyperlipidemia, protection

1. Introduction

Hyperlipidemia, hypercholesterolemia, and obesity are major risk factors for the development of cardiovascular diseases (CVDs), such as hypertension, atherosclerosis, coronary artery disease, and diabetes mellitus (*1-3*). One of the features of CVDs is vascular dysfunction, which involves an elaborate interplay between modified plasma lipoproteins (Lp), vascular endothelial cells (ECs) and smooth muscle cells (SMCs), migratory cells such as monocytes, T-lymphocytes, and platelets, and molecules produced by these cells (*4*). Furthermore, vascular endothelial dysfunction (VED) plays a key role in the development of many CVDs that are associated with a state of chronic, low-grade inflammation characterized by abnormal cytokine production and macrophage infiltration into tissues (*5*). A reasonable assumption is that these situations could be targeted to protect the vascular endothelium and maintain plasma homeostasis.

Many studies have indicated that the main etiology of VED involves a decrease in NO generation and bioavailability, leading to an increase in the generation of reactive oxygen species (ROS) (*6*). Oxidative stress may play a significant role in the development of metabolic syndrome (*7*). Accumulating evidence suggests that mitochondrial dysfunction might be associated with the response to inflammation. The excessive production of ROS and the release of oxidized mitochondrial DNA have been noted in inflammasomes (*8,9*). Vascular dysfunction is characterized by leukocyte recruitment, foam cell (FC) formation, endothelium dysfunction, and vascular inflammation. Inflammation is considered to be a hallmark of vascular dysfunction (*10,11*).

Some phytochemicals in certain plants have excellent pharmacological effects that have been studied extensively, and these effects are evident particularly in the antioxidant action of teas, spices, and herbs. Polyphenols are important additives in the food and pharmaceutical industries and these compounds have been widely studied because of their unique activities,

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*Address correspondence to:
Dr. You-Rui Suo, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, No. 23, Xinning Road, 810001 Xining, Qinghai, China.
E-mail: yrsuo@nwipb.cas.cn

Table 1. Major phenolic and flavonoid bioactives present in sea buckthorn

Chemical compound	Structures	Effective
Isorhamnetin-rutinoside		Anti-aging
Isorhamnetin-glycoside		Antioxidant
Quercetin-rutinoside	Catechin(CA) Isorhamnetin(IS)	Reduces LDL in blood (*16,17*)
Quercetin-glycoside		Antioxidant (*18,19*)
Catechin		Anti-atherogenic and hypocholesteremic (*19,20*)
Kaempferol	Kaempferol(KA) Quercetin(QA)	Antioxidant, reduces LDL in blood, anti-inflammatory (*20*)

such as anti-tumor activity, anti-diabetic activity, anti-inflammatory activity, hypolipidemic activity, and antioxidant activity, as well as immune regulation (*12,13*). Flavonoids are polyphenol compounds, and an epidemiological study has reported noting an inverse correlation between flavonoid consumption and the risk of CVD (*14*). Sea buckthorn (*Hippophae rhamnoides* L.), is a traditional Chinese medicine and Tibetan medicine and is thus one of the most important shrubs grown widely in China. In folk medicine, the fresh berries are used to relieve coughing, reduce phlegm, cure indigestion, stimulate the circulation of blood, and decrease hyperlipidemia (*15*). Since sea buckthorn is an excellent source of natural flavonoids such as isorhamnetin, quercetin, and aglycones (Table 1), many researchers have examined whether these components can effectively prevent and treat CVD. A study has examined the bioavailability and absorption of these components and their effect on emerging risk factors for CVD (*21*). Previous studies have reported that sea buckthorn mainly contains amino acids (*22*), triterpene derivatives, phytosteroids, and phenolic compounds such as oligomeric catechins and quercetin derivatives (*23-26*). According to vitro and vivo analyses of antioxidant activity, these active compounds possess anti-oxidative properties that can reduce the levels of free radicals and provide anti-diabetic activity, anti-inflammatory activity, hypolipidemic activity, and antioxidant activity (*27*). However, the role of polyphenols in vascular protection has not fully studied. In order to further investigate the pharmacological effects of polyphenols on vascular dysfunction, the current study evaluated the protection that polyphenols from sea buckthorn berries (SVP) provide against hyperlipidemia-induced VED in rats.

2. Materials and Methods

2.1. *Reagents and chemicals*

Sea buckthorn (*Hippophae rhamnoides* L.) berries were used to yield SVP, a compound containing phenolic actives such as flavonoids. Briefly, approximately 5 kg of sea buckthorn juice was dried with lyophilized, and then the powder was extracted 3 times with 1 L of 70% ethanol at a temperature of 60°C. Each extraction took 2 h. The supernatant was concentrated into a rough extract of about 1 kg. The rough extract was dissolved in 1 L of distilled water, and the resulting solution was subjected to column chromatography over D101 macro porous resin and eluted with 50-70% ethanol. Thirty-five g of refined SVP was prepared. The purity of SVP was determined using ultraviolet spectrophotometry. All reagents and chemicals used were of analytical grade.

2.2. *Animals and experimental design*

Four week-old male Sprague Dawley (SD) rats (n = 72) (specific pathogen-free grade, certification no. 62001000000134) weighing 90-100 g were purchased from the Animal Experimentation Center of Gansu University of Chinese Medicine. All animals were housed in groups of 12 per cage on a 12-h light/dark cycle. Rats were fed a basic diet in the experimental environment before the experiment was conducted. After one week of adaptation, all rats were randomly divided into 6 groups. Three of these groups were a normal control group (NC), a model control group (MC), and a positive control group (PC) receiving 2 mg/kg simvastatin (a dose corresponding to that administered to patients; simvastatin was from Lukang Drugs Group Co. Ltd., Lukang, Shandong, China). The remaining 3 groups received a low dose of SVP (7 mg/kg, equal of 1 g of herbs, SVPL), a medium dose of SVP (14 mg/kg, SVPM), or a high dose of SVP (28 mg/kg, SVPH). Each group consisted of 12 rats, and the chosen drug dose was based on the Chinese Pharmacopoeia (*15*). All groups except for the NC group were fed a high-fat diet (made with standard diet supplemented with 12 % lard, 10% yolk, 5% cholesterol, 1% bile salt, and 0.2% propylthiouracil) from Keaoxieli Co. Ltd. (Keaoxieli, Beijing, China). Every other day, rats were fed a high-fat emulsion preparation (10 mL/kg) as previously described (*28*). Body weight was recorded and blood was collected from the ocular fundus of two rats on a high-fat diet every two weeks to determine if

the model of hyperlipidemia was established. After 6 weeks, the NC and MC groups were orally administered the same volume of water while the other groups were administered the corresponding treatment once daily. After 5 weeks, animals were sacrificed, and the blood and tissues were collected for biochemical assays and structural analysis.

This experiment was carried out in accordance with local guidelines for the care of laboratory animals of Animal Experimentation Center and was approved by this institution's ethics committee for research on laboratory animal use.

2.3. *Morphology of the thoracic aorta*

The thoracic aorta was harvested and adherent connective tissue was removed. The thoracic aorta was initially fixed with a solution of 3% glutaraldehyde and then fixed in 1% osmium tetroxide, dehydrated with serial acetone solutions, and embedded in Epox 812. Semi-thin sections were stained with methylene blue. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (TEM; HITACHI, H-600IV, Japan).

2.4. *Biochemical analysis*

Rats were anesthetized with urethane. Blood samples were collected from the celiac artery and centrifuged at 4000 r/min for 10 min to obtain serum. All of the biochemical parameters were determined with colorimetry using commercially available kits (Jiancheng Biotech, Nanjing, China). Parameters related to antioxidant activity such as superoxide dismutase (SOD) activity in serum and the liver were measured using WAT-1 as a superoxide detector, the level of malondialdehyde (MDA) in serum and the liver was measured using the TBA method, and glutathione peroxidase (GSH-Px) in hepatic tissue was measured in a coupled system by measuring the decrease in NADPH at 340 nm. Biochemical parameters also included the levels of serum total cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL) and low-density lipoproteins (LDL).

Serum levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) were determined using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Jiancheng Biotech, Nanjing, China).

2.5. *Quantitative real-time PCR analysis of relative mRNA*

Aortic tissue was homogenized and total RNA was isolated using Trizol (Invitrogen, CA, USA). One microgram of total RNA from each rat sample was reverse-transcribed into cDNA using oligo (dt) 18 and reverse transcriptase (Fermentas, MA, USA). The cDNA of endothelial nitric oxide synthase (eNOS), lectin-like oxLDL receptor-1 (LOX-1), and intercellular adhesion molecule-1 (ICAM-1) was amplified using SYBR Green Mix (Thermo Fisher Scientific, MA, USA). PCR was performed with cycles of denaturation at 95°C for 15 s, annealing at 60°C for 45 s, and extension at 72°C for 30 s. The oligonucleotide primers used are listed in Table 2.

Table 2. Sequence of primers used in RT-PCR assays

Gene	Primer sequences	Amplicon size (bp)
eNOS	F: 5'-CTTTCGGAAGGCGTTTGAC-3' R: 5'-AACTCTTGTGCTGCTCAGG-3'	203
LOX-1	F: 5'-CAACCAGCCTTAGCGTATC-3' R: 5'-ATGCCCTCTTCTGACTTCC-3'	160
ICAM-1	F:5'-CCCACCTACATACATTCCTACC-3' R:5' TCTCCCAGGCATTCTCTTTG-3'	163
GAPDH	F: 5'- GTCGGTGTGAACGGATTTG-3' R: 5'-TCCCATTCTCAGCCTTGAC-3	181

2.6. *Western blotting analysis of relative protein*

Frozen aortas were homogenized with a Polytron homogenizer on ice in PIPA lysis buffer (Xinfan Biotech, Shanghai, China). After aortic tissue was fully lysed, the samples were centrifuged at 12000 g for 5 min at 4°C. Supernatants were collected and protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA). The supernatants were then denatured at 90°C for 10 min. Proteins were examined with 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then protein was transferred to polyvinylidene fluoride (PVDF) membranes using Bio-Rad Trans-Blot SD transfer cells (Bio-Rad Laboratories, CA, USA). The membranes were washed in TBST and blocked with 5% nonfat milk in TBST for 2 h at room temperature. Membranes were incubated overnight at 4°C with the primary antibodies (Polyclonal rabbit LOX-1 1:800, ICAM-1 1:500, eNOS 1:800 GAPDH 1:1500). The membranes were washed and incubated with horseradish peroxidase goat anti rabbit secondary antibody (Beyotime Institute of Biotechnology, Haimen, China) for 1 h at 37°C. The membranes were washed in TBST and then incubated in chemiluminescent substrate (ECL Western Blotting) for 30 s. Protein bands were detected on X-ray film. The software Image J was used to measure the integral optical density of protein bands from the scanned film and to calculate the integral optical density of the relative ratio of eNOS, LOX-1, ICAM-1, and GAPDH bands.

2.7. *Statistical analysis*

Data are presented as the mean ± SD. The statistical

analysis package SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA) was used for one-way analysis of variance (ANOVA) followed by a post-hoc test for multiple comparisons with a pre-determined significance level of $p < 0.05$.

3. Results

3.1. *The purity of SVP*

The main component of SVP was a flavonoid, and its activity significantly increased after column chromatography over D101 macroporous resin. The purity of the SVP was determined at 760 nm using UV with gallic acid as the standard control. The linear equation was $Y = 0.0504X + 0.0723$, $R^2 = 0.9998$, and the yield was 89.98 ± 0.032%.

3.2. *Effects of SVP on body weight*

All of the groups had a similar increase in body weight before receiving the corresponding treatment. Body weight was 299 ± 4.92 (g), and it began to significantly change once treatment with SVP and simvastatin began. The MC group had a significant increase in body weight compared to other groups, but there was no significant weight gain in the SVPM and SVPH groups in comparison to the MC group ($p < 0.05$). The rate of weight gain in rats in the SVPM and SVPH groups was similar to that in the PC group (Figure 1).

3.3. *Effects of SVP on TNF-α and IL-6 in serum*

The MC group had significantly higher levels of serum TNF-α and IL-6 in comparison to those in the NC group ($p < 0.05$). However, SVP decreased the levels of serum TNF-α and IL-6 in a dose-dependent manner (Figure 2). The level of serum TNF-α in the SVPH group was significantly lower than that in the PC group and that level was similar to that in the NC group (Figure 2A). However, the serum IL-6 level did not differ in the SVPH group and the PC group (Figure 2B).

3.4. *Effects of SVP on antioxidant enzyme activity in serum and the liver*

Results indicated that the activity of SOD in serum and the liver of rats with hyperlipidemia decreased ($p < 0.05$), and the level of SOD in serum was markedly lower than that in the liver. The activity of SOD in serum and the liver was markedly higher in the SVPL, SVPM, and SVPH groups in comparison to that in the MC group ($p < 0.05$) (Figure 3A). Similarly, a high-fat diet induced higher MDA levels, and the MDA levels in the SVPL, SVPM, and SVPH groups were distinctly lower than those in the MC group ($p < 0.05$). MDA levels in the SVPL, SVPM, and SVPH groups were similar to those in the PC group and slightly elevated in comparison to those in normal rats (Figure 3B). The level of GSH-Px in the liver decreased significantly in the MC group while the GSH-Px level increased significantly in the SVPM, SVPH, and PC groups ($p < 0.05$). The level of GSH-Px in the MC group was similar to that in the PC group (Figure 3C). In addition, the SVPM and SVPH groups had improved levels of serum SOD in comparison to the PC group. The SVPH group had improved levels of liver SOD in comparison to the PC group. The SVPH group had improved levels of liver MDA in comparison to the PC group. The SVPM and SVPH groups had improved levels of liver GSH-Px in comparison to the PC group. However, none of these differences were significant.

3.5. *The effect of treatment on blood lipids*

As shown in Figure 4, the serum levels of TC, TG, and LDL levels increased significantly in the MC group but

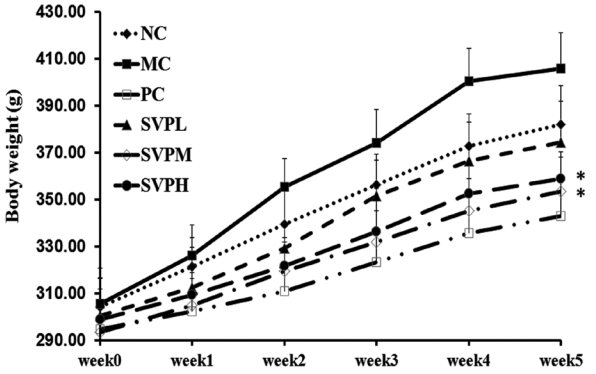

Figure 1. Effect of SVP on the body weight of rats. Data are presented as the mean ± SD ($n = 10$). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test).

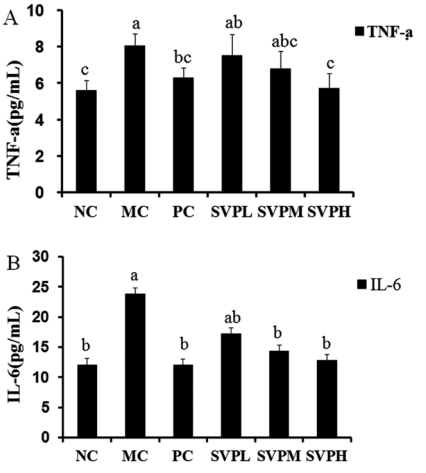

Figure 2. Effect of SVP on the level of serum TNF-α (A) and IL-6 (B) in rats. Data are presented as the mean ± SD ($n = 7$). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test).

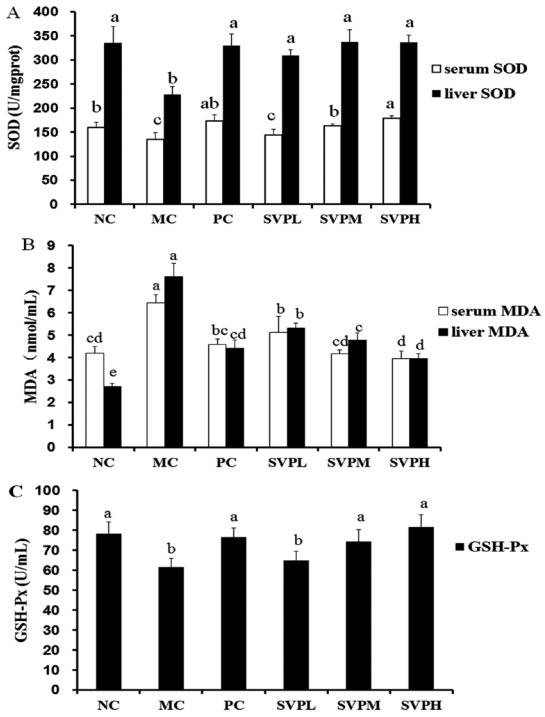

Figure 3. Effect of SVP on antioxidant activity in serum and the liver. Superoxide dismutase (SOD) **(A)** in serum and the liver, malondialdehyde (MDA) **(B)** in serum and the liver, and glutathione peroxidase (GSH-Px) **(C)** in serum from rats. Data are presented as the mean ± SD (n = 8). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test).

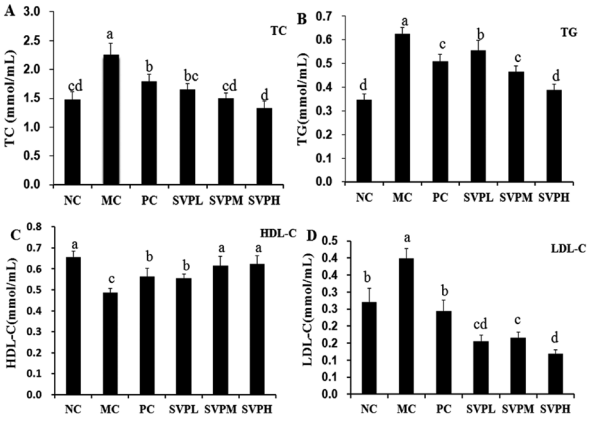

Figure 4. Effect of SVP on blood lipids in rats. Data are presented as the means ± SD (n = 8). **(A)** indicates TC, **(B)** indicates TG, **(C)** indicates HDL, and **(D)** indicates LDL. Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test)

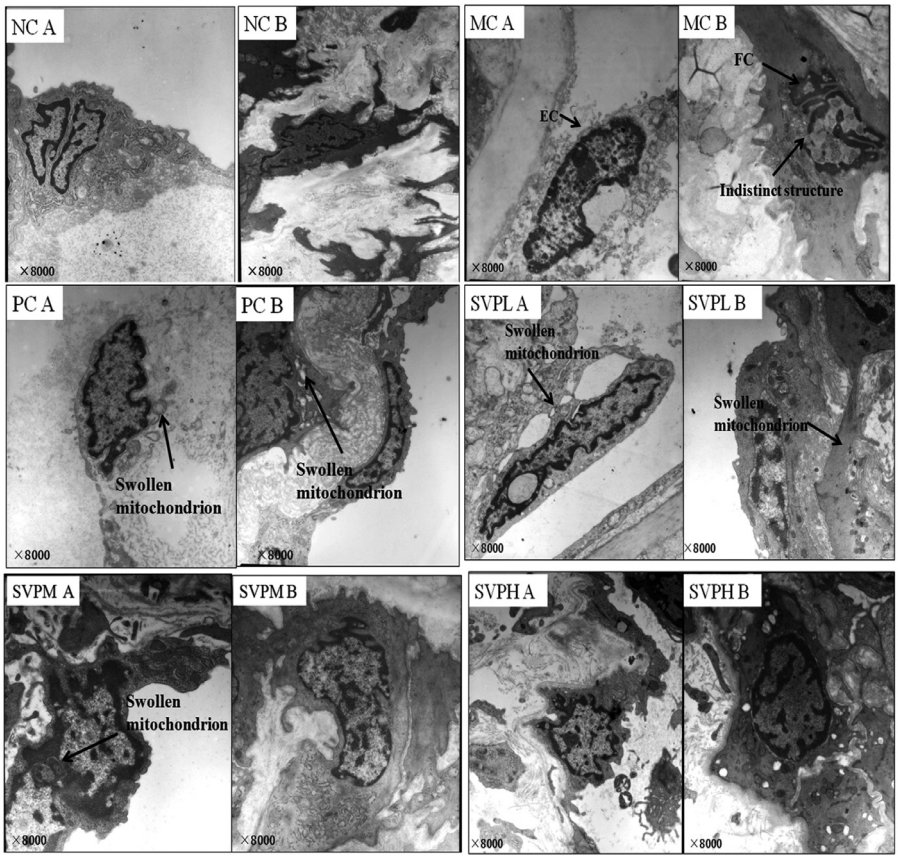

Figure 5. Effects of SVP treatment on histopathology in rats with hyperlipidemia. A indicates endothelial cells (ECs) in each group. **B** indicates smooth muscle cells (SMCs) in each group. **FC** indicates foam cells in proportion to ECs and SMCs in each group. Swollen mitochondria with an indistinct structure were noted. All images were taken at ×8000.

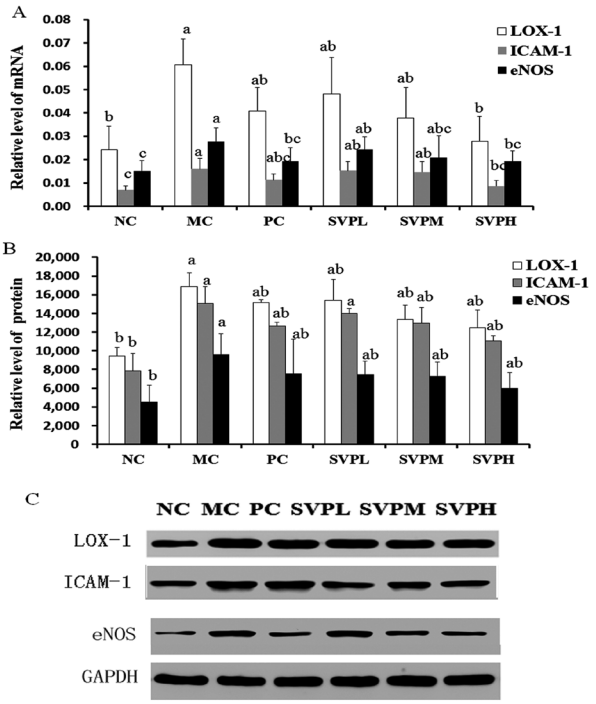

Figure 6. Effect of SVP on the expression of LOX-1, ICAM-1, and eNOS mRNA and proteins in aortic tissue from rats with hyperlipidemia. (A) are representative relative levels of LOX-1, ICAM-1, and eNOS mRNA. **(B)** and **(C)** are representative bands and relative levels of LOX-1, ICAM-1, and eNOS proteins. Data are presented as the mean ± SD ($n = 6$). Different letters indicate statistically significant differences; the same letter indicates that there were no significant differences between two groups. $p < 0.05$ (Turkey's test).

the level of HDL decreased significantly. In contrast, levels of TC, TG, and LDL were lower in the SVPL, SVPM, and SVPH groups than in model rats ($p < 0.05$). Levels of TC, TG, and LDL were considerably lower than those in positive control rats. The SVPL, SVPM, and SVPH groups had significantly increased HDL levels in comparison to the MC group. Effects were dose-dependent in the SVPL, SVPM, and SVPH groups (Figure 4)

3.6. *Histological changes in the aorta*

The histological appearance of the transverse section of the aorta is shown in Figure 5. In ECs from the MC group, abnormalities like abnormal chromatin assembly in the cell nucleus and an increased number of FCs were noted; moreover, organelles lacked a distinct structure and ECs were sloughed off. FCs were also noted among the SMCs. In comparison, these changes were not noted in the ECs and SMCs from the NC group. Cells were intact and had a normal cytoarchitecture, and organelles had a distinct structure. The arterial structure in the SVPL, SVPM, SVPH, and PC groups had significantly fewer FCs, and slight chondriosome swelling in ECs and SMCs was noted in the SVPL, SVPM, and PC groups. However, cytoarchitecture was intact in the SVPH group.

3.7. *Effect of SVP on aortic cytokines*

Hyperlipidemia induced expression of LOX-1, ICAM-1, and eNOS, and RT-PCR results indicating how SVP affected that expression are shown in Figure 6A. The expression of LOX-1, ICAM-1, and eNOS mRNA was barely detected in aortic tissue from the NC group while expression of these mRNAs increased significantly ($p < 0.05$) in the MC group. After treatment with SVP, the expression of these mRNAs was much lower than that in the MC group ($p < 0.05$). The regulating actions of SVP were found to proportionally decrease in a dose-dependent manner. Moreover, as shown in Figure 6B and 6C, the expression of ICAM-1, LOX-1, and eNOS proteins in aortic tissue from the MC group increased approximately 2.0-fold in comparison to that in the NC group. After treatment with SVP, the expression of these proteins decreased significantly ($p < 0.05$).

4. Discussion

The purpose of this study was to investigate the effects of SVP on vascular endothelial function in rats with hyperlipidemia. Results indicated that the vascular structure of the aorta was severely damaged in the MC group, such as sloughing off of ECs, formation of FCs, swollen mitochondria in ECs and SMCs, and destruction or disappearance of mitochondrial cristae. These phenomena were significantly abated in the SVPL, SVPM, SVPH, and PC groups in comparison to the MC group (Figure 5). Changes in vascular morphology coincided with diminished vascular dysfunction. eNOS expressed in ECs is the major source of endothelial NO (*29*). Early disruption of eNOS expression may have contributed to the reduced level of NO and even VED. In the current study, the levels of aortic eNOS expression (levels of both mRNA and protein) increased significantly in the MC group. This seems contradict the mechanism whereby VED is induced by eNOS/NO, and there is some evidence that uncoupling of endothelial NO synthase can decrease the bioavailability of NO, aggravating the development of atherosclerosis and vascular disease (*30,31*). According to the current findings, all 3 doses of SVP decreased expression of eNOS mRNA and protein (Figure 6A, 6B, and 6C). This suggests that SVP can increase the bioavailability of endothelium-derived NO and it can also protect ECs, ameliorating VED induced by *eNOS*/NO disorders.

Previous studies have indicated that high cholesterol (hypercholesterolemia) induces the generation of ROS, thereby decreasing the generation and bioavailability of NO (*29*). ROS may result in the instability of critical macromolecules and represent the molecular basis of many diseases including inflammation processes, cardiovascular alterations, and cancer (*32-34*). Antioxidant enzymes have an essential physiological

function, serving as the first line of defense against ROS by coordinating to reduce the generation of active oxygen radicals and by preventing lipid peroxidation and intermediate products of metabolization from undermining the body (*35*). The MDA level is closely associated with several components of metabolic syndrome (*36*). The current study examined the activity of antioxidant enzymes (SOD and GSH-Px) and the levels of the oxidation product MDA. As shown in Figure 3, SVP increased the activity of SOD in serum and the liver but it significantly decreased the levels of the oxide metabolite MDA in serum and the liver in comparison to levels in normal rats. However, SVP significantly increased the activity of the antioxidant enzyme GSH-PX in the liver. A previous study indicated that flavonoids have the ability to effectively reduce oxidative stress (*37*). Therefore, the current results suggest that SVP could improve the efficiency of antioxidant enzymatic systems and that SVP might be a compensatory way to counteract the possible detrimental effects associated with oxidative stress induced by exhaustive exercise.

In addition to the ROS and oxidative stress mentioned earlier, the inflammatory response is considered to be a common pathogenic factor in vascular impairment (*38*). A higher level of IL-6 may cause insulin resistance and metabolic disorders in patients with metabolic syndrome (*36*). TNF-α, another pro-inflammatory cytokine, is also closely associated with the pathogenesis of oxidant stress and vascular dysfunction (*39*). In order to investigate the possible involvement of the molecular mechanism of SVP in endothelial dysfunction induced by hyperlipidemia, the current study evaluated the expression of the inflammatory cytokines IL-6 and TNF-α in serum. Levels of serum TNF-α, and IL-6 in the MC group were significantly higher than those in the NC group ($p < 0.05$). However, SVP reduced the levels of serum TNF-α and IL-6 in a dose-independent manner (Figure 4). This suggests that vascular protection by SVP may result from its anti-inflammatory activity since it reduces the levels of TNF-α and IL-6 in rats with hyperlipidemia. These results coincide with the pathologic changes mentioned earlier.

Elevated serum LDL levels are thought to be related to the risk of CVD (*40*). Emerging evidence has implicated endothelial dysfunction induced by oxidized LDL (Ox-LDL) in atherogenesis. LOX-1 is a receptor for atherogenic Ox-LDL and it appears to mediate Ox-LDL-induced inflammation, which may be crucial in atherogenesis. LOX-1 is a lectin-like receptor for ox-LDL in ECs, and activation of those cells induces oxidative stress. Furthermore, a higher level of oxidative stress stimulates LOX-1 expression, and this have a positive effect by increasing intracellular production of ROS (*41*). In addition, another factor implicated in endothelial dysfunction is the activation of ECs, which is evident as an increase in the expression of specific cytokines and adhesion molecules ICAM-1 and the consequent penetration of monocytes into the intima (*42*). ROS are generated in response to a high-fat diet, but inhibition of those ROS could alleviate vascular oxidative stress and inflammation. Moreover, a previous study on an artichoke extract indicated that flavonoids retard LDL oxidation (*43*) and reduce oxidative stress in ECs stimulated with TNF-α and oxidized LDL (*44*). Furthermore, flavonoids have a positive cardiovascular effect, including decreased leukocyte adhesion and inflammation as well as vasodilatory properties (*45*). According to the current results, the modulating effects of SVP were evident as a decrease in LDL in a dose-dependent manner. Moreover, the expression of ICAM-1 and LOX-1 proteins in aortic tissue from the MC group was approximately 2.0-fold more than that in the NC group, as shown in Figure 6B. After SVP treatment, the expression of these proteins decreased significantly ($p < 0.05$). Therefore, findings indicate a molecular mechanism underlying the therapeutic effects of SVP and they highlight a new therapeutic intervention for the prevention of endothelial dysfunction induced by hyperlipidemia.

In conclusion, results indicated that oral administration of SVP at a dose of 7-28 mg/kg significantly improved the tolerance of hyperlipidemia in rats given a high-fat diet. More importantly, SVP prevented endothelial dysfunction of the aorta by enhancing the activity of antioxidant enzymes, attenuating the levels of inflammatory cytokines such as TNF-α and IL-6, and decreasing the level of eNOS, ICAM-1, and LOX-1 expression. These findings suggest that SVP has significant preventive and therapeutic action that could be explored as a promising food additive to prevent chronic CVD.

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Original Article

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Bile duct injuries after laparoscopic cholecystectomy: 11-year experience in a tertiary center

David Martin, Emilie Uldry, Nicolas Demartines*, Nermin Halkic

Department of Visceral Surgery, University Hospital CHUV, Lausanne, Switzerland.

Summary **Incidence of bile duct injuries (BDI) is low but remains a dramatic complication after laparoscopic cholecystectomy (LC). This study aimed to assess BDI incidence and management strategies. All patients treated in our institution for BDI after LC between 2000 and 2011 were retrospectively analyzed. Patients referred from others centers were excluded. Strasberg classification was used to determine the type of lesion. Thirteen patients presented iatrogenic BDI among 2,840 consecutive cholecystectomies performed (0.46%). Four cases were classified Strasberg type A, 4 type D, and 5 type E. Injury was recognized intraoperatively in 6 cases (46%). Three of these 6 required conversions to open surgery and all but one were primary sutured on a drain; the remaining patient required immediate biliodigestive anastomosis. In 7 patients, the injury was discovered postoperatively (54%). Among them, one was treated by direct closure of a cystic leak through immediate re-laparoscopy. Six underwent initially main bile duct stenting, but 4 required delayed secondary surgery (mean time 115 days), 2 to improve bile duct drainage and 2 for biliodigestive derivation. BDI incidence remains low but management depends on the time of diagnosis. BDI are complex and require tailored treatment usually in a tertiary center for a multidisciplinary approach.**

Keywords: Complications, primary suture, biliodigestive anastomosis

1. Introduction

From historical perspective, Langenbuch performed the first open cholecystectomy 1882, Kehr the first intraoperative biliary repair 1899, and Hepp and Couinaud the first biliodigestive anastomosis with detachment of the hilar plate 1956 (*1-3*). The first laparoscopic cholecystectomy (LC) was performed by Mühe in Germany 1985, followed by Mouret in France 1987 (*4*). Despite the absence of randomized controlled trials showing significant benefit of laparoscopic approach, it has now become the gold standard for more than 3 decades in the treatment of symptomatic cholelithiasis. Increased incidence of iatrogenic bile duct injuries (BDI) was reported, about 0.3% against 0.2% in laparotomy (*5,6*). The study aim was to analyze incidence and management strategies of these lesions in a single teaching center.

2. Materials and Methods

Single-center retrospective study of patients managed for BDI after LC between 2000 and 2011. Patients referred from others centers were excluded. Medical records were examined individually to extract data on demographics, LC indication, time of diagnosis, conversion to laparotomy, use of intraoperative cholangiography (IOC), length of stay and treatments. Strasberg classification was used to determine the type of lesion (*6*). Of note, cholecystectomies were performed using a standardized 3-trocars technique and IOC was used systematically until 2006 and then selectively only since 2007. Calot's triangle dissection was done using monopolar hook, followed by the application of two metal clips on the cystic duct with section between clips. In case of a wide cystic duct (> 5 mm diameter), Hem-O-Lok® was

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**Address correspondence to:*
Prof. Nicolas Demartines, Department of Visceral Surgery, University Hospital CHUV, Rue du Bugnon 46, 1011 Lausanne, Switzerland.
E-mail: demartines@chuv.ch

applied based on anatomy and surgeon's evaluation. The same technique was used for cystic artery. At the end of the procedure, no drain was placed. The institutional review board approved the study.

3. Results

Thirteen patients presented BDI among 2,840 consecutive cholecystectomies (0.46%), including 9 major wounds of the common bile duct (0.32%) and 4 minor wounds (0.14%). These injuries were observed in 6 men and 7 women, with a mean age of 67 years. Patient demographics are summarized in Figures 1-3. The distribution of body mass index (BMI) varied, with a predominance of 25-30 kg/m^2 category. The American Society of Anesthesiologists score II prevailed, and more than three quarters of patients were older than 60 years. The lesions were classified according to Strasberg in Table 1 (*6*).

Indication for surgery was acute cholecystitis in 9 cases, symptomatic cholecystolithiasis in 3, and choledocholithiasis with preoperative endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy in 1 case. The procedure was performed urgently in 3 cases and electively in 10 cases.

The main cause of BDI was anatomical misinterpretation (n = 5). This includes confusion of the common bile duct (CBD) with the cystic duct at the time of section (n = 2) or during IOC (n = 3). Other causes include postoperative cicatricial stenosis on a misplaced clip in 1 case, cystic stump dehiscence in 4 cases, direct injury to the CBD during dissection in 1 case and unknown mechanism in 2 cases.

On all 8 IOC performed, 3 identified directly a D type lesion, 3 confirmed a suspected lesion (2xE1 and D lesions), and 2 showed no abnormality at the time of interpretation (A and E1 lesions). Diagnostic methods were various: 6 injuries were recognized immediately in the operative field, 3 thanks to bile leak identification and 3 by IOC, while 2 were recognized postoperatively during secondary exploratory laparoscopy, and 5 detected by non-surgical methods – computed tomography (CT), ERCP, and magnetic resonance imaging (MRI).

Lesions were recognized intraoperatively in 6 patients and in 3 cases a conversion to laparotomy was performed. Injuries and their treatment are summarized in Table 2. Overall, 5 of 6 lesions were treated by primary suture on a drain. One biliodigestive anastomosis was performed immediately because of large substance loss. All lesions recognized intraoperatively underwent immediate reparation. As institutional policy, the surgeon involved never tried to repair the BDI himself, and HPB surgeon presence was required systematically.

Lesions identified postoperatively are described in Table 3. Six were treated initially by main bile duct stenting, and 4 underwent subsequent biliary surgery. One single lesion was treated by laparoscopy. For

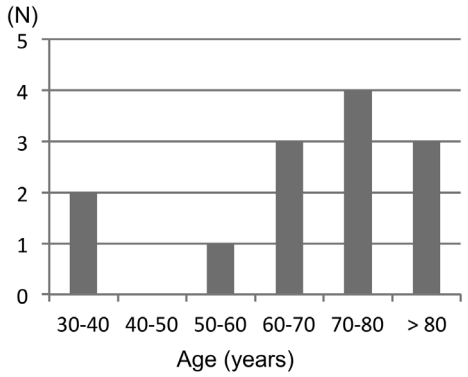

Figure 1. Patient's demographics: age distribution.

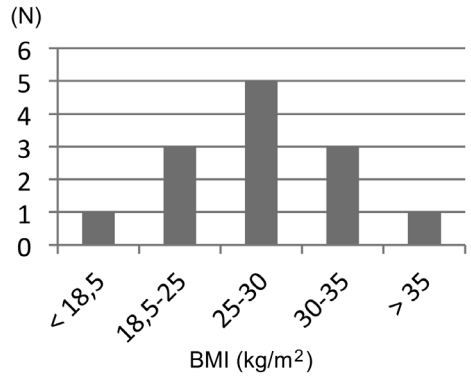

Figure 2. Patient's demographics: body mass index (BMI) distribution.

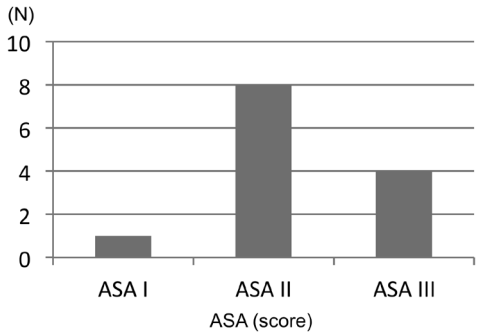

Figure 3. Patient's demographics: American Society of Anesthesiologists (ASA) score distribution.

all lesions discovered postoperatively, the average time between LC and definitive treatment was 115 days (range 1-480 days). Neither liver resection nor transplantation due to the BDI were necessary, and there were no operation-related deaths. One patient developed secondary biliary cirrhosis 2 years after a biliodigestive anastomosis repair (E1 lesion).

Pathological examination of the surgical specimen showed chronic cholecystitis in 10 cases, acute cholecystitis in 2 cases, and well-differentiated adenocarcinoma of the gallbladder in one case.

4. Discussion

Bile duct injury incidence remains low but precise

Table 1. Distribution of injuries according to the Strasberg classification

Type of injuries	*n* =13 (%)
A. Cystic duct leaks or leaks from small ducts in the liver bed	4 (31%)
B. Occlusion of part of the biliary tree, almost invariably the aberrant right hepatic ducts	-
C. Transection without ligation of the aberrant right hepatic duct	-
D. Lateral injuries to major bile duct	4 (31%)
E1. Low common hepatic duct (CHD) stricture, with the length of the CHD stump of > 2 cm	3 (23%)
E2. Proximal CHD stricture - hepatic duct stump < 2 cm	-
E3. Hilar stricture, no residual CHD, but the hepatic ductal confluence is preserved	-
E4. Hilar stricture, with involvement of confluence and loss of communication between right and left hepatic duct	-
E5. Involvement of aberrant right sectorial hepatic duct alone or with concomitant stricture of the CHD	2 (15%)

Table 2. Details of injuries and repairs in case of intraoperative diagnosis

Strasberg Lesion	Repair / surgical approach	Secondary treatment
E1	Primary suture on a drain / laparotomy	- None
D	Primary suture on a drain and surgical site drainage / laparoscopy	- None
D	Primary suture on a drain / laparotomy	- None
D	Primary suture on drain / laparotomy	- None
D	Primary suture on a drain and surgical site drainage / laparoscopy	- Percutaneous drainage of a bilioma
E1	Biliodigestive anastomosis / laparotomy	- Two laparotomies for resection and preparation of a new biliodigestive anastomosis

Table 3. Details of injuries and repairs in case of postoperative diagnosis

Strasberg lesion	Initial treatment	Secondary treatment
E1†	ERCP and stent	- 3 ERCP and stents, biliodigestive anastomosis
A*	ERCP failed	- Transcystic drainage by laparoscopy
A*	Percutaneous drainage	- 4 ERCP and stents
E5*	Percutaneous drainage	- 3 ERCP and stents, transcystic drainage by laparotomy, and new ERCP and stent
E5†	2 ERCP failed	- Biliodigestive anastomosis
A*	Ligation of cystic leak and surgical site drainage by laparoscopy	- None
A*	Percutaneous drainage	- ERCP and stent

ERCP, endoscopic retrograde cholangiopancreatography; * < 6 weeks, † ≥ 6 weeks

management depends on the time of diagnosis. The role of IOC is still controversial. In this series, IOC was used systematically until 2006, and then selectively only since 2007. IOC does not eliminate the risk for injuries, but rather helps to identify them earlier provided an adequate interpretation is done. In fact IOC is misinterpreted in up to 50% of cases, making the effectiveness of implementing it systematically questionable (*7*). A recently published systematic review displayed neither evidence in favor nor against the use of IOC (*8*).

There seems to be a relationship between the time when the injury is recognized, and the type of injury. Lesions recognized intraoperatively were related to confusion between cystic and CBD with partial or full section. Hugh demonstrated that up to 75% BDI were caused by such a misinterpretation (*9*). Another important cause of BDI is desperate attempts to control bleeding in the Calot's triangle with several clips or broad electrocautery (*10*). In cases of postoperative recognition, minor lesions due to cystic stump leakage are more likely. However, complex wounds can also be observed postoperatively; early in case of complete or partial transection, or later in case of cicatricial stenosis.

In case of intraoperative diagnosis, before immediate repair, complete assessment of biliary tract anatomy with IOC is mandatory, either by an open or by laparoscopic approach (*5*). A conversion to laparotomy is required if the operator's experience is limited, or if the anatomy is unclear. An HPB surgeon should be involved in the management and repair as outcomes are significantly better (*5,11,12*). For minor A lesion, applying a clip or a ligature combined with a transcystic drainage is recommended. In case of type D lesions, primary suture on a drain is the technique of choice. In case of complete CBD transection or aberrant duct (B, C and E types) and great substance loss, immediate biliodigestive anastomosis is recommended (*5*). If the defect is limited or absent, an end-to-end biliary anastomosis on a drain may be performed. DeReuver reported 91% stricture free results at 7 years follow up in 56 patients after end-to-end anastomosis (*13*). If the surgeon is not comfortable with the injury, drainage of the hepatic pedicle and sub-hepatic region should be performed, and patient transferred to a tertiary center (*14,15*). Mismanagement can result in extension of the lesions, and need for additional complex therapeutic procedures.

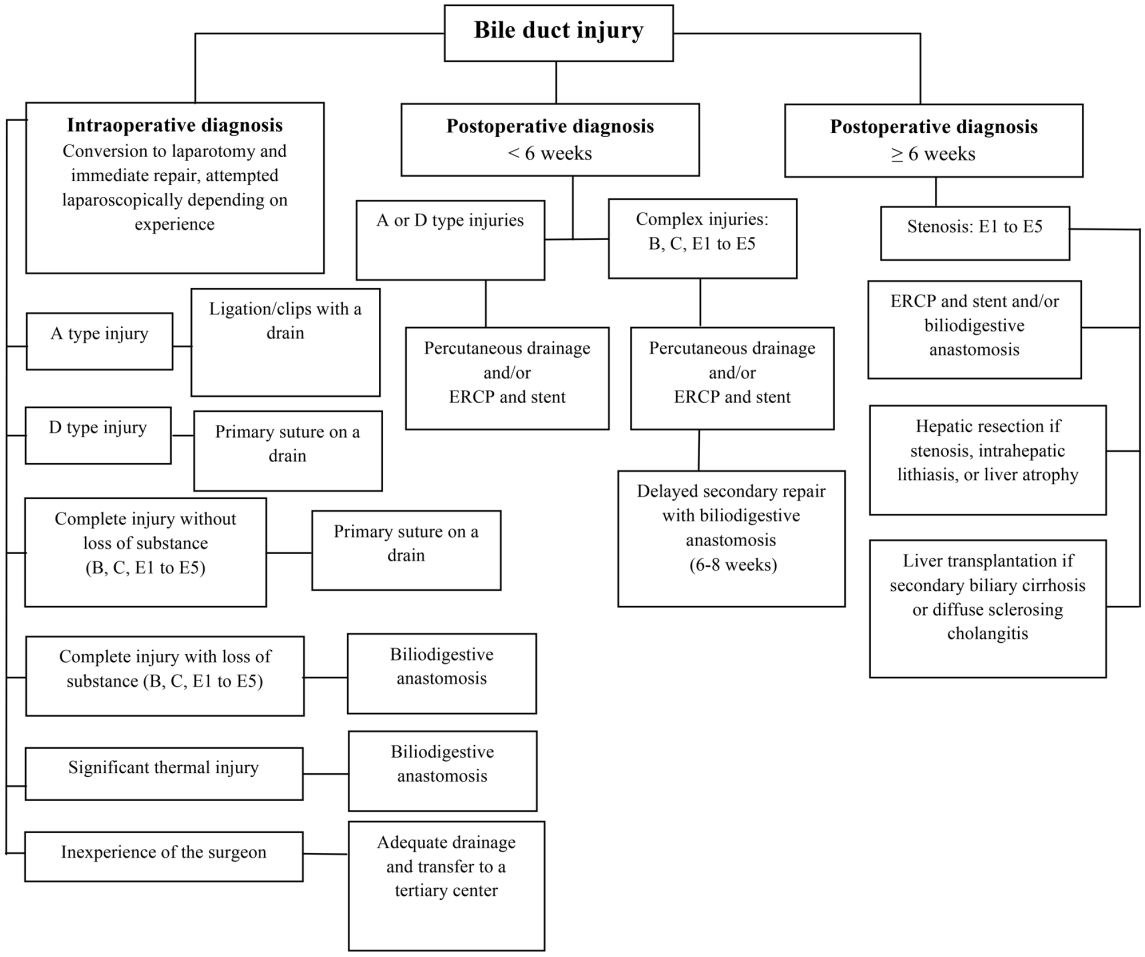

Figure 4. Treatment algorithm in case of bile duct injury according to Strasberg classification.

In case of postoperative diagnosis, the timing of bile duct repair is still a matter of debate. A full assessment of the lesion is essential before choosing an appropriate treatment. The choice of an interval of 6 weeks to define early and late diagnosis was based on the hypothesis that this time interval may differentiate sections from ischemic stenoses (*16*). If type A or D lesions are discovered early (< 6 weeks) by abscess, bilioma or cholangitis, immediate percutaneous drainage or ERCP with application of stent to calibrate the leak is recommended. If it fails, a surgical approach by laparoscopy or laparotomy becomes necessary. In case of complex lesion (B, C or E) and early diagnosis (< 6 weeks), immediate repair is not recommended, because of considerable risk of long-term complications (30% stricture rate) and mortality (*17*). Percutaneous drainage or ERCP to improve local conditions should be used, and then a biliodigestive repair performed 6-8 weeks later so that the inflammation process did regress. This approach is supported by data from several expert centers, and by the fact that the lesion may progresses to its final stage before final repair (*18,19*). In case of late postoperative diagnosis (≥ 6 weeks), injuries are mainly ischemic stenosis related to devascularization. Treatment remains controversial. ERCP with dilatation and stenting if the anatomical location allows it should be tried first, and biliodigestive anastomosis performed eventually in second line if stenting was not possible or the result insufficient. In the present series, neither resection nor liver transplantation were necessary.

Each case should be analyzed individually during multidisciplinary conference including interventional radiology, endoscopy, and HPB surgery. Stewart demonstrated that treating BDI in expert centers offered significantly better outcomes than if performed in the center where the injury was performed (94% *vs*. 17%) (*12*). On the other hand, there is less data on the results of HPB surgeons in high volume centers repairing their own injuries. Evaluation of the outcome of biliary tract repair is a difficult task. In our department, patients were examined by HPB surgeon 4 to 6 weeks after discharge, and then followed by their general practitioner with clinical and biological assessment every year. Long-term follow-up is mandatory because biliary stricture can be observed up to 10 to 20 years after initial repair (*19*). Moreover, it has been reported that such lesions may have significant impact on physical and psychological quality of life (*20*).

Based on BDI repair techniques analysis of our center, and based on data from several expert centers,

a treatment algorithm was developed and is presented in Figure 4. This algorithm however, needs further evaluation and validation.

The main limitation of the present study is its retrospective nature and a relatively small number of patients, thus limiting associations and comparisons with the literature.

In conclusion, BDI incidence remains low but their management depends on the time of diagnosis. These injuries are complex, and treatment needs to be individualized based on patient, anatomy and nature of the injury. Multidisciplinary management in tertiary centers should be recommended.

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Original Article

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Promoter hypomethylation of RAR-related orphan receptor α 1 is correlated with unfavorable clinicopathological features in patients with colorectal cancer

Hisao Kano[1,2], Tadatoshi Takayama[2], Yutaka Midorikawa[2,*], Hiroki Nagase[1,3]

[1] *Department of Cancer Genetics, Nihon University School of Medicine, Tokyo, Japan;*
[2] *Department of Digestive Surgery, Nihon University School of Medicine, Tokyo, Japan;*
[3] *Division of Cancer Genetics, Chiba Cancer Center Research Institute, Chiba, Japan.*

Summary **Retinoic acid receptor-related orphan receptor α (*RORA*) is a tumor-specific differentially methylated region. *RORA* mRNA expression is frequently downregulated in colorectal cancer (CRC) due to promoter methylation, and this methylation is correlated with the development of CRC. Here we investigated the correlation between the methylation status of the *RORA* promoter region and clinical CRC stages. The methylation status of *RORA* isoform 1 (*RORA1*) and isoform 4 (*RORA4*) promoters was investigated in 43 paired CRC specimens and adjacent normal tissues by quantitative DNA methylation analysis using the Sequenom MassARRAY system and bisulfite sequencing. The relationship between the methylation status of the *RORA1* promoter and the CRC pathological stage was analyzed. *RORA1* expression was evaluated using quantitative PCR. Sixteen of 43 CRC specimens (37%) and three CRC cell lines (Caco2, HT29, and HCT116) showed increased levels of methylation in the *RORA1* promoter region compared with adjacent normal tissues, whereas no methylation was observed in the *RORA4* promoter. Quantitative PCR showed downregulation of *RORA1* expression both in CRC samples and cell lines. Furthermore, the *RORA1* promoter hypomethylation status showed a significant correlation with unfavorable CRC stages (stages III and IV) compared with favorable stages (stages I and II, $p = 0.014$). Hypomethylation of the *RORA1* promoter may have important clinical implications in unfavorable CRC development, and therefore, the methylation status of the *RORA1* promoter may constitute a useful biomarker to determine an indication for postoperative therapy such as adjuvant chemotherapy in highly advanced CRC patients.**

Keywords: DNA methylation, RORA, colorectal cancer, prognostic factor

1. Introduction

The identification of prognostic and predictive markers in colorectal cancer (CRC) pathogenesis is of great importance for developing new therapeutic strategies. CRC typically develops over decades and involves multiple genetic and epigenetic alterations in cancer-related genes during carcinogenesis. One of the most common epigenetic alterations is aberrant methylation of cytosine-guanine (CpG) islands that encompass the promoter and transcription start site. Aberrant methylation of these sites can be accompanied by transcriptional repression. A variety of tumor suppressor genes such as *Rb*, *CDKN2A/p16*, *MGMT*, $p14^{ARF}$, and *HLTF* are aberrantly methylated in CRC (*1*).

Previously, we performed restriction landmark genomic scanning to identify novel genomic regions of mouse skin tumor-specific differentially methylated regions (DMRs), and found 14 DMRs that were highly conserved between mouse and human (*2-5*). Among these, we focused on retinoic acid receptor-related orphan receptor α (*RORA*), which is frequently downregulated in

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*Address correspondence to:
Dr. Yutaka Midorikawa, Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Oyaguchi Kami-machi, Itabashi-ku, Tokyo 173-8610, Japan.
E-mail: mido-tky@umin.ac.jp

CRC and is correlated with tumor progression (*6*).

ROR alpha, -beta, and -gamma are evolutionarily related transcription factors that belong to the steroid hormone receptor superfamily. ROR was cloned by virtue of its strong homology with the retinoic acid receptor. *RORA* generates four isoforms (RORA1–4), which differ in their N-terminal region and demonstrate distinct transactivation properties (*7*). Notably, only two of the four isoforms, *RORA1* and *RORA4*, are actually transcribed (*8*). Although some putative ligands have been proposed for RORA, the nature of RORA ligands remains elusive (*9,10*).

Previous reports have demonstrated significantly lower *RORA* mRNA and protein levels in CRC samples compared with normal mucosa. Furthermore, an inverse correlation was found between RORA protein levels and progression in CRC patients (*6*). The functional significance of RORA in CRC was proposed to be RORA inhibition of canonical Wnt/β-catenin signaling to suppress CRC cell growth through protein kinase Cα-dependent phosphorylation (*11*), and RORA enhancement of p53 stability and transactivation ability to increase apoptosis (*12*). Several studies have described the possible mechanism through which RORA contributes to human disorders. For example, RORA stimulates transcription of the NF-kB inhibitor IkB (*13*), and *RORA* regulates hypoxia-inducible factor 1 transcription (*14*). A study in the human hepatoma HepG2 cell line showed that RORA directly upregulates secreted protein, acidic, cysteine-rich (SPARC), which is associated with a highly aggressive tumor phenotype, and behaves as a tumor suppressor in several specific cancer types (*15*). All these studies have implicated RORA as a functional tumor suppressor. However, whether these findings could be used in clinical applications, such as a prognostic biomarker, for evaluation of therapy, or as an indication for treatment, remains unclear.

In the present study, we investigated the methylation status of the *RORA* promoter in CRC patient samples compared with adjacent normal tissues. In sharp contrast to previous studies obtained from gene and protein expression analyses, our results suggested that the *RORA1* promoter may be hypermethylated in early, favorable stages in CRC and subsequently hypomethylated as cancer becomes more advanced. Furthermore, our results indicate a significant correlation between the *RORA1* promoter methylation status and the CRC stage. Thus, hypomethylation of the *RORA1* promoter may be of great diagnostic value for determining an indication for postoperative chemotherapy for advanced CRC.

2. Materials and Methods

2.1. *Human surgical specimens and cell lines*

CRC specimens and adjacent normal mucosa were obtained from 43 patients who underwent a curative operation at Nihon University School of Medicine. The study was approved by the Institutional Review Board, and informed consent was obtained from all patients. All tumors were pathologically diagnosed CRC and staged according to the TMN staging system; two patients were Stage I, 17 were Stage II, 16 were Stage III, and eight were Stage IV. CRC cell lines (Caco2, colo205, HT29, HCT116) and a breast cancer cell line (MCF7) were obtained from the RIKEN BioResource Center (Tsukuba, Japan). CRC cell lines were grown in RPMI1640 containing 10% fetal bovine serum, and MCF7 cells were grown in DMEM containing 5% fetal bovine serum. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

2.2. *Bisulfite treatment and promoter methylation analysis*

Genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All primers were designed using Methprimer (*http://www.urogene.org/methprimer/index1.html*) or Methyl Primer Express Software v1.0 (Applied Biosystems, Foster City, CA) and purchased from Operon Biotechnology (Tokyo, Japan). Bisulfite modification was performed by the sodium bisulfite method with the EZ DNA Methylation Kit (Zymo Research, Orange, CA). The bisulfite-treated genomic DNA was amplified with HotStar Taq Polymerase (Qiagen) (15 min at 94°C followed by 45 cycles of 20 s at 94°C, 30 s at 56°C, and 1 min at 72°C, with a 3-min final extension at 72°C). For MassARRAY EpiTYPER, the reverse primer has a T7 promoter tag for *in vitro* transcription (5′-cagtaatacgactcacta tagggagaaggct-3′), and the forward primer was tagged with a 10-mer to balance the Tm (5′-aggaagagag-3′). The primer sequences for *RORA* were as follows: Rora-1F: aggaagagagTTGT AGAAAAATTAAAGTTAGGGGG and Rora-1R: cagt aatacgactcactatagggagaaggctCAAACAAAACTATTCC AACACCAACA, Tm 56°C; Rora-4F: aggaagagagTGT TGGTGTTGGAATAGTTTTGT and Rora-4R: cagtaata cgactcactatagggagaaggctTTTTTTAATACCATAAAATT ACTCTAA, Tm 56°C (Operon). The PCR products were analyzed by gel electrophoresis and then examined by Sequenom MassARRAY quantitative analysis using the Mass ARRAY Compact System (Sequenom, San Diego, CA), as described previously (*2,16*). The bisulfite-treated DNA was also directly sequenced on an Applied Biosystems 3130xl Genetic Analyzer using a BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems).

2.3. *Quantitative PCR*

The mRNA expression levels were analyzed by quantitative PCR (qPCR). Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA)

and the RNeasy Mini Kit (Qiagen). RNA integrity and quality were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from total RNA using the PrimeScript RT reagent Kit (Takara Bio, Tokyo, Japan). The generated cDNA was amplified on a Thermal Cycler Dice Real-Time System (Takara Bio) using SYBR Premix Ex Taq (Takara). The primer sequences and annealing temperatures for *RORA1* were as follows: HA057856-F: 5′-CAGAGCTATTCCAGCACCAGCA-3′ and HA057856-R: 5′-GGATTCCTGATGATTTGTCTCCAC-3′ (Takara Bio), Tm 63°C. The expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was measured using GAPDH-F: 5′-GCACCGTCAAGGCTGAGAA-3′ and GAPDH-R: 5′-TGGTGAAGACGCCAGTGGA-3′ primers at an annealing temperature of 60°C. *GAPDH* was amplified as a control to normalize the amount of input cDNA. The experiments were performed in triplicate.

2.4. *Statistical analysis*

Data collected from each experiment were statistically analyzed with the Pearson chi-square test and the ANOVA test. *p* values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. *Frequent methylation of the RORA1 promoter in colorectal tissue compared with RORA4*

To determine the methylation status of the *RORA* promoter in CRC, we used the Sequenom MassARRAY method and 43 fresh-frozen CRC surgical specimens, adjacent normal mucosa, four CRC cell lines, and a breast cancer cell line. Among the four different RORA isoforms, only *RORA1* and *RORA4* are actually transcribed, whereas the level of *RORA2* and *RORA3* are undetectable in normal tissue (*8*). Therefore, we focused our investigation on the methylation status of the *RORA1* and *RORA4* promoter regions. Methylation of the *RORA1* promoter was frequently observed in CRC samples and CRC cells (Figure 1). Comparing the epigrams of *RORA1* and *RORA4*, methylation of the *RORA1* promoter was more frequently observed than that of *RORA4* (Figure 2 and Figure 3). These results suggested that methylation of the *RORA1* promoter, but not the *RORA4* promoter, may occur as a distinct alteration in CRC. This finding prompted us to further analyze the methylation status of *RORA1* in CRC.

3.2. *Aberrant hypermethylation of the RORA1 promoter in CRC*

We then evaluated the methylation rate of the *RORA1* promoter in 43 paired CRCs and their adjacent normal mucosa. As shown in (Figure 4A), we defined hypermethylation as an average percent methylation of the entire target region of 38% or more, whereas hypomethylation was defined as less than 38%. Aberrant hypermethylation of the *RORA1* promoter was found in 16 of 43 CRC cases (37%). This finding was consistent with a previous study in which downregulation of *RORA* mRNA expression was frequently observed in CRCs (*6*). Three of the four CRC cell lines, Caco2, HT29, and HCT116, showed significantly increased *RORA1* promoter methylation compared with the MCF7 breast cancer cell line. To validate the *RORA1* promoter methylation data obtained from MassARRAY EpiTYPER, additional direct bisulfite sequencing analysis was performed using representative examples of the same samples. Consistent with the MassARRAY EpiTYPER data, direct bisulfite sequencing analysis clearly detected methylated cytosines in CRC samples. Representative examples of the paired samples of N9 and T9, and N11 and T11 are shown in (Figure 4B). Importantly, hypermethylation of N11 (normal mucosa), which was adjacent to T11 (tumor tissue), was also consistent with the MassARRAY EpiTYPER data showing peaks for both methylated cytosines and uracils at similar levels as T9.

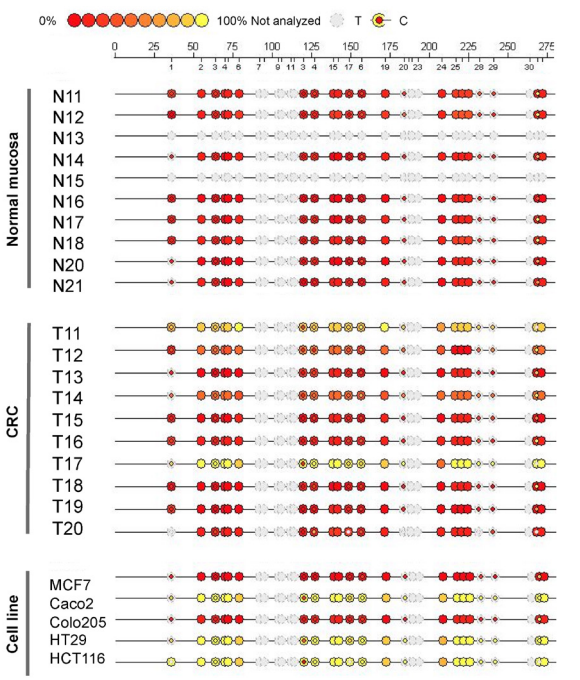

Figure 1. DNA methylation of the *RORA1* promoter region. The EpiTYPER program from Sequenom MassARRAY analysis presents the results of the percent of DNA methylation of the *RORA1* promoter region as an epigram. The epigram shows the percentage of DNA methylation at each CpG site of the target region. Different colors indicate relative methylation changes in 10% increments. The yellow circle indicates 100% methylation, and the red circle is 0% methylation at each CpG site. The number of CpG sites, target sequence length, and sample names are included in each epigram. *T*, primary tumor from a CRC patient; **N**, adjacent normal mucosa.

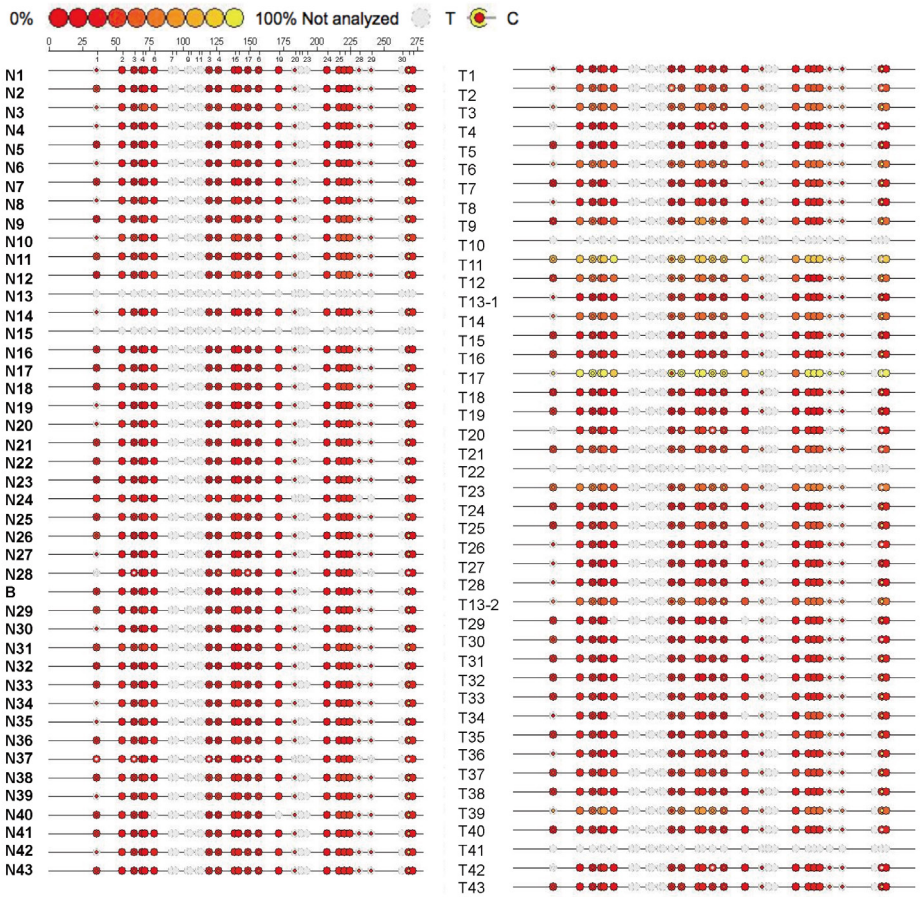

Figure 2. The rate of DNA methylation of the *RORA1* promoter in all CRC cases obtained from Sequenom MassARRAY analysis.

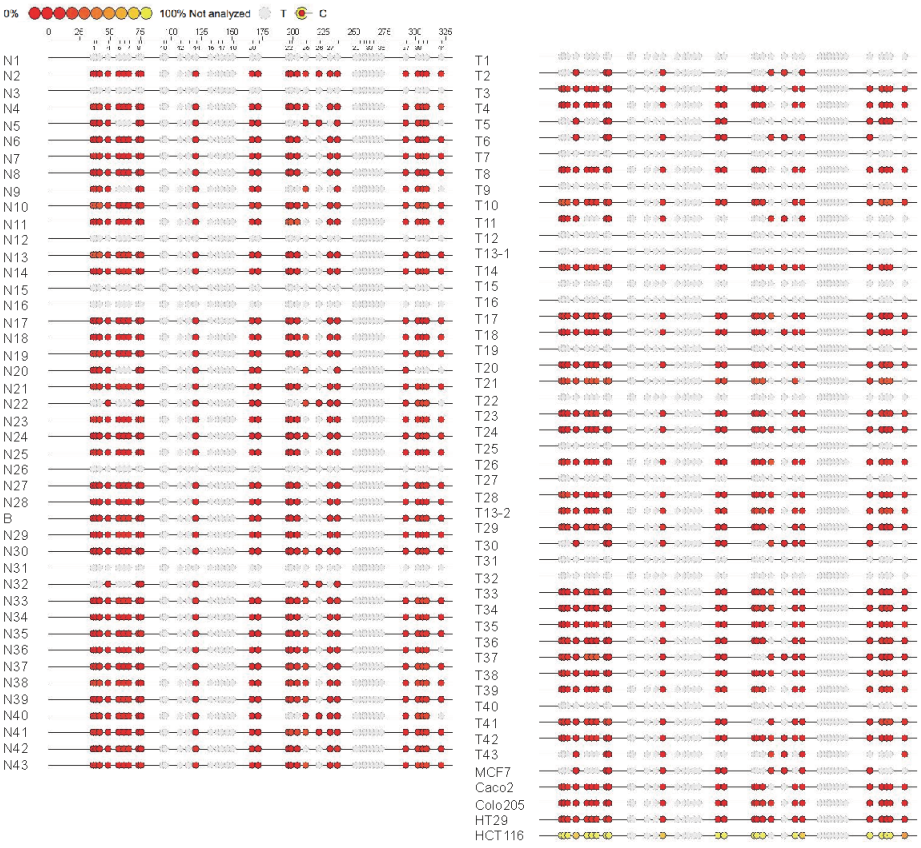

Figure 3. The rate of DNA methylation of the *RORA4* promoter in all CRC cases obtained from Sequenom MassARRAY analysis.

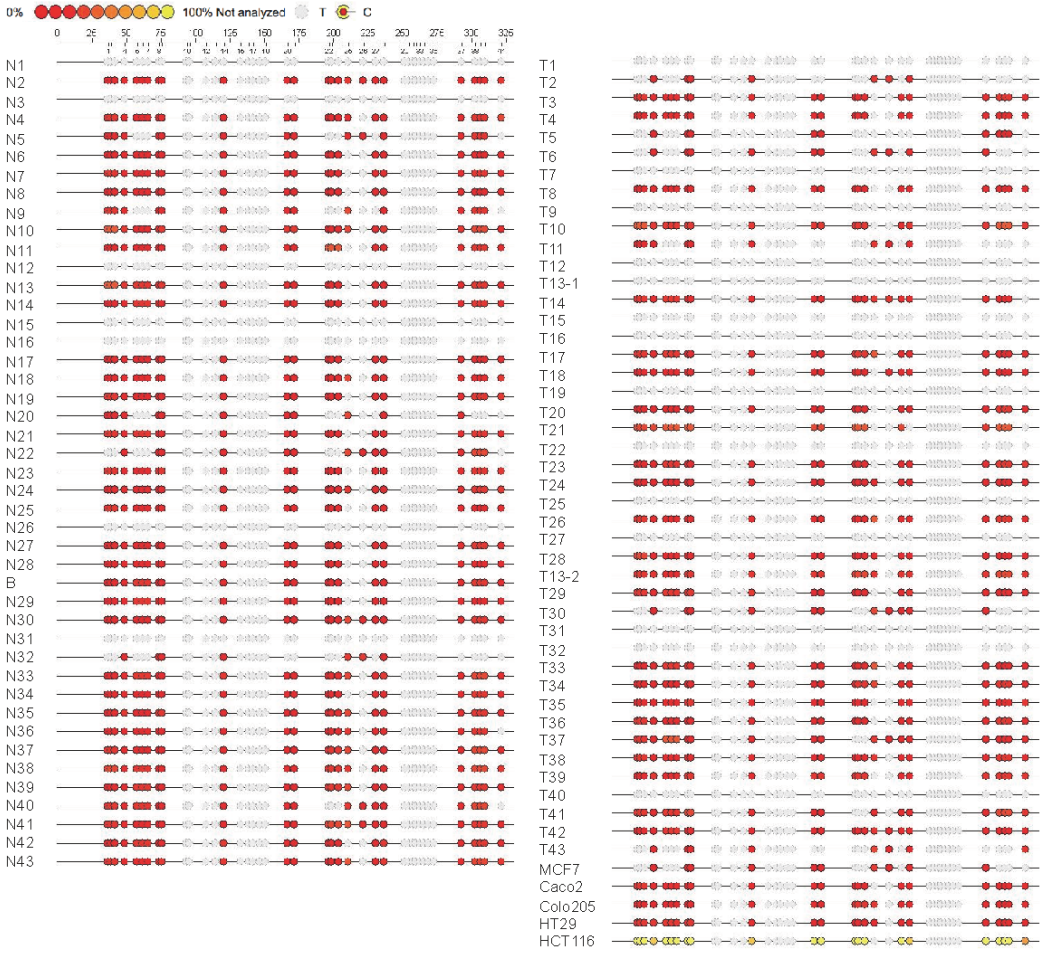

Figure 4. Promoter methylation of *RORA1* in CRC. (A) The y-axis represents the average methylation levels for the entire 354-bp target region of the bisulfite-treated fragment amplified by Rora-1F and Rora-1R primers. Error bars indicate the standard deviation of the mean. The black dashed line shows the average methylation levels for the entire target region of *RORA1*. **(B)** Histogram of bisulfite sequencing analysis depicting the methylation status of CpG sites. Blue arrows indicate the positions of methylated cytosine residues. Red arrows indicate the uracil residues converted from unmethylated cytosines by the bisulfite treatment. *T*, primary tumor from a CRC patient; *N*, adjacent normal mucosa.

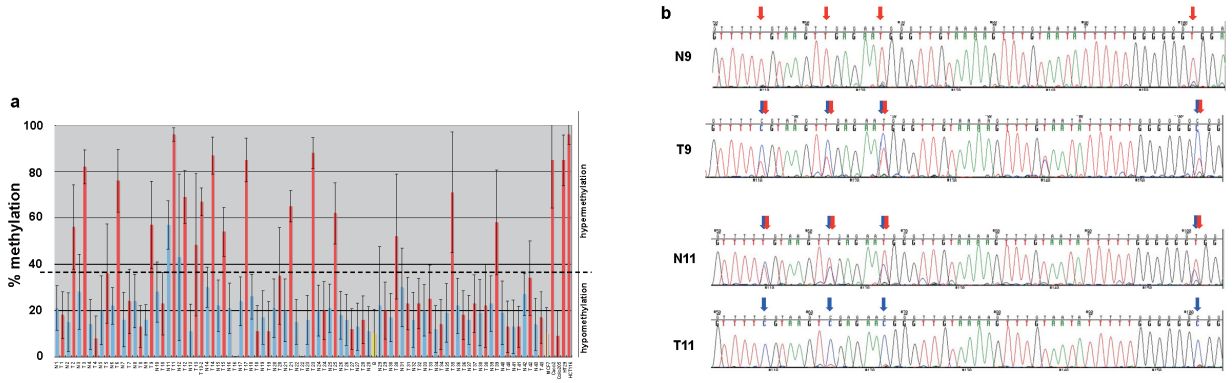

Figure 5. RORA1 mRNA levels in CRC cells and tumor tissues. Quantitative real-time PCR was performed to measure the expression of *RORA1* mRNA. The expression level was normalized to *GAPDH* expression. The y-axis represents the normalized value determined by the standard curve of each gene. The expression level was calculated as the mean of three independent experiments. Error bars indicate the standard deviation of the mean. **(A)** The relative expression of *RORA1* mRNA is shown as a histogram, and the methylation status of each cell line is shown below the graph. **(B)** Relative expression of *RORA1* in primary CRC and adjacent normal mucosa is shown as a histogram. *T*, primary tumor from a CRC patient; *N*, adjacent normal mucosa.

3.3. *Downregulation of RORA1 expression in CRCs*

To determine whether alteration in *RORA1* expression is associated with promoter methylation, we used qPCR to evaluate the level of *RORA1* mRNA in four CRC cell lines and the breast cancer cell line, as well as the 30 CRC surgical specimens and adjacent normal mucosa tissue for which RNA was available. *RORA1* expression

Table 1. Correlation between RORA1 methylation status and clinical CRC stage

Items	RORA1 methylation		*p*-value
	hypermethylation	hypomethylation	
Favorable CRC (*n* = 19)	9	10	0.014
Unfavorable CRC (*n* = 24)	7	17	

CRC, colorectal cancer.

was downregulated in the CRC cell lines (Caco2, colo205, HT29, HCT116) compared with the MCF7 breast cancer cell line (Figure 5A). Of note, although colo205 cells showed hypomethylation of the *RORA1* promoter, *RORA1* expression remained downregulated. Furthermore, low levels of *RORA1* expression were detected in almost every primary CRC compared with the adjacent normal mucosa, although we showed with MassARRY EpiTYPER that only 37% of the CRC samples were methylated (Figure 4B).

3.4. *3.4. Inverse correlation between methylation of the RORA1 promoter and the clinical stage of CRC*

Because promoter hypermethylation of *RORA1* was likely not correlated with its expression, we next assessed whether the methylation status of *RORA1* could be another prognostic indicator of CRC that is independent of gene expression. For this purpose, the 43 CRC patients were divided into two groups according to clinicopathological stage: 19 patients were grouped in favorable stages (stages I and II) and 24 patients in unfavorable stages (stages III and IV). We detected a significant difference between the two groups with respect to the methylation status of the *RORA1* promoter ($p = 0.014$) (Table 1). This result indicated that unfavorable, later stages of CRC were associated with decreased methylation of the *RORA1* promoter compared with favorable, earlier stages of CRC.

4. Discussion

Our study showed that the promoter region of *RORA1* was hypermethylated in CRC patients, and the methylation status of this gene was significantly associated with the clinical CRC stage. These results suggest that the methylation status of the *RORA1* promoter may constitute a useful biomarker to determine an indication for postoperative therapy such as adjuvant chemotherapy in highly advanced CRC patients.

We performed MassARRAY EpiTYPER analysis to quantitatively determine the methylation level at CpG sites in the *RORA1* promoter, which has been identified as a conserved tumor-specific DMR in a mouse model. A previous study showed that *RORA* expression is frequently inactivated in breast, prostate, and ovarian cancer. Notably, *RORA* is located in the middle of FRA15A, a common fragile site in chromosome 15q22.2 (*8*). Common fragile sites are highly unstable, and recombinogenic regions of the genome involve sister chromatid exchange, translocations, deletions, intrachromosomal gene amplification, and integration of DNA from tumor-associated viruses. Thus, *RORA* may behave as a tumor suppressor as described in the "two-hit" hypothesis, and promoter hypermethylation or a loss-of-function mutation coupled with loss of heterozygosity at the same locus may result in loss of tumor suppressive function. We assessed whether *RORA1* promoter methylation could play a causal role in *RORA1* gene silencing in CRCs. To the best of our knowledge, this is the first report describing the methylation status of the *RORA1* promoter in CRCs.

Consistent with a previous study, lower expression of *RORA1* was observed in CRCs compared with adjacent normal mucosa. Interestingly, *RORA1* was clearly downregulated in CRC cell lines compared with the MCF7 breast cancer cell line. Our study shows a slight discrepancy with a previous study that reported that *RORA* expression is frequently downregulated in breast cancer cell lines, including the MCF7 cell line, compared with the normal breast epithelium cell line MCF12F (8). This conflicting result may be due to the different primer sets used in each group. The previous study used primers that were designed to amplify a common region to detect all four isoforms of *RORA*, whereas we designed specific primers to amplify only *RORA1*.

CRC is well-known to result from multiple steps of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium to colon adenocarcinoma. Given the potential for functional involvement of RORA in canonical Wnt/β-catenin signaling, which is a genetic gatekeeper for CRC tumorigenesis, the increased methylation of *RORA1* and thus downregulation of expression may be required in an earlier step of the adenoma-carcinoma sequence. However, the "trigger" and mechanism that decrease *RORA1* methylation as CRC progresses are unknown. Regarding epigenetic alterations during CRC development, *RORA1* should be recognized not as part of the conventional two-hit model of "loss of heterozygosity (LOH) and methylation", but as an atypical tumor suppressor. Interestingly, a recent study using genome-wide single nucleotide polymorphism linkage arrays identified chromosome 15q22 as a novel CRC susceptibility locus (*17*). Additionally, an earlier linkage

study of autism spectrum disorder also demonstrated that chromosome 15q is an "epigenetic hotspot" or region that is susceptible to genomic imprinting that confers a risk for this disorder (*18*). As *RORA* is located on chromosome 15q, *RORA* gene expression likely involves a complex regulatory mechanism for expression, similar to that observed for loss of imprinting of *insulin-like growth factor 2*, a marker of the CpG island methylator phenotype in CRC (*19-21*). Indeed, a recent study revealed that RORA protein is destabilized by methyltransferase-mediated monomethylation of *zeste homolog 2* (*22*), which is frequently overexpressed in CRC (*23,24*). Taken together, we speculate that in favorable, early stages of CRC, *RORA1* expression is downregulated by methylation of CpG islands in the promoter region, and as CRC becomes advanced, along with accumulating genetic and epigenetic alterations in chromosome 15q, the methylation status of the *RORA1* promoter may become hypomethylated. *RORA1* hypomethylation is consistent with the observation of global hypomethylation in CRC from the discovery of large hypomethylated blocks in CRC that corresponds to more than half the genome (*25,26*). Additionally, decreasing *RORA1* expression may be no longer dependent on promoter hypermethylation but may be related to histone modification and/or posttranscriptional degradation in unfavorable CRC. Determining the acetylation and methylation of histone H3 at lysines 9 and 4, respectively, and methylation at lysine 9 in *RORA* promoter regions may provide mechanistic insight into the downregulation of *RORA1* expression in unfavorable, later stages of CRC.

In conclusion, we speculate that the methylation status of the *RORA* promoter may have important implications for prognosis of CRC that is independent of *RORA* expression. Identification of a useful marker for predicting the benefit of adjuvant chemotherapy for CRC patients is important. Therefore, we propose that hypomethylation of *RORA1* may be an attractive prognostic factor to identify patients with advanced CRC who require postoperative chemotherapy.

Acknowledgements

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Original Article

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iTRAQ-based quantitative proteomic analysis reveals potential early diagnostic markers of clear-cell Renal cell carcinoma

Limin Zhang[1,*], Haowen Jiang[1,*], Gang Xu[1,], Nan Chu[2,3,4], Ningxing Xu[5], Hui Wen[1], Bin Gu[1], Jun Liu[1], Shanghua Mao[1], Rong Na[1], Yan Jing[1], Qiang Ding[1], Yuanfang Zhang[1], Ling Wang[2,3,4,**]**

[1] *Department of Urology, Huashan Hospital, Fudan University, Shanghai, China;*
[2] *Laboratory for Reproductive Immunology, Hospital & Institute of Obstetrics and Gynecology, IBS, Fudan University Shanghai Medical College, Shanghai, China;*
[3] *Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, China;*
[4] *The Academy of Integrative Medicine of Fudan University, Shanghai, China;*
[5] *Shanghai Medical College, Fudan University, Shanghai, China.*

Summary **Early detection is the key to improve the prognosis of kidney cancer. This study profiled and identified differentially expressed serum proteins in stage T1a renal cell carcinoma (RCC) using isobaric tags for relative and absolute quantification (iTRAQ)-based mass spectrometry. A total amount of 99 serum samples including 29 patients with ccRCC, 24 patients with a benign kidney mass, 28 patients with another type of urological tumor (20 cases of transitional cell carcinoma and 8 cases of prostate cancer or a male genital tumor), and 18 healthy controls were subjected to iTRAQ-based mass spectrometry. ProteinPilot software was used to identify the differentially expressed serum proteins in RCC compared to the other three populations. Hierarchical clustering analysis according to The Cancer Genome Atlas (TCGA) RCC database was then performed as the cross-platform validation. Immunohistochemistry was performed to verify the expression of selected proteins in tissue samples from these subjects. iTRAQ identified 27 differentially expressed serum proteins in the RCC patients, and 11 of these proteins were cross validated in RCC tissues from the TCGA database. The expression of C1QC, C1QB, S100A8, S100A9, ceruplasmin, and lumican was verified and associated with the tumor stage and/or grade. There were 27 differentially expressed proteins in early-stage RCC identified by iTRAQ; among them, the expression of C1QC, C1QB, S100A8, S100A9, ceruplasmin, and lumican were associated with the tumor stage and/or grade. Further studies are needed to confirm these data for their use as biomarkers for the early detection of RCC.**

Keywords: Renal cell carcinoma, iTRAQ, proteomics, tumor marker, bioinformatics

1. Introduction

Renal cell carcinoma (RCC) is a significant health problem in adults, accounting for more than 100,000 worldwide cancer-related deaths each year (*1*), and is the most lethal of the common urological cancers (*2*). Clear-cell type compromises about 80% of RCCs. With the improvement of surgical techniques in radical and partial nephrectomy, the postoperative overall 5-year survival of patients with organ-confined disease has increased to 97.9% in stage T1aN0M0, 94.9% in stage T1bN0M0, and 88.4% in stage T2N0M0 (*3*). Favorable prognosis relies on the acute early diagnosis of RCC (5-year survival rate-85%). Unfortunately, RCC often presents with few signs, symptoms, or laboratory abnormalities; thus, RCC is frequently only diagnosed at the advanced stage of the disease, when the prospects for a cure are dismal (a five-year survival rate of 9%) (*4*). Early diagnosis of RCC is usually based on traditional manifestations such as pain,

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*These authors contributed equally to this works.
**Address correspondence to:
Dr. Gang Xu, Department of Urology, Huashan Hospital, Fudan University, 12 Wulumuqi Middle Road, Shanghai 200040, China.
E-mail: drxugang@163.com

Dr. Ling Wang, Obstetrics & Gynecology Hospital of Fudan University, 413 Zhaozhou Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

mass and hematuria, which are not always effective. Diagnosis and subsequent resection of the RCC are also not accurate because it only relies on the basis of imaging findings. The absence of biomarkers in early detection of RCC in clinical practices has complicated the early diagnosis and treatment of RCC. A non-invasive test in serum or urine will have a significant impact on patient management.

To date, many researchers have been dedicated to the identification of potential biomarkers for the early detection of RCC using different proteome-based techniques, including the newly emergent mass spectrometry method using isobaric tags for relative and absolute quantification (iTRAQ) (*5,6*). iTRAQ-based mass spectrometry, first introduced by Ross *et al*. (*7*) in 2004, is a technique of multiplexed protein quantitative mass spectrometry using amine-reactive isobaric tagging reagents. The advantage of iTRAQ is that it enables quantitation of multiple samples simultaneously but requires only a small amount of sample. It is utilized frequently in research of many other malignant diseases to investigate their unique proteomic profile (*8-12*) and thus provide a new method to explore the biomarkers in malignant tumors.

In this study, we used quantitative proteomic analysis by iTRAQ-based proteomic identification technology to identify proteins that are dysregulated in the serum and LC-MS of T1a clear-cell RCC (ccRCC) and healthy people. Bioinformatics analysis and cross-platform validation according to the Cancer Genome Atlas (TCGA) RCC database, which catalogs genetic mutations as well as the gene/microRNA expression/regulation responsible for renal cancer, were also used in this study. To the best of our knowledge, this study is the first to highlight that T1a stage ccRCC accounts for 70-80% of all RCCs and always has a worse prognosis compared with other histological types of RCC (*13*). Profiling of the differentially expressed serum proteins in the mentioned patient populations will help us to understand the etiology of ccRCC and even provide a new strategy for the early detection of ccRCC.

2. Materials and Methods

2.1. *Study population and serum samples*

The subjects included in this study were from the Department of Urology at Huashan Hospital, Fudan University from January 2010 to December 2015. A total number of 99 serum samples were obtained including 29 patients with ccRCC, 24 patients with a benign kidney mass, 28 patients with another type of urological tumor (20 cases of transitional cell carcinoma and 8 cases of prostate cancer or a male genital tumor), and 18 healthy controls. Patients with accompanying kidney diseases, cardiovascular disorders, or other cancers were excluded. All ccRCC patients had stage T1a cancer as confirmed by radiological evaluation and pathological assessment of the surgical specimens. Sera from each of these four groups of subjects were pooled and abbreviated as R for ccRCC, C for benign kidney masses, M for other urological tumors, and H for noncancer controls. The serum samples were preserved at -76°C before iTRAQ analysis. This study was approved by the Institutional Review Board of Huashan Hospital, Fudan University, China (#KY2011-026, version 01.2011.1.12 and #2011-017), and written consent was obtained from each participant before enrollment into this study. The clinical information and data were obtained from industrial or hospital records. The clinical characteristics of the subjects in this study are shown in Table S1 (*http://biosciencetrends.com/docindex.php?year=2016&kanno=3*).

2.2. *iTRAQ labeling and nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC-MS/MS)*

For iTRAQ analysis, 200 μL of each serum sample was dissolved and digested in a trypsin buffer (2 μg of trypsin in 40 μL of dissolution buffer) in a 37°C water bath for 16-18 h, then centrifuged, collected, and quantified by optical density (OD) measurements at an absorbance of 280 nm. Based on the OD_{280} values, an equivalent amount of peptide in each sample was subjected to iTRAQ labeling, according to the iTRAQ protocol (Applied Biosystems/MDS Sciex, Foster City, CA, USA). The samples were marked with iTRAQ tags as follows: iTRAQ117 for R, iTRAQ116 for C, iTRAQ115 for M, and iTRAQ114 for H. In order to obtain reliable results, the iTRAQ labeling experiment was replicated with the iTRAQ tags rearranged as follows: iTRAQ115 for R, iTRAQ117 for C, iTRAQ116 for M, and iTRAQ114 for H.

Next, the iTRAQ-labeled samples were pooled and fractionated by strong cation exchange chromatography on a polysulfoethyl 4.6 × 100 mm column (5 μm, 200 Å) (PolyLC Inc., Columbia, MD, USA) with a linear gradient of 0-500 mM KCl (10 mM KH_2PO_4, pH 3.0; 25% acetonitrile) over 75 min at a flow rate of 1 mL/min. According to the chromatography results, the collected fractions were recombined into 10 fractions and then freeze-dried. After that, each of the freeze-dried fractions from the SCX column was redissolved in 100 μL of 0.1% formic acid aqueous solution and then desalted using Vydac C18 supermicro-centrifugation chromatography. The sample was then extracted and analyzed by nano-LC-MS/MS. The nano-LC-MS/MS was completed by a system composed of an AB SCIEX Triple Time-of-Flight (TOF) 5600 mass spectrometer (Concord, Canada) and a liquid chromatograph with a cHiPLC nanoflex chip driven by an Eksigent nanoUltra 2D Plus nano-LC (Dublin, CA, USA). Each sample was run through a sampling course and subsequent separation with tandem MS analysis. The mode of

tandem MS was information-dependent acquisition (IDA). The resolving power of the screening performed by TOF MS was a full width at half maximum of 30,000, and the range was *m/z* 350-1250 in 250 ms. The top 30 abundant peptides with an ion peak greater than 120 counts/s and a range of charge from +2 to +5 were chosen to be analyzed by MS/MS with a range of *m/z* varying from 100 to 1800 in 100 ms for each TOF MS/MS screening time. The dynamic exclusion time was 18 s. When MS/MS was performed, the functions of enhanced iTRAQ splitting and auto-calculation of collision energy (AutoCE) were launched.

2.3. *Calculation of the ratios of differentially expressed proteins*

ProteinPilot (AB SCIEX, Framingham, MA, USA, 2012) software was then used to analyze the raw data. The signaling of the iTRAQ114 group served as the internal reference for the signal intensity, and all signals were normalized to it. The weighted average of the ratios of the respective peptides was calculated based on the protein quantitation results. False discovery rate analysis was conducted, and the detected protein threshold was set at less than 0.01. The proteomic database used in this study was the International Protein Index (IPI), Human v3.87. fasta (*http://www.ebi.ac.uk/*, Copyright© The European Bioinformatics Institute, 2013). Finally, the ratios of proteins of these four groups of samples with different iTRAQ tag labels were calculated as the averages of the ratios from two runs with different labeling sequences.

2.4. *Integration and hierarchical clustering analysis of iTRAQ data against the TCGA database on RCC*

The TCGA database catalogs genetic mutations as well as the gene/microRNA expression/regulation responsible for cancer risk and development using recently developed high-throughput genomic analysis techniques that were initiated in 2005. TCGA provides genomic characterization and sequence analysis of more than 20 different tumor types. In this study, the gene/microRNA expression pattern and clinical information of more than 500 patients with kidney renal clear-cell carcinoma (KIRC) were downloaded from the TCGA data portal (*https://tcga-data.nci.nih.gov/tcga/*).

For cluster profiling, the median expression value of each protein across the samples was set to zero. Cluster 3.0 and Tree View software (*http://rana.lbl.gov/EisenSoftware.htm*) were used for the cluster analysis and representation (*14*). The hierarchical clustering was performed on both genes and samples. Using a tree algorithm, these differentially expressed proteins were organized based on similarities in the expression profile. This allowed us to visualize and select genes based on individual expression profiles.

2.5. *Cross-platform validation vs. the TCGA database on RCC*

In the TCGA KIRC database, there are 31 normal control, 197 T1, 49 T2, 162 T3, and 6 T4 samples as well as 31 normal control, 5 G1, 173 G2, 169 G3, and 66 G4 samples. In this database, the expression levels of certain genes in these different grades or stages of KIRC were measured. The Student's *t*-test and analysis of variance were performed to statistically analyze the data using a cutoff of $p < 0.05$ and a fold change > 1.5 between different groups with different grades or T stages.

2.6. *Gene Ontology (GO) analysis*

The differentially expressed serum proteins in RCC were classified according to the GO category, including "biological process," "cell component," and "molecular function," respectively, by using the US National Institutes of Health (NIH) gene annotation software DAVID 6.7 (*15*) (*http://david.abcc.ncifcrf.gov/*; *http://david.abcc.ncifcrf.gov/gene2gene.jsp*). The differentially expressed protein list selected by ProteinPilot software was inputted into NIH DAVID 6.7 and thought to be significantly enriched if the *p* value was less than 0.05 using Fisher's exact test and the fold enrichment was more than 2-fold.

3. Results

3.1. *iTRAQ-based mass spectrometry profiling of the differentially expressed serum proteins from ccRCC patients*

In this study, we profiled differentially expressed serum proteins in 99 serum samples including 29 patients with stage T1a ccRCC, 24 patients with a benign renal mass, 28 patients with another type of urological tumor (18 for TCC), and 18 healthy controls using iTRAQ-based mass spectrometry. The basic clinical characteristics of subjects were collected as supporting information (Supplemental Data Table S1, *http://biosciencetrends.com/docindex.php?year=2016&kanno=3*)

Overall, 263 serum proteins were identified after two runs of iTRAQ and validated the ratios of proteins from both runs by a scatter diagram with ratios in the first run on the x-axis and ratios in the replicated run on the y-axis (Figure 1). The results from the two runs were consistent (Supplemental Data Table S2, *http://biosciencetrends.com/docindex.php?year=2016&kanno=3*). Using cutoff values of 1.5-fold for overexpression and 0.67-fold for underexpression of a protein, 74 differentially expressed proteins were identified in ccRCC vs. healthy controls (Table 1), with the identification of another 27 proteins (4 underexpressed and 23 overexpressed in ccRCC vs. the other three groups) according to the data from the TCGA database (Table 2; Figure 1).

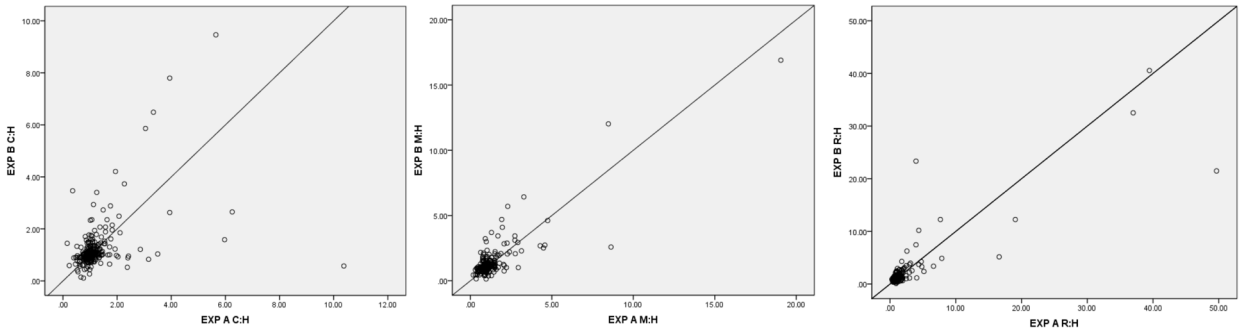


Figure 1. iTRAQ identification and validation of differentially expressed serum proteins in RCC. In this study, we first poured serum samples together from each of these four groups of subjects and performed the iTRAQ analyses two times. A total of 263 proteins after two iTRAQ runs were identified and validated, and the data were plotted on graphs. The x-axis indicates the value of the ratios in the first run, while the y-axis refers to those of the second run. Grouping of the differentially expressed proteins in this study. Note: R indicates patients with ccRCC, C indicates patients with a benign kidney mass; M indicates patients with another type of urological tumor; and H indicates noncancer controls.

Table 1. Differentially expressed serum proteins in ccRCC *vs.* healthy controls

IPI Serial No.	Gene symbol	Fold change for R:H	IPI Serial No.	Gene symbol	Fold change for R:H
IPI00290078.5	*KRT4 Cdna FLJ58275*	39.99825	IPI00329775.8	*Cpb2*	1.7606
IPI00009866.7	*Krt13*	35.56875	IPI00022392.1	*C1qa*	1.73835
IPI00022389.1	*Crp*	34.74575	IPI00010295.1	*Cpn1*	1.73765
IPI00909059.5	*KRT6A Cdna FLJ53910*	15.6504	IPI00021885.1	*Fga*	1.7024
IPI00643948.3	*C1qb*	13.6396	IPI00829636.2	*Ighd*	1.6924
IPI00218918.5	*Anxa1*	10.8741	IPI00879573.1	*Serpind1*	1.67605
IPI00783987.2	*C3*	9.9511	IPI00879709.3	*C6*	1.67525
IPI00022394.2	*C1qc*	7.27555	IPI00006114.5	*Serpinf1*	1.6597
IPI00025426.3	*Pzp*	6.3729	IPI00027235.1	*Atrn*	1.637
IPI01010737.1	*A2m*	5.69595	IPI00796990.4	*CFI Cdna FLJ58124*	1.62265
IPI00022974.1	*Pip*	4.9899	IPI00216065.3	*Proz*	1.6131
IPI00007047.1	*S100a8*	4.4943	IPI00914948.1	*Apol1*	1.5716
IPI01025667.1	*SERPINA3 Cdna FLJ35730 Fis*	4.40515	IPI00298828.3	*Apoh*	1.5514
IPI00641737.2	*Hp*	4.0558	IPI00023014.3	*Vwf*	1.53935
IPI00009867.3	*Krt5 Keratin*	4.03175	IPI00974055.1	*Crisp3*	1.52375
IPI00218407.6	*Aldob*	3.76455	IPI00032220.3	*Agt*	1.5207
IPI00022395.1	*C9*	3.5246	IPI00964994.1	*Habp2*	1.51505
IPI00027462.1	*S100a9*	3.33155	IPI00925621.1	*Il1rap*	0.65685
IPI00939824.1	*Cfb*	3.0278	IPI00479116.2	*CPN2 Carboxypeptidase N Subunit 2*	0.6502
IPI00029739.5	*Cfh*	2.9667	IPI00410333.2	*Treml1*	0.6474
IPI00553177.1	*Serpina1*	2.9385	IPI00296176.2	*F9*	0.64315
IPI00022417.4	*Lrg1*	2.6462	IPI00925635.1	*Igfals*	0.631
IPI00743766.2	*Fetub*	2.6123	IPI00010779.4	*Tpm4*	0.615
IPI00032291.2	*C5 Complement C5*	2.583	IPI00645849.1	*Ecm1*	0.6088
IPI00017601.1	*Cp*	2.52705	IPI00021856.3	*Apoc2*	0.60815
IPI00060800.5	*Zg16b*	2.3912	IPI00293925.2	*Fcn3*	0.58375
IPI00552768.1	*Txn*	2.2411	IPI00328609.3	*Serpina4*	0.57975
IPI00896380.1	*Ighm*	2.21655	IPI00021304.1	*Krt2 Keratin*	0.5755
IPI00019038.1	*Lyz*	2.1762	IPI00643348.4	*COMP Cdna FLJ60724*	0.56795
IPI00007240.2	*F13b*	2.0814	IPI00026314.1	*Gsn*	0.56025
IPI00023019.1	*Shbg*	2.07155	IPI00021854.1	*Apoa2*	0.559
IPI00019580.1	*Plg*	2.0611	IPI00647915.1	*Tagln2*	0.5075
IPI00021841.1	*Apoa1*	2.0512	IPI00940723.2	*Tnxb*	0.49205
IPI00953689.1	*Ahsg*	2.046	IPI00020986.2	*Lum*	0.48095
IPI00296608.6	*C7*	2.02405	IPI00163207.1	*Pglyrp2*	0.4501
IPI00218732.4	*Pon1*	1.9846	IPI00296099.6	*Thbs1*	0.43985
IPI00021727.1	*C4bpa*	1.87715	IPI00022446.1	*Pf4*	0.3987

R: patients with ccRCC; H: noncancer controls.

3.2. *Cross-platform analysis of the TCGA database vs. our differentially expressed proteins*

To compare these 27 differentially expressed serum proteins, we downloaded the kidney tissue gene expression patterns from 31 normal controls and 205 patients with T1 ccRCC from the TCGA data portal (*https://tcga-data.nci.nih.gov/tcga/*). We first analyzed the 27 gene expression patterns in the 30 normal controls and the 202 patients with T1 ccRCC, 1 normal control and 3 patients with T1a ccRCC (Figure 2A). Hierarchical and heat map analyses of 27 gene expression patterns showed that 9 (*C1qc, C1qb, Anxa1, Lyz, Cp, Agt, Zg16b, S100a8, And S100a9*) were upregulated and 2 (*Serpina4 And Lum*) were downregulated in both serum and tissue samples (Table 1). These 11 genes were selected for

Table 2. iTRAQ identification of differentially expressed serum proteins in RCC *vs.* the TCGA database

IPI serial No.	Proteins	Fold Ratios for differentially expressed proteins			Genes significantly increased/decreased in RCC tissues (TCGA)						Tissue genes	Serum proteins
					Normal Control		T1 RCC		T test			
		R:H	R:C	R:M	mean	standard deviation	mean	standard deviation	*p*	FC		
IPI00022394	C1QC	7.27555	2.213129	2.682626	15.24679	11.88908	134.6874	105.5283	1.59E-09	8.833825	up	up
IPI00643948	C1QB	13.6396	2.323038	2.81	20.60832	15.42208	196.4306	158.2369	2.96E-09	9.531614	up	up
IPI00218918	ANXA1	10.8741	9.902199	5.510198	42.0473	17.41306	103.3125	57.49486	1.39E-08	2.457055	up	up
IPI00019038	LYZ	2.1762	1.849881	1.518473	25.78253	22.49118	162.1104	131.2444	2.63E-08	6.287605	up	up
IPI00017601	CP	2.52705	1.936363	3.053099	4.599597	3.137652	165.0615	257.5653	0.000636	35.88608	up	up
IPI00032220	AGT	1.5207	2.268178	2.98821	14.15519	14.82322	34.54053	36.61214	0.002489	2.440132	up	up
IPI00060800	ZG16B	2.3912	2.046472	2.236962	0.160283	0.173884	0.410872	0.498079	0.00603	2.563412	up	up
IPI00007047	S100A8	4.4943	4.060075	2.269848	6.23674	6.064987	23.22495	34.21335	0.006364	3.723893	up	up
IPI00027462	S100A9	3.33155	3.610067	2.434098	13.1029	11.65109	36.49804	50.11201	0.010362	2.785493	up	up
IPI00328609	SERPINA4	0.57975	0.415815	0.534455	3.579333	3.009381	0.350075	1.142392	4.00E-23	0.097804	down	down
IPI00020986	LUM	0.48095	0.544061	0.632996	99.6874	75.92487	38.25532	64.23287	2.35E-06	0.383753	down	down
IPI00022389	CRP	34.74575	18.17294	1.93252	0.065947	0.127943	10.21652	91.41613	0.5377624	154.9205	up	up
IPI00009866	KRT13	35.56875	74.85006	64.85914	0.117634	0.344086	1.591729	18.09628	0.6511775	13.53121	up	up
IPI00290078	KRT4	39.99825	49.96658	54.30855	0.009406	0.015682	0.054731	0.167793	0.1348779	5.818911	up	up
IPI00909059	KRT6A	15.6504	2.858338	14.81905	0.048689	0.217767	0.248698	1.004503	0.2712461	5.107877	up	up
IPI00022417	LRG1	2.6462	2.094756	2.142672	2.409539	2.371319	7.391328	15.39787	0.0739449	3.067528	up	up
IPI00009867	KRT5	4.03175	2.778984	6.042791	0.314106	1.006641	0.520658	1.560287	0.4758168	1.657587	up	up
IPI00022974	PIP	4.9899	2.956102	3.536804	0.063886	0.0992	0.086039	0.292671	0.6769375	1.34676	up	up
IPI00552768	TXN	2.2411	3.140335	2.834862	197.7238	82.62611	207.598	107.3115	0.6242722	1.049939	up	up
IPI00021854	APOA2	0.559	0.611966	0.444427	1.883181	9.391258	1.823045	22.01121	0.988057	0.968066	down	down
IPI00218407	ALDOB	3.76455	4.097023	1.634345	1380.982	1424.919	64.59174	254.2692	1.27E-26	0.046772	down	up
IPI00743766	FETUB	0.4501	0.371523	0.524042	0.231238	0.197704	0.045323	0.237875	4.88E-05	0.196001	down	up
IPI00218732	PON1	1.9846	0.403858	0.424259	0.235652	0.507248	0.051987	0.168255	9.61E-05	0.220607	down	up
IPI00953689	AHSG	2.046	0.665561	0.51198	1.873805	9.962775	0.229014	2.373435	0.0432425	0.122219	down	up
IPI00021841	APOA1	2.0512	0.542983	0.36589	1.162947	4.175419	0.414824	2.427988	0.1541711	0.356701	down	up
IPI01025667	SERPINA3	4.40515	2.090275	1.718748	14.25598	22.14037	12.79282	27.03231	0.7743703	0.897365	down	up
IPI00163207	PGLYRP2	0.4501	0.371523	0.524042	0.010583	0.031642	0.210003	0.579237	0.0569706	19.84334	up	down

There were 4 downregulated and 23 upregulated serum proteins in T1a stage RCC, the top 11 of which were consistent with the tissue data from the TCGA database. R: T1a RCC; H: healthy subjects; C: patients with a benign mass lesion in the kidney; M: patients with another type of malignant urological tumor.

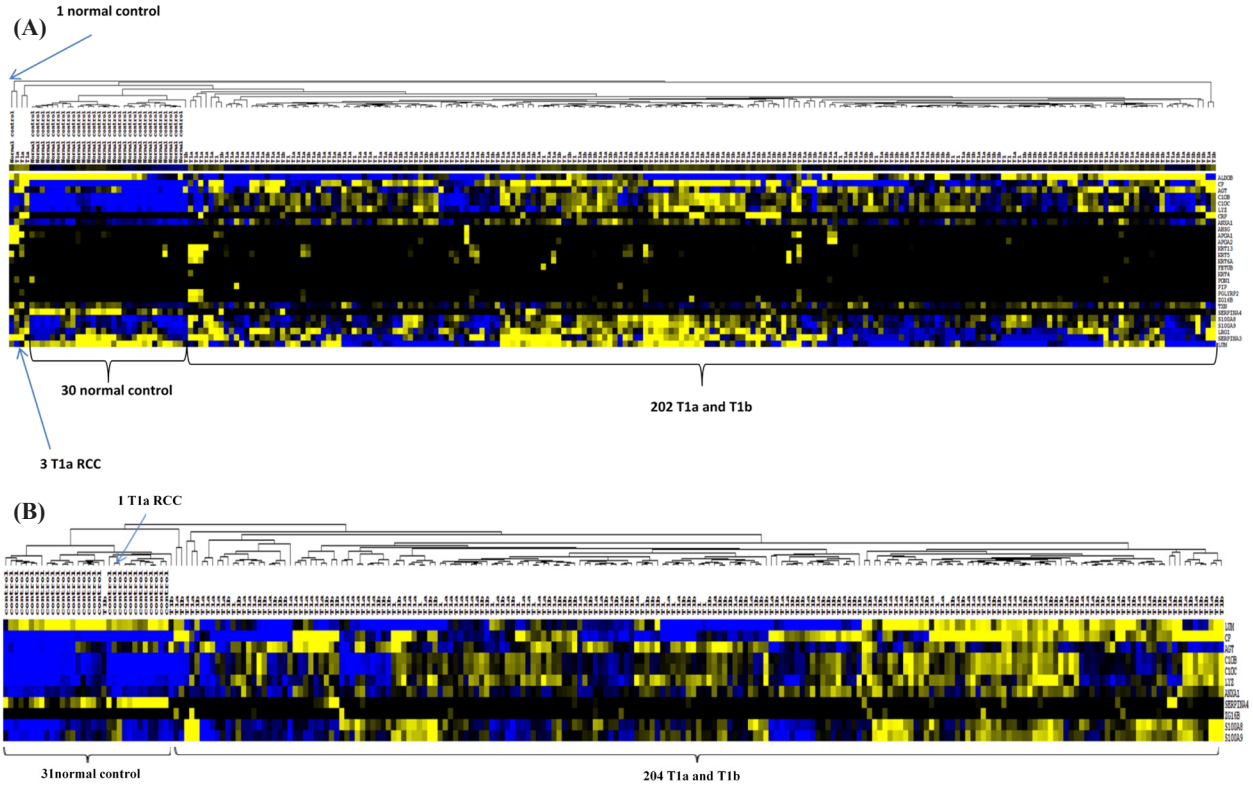


Figure 2. Analysis of differentially expressed serum proteins in RCC. (A), Hierarchical analysis of the 27 genes against data from the TCGA database including 31 normal controls and 205 patients with T1 RCC. The data indicate that the expression pattern of these 27 genes can distinguish normal controls from those with T1 ccRCC. **(B)**, Hierachical analysis of the selected 11 genes against data from the TCGA database including 31 normal controls and 205 patients with T1 RCC. The data indicate that the expression pattern of these 11 genes can distinguish the normal controls from patients with T1 RCC.

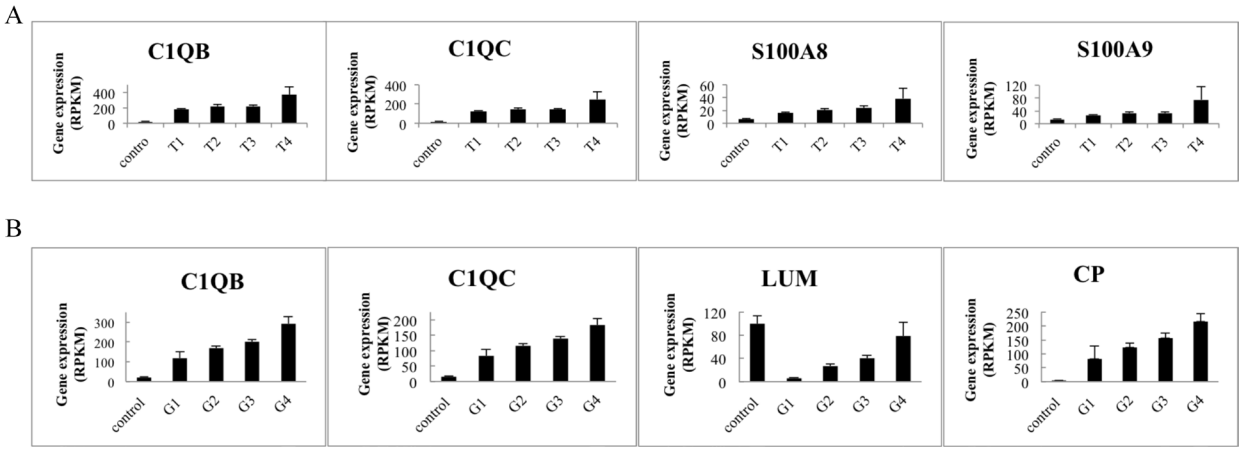
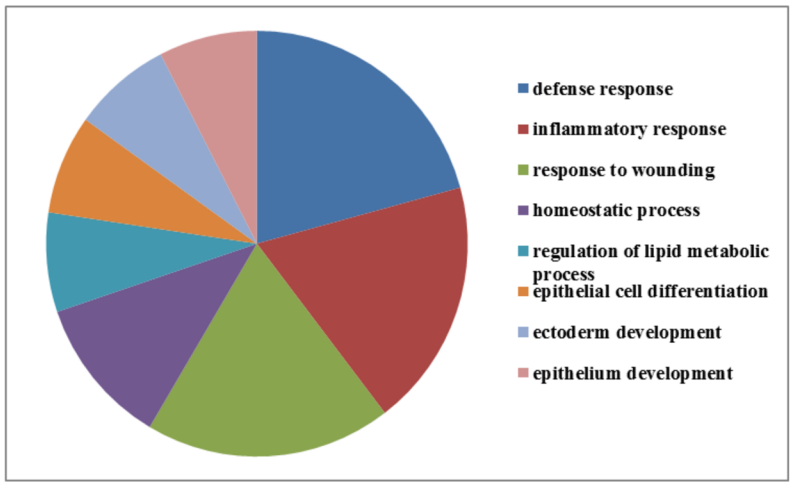


Figure 3. Association of different protein expression with the RCC stage and grade. (A), Association of C1QC, C1QB, S100A8, S100A9, and LUM expression with the RCC stage. We selected these differentially expressed serum proteins for association with the RCC stage using data from the TCGA database including 31 normal controls and 205 patients with T1 RCC. **(B)**, Association of C1QC, C1QB, CP, and LUM expression with the RCC grade. We selected these differentially expressed serum proteins for association with the RCC grade using data from the TCGA database including 31 normal controls and 205 patients with T1 RCC.

further analyses. Hierarchical analysis of 11 genes in the 31 normal controls and 205 patients with T1 RCC from the TCGA database indicates that the expression pattern of the 11 proteins can better distinguish normal controls from T1a RCC (Figure 2B).

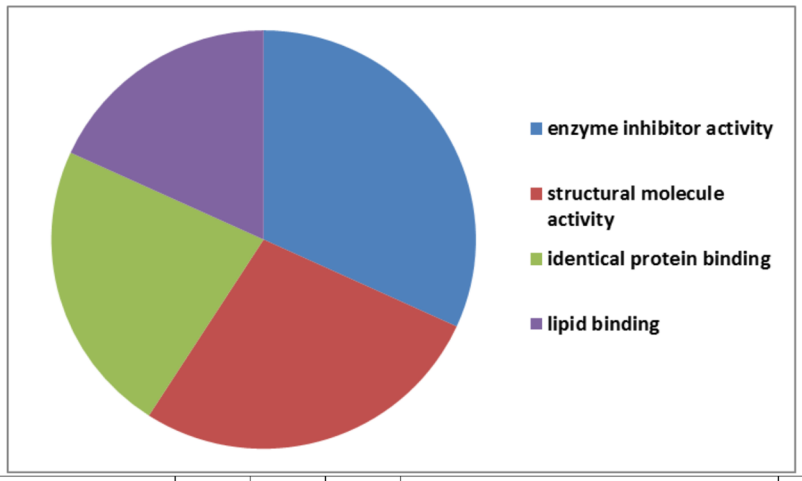
3.3. *Association of selected proteins with the tumor stage and grade in comparison to the TCGA database*

Next, we selected 11 proteins from these 27 differentially expressed serum proteins for confirmation



Category	Term	Count	%	PValue	Genes	Fold Enrichment	FDR
GOTERM_BP	defense response	11	40.74074	1.38E-08	C1QB, APOA2, S100A8, PGLYRP2, CRP, S100A9, ANXA1, SERPINA3, LYZ, C1QC, AHSG	10.52018381	2.07E-05
GOTERM_BP	inflammatory response	10	37.03704	9.02E-10	C1QB, APOA2, S100A8, CRP, S100A9, ANXA1, SERPINA3, LYZ, C1QC, AHSG	18.09765886	1.35E-06
GOTERM_BP	response to wounding	10	37.03704	6.41E-08	C1QB, APOA2, S100A8, CRP, S100A9, ANXA1, SERPINA3, LYZ, C1QC, AHSG	11.097621	9.6E-05

Figure 4. Gene ontology (biological process) analysis of the differentially expressed serum proteins in ccRCC *vs.* that of NIH DAVID. We performed gene ontology analysis to group the functions of these differentially expressed serum proteins in ccRCC using data from the NIH DAVID database.



Category	Term	Count	%	PValue	Genes	Fold Enrichment	FDR
GOTERM_MF	enzyme inhibitor activity	7	25.92593	5.74E-06	APOA2, FETUB, AGT, SERPINA4, ANXA1, SERPINA3, AHSG	14.02485	0.006241
GOTERM_MF	structural molecule activity	6	22.22222	0.004416	KRT6A, KRT5, LUM, ANXA1, KRT13, KRT4	5.119479	4.701003
GOTERM_MF	identical protein binding	5	18.51852	0.024534	C1QB, APOA2, APOA1, ALDOB, PON1	4.226237	23.67922
GOTERM_MF	lipid binding	4	14.81481	0.043804	APOA2, APOA1, ANXA1, PON1	4.808519	38.57092

Figure 5. The molecular functions of the differentially expressed serum proteins identified by the iTRAQ technique. They are mainly categorized into four classes: 1) enzyme inhibitor activity, 2) structural molecule activity, 3) identical protein binding, and 4) lipid binding.

and association with the clinicopathological data from the TCGA database. Our data showed that the expression of C1QC, C1QB, S100A8, and S100A9 proteins was significantly increased in RCC and associated with an advanced stage of disease ($p < 0.01$; Figure 3A; Supplemental Data Table S3, *http://biosciencetrends.com/docindex.php?year=2016&kanno=3*). The expression of ceruplasmin (CP) was also significantly increased, whereas lumican (LUM) was significantly decreased in the RCC samples vs. the controls ($p < 0.01$; Figure 3B; Supplemental Data Table S4, *http://biosciencetrends.*

com/docindex.php?year=2016&kanno=3).

3.4. *Identification of the functions of these differentially expressed serum proteins in ccRCC*

After that, we employed NIH DAVID 6.7 software (http://david.abcc.ncifcrf.gov/home.jsp) to assess the potential functional categories of these 27 differentially expressed serum proteins in early ccRCC. The cell component analysis in GO by NIH DAVID (Supplemental Data Figure S1, *http://biosciencetrends.com/docindex.php?year=2016&kanno=3*) showed that S100A8, S100A9, LUM, ZG16B, C1QC, C1QB, SERPINA4, and CP proteins are secreted proteins and that LUM, LYZ, AGT, ZG16B, C1QB, CP, and SERPINA4 proteins are found in the extracellular region. Biological process analysis presented that most of these 11 genes are involved in the host defense response, inflammatory response, and the response to wounding, indicating that the identified proteomic expression pattern might reflect the early change in the serum microenvironment of ccRCC (Figure 4). As for the molecular functions of the differentially expressed serum proteins, they might be involved in four kinds of biological processes including enzyme inhibitor activity, structural molecule activity, identical protein binding, and lipid binding (Figure 5).

4. Discussion

The treatment and prognosis of ccRCC largely depends on the tumor stage and the pre-existing conditions of the patients. In other words, surgery is still the best option for treating these patients, if diagnosed at an early stage. The lack of biomarkers to distinguish the malignant and benign lesions is the biggest challenge in ccRCC. Imaging has limited accuracy and might lead to unnecessarily surgery. Biopsy is a kind of accurate detection method for ccRCC but the invasive procedure and other side effects might limit its use. A non-invasive test such as urine or a blood-based test might provide a new idea for RCC diagnosis. In the current study, we performed proteomics analysis by using iTRAQ and several proteins have been identified as promising biomarkers for the accurate diagnosis of ccRCC although further investigation is still needed. In our study, we identified 27 differentially expressed serum proteins, 11 of which were cross-validated in RCC tissues against the TCGA database. Moreover, we found that expression of C1QC, C1QB, S100A8, S100A9, CP, and LUM proteins was associated with the RCC stage and/or grade. Of the dysexpressed proteins, 8 of them including S100A8, S100A9, LUM, ZG16B, C1QC, C1QB, SERPINA4, and CP proteins were secreted proteins and thus have the potential to be used as a serum biomarker in the diagnosis of ccRCC, especially in the early stage.

iTRAQ technology, the proteomic technology that we chose to use in this study, has been utilized frequently for the assessment of differentially expressed proteins in many other diseases (*8-12*), and previous publications have shown promising findings in the field of oncologic proteomic research. In RCC, to date, there have been two pioneering studies using the iTRAQ technique: one was related to the von Hippel-Lindau gene in RCC cells (*5*), and the other one investigated gene profiling in RCC tissues at different stages (*6*). To the best of our knowledge, our current study is the first to highlight differentially expressed serum proteins from patients with stage T1a disease, since these patients are usually diagnosed during a health check-up. Moreover, we also utilized the IPI human proteomic database to identify certain differentially expressed proteins and the NIH DAVID database to functionally analyze these differentially expressed serum proteins. The TCGA KIRC database was used for cross-platform validation of the selected serum proteins at the relevant genetic level in a totally different population. Thus, we identified 27 differentially expressed serum proteins in ccRCC patients vs. healthy controls, those with benign renal masses, and those with another type of urological tumor. Four proteins were underexpressed and 23 were overexpressed. Hierarchical analysis showed that these 27 genes could constitute a proteomic expression pattern that is able to distinguish T1 ccRCC from normal controls. However, the regulation of these 27 proteins in the tissue samples was not exactly consistent with the levels of serum proteins measured by the iTRAQ test. This might be attributed to the possibility of post-transcriptional modifications and the inherited inaccuracy of this protein quantification.

In addition, we found that the expression of the 11 differentially expressed serum proteins (C1QB, C1QC, ANXA1, LYZ, CP, ACT, ZG16B, S100A8, S100A9, SERPINA4, and LUM) was associated with the RCC tumor stage and grade. Functionally, C1QB and C1QC are complement subcomponent subunits (*16*) and are related to antibody-dependent and -independent immune responses in the human body (*17*). They are able to induce apoptosis of prostate cancer cells by targeting the tumor suppressor WWOX and hampering cell adhesion *via* a mechanism which is still unknown (*18*). As RCC has been demonstrated to be immunogenic, the association between C1q and the carcinoma is worthy of further study. Moreover, S100A8 and S100A9 are EF-hand Ca^{2+} binding proteins. They are abundant in the cytosol of phagocytes and play a critical role in numerous cellular processes, such as motility and danger signaling, by interacting and modulating the activity of target proteins. The expression levels of S100A8 and S100A9 are increased in many types of cancer, including gastric, colon, pancreatic, bladder, ovarian, thyroid, breast, skin, and prostate cancers, but not in ccRCC according to previous studies (*19-24*). All four of these proteins are secreted proteins and are involved in the

defense response, according to the GO analysis by NIH DAVID.

Our study suggested that ccRCCs might have 8 protein biomarkers that play important roles in the immune response, especially the defense response, although larger scale studies are needed to get more specific conclusions. The relationship between immune response including defense response and RCC needs further investigation and whether other defense response related proteins could also be biomarkers for early detection are also of great value. Dysregulated immune response is thought to be related to the rapid cell proliferation, metastasis and lower apoptosis of cancer cells.

Our findings are consistent with previous studies on functional analyses in ccRCC. The link between dysregulated immune response and ccRCC is not surprising. The reaction of the immune system plays a dual role in the development of carcinoma including ccRCC. The immune system could identify and control the proliferation of cancer cells to play the immunosurveilance function (*25*). However, on the other hand, it could also promote chronic inflammation, immunoselection of defective immunogenic variants and even suppress antitumor immunity and thus provide a beneficial microenvironment for progression of carcinoma. Defective immune-editing to balance activation and inhibition is responsible for the occurrence, angiogenesis, metastasis, apoptosis and inhibition of malignant lesions including in ccRCC (26).

Global analysis through protein analysis and pathway analysis may provide new significant applications in clinical practice. It might provide more than diagnostic markers. The pathway-derived metabolic products especially those proteins that are involved in the defense response might be predictive or even prognostic markers in patients and thus offer new methods for a deeper comprehension of malignant lesions (*27*).

In conclusion, we identified several serum proteins that can distinguish ccRCC and other controls in serum. Most of them are involved in the biological defense response. If the promising proteins could be confirmed as dysregulated in the serum of ccRC patients that would suggest their potential as noninvasive biomarkers for early detection of RCC. The functional analysis of these proteins in RCC may lead to a novel mechanism of RCC development and progression, possibly revealing a novel strategy to treat RCC patients in the future.

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Original Article

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Analysis of p.V37I compound heterozygous mutations in the *GJB2* gene in Chinese infants and young children

Yating Du[1,2,3], Lihui Huang[1,2,3,*], Xiaohua Cheng[1,2,3], Liping Zhao[1,2,3], Yu Ruan[1,2,3], Tingting Ni[1,2,3]

[1] *Beijing Tongren Hospital, Capital Medical University, Beijing, China;*
[2] *Beijing Institute of Otolaryngology, Beijing, China;*
[3] *Key Laboratory of Otolaryngology, Head and Neck Surgery, Ministry of Education, Beijing, China.*

Summary **The p.V37I (c.109G>A) mutation in the *GJB2* gene is the common frequent cause of congenital deafness; however, its pathogenicity is debated. The present study investigated the prevalence of p.V37I in Chinese infants and young children and associated clinical characteristics. The subjects of the present study were screened for mutations in *GJB2* (235delC, 299delAT, 176dell6, 35delG), *SLC26A4* (IVS7-2A>G, 2168A>G), *GJB3* (538C>T), and in the mitochondrial *12S rRNA* gene (1555A>G, 1494C>T). Subjects with p.V37I underwent an audiological evaluation. *GJB2* exon sequencing revealed that 20 subjects had p.V37I compound heterozygous mutations, one of whom had a family history; the mutations included c.235delC/p.V37I (*n* = 12), c.299AT/p.V37I (*n* = 7), and c.176del16/p.V37I (*n* = 1). Of the 20 subjects, 12 were referred for Universal Newborn Hearing Screening (UNHS). Nine of the 20 subjects had mild hearing loss in the better ear and 5 had moderate hearing loss in the better ear while 4 had normal hearing. Among subjects with the c.235delC/p.V37I mutation, 5 had mild hearing loss and 2 had moderate hearing loss while 3 had normal hearing. Among subjects with the c.299AT/p.V37I mutation, 3 had mld hearing loss and 3 had moderate hearing loss while 1 had normal hearing. One subject with the c.176del16/p.V37I mutation had mild hearing loss. Few studies have reported on the clinical characteristics of Chinese infants with p.V37I compound heterozygous mutations identified *via* screening for deafness genes and *GJB2* sequencing. The c.235delC/p.V37I mutation was the most prevalent mutation found in subjects. The degree of hearing loss associated with p.V37I compound heterozygous mutations was mainly mild to moderate.**

Keywords: Infant, children, gene, mutation, audiological evaluation, hearing loss

1. Introduction

Deafness, which refers to varying degrees of hearing loss, is one of the most common sensory disorders. Although there are many causes of deafness, genetic factors account for approximately 50-60% of cases (*1*). The *GJB2* gene is the most frequently mutated gene in cases of hereditary hearing loss, but the mutation spectrum varies among ethnic groups. For example, among Caucasians the most common *GJB2* mutation is c.35delG, with a carrier frequency of 2-4% (*2*). However, c.235delC is most frequently observed among Asians, with an allele frequency in the hearing impaired ranging from 5-22% (*3-5*). Notably, p.V37I (c.109G>A) also has a high incidence in Asia, with a frequency of 8.5% in Thailand (*6*), 1.75% in Japan (*7*), and 6.2% in China (*8*) in patients with hearing loss.

The p.V37I mutation was first identified as a polymorphism because it was present on one chromosome in normal individuals but not in the deaf (*9*). However, compound heterozygous mutations (*i.e.*, p.V37I and R143W heterozygous mutations) were later observed in a Japanese family that exhibited deafness (*10*). R143W was later confirmed to cause recessive nonsyndromic

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**Address correspondence to:*
Dr. Lihui Huang, Key Laboratory of Otolaryngology, Head and Neck Surgery, Ministry of Education, Beijing Institute of Otolaryngology, Beijing Tongren Hospital, Capital Medical University, No.17 Hougou Lane, Chongnei Street, Beijing 100005, China.
E-mail: huangpub@126.com

Table 1. Comparison of previous studies investigating the relationship between the *GJB2* p.V37I mutation and hearing status

Study	PT	Subjects	GT	MT	Conclusion
Snoeckx *et al*. (*19*)	2005	Persons with congenital HL. Patients were ages 0–70 years, with a median age of 8.	Not described	Hom M	The p.V37I homozygous mutation is associated with mild to moderate hearing loss.
Dai *et al*. (*22*)	2009	Patients with HL and an average age of 13.7 ± 4.5.	*GJB2* sequencing	Hom M	Revealed a unique *GJB2* mutation spectrum in Chinese patients with HL. The p.V37I mutation may be pathogenic, with an allele frequency of 6.7%.
Kim *et al*. (*23*)	2013	Children under the age of 15 with HL (n = 103). Five carried the p.V37I variant of *GJB2*.	Sanger sequencing of *GJB2* and targeted capture of exons and flanking sequences of 82 deafness genes	Het M	The p.V37I variant of *GJB2* contributes to the pathogenesis of mild HL and may justify sequencing of *GJB2* in Korean patients with mild to moderate hearing loss.
Chai Y *et al*. (*24*)	2015	945 subjects with HL and a mean age over 6. Twenty-five subjects were identified as p.V37I homozygous.	*GJB2* sequencing	Hom M	The homozygous p.V37I variant of *GJB2* is associated with diverse hearing phenotypes
Huang Y *et al*. (*25*)	2015	Of a total of 300 infants ages 0-3 months diagnosed with HL, 26 exhibited the p.V37I mutation of *GJB2*.	*GJB2* sequencing	Hom M, Het M	The p.V37I mutation of *GJB2* is also strongly correlated with SNHL in infants, whose hearing pheno-types ranged from mild to profound. In addition, 69.6% of p.V37I carriers exhibit mild to moderate HL, indicating that even patients with mild or moderate HL must be tested for *GJB2*.
Bakhchane A *et al*. (*26*)	2016	A large cohort of 152 Moroccan families with HL.	*GJB2* sequencing	Hom M, CH M	*GJB2* mutations may play a role in 43.42% of the Moroccan patients diagnosed with HL. p.V37I had an allele frequency of 3.29%. This mutation was homozygous and compound heterozygous in patients with moderate HI.

PT: time of publication; GT: Genetic testing; MT: mutation type; HL: hearing loss; Hom M: homozygous mutation; Het M: heterozygous mutation; CH M: compound heterozygous mutation; SNHL:sensorineural hearing loss; HI:hearing impaired.

sensorineural deafness (*11*). A study of the p.V37I genotype of *GJB2*, a genetic risk factor for permanent childhood hearing impairment, revealed that the mutation was closely related to late-onset hearing loss in Chinese children (*8*). Many studies (*12-14*) have also found that the mutation is more prevalent in patients than in control subjects; it has therefore been classified as a recessive missense mutation.

GJB2 has two modes of inheritance, *i.e.*, dominant and recessive, that often manifest as syndromic and nonsyndromic deafness, respectively. Most individuals with *GJB2*-associated deafness are nonsyndromic and exhibit autosomal recessive inheritance (*15*); *GJB2*-associated deafness was initially described as bilateral, severe-to-profound, autosomal-recessive, nonprogressive, sensorineural hearing loss (*16,17*). However, there is growing recognition of a wide range of phenotypes associated with *GJB2* mutations, ranging in severity from mild to profound hearing loss with varying degrees of progression (*18,19*). Given that p.V37I is associated with mild and progressive hearing loss, clinical identification of affected individuals is vital (*20,21*).

Recent studies on p.V37I have focused on the incidence of p.V37I (*6-8*) and the hearing status of individuals with a p.V37I homozygous mutation or heterozygous mutation (Table 1). Subjects in those studies generally had a mean age over 6 except those in the study by Huang *et al*. (*25*), where subjects had a mean age of 0-3 months. Nonetheless, the principle was the same: subjects were initially the hearing impaired; *GJB2* sequencing was performed as part of genetic testing, and results suggested that p.V37I-associated hearing loss was mild to moderate or diverse hearing loss (*19,22-26*). In China, newborn screening for deafness genes was first instituted in Beijing in 2012, and newborns are screened for 9 loci in 4 genes, including *GJB2* c.235delC, c.299delAT, c.176dell6, and c.35delG; *GJB3* c.538C>T; *SLC26A4* c.IVS7-2A>G and c.2168A>G; and mitochondrial *12S rRNA* m.1555A>G and m.1494C>T. Numerous newborns have been referred for screening for deafness genes. In the process of genetic counseling and clinical diagnosis, a large number of infants with a single locus mutation in *GJB2* have been identified. In a preliminary study, the current authors screened 915 newborns for 4 *GJB2* mutant alleles, *i.e.*, c.235delC, c.299delAT, c.176del16, c.35delG, and *GJB2* gene sequencing revealed that

p.V37I had a frequency of 4.04%. However, no study has examined the clinical significance of the p.V37I compound heterozygous mutation in deafness (*27*). This was addressed in the present study by examining the clinical characteristics of children with this mutation.

2. Materials and Methods

Parents provided written, informed consent for subjects to participate in this study. The protocol was in accordance with the Declaration of Helsinki principles and was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

2.1. *Subject recruitment*

From 2012 to 2016, 1,348 Chinese newborns who underwent screening for deafness genes were recruited from among patients seeking genetic testing and counseling at the Department of Otolaryngology, Head and Neck Surgery, Tongren Hospital (Beijing, China). Newborns were screened for 9 loci in 4 genes, including *GJB2* c.235delC, c.299delAT, c.176dell6, and c.35delG; *GJB3* c.538C>T; *SLC26A4* c.IVS7-2A>G and c.2168A>G; and mitochondrial *12S rRNA* m.1555A>G and m.1494C>T. After sequencing of *GJB2*, 20 children with compound heterozygous mutations in *GJB2*, *i.e.*, c.235delC, c.299delAT, c.176del16, or c.35delG, and p.V37I, were included in the study.

Inclusion criteria were as follows: 1 of the 4 mutations in *GJB2* identified in screening for deafness genes and the presence of the p.V37I mutation according to sequencing. Exclusion criteria were as follows: carriers of *GJB3* c.538C>T, *SLC26A4* c.IVS7-2A>G or c.2168A>G, or mitochondrial *12S rRNA* m.1555A>G or m.1494C>T mutations; *GJB2* homozygous or compound heterozygous mutations; familial segregation of hearing loss in an autosomal dominant, maternally transmitted, or X-linked manner; and individuals with syndromic hearing loss, conductive deafness, or secretory otitis media.

2.2. *Clinical evaluation*

The following demographic information was collected for each patient: sex, date of birth and birth history, history of maternal pregnancy, family history, date of initial otolaryngological consultation, and major comorbidities. All subjects were Chinese.

2.3. *DNA analysis*

Genomic DNA was extracted from 2 mL of whole blood from each patient, using the Blood DNA kit (Tiangen Biotech, Beijing, China). All exons and flanking splice sites of *GJB2* were screened for mutations using PCR amplification and bidirectional sequencing (*22*).

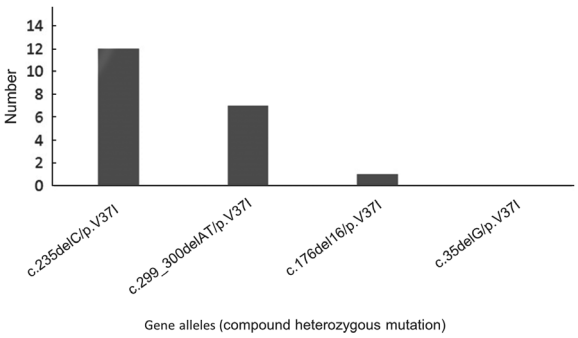

Figure 1. Distribution of *GJB2* genotypes among subjects.

2.4. *Auditory evaluation*

Subjects carrying known compound heterozygous mutations in *GJB2* (c.235delC, c.299delAT, c.176del16, or c.35delG and p.V37I) underwent a physical examination, including an otoscopic examination, with special attention to hearing. Comprehensive audiological evaluation included tympanometry, distortion product otoacoustic emission test, auditory brainstem response, auditory steady-state response (ASSR), and conditioned orientation reflex audiometry. The hearing threshold was calculated as the average hearing level at 0.5, 1.0, 2.0, and 4.0 kHz for the better ear according to the standards of the World Health Organization (1997). The severity of hearing impairment was defined as mild (26-40 dB), moderate (41-60 dB), severe (61-80 dB), or profound (> 80 dB) hearing loss. Given the subjects' young age, the auditory brainstem response threshold and/or ASSR were recorded, and mean thresholds at frequencies in the 0.5- to 4-kHz range were averaged to obtain an approximation for the conditioned orientation reflex (*23*). Subjects were considered as lost to follow-up if hearing results could not be obtained.

3. Results

3.1. *Demographic data*

DNA sequencing identified 20 unrelated children with a p.V37I compound heterozygous mutation, with the other locus being either c.235delC, c.299delAT, c.176del16, or c.35delG. Subjects included 14 males and 6 females with a mean age of 24.2 ± 14.7 months. One subject had a family history of hearing loss. None had an abnormal birth history or abnormal history of maternal pregnancy. The age at first visit was 3-6 months for 12 subjects, younger than 3 months for 3 subjects, and 6-12 months for 5 subjects.

3.2. *Genetic testing*

Three different sequence variants were identified in the coding region of *GJB2* (Figure 1). The most common mutation was the c.235delC/p.V37I compound

Table 2. Demographic information and audiological diagnoses of subjects (*n* = 20)

No.	Sex	Age (m)	Fvma (m)	Gene (p.V37I/X)	UNHS (L)	UNHS (R)	DP	AI	DHL (L)	DHL (R)
01	M	42	4	c.235delC	1	1	1	A	mild	mild
02	M	22	3	c.235delC	1	1	1	A	moderate	moderate
03	M	56	3	c.235delC	1	1	1	A	moderate	moderate
04	M	19	12	c.235delC	0	1	1	A	0	0
05	M	15	3	c.235delC	1	1	down	A	mild	mild
06	M	39	2	c.299delAT	1	1	down	A	mild	mild
07	M	26	9	c.299delAT	1	1	1	A	moderate	moderate
08	M	25	6	c.176del16	1	1	1	A	mild	mild
09	M	38	2	c.235delC	1	1	down	A	mild	mild
10	M	10	2	c.235delC	1	1	down	A	mild	mild
11	M	13	5	c.235delC	0	0	1	A	0	0
12	M	9	4	c.299delAT	0	1	down	A	moderate	moderate
13	M	6	4	c.299delAT	0	0	down	A	0	0
14	F	31	5	c.299delAT	1	1	1	A	moderate	moderate
15	F	8	3	c.235delC	0	0	down	A	mild	mild
16	M	11	3	c.235delC	0	0	down	A	0	0
17	F	7	3	c.299delAT	0	0	down	A	mild	mild
18	F	14	4	c.299delAT	0	0	down	A	mild	mild
19	F	44	8	c.235delC	0	0	/	/	/	/
20	F	40	12	c.235delC	0	0	/	/	/	/

Fvma: age at first visit (months); DP: distortion product otoacoustic emission test; AI: tympanometry; DHL: degree of hearing loss; M: male; F: female; m: months; L: left; R: right; 0: normal; 1: abnormal; /: not determined; A: normal tympanometry type.

Table 3. Patient characteristics

Fvma (m)	Sex		Alleles (with p.V37I)			UNHS		DHL (*n* = 18)		
	M	F	c.235delC	c.299delAT	c.176del16	Pass	Refer	Mild	Moderate	Normal
0-3	3	0	2	1	0	0	3	3	0	0
3-6	8	4	7	5	0	6	6	4	4	3
6-12	3	2	3	1	1	2	3	2	1	1
Total	14	6	12	7	1	8	12	9	5	4

Fvma: age at first visit; m: months; F: female; M: male; DHL: degree of hearing loss.

heterozygous mutation, which was detected in 12 of 20 subjects. Seven had the c.299AT/p.V37I compound heterozygous mutation and 1 had the c.176del16/p.V37I compound heterozygous mutation. No subjects had a c.35delG mutation.

3.3. *Audiological evaluation*

Clinical examination and medical history ruled out the involvement of environmental factors in the hearing loss of the 20 subjects and did not reveal clinical evidence of syndromic features. No pre- or postnatal risk factors were identified. Twelve subjects were referred for hearing screening, including two subjects who were referred for unilateral screening (right ear). Eight subjects passed the screening. Tympanometry revealed normal tympanic membranes in 18 subjects. Audiological results were obtained for 18 subjects; 2 subjects were lost to follow-up. Of the 18 subjects, 9 had mild hearing loss and 5 had moderate hearing loss while 4 had normal hearing (in the better ear). The 2 subjects who were lost to follow-up passed the hearing screening and were female (Table 2).

3.4. *Relationship between mutations and patient characteristics*

Table 4. Results of hearing screening by *GJB2* alleles (*n* = 20)

Alleles (with p.V37I)	UNHS Pass	UNHS Refer
c.235delC	5	7
c.299delAT	3	4
c.176del16	0	1

The age at first visit, sex, mutation, hearing screening, and degree of hearing loss were compared among subjects (Table 3). Most patients had their first visit at the age of 3-6 months (7 of those had the c.235delC/p.V37I compound heterozygous mutation and 5 had the c.299delAT/p.V37I compound heterozygous mutation). However, only 1 subject had the c.176del16/p.V37I mutation, and the subject's first visit was after the age of 6 months. The age at first visit was most often 3-6 months for subjects who passed the hearing screening ($n = 6$). A point worth noting is that 3 subjects who were referred for the hearing screening test were under the age of 3 months at first visit. The degree of hearing loss was determined for 18 subjects; mild hearing loss occurred irrespective of age at first visit.

Hearing screening test results were examined with respect to *GJB2* mutations (Table 4). Among subjects

Table 5. Degree of hearing loss by compound heterozygous GJB2 mutations (*n* = 18)

Degree of hearing loss	genetic loci		
	c.235delC/p.V37I	c.299delAT/p.V37I	c.176del16/p.V37I
Mild	5	3	1
Moderate	2	3	0
Severe	0	0	0
Profound	0	0	0
Normal	3	1	0
Total	10	7	1

with the c.235delC/p.V37I compound heterozygous mutation, 7 were referred for the hearing screening test and 5 passed. Among subjects with the c.299delAT/p.V37I mutation, 4 were referred for testing and 3 passed. One subject with the c.176del16/p.V37I mutation was referred for testing.

The degree of hearing loss in subjects was examined with respect to *GJB2* compound heterozygous mutations (Table 5). Among subjects with the c.235delC/p.V37I compound heterozygous mutation, 5 had mild hearing loss and 2 had moderate hearing loss while 3 had normal hearing. Among subjects with the c.299delAT/p.V37I mutation, 3 had mild hearing loss and 3 had moderate hearing loss while 1 had normal hearing. One subject with the c.176del16/p.V37I mutation exhibited mild hearing loss.

4. Discussion

Hearing impairment is a common disorder with a major impact on public health. Many factors can cause deafness, but genetics account for approximately 50-60% of cases (*1*). *GJB2* is the gene that is most frequently implicated in hearing impairment, and its normal expression in the inner ear is required for normal development and signal transduction between inner ear sensory cells and supporting cells (*28*). The *GJB2* gene mutation spectrum varies according to ethnicity. For example, the most common *GJB2* mutation among Caucasians is c.35delG (*2*), whereas c.235delC is the most prevalent mutation among Asians (*3-5*). The spectrum of *GJB2* gene mutations associated with autosomal recessive nonsyndromic hearing loss is continually expanding (*29*).

The p.V37I (c.109G>A) mutation is highly prevalent in Asia (*6-8,27*), but its pathogenicity is debated. Up to 6% of Han Chinese are carriers of p.V37I, suggesting that there are over 4 million people with p.V37I homozygous mutations in China (*24*). A previous study reported the occurrence of a compound heterozygous mutation (p.V37I and R143W) in a Japanese family with hearing impairment (*10*). R143W has been confirmed to cause recessive non-syndromic sensorineural deafness (*11*). However, p.V37I is also thought to be linked to hearing impairment, as it is found more often in patients than in normal controls (*10,12-14,30*). Therefore, p.V37I is classified as a recessive missense gene.

First, studies on p.V37I have focused on the incidence of p.V37I (*6-8*). Recent studies have increasingly revealed the hearing status of individuals with p.V37I homozygous mutation or heterozygous mutation (Table 1). In most of those studies, subjects generally had a mean age over 6 except those in the study by Huang *et al*. (*25*), where subjects had a mean age of 0-3 months. Nonetheless, the principle was the same: subjects were initially the hearing impaired, *GJB2* sequencing was performed as part of genetic testing, and results suggested that p.V37I-associated hearing loss was mild to moderate or diverse hearing loss (*19,22-26*). In China, newborn screening for deafness genes was first instituted in Beijing, in 2012, and newborns are screened for 9 loci in 4 genes, including *GJB2* c.235delC, c.299delAT, c.176dell6, and c.35delG; *GJB3* c.538C>T; *SLC26A4* c.IVS7-2A>G and c.2168A>G; and mitochondrial *12S rRNA* m.1555A>G and m.1494C>T. Numerous newborns have been referred for screening for deafness genes. In the process of genetic counseling and clinical diagnosis, a large number of infants with a single locus mutation in *GJB2* have been identified. In a preliminary study, the current authors screened 915 newborns for 4 *GJB2* mutant alleles, *i.e*., c.235delC, c.299delAT, c.176del16, c.35delG, and *GJB2* sequencing revealed that p.V37I had a frequency of 4.04%. However, no study has examined the clinical significance of the p.V37I compound heterozygous mutation in deafness (*27*). This was addressed in the present study by examining the clinical characteristics of children with this mutation.

The present study examined the rate of *GJB2* compound mutations, *i.e*., p.V37I concurrent with either c.235delC, c.299delAT, c.176del16, or c.35delG, in Chinese children under the age of 5. Results revealed that more males than females were compound heterozygotes. The most common compound heterozygous mutation was c.235delC/p.V37I, which was detected in 12 of 20 subjects, while no subjects had the c.35delG mutation. This is consistent with previous findings that c.235delC is common among Chinese whereas c.35delG is rare (*2-5*).

More subjects were referred for the hearing screening test than subjects who passed. Of those who

underwent screening, only 1 had normal hearing; the others exhibited mild to moderate hearing loss. Of those that passed the hearing screening test and were followed up, half had mild hearing loss. A study found that biallelic mutations in *GJB2* are a rare occurrence among newborns that pass the screening test if p.V37I is screened for (*31*). The inability to identify an infant who is most likely deaf based on genetic testing may be attributable to technical limitations or to the late onset of hearing loss. This would contradict the contention that the hearing loss caused by *GJB2* mutations is usually congenital, moderate to profound, and nonprogressive (*16,17*).

The p.V37I mutation is associated with mild to moderate hearing loss (*30-32*), which was corroborated by the current findings. Biallelic truncating mutations (*i.e.*, frameshift and nonsense mutations such as c.35delG, c.235delC, and c.167delT) are detected more frequently in individuals with severe to profound hearing loss, whereas biallelic amino acid substitutions (missense mutations; *e.g.*, p.V37I) occur more frequently in individuals with mild to moderate hearing loss. Mutations in the *GJB6* gene should be considered when infants present with hearing impairment since these mutations are significantly correlated with *GJB2* mutations in individuals with nonsyndromic sensorineural hearing loss (*33*).

5. Conclusion

The *GJB2* c.235delC/p.V37I compound heterozygous mutation was the mutation that was detected most often in the Chinese children in this study, and that mutation was mainly associated with mild to moderate hearing loss. Therefore, children need to undergo hearing screening or screening for the *GJB2* p.V37I mutation. Although the current findings need to be verified in a larger sample, they nonetheless provide a reference for clinical diagnosis and treatment of hearing loss in children.

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Brief Report

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Simvastatin downregulated C35 expression and inhibited the proliferation of colon cancer cells Lovo and HT29 *in vitro*

Min Li[1,2], Yong Huang[1], Xuan Dong[1], Qingkuan Wei[1], Jin Li[1], Hui Sun[1], Chenchen Li[1,2], Conghu Qi[3], Jingyu Yang[1,*]

[1] *Shandong Academy of Medicine Sciences, Shandong Provincial Institute of Parasitic Diseases, Ji'ning, Shandong, China;*
[2] *School of Medicine and Life Sciences, Ji'nan University, Shandong Academy of Medicine Sciences, Ji'nan, Shandong, China;*
[3] *Zoucheng People's Hospital, Ji'ning, Shandong, China.*

Summary **The aim of this study was to investigate the antitumor effect of simvastatin in human colon cancer and the possible underlying mechanism. We found that simvastatin dose-dependently inhibited the proliferation of human colon cancer cells Lovo and HT29 using a MTT assay. Real-time PCR and Western blotting assays showed that simvastatin significantly suppressed C35 expression at both mRNA and protein levels. Since C35 is known to have a significant oncogenic role in cancer development *via* promoting cell proliferation and migration, results obtained in the current study imply that downregulation of C35 expression might be involved in the antitumor effect of simvastatin on colon cancer.**

Keywords: Colon cancer, C35, simvastatin, mevalonate, HMG-CoA

1. Introduction

Colon cancer is one of the leading cancer deaths worldwide (*1*). Chemotherapy drugs including cytotoxic agents such as irinotecan and capecitabine and targeted drugs such as cetuximab and panitumumab are usually recommended for advanced colon cancer, however, most of the patients die from metastatic diseases eventually (*1*). Clarification of mechanisms underlying the initiation and progression of colon cancer and thus development of novel targeted drugs may lead to better treatment of this disease.

Statins such as hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, are commonly used to treat hyperlipidemia and show benefits of reducing cardiovascular events (*2*). Studies in recent years showed that this type of drug also has anti-tumor effects through inhibiting cell proliferation, inducing cell differentiation, or soliciting cell apoptosis (*3,4*). In colon cancer, it was reported that simvastatin induced apoptosis in human colon cancer cells and in tumor xenografts, and attenuated colitis-associated colon cancer in mice (*5*). Simvastatin could synergize with irinotecan in suppressing growth of colon cancer cells as well (*6*). In a colorectal cancer epidemiology survey, it was found that long-term treatment with statins reduced the prevalence of colorectal cancer by 47% (*7*). These studies reveal the usefulness of simvastatin against colon cancer. However, the antitumor mechanisms of this drug have not yet been fully elucidated.

C35 (also termed C17orf37) is a novel tumor biomarker abundantly expressed in breast cancer (*8*). Our previous studies demonstrated that C35 was also overexpressed in colorectal cancer and correlated with tumor serosal invasion, lymph node metastasis, and an advanced Dukes stage (*9*), implying C35 might serve as a potential biomarker for colorectal cancer. The conserved canonical immunoreceptor tyrosine-based activation (ITAM) motif located in its C-terminal end and the last four amino acids CVIL of C35 were proved to have an important role in cancer progression and metastasis (*10,11*), suggesting C35 functions as an oncogene and thus can serve as a potential therapeutic drug target. It is worth noting that the maturation of C35 involves polyisoprene groups such as farnesyl and geranylgeranyl which are dependently generated on

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**Address correspondence to:*
Dr. Yingyu Yang, Shandong Provincial Institute of Parasitic Diseases, Ji'ning 272033, Shandong, China.
E-mail: yangjingyu3368@sina.com

HMG-CoA (*12*). We speculate that simvastatin may suppress the maturation and expression of C35 and thus inhibit the proliferation of colon cancers. This hypothesis was explored in the present study.

2. Materials and Methods

2.1. *Agents*

Simvastatin was obtained from Lukang Pharmaceutical Co., Jining, Shandong, China.

2.2. *Cell lines and cell culture*

Human colon cancer cell lines Lovo and HT-29 were obtained from the cell bank of Chinese Academy of Sciences. Cells were maintained in Dulbecco Modified Eagle Medium (DMEM, Hyclone, Thermo scientific, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Evergreen Biotechnology, Hangzhou, Zhejiang, China) at 37°C in a humid atmosphere (5% CO_2-95% air).

2.3. *Cell proliferation assay*

Cells (5×10^3 per well) seeded in 96-well plates were exposed to simvastatin for a specified time. Then the medium was removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μL of MTT (5 mg/mL, Sigma, USA) for 4 h. Light absorbance of the solution was measured at 570 nm on a microplate reader (Perkin-Elmer, USA).

2.4. *Real-time PCR*

Total RNA was extracted with Trizol reagent (Invitrogen, USA), and reverse-transcribed with oligo-dT using a reverse transcription kit (TaKaRa, Dalian, Liaoning, China) according to the manufacturer's protocol. Quantitative real time PCR was carried out by monitoring the increase in fluorescence of SYBR green (SYBR Green Kit, Genstar, Beijing, China). The primer sets were synthesized by Sangon Biotech (Shanghai, China). C35: upstream primer 5'-GCCATCCGAAGAGCCAGTA-3'; downstream primer 5'-ATTACCGAGGCGAAGAGTGG-3'. GAPDH: upstream primer 5'-GACTCACCCTGCCCTCAATA-3'; downstream primer 5'-CCCTGTAGCCTGGACCTGAT-3'. PCR reaction conditions: denaturation at 95°C for 10 min, denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec, extension at 72°C for 35 sec, for a total of 40 cycles. Each sample was amplified in triplicate for quantification. Data were analyzed by relative quantitation using the $\Delta\Delta C_t$ method and normalized to GAPDH.

2.5. *Western blotting*

Cells seeded in 6-well plates were exposed to simvastatin for a specified time period. Cells were harvested and cell lysates (30 μg of protein per lane) were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto nitrocellulose membrane and the protein levels were detected using the primary antibodies against XTP4 and GAPDH (Abcam, Cambridge, England) with appropriate dilution. The bound antibodies were visualized using an enhanced chemiluminescence reagent and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad, Hercules, CA, USA). Densitometric analyses of bands were adjusted with GAPDH as loading control. The percentages of increase or decrease of protein were estimated by comparison to the vehicle control (100%).

2.6. *Statistical analyses*

Data was expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests for the MTT assay and by Mann-Whitney *U* test for real-time PCR and Western blotting assays. $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using the SPSS/Win 17.0 software (SPSS, Inc, Chicago, IL, USA).

3. Results and Discussion

We first examined the effect of simvastatin on cell proliferation of Lovo and HT29 cells. Cells were incubated with increasing concentrations of simvastatin (10, 20, 40, 60, and 80 μM) and subjected to MTT assay at 24, 48, 72, and 96 h, respectively. Results showed that simvastatin inhibited proliferation of Lovo and HT29 cells in a both time- and dose-dependent manner (Figure 1). IC_{50} values of simvastatin determined at 96 h were 45.6 and 33.7 μM, respectively, for Lovo and HT29 cells. Maximum inhibition rates on Lovo and HT29 cells were determined to be 69.8 and 89.7%, respectively, at 96 h by 80 μM simvastatin. These results suggested that simvastatin has certain antitumor effects on colon cancer.

We next determined the effect of simvastatin on C35 expression at both mRNA and protein levels. Lovo and HT29 cells were exposed to 80 μM simvastatin for 48 h. C35 mRNA and protein expression were examined by real-time PCR and Western blotting assays, respectively. Results demonstrated that the mRNA level of C35 was significantly lower in Lovo ($2^{-\Delta\Delta ct} = 0.08 \pm 0.0022$) and HT29 ($2^{-\Delta\Delta ct} = 0.05 \pm 0.0053$) cells exposed to simvastatin than that in cells treated with vehicle (Figure 2A). We found that C35 protein expression was also obviously downregulated in Lovo and HT29 cells

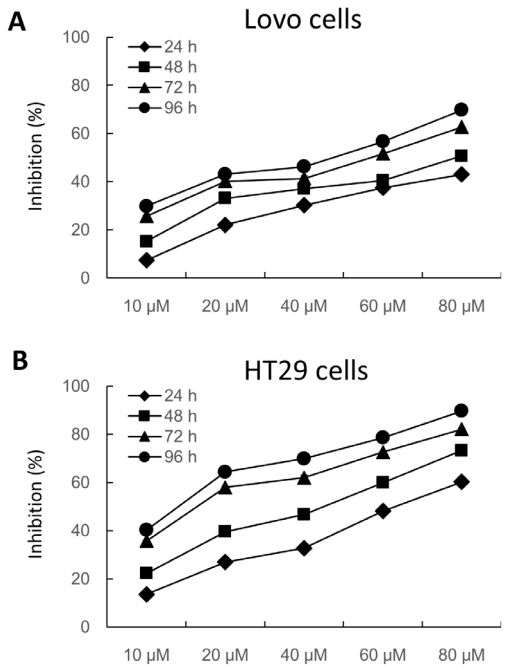

Figure 1. Simvastatin dose- and time-dependent inhibition of proliferation of Lovo (A) and HT29 cells (B). Cells were incubated with increasing concentrations of simvastatin for 96 h and subjected to MTT assay at an interval of 24 h.

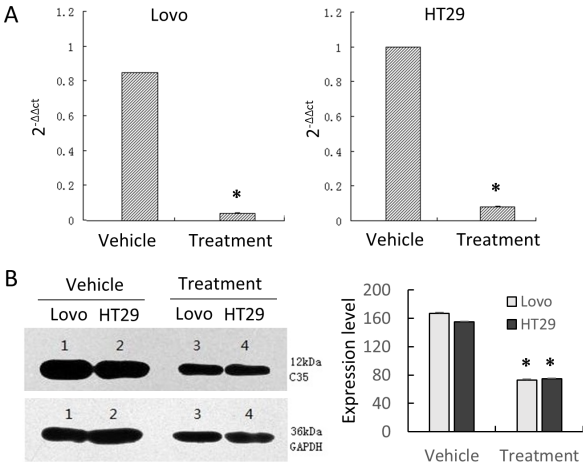

Figure 2. Simvastatin inhibited C35 expression at both mRNA (A) and protein (B) levels in Lovo and HT29 cells. Cells were incubated with 80 μM simvastatin for 48 h and then subjected to real-time PCR and Western blotting assays, respectively. $p < 0.05$ *vs*. vehicle control.

exposed to simvastatin compared to control cells (Figure 2B). These results suggested that simvastatin is capable of inhibiting the expression of C35 at both mRNA and protein levels in Lovo and HT29 cells.

The present study provides a possible mechanism underlying the antitumor effect of simvastatin. Previous studies showed that C35 has a significant oncogenic role in cancer, promoting cell growth, cell migration, and transformation, and that knockdown of C35 inhibited cell motility and cell growth (*13*). Therefore, it is reasonable that downregulation of C35 might be involved in the effect of simvastatin in suppressing the proliferation of Lovo and HT29 cells. Sequencing of C35 revealed a 'CaaX' prenylation motif consisting of the last four amino acids, 'CVIL,' at the C-terminal end (*10*). The 'CaaX' group of proteins are known to be farnesylated by the enzyme farnesyltransferase or geranylgeranylated by the enzyme geranylgeranyl transferase type I (GGTase-I) (*14*). This post-translational modification of the C-terminal prenylation domain of C35 is essential for its membrane association, which facilitates the induction of filopodia formation (*15*). Because farnesyl and geranylgeranyl are generated from mevalonate (MVA) which is synthesized by HMG-CoA (*16*), inhibition of HMG-CoA will reduce the production of MVA and in turn decrease the generation of farnesyl and geranylgeranyl, which finally influences the maturation and expression of C35. These studies provide an explanation of the effect of simvastatin in reducing the expression of C35 in Lovo and HT29 cells.

In conclusion, the present study found that HMG-CoA inhibitor simvastatin inhibited the proliferation of colon cancer cells Lovo and HT29, which might be associated with the activity of simvastatin in suppressing C35 expression. Further detailed mechanisms underlying the anticancer effect of simvastatin is currently undergoing investigation.

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Brief Report

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Dual modulating functions of thrombomodulin in the alternative complement pathway

Koichiro Tateishi, Mio Imaoka, Misao Matsushita*

Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa, Japan.

Summary **Thrombomodulin (TM) is a transmembrane protein expressed on vascular endothelial cells. TM has anticoagulant and anti-inflammatory properties. It has recently been reported that TM modulates complement, an immune effector system that destroys pathogens and is also involved in inflammation. TM was demonstrated to enhance the degradation of C3b into iC3b by factor I and factor H, indicating that its role is in negative regulation in the alternative pathway of the complement system. In this study, we examined the effects of recombinant human soluble TM protein composed of the extracellular domains (rTM) on the alternative pathway. The degradation of C3b into iC3b by factor I and factor H was enhanced by rTM as assessed by SDS-PAGE, confirming the previous observation. We also found that rTM enhances the cleavage of C3 into C3b as a result of activation of the alternative pathway. These results indicate that TM has both activating and inactivating functions in the alternative pathway.**

Keywords: Thrombomodulin, complement, alternative pathway, modulation

1. Introduction

Thrombomodulin (TM) is a transmembrane protein expressed on vascular endothelial cells and is composed of a lectin-like domain, six tandemly repeated epidermal growth factor (EGF)-like domains, a Ser/Thr-rich domain, a transmembrane segment and a cytoplasmic tail (*1*). TM has an anticoagulant role by acting as a cofactor for thrombin, which activates protein C. Activated protein C in turn inactivates factor Va and factor VIIIa of the coagulation system. TM also exhibits anti-inflammatory properties.

The complement system is an immune effector mechanism, which is composed of many plasma and membrane proteins (*2*). It is activated in three ways, the classical, lectin, and alternative pathways. Once complement is activated, a chain reaction of cleavage and assembly of complement components in plasma occurs, leading to destruction of pathogens. C3a and C5a, fragments generated from C3 and C5 by their degradation during complement activation, have a role in inducing inflammation by acting as a chemotactic factor and by activating mast cells to release histamine. It has recently been reported that TM has the ability to modulate complement. The lectin domain of TM possesses a function that inhibits the activation of the classical and lectin pathways but not the alternative pathway (*3*). It also supports thrombin-mediated activation of procarboxypeptidase B (thrombin-activatable fibrinolysis inhibitor), which inactivates C3a and C5a. Delvaeye *et al*. reported that TM binds to C3b and factor H, and thereby enhances the degradation of C3b into iC3b by factor I and factor H, suggesting that it has a negative modulatory function in the alternative pathway (*4*).

Systemic inflammation results in activation of coagulation due to tissue factor-mediated thrombin generation. Disseminated intravascular coagulation (DIC) is an acquired syndrome involving systemic activation of blood coagulation, which leads to microvascular thrombosis in various organs. Based on its anticoagulant activity, TM has recently been used in clinical medicine for the treatment of DIC and also hemolytic uremic syndrome (HUS), a syndrome

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**Address correspondence to:*
Dr. Misao Matsushita, Department of Applied Biochemistry, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan.
E-mail: mmatsu@keyaki.cc.u-tokai.ac.jp

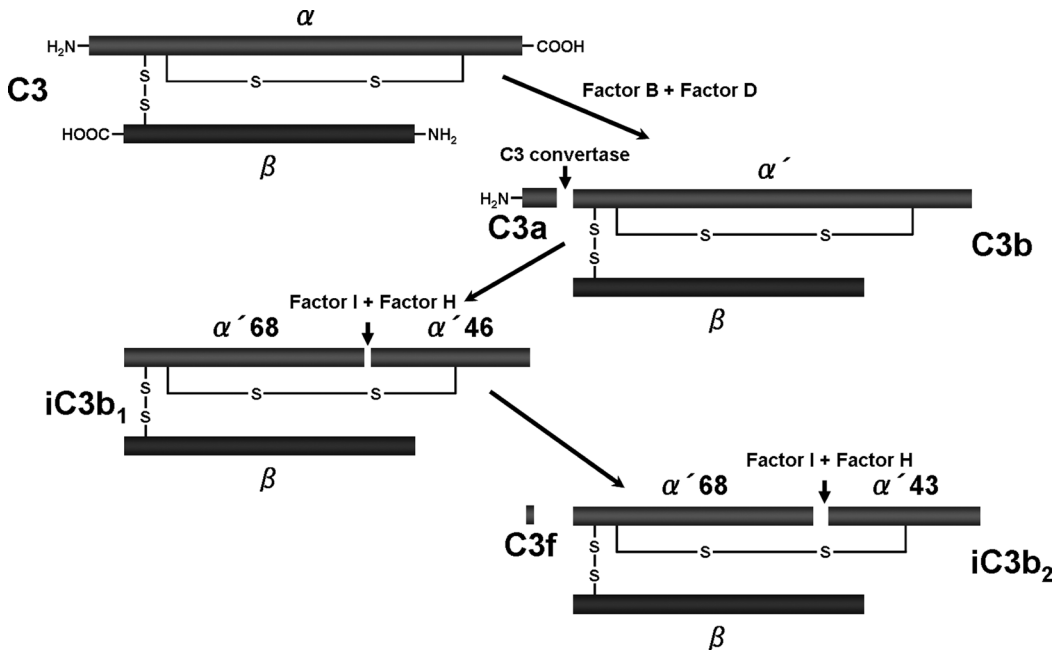

Figure 1. The cleavage process of C3 in the alternative pathway. The details of the cleavage process of C3 are described in the text.

characterized by microvascular thrombosis (*5-10*). Recent findings suggest that the complement-inhibitory activity of TM might also be one of the mechanisms by which it exhibits therapeutic effects against DIC and HUS.

In this study, we investigated the effects of recombinant human soluble TM protein composed of the extracellular domains (rTM) (*i.e.*, a lectin-like domain, EGF-like domains and a Ser/Thr-rich domain) on the alternative complement pathway and confirmed Delvaeye's report that TM enhances the degradation of C3b into iC3b by factor I and factor H. We also found that rTM enhances activation of the alternative pathway.

2. Materials and Methods

2.1. *Reagents*

Human complement components (C3, factor B, factor D, factor H, and factor I) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other general reagents were purchased from Wako Pure Chemicals (Osaka, Japan). rTM (thrombomodulin α) (*6*) was provided by Asahi Kasei Pharma (Tokyo, Japan).

2.2. *Assay for the effects of rTM on the activation of C3 by factors B and D*

C3 (50 μg/mL), factor B (8 μg/mL), factor D (1 μg/mL), and various concentrations of rTM in 20 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 2 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (Tris buffer) were incubated at 37°C for 1 h. After incubation, the reaction mixture was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 8% gel) under reducing conditions. Protein bands were visualized by CBB staining and semi-quantified by a densitometer. The effect of rTM on the activation of C3 by factor B and factor D was evaluated by calculating the ratio of α to β.

2.3. *Assay for the effects of rTM on the degradation of C3b by factors H and I*

C3 (75 μg/mL), factor B (12 μg/mL), and factor D (1 μg/mL) in Tris buffer were incubated at 37°C for 1 h. After incubation, factor H (6 μg/mL), factor I (0.5 μg/mL), and various concentrations of rTM were added to the reaction mixture. After incubation at 37°C for 1 h, the reaction mixture was subjected to SDS-PAGE (8% gel) under reducing conditions. Protein bands were visualized by CBB staining and semi-quantified by a densitometer. The effect of rTM on the degradation of C3b by factors H and I was evaluated by calculating the ratio of α'68 to α', α'46 to α', and α'43 to α'.

2.4. *SDS-PAGE*

SDS-PAGE was performed using the Laemmli method (*11*). 2-Mercaptoethanol was
used as a reducing reagent.

3. Results and Discussion

When C3, factor B, and factor D are incubated in the fluid phase, the alternative pathway of complement is activated. C3 is composed of two polypeptide chains (α and β) linked by disulfide bonds (Figure 1).

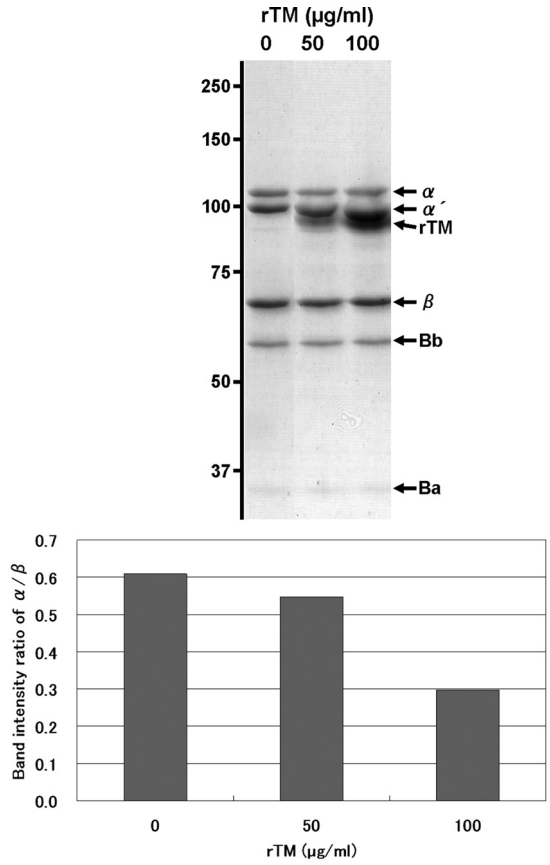

Figure 2. SDS-PAGE analysis of the effects of rTM on the activation of C3 in the alternative pathway. C3, factor B, and factor D were incubated in the absence or presence of various amounts of rTM. After incubation, the reaction mixtures were subjected to SDS-PAGE under reducing conditions followed by CBB staining. Positions of molecular weight markers (kDa) are indicated on the left (upper). Effects of rTM on the activation of the alternative pathway were evaluated by calculating the ratios of band intensity of α to β (lower).

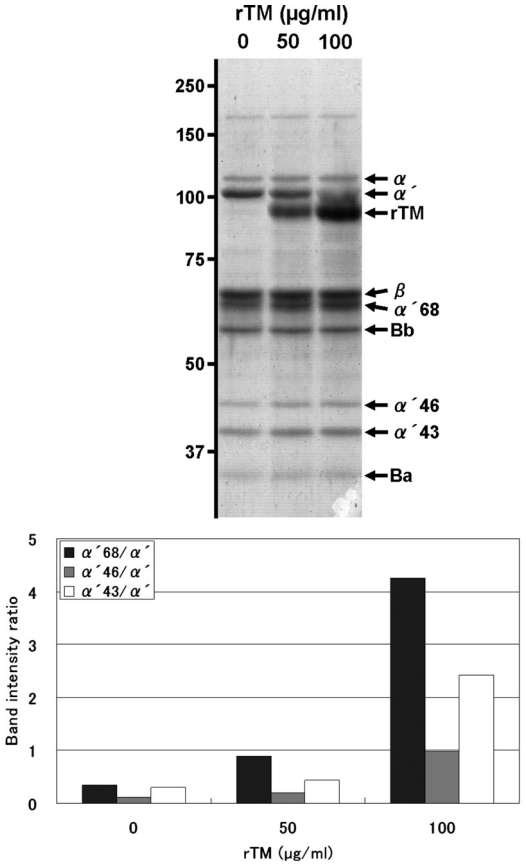

Figure 3. SDS-PAGE analysis of the effects of rTM on the degradation of C3b by factor I and factor H. C3b preparations which had been obtained by alternative pathway activation were incubated with factor I and factor H in the absence or presence of various amounts of rTM. After incubation, the reaction mixtures were subjected to SDS-PAGE under reducing conditions followed by CBB staining. Positions of molecular weight markers (kDa) are indicated on the left (upper). Effects of rTM on the degradation of C3b by factor I and factor H were evaluated by calculating the ratios of band intensity of α'68 to α', α'46 to α', and α'43 to α' (lower).

C3 has a thioester bond in the α chain. The thioester bond is partially hydrolyzed in the fluid phase and the hydrolyzed form of C3 is called C3(H_2O). Factor B is a zymogen and binds to C3 (H_2O). The factor B molecule on C3(H_2O) is cleaved by factor D to generate Bb. As a result, the bimolecular complex C3(H_2O)Bb acts as the initial C3 convertase and is able to cleave C3 into C3a and C3b. C3b is composed of the α' chain and β chain. Factor B binds to C3b generated by the initial C3 convertase. Factor B on C3b is cleaved by factor D to generate Bb. The bimolecular complex C3bBb acts as the C3 convertase capable of cleaving C3 into C3a and C3b. Once C3b is generated, therefore, alternative pathway activation is amplified. Factor I is a serine protease that degrades C3b. Factor H acts as a co-factor for factor I-mediated cleavage of C3b. C3b is cleaved by factor I in association with factor H to generate $iC3b_1$ consisting of a 68 kDa-chain (α'68), a 46 kDa-chain (α'46) and β. $iC3b_1$ is further cleaved to generate $iC3b_2$ and C3f. $iC3b_2$ is composed of α'68, a 43 kDa-chain (α'43) and β. Unlike C3b, $iC3b_1$ and $iC3b_2$ are not able to activate the alternative pathway (*12,13*).

Under the condition in which C3 was partially cleaved to generate the α' chain by incubation of complement components of the alternative pathway (C3, factor B, and factor D), co-incubation of various concentrations of rTM with these complement components resulted in a decrease in the intensity of a band corresponding to the α chain (Figure 2, upper). Densitometric analysis shows that the ratio of band intensity of the α' chain to that of the β chain decreased in the presence of rTM in a dose-dependent manner (Figure 2, lower). These results indicate that rTM enhances the cleavage of C3 by factor B and factor D.

As described in Introduction, TM was reported to enhance the degradation of C3b by factor I and factor H. We, therefore, tried to confirm this modulating activity of TM. C3b was first generated by incubation of C3, factor B and factor D. C3b preparations generated in this way were then incubated with factor H and factor I in the absence or presence of various amounts of rTM. The reaction mixtures were analyzed by SDS-PAGE.

When C3, factor B and factor D were incubated, a band corresponding to the α' chain appeared, indicating a generation of C3b from C3 by alternative pathway activation. The intensity of a band corresponding to the α' chain decreased by co-incubation of rTM in a dose-dependent manner (Figure 3, upper). Densitometric analysis clearly demonstrates that all of the ratio of band intensities of α'68 to α', α'46 to α', and α'43 to α' increased with increasing concentration of rTM (Figure 3, lower). These results indicate that rTM enhances the degradation of C3b by factor I and factor H and are consistent with a report by Delvaeye *et al*. (*4*).

It has been demonstrated that compared with control Chinese hamster ovary (CHO) cells, TM expressed on CHO cells enhances the cleavage of C3b into iC3b in human serum after complement activation, indicating that TM negatively regulates the alternative pathway (*4*). The present study suggests that rTM modulates the alternative pathway by two distinct ways; rTM enhances the activation of C3 and enhances the inactivation of C3b as well. It is, therefore, possible that TM enhances the inactivation of C3b more efficiently than the activation of C3.

The mechanisms underlying the dual modulating effects of TM on the alternative pathway remain unknown at the moment. It has been shown that TM binds to C3b and its binding is increased in the presence of factor H (*4*). TM also binds to factor H alone (*4*). These facts suggest that TM bound to C3b enhances the factor I-mediated cleavage of C3b by recruiting factor H on C3b. Properdin is a positive regulatory component of the alternative pathway (*14*). It binds to C3b and enhances the activation of the alternative pathway by slowing the intrinsic decay of C3 convertase C3bBb. The modulating effect of TM on the activation of C3 in the alternative pathway seems to resemble that of propredin. It is, therefore, possible that TM enhances the activation of C3 in a similar fashion to properdin.

In conclusion, TM was found to modulate the alternative pathway by enhancing the activation of C3 and also by enhancing factor I-mediated inactivation of C3b.

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Commentary

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E-learning for grass-roots emergency public health personnel: Preliminary lessons from a national program in China

Wangquan Xu[1,2], Qicheng Jiang[2], Xia Qin[1], Guixia Fang[1], Zhi Hu[1,*]

[1] *Health Management School, Anhui Medical University, Hefei, Anhui, China;*
[2] *Public Health School, Anhui Medical University, Hefei, Anhui, China.*

Summary **In China, grass-roots emergency public health personnel have relatively limited emergency response capabilities and they are constantly required to update their professional knowledge and skills due to recurring and new public health emergencies. However, professional training, a principal solution to this problem, is inadequate because of limitations in manpower and financial resources at grass-roots public health agencies. In order to provide a cost-effective and easily expandable way for grass-roots personnel to acquire knowledge and skills, the National Health Planning Commission of China developed an emergency response information platform and provided trial access to this platform in Anhui and Heilongjiang provinces in China. E-learning was one of the modules of the platform and this paper has focused on an e-learning pilot program. Results indicated that e-learning had satisfactorily improved the knowledge and ability of grass-roots emergency public health personnel, and the program provided an opportunity to gain experience in e-course design and implementing e-learning. Issues such as the lack of personalized e-courses and the difficulty of evaluating the effectiveness of e-learning are topics for further study.**

Keywords: Online training, public health workers, e-course, pilot study

1. Introduction

Grass-roots emergency public health personnel (GEPHP) are responsible for dealing with a large number of public health emergencies in the early stages, and their capabilities directly affect the occurrence of emergencies and their impact on society (*1*). Therefore, the knowledge and capabilities of GEPHP should be enhanced. In China, there are nearly 200,000 GEPHP, and these GEPHP face difficulties: *i*) GEPHPs have relatively limited emergency response capabilities; and *ii*) recurring and new public health emergencies require GEPHP to constantly update their professional knowledge and skills (*2*). Professional training is a principal solution to these problems (*3,4*). After outbreaks of severe acute respiratory syndrome (SARS), the Chinese Government invested heavily in the creation of a public health system, with a particular emphasis on manpower. However, manpower and financial limitations in basic public health agencies often preclude GEPHP from receiving sufficient training. At present, GEPHP receive formal training only 1-2 times a year, less than one week in total. In addition, the heavy workload at grass-roots agencies also increases the difficulty of organizing training programs. In general, learning tends to be ineffective. Learning is primarily affected by limited learning opportunities and a lack of systematic and long-term programs. As a result, GEPHP have been unable to update their professional knowledge in a timely manner and they have limited emergency response capabilities, and this situation has changed little. The inadequate training of GEPHP in China remains a challenge (*5*). Self-training and self-improvement through e-learning is an ideal way to overcome this challenge (*6-8*).

Compared to traditional face-to-face forms of centralized learning, e-learning has the advantages of no time and site restrictions, low costs, and ease of

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**Address correspondence to:*
Dr. Zhi Hu, Health Management School, Anhui Medical University, No.81 Meishan Road, Hefei, Anhui, China.
E-mail: huzhi5@yahoo.com

organization, and e-learning has become an important form of vocational education in various fields in developed countries (*9-12*). In China, however, e-learning is primarily focused on degree education (*e.g.* correspondence education) at present. Wide-scale use of e-learning in vocational education seldom occurs in the field of public health. Existing methods of instruction rely primarily on traditional face-to-face learning. Simple video presentations and e-courses delivered via the Internet are occasionally used. To the extent known, a systematic and formal e-learning program for public health personnel has yet to be reported in China.

In order to provide a cost-effective and easily scalable way for GEPHP to acquire knowledge and skills, the National Health and Family Planning Commission of China developed an emergency response information platform (ERIP) in 2011. The Commission used Microsoft Visual Studio 2008 as the develop platform, SQL Server 2008 as the data management tool, and java as the programming language. The ERIP was completed and opened to the public in April 2013. Trial access to the ERIP was provided in Anhui and Heilongjiang provinces. The ERIP consists of five modules: information searches, e-learning, outbreak maps, incident news, and training. This paper describes the e-learning component of ERIP e-learning (hereafter refer to as the ERIP-EL).

2. Development of the ERIP-EL

The ERIP-EL was intended for GEPHP, and a key aspect of the creation of the ERIP-EL was the development of suitable e-courses for GEPHP. E-courses featured in the ERIP-EL are based on two principles: the knowledge and skills that GEPHP lack and the knowledge and skills that GEPHP should acquire. Here, public health emergencies handled by GEPHP were classified into eight types. An investigation of requirements and Delphi consultation were both used to identify the key knowledge and skills for GEPHP.

An *investigation of requirements* was used to identify the knowledge and skills that GEPHP lacked. This investigation was accomplished via an on-site survey methodology including quantitative and qualitative research. Random cluster sampling was used to select more than 2,000 respondents from databases at the Centers for Disease Control (CDC), medical facilities, and health administration agencies at the city or county/district level in Heilongjiang and Anhui provinces in 2012. A questionnaire was individually administered to each respondent. In addition, individual in-depth interviews were conducted. Frontline emergency health personnel who were responsible for responding to and managing emergencies were interviewed in group interviews.

Delphi Consultation was used to identify the knowledge and skills that GEPHP should acquire. In March, May, and June 2012, consultation was conducted three times. Twenty-four experts were invited from 13 sub-provincial institutions, including health administration departments, grass-roots health institutions, public health departments in universities, and other related fields. Experts were asked to score various incidents (out of 100) based on three aspects: the frequency of the emergency, incident hazards, and prompt improvement of emergency response capabilities. Required skills for GEPHP were determined based on the final score according to the technique for order performance by similarity to the ideal solution (TOPSIS).

Fifty-nine categories of core skills and knowledge were comprehensively identified, as shown in Table 1, and corresponding e-courses were developed by experts in different fields who were part of the ERIP-EL program team. Each course was presented to learners in the form of a digital slide show, a digital document, a video lecture, a game, a role-playing drill, or a toolkit depending on its characteristics (*13-15*).

Refinement of the ERIP-EL The adjustability of e-courses for GEPHP is crucial, so frontline health emergency experts and specialists in public health emergencies were invited to view and amend the e-course content to make sure that the content was factually correct and also that it met the practical needs of GEPHP. The forms in which e-courses were presented was also evaluated and constantly modified to presentation the course most effectively.

The ERIP-EL uses a browser/server architecture, and learners can access the ERIP-EL by typing a URL in their Internet browser. After entry of their personal information and registration, learners can access the platform to start learning.

In 2014, trial access to the ERIP-EL was provided to grass-roots public health agencies (GPHA) in Anhui and Heilongjiang provinces in China for one year, and a total of 441 GEPHP participated in and completed the e-learning program. Of these learners, 210 (45.4%) were male. The one-year e-learning period was divided into two six-month stages: unspecified study and specified study. In the first stage, participants were allowed to choose e-courses to study depending on their interests and actual work requirements. In the second stage, participants were required to complete prescribed e-courses including elective and compulsory courses. After the e-learning period, a t-test was used to compare the average pre-learning score and post-learning score for GEPHP, and self-evaluation was used to assess the improvement of capabilities.

3. Trial access to the ERIP-EL

Use of the ERIP-EL. During the e-learning period, most learners (56.9%) logged on to the platform 1-2 times a week, and their favorite form of e-course presentation was a digital slide show (55.6%) (Table 2). Learning

Table 1. List of core skills and knowledge that GEPHP should acquire

Category	Key skills and knowledge
Infectious disease	1) On-site investigation (P,V,T,G) 2) Team creation (P,W) 3) Response to report (P,V,W,D) 4) Communication of risk (P,V,T) 5) Health education (P,V,W) 6) Personal protection (W,V) 7) Incident report (P,V,W) 8) Monitoring (P,V) 9) Sampling (P,W) 10) Incident verification (P,V,W) 11) Incident detection (P,V)
Food poisoning	1) Response to report (P,V,W,D) 2) Incident verification (P,V,W) 3) On-site investigation (P,V,T) 4) Sampling (P,W) 5) Team creation (P,W) 6) Communication of risk (P,V,T) 7) On-site medical aid (P,V) 8) Technical preparations (P)
Occupational poisoning	1) Incident report (P,V,W) 2) Incident verification (P,V,W) 3) Response to report (P,V,W,D) 4) Communication of risk (P,V,T) 5) Technical preparations (P,V)
Environmental pollution	1) Communication of risk (P,V,T) 2) Public health education on-site (P,V) 3) On-site medical aid (P,V,D) 4) Personal protection (W,V)
Mass psychogenic reaction	1) Communication of risk (P,V,T) 2) Incident verification (P,V,W) 3) Response to report (P,V,W.D) 4) Incident report (P,V,W)
Nuclear contamination and radiation	1) Personal protection (W,V) 2) Incident report (P,V,W) 3) Response to report (P,V,W,D) 4) Training (P,V)5) On-site medical aid (P,V)
Large incident support	1) On-site medical aid (P,V,D) 2) Response to report (P,V,W,D) 3) Emergency planning (T) 4) Supplies (V) 5) Training (P,V) 6) Technical preparations (P)
Medical aid	1) On-site medical aid (P,V,D) 2) Team creation (P,W) 3) Supplies (V) 4) Response to report (P,V,W,D) 5) Training (P,V) 6) Drills (P) 7) Incident report (P,V,W) 8) Communication of risk (P,V,T)

D, role-playing drill; G, game; P, digital slide show; T, toolkit; V, video (lecture or cartoon); W, digital document. GEPHP: grass-roots emergency public health personnel.

times were mainly in the afternoon (77%).

Attitudes towards the ERIP-EL. After a year of e-learning, most participants (74.8%) had a positive attitude towards the platform and they felt that

Table 2. E-learning times for GEPHP and their favorite forms of e-courses

Variable	*n* (%)
Frequency of use	
Less than 1 time a week	141 (32)
1-2 times a week	250 (56.9)
3-4 times a week	44 (10)
More than 5 times a week	5 (1.1)
Favorite form of e-course	
Digital slide show	245 (55.6)
Digital document	112 (25.4)
Video	171 (38.8)
Toolkit	90 (20.4)
Game	25 (5.7)

GEPHP: Grass-roots emergency public health personnel.

e-learning was "useful/very useful" for their work, with only 0.9% responding that it was "bad/very bad." Compared to pre-learning scores, participants' average scores improved substantially after e-learning (58.5 *vs*. 85.5, $p < 0.01$), as did their pass rate (26.2% *vs*. 92.3%, $p < 0.01$). Participants rated the improvement in their capabilities as "general improvement," indicating improvement to a certain extent (*16,17*). One reason for this finding may be because this e-learning pilot program lasted 1 year and the effects of the program have yet to appear. Therefore, long-term and systematic learning are more helpful to GEPHP.

After the formal e-learning period, 67.98% of the participants were logging on to the ERIP-EL to actively learn depending on their needs, and participants who had 1-2 years of work experience more actively participated in e-learning than those with less than 1 year of work experience or those with more than 2 years of work experience. Of the participants, 73.52% regarded the ERIP-EL as a tool to solve daily work-related problems. The most influential factor for participants was whether the e-course met their needs (Table 3).

4. Lessons and issues

This attempt to provide training in the field of public health emergency response provided an opportunity to gain experience and identified several problems with the development of e-learning that will help with the future development of related programs.

Development of the ERIP-EL. The survey indicated that a digital slide show was the most popular form of e-learning, while game learning was favored little. This may be because GEPHP were unfamiliar with this new form of e-learning. Therefore, attention should be paid to informing potential learners about the development of new forms of e-courses. An interface should also be developed to ascertain the needs of learners after they complete an e-course so that e-courses can be continually improved based on that feedback.

Results also suggested that communication among

Table 3. Factors influencing e-learning by GEPHP

Influence factors	*n* of answers (%)	
	Yes	No
Do hardware conditions influence e-learning?	326 (73.9)	115 (26.1)
Do basic computer skills influence e-learning?	298 (67.6)	143 (32.4)
Do e-courses meet the needs of GEPHP?	382 (86.6)	59 (13.4)
Do administrators support e-learning?	289 (65.6)	152 (34.4)
Is there sufficient motivation to participate in e-learning?	277 (62.9)	164 (37.2)

GEPHP: grass-roots emergency public health personnel.

GEPHP is essential for self-learning. Therefore, a module should be incorporated in the ERIP-EL so that learners can indicate which courses they selected, search posted test scores on e-courses, exchange learning experiences, and even communicate instantly with other learners. This environment will help to establish a friendly atmosphere among learners, increase their enthusiasm, and make learning more effective.

At present, the ERIP-EL is just a platform for presenting e-courses that learners can choose to study. Results of the pilot study indicated that some GEPHP had difficulty determining the right e-courses to study, and this was especially true for new personnel. E-courses need to be tailored to learners. Tailoring e-courses can be based on two principles: *i*) compulsory and elective courses depending on the learner's position and *ii*) courses that learners perform poorly on according to their test scores. These steps will save learners' time in searching for courses and also make learning more effective by identifying learners' weaknesses.

Motivation to participate in e-learning and evaluation of its effectiveness. Self-learning depending on needs is one of the advantages of e-learning. In the provinces studied, however, a moderate proportion (67.98%) of GEPHP logged on to the ERIP-EL and took part in e-learning. Reasons for this may include a heavy work load, family duties, and limited self-control. In the absence of monitoring, the ERIP-EL may fail to teach effectively by relying solely on self-learning. External requirements and incentives, such as credits, certificates, or benefits, may be necessary for successful e-learning. Moreover, obtaining support from administrators of grass-roots agencies is essential when starting an e-learning program since they can create a conducive e-learning environment for GEPHP.

Evaluation of the effectiveness of learning can be divided into multiple types depending on the purpose. Because of the inherent characteristics of e-learning, evaluating its effectiveness is a challenge for the evaluator (*18*). According to Kirkpatrick's training evaluation model, evaluation can be clarified into four levels: reaction, learning, behavior, and results (*19*). The developed program had difficulty determining whether learners had actually enhanced their ability to cope with public health emergencies during the e-learning period. Thus, the effectiveness of e-learning was assessed using a test of knowledge test and self-evaluation of ability, which are the first two levels of Kirkpatrick's model. Plans are to assess the effectiveness of e-learning at the levels of behavior and results in a long-term study in the future.

Sustainability of the ERIP-EL. Operation of the ERIP-EL requires personnel and funding. The result of a national project, ERIP-EL is free for registrants to access. Once the project ends and funding and operation & maintenance personnel are no longer provided, a pressing problem will be how to sustain the ERIP-EL. One suggestion is that departments, such as public health departments or emergency response organizations, could take over management of the ERIP-EL.

Prospects. If administrative measures conducive to e-learning are implemented, e-learning can effectively improve the capabilities and problems-solving ability of GEPHP. Given the limited training resources of GPHA, e-learning can be a feasible and alternative form of education.

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Commentary

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Continuing violence against medical personnel in China: A flagrant violation of Chinese law

Wei Peng[1,2], Gan Ding[2], Qi Tang[2], Lingzhong Xu[2,*]

[1] *Department of Scientific Research, Shandong University of Traditional Chinese Medicine Hospital, Ji'nan, China;*
[2] *Department of Social Medicine and Health Services Management, School of Public Health, Shandong University, Ji'nan, China.*

Summary **Over the past few years, China has witnessed a surge in violence against medical personnel, including widely reported incidents of violent abuse, riots, attacks, and protests in hospitals, where doctors suffer from heavy workloads and little protection. China has engaged in serious efforts, such as investing large amounts into the healthcare system and implementing several decades of healthcare reform, to make medical care more accessible to and affordable for the public. However, incidents of violence against medical personnel have increased in intensity, reflecting deteriorating relations between medical staff and their patients in China over the past few decades. Hence, the effectiveness of healthcare legislation needs to be examined and medical reform and development of the healthcare system need to be reevaluated. Only by enhancing oversight, promoting healthcare reform, and improving the healthcare system can we repair the doctor-patient relationship and decrease violence against doctors in China.**

Keywords: Incidents of violence, oversight of the medical system, healthcare reform, healthcare system

1. Introduction

Over the past few decades, Chinese medical personnel have received international attention because of the dilemma the face, not because of their medical skill or concerns about patient care, but because of the high risk of serious injuries or murder by patients or their family members (*1,2*).

In May 2016, Chen Zhongwei, a retired dentist at Guangdong Provincial People's Hospital, died after being stabbed with a knife over 30 times from head to toe by a former patient he treated 25 years ago; this incidence once again raised serious questions about the Chinese healthcare system (*3*). Although this type of death is nothing new for doctors in China, the tragic senselessness of Chen's murder led to a strong public outcry from medical professionals, citizens, and the Internet community (*4*).

However, no one could ever have imagined the "Black May" that Chinese doctors went through in 2016. Only few days after the attack on Chen, a surgeon in Chongqing in Southwest China was still in danger after being stabbed in the face and back by a 19-year-old patient and two of his friends, while another doctor from Jiangxi Province was beaten by a patient's family members after the patient died (*5*). In addition, a doctor named Wang Jun in Hunan Province died after he was attacked by relatives of a patient on May 18 (*6*).

From 2003 to 2013, 101 incidents of violence against medical personnel occurred, including 23 incidents that resulted in the death of 24 doctors or nurses (*7*). According to the White Paper on the Practice of Medicine by Chinese Physicians by the Chinese Medical Association in 2014, nearly a quarter of Chinese doctors were victims of violence in the workplace in varied forms and 32.7% of doctors had an average workweek over 60 hours (Figure 1) (*8,9*).

China has engaged in serious efforts, such as investing large amounts into the healthcare system and

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**Address correspondence to:*
Dr. Lingzhong Xu, Department of Social Medicine and Medical Service Management, School of Public Health, Shandong University, Mailbox No. 110, 44 Wenhuaxi Road, Ji'nan, 250012, Shandong, China.
E-mail: lzxu@sdu.edu.cn

implementing several decades of healthcare reform, to make medical care more accessible to and affordable for the public (*10*). However, all of the incidents of extreme violence against medical personnel over the past few decades reflect an increasing number of disputes between medical personnel and their patients in China (*11*). Given these circumstances, the effectiveness of legislation and oversight of the medical system and reevaluation of healthcare reform and development of the healthcare system should be discussed.

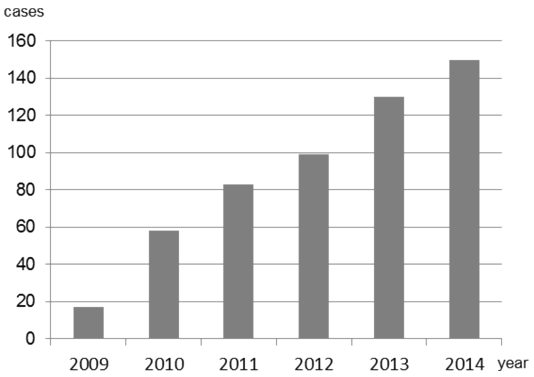

Figure 1. Incidents of violence against medical personnel in China from 2009-2014. A survey by the Chinese Medical Association in 2014 reported that nearly a quarter of Chinese doctors were victims of violence in the workplace in varied forms, and almost 60% of reported cases in China involved doctors receiving verbal abuse from their patients. As shown in the figure, violence occurred in hospitals every year, and the number of incidents has increased rapidly over the past 5 years.

2. Legislation, enforcement, and oversight in the healthcare sector

A few cases of violence might have occurred because of medical negligence or malpractice by doctors 20 years ago, but the public is now expressing worry and patients are resentful over their medical care (*12*). Given these issues, laws, rules, and regulations have been gradually unveiled to curb violence against medical personnel (Table 1) (*13-18*).

Although relatively clear laws and regulations have been unveiled, the issues of failure to follow those laws, lax enforcement, and a reticence to punish violators have existed in relevant bureaus and departments, indicating that medical facilities are still very passive when faced with legal issues (*19*). Therefore, laws and regulations need to be implemented and revised, and obedience of

Table 1. Major laws, rules, and regulations governing violence against medical personnel in China

Time (*Ref.*)	state	Title	Highlights	Notes
2002.09 (*13*)	State Council of the People's Republic of China	Regulations on the handling of medical malpractice	Stipulates that scientific societies (medical associations) determine if medical malpractice has occurred.	Used to identify medical malpractice to resolve disputes over medical care.
2009.11 (*14*)	Ministry of Health*	Procedures for the management of hospital complaints (For trial implementation)	Requires hospitals to create a doctor-patient relationship office or specify a department to manage complaints.	Might not work well because of differing conditions at different facilities.
2009.12 (*15*)	Standing Committee of National People's Congress	Tort Liability Law	Provisions cover medical damages (*i.e.* medical malpractice), eliminating prior requirements that established when hospitals would be liable for damages.	Ensuring that the Law and judgements based on it apply to medical malpractice will be difficult.
2012.05 (*16*)	Ministry of Health*; Ministry of Public Security	Notice on maintaining order at healthcare facilities	Notice jointly issued by the Ministry of Health* and Ministry of Public Security.	Not fully implemented.
2013.10 (*17*)	National Health and Family Planning Commission; Ministry of Public Security	Advice on instituting enhanced hospital safety and security systems	National Health and Family Planning Commission and Ministry of Public Security jointly issued the Notice.	Specifies zero tolerance for incidents of violence against medical personnel.
2014.04 (*18*)	Supreme People's Court; Supreme People's Procuratorate; Ministry of Public Security; Ministry of Justice; National Health and Family Planning Commission	Views on punishing crimes against medical personnel and maintaining normal order in healthcare settings	*i*) Articulated by the Supreme People's Court and Supreme People's Procuratorate, which accelerated the adjudication process and clarified the roles of different bodies, greatly increasing enforcement. *ii*) Enables crimes against medical personnel to be legally punished in accordance with the Criminal Code and the Law on Penalties for Obstruction of the Administration of Public Security.	The breakthrough in laying out "penalties for violence against medical personnel" has extremely important significance in terms of establishing order in healthcare settings and protecting the legal rights and interests of both doctors and patients.

*The Ministry of Health was reformed into the National Health and Family Planning Commission of the People's Republic of China in 2013.

the law and education of the public with regard to the law are also indispensable. In addition, most hospitals expect the Public Security Bureau to provide hospitals with more security by, for example, providing a security office in primary hospitals.

Generally, disputes over medical care are the responsibility of the hospital and medical personnel. However, blaming individual medical personnel for medical mishaps is inappropriate since those errors are the combined result of organizational and individual factors. Identifying the causes of a mishap and preventing errors from reoccurring by simply blaming and punishing an individual is not conducive to solution of the greater problem, i.e. the systemic factors that allowed the mishap to occur in the first place (*20*). Hence, resolving disputes over medical care should pay closer attention to the individual responsible for a mishap and also help to create a safer system of medical care, reducing the possibility of mistakes and providing medical facilities with the opportunity to make structural reforms.

In short, the improvement of medical systems and processes must be considered as a way to effectively improve safety.

3. Healthcare reform could be the solution

Although China has reformed its healthcare system for more than 30 years, including 7 years of new healthcare reforms, violence against medical personnel still continues (*21*). Violence against medical personnel needs to be resolved by sustained and innovative healthcare reform (*22*).

Big hospitals in large cities have "a massive patient load" and provide "expensive care," but not all facilities face these same problems because of an imbalance in medical resources. Important measures for healthcare reform in China are increasing the supply of skilled medical personnel and encouraging social medicine, since these measures can lead to a greater supply of medical services, satisfy the diverse requirements for medical services, promote market competition, and optimize the healthcare system.

Important measures for reform of the medical system are reform of doctors' groups, such as multi-site physician practice, medicine prices, and remuneration system; these measures can improve the allocation of healthcare resources and the doctor's level of expertise and remuneration (*23*). However, an online survey by Ding Xiang Yuan, the most popular biomedical website in China, found that 91% of doctors believed that China's healthcare reforms would not be successful unless the social and economic status of doctors was improved (*24*). Measures to improve medical care are important, but so are measures to improve the salaries of and working conditions for Chinese doctors (*25*).

The status of patients needs to be defined and their rights need to be protected, but patients also need to be mindful of their responsibilities. Let us look at the University of Tokyo Hospital as an example. The Hospital contributes to the advancement of clinical medicine and it fosters healthcare professionals in order to provide each patient with the best medical care. The Hospital's Guidebook clarifies the obligations of patients: *i*) The patient is obligated to provide accurate information about his/her health; *ii*) The patient is obligated to follow the regulations of this hospital; *iii*) The patient is obligated to avoid being a public nuisance; *iv*) The patient is obligated to pay for the medical services that he/she received (*26*). Therefore, by fulfilling these obligations, the patient has the right to receive the best medical care.

As healthcare reform continues, there is a chance to repair relationship between doctors and patients.

4. Compensation for medical malpractice

When longstanding practices lead to a problem, finding a solution can be difficult. This dilemma is evident in the key question of "Who pays for healthcare in China?" as asked by Chen *et al*. They found that the tax burden associated with healthcare costs was disproportionately borne by low-income earners due to several causes, including tax brackets and health insurance schemes (*27*). Some patients are unable to bear medical expenses, which suggests that social health insurance has strongly influenced society. Given the situation in Japan and the US, basic medical insurance should be paid by the Chinese Government and private medical insurance should be used as a direct and efficient way to ensure patients' livelihoods and improve the doctor-patient relationship in China (*28*).

In conclusion, continuing violence against medical personnel is a violation of Chinese law that warrants an examination of the effectiveness of medical legislation and reevaluation of healthcare reform and development of the healthcare system. Only by enhancing oversight, promoting healthcare reform, and improving the healthcare system can we repair the doctor-patient relationship and decrease violence against doctors in China.

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Title Page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of

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Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For article types including Original Article, Brief Report, Review, Policy Forum, and Case Report, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For News and Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

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Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

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Example 2 (Sample journal reference with more than 15 authors):
Darby S, Hill D, Auvinen A, *et al.* Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

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Editorial and Head Office:
Pearl City Koishikawa 603
2-4-5 Kasuga, Bunkyo-ku
Tokyo 112-0003 Japan
Tel: +81-3-5840-8764
Fax: +81-3-5840-8765
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