ISSN 1881-7815 Online ISSN 1881-7823

# BST BioScience Trends

Volume 11, Number 2
April, 2017

IRCA BSSA

IACMHR

www.biosciencetrends.com

BST
BioScience Trends

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

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

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## Guide for Authors

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Editorial

DOI: 10.5582/bst.2017.01102

# Propelling medical humanities in China

**Wei Tang**[§,*]

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**Summary** **Advances in the study of the medical humanities and medical humanities education have been made over the past few decades. Many influential journals have published articles examining the role of medical humanities and medical humanities education, the development and evaluation of medical humanities, and the design of a curriculum for medical humanities education in Western countries. However, most articles related to medical humanities in China were published in Chinese, moreover, researchers have worked in relative isolation and published in disparate journals, so their work has not been systematically presented to and evaluated by international readers. The six companion articles featured in this issue describe the current status and challenge of medical humanities and medical humanities education in China in the hope of providing international readers with a novel and meaningful glimpse into medical humanities in China. This Journal is calling for greater publication of research on medical humanities and medical humanities education to propel medical humanities in China.**

***Keywords:*** Medical humanities, medical humanities education, academic literature

Defined as an inter- and multidisciplinary field that explores contexts, experiences, and critical and conceptual issues in medicine and health care (*1*), medical humanities ought to be a source of encouragement, illumination, and understanding in support of the detailed manifestations of the idea of humane health care (*2*). Advances in the study of the medical humanities and medical humanities education have been made over the past few decades.

The literature on medical humanities is relatively extensive. From the end of the 19th century to the early 20th century, research began to focus on the interaction between medicine and society. In 1919, William Osler put forward the concept of scholars in medical humanities. In the 1940s, George Sarton first used the term "medical humanities" in the journal *ISIS* (*3*). Since the 1970s, new journals such as the *Journal of Medicine and Philosophy*, the *Journal of Medical Humanities*, and *Literature and Medicine* began to outline the contours of the intersection between humanities and medicine interface in the United States (US). Medical humanities has also had an equally fruitful past and achieved academic recognition in the United Kingdom (UK). The BMJ Group publishes *Medical Humanities*, the *Journal of Medical Ethics*. And several peer-review journals devoted to exploration of particular facets of humanities and medicine, such as *Medical History* and *Social History of Medicine*, that predate the appearance of publications devoted to the generic area of medical humanities.

Medical humanities gained institutional recognition with the founding of the Institute of Medical Humanities at the University of Texas Medical Branch at Galveston (UTMB) in 1973 to ensure that humanities teaching and research became an integral part of the education of future scientists and healthcare professionals at UTMB (*4*). In 1993, the General Medical Council in UK also highlighted the importance of the humanities in medicine by suggesting its integration into the undergraduate medical curriculum to foster communication skills, study of ethical and legal issues relevant to clinical practice, respect for patients and colleagues, and patients' rights in all respects (*5*).

Besides efforts in the US and UK, many medical universities in other Western countries, such as Canada,

Released online in J-STAGE as advance publication April 30, 2017.

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Germany, and Sweden, and in some Asian countries like Japan and South Korea, have already recognized the value of medical humanities by integrating humanities teaching into their medical education.

Over the past 40 decades, articles on medical humanities and medical humanities education have been published in many influential journals including *The Lancet*, *Academic Medicine*, *Medical Teacher*, *BMC Medical Education*, *The Journal of Medical Humanities*, and *Medical Humanities*. These articles have extensively examined the role of medical humanities and medical humanities education, the development and evaluation of medical humanities, and the design of a curriculum in medical humanities education in Western countries.

In China, instruction in and research on medical humanities has increased at many medical colleges since the 1980s. The reform of medical education over the past decade has emphasized topics such as medical humanities, life-long learning, and patient-centered learning in an effort to increase the professionalism of future physicians (*6*). However, obstacles such as a lack of organizational independence and a shortage of instructors have delayed the integration of medical humanity courses in medical universities in comparison to the pace in Western countries (*7*). Moreover, many articles have featured a long-running commentary on the medical humanities and frequent calls for a greater emphasis on the humanities in medical education, but most of those articles were in Chinese. In China, researchers have worked in relative isolation and published in disparate journals, so their work has not been systematically presented to and evaluated by international readers.

The six companion articles featured in this issue describe the current status and challenge of medical humanities and medical humanities education in China in the hope of providing international readers with a novel and meaningful insight into medical humanities in China.

In this issue, Yun *et al*. emphasized the importance of using medical humanities in medical policy-making and to guide clinical practice, and those authors called for the launch of a national foundation to support research on medical humanities in China. Liu *et al*. emphasized the importance of incorporating medical humanities education into education for Chinese medical students, arguing that comprehensive medical education that includes both medical skills and humanities could greatly improve medical care. Yun *et al*. described centers and institutes of medical humanities education established in China over the past few decades. Courses such as an online course entitled Introduction to Medical Humanities created at Fudan University in 2015 have benefited almost half a million students from 140 universities.

Given the current tensions in the doctor-patient relationship in China, Wang *et al*. have emphasized the role of medical humanities in clinical practice. They argue that the most efficient way to improve the doctor-patient relationship is to change the emphasis on medical science and to reshape medical humanities. Liu *et al*. raise similar concerns about training in communications skills for Chinese doctors to improve the doctor-patient relationship. They suggest that efforts should be made to teach medical communication in accordance with the requirements of competency-based education.

A thorny problem in China is the mindset that "medical technology comes first", leading to technology-oriented medicine that overlooks humanity in medical practice. This is one of the most pressing issues that China must address. An article in this issue by Song *et al*. goes straight to the heart of this question, insisting that medical humanities play a greater role in medical education to foster medical personnel with humanistic spirit, and measures should be taken to promote the integration of medical scientific spirit and medical humanistic spirit. Moreover, Chen *et al*. recommends the use of films and television shows as a medium to accelerate the spread of medical humanistic spirit.

In the era of biological-psychological-social medicine model, an exclusively technical-scientific approach to education is increasingly considered inadequate for the 21st century doctor. Medical humanities must be added to the medical curricula to foster sensitivity, empathy, and understanding of the human condition among medical students. In China, the integration of medical humanities courses in medical universities has occurred later than in Western countries. The field of education differs considerably between Western countries and China. Here in China, medical students need to be reminded that they need both knowledge of medical science as well as medical humanistic spirit in the care they provide.

The six companion articles featured in this issue are just the beginning. This Journal is calling for greater publication of research on medical humanities and medical humanities education to propel medical humanities in China. These articles and future publications should stimulate ideas regarding both the teaching and learning of medical humanity, thus helping to create better conditions for medical education and to give medical students in China a more well-rounded professional identity.

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*(Received April 25, 2017; Accepted April 27, 2017)*

Policy Forum

DOI: 10.5582/bst.2017.01092

# Emphasizing humanities in medical education: Promoting the integration of medical scientific spirit and medical humanistic spirit

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**Summary** **In the era of the biological-psychological-social medicine model, an ideal of modern medicine is to enhance the humanities in medical education, to foster medical talents with humanistic spirit, and to promote the integration of scientific spirit and humanistic spirit in medicine. Throughout the United States (US), United Kingdom (UK), other Western countries, and some Asian countries like Japan, many medical universities have already integrated the learning of medical humanities in their curricula and recognized their value. While in China, although medical education reform over the past decade has emphasized the topic of medical humanities to increase the professionalism of future physicians, the integration of medical humanity courses in medical universities has lagged behind the pace in Western countries. In addition, current courses in medical humanities were arbitrarily established due to a lack of organizational independence. For various reasons like a shortage of instructors, medical universities have failed to pay sufficient attention to medical humanities education given the urgent needs of society. The medical problems in contemporary Chinese society are not solely the purview of biomedical technology; what matters more is enhancing the humanities in medical education and fostering medical talents with humanistic spirit. Emphasizing the humanities in medical education and promoting the integration of medical scientific spirit and medical humanistic spirit have become one of the most pressing issues China must address. Greater attention should be paid to reasonable integration of humanities into the medical curriculum, creation of medical courses related to humanities and optimization of the curriculum, and actively allocating abundant teaching resources and exploring better methods of instruction.**

***Keywords:*** Medical humanities, medical education, Chinese medical students, healthcare

## 1. Introduction

"To Cure Sometimes, To Relieve Often, To Comfort Always", the aphorism engraved on the tombstone of Dr. Edward Livingston Trudeau (1848-1915) has witnessed a century's time and been shining the light of medical humanities. It is a reminder that the duties of medical practitioners are not limited to the treatment of disease, but also include relieving and comforting patients. "To Relieve Often, To Comfort Always" is an expression of humanistic spirit; if humanistic spirit were to be divorced from medical practice, then the essential goal of medicine would be betrayed.

The past 40 years have witnessed tremendous advances in medical science, including the development of new approaches and devices that have substantially altered the courses of various diseases, the sequencing of the human genome, the development of new biologic agents and approaches to treat cancers and autoimmune diseases, and advances in transplantation (*1,2*). Furthermore, advances in electronics, optics, mechanical equipment, and technology have been extensively incorporated into medical practice, changing ways of thinking, routes of and algorithms for treatment

Released online in J-STAGE as advance publication April 29, 2017.

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of disease, and even the ultimate aims of clinical care to a certain extent. Modern medical technology has saved the lives of countless patients, but medical humanistic spirit has been gradually fading from medicine as a result of the fight against disease. This trend is due to several common phenomena: emphasizing the disease but ignoring patients; emphasizing the treatment but ignoring patient care; emphasizing lab tests but ignoring patients' subjective experiences; emphasizing physical recovery but ignoring psychological changes; and emphasizing the use of technology but ignoring ethical and social considerations (*3-5*).

The mindset that "medical technology comes first" leads to technology-oriented medicine that overlooks the humanistic spirit in medical practice. In the era of the biological-psychological-social medicine model, an exclusively technical-scientific approach to education is increasingly considered inadequate for the 21st century doctor. Any clinician who wishes to be fully prepared to understand and tackle many of the inevitable problems to come cannot avoid the concept that the aim of medicine is always the investigation of disease, and the clinician should always remember that the patient is a human being. An ideal of modern medicine is to enhance the humanities in medical education, to foster medical talents with humanistic spirit, and to promote the integration of medical scientific spirit and medical humanistic spirit.

## 2. Medical humanities and humanities in medical education

In the West, there is now a relatively long history of academic literature on medical humanities (Table 1), definable as an inter- and multidisciplinary field that explores contexts, experiences, and critical and conceptual issues in medicine and health care (*6,7*). As an interdisciplinary group of subjects, medical humanities includes philosophy, law, history, cultural studies, anthropology, religion, arts, and so on (*8,9*).

Research has shown that medical university students with a humanities and science background perform better in practice than those with a science background alone (*10*). Medical humanities provide insight into the human condition, illness and suffering, the perception of one's self, professionalism, and responsibilities to one's self and to others, colleagues, and patients. All of the sciences in medical humanities are key to the quality education of future doctors. The medical humanities can have both instrumental and non-instrumental functions in a medical school curriculum (*11*). Humanities can have an instrumental function when directly applied to the daily work of the clinician. For example, study of the visual arts has been used to improve the ability of the clinician to recognize visual clinical signs of disease in a patient (*12,13*). The humanities also have a non-instrumental function when they lead to general education, personal development, or new ways of thinking beyond the biomedical perspective (*14,15*). For example, study of the humanities has been used to develop self-reflexivity and understanding of the role of the professional in society (*16*).

Medical humanities ought to be a source of encouragement, illumination, and understanding in support of the detailed manifestations of the idea of humane health care (*17*). Famed bioethicist Edmund Pellegrino suggested that the humanities should have a reasonable role in medicine. According to Pellegrino, the humanities should not be regarded as a sign of the gentlemanly character of a physician, a polite veneer overlying medical practice, or a sign of the physician's upbringing; instead, the humanities should be a basic component that physicians possess to make prudent and correct decisions, and that component should be as important as scientific knowledge and skills (*18*). Hence, humanities need to be added to the medical curriculum in light of moral and ethical dilemmas that clinicians face. In order for medical students to develop sensitivity to, empathy for, and understanding of the human condition, humanities must be incorporated into the existing curriculum to balance the largely scientific content, since such subjects act as a vehicle for exploring what it means to be human.

**Table 1. Three stages of development of medical humanities in the West**

| Period: Stage | Typical events |
|---|---|
| End of 19th century ~ Mid-20th century:<br>Initial appearance | ♦ From the end of the 19th century to the early 20th century, academic research began to focus on the interaction between medicine and society;<br>♦ In 1919, William Osler put forward the concept of medical humanities scholars;<br>♦ In the first half of the 20th century, the modern medical system was basically completed;<br>♦ In 1948, Sutton pointed out that medical humanities had an important impact on medical development;<br>♦ In 1951, Wick reaffirmed the important role of the humanities in medical education. |
| Mid-20th century ~ End of 20th century:<br>Rising tide of bioethics | ♦ The mid-20th century featured the turning point of modern medicine;<br>♦ In the 1960s, there was reflection on the negative impact of scientific and technological development;<br>♦ In 1969, the American Society for Health and Human Values was founded with the goal of including human values as a fundamental and definitive component of the education of health professionals;<br>♦ Humanities education was gradually integrated into medical education. |
| End of 20th century ~ The present:<br>Globalization | ♦ Diversification and globalization of medical humanities;<br>♦ Medical humanities education has become an important component of medical education reform;<br>♦ The concept of a healthy humanity was proposed. |

**3. Emphasizing humanities in medical education to foster medical talents with humanistic spirit**

The goal of medical education is to train knowledgeable, compassionate, and well-informed physicians who will serve as healers and leaders by caring for the sick, acquiring medical knowledge, and promoting public health through service to the community and the nation. In the medical school curriculum, the medical humanities are intended to promote a way of being that incorporates personal convictions about one's obligations to others and to the development of a professional identity as a humanistic physician with personal attributes such as compassion, engagement, integrity, respect for patients, and a commitment to their own human flourishing (*19,20*).

Medical humanities can foster medical students' critical thinking as well as their understanding of personal values, empathy, cultural competence, leadership, and teamwork, thus preparing medical students to respond appropriately to complicated clinical problems (*21*). Since incorporating medical humanities into the medical education curriculum promotes the development of empathetic, compassionate, and culturally sensitive physicians, medical humanities are attracting greater attention as part of current medical education (*9,22,23*).

Medical humanities gained institutional recognition with the founding of the Institute of Medical Humanities of the University of Texas Medical Branch at Galveston (UTMB) in 1973 to ensure that humanities teaching and research became an integral part of the education of future scientists and healthcare professionals at UTMB (*9*). The multidisciplinary faculty of the Institute--who currently represent the disciplines of art, drama, history, law, literature, philosophy, and religious studies--teach in all four years of the undergraduate medical curriculum as well as in various residency programs. In addition to its focus on students and residents in the School of Medicine, the Institute has a vibrant graduate program in medical humanities with several joint degree options, including an MD/MA and an MD/PhD program, and the Institute has always included the School of Nursing, the School of Allied Health Sciences, and the Graduate School of Biomedical Sciences in its activities (*24*). Currently, 69 of 133 accredited schools in the United States (US) require that medical students take a course in the medical humanities (*25*).

In 1993, the General Medical Council in the United Kingdom (UK) also highlighted the importance of the humanities in medicine by suggesting its integration into the undergraduate medical curriculum to foster communication skills, study of ethical and legal issues relevant to clinical practice, respect for patients and colleagues, and patients' rights in all respects (*26*). Three professorial chairs are now established in the UK: at the University of Swansea in 'Healthcare and Medical Humanities', at the University of Durham in 'Humanities in Medicine', and at King's College London in 'Medicine and the Arts'. Specialized degrees are offered by Leicester (MA in Medical Humanities), Swansea (MA and PhD programs in Medical Humanities), Bristol (BA in Medical Humanities) and by King's College London (MA in Literature and Medicine) (*27*).

Besides efforts in the US and UK, many medical universities in other Western countries, such as Canada, Germany, and Sweden, have already integrated the learning of medical humanities in their curricula and recognized their value (*28-31*).

In Japan, there are currently 79 undergraduate medical schools, including 50 national/prefectural ones and 29 private schools (*32*). Japanese medical education is 6 years in duration, typically consisting of 2 years of general liberal arts, 2 years of pre-clinical education, and 2 years of clinical education. Most medical students in Japan are fresh graduates from high schools. Although college graduates are also allowed admission at 36 schools, they account for fewer than 10% of incoming students (*32,33*). In Japan, the core curriculum for humanities in medical education includes medical ethics and bioethics, a patient's rights and informed consent, a doctor's obligations and responsibilities, medical safety and risk management, communication and group medical care, and problem-solving and logical thinking. According to the White Paper on Medical Schools in Japan published by the Association of Japan Medical Colleges in 2007 (*34*), 92% (73/79) of medical schools have provided medical humanities education, and 2 schools were preparing to do so. Around 80% of the medical schools created medical humanities courses for incoming students, and 40% did so in the second or fourth year of undergraduate study. In Japanese undergraduate medical education, the first 2 years primarily focus on general education, while the latter years focus on professional medical education and clinical education. Among the medical schools, 29 established a medical ethics courses, 14 provided a course in doctor and patient communication, and 11 provided courses related to literature and medicine, medical anthropology, professional ethics of physicians, and the doctor and patient relationship.

Since the advent of the 21st century, medical humanities has become more diverse and more globally oriented, with increasing attention to exchanges and dialogues between different cultures. This has meant that medical humanities education has become an important part of medical education reform. A 2010 Carnegie Foundation report called for changes in the medical curriculum, insisting that clinical education become more learner-focused and experiential and that clinical training be merged with social sciences and humanities to develop professional values and to encourage students to take a "holistic view of the patient experience" (*35*). In 1998, with the approval of the World Health Organization and the World Medical Association, the World Federation

for Medical Education initiated a program entitled the "International Standard for Medical Education". Pursuant to this program, the "Global Standard for Undergraduate Education" was enacted in 2003 and revised in 2013 (*36*). The "Global Standard for Undergraduate Education" implies that core medical courses should include both basic medical theory and medical practice, and especially basic biomedical, behavioral, and social sciences, basic clinical skills, clinical decision-making skills, communication skills, and medical ethics. These core courses should be established at all medical schools to foster competent medical practitioners.

**4. Emphasizing humanities in medical education is one of most pressing issues China faces**

Since the 1980s, instruction in and research on medical humanities has increased at many medical colleges in China. Teachers and researchers in medical history, natural dialectics, medical ethics, and political theory created a number of medical humanities courses and they developed new areas of research, such as medical cultural anthropology, bioethics, medical aesthetics, and medical literature. Instruction in and research on medical humanities has attracted growing attention from the rest of society. In 2002, the Chinese Ministry of Education held an international symposium on medical education standards to study international standards for medical education and to determine how to adapt international standards to practical research work in China. In 2008, the Ministry of Education and the Ministry of Health jointly issued the "Undergraduate Medical Education Standards - Clinical Medicine (Trial program)" (*37*), which clearly advocates enhancing "behavioral science, humanities and social sciences, and medical ethics courses." The Standards are attempting to comprehensively improve the humanity of and social interaction by medical students in an attempt to foster the next generation of medical and health personnel to develop medical science and provide care.

In line with national guidelines, medical education reform in China over the past decade has emphasized topics such as medical humanities, life-long learning, and patient-centered learning in an effort to increase the professionalism of future physicians. However, the integration of medical humanity courses in Chinese medical universities has lagged behind the pace in Western countries (*38-40*). Although some colleges and universities in China have established institutes or centers of medical humanities, such as the Institute of Medical Humanities of Peking University that was founded in 2008, most schools have not yet established an independent system for medical humanities education (*41*). In Europe and the US, new disciplines are mostly created in the form of projects/programs. Interdisciplinary projects/programs like medical humanities are mostly implemented by teachers from different faculties, and the programs cover course instruction, academic research, and postgraduate training. After a certain period, a special or independent organization will emerge, and this flexible model is conducive to the development of new disciplines. China places considerable emphasis on distinct disciplines, but medical humanities scattered in the disciplines of philosophy, sociology, political theory, education, and traditional Chinese medicine.

In addition, current courses in medical humanities were arbitrarily established due to a lack of organizational independence. For various reasons like a shortage of instructors, medical universities have failed to pay sufficient attention to medical humanities education given the urgent needs of society. In the medical curriculum, humanities account for less than 5% of a student's university education. Moreover, research concerning the current state of humanities education revealed that 55.26% of students chose humanities courses only to earn academic credit (*42*). Courses related to medical humanities, including medical history, medical ethics and law, patient-physician communication, and medical social science, are often considered to be electives, so they are thus ancillary and less important than core courses. These courses tend to be taught separately, with little integration and no overall consideration of their position in the curriculum. Another major issue is the faculty in Chinese medical universities, where lecturers are more likely to specialize in law, psychology, or social sciences but rarely in medicine. Moreover, the current curriculum emphasizes theory but ignores practice. Medical schools are not concerned about the importance of medical humanistic spirit and practice, so they thus fail to include medical humanities in the evaluation system. In light of the urgent need for development of medical humanities, the field has a long way to go in China.

A relatively ideal system of medical humanities education has been established in Europe and the US. Drawing on that example, the following three aspects of medical humanities education should be promoted in China: *i*) new nationwide goals for medical education should be established and the integration of medical science spirit and medical humanistic spirit should be promoted; *ii*) reasonable integration of humanities into the medical curriculum should be vigorously promoted, medical courses related to the humanities should be created, and the curriculum should be optimized; and *iii*) actively allocating abundant teaching resources and exploring better methods of instruction, including problem-based learning, case-based learning, task-based learning, and online teaching and learning.

**5. Conclusion**

The integration of humanities education and medical education is already occurring around the world. Incorporating humanities education in medical education

and fostering competent medical talents with profound humanity are the acknowledged goals of such efforts worldwide. Biomedical technology alone cannot solve the medical problems in contemporary Chinese society. Aspects of and flaws in the social and political culture pose bigger obstacles, and a multi-faceted, multi-disciplinary system of knowledge is needed to overcome those obstacles. Only under the guidance of medical humanistic spirit, medical science can shed its predilection for medical technology and instead provide humane care. This approach is the only way modern medicine can stay true to its original mission of humanely caring for others.

Emphasizing the humanities in medical education and promoting the integration of medical scientific spirit and medical humanistic spirit have become one of the most pressing issues China must address. Greater attention should be paid to reasonable integration of humanities into the medical curriculum, creation of medical courses related to humanities and optimization of the curriculum, and actively allocating abundant teaching resources and exploring better methods of instruction.

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*(Received March 16, 2017; Accepted April 10, 2017)*

Policy Forum

DOI: 10.5582/bst.2017.01087

# Medical humanities play an important role in improving the doctor-patient relationship

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**Summary** **Doctors in China have been wounded or even killed in frequent violence as conflict between doctors and patients has intensified. China has had a massive dearth of medical students over the past decade and doctors are dissatisfied with conditions in their profession. Conditions in medicine are not conducive to medical reform. This paper notes that the main factors affecting the doctor-patient relationship are a lack of humanity in medicine, the predominance of techniques and technologies, and inappropriate administration of hospitals. These factors are related to a lack of medical humanities. This paper describes several steps to make medicine more humane and to help establish a harmonious doctor-patient relationship, including improved humanities education for doctors and medical students, ending the predominance of techniques and technologies, bringing back "humanity" in medicine, and improving the administration of hospitals.**

***Keywords:*** Doctor-patient conflict, medical humanities, doctor-patient relationship

## 1. Introduction

Over the past few years, violence against doctors and other medical personnel has become a serious problem in China (*1*). A 2014 survey by the Chinese Medical Doctor Association indicated that the annual incidence of injuries to doctors rose each year from 2009 to 2014 (*2*). According to the survey, only 27.14% of medical personnel had not experienced verbal abuse or physical injury. The media reported 51 instances of injuries to medical personnel in 2015, with 21 occurred in the month of June alone (*3*). Statistics from the official website people.com.cn indicated that there were 42 instances of injuries to medical personnel in 2016, resulting in the injury or death of more than 60 medical personnel (*4*).

The current trends are evident in China's doctor-patient conflicts: *i*) Conflicts are becoming more violent. There were 7 doctors killed in 2016, including 2 consecutive incidents occurring in May: Dr. Zhongwei Chen, head of Stomatology at Guangdong General Hospital, and Wang Jun, a young doctor at Shaodong People's Hospital, both died as a result of violence by patients or their family. Statistics indicate that such incidents of violence have occurred in 20 provinces. In addition, the frequency of violence has increased, with an injury occurring almost every month (*3*) (Figure 1). *ii*) Some incidents of violence occur in the heat of the moment while others are premeditated. In 2012, a patient who was unable to control his anger murdered a young interne and wounded several doctors at the First Affiliated Hospital of Harbin Medical University. In contrast, the murder of doctors in Wenling and Guangzhou was highly planned. Medical care has become fraught with hostility and violence as doctor-patient conflicts intensify. In addition to direct injury of doctors, family members of doctors have also been injured. In June 2016, the child of a doctor in the City of Yiyang, Hunan Province was lacerated multiple times on the bus by a patient (*5*).

The career choices of medical students have been affected by the violence against doctors. According to a Lancet article, there were 4.73 million medical graduates in China from the beginning of 2005 to the end of 2014,

Released online in J-STAGE as advance publication April 29, 2017.

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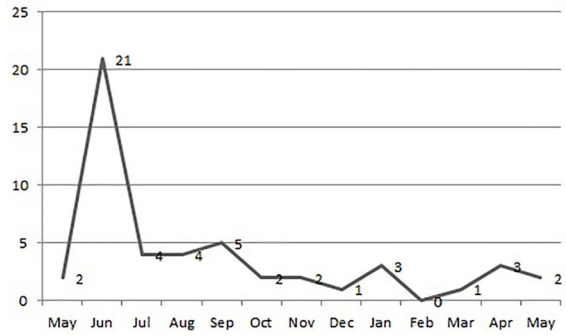


**Figure 1. Frequency of incidents from May 2015-May 2016.** The Beijing News: Deciphering big data reveals general "rules" regarding incidents of violence against medical personnel.

but the number of physicians only increased by 752,000 in the same period. Meanwhile, rural areas in China had a shortfall of over 500 000 physicians (*6*). Moreover, the shortage of doctors may worsen in the future if a large number of medical students have chosen not to pursue medicine.

A 2015 survey indicated that medical personnel are less satisfied with hospital conditions than patients (*7*). Since medical personnel are the cornerstone of China's medical care system, doctors' doubts about their career choices will negatively affect the long-term development of medical care and medical reform in China.

## 2. A combative doctor-patient relationship mainly results from a lack of humanity in medicine, the predominance of techniques and technologies, and inappropriate administration of hospitals

The doctor-patient relationship is a complex social relationship that is affected by numerous factors. Three such factors are a lack of humanity in medicine, the predominance of techniques and technologies, and inappropriate administration of hospitals. All three of these problems are related to the absence of medical humanities. Hence, most efficient way to improve the doctor-patient relationship is to change the emphasis on medical science and to reshape medical humanities (*8*).

### 2.1. *A lack of humanity in medicine*

In a survey to determine the cause of violent behavior by patients, 59.78% of physicians cited patient dissatisfaction with doctors' attitude and poor communication (*2*). According to research on medical complaints in Beijing, Shanghai, and Guangzhou, the lack of humanity in medicine triggered most medical complaints against medical personnel. Incidents often occur when doctors and nurses have improper attitudes towards patients or communication between the doctor and patient is insufficient or poor (*9-11*).

Chinese researchers examined Chinese literature on the doctor-patient relationship from 2003-2012 (*12*). They found that doctor-patient conflicts and disputes mainly stemmed from a lack of humanity among medical personnel. There are numerous other ethical problems in hospitals, such as absence of medical ethics, a lack of empathy from doctors and shirking of responsibilities, and ignoring patients.

Thus, a lack of humanity in medicine is a significant factor contributing to the current conflict between physicians and patients. Physicians should provide humane care, and this approach is the best way to improve their relationship with patients (*12*).

### 2.2. *The predominance of techniques and technologies*

Since the 20$^{th}$ century, medical technology has advanced rapidly and profoundly. On the one hand, doctors are able to use medical equipment to diagnose and treat patients. Over the past few years, various technologies have been used, such as MRI, CT, and radiography. The use of these technologies make diagnosis and treatment more formal and impersonal (*13*). This alters the interaction between physicians and patients. As physicians increasingly rely on medical equipment and data, they gradually communicate less with patients. Doctors pay more attention to medical data for research than caring for patients (*14*). Physicians often ignore patients' wishes and feelings during treatment.

On the other hand, patients mistakenly believe that advanced medical techniques and technologies can be used to cure everything. They also believe that modern medicine can triumph over any disease. When patients believe that medical technologies are a panacea and they place unrealistic expectations on physicians, conflicts and disputes between physicians and patients are more likely to occur. In order to improve the doctor-patient relationship, both doctors and patients should view medical technologies realistically and better communicate through mutual respect and understanding.

### 2.3. *Inappropriate administration of hospitals*

The absence of humanity in medical care also influences patients' experiences. Completing administrative procedures is time-consuming for patients, and this is compounded by unreasonable setting of different departments and unclear instructions (*15*). Patients also complain about inefficient personnel, the disconnect between different departments, and inadequate support, all of which are related to poor hospital administration (*9*).

Moreover, many hospitals are focused on improving facilities (*9*) while not providing adequate support to patients. According to the concept of health promoting hospital, hospitals should improve medical conditions, provide health education tailored to different people's requirements, and interact with the public (*16*). These aspects require hospitals to become more humane in order to meet patients' needs.

## 3. The route to improving humanity in medicine

### 3.1. *Improving medical education by enhancing humanity*

The key to resolving the tension between doctors and patients is reshaping the humanity of medical personnel. Improving medical humanities education is key to reshaping the humanity of doctors (*17*). There are inadequacies with medical humanities education in China. According to a survey, 88.7% of medical students indicated that their medical college does not have a framework for medical humanities education (*18*). In addition, medical education emphasizes knowledge about diseases, anatomy, and other technical subjects, and medical humanities courses account for only 8% of all medical courses (*19*).

Therefore, steps can be taken to enhance medical humanities in different stages of education. Medical colleges should expand their offerings in the humanities and increase the resources they devote to medical humanities education (*20*). Medical humanities must be promoted in conjunction with clinical training (*21*). Medical education should be problem-oriented and combined with clinical case studies. Simulated cases and role-playing are suited to medical humanities education and have gradually increased the level of thinking and behavior by medical students (*22*).

Medical humanities can be promoted in several ways during on-the-job training. Fostering humanity in medicine is a long-term process, and medical personnel can play a significant role in advocating humanity. Colleagues can learn from and compete with one another, thus enhancing their clinical skills and humanity. In addition, medical associations can advocate humanity among medical personnel and establish a standard for humane practices (*23*).

### 3.2. *Ending the predominance of techniques and technologies and bringing back humanity to medicine*

The absolute emphasis on science and reason leads to a predilection for "scientific thought" that obscures the ultimate meaning of medicine, which revolves around "humans"(*24*). Medicine is a combination of natural science and human science in which both are essential. Medical development needs to find a proper balance between technology and humanity, and this requires the joint efforts of all sectors of society.

Medicine itself must return to its origins, and medical ethics must be actively promoted to end "the predominance of techniques and technologies" (*25*). The Doctor-Patient Relationship Course at the University of Chicago examine topics related to the doctor-patient relationship, communication between doctors and patients, and societal issues related to health (*26*). This course places medicine in a broader context in order to end "the predominance of technology" and to heighten awareness that technical and professional development are not the only parts of medicine.

Public discussions are also important since they affect how people view the human side of medicine. In China, the country's most largely read medical newspaper Health News, runs a special column on the human side of medicine. On television, medical documentaries such as "Life Matters" and "The Story in ER" present various aspects of the human side of medicine, helping the public to look at medicine from a fresh perspective. The media also has substantial ability to shape public opinion about doctors by dispelling the image of a doctor as an "angel" and instead providing the public with a more comprehensive view and by explaining the limitations of modern medicine since "medicine is not a panacea."

### 3.3. *Providing humane administration to improve the patient experience*

Only 20% of doctor-patient disputes are caused by medical technology. According to one study, medical equipment has become a greater part of the diagnostic process than communication (*27*). The patient experience is greatly affected by physical conditions, administrative procedures, and the medical procedure the patient is undergoing. Therefore, humanity needs to be fostered in hospital administration.

The internal administration of a hospital should focus on the "patient" and a method of assessment should be established to indicate the subjective quality of care. During treatment, the patient's views should be heeded and the patient's needs should be incorporated in hospital administration. A hospital should also enhance the humanity of medical personnel since their attitudes and mood will affect the relationship between doctors and patients (*28*). As an example, an employee assistance program (EAP) should be incorporated in hospital administration to help hospital employees address personal problems (*29*). Attending to the psychological and emotional needs of staff will help to resolve issues with the quality and efficiency of their work, reduce complaints and negative emotions, enhance the effectiveness of communication, and lead to a harmonious relationship.

Moreover, a hospital can use the concept of "health promoting hospital" to enhance its collaboration with the community and to involve family members and medical personnel in a patient's care. Several Chinese hospitals have implemented such projects. As an example, the Children's Hospital of Fudan University in Shanghai created a "patient counter" to accept feedback from patients at the start of treatment. Hunan Cancer Hospital started a clinical psychiatry project that routinely cares for the mental state of patients. The Second Hospital of Shandong University has cooperated with the community of Jinan to provide personalized health instruction and

health management for residents over the age of 65. The establishment of community-based hospice care is another popular aspect of humane medical care in Shanghai (*30*). Efforts have been made to improve the humane side of medicine and the relationship between doctors and patients, and these efforts should have a positive effect in the future.

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*(Received March 4, 2017; Revised April 16, 2017; Accepted April 18, 2017)*

Policy Forum

DOI: 10.5582/bst.2017.01099

# Using films and television shows with a medical theme as a medium to accelerate the spread of medical humanities

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**Summary** **People have more visual experiences than ever before, and the same is true for situations in medicine. More mature films and television shows with a medical theme have been available over the past 20 years. In mainland China, the TV series "Angel Heart" has generated a wave of universal concern since it truly depicts the work of health care workers and it reflects the sharp distinction between doctors and patients to a certain extent. Riding this wave, many medical documentaries like *The Human World* have also been launched in China and have garnered sizable audiences. Such films and television shows with a medical theme strive to depict the lives of ordinary people. When watching these medical documentaries, audiences are able to better comprehend the work of health care workers in light of their life experiences and feelings towards current society. Audiences can gain a profound understanding of the medical humanities through films and television shows with a medical theme. We look forward to more such films and television shows with a medical theme that depict "hospitals-the realest place" on camera. Films and television shows with a medical theme can serve as a storytelling medium to accelerate the spread of medical humanities and to promote harmony among doctors, patients, and the public.**

***Keywords:*** Medical humanities, television programs, doctor-patient relationship

## 1. Introduction

According to one saying, "Modern life happens on the screen" (*1*). Nowadays, images have become an indispensable part of human life, and people have more visual experiences than ever before. In place of words, images have become a feature of contemporary culture, influencing our attitudes towards everything and changing our views on the world (*2*). The multi-dimensionality, vitality, and directness of images have a deep impact on all spheres of society. Looking at medical practices within the broad context of society and history, medicine has been shaped by social sciences and the humanities for only a century. People started using films and television programs to depict the medical community and society just 3o years ago. An outstanding television program about medicine that successfully integrates medical topics, social values, and views on life will catch the eye of the public, providing a way for people from all walks of life to ponder upon social reality (*3*). People undoubtedly have an easier time understanding medical concepts and ideas conveyed by television programs, so those programs can overcome the barriers between different cultures and become a global language.

More mature films and television shows with a medical theme have become available over the past 20 years (Table 1). As a milestone in the field of medical TV shows, the American series *E.R.* has been continuously shown on TV for 15 years since its debut in 1994. The series House M.D. that debuted in 2004 and the series *Grey's Anatomy* that debuted in 2005 have also won public acclaim not only in the United States but also around the world.

In China, Television Broadcasts Limited (TVB) launched the series *Healing Hands* in Hong Kong in 1998. The series called attention to the practices of medical personnel since it moved its audience through the emotions and feelings of its characters. Starting in 2012, the mainland TV series "*Angel Heart*" really generated a wave of universal concern in China (*4*) because of the

Released online in J-STAGE as advance publication April 30 2017.

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**Table 1. Television programs with a medical theme**

| No. | Program name | Genre | Number of episodes | Country | Debuted | Tagline | Website where the program can be viewed | Number of Clicks on that website |
|---|---|---|---|---|---|---|---|---|
| 1 | *The Human World* | Medical documentary | 10 | China | 2016 | Directly face this imperfect world. | *www.iqiyi.com* (Official link: *www.kankanews.com/z/renjianshi/index.shtml*) | 815 thousand (*Facing This Imperfect World*)<br>961 thousand (*Salvage on the Operation Table*)<br>606 thousand (*Persistence Originates from the Respect for Life*)<br>547 thousand (*A Mother-to-be with Cancer Faces Death*)<br>387 thousand (*Newborns, Pre-mature Infants. and Cesarean Sections*)<br>434 thousand (*Diseases Never Show Children Mercy)*)<br>515 thousand (*A Tough Choice Regarding the Uterus*)<br>535 thousand (*Palliative Care and the Cycle of Life and Death*)<br>479 thousand (*The Difficulty of Receiving A Donated Organ*)<br>566 thousand (*Life Stories in Ambulances*) |
| 2 | *Come, My Child* | Medical documentary | 13 | China | 2014 | A heartwarming and extraordinary glimpse into the process of giving birth. | *www.iqiyi.com* | Total times played: 1.79 million; Score on the website: 8.5 |
| 3 | *Fate of Life* | Medical documentary | 5 seasons | China | 2014 | There is a kind of love called "never giving up". | *www.youku.com* | Total times played (Season 1): 3,092,445; Score on the Website: 8.1.<br>Total times played (Season 2): 8,640,088; Score on the Website: 8.9.<br>Total times played (Season 3): 824,073; Score on the Website: 8.5.<br>Total times played (Season 4): 13,985,386; Score on the Website: 9.2.<br>Total times played (Season 5): 65,284; Score on the Website: 7.3. |
| 4 | *The Story in the ER* | Medical documentary | 2 seasons | China | 2014 | Hospitals are the realest place. | *www.youku.com* | Number of clicks: 241674; Score on the website: 8.0 |
| 5 | *Healing Hands* | Fictional TV series | 3 | Hong Kong, China | 1998 | Real depictions of medical practitioners are shown. They face various relationship conflicts and save countless lives at work. | *www.iqiyi.com* | Number of clicks: 9.441 million; Score on the website: 9.4 |
| 6 | *Angel Heart* | Novel/Fictional TV series | 36 | China | 2002 | Medical practitioners deal with life and death every day. Influenced by love, compassion, and morality, they experience all sorts of vicissitudes of life. | *tv.sohu.com* | Number of clicks: 890 million; Score on the website: 9.0 (scored by 2,699,384 people) |
| 7 | *E.R.* | Fictional TV series | 10 seasons | America | 1999 | Health is linked to disease while life is associated with death. This series depicts realistic and moving stories happening in the emergency room. | Official website: *www.nbc.com/ER* | |
| 8 | *House M.D.* | Fictional TV series | 8 seasons | America | 2004 | | Official website: *www.fox.com/house* | |
| 9 | *Grace's Anatomy* | Fictional TV series | 13 seasons | America | 2005 | Personal relationships among a group of young medical interns and their progression and career challenges. | Official website: *abc.go.com/primetime/greysanatomy/* | |

extent to which it truly reflects the work of health care workers and it reflects the sharp distinction between doctors and patients to a certain extent. Riding this wave, many medical documentaries have also been launched in China and have garnered sizable audiences, including *The Story in the ER* (2014), *Fate of Life* (2014), *Come, My Child* (2014) and *The Human World* (2016). Filmed in a hospital, *The Human World* focuses on the critical choices of doctors and patients in situations involving disease, life, and death. The documentary captures real scenes that are usually inaccessible to the public and it portrays the interactions between doctors and patients in a humane manner. Moreover, the documentary spurs public commentary and discussion. On *iqiyi.com*, the highest click volume of a single episode has reached 961,000, while the average click volume is about 580,000.

These medical TV programs have been well received because they attract the emotional interest of the public. Medical documentaries like *The Human World* accurately reflect the current medical environment and the doctor-patient relationship in China. Instead of focusing on serious medical incidents, *The Human World* opts for topics that are more familiar to the public, thus moving the audience and generating interest in the topic. Such films and television shows with a medical theme strive to depict the lives of ordinary people. When watching these medical documentaries, audiences are able to better comprehend the work of health care workers in light of their life experiences and feelings towards current society. Audiences can gain a profound understanding of the medical humanities throughout films and television shows with a medical theme.

**2. Cultural recognition of the value of life**

Confucius said, "Death will not be understood unless life itself is comprehended". A Taoist proverb says that "Life and death are all people's destiny". Views on life and death are a crucial part of a person's world view, outlook on life, and values. However, death profoundly disrupts values since those values crumble in the face of death. Chinese culture spares no effort to conceal death, and death has become a taboo in daily discourse. Most modern people encounter death only when a close relative dies.

The aforementioned medical TV programs look directly at life and death and extensively cover death in order to describe the possibilities, limits, and risks of medicine. Those programs also depict the concept that life is an accident while death is a must. One such scene can be found in *Grey's Anatomy*, where a patient is on the brink of death. Upon pronouncing the patient's death, Dr. Izzie whispers goodbye, saying "I know you've tried hard, so don't feel sad about it". In some sense, these medical TV programs make up for a shortcoming in Chinese culture by teaching views on life and death. They also teach people to appreciate the joys of life and the serenity of death by depicting life through death, thus making them aware of their humanity.

**3. A humanity that resonates beyond medicine**

For many ordinary Chinese audiences, the experience of going to the hospital may involve "receiving a prescription full of professional terms and symbols and entering through the door of an operating room that is inaccessible to relatives". Medial expertise imbues doctors with mystery and it causes anxiety in patients (*5-7*). When patients place unrealistically high expectations on doctors, they are prone to overreact when faced with disappointment. Against a backdrop of escalating doctor-patient tension, *The Human World* enters the hospital, where it extensively documents the real doctor-patient relationship. Instead of selectively depicting successful cases to imply that man can conquer nature, the documentary presents both successes and failures in an attempt to place doctors and patients on even footing, revealing patients' hardships and yearning for life while revealing doctors' efforts and feelings of helplessness. *The Human World* also enhances people's comprehension of the essence of medical practice, which is "sometimes healing, often helping, and always comforting". The documentary encourages the public to recognize the possibilities and limits of modern medicine, prompting them to reflect on and be cognizant of the vicissitudes of life, thus helping to bridge the gap between doctors and patients.

**4. Multi-channel media have combined forces to promote the spread of medical humanities**

An outstanding TV program is not significant if it cannot be shared with people. Every medical TV program mentioned thus far has garnered recognition from both online media and mainstream media. With the support of various channels of communications, medical concepts can be propagated, doctors and patients can better understand each other, and medical humanities can begin to shine.

New media such as Weibo, WeChat, and video networks are becoming new means of disseminating content, and medical documentaries are using the same approach to attract audiences. Online media helped *The Human World* to become the latest Internet sensation in China in 2016. According to data from *douban.com* (an influential film critic website in China) (*8*), the documentary scores 9.6 out of 10, ranking first in documentaries of the same genre. Weibo posts regarding *The Human World* have sparked thousands of comments, and popular posts forwarded more than 100,000 times continually emerge on WeChat. As one netizen commented, "I was often moved to tears when watching this documentary. There are points that moved

me in every single episode. Though it makes me sad, I will stick to watching this documentary". As another netizen remarked, "There are people who are doing their jobs in hospitals. Many people have endeavored to tell us there are too many unknown stories in a city, too many gaps that medicine cannot fill in an emergency room, and too many interactions that people cannot engage in calmly".

In addition to the extensive attention from audiences and netizens, *The Human World* is also spoken highly of by Chinese authorities and mainstream media. Notices and proclamations have been made by officials from the Shanghai Municipal Government and the Propaganda Department of the Central Committee of the CPC. According to these pronouncements, "A harmonious social atmosphere has a significant impact on further medical reform. *The Human World* looks directly at the limits and problems of medicine as well as sensational topics such as successes and failures of medical practices and life and death. This promotes a correct, objective, and comprehensive understanding of medicine and doctors by the public and it also helps enhance the professionalism of medical practitioners and give them hope. We hope that more programs like this are created to foster a harmonious atmosphere for further medical reform". Authorities have required that domestic media promote the documentary. A spot on the *News Network Show* on July 21, 2016 (*9*) described *The Human World* with the caption "The documentary *The Human World* has garnered extensive public attention". The spot featured remarks like "portraying the real doctor-patient relationship and presenting all aspects of life in a hospital" and "looking directly at problems with and limits of modern medicine as well as sensitive topics like life and death, and garnering considerable attention from the public". Quality coverage has also been provided by *People's Daily*, *Guangming Daily*, *Wen Hui Bao*, *China Youth News*, *Beijing Youth Daily*, *Youth Travel*, *Southern Weekly*, *Xinhua Daily*, *Guangzhou Daily*, *Dazhong Daily*, *Xinmin Evening News*, *Qianjiang Evening News*, *Yangcheng Evening News*, *xinhuanet.com*, t*hepaper.cn*, and *eastday.com*.

## 5. Using films and television shows with a medical theme as a medium to accelerate the spread of medical humanities

The launch of every successful medical TV program starts a public conversation on medicine and lays the foundation to reach a consensus. *The Human World* provides a bridge between doctors and patients and it fosters a better relationship between the two. The documentary was well crafted, as indicated by its veracious coverage and earnest depictions, just like computerized tomography equipment in a hospital. Although fictional, *E.R.* was a landmark among TV series with a medical theme. Every time a new season was launched, audiences and netizens were drawn to it and discussed it passionately. Mainstream media highlighted and pondered over it, and it prompted emotions and pride among medical practitioners. An adaptation from a Chinese bestseller, *Angel Heart* has provided a pathway for communication between hospitals and patients. The Chinese documentary *The Story in the ER* motivates hospitals to open themselves to the public, while patients suffering from diseases are inspired to express their real emotions despite their fear over loss of privacy.

Television programs shape how people spend their time and they also shape the views of audience members. The American TV series *E.R.* and the Chinese documentary *The Human World* have provided successful models for TV programs with a medical theme to deal with topics and morals and to depict expressions of emotion. These programs provide examples of how a program should look and feel and how it can be disseminated. We look forward to more such films and television shows with a medical theme that depict "hospitals- the realest place" on camera. Films and television shows with a medical theme can serve as a storytelling medium to accelerate the spread of medical humanities and to promote harmony among doctors, patients, and the public.

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*(Received April 2, 2017; Revised April 21, 2017 ; Accepted April 25, 2017)*

Review

DOI: 10.5582/bst.2017.01095

# Communication skills training: Adapting to the trends and moving forward

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**Summary** **Communication ability is one of the core requirements of doctors' competency. Teaching communication to medical students and junior doctors has attracted much attention. With the challenge of escalating demands, the status of training communication skills has been promoted in the past several decades. The training content was integrated with other courses and various pedagogic approaches have been applied and proved to be effective. Practical strategies and mixed types were highly recommended. However, there are still many problems, including the fragmentation of the training, insufficient practice, inadequate qualified teachers, case adaptation, course localization and impediment from the environment. This paper proposes some suggestions to solve the problems.**

***Keywords:*** Communication skills, medical education, doctor-patient relationship, curriculum integration

## 1. Introduction

Communication ability is one of the core requirements of doctors' competency in various countries and regions all over the world (*1*). It is also one of the primary fields for medical ethics and medical humanities (*2*). Enhancing medical communication is key to achieve high-quality health care (*3*). With regard to the upgrading of tensions between doctors and patients all over the world, especially in some developing countries, improving doctor-patient communication is becoming a pressing matter of the moment (*4,5*).

Teaching communication to medical students and junior doctors has attracted much attention. How to make the learners achieve a high level of knowledge, attitude and skills of communication in order to better meet current medical needs, is worthy of thinking, research and practice (*6*). The purpose of this paper is to clarify the current state of medical communication and communication teaching, and also to propose some suggestions for the future development of communication education.

## 2. Doctor-patient relationship: changing times and future mission

"Doctor" was once an appellation with halo. Asklepios, the ancient mythical god of medicine, and Chinese legendary ancient empire, Shennong, who was said to taste hundreds of herbs to test the medical effects, both represented the image of divinity, authority and power. Since the Middle Ages of Europe, with the introspection and criticism of the past view on medical philosophy and the mechanism of diseases, the prototype of modern medicine was gradually founded (*7*). Over the past hundreds of years, advanced science and technology provided insights into the nature of human diseases. Effective and safe drugs were developed and treatment experience was accumulated. Consequently, more and more previously untreatable diseases could be treated or even cured. As a matter of course, doctors were to be admired and appreciated due to their life-saving duties.

However, the truth is not that simple. Due to the rapid development of medicine, medical expenses have increased significantly; and the application of information technology provided convenient tools

Released online in J-STAGE as advance publication April 29, 2017.

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for the general public to access medical professional knowledge. As a result, the contradiction between the growth of demands and the relative shortage of medical resources has become increasingly prominent (*8*). The healthcare system is expected to provide a high quality of professionals and services (*9,10*). Unfortunately, doctors are facing much more pressure while the reputation of medical professionalism is not as good as previously seen. It takes more effort for the doctor to gain trust from the patient. The noble professions fall into the secular world unexpectedly, accompanied with escalating demands of communication.

Lewis Thomas (1913-1993), a distinguished physician, medical scientist and medical educator in the United States, wrote the book entitled "The Youngest Science". Medicine deserved the name of "The Youngest Science", accompanied by rapid alterations in its educational philosophy and theories. Experiencing the shift from the first generation of discipline-bound curriculum to the second generation of disciplinary integrated curriculum and problem-based learning, the global medical education was brought to take the third generation of reform, competency-based education (*11*). Every generation is a radical change, ripping the old system while establishing a new one. Currently, competency based education has become a standard for health professional talent training (*12*). Many organizations, including AAMC (American Medical Colleges), GMC (General Medical Council), RCPSC (Royal College of Physicians and Surgeons of Canada), LCME (Liaison Committee on Medical Education) and European Definition of General Practice/Family Medicine outlined communication skills as an essential competency and one of the vital constituents of the medical curriculum. As a matter of fact, the education of medical communication has been entering into the mainstream of the curriculum in many medical schools and is achieving more concern and a positive attitude toward its core value, reflecting a growing trend that could hardly be ignored (*13*).

## 3. Upgraded communication education

The theory and implementation of communication education at medical schools have been developed for over forty years. During its improvement various teaching patterns and teaching methods have been applied, and its educational significance has been recognized by more and more educators.

### 3.1. *Promoted status and integration*

At many medical schools, communication used to be bound to the course of medical ethics or slightly involved in medical psychology (*14*). Perhaps it was provided simply as an optional course or program, implying its dispensable and awkward status. It may show up as an isolated course separated from other courses, standing alone and self-sufficient. It may be provided only in the early stage of the educational plan and disappeared thoroughly subsequently, since it was believed that the students could get the best training automatically when they enter into the clinical setting with a real environment and patients (*15*). However, more and more medical educators realize that what we have done as mentioned above are not enough to meet the demands of fostering qualified doctors.

Nowadays, more and more medical schools have been concerned with the issue of conducting communication skills courses. Relevant programs were developed, thus increasing the course numbers and the proportion of total credit hours for the curriculum. In addition, communication skills training was integrated into the framework of other courses. The most common model for integration is to combine with the teaching of history taking and the training of clinical practical skills (*16*). Although the combination courses usually do not include "communication" as its course name (such as "Doctoring", "the Art of Interview"), the teaching processes present scenarios suited for training communication skills and the teaching objectives target the aims of acquisition of correct knowledge, skills and attitudes of communication. Obviously, communication skills could be taught along with all the activities of doctor-patient interaction (*17*). Moreover, such integration models are more effective and believed to bring more feelings of self-efficacy than the stand-alone courses in the past (*18*).

### 3.2. *Various forms and approaches*

There are many pedagogical patterns applied in medical communication skills training, such as moral education, accumulation of apprenticeship experience, didactic lectures, skill practice and utilization of informed handbooks, *etc*. Since the education of communication was based on skill acquisition, reflecting the intrinsic value of ethics and medical professionalism by means of practice and application, it should be learner centered and conducted based on skill practice. This has been proved to be highly effective.

Traditional didactic lecture are suitable to give outlines and make a brief introduction concerning the concepts and fundamental principles. Other forms using case based learning and practice have the superiority of facilitating the process of internalization, thus reinforcing the training effects. By organizing group discussion, using role-playing, simulated patient/standardized patient (SP) or virtual patients, as well as a variety of simulations or real environments and real patients, the training courses have proved to help students be familiar with the process, strengthen skills, and develop mature strategies to deal with difficulties (*19,20*).

Additionally, formative assessment is carried out in the teaching process. Prompt feedback of students' performance based on practice can help them adjust and improve their behavior and consolidate their achievements. With regard to summative assessment, educators prefer to use self-developed questionnaire, OSCE, SP-based evaluation and some instruments such as SEGUE framework, PCAS, CPCI, PPOS scales, *etc* (*21-24*). They were proved to be high in reliability and validity.

### 3.3. *Booming research*

The training of medical communication skills has been carried out for years, fostering a great deal of relevant research, including studies of effectiveness, evaluations of programs and courses, teaching approaches, assessment of learners, *etc.* (*25*). Annually increasing paper work reflects the growth of interest and achievements in this area. PubMED data showed that the number of articles titled "communication skills" steadily increased during the period from 1997 to 2016, suggesting communication skills have been getting more and more attention (Figure 1). Evidence based research provided guidelines for teaching design and program improvement, also giving some clues to developmental direction.

## 4. Problems and solutions

As educators, we are goal oriented and sincerely hope that we can find the best ways to improve communication, thus leading to better clinical practice. Being conscious about the flaws and disadvantages within and without the educational system help us to find the solution.

### 4.1. *Fragmented instead of systematic*

The training of communication skills at many medical schools are fragmented and less integrated (*16*). Sometimes due to poor top-level design, it is not deliberately considered and properly arranged in the curricular framework. It may only be involved in courses of medical ethics or psychology or just an optional isolated course. Although its importance may be repeatedly mentioned by teachers, whether the learners could acquire the relevant knowledge and skills is doubtful, because the teaching objectives are unclear and the position is improper.

This unsatisfactory situation was caused by outmoded educational concepts about medical communication skills. Even if the administrators and experts realize the importance and urgency, most of them are not likely to have received formal training themselves, thus lacking perceptual experience and rational deduction. Their own learning experience relying on observation, self-perception, trials and errors, and adjustments may lag behind the urgent demands of today.

While designing the curricular framework, the educator should reserve some space for training of communication skills, especially in the parts of doctor-patient interaction, whether it is an integrated curriculum or disciplinary-bound curriculum. The training of communication skills should be arranged in all stages of the educational plan (not only in the early stage) in order to stimulate and support the medical students and junior doctors as well (*26*). Programs implemented during clinical years including the specialty training period, as continuous medical education, could target specific communication problems existing in clinical setting, which may directly benefit the doctor-patient relationship (*13*).

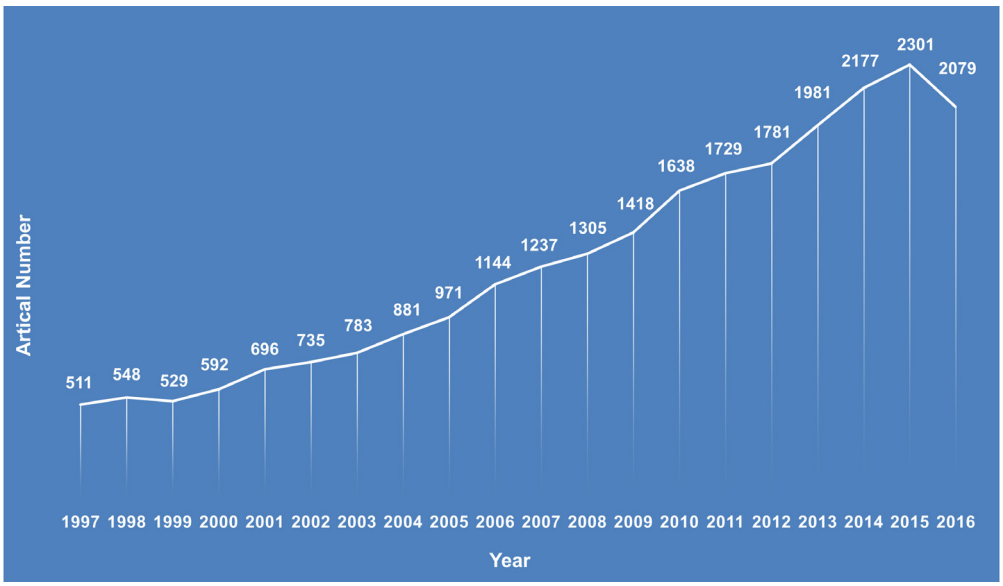


**Figure 1. The number of articles titled "communication skills" between the years of 1997-2016.**

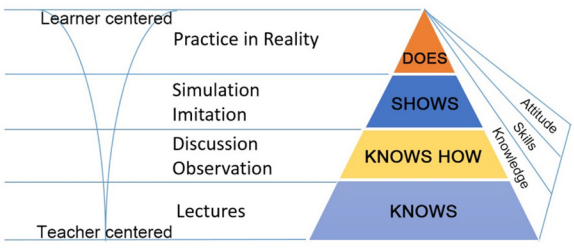


**Figure 2. Miller's pyramid for communication skills training.**

Explicit teaching goals should be set and outcome based requirements mentioned in the grading scheme. The student assessment needs to be included in the evaluation criteria system. Finally we should get a multistage, multilevel and multiple link teaching system of medical communication throughout the whole education process (*27*). The learners could refine their communication skills through repeated training in recognizing and understanding ideas, theories and the processes.

4.2. *Insufficient practice*

Concerning the teaching approach, in China there are still quite a lot of medical schools mainly using didactic lectures. For sure didactic lecture has the advantage of cost efficiency and is easy to design and organize, but its disadvantage is obvious due to an insufficient process of internalization and application (*28*). Derived from Miller's pyramid, didactic lectures could just lead the learners onto the first step, "knows". However, it is the top triangle "does" that we want the doctors to perform. Focusing on practical training, we should also note that different approaches (observation, discussion, imitation, simulation or practice in reality) lead to different effects, because its virtual degree affects the facilitation of self-reflection and internalization (Figure 2).

Written information on paper could simply generate imagination in the learners' brain, so we could add group discussion to enrich the vision and perspectives. Video tapes of simulated patient interviews are provided for perpetual recognition. Visiting clinics and outpatient departments as well as taking clerkships in hospitals bring the students to the real environment and give them observational experience. Simulated medical interviews with standardized patient train and test the learners' abilities of principle adoption and responding on site. Moreover, self-evaluation by reviewing their own videos assists the learners reflecting upon their performance and adjusting. These methods bring various levels of learning experience in an order from low to high. A mixture of the approaches is recommended and the general teaching design is of great importance (*29*).

Teaching designs and methods should match teaching objectives, which need to be deliberately considered. The educational goal is to facilitate deep learning of communication skills. From theory to practice, virtual environment to reality, the road map of communication skills development and progression is constructed by sequentially recognizing, reflection, adjustment and internalization.

4.3. *Inadequate qualified teachers and cases*

Communication skills training has been widely adopted in medical education in various countries and regions for years. However, due to the time of implementation being short and still in continuous improvement, most of the teachers haven't received systematic and formal training as the students do (*30*). In the past, they observed the performance of the supervisors and colleagues in daily working, practiced with real patients, and accumulated valuable experience through trial and error. Undoubtedly, some of them have extraordinary perception and sharp insight. Nevertheless, apprenticeship experience could not ensure them to be qualified communication teachers. And whether their performance in daily work reaches the expert level and is consistent with the principles and norms is unknown. This is particularly the case that teachers might mislead the students if they do not receive regular and formal training.

In addition, no matter which approach was adopted, teaching communication skills could not succeed without proper case utilization (*31*). Certain scenarios should be set to arouse the communication dialogue. Given the task to explain the state of illness or inform the risk of operation, the nature of the disease, the communication objective and the complexity of treatment predetermines the difficulty level of communication. Restricted teaching time should be fully used to maximum teaching effectiveness. As a result, appropriate cases should be developed on purpose, instead of occasional cases generated by chance. Cases presented in books from abroad may not be suitable for direct use due to diversity in culture and background. Case development should take the teaching goal into consideration. It should be based on the level and prior knowledge of the learners and closely relate to the application in reality in order to help solve the practical problems effectively.

4.4. *Impediment from the environment*

Medical students are often influenced by hidden curriculum unconsciously, which shape their professional cognition, attitude and behavior (*32*). A supportive learning environment and appropriate working pressure are helpful for medical students and junior doctors to refine their communication skills (*33*). No matter how well the learner knows and performs in the course of communication, if the supervisor doesn't care about his/her communication endeavor and if the relevant instruction and discussion are insufficiently provided, the

good communication behavior fades even if he/she has the correct ideas and concepts (*34*). In order to solve this problem, a supportive learning environment should be built up. Moreover, the opportunities for self-reflection and critical thinking should also be provided.

4.5. *Adaptation and Localization*

Medical communication skills training belongs to medical ethics, which must be related to the condition of the nation and public, history and culture, religion and customs, and social phenomena. Even language expression habits influence the effectiveness of communication. Although the key issues of medical communication to be solved in different countries are similar, there might be some differences in the specific details and coping strategies. Western and eastern countries have great differences in cultural background, so the teaching materials, cases and communication curriculum from European and American medical schools could not be directly applied to medical schools in Asia. It is really a dynamic process to tailor a curriculum and it needs continual evaluation, feedback, and improvement (*35*).

Additionally, it is essential to have thorough studies on the native medical communication phenomena and existing problems, as well as studies on diagnostic evaluations of the implemented programs and courses (*36*). Taking the results of investigation and evaluation as the reference for curriculum design and teaching arrangements, we are able to better solve the problem of localization for communication skills training.

## 5. Conclusions

The progressions of medical communication education in different countries are not balanced. The western developed countries started early, accumulating much valuable experience based on innovative reforms. Some developing countries and regions, such as China, are still in the initial stage both in research and education, continuously exploring a feasible scheme (*37,38*).

Asia has a dense population and the health care system is far from properly constituted. Lacking human resources, health professionals are facing tremendous pressure. If we do not pay enough attention to the medical needs of the general public at present and in the near future, the cost may be unaffordable.

The third generation of medical education reform emphasizes patient and population centeredness. The reform is featured by competency-based curriculum applied to medical schools globally (*11*). For those who just set out, great efforts should be made on research and practice in teaching medical communication according to the requirements of competency-based education. Only in this way, can we better cope with the changing medical environment in the forthcoming age.

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*(Received April 5, 2017; Revised April 18, 2017; Accepted April 21, 2017)*

Mini-Review

DOI: 10.5582/bst.2017.01085

# Preliminary thoughts on research in medical humanities

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**Summary** **Medical humanities (MH) is an interdisciplinary field of medicine which includes the humanities (literature, philosophy, ethics, history, and religion), social sciences (anthropology, cultural studies, psychology, sociology, and health geography), and the arts (literature, theater, film, and visual arts) and their application to medical education and practice. Studies of MH should not be limited to theoretical discussions. Research results must be translated into use of methodologies to formulate medical policies, guide clinical practices, and help resolve physical or mental problems. MH has a critical role in addressing medicine-related issues, such as human cloning legislation and the treatment of Ebola virus infection. Recently, MH has also been included in the "Healthy China 2030" project, indicating that MH has garnered more attention in China. Medical colleges, research institutes, and non-profit organizations are focusing on MH studies. Over the past few years, financial support for MH studies has also increased. Although the development of MH currently lags behind medicine and health sciences, MH has promise.**

***Keywords:*** Medical humanities, medical education, medical humanities research, China

## 1. Introduction

Medical humanities (MH) provides a humanistic problem-based approach to medicine aiming at influencing its nature and practice (*1*). A combination of medicine and the humanities, MH has arisen along with the rapid development of medicine and health sciences since the 1960s (*2*). With MH increasingly characterized as an interdisciplinary endeavor that draws on the creative and intellectual strengths of diverse disciplines in pursuit of improved medical education and practice, MH plays an important role. However, the worldwide development of MH is still relatively slow. In China, MH started in 80s and has yielded some certain results thus far (*3*). However, MH in China is still mainly focused on MH education and courses. Although studies of MH have been conducted, most are theoretical discussions, limiting their ability to guide health policy or clinical practice. Like medicine, MH must be translated into use of methodologies to formulate medical policies, guide clinical practices, and help resolve physical or mental problems.

## 2. The use of MH in medical policy-making

Cloning was the most controversial medical topic at the beginning of this century (*4*). With the development of cloning technology, human cloning could be performed. As promising as it may be scientifically, human cloning poses a variety of problems that cannot be resolved through an approach defined by narrow disciplinary boundaries but rather through an approach grounded in multiple fields like MH. An anthropological concern is that human cloning violates the principle of sexual reproduction and instead represents asexual reproduction, provoking philosophical and religious debates. In addition, human cloning would disrupt the normal ethics of families. In existing human reproduction, the reproductive model depends on male and female contributions, but cloning only requires genetic material from a single parent, which means that humans can continue to reproduce as long as there are women (*5*). Most countries have prohibited human reproductive cloning experiments through legislation or statements since around 2001 (*6*). MH studies on

Released online in J-STAGE as advance publication April 29, 2017.

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cloning limited the irrational use of the technology and avoided its unintended consequences.

MH also guides government policies. In 2016, the Chinese Government launched the "Healthy China 2030" project, a bold declaration that made public health a precondition for all future economic and social development (*7*). An "expanded concept of health" has also been put forth with an emphasis on medical science as well as on issues of public health, nutrition, and tobacco cessation. A recent effort of this project was the banning of cigarette smoking in public areas. Smoking is not limited to medicine since it also involves sociology and public communication & education. As of March 1st, smoking was banned in public spaces indoors, workplaces, mass transport, and other areas of Shanghai, following Beijing and Shenzhen (*8*). In support of Shanghai's effort, Bernhard Schwartländer, the World Health Organization's (WHO) Representative in China, remarked that "Making Shanghai smoke-free is a necessary decision and China's responsibility as great power." Widespread public support, extensive public education, and public participation are key to the effective prohibition of smoking (*9*), according to Professor Hua Fu, Director of the Institute of Health Communication, Fudan University. The Institute, sponsored by Fudan University's School of Public Health in line with the concept of resource integration and multidisciplinary cooperation, has conducted innovative research on major health problems in the course of social and economic development. In March 2016, the Institute set up a tobacco control research center on the basis of years of tobacco control work and it launched the smoking-free Shanghai Health Communication Project under the leadership of the city government, which in turn lead to the final ban on smoking (*10*).

### 3. The use of MH to guide clinical practices

MH also plays a critical part in clinical practices. One example of this is the response to the Ebola epidemic in West Africa. There was no cure for the disease until mid-2014 when ZMapp, an experimental drug to combat the Ebola virus, was used on a limited basis. In 2014, ZMapp was used to treat 7 individuals infected with the Ebola virus, and 2 of those individuals died (*11*). Due to the small sample size, there is no evidence that the drug is significantly efficacious. ZMapp has demonstrated some benefit thus far, but the drug does not meet the pre-specified statistical threshold for efficacy or further studies (*12*).

In accordance with those findings, some experts have contended that any use of drugs that have yet to be tested for safety in large-scale human trials is unethical and potentially catastrophic. Despite widespread disapproval of ZMapp, Peter Piot, co-discoverer of the Ebola virus, supported its use as an experimental drug, given the unprecedented epidemic in West Africa. From an MH perspective, the unprecedented use of ZMapp is grounded in the principle of compassion, i.e. when a patient is faced with a life-threatening condition, the patient can receive an untested intervention in emergency circumstances (*13*). Therefore, the WHO convened a panel on August 11, 2014 to discuss and assess the ethical implications of use of unregistered interventions that have shown promising results in the laboratory and in animal models but that have not yet been evaluated for safety and efficacy in humans. The panel members unanimously concluded that on both ethical and evidentiary grounds the use of unregistered interventions, *e.g.* ZMapp, would be acceptable, provided that certain conditions are met. In reaching this conclusion, the panel members were mindful that their decision was a departure from the well-established, historically evolved system of regulation and governance of therapies and interventions, an unprecedented event in the history of MH (*14*).

Just as Dr. E.L Trudeau's epitaph famously declares, "To cure sometimes, to relieve often, to comfort always" (*15*). In clinical practices, humane care is considered to be equally as important as the treatment itself. MH education has received a great deal of attention in clinical training of doctors. MH heightens awareness of and appreciation for the "patient as a whole" in medicine. Medical students are also required to graduate with a greater understanding of the art of medicine, the importance of the doctor-patient relationship, the spiritual and emotional dimensions of disease, and the human experience of illness. Exploring the many meanings of the word 'critical', Viney et al. argued for a critical MH characterized by: (*i*) a widening of the aspects of 'the medical' beyond the primal scene of the clinical encounter; (*ii*) greater focus not simply on the context and experience of healthcare but also on their composition at a variety of levels; (*iii*) closer engagement with studies of critical theory and disability, activist politics, and other relevant areas; (*iv*) a belief that the arts, humanities, and social sciences are best regarded not as in service or in opposition to the clinical and life sciences but are productively entangled in a 'biomedical culture'; and, following on from this, (*v*) a commitment to new types of interdisciplinary and intersectional collaboration (*16*).

### 4. Support for MH

At present, medical schools or institutes, such as the Yale School of Medicine and the NYU School of Medicine, mainly provide MH education or conduct MH research. This requires that MH programs consist of experts with different backgrounds. For example, the Medical Humanities Program at Baylor University consists of experts from different backgrounds, such as clinical medicine, philosophy, and art. Think tanks

**Table 1. Top 20 Institutions ranking in the top 1% on Essential Science Indicators (ESI) in clinical medicine and their ranks in social sciences, general in China**

| No. | University | Clinical Medicine | Social Sciences, General |
|---|---|---|---|
| 1 | Shanghai Jiaotong University | 184 | 525 |
| 2 | Sun Yat-sen University | 253 | 661 |
| 3 | Peking Union Medical College | 268 | 833 |
| 4 | Fudan University | 270 | 416 |
| 5 | Peking University | 280 | 258 |
| 6 | Zhejiang University | 405 | 622 |
| 7 | Capital Medical University | 411 | - |
| 8 | Sichuan University | 439 | 1130 |
| 9 | Nanjing Medical University | 469 | - |
| 10 | Huazhong University of Science and Technology | 477 | 938 |
| 11 | The Second Military Medical University | 479 | - |
| 12 | Shandong University | 509 | 1008 |
| 13 | The Fourth Military Medical University | 522 | - |
| 14 | Central South University | 533 | 1170 |
| 15 | China Medical University | 607 | - |
| 16 | Nanjing University | 639 | 894 |
| 17 | Southern Medical University | 675 | - |
| 18 | The Third Military Medical University | 677 | - |
| 19 | Harbin Medical University | 682 | - |
| 20 | Wuhan University | 709 | 802 |

can also fulfill this role (*17*). In a general sense, the most important function of a think tank is to provide the government with intellectual support to assist with policy-making. However, think tanks with an MH background serve in additional roles. In addition to providing theoretical support, think tanks involved in MH help to address specific aspects of medical and health problems and to educate the public about the concept of MH. In other words, these think tanks should become a database for government decision-making as well as a platform for the spread of MH in medical and health practices.

In around the year 2000, medical colleges began to increasingly join with full-fledged universities in China. For example, Shanghai Medical College was incorporated into Fudan University, and Beijing Medical University was incorporated into Peking University. This trend has greatly benefited the development of MH because of academic exchanges between medical and humanities faculties at universities. Over the past few years, more and more centers and institutes of MH have been launched. The Institute of Medical Humanities was established at Peking University in 2008, and it mainly focuses on studying the value of life through an interdisciplinary approach. An online course entitled Introduction to Medical Humanities was created at Fudan University in 2015, and it has attracted considerable attention from around the country in the two years that followed. This course is taught by academics, medical scientists, philosophers, educators, and doctors. A data analysis has indicated that almost half a million students from 140 universities have benefited from this course, reflecting the wide demand for knowledge on MH. Universities undoubtedly provide better support to the humanities. The current authors examined the top 20 Chinese educational institutions ranking in the top 1% on Essential Science Indicators (ESI) in clinical medicine. Thirteen of the institutions were universities and 7 were medical colleges. All 13 of the universities ranked in the top 1% on ESI in social sciences (general) while only one medical college ranked in the top 1% in this field (Table 1). This does not preclude MH from being improved at medical colleges. In fact, MH education needs to be provided and MH research needs to be conducted both in medical colleges and in universities with a medical college. The equivalent academic strength of medical and social sciences offers additional options and chances to develop MH.

MH is not limited to universities and colleges. Some non-governmental organizations participate in policy-making and conduct research. In China, a non-profit organization entitled the China-Dolls Center for Rare Disorders (CCRD, formerly known as the China-Dolls Care and Support Association) was founded by patients with osteogenesis imperfecta (OI) and other rare disorders in 2008. The CCRD provides medical rehabilitation and individualized support to people with OI and other rare disorders, it facilitates self-exploration and empowerment, it engages in public advocacy and policy-making, and it conducts research. The CCRD is working to eliminate the stigma of having a rare disorder, to improve social security, to sponsor legislation, and to promote equal access to medication, education, employment, and society for patients (*18*).

The development of MH is lagging behind medicine, as was mentioned earlier, and the same is true for financial support. Financial support for MH comes mainly from government investment or private donations. More attention has been paid to MH

research in recent years. In 2011, the Royal Society of Edinburgh and the Scottish Government funded the launch of the Medical Humanities Research Network Scotland (MHRNS) in order to enable greater and more sustained collaborative research on MH in Scotland (*19*). The NIH also devised special support for MH. The Glasgow University Medical Humanities Network, supported by the Wellcome Trust, acts as a forum to connect individuals working across a range of disciplines and practices at the University of Glasgow, providing an intersection of medicine, culture, and the arts and humanities. As a global charitable foundation, the Wellcome Trust supports areas such as biomedical science, population health, the humanities, and social sciences. Private endowments are also facilitating MH research at institutions such as the University of California, San Francisco (*20*). The National Science Foundation of China or the National Natural Science Fund of China need to launch special projects to support research on MH in China.

## 5. Conclusion

Critical MH is an approach that argues that the arts and humanities have more to offer to healthcare than simply improving medical education. It proposes that the arts and humanities offer different ways of thinking about human history, culture, behavior and experience that can be used to dissect, critique, and influence healthcare practices and priorities. MH balances medical technology and humanity. On one hand, MH limits the irrational use of technology and it avoids unintended consequences of that use. On the other hand, MH helps to formulate medical policies, guide clinical practices, and resolve physical or mental problems. Achieving such an ambitious goal requires the establishment of an interdisciplinary field in which different people can actually interact and produce worthwhile results. Institutions and organizations are increasingly interested in MH education and research, and financial support is increasingly being given to MH. These trends are promising. Universities should promote MH because they have sufficient resources to do so in terms of medical, social science, and humanities faculties. More financial funds should be allocated to MH. Much is required of MH, and much is expected of it. There is still much work to do.

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*(Received April 5, 2017; Revised April 17, 2017; Accepted April 18, 2017)*

Communication

DOI: 10.5582/bst.2017.01094

# An upcoming program for medical humanities education in Fudan University's School of Basic Medical Sciences

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**Summary** **Ideal medical care requires professional skills as well as appropriate communication skills. However, traditional medical education in medical schools mostly emphasizes the former. To remedy this situation, medical humanities education will be incorporated into education for medical students at Fudan University. Comprehensive medical education that includes both medical skills and humanities may greatly improve medical care.**

***Keywords:*** Medical humanities, medical education, China

An author has opined that if we wish to have a truly humanistic ethic, we need a truly humanistic medicine first (*1*). It will be foreseen that the socio-humanistic approach on a medicine or biological perspective population health science fields will be absolutely necessary for the development of socio-humanistic competences. Humanities faculty, like faculty in undergraduate programs, were expected to focus in teaching and collegial activities at both local and national levels, and promotion were a function of excellence in medicine areas. However, the health-illness process is primarily a social and cultural process where the biological and the psychological are subsumed, and the health-illness process is socially and culturally determined (*2,3*). A program in Humane Medicine has been established in the School of Basic Medical Sciences at Fudan University, and presumably this program will be filled with clinicians with a particular interest in the humane aspects of medicine. Under the new program, the School of Basic Medical Sciences hopes to educate medical students who can reform China's healthcare system and thus increase the public's wellbeing. The program was long anticipated but it was delayed because of a certain level of ignorance regarding developments in medical humanities and scientific conventions. A key aspect of this program is a focus on the potential contributions of medical humanities in clinical practice. Humanities should be clearly connected to medical practice and medical research so that medical students and clinicians comprehend the human aspects of their work. An optimal mixture of professional competencies is needed to teach medical humanities, including personnel with a combination of medical and humanities training and personnel specializing solely in the humanities.

In the Humane Medicine program, learning activities will be designed to meet medical knowledge, skills, and attitudinal objectives. Traditionally, clinical knowledge was taught a lecture-based or problem- or case-based approach, and uncompleted humane medicine knowledge were acquired during in the clinical years. The Humane Medicine program has facilitated an earlier or more extensive approach to learning. Another priority has been introducing medical students to humane medicine activity or course development early in their course. The trend away from the traditional medical student's curriculum approach to humane medicine course learning in medicine has forced to develop innovative strategies in junior medical student's education system.

Implementation of the program must be accelerated to realize the potential of humane medicine. This is especially important because humanistic skills can be learned. Medical schools might consider integrating courses that teach humanistic qualities into their full curriculum and student evaluations like those used at other universities or institutions (*4-6*). The Humane

Released online in J-STAGE as advance publication April 29, 2017.

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Medicine program will provide an essential avenue for lifelong learning by medical doctors and clinicians. It aims to integrate humanistic qualities of individual components of critical appraisal by simulating a real-life event. Healthcare personnel need to focus on and improve their communication with patients and their families; basically, they need to learn how to unite the humanistic aspects of care with its technical aspects; how to be professionals without losing their humanistic identity. The Humane Medicine program also allows students to practice giving and receiving peer review, an opportunity that was not previously available to them.

Students have been sent a very clear message regarding the need for a specific program in humane medicine, and this program is driven by assessment. The program's results and impact will be evaluated by an independent evaluation system. Student progress within the program will be assessed using written examinations, and students will be rated on their ability to apply their knowledge of the basic sciences and skills that underpin clinical medicine to the practice of medicine at an appropriate level. Presumably, a practical examination in medical humanities would be a more accurate test of whether students have the clinical communication skills they need to succeed at the next level of their medical training.

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*(Received April 2, 2017; Revised April 17, 2017; Accepted April 19, 2017)*

Review

DOI: 10.5582/bst.2017.01017

# Transforming growth factor-beta and Forkhead box O transcription factors as cardiac fibroblast regulators

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**Summary** **Fibroblasts play several homeostatic roles, including electrical coupling, paracrine signaling and tissue repair after injury. Fibroblasts have low secretory activity. However, in response to injury, they differentiate to myofibroblasts. These cells have an increased extracellular matrix synthesis and secretion, including collagen fibers, providing stiffness to the tissue. In pathological conditions myofibroblasts became resistant to apoptosis, remaining in the tissue, causing excessive extracellular matrix secretion and deposition, which contributes to the progressive tissue remodeling. Therefore, increased myofibroblast content within damaged tissue is a characteristic hallmark of heart, lung, kidney and liver fibrosis. Recently, it was described that cardiac fibroblast to myofibroblast differentiation is triggered by the transforming growth factor β1 (TGF-β1) through a Smad-independent activation of Forkhead box O (FoxO). FoxO proteins are a transcription factor family that includes FoxO1, FoxO3, FoxO4 and FoxO6. In several cells types, they play an important role in cell cycle arrest, oxidative stress resistance, cell survival, energy metabolism, and cell death. Here, we review the role of FoxO family members on the regulation of cardiac fibroblast proliferation and differentiation.**

***Keywords:*** TGF-β, FoxO, transcription factor, fibroblast, myofibroblast, fibrosis

## 1. Introduction

In cardiac tissue there are three main cell types: cardiomyocytes, vascular cells, and fibroblasts. Fibroblasts represent around 30% of the total heart cell population (*1*). Fibroblasts are flat spindle-shaped cells of mesenchymal origin responsible for extracellular matrix homeostasis (*2,3*). Fibroblasts do not possess a definitive cell marker, so their characterization relies on morphological, proliferative, and phenotypical characteristics (*2*). Although, a protein that is expressed exclusively in fibroblasts is not described, in human and mouse cardiac tissue, fibroblasts can be identified by the expression of collagen-activated receptor tyrosine kinase discoidin domain receptor 2 (DDR2) and intermediate-filament associated calcium-binding protein S100A4 (or fibroblast-specific protein 1 [FSP-1]) (*1,4*). Cardiac fibroblasts play various roles among which electrical coupling, paracrine signaling and tissue repair after injury are the most described (*5*).

In a normal situation, cardiac fibroblasts have low secretory activity and produce extracellular matrix proteins, such as collagen types I and III, laminin and fibronectin. They also synthesize and secrete various metalloproteinases that are responsible for extracellular matrix degradation, and several growth factors and cytokines (*6*). However, upon tissue injury, cardiac fibroblasts differentiate to cardiac myofibroblasts.

Released online in J-STAGE as advance publication February 24, 2017.

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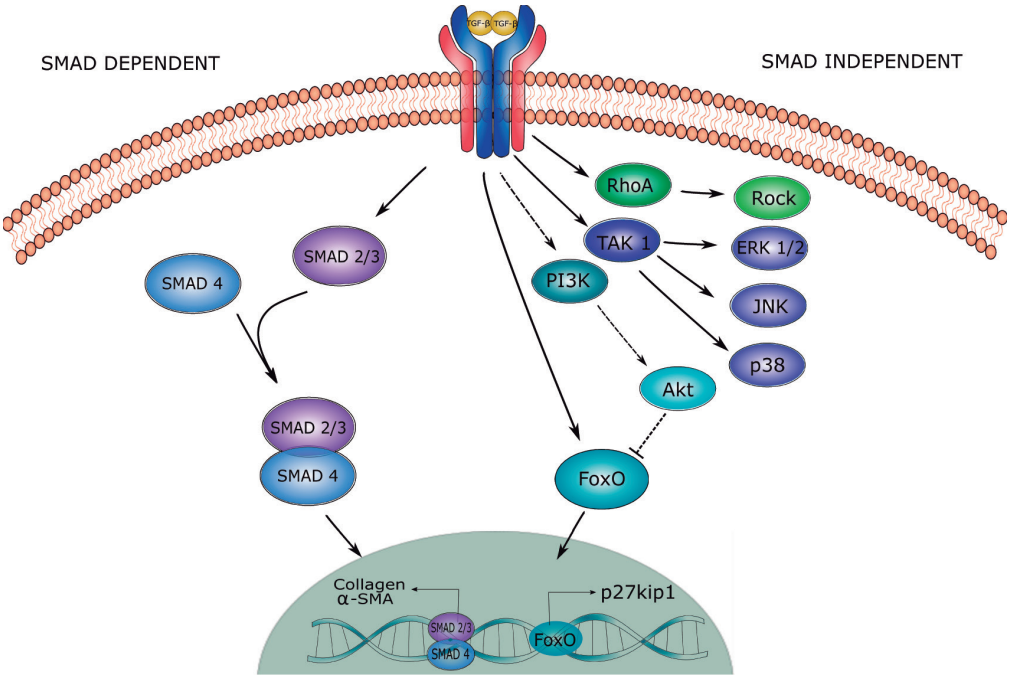


**Figure 1. Smad-dependent and Smad independent signaling pathways triggered by TGF-β.** The canonical transductional pathway for TGF-β involves the Smad-dependent signaling pathway. Once activated, TβRI phosphorylates Smad 2/3 which dissociates from the receptor and binds to Smad 4. Later, Smad 2/3-Smad 4 complex translocates to the nucleus and regulates the expression of several genes, *e.g.* collagen, fibronectin, α-SMA. On the other hand, in the Smad-independent pathway, TGF-β activates TAK1, which phosphorylates and activates ERKs, p38, and JNK. Also, TGF-β activates ROCK by RhoA. New evidence has shown that in T cells, TGF-β1 activates the PI3K/Akt pathway, which phosphorylates and inhibits FoxO transcription factors. But in cardiac fibroblast, TGF-β1 activates FoxO promoting gene expression, *e.g.* p27kip1.

Myofibroblasts are characterized by an augmented capacity to synthesize and secrete collagen and other extracellular matrix proteins important for scar formation and wound healing (*5*). They have lower migratory and proliferative capacity than cardiac fibroblasts (*7*) but with an increased resistance to apoptosis (*8*). Furthermore, from a structural point of view, myofibroblasts have a specialized contractile protein, α-smooth muscle actin (α-SMA), commonly used as a marker for differentiation (*9,10*). Due to the reduced susceptibility to apoptosis, myofibroblasts remain in the myocardial tissue, causing excessive secretion and deposition of extracellular matrix components, increasing myocardial tissue stiffness (*4,11*), which can contribute to the progressive cardiac pathological remodeling (*12,13*). Furthermore, the fibrotic scar can cause disruption of electrical signaling and muscle contraction which eventually result in heart failure (*5*). Participation of myofibroblasts in pathological fibrosis is not limited to the heart (*14*), but is a common hallmark of all fibrotic processes, including lung (*15*) and liver (*16*) among others. Therefore, the control of fibroblast to myofibroblast differentiation is an interesting target in order to prevent adverse consequences of tissue fibrosis and remodeling.

## 2. TGF-β

Transforming growth factor-β (TGF-β) are cytokines belonging to a peptide family with multiple physiological and pleiotropic functions, including inflammatory and immune responses, cell proliferation, growth, differentiation and collagen production (*17-20*). In mammals, three isoform (*TGF-β1*, *TGF-β2*, *TGF-β3*) have been described, each isoform encoded by different genes (*21*). TGF-β1 is expressed in a broad range of cells including fibroblasts, T-cells, B-cells, macrophages, and epithelial and endothelial cells and almost all of them have specific receptors for this peptide (*22-24*). TGF-β1, TGF-β2 and TGF-β3 are released in a non-active latent form (L-TGF-β) that binds to the L-TGF-β binding protein 1 (LTBP1) in the extracellular matrix. In order to be activated L-TGF-β is proteolyzed by thrombospondin-1 or plasmin (*25*). TGF-β1, TGF-β2, and TGF-β3 all function through the same receptor signaling system (*22*). TGF-β1 receptors (TβR) are single transmembrane serine/threonine kinase receptors (*17*). TGF-β1 binding to type II receptor (TβRII) homodimer induces the recruitment of a receptor type I (TβRI) homodimer resulting in a heterotetramer complex. The TβRII serine/threonine kinase catalyzes unidirectional phosphorylation of TβRI, which activates the receptor (*26,27*).

Subsequently, Smad-dependent and Smad independent signaling pathways are activated (Figure 1). The Smad-dependent signaling pathway is the canonical transductional pathway for TGF-β. Activated TβRI phosphorylates Smad 2/3 at the SSXS C-terminal motif. Later, Smad2/3 dissociates from the receptor

and binds to a coSmad, *e.g*. Smad 4. This complex translocates to the nucleus and acts as a transcription factor controlling the expression of several genes (*28*). In the Smad-independent pathway many signaling cascades are described. TGF-β rapidly activates Rho family guanosine triphophatases (GTPases) (*29*). TGF-β dependent activation of RhoA pathway induces the translocation of myocardin related transcription factor A (MRTF-A) to the nucleus to regulate a profibrotic response (*30,31*). TGF-β activates mitogen-activated protein kinases, including (extracellular signal-regulated kinases) ERKs, p38, and c-Jun N-terminal kinases (JNKs) through their upstream kinase activators such as transforming growth factor-β activated kinase-1 (TAK1) (*29*). In human lung fibroblasts, TGF-β induction of endothelin-1 occurs *via* activation of JNK, and endothelin-1 acts *via* the endothelin-A/B receptors to initiate Rac/Akt/Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling, resulting in expression of α-SMA. TGF-β1 can also activate the PI3K pathway which in turn induces Akt by selective phosphorylation at S473, but not at T308. Akt, in turn, phosphorylates and inhibits FoxO transcription factors, associated with the prevention of regulatory T cell differentiation (*32*).

## 3. TGF-β and fibroblasts

TGF-β1 plays an important role in fibrosis, because it mediates the differentiation of fibroblast into myofibroblast (*7,33*). Several line of evidences support the importance of TGF-β in the induction and maintenance of the fibrotic response. In cultured fibroblasts, TGF-β directly induces collagen production and contraction by fibroblasts, including those isolated from heart tissue (*34*). Subcutaneous injection of TGF-β results in enhanced deposition of extracellular matrix (*35*). Treatment of wounds with TGF-β promotes wound closure and scarring (*36*). Moreover, blocking TGF-β with antibodies (*37*) or anti-sense oligonucleotides (*38*) reduces collagen deposition and scarring. Overexpression of TGF-β in heart induces myocardial fibrosis (*39*), while blunting TGF-β1 expression, using TGF-β knock out (KO) mice or dominant negative TGF-β receptor mice, markedly reduced collagen deposition (*40*).

Besides TGF-β1, fibroblast to myofibroblast differentiation also requires mechanical tension, which is regulated by the interaction between the contractile activity of myofibroblasts and the stiffness of the extracellular matrix (*41,42*). Inhibition of mechanical stress triggers α-SMA protein level decrease (*42*), a widely used marker of myofibroblast differentiation. Also, during wound healing, fibroblasts express the ED-A fibronectin, which is crucial for fibroblast to myofibroblast differentiation and for a normal scar formation (*43,44*). Furthermore, it is demonstrated that ED-A fibronectin is necessary, but not sufficient, to induce the myofibroblast phenotype, but it exerts a permissive effect on TGF-β activity (*43*).

## 4. FoxO transcription factors

A recent study in cardiac fibroblasts, Vivar *et al*. demonstrated that TGF-β1 induces differentiation of fibroblast into myofibroblast in a FoxO1 dependent manner (*34*). The forkhead transcription factor superfamily is characterized by a winged-helix DNA binding motif and the forkhead domain (*45*). The mammalian forkhead transcription factors of the O class (FoxO) have four members: FoxO1, FoxO3, FoxO4, and FoxO6. FoxO1 and FoxO3 are expressed in most tissues and cells, including fibroblasts (*46*). FoxO4 is highly expressed in muscle, kidney, and colorectal tissue while FoxO6 has been seen only in the central nervous system (*47*). FoxOs recognize two response elements and binds to the insulin-responsive element (5'-(C/A)(A/C)AAA(C/T)AA-3') with higher affinity than to the Daf-16 family member binding element (5'-GTAAA(T/C)AA-3') (*48*).

Hosaka *et al*. developed KO mice for FoxO1, FoxO3 and FoxO4 (*49*). FoxO1 KO is lethal in embryonic stages due to lack of vascular development. FoxO3 and FoxO4 KO mice are viable and show normal growth in appearance, but have altered lymph proliferation, widespread organ inflammation (*50*) and decline in the neural stem cell pool (*51*), as compared to wild type mice. FoxO3 KO female mice showed irregular ovarian follicle growth causing infertility (*49*). FoxO4 KO exacerbates colitis in response to inflammatory stimuli (*52*). FoxO6 KO mice display normal learning but impaired memory consolidation (*53*).

FoxO transcriptional activity is regulated by different post-translational modifications, mainly through phosphorylation, acetylation and ubiquitination (*54,55*). These modifications can activate or inactivate the function as a transcription factor. They alter its subcellular localization, modify the DNA binding affinity, and change the pattern of transcriptional activity for specific target. The main pathway regulating FoxO activity is the phosphorylation by Akt in three different sites (T24, S256 and S319), which favors their interaction with 14-3-3 adapter protein inhibiting its interaction with DNA and promoting their nuclear export and subsequent degradation by proteasome (*55,56*). Also, deacetylase protein sirtuin, such as SIRT1, can deacetylate FoxO, preventing its phosphorylation by Akt, leading to its activation (*57*).

Additionally to Akt, there are other kinases that can phosphorylate FoxO. ERK (*58*), IkB kinase (IKK) (*59*) and casein kinase 1 (CK1) (*60*) negatively regulate FoxO activity by inducing active exportation of the nucleus to the cytosol. On the other hand, under general stress conditions, other kinases can promote FoxO nuclear import. Oxidative stress induces FoxO

phosphorylation by JNK and Mammalian Ste20-like kinase (MST1), and activates the expression of antioxidant genes such as superoxide dismutase. Also, by nutrient stress, AMP-activated protein kinase (AMPK) phosphorylates FoxO, and induce energy metabolism related gene expression (*45*).

## 5. FoxO and fibroblasts

Several articles have described the role of FoxO in fibroblast physiology. There are only few articles describing role of FoxO in cardiac fibroblast. In order to obtain a more wide perspective of FoxO actions, fibroblast from other sources were included in this review. Therefore, here we summarize the available information and highlight its relevance for fibroblast to myofibroblast differentiation.

### 5.1. *FoxO1*

In a model of iron-overload cardiomyopathy, cardiac fibrosis was observed associated with an increase of α-SMA levels. These data suggest that iron overload induces cardiac fibroblast to myofibroblast differentiation. Interestingly, in these cells an increase in FoxO1 nuclear localization was observed (*61*). In neonatal cardiac fibroblast TGF-β1 induces fibroblast to myofibroblast differentiation, which is completely prevented by FoxO1 inhibition (*34*). In this cell model, TGF-β1 increases in a time and dose dependent manner FoxO1 mRNA and protein levels, induces FoxO1 dephosphorylation, increases FoxO1 nuclear translocation and increases FoxO1 target gene transcription (*34*). Moreover, FoxO1 overexpression enhances TGF-β1 effects on cardiac fibroblasts (*34*). Taken together, these data suggest that FoxO1 is required for TGF-β1 dependent fibroblast to myofibroblast differentiation. Remains to be elucidated whether the participation of FoxO1 is exclusively associated to TGF-β1 or is a general mediator of fibroblast to myofibroblast differentiation.

One of the main skin alteration that take place during wound healing remodeling is the elimination of fibroblasts by apoptosis (*62,63*). FoxO transcription factors play a key role in the regulation of this process (*64*). In primary human dermal fibroblasts, TNFα activates FoxO1 expression, decreasing fibroblast proliferation (*64,65*). Silencing of FoxO1 using a siRNA, reduced TNFα induced fibroblast apoptosis as well as a wide range of TNFα-induced pro-apoptotic genes (*64*). Both results suggest that the ability of TNFα to induce dermal fibroblast apoptosis and to inhibit their proliferation requires FoxO1 activation. Skin damage by arsenic acid activates FoxO1 through a mechanism involving MST1 activation by a reactive oxygen species (ROS) dependent mechanism. Consequently, FoxOs translocate to nucleus and inhibit cell proliferation of mouse skin fibroblasts (*66*). In human foreskin fibroblasts, growth factors such as PDGF, FGF and IGF-I can inhibit the expression of FoxO genes, promoting their proliferation (*67*). PDGF promoted FoxO1 phosphorylation and translocation from the nucleus to the cytosol, and FoxO1 inhibition using a shRNA, led to fibroblast proliferation (*67*). FoxO1 inhibition of human foreskin fibroblast proliferation is partially due to an increase in high-mobility group-box protein 1 expression (*68*). Additionally, in primary human adult dermal fibroblasts, dehydroabietic acid can reverse TNFα stimulated cell responses, including FoxO1 activation, and increase fibroblast proliferation (*65*). Interestingly, in excisional wounds from mice with type 1 or type 2 diabetes, increased levels of apoptosis, TNFα and FoxO1 activation was observed in fibroblasts (*69,70*). In diabetic *db/db* mice, FoxO1 regulates cell cycle genes in a TNFα-independent manner, and FoxO1 inhibition using a siRNA, blocked the TNFα-induced pro-inflammatory genes (*70*). Therefore, in patients with diabetes, FoxO1 could be an important target for the treatment of diabetic foot and other skin complications. Taken together, these results suggest that FoxO1 is an important regulator of wound healing, through the control of fibroblast apoptosis and proliferation. However, in none of these works a relationship between proliferation and apoptosis with fibroblast to myofibroblast differentiation was studied.

Prostaglandin E2 (PGE2) signaling is an important inhibitor of primary fetal and adult lung fibroblast to myofibroblast differentiation, by counteracting the effects of TGF β1 (*71*). Additionally, PGE2 can also reverse the established myofibroblast differentiation (*72*). On the other hand, PGE2 promotes FoxO1 phosphorylation and nuclear export by PI3K/Akt activation (*73*). However, whether FoxO1 is involved in the PGE2-induced reversion of myofibroblast differentiation remains unexplored.

In the liver, a relationship between FoxO1 and the differentiation of hepatic stellate cells to myofibroblasts has also been found. In a model of mice hepatic fibrosis, FoxO1 inhibition in hepatic stellate cells, assessed by an increase in FoxO1 phosphorylation and its subsequent exclusion of the nucleus, induces an increase in α-SMA levels (*74*). This data suggest that in this mice model, FoxO1 inhibits the hepatic stellate cells to myofibroblast differentiation. However, an inversed relationship was observed in humans. Patients with nonalcoholic steatohepatitis had low levels of phosphorylated FoxO1 associated with an increased type 1 collagen levels (*75*). These contradictory evidences point out that the role of FoxO1 in the hepatic stellate cells to myofibroblasts differentiation needs to be further analyzed.

### 5.2. *FoxO3*

In adult rat cardiac fibroblasts, FoxO3a regulates cell

cycle progression by increasing p27kip1 expression, an inhibitor of cyclin dependent kinase, through an ERK1/2 dependent signaling pathway (*76*). In the heart of diabetic mice elevated collagen levels is associated with low levels of FoxO3 (*77*). Additionally, FoxO3 inactivation by resveratrol decreases cardiac fibrosis produced by exercise (*78*). However, in this model, exercise-induced fibrosis is not related with FoxO3 phosphorylation (*78*). All these data suggest that FoxO3 inactivation is associated with a decreased fibrosis, probably by regulating proliferation and apoptosis sensitivity.

In a model of renal fibrosis induced by unilateral ureteral obstruction, accumulation of collagen and increased expression of α-SMA is associated with FoxO3 inactivation (*79*). Moreover, increased FoxO3 expression by silencing microRNA-132, a microRNA that downregulates FoxO3 mRNA, selectively inhibits fibroblast proliferation and decrease renal fibrosis (*80*). Primary fibroblast cultures obtained from idiopathic pulmonary fibrosis (IPF) are characterized by their ability to elude the proliferation-suppressive properties of polymerized type I collagen. This ability involves the aberrant activation of the PI3K/Akt signaling pathway that inactivates FoxO3a, resulting in p27kip1 down regulation and a decreased sensitivity to apoptosis (*81,82*). Moreover, in IPF fibroblasts FoxO3a is further inhibited by microRNA-96 (*83*). Decreased sensitivity to apoptosis is due to caveolin 1 downregulation which decrease Fas levels (*81*). On the other hand, reduced FoxO3 expression is sufficient to generate a hyperproliferative state in IPF fibroblasts (*84*). All these findings show that inhibition of FoxO3 is responsible for the maintenance of the proliferative pathological fibroblast phenotype, which contributes to the development of renal or pulmonary fibrosis.

In the liver, FoxO3 also regulates the proliferation and apoptosis of hepatic stellate cells (*85,86*). TRAIL, a member of the TNFα family, induces FoxO3 activation by promoting its nuclear translocation and thereby increasing apoptosis in the LX-2 cell line, an immortalized human hepatic stellate cells (*86*). Moreover, FoxO3 activation also promotes an increase in p27kip1 expression, causing a decrease in LX-2 proliferation (*85*). Therefore, data support the notion that FoxO3 regulates proliferation and apoptosis sensitivity rather than hepatic stellate cell to myofibroblast differentiation.

In primary culture of human dermal fibroblasts, FoxO3a down regulation by siRNA induces a senescent phenotype (*87*). In the same way, UV treatment of human dermal fibroblasts also induce the senescent phenotype associated with FoxO3 phosphorylation. Prevention of FoxO3 phosphorylation impedes block senescent phenotype induced by UV treatment (*88*). In a model of aging induced by DNA damage in mice embryonic fibroblast (MEF) cells, activation of p53 inhibits FoxO3a transcriptional activity by phosphorylation and subcellular localization change

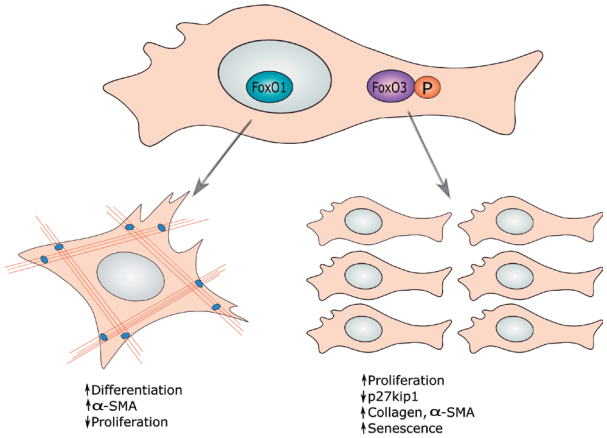


**Figure 2. Proposed actions of FoxO transcription factors in fibroblasts.** FoxO1 regulates fibroblast to myofibroblast differentiation, and increases both α-SMA protein levels and cell proliferation. On the other hand, phospho-FoxO3 (p FoxO3) translocates to the cytosol and promotes cell proliferation by decreasing p27kip1 gene expression. Decrease of FoxO3 activity also increases collagen and α-SMA protein levels and triggers fibroblast senescence.

(*89*). This phosphorylation could be mediated by serum- and glucocorticoid-inducible kinase 1 (SGK1) due to p53-dependent activation of ERK1/2 (*89*). These data indicate that FoxO3a regulates senescence and cell cycle progression in fibroblast.

### 5.3. *FoxO4*

There are only few studies with FoxO4 and fibroblasts. In 3T3L1 fibroblasts, FoxO4 regulates the late steps of cholesterol biosynthesis due to repression of cytochrome P450 sterol 14α-demethylase (CYP51) (*90*). The mechanism involves FoxO4 binding to sterol regulatory element-binding protein 2 to upregulate CYP51 and FoxO4 binding to hypoxia-inducible factor-2 to regulate CYP51 repression (*91*). Therefore, until now, FoxO4 has only been associated to the regulation of cholesterol metabolism in fibroblast.

## 6. Conclusions

Figure 2 shows the general actions that are regulated by FoxO transcription factors in fibroblasts. The main action of FoxO1 seems to be the regulation of the fibroblast to myofibroblast differentiation process, especially in cardiac fibroblasts. FoxO1 is regulated by a SMAD independent pathway downstream TGF-β1 receptor. Moreover, FoxO1 has also been involved in the hepatic stellar cell to myofibroblast differentiation in the liver, and possibly in the PGE2-induced reversion of myofibroblast differentiation. In the other hand, FoxO3 shows a close relationship with proliferation, senescence, and apoptosis sensitization in fibroblasts. FoxO3 deregulation is associated with an exacerbated proliferation of fibroblasts leading to renal, pulmonary, skin and cardiac fibrosis. In liver, FoxO3 has also

been linked to fibrosis by regulating hepatic stellar cell proliferation. Finally, FoxO4 has only been described as a regulator of cholesterol metabolism. Deciphering the role of FoxO transcription factors could be useful to design effective therapeutic approaches for the treatment of different types of fibrosis.

**Acknowledgements**

This research was funded in part by Comision Nacional de Ciencia y Tecnologia (CONICYT), Chile (FONDECYT 1140329 to M.C.; FONDECYT 11160531 to R.V., FONDECYT 1130300 to G.D.A.). IN-S and DM-R hold CONICYT PhD fellowships.

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*(Received January 20, 2017; Revised February 18, 2017; Accepted February 19, 2017)*

Review

DOI: 10.5582/bst.2017.01007

# Key role of liver sinusoidal endothelial cells in liver fibrosis

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**Summary** **Because of the prevalence of viral hepatitis and nonalcoholic fatty liver disease (NAFLD), liver fibrosis has become a very common disease in Asia and elsewhere in the world, constantly increasing the burden of care borne by society. Hepatic sinusoidal capillarization, characterized by gradually shrinking fenestrae on the surface of liver sinusoidal endothelial cells (LSECs) and the formation of an organized basement membrane, is an initial pathologic change associated with liver fibrosis. Basic and clinical studies have indicated that LSECs play a key role in hepatic sinusoidal capillarization by affecting various aspects of the development and progression of liver fibrosis. Reviewing studies on the effect of LSECs on liver fibrosis is essential to better understanding the pathogenesis of liver fibrosis and its mechanism of progression. Moreover, such a review will provide a theoretical basis for identifying new methods to promote the regression or even inhibition of fibrosis. This review will focus on structural and functional changes in LSECs during hepatic sinusoidal capillarization and the interaction between the micro-environment of the liver and the body's immune system.**

***Keywords:*** Liver sinusoidal endothelial cells, capillarization, liver fibrosis, fenestrae, immune system

## 1. Introduction

Liver fibrosis, characterized by excessive deposition of extracellular matrix (ECM),is a common outcome of chronic liver diseases including viral hepatitis, metabolic diseases, and nonalcoholic fatty liver disease(NAFLD) (*1-3*). Thanks to antivirals and hepatitis B vaccines, liver fibrosis caused by viral hepatitis has decreased in recent years. However, other liver diseases that are prevalent worldwide, such as NAFLD, are gradually becoming key pathogenic factors, particularly in Western countries. Thus, a decline in the incidence of liver fibrosis in the near future seems unlikely and liver fibrosis remains prevalent (*4,5*).Early-stage fibrosis can regress to a nearly normal level when its cause is eliminated; in fact, the self-protective behavior of the body allows it to fight pathogenic factors in that it can limit damage to a particular region (*6-8*). However, advanced fibrosis and cirrhosis can lead to irreversible damage to the liver and eventually cause portal hypertension, bleeding of the digestive tract, and even hepatocellular carcinoma (*2,9*). Hepatic sinusoidal capillarization is a basic pathological change associated with hepatic fibrosis and cirrhosis (*10-13*). Hepatic sinusoidal capillarization plays a key role in the pathogenesis and progression of this process, and it can also induce hepatocellular carcinoma along with a newly forming arterial blood supply (*14-16*). Defenestration of LSECs, a typical phenomenon that occurs during hepatic sinusoidal capillarization, plays a unique role in liver fibrosis and cirrhosis. However, the specific mechanism of capillarization and when defenestration occurs remain unclear.

Released online in J-STAGE as advance publication February 28, 2017.

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## 2. Role of LSECs in the progression of liver fibrosis/cirrhosis

### 2.1. *Structure of LSECs and their physiological and pathological function*

LSECs are highly specialized endothelial cells in the human liver. Under normal physiological conditions, LSECs are gateways between hepatocytes and hepatic

sinusoids, they mediate the exchange of plasma, nutrients, lipids, and lipoproteins between hepatic sinusoids and hepatocytes through an ultrafiltration system or the so-called "liver sieve plates", consisting of fenestrae, non-diaphragmed pores that traverse the endothelial cytoplasm (Figures1 and 2) (*17-20*). Generally speaking, the fenestrae are 100-150 nm in size and lack a basement membrane. Their distribution follows some kind of rule, namely larger but fewer fenestrae per sieve plate are seen in the periportal region and smaller but more numerous fenestrae are seen in the centrilobular region (*21,22*). Under some pathological situations, however, their structural and functional features change markedly.

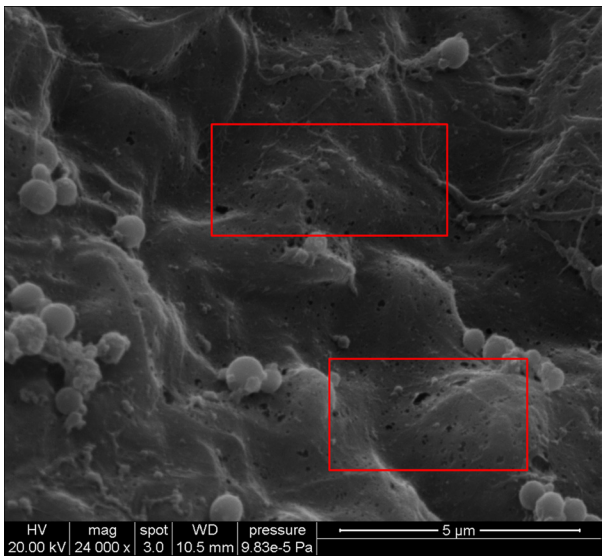


**Figure 1. Fenestrae on liver sinusoidal endothelial cells.** Numerous fenestrae on the surface of normal LSECs were apparent under a scanning electron microscope.

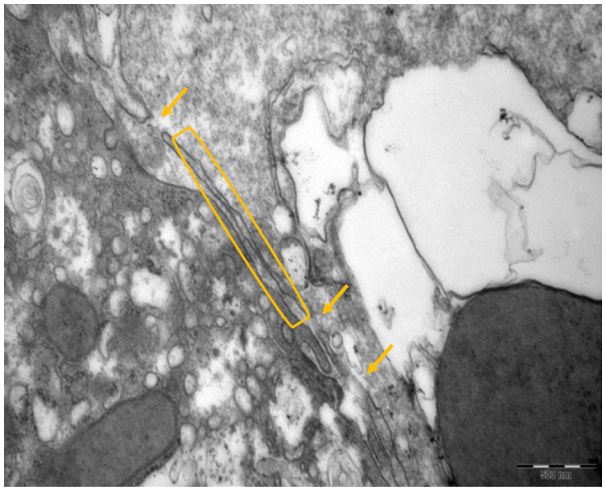

**Figure 2. Absence of a basement membrane underneath LSECs.** Scattered fenestrae (indicated by yellow arrows) on the surface of normal LSECs were apparent under a transmission electron microscope. This photograph also shows the lack of a basement membrane underneath LSECs (the area enclosed with a yellow line).

2.2. *Capillarization of LSECs*

Capillarization, characterized by defenestration and formation of a continuous basement membrane, is a very common phenomenon in chronic liver diseases. Defenestration is a distinctive structural change during this process. Defenestration accounts for a reduction in the number of fenestrae and fenestrae with a smaller diameter. When capillarization occurs, LSECs actively adjust their structures to adapt to pathogenic factors, such as chronic viral infection and toxins that damage the liver. This change can protect the liver from continuing damage by restricting toxins to a specific area, but it also alters the physiological structure of hepatic sinusoids by promoting the formation of a continuous basement membrane. The bidirectional exchange of molecules between hepatocytes and hepatic blood sinus is disrupted, causing problems such as decreased sinusoidal compliance and increased resistance to blood flow that can affect the physiology of the liver. Disruption of that exchange of molecules may also contribute to the development of portal hypertension during cirrhosis by inducing ischemic atrophy of hepatocytes, leading to increased fibrogenesis and compensatory hypertrophy of surrounding hepatocytes. All of these changes may result in the development of hepatic failure.

## 3. Structural changes and triggers of defenestration

Thus far, hepatic sinusoidal capillarization has been regarded as a basic pathological change of liver fibrosis, and defenestration of LSECs is viewed as the main characteristic of this pathological change. As described earlier, shrinking fenestrae and formation of a basement membrane are evident during defenestration. However, what initiates this change, in other words what triggers defenestration, has yet to be fully ascertained. Several studies have indicated that aflatoxin, a risk factor for liver cirrhosis and liver cancer, can significantly damage LSECs and reduce their number (*23-25*). A study by Venkatraman *et al*. indicated that the C-terminal fragment of thrombospondin-1 (P4N1), the ligand of CD47, is involved in defenestration of LSECs through the Rho/Rho kinase-myosin signaling pathway (*26*). CD47 can indirectly induce the decrease or even disappearance of LSECs' fenestrae by inhibiting the eNOS-NO-cGMP system (a key signaling pathway during the formation of LSECs' fenestrae) (*27,28*). Additionally, Addo *et al*. indicated that an iron overload in chronic hepatitis could lead to the formation of nerve growth factors that eventually lead to defenestration by binding to TrKA receptors of LSECs (*29*). Although there are differing opinions on the factors that influence LSECs' defenestration, the exact mechanism remains unknown and needs to be examined further.

**4. LSECs and their microenvironment**

Liver fibrosis is a gradual process that affects the hepatic parenchyma and its microenvironment. In general, the defenestration of LSECs occurs earlier than the formation of fibrous septa. After fibrous septa form, abasement membrane begins to appear. This process indicates that defenestration may be the starting point for liver injury, and the formation of a sub-endothelial basement membrane could be the result of the deposition of ECM during liver fibrosis. By creating a barrier between the hepatic sinusoids and hepatocytes, hepatic sinusoid capillarization decreases the exchange of oxygen and nutrients in hepatic cells, thus worsening damage to the liver. ECM continues to be deposited in Disse's space as the process of hepatic fibrosis progresses. This vicious cycle of liver damage eventually leads to hepatic atrophy and collapse of hepatic sinusoids.

4.1. *LSECs and hepatic stellate cells*

Hepatic stellate cells (HSCs) are one type of nonparenchymal cells of the liver that are close to sinusoids in Disse's space. Since HSCs store retinoids and produce glial fibrillary acidic protein (GFAP), they are also called fat-storing cells or vitamin A-rich cells (*30-33*). Generally speaking, activated-HSCs are known to be a key factor in fibrogenesis. When pathologic liver injury occurs, HSCs convert cellular phenotypes from a quiescent to an activated myofibroblastic state and cause liver fibrosis by secreting fibrogenic cytokines, including tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β) (*34,35*).Normally, LSECs can keep HSCs quiescent through nitric oxide (NO) production stimulated by vascular endothelial growth factor (VEGF) (*36*). However, defenestration and capillarization of LSECs due to liver injury promotes the activation of HSCs, thereby inducing liver fibrosis through loss of VEGF-stimulated NO production. Studies have indicated that LSECs in different states of differentiation affect HSCs differently. That is, differentiated LSECs promote the quiescence of HSCs and they accelerate the regression and prevent the progression of fibrosis, while capillarized LSECs do the opposite (*12,22,36*).

4.2. *LSECs and macrophages*

Hepatic macrophages are important to the pathogenesis of chronic liver injury. The general consensus is that hepatic macrophages can either arise from circulating monocytes or from self-renewing embryo-derived local macrophages, which are also called Kupffer cells (KCs). KCs are usually considered to be a key factor for the initiation and progression of fibrosis. When the human body is subjected to harmful influences such as viral hepatitis and alcohol consumption, KCs can be induced into an activated state in which they secrete a wide variety of proinflammatory cytokines, such as IL-6, IL-10, IL-13, TNF-α, and TGF-β, further activating HSCs. Macrophages can play an indirect role in the development of liver fibrosis (*37-40*). You *et al*. found that KCs can substantially affect liver blood vessel repair by expressing various angiogenic factors and inducing the proliferation and migration of LSECs, thereby accelerating tissue recovery from acute injury (*41*). However, the correlation between LSECs and macrophages in chronic liver diseases and the mechanism of their interaction remains unclear and needs to be examined further.

4.3. *LSECs and T lymphocytes*

There are many classifications of T lymphocytes. In accordance with the differential markers of T cells, T lymphocytes can be divided into two groups, namely $CD4^+$ T cells and $CD8^+$ T cells. Depending on the different functions of T cells in the immune response, T lymphocytes can be divided into helper T lymphocytes (Th cells), cytotoxic T lymphocytes (CTL, or Tc cells), and regulatory T cells (Tr cells). Various T cells have different effects during the progression of chronic liver diseases. Several studies have indicated that $CD8^+$ T and $CD4^+$ T lymphocytes are recruited within the liver in ALD and NAFLD and that T lymphocytes are associated with the prolonging of intralobular inflammation, piecemeal necrosis, and septal fibrosis (*42-45*). In additional, Th cells, and especially the conventional Th1 and Th2 subtypes, also play a key role in fibrosis. Under normal conditions, Th1 and Th2 lymphocytes maintain a dynamic balance that helps to facilitate the body's immune response. When the liver is exposed to infection or toxins for a prolonged period, this balance is disrupted. Studies have indicated that Th2 lymphocytes stimulate the development of hepatic fibrosis after liver injury while Th1 lymphocytes do the opposite (*46-50*). A study by Bonder *et al*. found that Th1 and Th2 cells respectively recruit and use α4β1-integrin and vascular adhesion protein (VAP)-1,two cell surface molecules expressed on LSECs, to adhere to liver sinusoids during liver fibrosis (*51*) (Figure 3).In addition, LSECs can be activated, express adhesion molecules, and synthesize chemokines that are exposed on their luminal surface during inflammation whileVAP-1 can be upregulated by chronic inflammation (*52-55*). However, the relationship between T cells and defenestration of LSECs during hepatic sinusoid capillarization still needs to be explained further.

**5. Liver fibrosis and immunoregulation**

The anatomical organization of the liver is crucial to its immune functions. As everyone knows, the liver

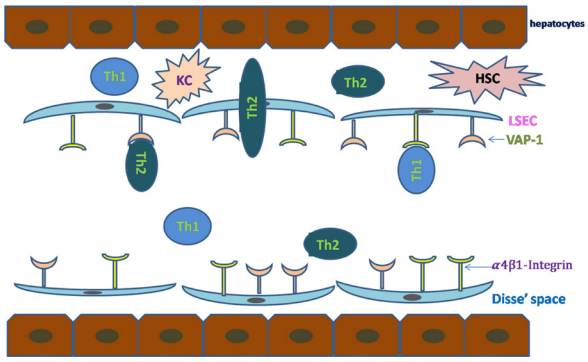


**Figure 3. Diagram of interaction between Th cells and liver sinusoidal endothelial cells.** Note that Th1 and Th2 cells respectively recruit using α4β1-integrin and vascular adhesion protein-1 and adhere to liver sinusoids during liver fibrosis. HSC, hepatic stellate cells; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells; Th1/Th2, helper T lymphocytes; VAP-1, vascular adhesion protein-1.

has two blood supply systems, an arterial system and a portal vein system. Ordinarily, about 30% of all blood passes through the liver per minute, carrying about 108 peripheral blood lymphocytes a day (*56,57*). Because of the unique structure of LSECs, infiltrating lymphocytes can penetrate into Disse's space and even enter hepatocytes by crossing scattered fenestrae; this behavior allows immune cells in the liver to function (*58*). Currently, an innate immune response is viewed as the main factor for the onset of hepatic inflammation in both alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH) (*59*).During HBV-related liver fibrosis, however, the homeostasis of $CD4^+$ T cells is pivotal (*60*).Another critical balance that has attracted increasing attention involves helper T17 (Th17) cells and Tr cells. Several studies have reported that IL-17,a pro-inflammatory factor mainly secreted by Th17, is correlated with the severity of liver diseases(*61,62*). The severity of liver fibrosis is also closely related to the number of Th17 cells (*63,64*). Tr cells are thought to limit liver fibrosis by inhibiting HSC activation and proliferation (*65,66*). Nevertheless, the underlying mechanisms regulating the Tr/Th17 balance during liver fibrosis have yet to be fully explained.

## 6. Conclusion

Thanks to the use of hepatitis B vaccines, hepatitis B-related liver fibrosis will decrease, but liver fibrosis will remain a serious problem because of the prevalence of NAFLD. Hepatic sinusoidal capillarization is a basic pathological feature of hepatic fibrosis and cirrhosis, and defenestration of LSECs is garnering increasing attention as an essential characteristic of that capillarization. However, the exact mechanism of defenestration, the interaction between LSECs and other interstitial cells, and the role of immune regulation in the progression of liver fibrosis still need to be explained further, so substantial work needs to be done in the future.

## Acknowledgements

This project was supported by the National Natural Science Fund of China (81470860, Yuesi Zhong), the Science and Technology Planning Project of Guangdong Province, China (2014A020212575, Yong Zou), and Natural Science Foundation of Guangdong Province, China (2016A030313357, Yong Zou).

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*(Received January 10, 2017; Revised February 16, 2017; Accepted February 23, 2017)*

Original Article

DOI: 10.5582/bst.2016.01252

# Livebearing or egg-laying mammals: 27 decisive nucleotides of FAM168

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**Summary** **In the present study, we determine comprehensive molecular phylogenetic relationships of the novel myelin-associated neurite-outgrowth inhibitor (MANI) gene across the entire eukaryotic lineage. Combined computational genomic and proteomic sequence analyses revealed MANI as one of the two members of the novel family with sequence similarity 168 member (FAM168) genes, consisting of FAM168A and FAM168B, having distinct genetic differences that illustrate diversification in its biological function and genetic taxonomy across the phylogenetic tree. Phylogenetic analyses based on coding sequences of these FAM168 genes revealed that they are paralogs and that the earliest emergence of these genes occurred in jawed vertebrates such as *Callorhinchus milii*. Surprisingly, these two genes are absent in other chordates that have a notochord at some stage in their lives, such as branchiostoma and tunicates. In the context of phylogenetic relationships among eukaryotic species, our results demonstrate the presence of FAM168 orthologs in vertebrates ranging from *Callorhinchus milii* to *Homo sapiens*, displaying distinct taxonomic clusters, comprised of fish, amphibians, reptiles, birds, and mammals. Analyses of individual FAM168 exons in our sample provide new insights into the molecular relationships between FAM168A and FAM168B (MANI) on the one hand and livebearing and egg-laying mammals on the other hand, demonstrating that a distinctive intermediate exon 4, comprised of 27 nucleotides, appears suddenly only in FAM168A and there in the livebearing mammals only but is absent from all other species including the egg-laying mammals.**

***Keywords:*** Central nervous system, genomics, evolution, eukaryotes, FAM168, myelin, neuron, phylogenetic, orthologs

## 1. Introduction

Understanding phylogenetic gene distributions is one of the most challenging aspects of modern genomics-supported taxonomy (*1-3*). A primary goal is to understand the molecular basis of phylogenetics, which is necessary to determine the origins of species (*4,5*). In this context, species specification is crucial for the determination of a potential evolutionary process (*3,6*). Large-scale comparative genomics analyses have revealed that gene duplication and mutations are pervasive sources of genetic changes that underlie phenotypic diversity among species (*7,8*). Despite a longstanding interest in the genetic basis of speciation, little is known about genetic changes in the human lineage or their implications in human evolution theory (*9,10*).

Recent progress in sequencing technologies has provided unprecedented opportunities for exploring genetic differences between primitive and derived species (*11*). The increased availability of new sequence data, *e.g.*, DNA sequences, mRNA expression, and proteins, may not directly provide fundamental knowledge about speciation or interspecies relationships (*12*). However, comparative analyses of sequence data across the phylogenetic tree can provide insights into detailed speciation pathways (*13-16*).

Recently, our group identified and characterized the

Released online in J-STAGE as advance publication April 3, 2017.

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novel human neuronal protein, family with sequence similarity 168 member B (FAM168B also known as myelin-associated neurite-outgrowth inhibitor (MANI)), which is a member of the FAM168 family (*17,18*). FAM168B is localized to neuronal cell membranes and has potential for inhibition of neurite-outgrowth and axonal guidance in the central nervous system (CNS). Our findings also suggested that FAM168B plays an important role in neuronal differentiation of neural stem cells (NSCs) into catecholaminergic neurons (*17,18*). Other studies have recently characterized the human FAM168A gene (also known as tongue cancer resistance-associated protein 1(TCRP1)) in oral squamous cell carcinoma (OSCC) cells (*19-21*). These studies demonstrated that FAM168A mediates specific resistance to cisplatin in Tca8113 cells by reducing cisplatin-induced apoptosis (*20,22*). Available data show that FAM168A and FAM168B have distinct physio-biological functions, even though they belong to the same gene family and exhibit very high gene homology. Accordingly, we performed comparative genomic and proteomic sequence analyses to explore further potential functional implications of FAM168. Phylogenetic analyses of this gene family across the entire eukaryotic tree of life revealed the phylogenetic origins and taxonomic relationships of and among the species carrying these genes, demonstrating that a distinctive intermediate exon, comprising 27 nucleotides (nts) only, appears in FAM168A and defines livebearing mammals.

## 2. Materials and Methods

### 2.1. *Materials*

The human chromosome (chr) dataset used for the genomic, proteomic, and phylogenetic analyses in the present study was collected from the public database of the National Center for Biotechnology Information (NCBI). The dataset for FAM168A, which is located on chr 11, has access number NC_000011.10 (chr 11, GRCh38), and the dataset for FAM168B, which is located on chr 2, has access number NC_000002.12 (chr 2, GRCh38). Details of FAM168 gene IDs, mRNA, and protein sequence sources used in this study are provided as Supplementary materials (Figure S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*).

### 2.2. *Data analysis and sequence alignments*

In the present study, we used several genome viewer tools, including Integrative Genomics Viewer (IGV) (*23,24*), NCBI Map Viewer (*25-28*), the UCSC genome browser (*29,30*), and ClinVar (*31*) for the visualization and analysis of genomic data. For local alignments, a whole human genome analysis of FAM168 gene family homology search was performed using NCBI's BLAST program, which finds regions of local similarity between sequences (*32,33*). For global multi-alignments, the retrieved mRNA and protein sequences were aligned using multiple alignment tools, including Clustal Omega (*34*) and MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (*35*). All standard parameters were unchanged unless stated otherwise. The alignment was optimized manually according to previous knowledge of exons and coding sequences (CDSs) based on visualizations using genome viewing tools such as IGV and NCBI Map Viewer.

### 2.3. *FAM168 analysis in the genus Homo*

In order to analyse FAM168 genes in *Homo*, including *Homo Neandertalensis* and Denisovan samples, FASTQ reads provided as BAM files by the Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany (eva.mpg.de), were extracted and realigned locally to the GRCh38 assembly of *H. sapiens* with the Bowtie2 aligner. All standard parameters were unchanged unless stated otherwise (*15,36*) (Supplementary methods).

### 2.4. *FAM168 in phylogenetic analysis*

We developed a special tailored C++11 application with an embedded Burrows-Wheeler Aligner as the central component (*15,37*) (Supplementary methods). Using the integrated aligner, our application allowed for the tracing of individual sequences within a taxonomic context. We relied on a taxonomy offered by NCBI that can be reconstructed based on a foundation of publically available database dumps (*38,39*). The generated phylogenetic trees were visualized and analyzed using Archaeopteryx (*40*). Additionally, we retrieved the genomes of species within the taxonomy from assemblies offered by NCBI (*28,38,39*), and all data retrievals were conducted automatically by evaluating database-information available from NCBI (*15,41,42*).

## 3. Results

### 3.1. *Chr loci of FAM168 genes*

Whole human genome analysis of the FAM168 gene family using a small nt homology search in BLAST revealed that there are two members of this gene family, FAM168A and FAM168B. Both FAM168A and B are transcribed on the reverse strand (Figure 1). Comparisons of all exons of FAM168A and FAM168B showed that the two gene members are paralogous in humans, and that two intermediate exons are missing in the longest isoform of member B (NM_001009993.3) having 5449 nts in its mRNA and 195 amino acids (aa) in its protein with respect to the longest isoform of member A (NM_001286050.1) and having 7305 nts in its mRNA and 244 aa in its protein (Figures

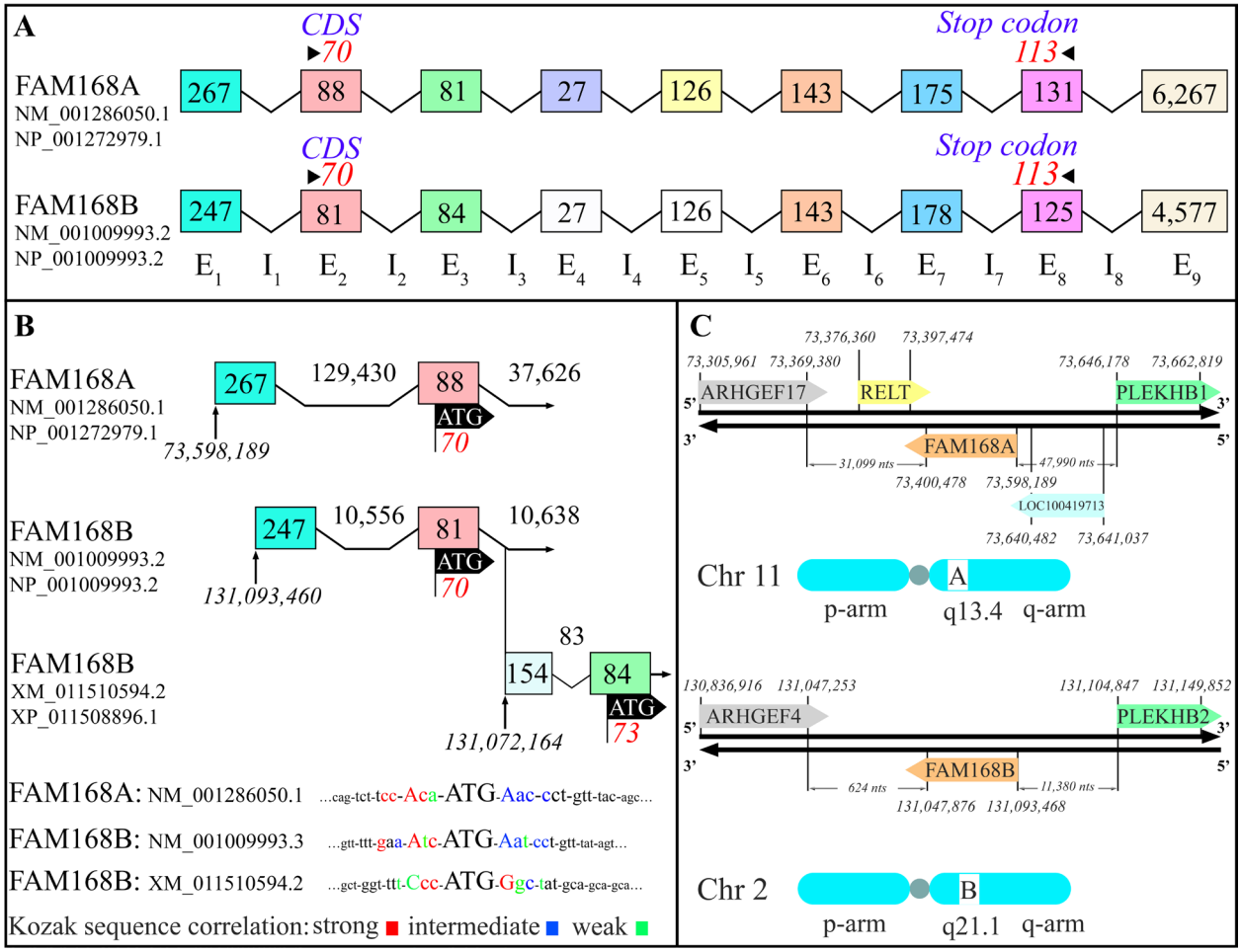


**Figure 1. Gene structures of FAM168A and FAM168B. (A)** Homology between FAM168A and FAM168B. The CDSs start from exon 2 (E2) and stop at E8 for both genes. Filled boxes of different colors indicate homologous sequences present in both genes, whereas the empty boxes of FAM168B (E4, E5) indicate that these two exons are absent in FAM168B but present in FAM168A. For consistency, exon and intron counts for FAM168B were made with respect to FAM168A. The intermediate two exons and two introns of FAM168B (E4, E5, I4, and I5) are missing with respect to FAM168A in the livebearing mammals. **(B)** Transcripts of start codons and Kozak consensus sequence of FAM168s. A newly predicted short transcript of FAM168B (168 aa) has a relatively strong Kozak consensus sequence, as do the longer transcripts. This short version remains to be confirmed by further experiments. **(C)** Comparative human genomic loci of FAM168A and FAM168B. FAM168A is located in the q arm of chr 11, whereas FAM168B is located in the q arm of chr 2. Neighboring genes of FAM168s belong to members of the ARHGEF and PLEKHB families, respectively. An additional gene, RELT is present between FAM168A and ARHGEF17, but not between FAM168B and ARHGEF4, thus suggesting a possible function of FAM168A in the immune system.

1 and S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). According to isoform analysis, all isoforms of FAM168A have the same start and stop codons, whereas variation was observed in the intermediate exons for both validated and predicted isoforms (Figures 1 and S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Similarly, all validated and predicted isoforms of FAM168B have the same start and stop codons, except the predicted short isoforms XM_017003328.1 and XM_011510594.2 (Figures 1 and S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*).

### 3.2. *FAM168 transcripts*

According to NCBI's GRCh38.p7 gene assembly, FAM168A has validated short mRNA transcripts of 7305 nts (244 aa), 7278 nts (235 aa), and 6960 nts (129 aa) as well as predicted (using NCBI's Gnomon software (*43,44*)) short isoforms of 6571 nts (193 aa) and 6406 nts (138 aa) using the same ATG-start codon with an alternative splicing pattern (Figures 1B, S2, and S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). This observation is similar to the earlier GRCh38.p2 gene assembly, which also predicted a short FAM168A transcript (6776 nts, 193 aa) using the same start codon as the long transcript (7305 nts, 244 aa).

For FAM168B, GRCh38.p7 gene assembly analysis showed multiple transcripts with CDSs for 195 aa (*e.g.*, 5449 nts, 5590 nts, 5884 nts, 5331 nts, 5338 nts, 5258 nts, 5207 nts, and 5927 nts) using the same ATG-start codon with an alternative splicing pattern

**Table 1. Lengths and GC and AT contents of FAM168A (NM_001286050.1) and FAM168B (NM_001009993.3)**

| Exon | # nts in FAM168A | | | # nts in FAM168B | | |
|---|---|---|---|---|---|---|
| | length | GC% | AT% | length | GC% | AT% |
| 1 | 267 | 73.40 | 26.60 | 247 | 78.94 | 21.06 |
| 2 | 88 | 55.68 | 44.32 | 81 | 39.50 | 60.50 |
| 3 | 81 | 58.02 | 41.98 | 84 | 51.19 | 48.81 |
| 4 | 27 | 40.74 | 59.26 | – | – | – |
| 5 | 126 | 57.14 | 42.86 | – | – | – |
| 6 | 143 | 56.64 | 43.36 | 143 | 62.23 | 37.77 |
| 7 | 175 | 61.14 | 38.86 | 178 | 62.92 | 37.08 |
| 8 | 131 | 60.30 | 39.70 | 125 | 63.20 | 36.80 |
| 9 | 6,267 | 48.69 | 51.31 | 4,577 | 45.48 | 54.52 |
| 5'UTR | 285 | 71.92 | 28.08 | 258 | 75.98 | 24.02 |
| CDS | 735 | 57.95 | 42.05 | 588 | 59.18 | 40.82 |
| 3'UTR | 6,285 | 48.73 | 51.27 | 4,589 | 45.47 | 54.53 |

(Figure S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Additionally, the predicted short transcripts of 168 aa (*e.g.*, 5541 nts and 5250 nts) were observed using the fourth in-frame ATG-start codon of its full-length transcript (Figure S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Importantly, the start codon of the short prediction of 168 aa also has a relatively strong Kozak sequence compared with the start codon used by the longer transcript (Figure 1B).

### 3.3. *FAM168 CDSs and protein sequence comparisons*

A comparative CDS analysis of the longest isoform of FAM168A (NM_001286050.1, NP_001272979.1) and FAM168B (NM_001009993.3, NP_001009993.2) showed that two intermediate exons comprised of 27 and 126 nts are missing in FAM168B compared to FAM168A (Figures 1A, S2, and S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Protein sequence comparison of the two FAM168 proteins encoded by chr2 and chr11 also revealed significant differences at the protein level based on nt differences in the CDSs (Figure 1 and Table 1).

### 3.4. *5'- untranslated regions (5'-UTRs) and 3'-UTRs of FAM168*

According to UTRs analysis, 5'-UTRs are significantly shorter than 3'-UTRs for both FAM168A and FAM168B (Table 1). A comparison of the 5'-UTR of FAM168A (285 nts of NM_001286050.1) with the 5'-UTR of FAM168B (258 nts of NM_001009993.3) indicated that they are not significantly homologous (Figures S4 and S5, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Likewise, 3'-UTRs of FAM168A (6,285 nts of NM_001286050.1) and FAM168B (4,589 nts of NM_001009993.3) were also not significantly homologous (Figures S4 and S5, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*).

### 3.5. *GC and AT content of FAM168*

A comparative analysis of GC and AT contents of FAM168A and FAM168B showed that their respective percentage contents have high similarity except for two intermediate exons of FAM168A containing 27 and 126 nts, which are missing in FAM168B (Table 1). In the 5'-UTR regions, GC% (71.92% for FAM168A and 75.98% for FAM168B) are significantly higher than AT% (28.08% for FAM168A and 24.02% for FAM168B) for both FAM168A and FAM168B. In the CDSs, GC and AT contents are almost identical for FAM168A and FAM168B, respectively. However, GC content is higher than AT content in both FAM168A and FAM168B. Interestingly, exon 2 of FAM168B, containing the start codon, has a relatively low GC content of about 39.5%, while the average GC content of a gene is usually in the range of 50%-60% (*45*). On the other hand, GC contents (48.73% for FAM168A and 45.47% for FAM168B) are lower than AT (51.27% for FAM168A and 54.53% for FAM168B) contents in the 3'-UTRs (Table 1).

### 3.6. *Closest neighboring genes of FAM168*

Analyses of neighboring loci of human FAM168 revealed complex relationships of FAM168A and FAM168B with their neighbor genes. We observed that the common genes pleckstrin homology domain containing B1 (PLEKHB1) and PLEKHB2 reside upstream of FAM168A and FAM168B, respectively (Figure 1C). The intermediate length between FAM168A and PLEKHB1 (47,990 nts) differs from that between FAM168B and PLEKHB2 (11,380 nts). Moreover, based on NCBI's GRCh38 gene assembly, we observed that the cutaneous T-cell lymphoma-associated antigen (CTAGE) family member 5, a pseudogene (LOC100419713), is situated between FAM168A and PLEKHB1, whereas no other genes were observed between FAM168B and PLEKHB2. A similar phenomenon is observed in the downstream analysis of FAM168A and FAM168B (Figure 1C). The common genes Rho guanine nucleotide exchange

factor 17 (ARHGEF17) and ARHGEF4 (members of the ARHGEF gene family) reside downstream of both FAM168A and FAM168B. However, the receptor expressed in lymphoid tissues (RELT) gene is located only between FAM168A and ARHGEF17, not between FAM168B and ARHGEF4, thus indicating a possible function of FAM168A in the immune system. The intermediate length between FAM168A and ARHGEF17 (31,099 nts) significantly differs from that between FAM168B and ARHGEF4 (624 nts).

### 3.7. *FAM168 in Homo*

In search of the closest hominin relative of *H. sapiens*, recent discoveries of genomic data obtained by sequencing ancient DNA from Neandertal and Denisovan fossils might enable us to answer longstanding questions about the relationships between archaic and modern humans (*46,47*). In the present study, we analyzed the FAM168 gene family of *H. sapiens* and compared it with data from Neandertals and Denisovans. FAM168A and FAM168B are found in both Neandertals and Denisovans, although with different mutation patterns (see Tables S1 and S2 for details, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Exon analyses among groups indicate that all exons of FAM168A are identical among the three *Homo* genomes except E7 and E9, whereas all introns of FAM168A display a number of mutations (Figure S6, Tables S1 and S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). *H. sapiens* differs by one nt from Neandertals and Denisovans in E7 of FAM168A. However, variations in E9 of FAM168A remain elusive, where three nts of *H. sapiens* differ from Neandertals and Denisovans, six nts of Neandertals differ from *H. sapiens* and Denisovans, and four nts of Denisovans differ from *H. sapiens* and Neandertals (see Tables S1 and S2 for details, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). In the case of FAM168B, all exons are identical among the three *Homo* genomes except E9. For consistency, exon and intron counts for FAM168B were made with respect to FAM168A. The intermediate two exons and two introns of FAM168B (E4, E5, I4, and I5) are missing with respect to FAM168A in the genus *Homo*. The variations in E9 of FAM168B include *i*) one nt difference in *H. sapiens* compared with Neandertals and Denisovans, *ii*) Neandertals differ by three nts from Denisovans and *H. sapiens*, and *iii*) Denisovans differ by one nt from *H. sapiens* and Neandertals. Intron analysis of FAM168B showed a number of mutations in all introns except I6 and I8 (see Figure S6, Tables S1 and S2 for details, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Although Neandertals and Denisovans are considered the closest hominin relatives of *H. sapiens* (*48*), our genomic comparison does not indicate which archaic human is more closely related to *H. sapiens*. Nevertheless, growing evidence suggest that Neandertals are more closely related to *H. sapiens* than are Denisovans (*15,49,50*).

### 3.8. *FAM168 in phylogenetic analyses*

We conducted a comparative genomic analysis to explore the phylogenetic distribution of the FAM168 gene family among the eukaryotes. The phylogenetic relationships of FAM168 gene families among different species are displayed in the form of taxonomic clusters, or dendrograms, through CDS sequence alignments (*15,41,42*) (Figures S7 and S8, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Aligned sequences appear as taxonomic cluster blocks of high correlation along the diagonal axis and correspond to taxonomic groups (Figure 2). According to the phylogenetic tree, both FAM168A and FAM168B formed five distinct ortholog clusters of fish, amphibians, reptiles, birds, and mammals. Interspecies correlations showed that both genes have similar phylogenetic patterns from lower species to higher species. The sequence similarity matrix in the dendrograms is colorized as a heat map (Figure 2). Within the mammals, FAM168A shows higher interspecies correlation than FAM168B. Interestingly, among the birds, *Passeriformes* showed a distanced sub-cluster ortholog for both genes with higher sequence similarity (Figure 2). Within the reptiles, two distinct sub-clusters are formed by the homologs of FAM168 genes, one including turtles and crocodilians and another within lepidosaurs. Unlike the mammals, available genomic data reflects that FAM168B has higher interspecies correlation in reptiles than does FAM168A. In the context of phylogenetic relationships between fish and reptiles, the intermediate class of amphibians (*e.g.*, tropical clawed frog) also contains both genes (*51*). The earliest apparent emergence of both FAM168A and FAM168B is observed in the jawed vertebrates, represented by *Callorhinchus milii* (elephant shark) (gene ID: 103177153 for FAM168A and gene ID: 103174665 for FAM168B) (*25,52*) (Figures 2, S7 and S8, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Analysis of the available molecular phylogenetic data suggests that the FAM168 gene pair emerged in vertebrates with a notochord and neural tube (*53*).

Our comparative genomic and proteomic analyses showed that two intermediate exons comprised of 27 and 126 nts (E4 and E5), respectively, are missing in FAM168B with respect to FAM168A in humans (Figures 1A, S4 and S8, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Further phylogenetic analysis of the individual exons in the eukaryotic lineage revealed that exon 4 of FAM168A, comprised of 27 nts (E4, translated into nine aa: EFQFLHSAY), is present in the livebearing marsupial *Monodelphis domestica*

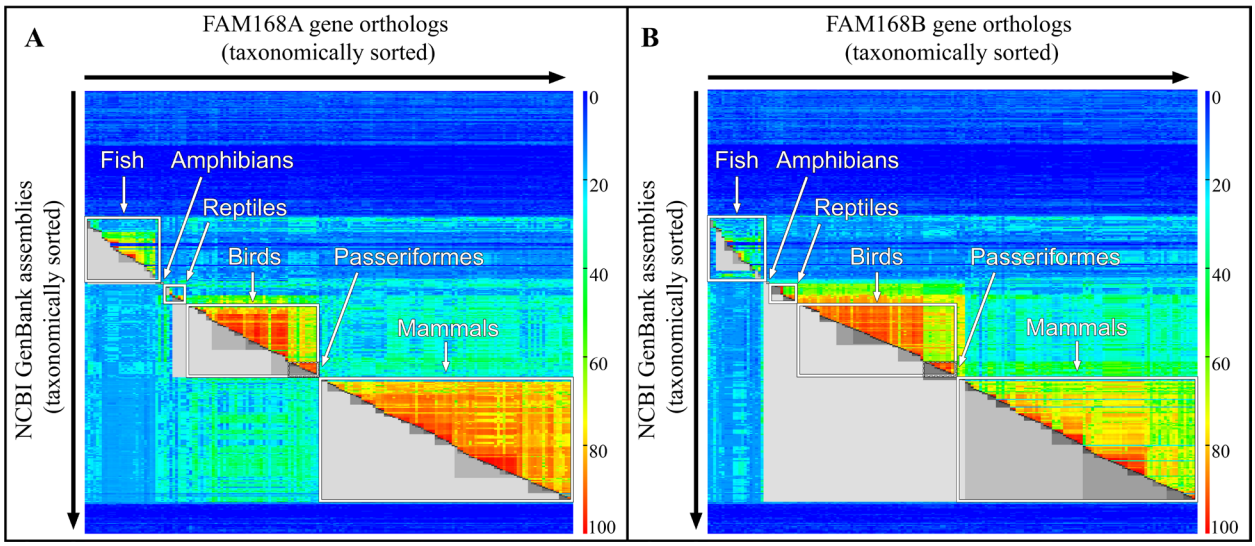


**Figure 2. Cluster representation of phylogenetic analysis of FAM168A and FAM168B in the eukaryotic lineage, based on the NCBI GenBank. (A)** Orthologs of FAM168A displayed discrete taxonomic cluster groups with higher correlation along the diagonal (red = high homology, blue = low homology). Taxonomic cluster blocks along the diagonal correspond to (*i*) fish, (*ii*) amphibians, (*iii*) reptiles, (*iv*) birds, and (*v*) mammals. **(B)** Orthologs of FAM168B displayed a similar pattern of sequence similarity with discrete taxonomic cluster groups with higher correlation along the diagonal. Interestingly, FAM168B showed cross-clustering or cluster overlapping between reptiles and birds as well as between birds and mammals. *Passeriformes* showed a sub-cluster within the birds. The gray triangles below the diagonal display clusters obtained when using a threshold of 60 as described previously (*54*).

(gray short-tailed opossum) but is absent in the egg-laying mammal *Ornithorhynchus anatinus* (platypus) and in all other species in the analysis, including birds (*e.g.*, *Ficedula albicollis*), reptiles (*e.g.*, *Alligator mississippiensis*), amphibians (*e.g.*, *Xenopus tropicalis*), and fish (*e.g.*, *Danio rerio* and *C. milii*) (Figures 3, S9, and S10, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). In contrast, exon 5 of FAM168A, comprised of 126 nts (E5, translated into 42 aa in *H. sapiens*), is conserved in the mammals as well as in birds (*e.g.*, *Ficedula albicollis*), reptiles (*e.g.*, *Alligator mississippiensis*), amphibians (*e.g.*, *Xenopus tropicalis*), and fish (*e.g.*, *Danio rerio* and *C. milii*) (Figures 3, S9, and S10, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Protein sequence comparisons indicate that significant differences exist between these two proteins, FAM168A and FAM168B (Figures 3 and S4, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). These observations suggest that higher mutations occurred in regions located closer to the N-terminus for FAM168 proteins during possible evolutionary and speciation events.

## 4. Discussion

### 4.1. *Genomics and proteomics analyses of FAM168*

We provide for the first time a comprehensive genomics and proteomics feature overview of the FAM168 gene family. We found that the human FAM168A and FAM168B paralogs are located on chr 11 and chr 2, respectively, and show significant sequence differences (Figures 1 and 3). In particular, protein sequence comparisons showed that most variations appear toward the N-terminus of FAM168 proteins. Analyses of the individual exons suggest that deletion and/or insertion occurred during gene duplication events, leading to the emergence of new genes within the FAM168 gene family (*55-58*) (Figures 3, S9, and S10, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). Experimental data suggest that FAM168B (MANI) plays a role in neuro-axonal guidance and neuronal differentiation in the CNS (*17,18*), whereas FAM168A functions in chemoresistance by reducing cisplatin-mediated apoptosis (*20,22*). Thus, genetic differences between the FAM168A and FAM168B may explain why these two genes have distinct functions (*2,56,59*).

### 4.2. *Phylogenetic analysis of FAM168*

This is the first detailed phylogenetic analysis of the FAM168 gene family across the eukaryotic lineage. Our phylogenetic analyses, based on the CDSs of the two FAM168 genes (A and B), outline deep relationships among eukaryotes. The earliest emergence of the FAM168 genes may have occurred in the jawed vertebrates, represented by *C. milii*, which is surprising as these two genes are absent in the other main chordate sub-group (non-vertebrate chordates) that also have a notochord at some stage in their lives, for example, branchiostoma (*e.g.*, *Branchiostoma belcheri* and *Branchiostoma floridae*) and tunicates (*e.g.*, *Botryllus schlosseri*, *Ciona intestinalis*, *Oikopleura dioica*, and *Ciona savignyi*) (*52,60,61*). Accordingly, FAM168A

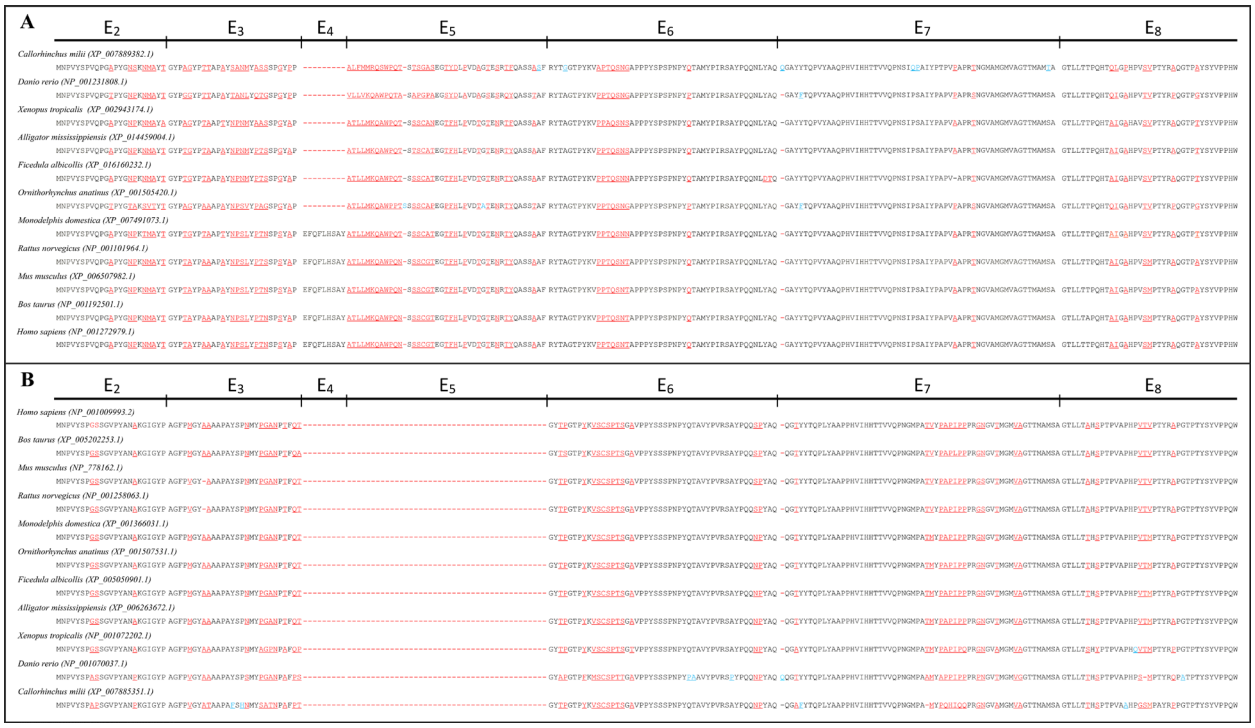


**Figure 3. Comparative protein sequence analysis of FAM168A and FAM168B.** FAM168A and FAM168B proteins were observed earliest in the jawed vertebrates, represented here by *C. milii*. **(A)** Protein sequence analysis of FAM168A for a few species, from *C. milii* to *H. sapiens*. Analyses showed that exon 4 of FAM168A, comprised of 27 nts (E4, translated into nine aa: EFQFLHSAY), is present in livebearing marsupials, represented by *M. domestica*, but is absent in egg-laying mammals such as *O. anatinus* and in all other groups including fish, represented by *C. milii*. Exon 5 of FAM168A, comprised of 126 nts (E5, translated into 42 aa of *H. sapiens*), is conserved across all groups. **(B)** Protein sequence analysis of FAM168B is shown for a few representative species across the phylogenetic tree, from *C. milii* to *H. sapiens*. All exons (E1-E9) are presented in Figures 1A and S4.

and FAM168B seem to be distinctive features of vertebrates (Figure 4).

Individual cluster analysis demonstrates that both genes are highly conserved within each cluster of species; however, FAM168B showed cross-clustering for birds with reptiles and birds with mammals (Figure 2). Although both FAM168 genes show a similar phylogenetic distribution in the Callorhinchidae, a derived mutation pattern is observed in *H. sapiens*. However, we also identified a few species within the fish, amphibians, reptiles, birds, and mammals (*e.g.*, *Melanochromis auratus*, *Nanorana parkeri*, *Apalone spinifera*, *Phoenicopterus ruber*, *Megaderma lyra*, *Manis pentadactyla*, and *Apodemus sylvaticus*) without either FAM168A or FAM168B. We cannot rule out that we failed to detect these genes in some species because genome data for some species remain incomplete or at the scaffold level only.

The exon–intron architecture is a longstanding question in evolutionary genomics (*62-65*). Changes in the splicing of exons and introns are a major driving force in proteomic diversification and the generation of new gene functions (*64,66,67*). In our molecular phylogenetic analysis, we observed that FAM168A contains additional exons E4 and E5 (with respect to FAM168B) across all vertebrate species (Figures 3, S9, and S10, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=9*). However, while exon E5 is present in FAM168A across all vertebrates, the jawed vertebrates, represented by *C. milii*, do not contain E4, whereas *H. sapiens* contains E4 in FAM168A (Figures 3 and 4). Thus, we sought to identify the phylogenetic origin of this distinctive 27 nts-containing exon E4 in FAM168A. Surprisingly, we observed this exon in the livebearing mammal *M. domestica*, but not in the egg-laying mammal *O. anatinus*. Thus, this intermediate exon E4 seems to be a distinctive feature of livebearing mammals. Moreover, insertion of this new exon may have led to proteomic diversification by generating new gene functionality in FAM168A, the development of a higher immune system, which is essential for maturation in livebearing mammals (*56,67,68*) (Figure 4). Although the immune system is relatively undeveloped at birth and is developed during a lifetime of exposure to multiple foreign challenges (the so-called adaptive immune system), the development of the immune system, in particular innate immunity, starts early in fetal life for livebearing mammals (*69-71*). Considering this finding and our previous discoveries, the FAM168 gene family may contain crucial genes involved in the higher immune system and brain functions that are essential for mammals giving birth to live young (*15,17,18,68*).

Concluding, our results reflect a comprehensive

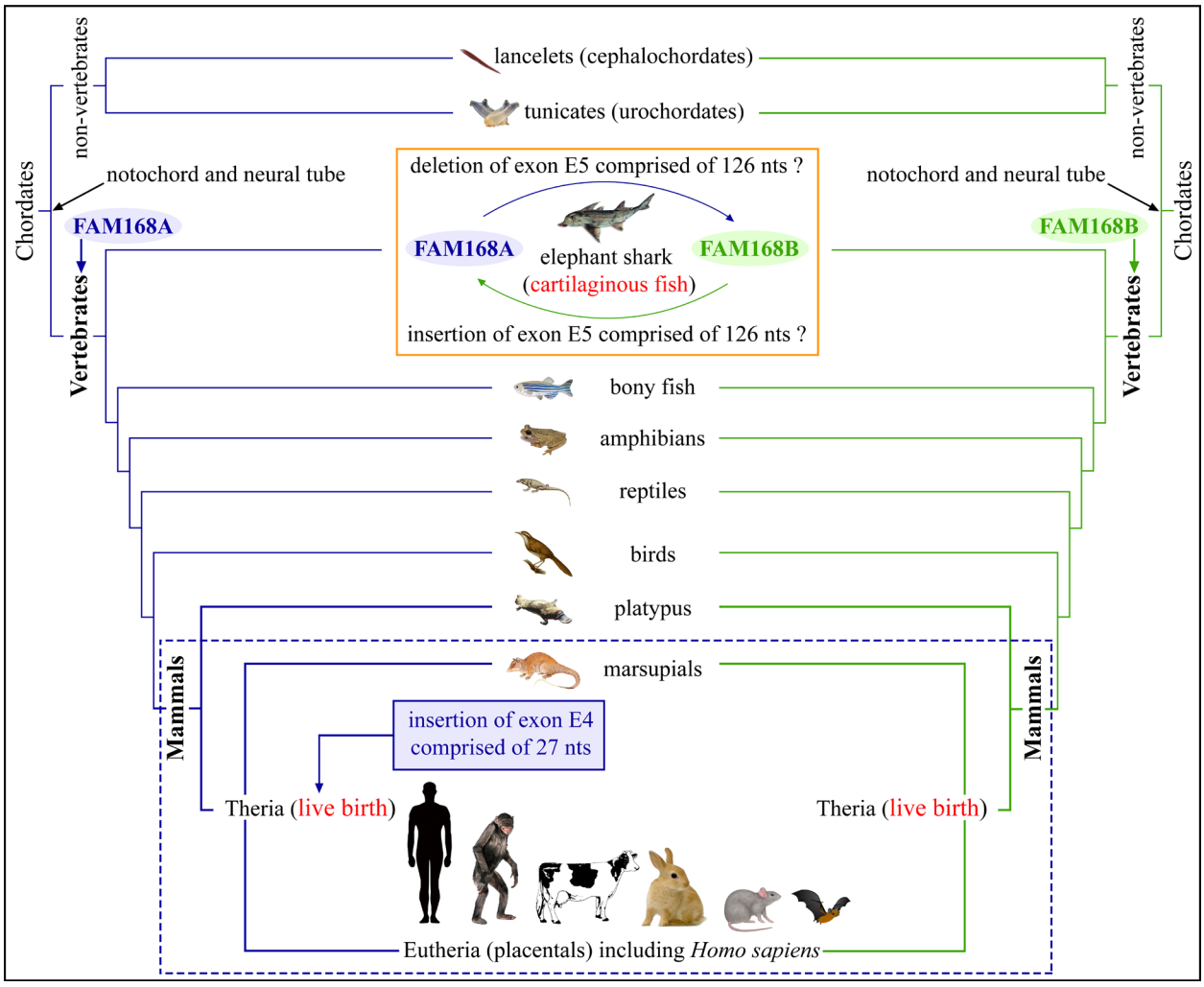


**Figure 4. Phylogenetic overview of FAM168A and FAM168B from elephant shark to human in the eukaryotic lineage.** Both FAM168A and FAM168B appear only in the vertebrates. Interestingly, only animals that give birth to live young have the distinctive exon E4, comprising 27 nts, in FAM168A, implying a functional significance in terms of higher immune system and brain function development.

picture of the genomic, proteomic, and phylogenetic features of the FAM168 gene family. We identified significant differences between FAM168A and FAM168B, in particular the incorporation of exon E5 into FAM168A in lower vertebrate species, such as *C. milii*, and the sudden incorporation of the distinctive exon E4 into FAM168A in higher vertebrate species (*i.e.* mammals) that give birth to live young. These patterns may illustrate functional diversification and species-dependent functional specification across the phylogenetic tree of the eukaryotic lineage (*62,64,65*). The phylogenetic distributions of FAM168A and FAM168B from *C. milii* to H. sapiens comprise distinct taxonomic clusters of species, thus indicating that morphology-based analyses remain insufficient to accurately define the relationships among species (*2*). Genomic analysis across a large sample of species may allow the identification of interspecies relationships and phylogenetic hot spots of distinctive gene origins (*8,42*). Future experimental studies investigating the regulation of gene expression and function of FAM168 along with large-scale gene datasets from additional diverse lineages using an even more global perspective may provide further insights into the functional significance of the two FAM168 genes across the entire phylogenetic tree of life, particularly in terms of specific higher immune and brain functions.

## Acknowledgements

This study was supported by Hanyang University. We thank the Max Planck Institute, Evolutionary Anthropology, Leipzig, Germany (eva.mpg.de) for providing the Neandertal and Denisova genome data. We thank Mr. Markus Schmidt for technical assistance.

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*(Received December 31, 2016; Revised February 22, 2017; Accepted March 7, 2017)*

Original Article

DOI: 10.5582/bst.2017.01009

# The evolutionary appearance of signaling motifs in PGRMC1

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**Summary** **A complex PGRMC1-centred regulatory system controls multiple cell functions. Although PGRMC1 is phosphorylated at several positions, we do not understand the mechanisms regulating its function. PGRMC1 is the archetypal member of the membrane associated progesterone receptor (MAPR) family. Phylogentic comparison of MAPR proteins suggests that the ancestral metazoan "PGRMC-like" MAPR gene resembled PGRMC1/PGRMC2, containing the equivalents of PGRMC1 Y139 and Y180 SH2 target motifs. It later acquired a CK2 site with phosphoacceptor at S181. Separate PGRMC1 and PGRMC2 genes with this "PGRMC-like" structure diverged after the separation of vertebrates from protochordates. Terrestrial tetrapods possess a novel proline-rich PGRMC1 SH3 target motif centred on P64 which in mammals is augmented by a phosphoacceptor at PGRMC1 S54, and in primates by an additional S57 CK2 site. All of these phosphoacceptors are phosphorylated *in vivo*. This study suggests that an increasingly sophisticated system of PGRMC1-modulated multicellular functional regulation has characterised animal evolution since Precambrian times.**

***Keywords:*** Phosphorylation, evolution, steroid signalling, kinases, metazoan

## 1. Introduction

Progesterone Receptor Membrane Component 1 (PGRMC1) is the archetypical protein of the Membrane Associated Progesterone Receptor (MAPR) family, which consists of proteins which share a basic cytochrome $b_5$ ($Cytb_5$) domain fold, with the common insertion of a strectch of amino acids between helices 3 and 4 of the domain fold of cytochrome b5 itself (*1*) (hereafter referred to as the MAPR-specific inter-helical insertion region, or MIHIR). Humans possess four MAPR proteins: PGRMC1, PGRMC2, Neudesin, and Neuferricin (*2-4*).

PGRMC1 is associated with an uncharacteristically large number of attested functions, including association with cytochrome P450 (CyP450) enzymes (activating steroidogenic CyP450s and repressing xenobiotic metabolizing CyP450s), regulation of sterol synthesis (conversion of lanosterol to cholesterol) and interaction with the INSIG1/SCAP/SREBP complex that regulates synthesis of sterol precursors, conferring responsiveness to progesterone (pregn-4-ene-3,20-dione, hereafter: P4), activating vesicle trafficking, regulating entry into G0 stage of cell cycle, association with Aurora kinase on the mitotic spindle kinetochore, participation in the protein complex containing the Sigma 2 Receptor, angiogenesis, invasive growth, motility, anchorage-independent growth, and hypoxic biology (reviewed by (*5*)). It is also localized to the outer mitochondrial membrane, where it interacts with Ferrochelatase (FECH), the final enzyme in the heme synthetic pathway (*6*). The yeast PGRMC1 homolog Dap1 is thought to be involved in either heme synthesis, or its cellular transport to Erg11p, the yeast sterologenic CyP450 enzyme (*7*). Based upon these observations, PGRMC1 has been proposed to be involved in the transport of heme (*6*) and perhaps other hydrophobic ligands (*8*) between subcellular locations.

The acquisition of multiple functions by PGRMC1 presumably occurred throughout evolution in a successive manner to produce a set of stratified influences. For instance PGRMC1 association with the mitotic spindle could represent a universal eukaryotic MAPR trait, or may represent a metazoan, vertebrate, or mammalian innovation. Sterol or heme synthesis are ancestral traits of all eukaryotes, and are indeed also widespread in bacteria (*9,10*). The involvement

Released online in J-STAGE as advance publication February 28, 2017.

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of PGRMC1 and its yeast homolog Dap1 in the conversion of lanosterol to cholesterol (animals) or ergosterol (yeast) (*11*) argues strongly for an ancestral eukaryotic role of MAPR proteins in sterol production, and perhaps also in heme synthesis based upon a role proposed by Ghosh *et al*. for yeast (*7*) and by Piel *et al*. in mammals (*6*).

On the other hand the presence of PGRMC1/MAPR proteins in the complex containing INSIG/SCAP/SREBP that regulates the mevalonate pathway leading to sterol precursor synthesis in animals (*12*) (reviewed in (*13*)), but not reported in yeast, argues that this should be an acquired higher organism PGRMC1-function. Similarly, steroid hormone signaling first evolved in metazoans. The Estrogen Receptor (ESR), the original classical steroid receptor, appeared in the lineage that gave rise to vertebrates, with subsequent classical steroid receptors such as the P4 receptor evolving later (*14-16*). Therefore, conferring P4 responsiveness onto cells is presumably an acquired secondary function (unless a PGRMC1-like P4-response predates the estrogen response).

Finally, both mammalian PGRMC1 and its nematode homologue VEM-1 fulfil conserved roles in animal embryogenesis by regulating the fidelity of nerve chord axonal guidance along the ventral midline of nematodes and the spinal chord of rats (*17-19*). While this function must be ancient in animals, axonal guidance must also be acquired by animals and absent from protists. I have previously proposed that this function relies on the vesicle trafficking properties of PGRMC1 to expose specifc cell surface receptors required axonal guidance (*5*). If so, then vesicle trafficking may be another acquired metazoan PGRMC1 function.

I have previously proposed that PGRMC1 phosphorylation plays a paramount role in the regulation of its function (*20,21*). In this paper I have assessed the evolutionary appearance of PGRMC1 phosphorylation sites using BLASTp and CLUSTAL alignments by sampling MAPR proteins in some strategically selected species thought to be phylogentically separated by differing evolutionary periods. This study creates a novel systematics and rationale in this field for the future experimental stratification and functional characterization of PGRMC1 and other MAPR protein functions.

## 2. Materials and Methods

### 2.1. *Phosphosite post-translational modifications*

The Phosphosite data base of post translational modifications (PTMs) detected by high throughput mass spectrometry analyses (*22*) was queried with UniProt IDs for human PGRMC1 (O00264), PGRMC2 (O15173), Neudesin (Q9UMX5) and Neferricin (Q8WUJ1). Any PTM scored by Phosphosite with a frequency of once or more was marked on the CLUSTAL multiple sequence alignment of Figure 1A.

### 2.2. *BLAST and CLUSTAL analysis*

For Figure 1, PGRMC1 (UniProt O00264) used as search sequence in the UniProt Protein Basic Local Alignment Search Tool (BLASTp) (*http://www.uniprot.org/blast/*). In the results, organisms were deliberately restricted to *Homo sapiens*, the flowering plant *Arabidopsis thaliana*, the fusion yeast *Schizosaccharomyces pombe*, and the budding yeast *Saccharomyces cerevisiae*. The results (4 human proteins, 4 *Arabidopsis* proteins, one protein for each yeast species) were aligned by inputting their UniProt IDs to the UniProt CLUSTAL O (1.2.3) (*23*) multiple sequence alignment tool (UniProt Align) (*http://www.uniprot.org/align/*). The CLUSTAL guide tree for Figure 1B was recreated and coloured in Powerpoint to accurate relative scale as the original. For Figure 1C-D, the human Neudesin UniProt ID (Q9UMX5) was used as BLASTp input, and selected phylogentically diverse species were chosen.

For Figure 2 and Table 1, the Uniprot FASTA sequences for the four human MAPR proteins were BLASTed against the indicated individual specific organisms using the NCBI BLASTp page (*https://blast.ncbi.nlm.nih.gov*). The FASTA sequences of all identified MAPR proteins were then aligned with the Uniprot FASTA sequences for the four human MAPR proteins using the UniProt CLUSTAL multiple sequence alignment tool, as described above, and designated as Neudesin-like, Neuferricin-like, PGRMC-like, PGRMC1-like or PGRMC2-like by closest alignment to human MAPR proteins in CLUSTAL tree topology.

Subsequent BLAST analyses were performed with NCBI BLASTp with query sequences and Organism taxid ID as described for each study. The top resulting BLASTp results were chosen, and FASTA sequence files added with NCBI identifiers to UniProt Align. The BLAST Query sequences were entered with UniProt Identifiers to color highlight them in UniProt Align results.

## 3. Results

### 3.1. *Phosphorylation sites are variously conserved among MAPR proteins*

PGRMC1 phosphorylations as documented in the Phosphosite data base (*22*) have been presented previously (*21*), as have PGRMC2 sites (*8*). Figure S1 shows the complete set of Phosphosite post-translational modifications (PTMs) documented for all four human MAPR proteins. Figure 1A maps the PTMs from Figure S1 on human MAPR proteins aligned against the sequences of two phylogentically divergent yeast Dap1p MAPR family members, and four MAPR family members from the plant *Arabidopsis thaliana*. Phosphorylation of a Casein Kinase 2 (CK2) consensus motifs adjacent a C-terminal Src Homology

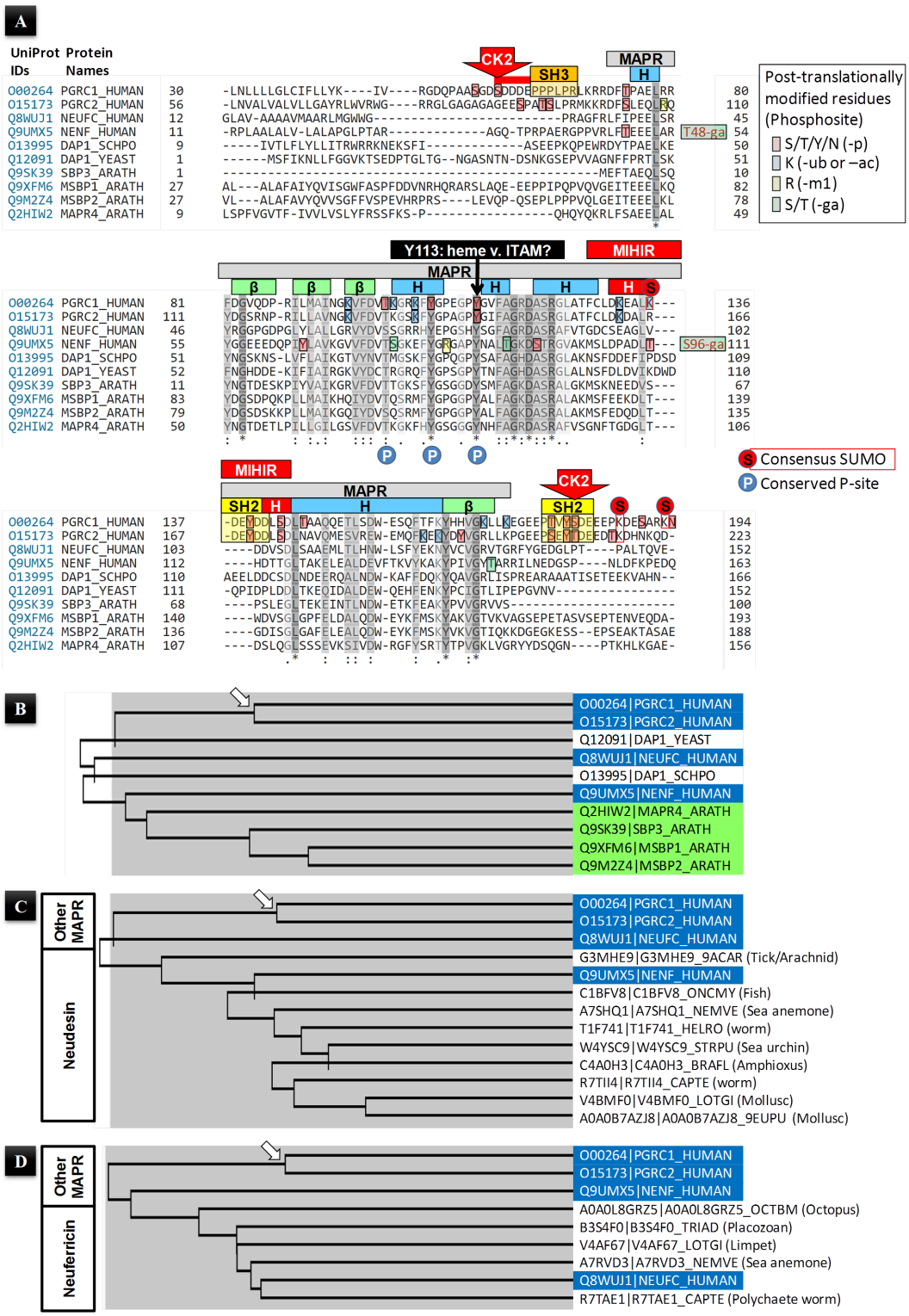
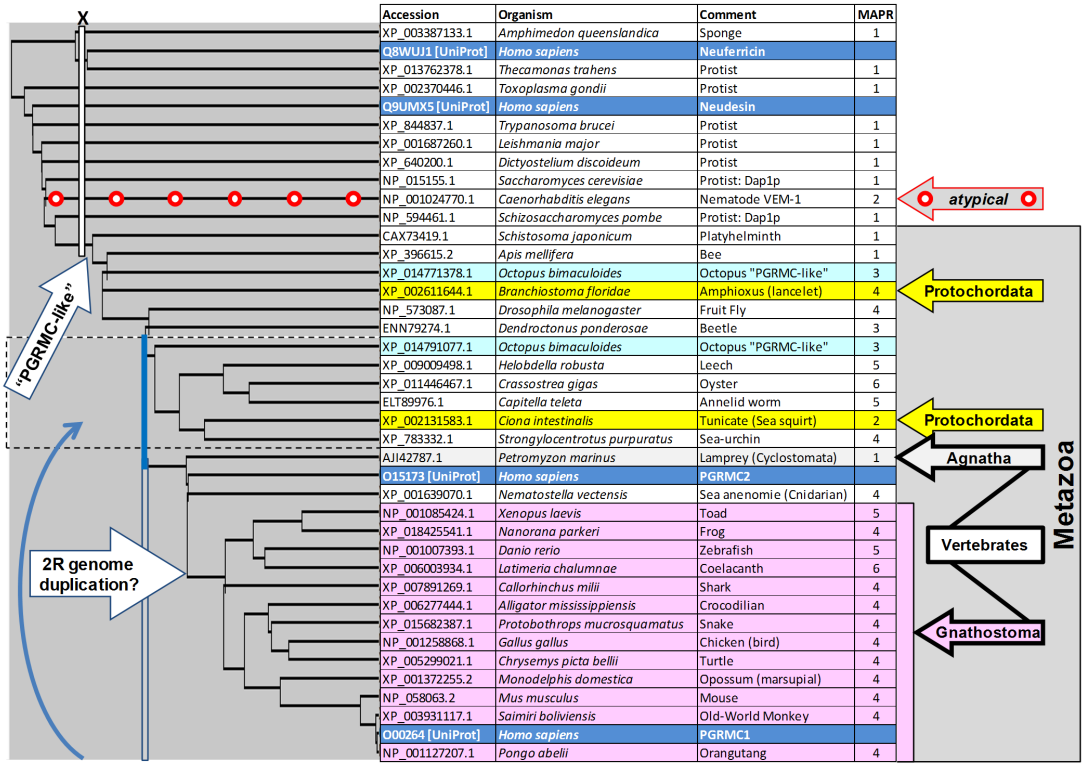



**Figure 1. Sequence alignment of selected phylogenetically disparate MAPR family members, showing known post-translational mofifications of human proteins from Phosphosite. (A)** Post-translational modifications in the vicinity of the MAPR domain from **Figure S1** are mapped to the amino acid sequence of the four human proteins, and aligned against 2 yeast and 4 plant MAPR proteins. The location of predicted SH2 target sequences centred on Y139 and Y180 (numbering refers to PGRMC1), and the SH3 target centred on P63 are marked. All four MAPR family proteins from human, four members from the flowering plant *A. thaliana*, and the single Dap1 family members from yeasts *S. pombe* and *S. cerevisiae* were aligned using the UniProt BLASTp tool, entering the UniProt identifiers (IDs) indicated in the figure. Similarity of amino acid properties are highlighted. The conserved cytochrome b5 domain of the MAPR family is marked by the grey box above the alignment. The positions of beta sheets (β) or alpha helices (H) are indicated. The half inset red bar in the cytochrome b5 domain (PGRMC1 amino acids 128-146) represents the MIHIR insertion (see text) between cytochrome b5 domain fold helices which characterizes the MAPR family (*1,13*). Known post-translational modifications from the Phosphosite data base **(Figure S1)** are included for the human proteins as indicated in the key. Blue circled P depicts phosphorylation acceptor sites conserved across all species. Red circled S depict consensus Sumoylation sites in PGRMC1 (*51,52*). **(B)** The CLUSTAL similarity tree corresponding to **(A)**. **(C and D)** Divergence of PGRMC1-like proteins from other MAPR proteins predates the metazoan radiation. CLUSTAL alignment of MAPR proteins from phylogenetically diverse metazoans indicates that the PGRMC1/2 clade is distinct from the Neudesin **(C)** and the Neuferricin clades **(D)**. None of these families have been detected in unicellular eukaryotes **(Table 1)**.




| Accession | Organism | Comment | MAPR |
|---|---|---|---|
| XP_003387133.1 | *Amphimedon queenslandica* | Sponge | 1 |
| Q8WUJ1 [UniProt] | *Homo sapiens* | Neuferricin | |
| XP_013762378.1 | *Thecamonas trahens* | Protist | 1 |
| XP_002370446.1 | *Toxoplasma gondii* | Protist | 1 |
| Q9UMX5 [UniProt] | *Homo sapiens* | Neudesin | |
| XP_844837.1 | *Trypanosoma brucei* | Protist | 1 |
| XP_001687260.1 | *Leishmania major* | Protist | 1 |
| XP_640200.1 | *Dictyostelium discoideum* | Protist | 1 |
| NP_015155.1 | *Saccharomyces cerevisiae* | Protist: Dap1p | 1 |
| NP_001024770.1 | *Caenorhabditis elegans* | Nematode VEM-1 | 2 |
| NP_594461.1 | *Schizosaccharomyces pombe* | Protist: Dap1p | 1 |
| CAX73419.1 | *Schistosoma japonicum* | Platyhelminth | 1 |
| XP_396615.2 | *Apis mellifera* | Bee | 1 |
| XP_014771378.1 | *Octopus bimaculoides* | Octopus "PGRMC-like" | 3 |
| XP_002611644.1 | *Branchiostoma floridae* | Amphioxus (lancelet) | 4 |
| NP_573087.1 | *Drosophila melanogaster* | Fruit Fly | 4 |
| ENN79274.1 | *Dendroctonus ponderosae* | Beetle | 3 |
| XP_014791077.1 | *Octopus bimaculoides* | Octopus "PGRMC-like" | 3 |
| XP_009009498.1 | *Helobdella robusta* | Leech | 5 |
| XP_011446467.1 | *Crassostrea gigas* | Oyster | 6 |
| ELT89976.1 | *Capitella teleta* | Annelid worm | 5 |
| XP_002131583.1 | *Ciona intestinalis* | Tunicate (Sea squirt) | 2 |
| XP_783332.1 | *Strongylocentrotus purpuratus* | Sea-urchin | 4 |
| AJI42787.1 | *Petromyzon marinus* | Lamprey (Cyclostomata) | 1 |
| O15173 [UniProt] | *Homo sapiens* | PGRMC2 | |
| XP_001639070.1 | *Nematostella vectensis* | Sea anenomie (Cnidarian) | 4 |
| NP_001085424.1 | *Xenopus laevis* | Toad | 5 |
| XP_018425541.1 | *Nanorana parkeri* | Frog | 4 |
| NP_001007393.1 | *Danio rerio* | Zebrafish | 5 |
| XP_006003934.1 | *Latimeria chalumnae* | Coelacanth | 6 |
| XP_007891269.1 | *Callorhinchus milii* | Shark | 4 |
| XP_006277444.1 | *Alligator mississippiensis* | Crocodilian | 4 |
| XP_015682387.1 | *Protobothrops mucrosquamatus* | Snake | 4 |
| NP_001258868.1 | *Gallus gallus* | Chicken (bird) | 4 |
| XP_005299021.1 | *Chrysemys picta bellii* | Turtle | 4 |
| XP_001372255.2 | *Monodelphis domestica* | Opossum (marsupial) | 4 |
| NP_058063.2 | *Mus musculus* | Mouse | 4 |
| XP_003931117.1 | *Saimiri boliviensis* | Old-World Monkey | 4 |
| O00264 [UniProt] | *Homo sapiens* | PGRMC1 | |
| NP_001127207.1 | *Pongo abelii* | Orangutang | 4 |

**Figure 2. The phyogentic representation of PGRMC1-like MAPR proteins.** Either the sole MAPR family, or the most PGRMC1-like protein from each species (see **Table 1**) were grouped by similarity by CLUSTAL. A single exception is the octopus, where two PGRMC1-like (PGRMC-like: see **Table 1**) proteins are included to demonstrate that these are paralogous and not homologous to PGRMC1 and PGRMC2. *i.e.* octopus split from the vertebrate lineage before the divergence of PGRMC1 and PGRMC2. Therefore the octopus proteins represent a separate duplication of PGRMC-like proteins. It is not valid to speak of PGRMC1 and PGRMC2 for organisms which are not jawed vertebrates. PGRMC-like proteins appear to have arisen with metazoans. *C. elegans* VEMA protein is an atypical animal MAPR protein which has diverged from its metazoan ancestor (see **Figure 3**). PGRMC1 and PGRMC2 appear to have diverged correspondently with the emergence of jawed vertebrates, perhaps with the 2R genome duplication thought to have been associated with that lineage (*53*). For identities of MAPR proteins see **Table 1**. One region of the CLUSTAL alignment tree (dotted lines and curved arrow) was manually repositioned. Otherwise the CLUSTAL similarity topology has not been altered. The white bar marked "X" indicates phylogenetic separations thought by the author too deep for alignment to reasonably represent phylogeny.

2 (SH2) target motif is conserved between PGRMC1 and PGRMC2, as are various other tyrosine (Y) and serine/threonine (S/T) phosphorylation sites. The SH3-target motif centred on P64, along with phosphoacceptor residues at S54 and S57, are absent from PGRMC2, and none of these motifs are present in Neudesin or Neuferricin. Alignment with other MAPR family members from human, yeast, and plant (*A. thaliana*) show that some potential sites of phosphorylation (PGRMC1 T101, Y107, Y113) are conserved across these MAPR proteins, whereas PGRMC1 and PGRMC2 share multiple common sites (Figure 1A).

### 3.2. *Posttranslational modifications of Neudesin and Neuferricin*

PTMs are documented less frequently for Neudesin and Neferricin than for PGRMC1 and PGRMC2. This may be because Neudesin and Neferricin are present in the luminal compartment and secreted extracellularly (*2,3,24-28*), however the presence of some observed phosphorylation events suggests that the MAPR domain of these proteins may also be found in the cytoplasm. The detection of O-N-acetylgalactosamine (O-GalNAc) glycosylation does not necessarily imply an extracellular location for Neudesin, since 14% of O-GalNAc proteins are annotated with nuclear Gene Ontogeny (GO) assignments (*29*). Indeed the Human Protein Atlas (*30*) lists the main subcellular location of Neudesin as nuclear based on two antibodies using immunofluorescence and confocal microscopy in human cells (*http://www.proteinatlas.org/ENSG00000117691-NENF/subcellular*). It is not unknown for proteins to exhibit dual orientation of membrane toplogy, such as human CD38 antigen which exists 90% with its C-terminus and active site in the extracellular/luminal compartment, with another 10% of protein molecules where these elements are cytoplasmic (*31*). Indeed the Cytb5 domain of PGRMC1 itself has been reported to be both cytoplasmic and extracellular (reviewed by (*5*)), such that dual membrane topology is conceivably a common feature of animal MAPR proteins.

### 3.3. *The invertebrate divergence of Neudesin and Neuferricin predates that of PGRMC1/PGRMC2*

Figure 1B depicts the Clustal guide tree corresponding to the alignment of Figure 1A. This is entirely based on

**Table 1. PGRMC1/2-like (PGRMC-like) proteins (which CLUSTAL groups with both PGRMC1 and PGRMC2) are found in metazoans higher than Platyhelminthes**

| Accession | Organism | Comment | MAPR | NEUFC-like | | NENF-like | | "PGRMC"-like | | PGRMC1 | PGRMC2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| XP_003387133.1 | *Amphimedon queenslandica* | Sponge | 1 | | | | | | | | |
| **Q8WUJ1** | ***Homo sapiens*** | **Neuferricin** | | | | | | | | | |
| XP_013762378.1 | *Thecamonas trahens* | Protist | 1 | | | | | | | | |
| XP_002370446.1 | *Toxoplasma gondii* | Protist | 1 | | | | | | | | |
| **Q9UMX5** | ***Homo sapiens*** | **Neudesin** | | | | | | | | | |
| XP_844837.1 | *Trypanosoma brucei* | Protist | 1 | | | | | | | | |
| XP_001687260.1 | *Leishmania major* | Protist | 1 | | | | | | | | |
| XP_640200.1 | *Dictyostelium discoideum* | Protist | 1 | | | | | | | | |
| NP_015155.1 | *Saccharomyces cerevisiae* | Protist: Dap1p | 1 | | | | | | | | |
| NP_001024770.1 | *Caenorhabditis elegans* | Nematode VEM-1 | 2 | 1 | NP_497868.1 | 0 | | 1 | NP_001024770.1 | | |
| NP_594461.1 | *Schizosaccharomyces pombe* | Protist: Dap1p | 1 | | | | | | | | |
| CAX73419.1 | *Schistosoma japonicum* | Platyhelminth | 1 | 1 | | 1 | | 1 | CAX73419.1 | | |
| XP_396615.2 | *Apis mellifera* | Bee | 1 | 1 | XP_006566840.1 | 0 | | 1 | XP_396615.2 | | |
| XP_014771378.1 | *Octopus bimaculoides* | Octopus "PGRMC-like" | 3 | 1 | XP_014778686.1 | 0 | | 2 | XP_014791077.1, XP_014771378.1 | | |
| XP_002611644.1 | *Branchiostoma floridae* | Amphioxus (lancelet) | 4 | 2 | XP_002605720.1, XP_002605719.1 | 1 | XP_002585704.1 | 1 | XP_002611644.1 | | |
| NP_573087.1 | *Drosophila melanogaster* | Fruit Fly | 4 | 1 | NP_572535.1 | | | 3 | NP_723757.1, NP_609650.1, NP_573087.1 | | |
| ENN79274.1 | *Dendroctonus ponderosae* | Beetle | 3 | 2 | ERL94051.1, ENN78869.1 | | | 1 | ENN79274.1 | | |
| XP_014791077.1 | *Octopus bimaculoides* | Octopus "PGRMC-like" | 3 | 1 | XP_014778686.1 | 0 | | 2 | XP_014791077.1, XP_014771378.1 | | |
| XP_009009498.1 | *Helobdella robusta* | Leech | 5 | 1 | XP_009019434.1 | 1 | XP_009018536.1 | 3 | XP_009009498.1, XP_009012871.1, XP_009024185.1 | | |
| XP_011446467.1 | *Crassostrea gigas* | Oyster | 6 | 3 | EKC42144.1, XP_011418616.1, XP_011418617.1 | 1 | XP_011445092.1 | 2 | XP_011446467.1, EKC28875.1 | | |
| ELT89976.1 | *Capitella teleta* | Annelid worm | 5 | 1 | ELT90457.1 | 1 | ELT93549.1 | 3 | ELT89976.1, ELU07585.1, ELU07586.1 | | |
| XP_002131583.1 | *Ciona intestinalis* | Tunicate (Sea squirt) | 2 | 1 | XP_002126672.1 | | | 1 | XP_002131583.1 | | |
| XP_783332.1 | *Strongylocentrotus purpuratus* | Sea-urchin | 4 | 2 | XP_795139.1, XP_797342.1 | 1 | XP_003727182.1 | 1 | XP_783332.1 | | |
| AJI42787.1 | *Petromyzon marinus* | Lamprey | 1 | | | | | 1 | AJI42787.1 | | |
| **O15173** | ***Homo sapiens*** | **PGRMC2** | | | | | | | | | |
| XP_001639070.1 | *Nematostella vectensis* | Sea anenome (Cnidarian) | 4 | 2 | XP_001636685.1, XP_001619521.1 | 1 | XP_001628837.1 | 1 | XP_001639070.1, XP_001641507.1 | | |
| NP_001085424.1 | *Xenopus laevis* | Toad | 5 | 1 | XP_018102462.1 | 1 | XP_018118068.1 | 3 | | NP_001085424.1 | NP_001089766.1, NP_001087737.1 |
| XP_018425541.1 | *Nanorana parkeri* | Frog | 4 | 1 | XP_018408871.1 | 1 | XP_018409295.1 | 2 | | XP_018425541.1 | XP_018421456.1 |
| NP_001007393.1 | *Danio rerio* | Zebrafish | 5 | 1 | NP_001096144.1 | 2 | NP_001032793.2, BAE48265.1 | 2 | | NP_001007393.1 | NP_998269.1 |
| XP_006003934.1 | *Latimeria chalumnae* | Coelacanth | 6 | 3 | XP_006007296.1, XP_006007297.1, XP_006007298.1, XP_006007299.1 | 1 | XP_006013354.1 | 2 | | XP_006003934.1 | XP_005991348.2 |
| XP_007891269.1 | *Callorhinchus milii* | Shark | 4 | 1 | XP_007909304.1 | 1 | XP_007903232.1 | 2 | | XP_007891269.1 | XP_007882916.1 |
| XP_006277444.1 | *Alligator mississippiensis* | Crocodilian | 4 | 1 | XP_014463495.1 | 1 | XP_014453499.1 | 2 | | XP_006277444.1 | XP_006274062.1 |
| XP_015682387.1 | *Protobothrops mucrosquamatus* | Snake | 4 | 1 | XP_015676553.1 | 1 | XP_015670150.1 | 2 | | XP_015682387.1 | XP_015674943.1 |
| NP_001258868.1 | *Gallus gallus* | Chicken (bird) | 4 | 1 | XP_415743.1 | 1 | XP_004935389.1 | 2 | | NP_001258868.1 | NP_001006441.1 |
| XP_005299021.1 | *Chrysemys picta bellii* | Turtle | 4 | 1 | XP_007054253.1 | 1 | XP_007058066.1 | 2 | | XP_007060285.1 | XP_007059215.1 |
| XP_001372255.2 | *Monodelphis domestica* | Opossum (marsupial) | 4 | 1 | XP_001370477.1 | 1 | XP_001374471.1 | 3 | | XP_001372255.2 | XP_001365574.1, XP_007495566.1 |
| NP_058063.2 | *Mus musculus* | Mouse | 4 | 1 | AAH86682.1 | 1 | NP_079700.1 | 2 | | NP_058063.2 | NP_081834.1 |
| XP_003931117.1 | *Saimiri boliviensis* | Old-World Monkey | 4 | 1 | XP_003933104.1 | 1 | XP_010339346.1 | 2 | | XP_003931117.1 | XP_010336708.1 |
| **O00264** | ***Homo sapiens*** | **PGRMC1** | | | | | | | | | |
| NP_001127207.1 | *Pongo abelii* | Orangutang | 4 | 1 | XP_002826904.1 | 1 | XP_002809527.1 | 2 | | NP_001127207.1 | XP_002815179.1 |

Neuferricin-like proteins are present in Cnidarians, nematodes, and higher animals. Neudesin-like proteins are present in worms, molluscs and chordates, but not in sampled insects. Distinct PGRMC1 and PGRMC2 proteins (as grouped by CLUSTAL) are present in jawed vertebrates (gnathostoma), but not agnathan vertebrates (lamprey) or protochordates (Amphioxus, tunicate/sea squirt).

the pairwise alignments, and should not be confused with an evolutionary phylogeny, which it vaguely approximates for more closely related species. However it quantitatively depicts the degree of similarity between proteins (*23*). The degree of similarity between Neudesin and Neuferricin is similar to that between the two yeast species, which are separated by a deep phylogenetic distance (*32,33*). All four MAPR family proteins from the plant species clustered together, consistent with their having shared a common ancestor after the monophyletic separation of plants from fungus and animals. This result suggested that the common ancestor of animal MAPR proteins was evolutionarily ancient. This was somewhat surprising, because Kimura *et al*. (*26*) reported that Neudesin was present in vertebrates, but not in the invertebrates *Caenorhabditis elegans* (a nematode roundworm) or *Drosophila melanogaster* (an insect), nor in the primitive chordate *Ciona intestinalis* (a sea squirt). That phylogenetic distribution would suggest that the gene for Neudesin originated in the chordate lineage leading to animals.

That interpretation seemed incompatible with the tree topology of Figure 1B. The UniProt Eumetazoan protein sequence data base was interrogated by BLASTp against human Neudesin (Figure 1C) and Neuferricin (Figure 1D) as query, revealing that sequences with greater similarity to both of these proteins are distributed across the animal kingdom, and therefore were present as separate proteins in the common ancestor of those organisms. Paradoxically, There were no BLASTp hits returned from *C. elegans* or *D. Melanogaster*, suggesting that insects and nematodes (but not arachnids or annelid worms) have lost their ancestral Neudesin genes. Therefore Kimura *et al*. (*26*) correctly reported Neudesin's absence in these species, but did not appreciate that the gene was probably secondarily lost in both lineages. Neuferricin was also widely distributed across the Eumetazoa (Figure 1D), including primitive groups such as Placazoans (B3S4F0), sea anemone (A7RVD3) Polychaete worm (R7TAE1) and molluscs (A0A0L8GRZ5, V4AF67), as well as a wide range of chordates and vertebrates but not insects (not shown), indicating that Neudesin- and Neuferricin-like MAPR proteins were present in an early metazoan ancestor.

### 3.4. *The ancestral Metazoan possessed a PGRMC-like MAPR protein*

To examine the phylogenetic distribution of these MAPR families a series of single celled protists, and animals (metazoans) of various selected indicative phylogenetic affinities were BLASTed with NCBI BLASTp (which provides greater phylogenetic representation than UniProt) using all four human MAPR proteins together as input query sequence. The results are presented in Table 1, which indicates that all single cell level organisms screened possessed only one MAPR protein. Platyhelminths (flat worms) possess only one PGRMC-like protein that aligns most closely with the PGRMC1/PGRMC2 group (subsequently "PGRMC-like", to denote proteins from organisms not descended from the earliest vertebrate ancestor in which PGRMC1 and PGRMC2 had diverged). Cnidarians (jellyfish and corals) possess Neferricin-like and Neudesin-like proteins by CLUSTAL alignment. Loss of Neudesin-like proteins in several higher lineages is suggested since they are absent from nematodes, insects, and octopus (mollusc), although the Oyster possesses a Neudesin-like protein, indicating that the common mollusc progenitor possessed one. Neudesin must have been secondarily lost from Octopus, which interestingly may have accommodated the loss by evolving multiple PGRMC-like proteins (Table 1).

### 3.5. *The PGRMC-like ancestrally inherited metazoan protein diverged into PGRMC1 and PGRMC2 in the lineage leading to vertebrates*

The jawless vertebrates Amphioxus and the lamprey have only one PGRMC-like protein. The lamprey has presumably secondarily lost its other ancestral complement of MAPR genes, since it has only a single PGRMC-like MAPR gene (Table 1). Jawed vertebrates exhibit at least two distinct PGRMC-like proteins which each clustered closer to either PGRMC1 or PGRMC2, indicating that the PGRMC1/PGRMC1 gene duplication event occurred in the lineage that gave rise to jawed vertebrates.

The same organisms as Table 1 are shown in Figure 2 along with the CLUSTAL alignment trees for the alignment of the most PGRMC1-like protein in each organism. Table 1 includes the single most PGRMC1-like protein per organism (with two exceptions, being human, where all four MAPR proteins are included, and octopus, to demonstrate that the two anciently diverged PGRMC-like proteins do not cluster more closely to either PGRMC1 or PGRMC1). The gene divergence that gave rise to PGRMC1 and PGRMC1 had not occurred in the organism that gave rise to molluscs and vertebrates. Therefore it is fallacious to refer to separate homologues of PGRMC1 and PGRMC2 for non-vertebrate organisms.

Figure 2 and Table 1 reveal that PGRMC-like proteins are shared by metazoans but not protists, and that PGRMC2 is most similar to lamprey, sea anenome and then the vertebrate PGRMC1 group (Figure 2), reinforcing that the PGRMC1/2 divergence occurred prior to the vertebrate radiation. Note that the *Ceanorhabditis elegans* VEM-1 protein atypically clusters with protist MAPR proteins, which will be considered below.

### 3.6. *The ancestral animal PGRMC-like protein contained a tyrosine C-terminally to the MAPR domain corresponding to PGRMC1 Y180*

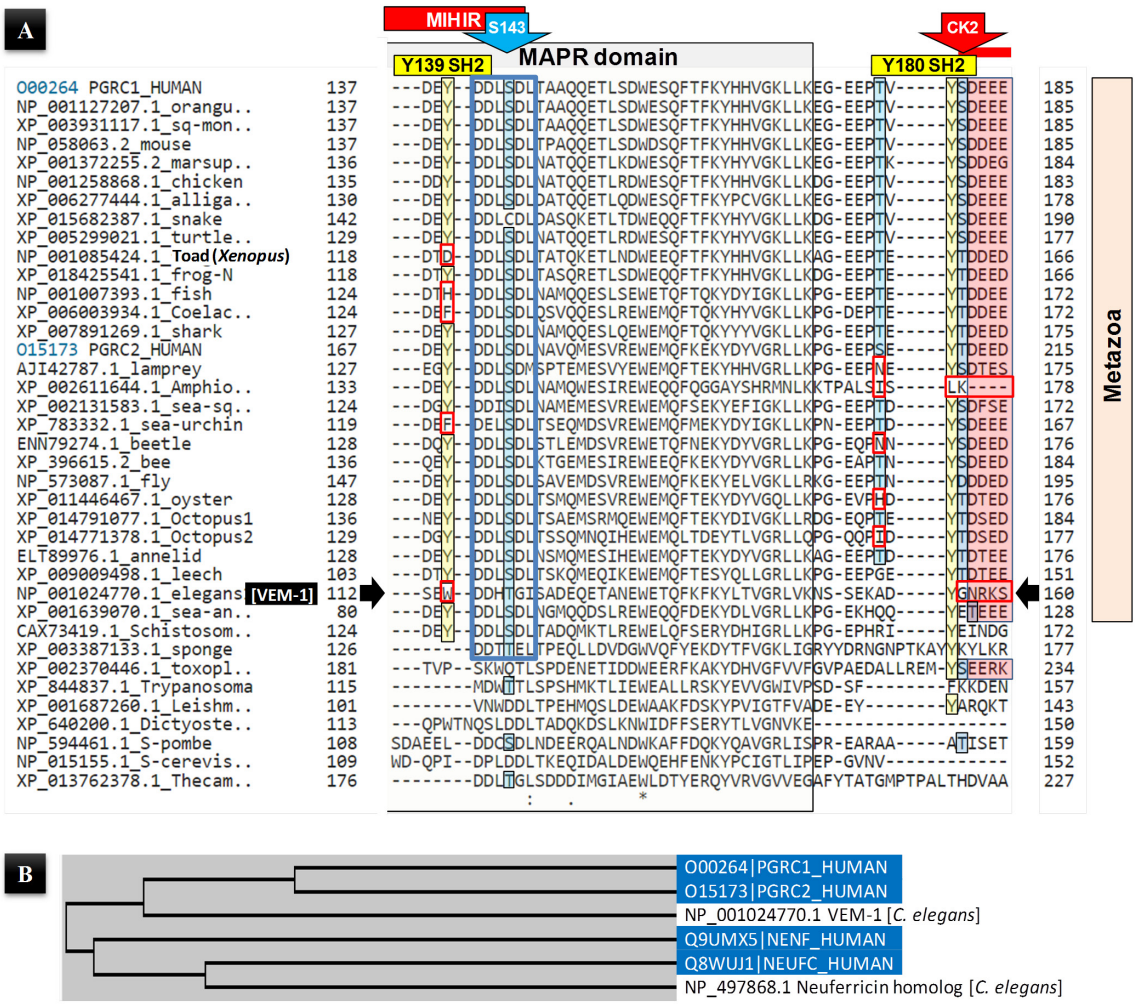



**Figure 3. SH2 target motifs centred on Y139 (numbering refers to PGRMC1) with adjacent phosphorylatabel residue at S143 and at Y180 C-terminal to the MAPR domain and an adjacent CK2 site present at the dawn of metazoan evolution. (A)** The appearance of PGRMC1 S/T position 143 and Y180 predated metazoan evolution. Proteins and species are the same as **Table 1** and **Figure 2**. Note that *C. elegans* VEM-1 (black arrows) is an atypical animal PGRMC-like protein, exhibiting disruptions in both elements. **(B)** Although atypical **(A)**, **C. elegans** VEM-1 is a "PGRMC-like" protein. Note that NCBI BLAST revealed a *C. elegans* 964 residue protein sequence "PIR: H89582 protein K07E3.6" [imported from UniProt] with a region of 100% identity to VEM-1. BLAST of UniProt version August 4, 2016 with H89582 revealed a protein Q21286 Probable cation-transporting ATPase K07E3.7 (Gene K07E3.7/K07E3.6) lacking the VEM-1 sequence. Therefore NCBI sequence H89582 is a presumed database artefact and is excluded from this study.

Figure 3A shows the CLUSTAL amino acid alignment of the MAPR proteins from Figure 2 and Table 1, for the region aligned with human PGRMC1 residues 137-185 (all amino acid numbering refers to the corresponding aligned residue of human PGRMC1), which contains both SH2 target motifs, and includes conserved phosphorylation sites at Y139, S143, T178, Y180 and S181 (Figure S1, Figure 1).

The appearance of a Y residue corresponding to Y180 (or at least proximally C-terminal to the MAPR domain) is shared by animals and some protists, suggesting that tyrosine phosphorylation immediately C-terminal of the MAPR domain may have provided a level of regulation that was important for an inferred ancestral PGRMC-like function in some protists, including those which gave rise to animals, which has retained selectional importance throughout animal evolution. To my knowledge, this is the first such phosphorylation event inferred to have been developed for any protein in the protist eukaryotic group that gave rise to animals, and has been conserved throughout animal evolution.

### 3.7. *The ancestral animal PGRMC-like protein contained both SH2 target motifs*

Interestingly, animals and sponges share a PGRMC1 "DDLSDL" motif containing S143 (Figure 3A). The phylogentic appearance of PGRMC-like proteins in the animal lineage (*Schistosoma* and higher) corresponds with the appearance of the DEY motif of Y139 at the level of the platyhelminth *Schistosoma*. Sea anenome and higher organisms also share a patch of negatively charged residues and a phosphorylatable residue immediately C-terminal to Y180, corresponding to the

| Accession | Organism | Comment | Start AA |
|---|---|---|---|
| O00264 [UniProt] | *Homo sapiens* | Human PGRMC1 | 52 |
| NP_001127207.1 | *Pongo abelii* | Orangutang | 52 |
| XP_003931117.1 | *Saimiri boliviensis* | Old-World Monkey | 52 |
| XP_014439595.1 | *Tupaia chinensis* | Chinese tree shrew | 51 |
| NP_058063.2 | *Mus musculus* | Mouse | 52 |
| XP_001372255.2 | *Monodelphis domestica* | Opossum (marsupial) | 51 |
| NP_001258868.1 | *Gallus gallus* | Chicken (bird) | 52 |
| XP_006277444.1 | *Alligator mississippiensis* | Crocodilian | 46 |
| XP_015682387.1 | *Protobothrops mucrosquamatus* | Snake | 57 |
| XP_005299021.1 | *Chrysemys picta bellii* | Turtle | 47 |
| NP_001085424.1 | *Xenopus laevis* | Toad | 37 |
| XP_018425541.1 | *Nanorana parkeri* | Frog | 37 |
| NP_001007393.1 | *Danio rerio* | Zebrafish | 43 |

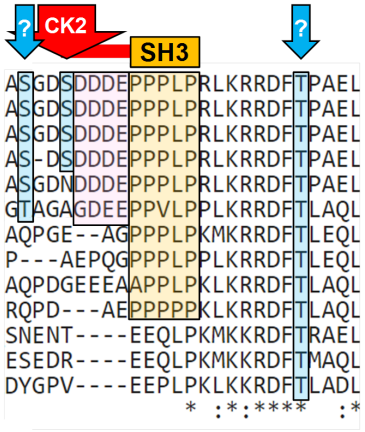



**Figure 4. The PGRMC1 SH3 target motif centred on P64 (numbering refers to PGRMC1) with an adjacent CK2 site is present in the primate lineage.** The proline-rich SH3 target motif is present in all land vertebrates which evolved from amphibians. A phosphorylatable residue appears C-terminally at the S54 position in marsupials and mice, which is augmented by an adjacent CK2 consesus site (SDDDE) present in the primate lineage from shrews to apes. The phosphorylation site at T74 is highly conserved **(Figure S1A)**, but of unknown function.

superposition of the S181 CK2 site motif upon the Y180 SH2 target motif (Figure 3A).

Annelid worms and molluscs, and other higher organism in Figure 3A additionally exhibit the adjacent T178 phosphorylation site in their PGRMC-like protein, which was variously lost in some lineages, but which became absolutely conserved in vertebrates, and therefore presumably performs some function enabling the complexity of PGRMC-like protein regulation required for vertebrate biology.

### 3.8. *C. elegans VEM-1 is an atypical PGRMC-like protein*

It becomes apparent why VEM-1 clusters away from other animal PGRMC-like proteins in Figure 2, because it lacks the Y139 and C-terminal negatively charged CK2-like motifs, which we can deduce were secondarily lost from the ancestral PGRMC-like condition in the nematode lineage. Alternatively, VEM-1 could be descended from a non-PGRMC1-like protein. To test this situation both *C. elegans* MAPR proteins were aligned with all four human MAPR proteins (Figure 3B). The alignment of VEM-1 with PGRMC1 and PGRMC2 clearly identifies it as a PGRMC-like protein which is distinct from the Neuferricin homologue (Figure 3B), but which has lost some hallmark PGRMC1-like features (Figure 3A). Note that *C. elegans* therefore does not have PGRMC1 and PGRMC2 homologues, but rather a PGRMC-like gene and a Neuferricin-like gene. It has also lost its ancestral Neudesin-like gene (Table 1).

### 3.9. *The PGRMC1-specific SH3 target motif is shared by terrestrial tetrapods*

The N-terminal regulatory cytoplasmic region of PGRMC1 is not shared with PGRMC2, and therefore arose after their gene divergence. The conserved PGRMC1 phosphorylation site at T74 in Figure 4 is shared with PGRMC2 (Figure 1A), however with the appearance of terrestrial tetrapods (reptiles including birds, and mammals, but not amphibians) a proline-rich sequence centred on P64 appeared. Placental and marsupial mammals, but not birds or reptiles, possess the phosphorylatable reside at T54, and all placental mammals have acquired the negatively charged DDDE motif immediately C-terminal to the proline-rich patch. The poly-negative charge probably influences the specificity of SH3 domain proteins that can interact with the adjacent proline-rich sequence. In the primate lineage this motif is further garlanded by the appearance of S57, to create the CK2 motif that is thought to sterically prevent interaction of SH3-domain proteins to the proline-rich SH3-target motif when phosphorylated (*13,20,21*), and provide new levels of finesse to the regulation of PGRMC1 function.

In summary, a new binding site for one or more SH3 domain proteins appeared on PGRMC1 as vertebrates colonised the land. In the mammalian lineage leading to humans this motif was equipped with the means for increasingly sophisticated regulation. It is currently unknown which biological processes are governed by such regulations. Possible functions which differ between the organisms involved include embryogenesis, skin ultrastructure including hair formation, properties of the ovaries/eggs, lactation, oestrus, and many more. Clearly an imperative research priority should be to develop reagents to assay for the state of phosphorylation of this and other PGRMC1 motifs to gain deeper understanding of its potential role in these foundational vertebrate processes.

PGRMC2 is fundamentally different to PGRMC1 in this region (Figure 1A). It should be equally important to determine which processes these two proteins can affect uniquely or in common, whether their effects on common processes are in the same directions, and the

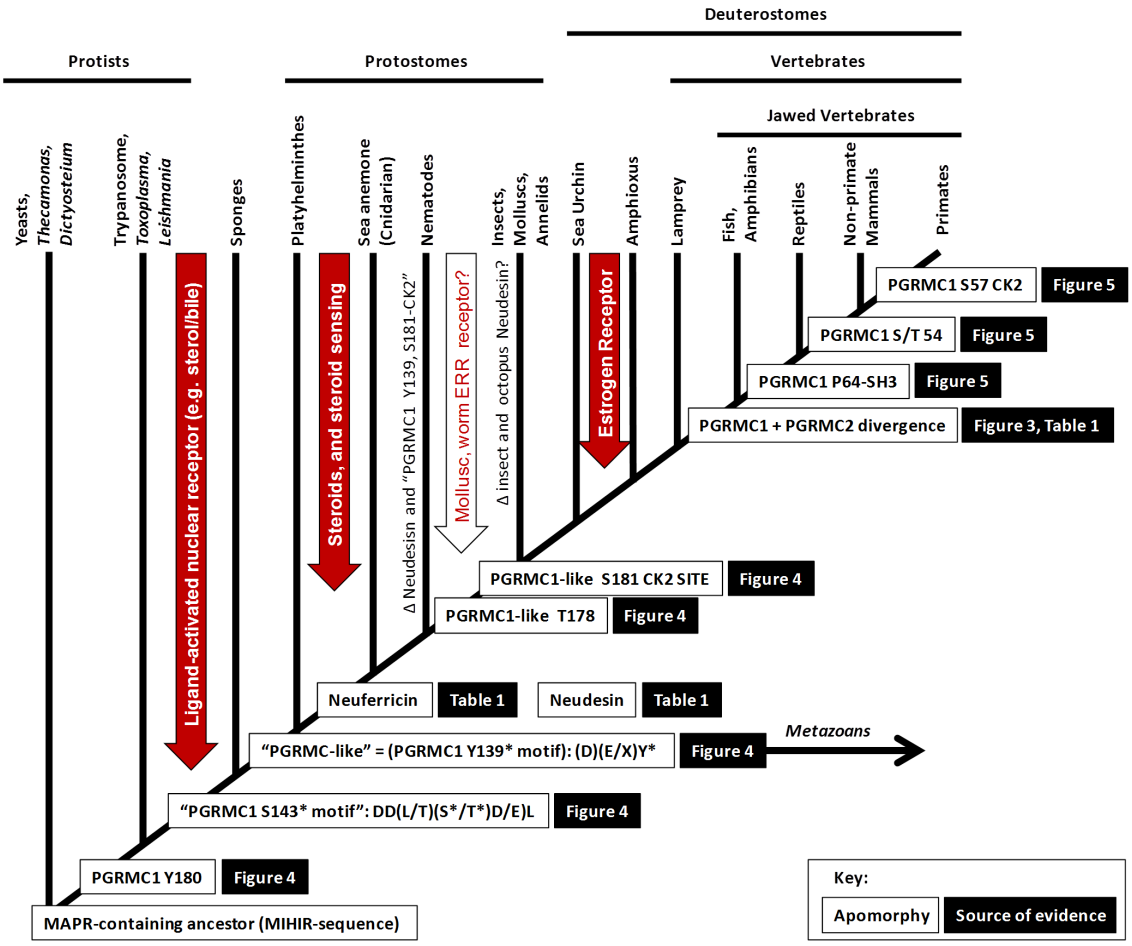



**Figure 5. A cladogram of eukaryotic organisms considered in this study based upon MAPR-family traits.** The single celled eukaryotic ancestral organism to all organisms considered contained a MAPR family gene, called the the plesiomorphic condition. Apomorphies, or shared derived character states, are indicated on the cladogram stem with their source in black boxes. Amino acid numbers refer to motifs with reference to PGRMC1. The question mark (?) is present because a symmetrical segmented worm-like body plan is thought to have predated insect development, and because octopus and oysters are thought to be part of the same monophyletic evolutionary mollusc clade. This emphasizes the point that this is not a valid evolutionary tree, but a cladogram based solely upon inherited MAPR-related traits, and the appearance of steroids and steroid receptors (ERR, Estrogen related receptor; ESR estrogen receptor) following Baker (*16*) and Markov *et al*. (*14*) (vertical downward arrows) to separate some clades. Sea urchin is separated from protostomes by virtue of being a deuterostome (*i.e.* not by MAPR- or steroid-related traits).

molecular basis underlying any different functions.

### 3.10. *Animal evolution topologically aligns with MAPR apomorphies*

The above derived character traits of the MAPR family can be used to construct a cladogram based upon the appearance of new character states (apomorphies). Different organisms which inherit the same apomorphy share a synapomorphy which defines monophyletic groups of taxa (clades) which are descendants of the ancestral organism in in which the apomorphy first appeared (*34*). The cladogram of Figure 5 relatively accurately corresponds with known phylogeny of evolutionary divergence of these groups. Because of the association of PGRMC1 with P4 responsiveness, the cladogram has also been augmented by information about the evolutionary origins of ligand activated receptors, steroid production and sensing, and the appearance of the Estrogen receptor in protochordates (Amphioxus in Figure 5), as referenced from Markov *et al*. (*14*). One interpretation of this cladogram is that the development of increasingly sophisticated regulation of PGRMC-like protein function throughout animal evolution has been intimately involved with the development of the key features which define these different lineages, right up to primates. In this model, PGRMC-like proteins provided some quintessential function to higher eukaryotic cell biology, and the tweaking and fine tuning of the MAPR/PGRMC1 functional axis has been a powerful driving force throughout the diaspora of animal evolution. If so, it appears that the regulation of PGRMC-like proteins by phosphorylation (*21*) has been at the very heart of this evolutionary process.

This concept implies that animal evolution has involved regulated phosphorylation of unknown

signalling events based upon the Y180 motif of PGRMC-like proteins since our Precambrian ancestors. The motif was subject to increasingly sophisticated regulation during early animal evolution, particularly involving gain of the T178 site and its subsequent loss in several lineages. However, it appears to have been absolutely conserved in both PGRMC1 and PGRMC2 since their divergence prior to the origin of jawed vertebrates (Figure 3A, Figure 5), after which protein interactions with the PGRMC1 proline-rich SH3 motif appeared and became subject to increasingly complex regulatory possibilities (Figure 4, Figure 5).

## 4. Discussion

### 4.1. *PGRMC or MAPR family?*

McCallum *et al*. (*35*) referred to the MAPR family as the "PGRMC family". In this present paper I argue for the presence of PGRMC-like, Neuferricin-like, and Neudesin-like metazoan MAPR proteins, which are specific for animals (metazoan) but not other eukaryotes. "PGRMC-like" proteins (as defined here) are then a specific sub-clade of the MAPR family which does not include protist MAPR proteins. To avoid terminological confusion I refer to the MAPR family to denote the group of MIHIR-containing Cytb5 proteins. "PGRMC-like" better refers to those metazoan MAPR proteins which ancestrally contained the equivalent of PGRMC1 Y139, S143, and Y180. Neuferricin appears to have evolved by gene duplication of the PGRMC-like gene followed by loss of the Y180 homologous residue. Neudesin appears to have similarly later diverged prior to the divergence of Protostomes and Deuterostomes, from probably Neuferricin however that is uncertain (Figure 1B-D, Figure 3B, Figure 5, Table 1). All four MAPR proteins were present in the ancestor of annelids and molluscs, whereas ancestral Neudesin appears to have been lost from insects. Therefore use of the term "PGRMC family" (*35*) to denote the MAPR family appears to introduce room for potential systematic confusion.

### 4.2. *Progesterone responsiveness of PGRMC-like proteins is likely a vertebrate phenomenon*

Participation of MAPR proteins in cholesterol synthesis is a function shared from protist to metazoan eukaryotes (*11*), which we can conclude was inherited from the earliest eukaryotic cell. Steroid hormones arose much later in evolution, presumably in the metazoan lineage. Both vertebrates and invertebrates possess ligand-activated classical/nuclear receptors for hydrophobic ligands such as terpenes, fatty acids, eicosanoids, sterols and bile acids, most of which are products of the mevalonate/isoprene pathway (*15,36*). From such a commonly inherited sterol sensing mechanism, separate clades are thought to have convergently evolved steroid signalling mechanisms based upon cnidosteroids (cnidarians such as jellyfish and corals), lophosteroids (annelid worms, molluscs, *etc*), ecdyosteroids (insects, other arthropods, and nematode worms), and vertebrosteroids (vertebrate lineage, commencing from an original ESR gene) (*14*). These steroid signalling systems evolved independently from one another in those clades in a striking example of parallel evolution.

The main steroidogenic CyP450 enzymes (*37*) as well as adrenal and sex steroid receptors (*15*) both recognizably arose in the protochordate lineage leading to vertebrates, with subsequent receptor gene duplications and functional diversification throughout vertebrate evolution (*15*). The estrogen receptor (ESR) was the first vertebrate steroid receptor (Figure 5), from which subsequent gene duplications eventually produced the classical progesterone receptor (*14*).

The enzyme P450 side-chain cleavage enzyme (P450scc, CYP11A1), which catalyzes the synthesis of the first vertebrate steroid hormone pregnenolone from cholesterol in mitochondria, has low sequence conservation (*37*), and therefore may not be recognizable in lower organisms. However, extensive BLAST analysis of lower organisms has not identified CYP11A1 in organisms lowere than *Amphioxus* (*14*), and pregnenolone has not been convincingly reported in invertebrates to date (*14*).

From the above consideration, we can conclude by process of deductive logic that if sterol sensing arose in metazoans, and if steroid synthesis involving P4 appeared first in the vertebrate lineage, then any P4 or even progestogen sensing or signalling function of PGRMC1 is probably not an ancestral MAPR function. MAPR proteins do share an ancestrally inherited role in sterol synthesis (*11*). If an ancestral PGRMC-like protein participated in sterol sensing or sterol transport that involved low affinity interactions with cholesterol or derivatives, it is quite feasible that it could have secondarily acquired responsiveness to progesterone/progestagens once they appeared. (However whether PGRMC1 itself acts as a direct P4 receptor itself is still unclear (*5*).) Indeed, the ancestral metazoan nuclear steroid receptor is thought to have similarly originally been involved in sterol sensing, as well as being able to bind a broad range of metabolites including dietary sterols and xenobiotics. That activity had evolved into an ESR activity by the time the vertebrate estrogen synthetic pathway had evolved, and there is still some overlapping ligand affinity of certain modern steroid receptors for specific steroids and other hydrophobic ligands (*14*).

### 4.3. *Axonal migration probably does not involve PGRMC-like SH3 or SH2 target motifs*

PGRMC1 is involved in directing the axonal migration of nerve cells in the embryologically developing nerve chord of nematodes and humans (*17-19*). Interestingly,

the lack of the Y139 motif and the S181 CK2 site from VEM-1 (Figure 3A) most probably mean that these motifs are not involved in that function exerted by PGRMC1, although we cannot be certain. VEM-1 interacts with members of Netrin-receptor family in *C. elegans* (*18*), and presumably in other animals in such a conserved process of body plan definition. The membrane trafficking function of PGRMC1 (reviewed by (*5*)) may be involved in regulating the cell surface expression of Netrin receptors to enable axon guidance in this process. Because they are the only major MAPR phosphorylation sites conserved between mammals and nematodes, presumably either Y113 (which contains a presumed membrane trafficking motif (*5,21*)) or Y107 are involved in this process. However that hypothesis requires experimental confirmation. This thought process demonstrates how systematic approaches such as this study can guide us to begin to functionally separate and stratify different pathways of the mutinodal signal integration web that is suspected to revolve around the fulcrum of PGRMC1 function (*5*). Indeed a central axis involving PGRMC1 Y180 is now revealed as running right back to single celled Precambrian biology.

Other intruiging PGRMC1 functions, in terms of cellular life as part of a multicellular organism, are controlled cell death, and hormonal signalling (reviewed by (*5*)). PGRMC1 confers resistance to death-inducing signals to some cells, and also confers responsiveness to progesterone (P4) and related progestogens. It is highly likely that these functions are separate to axonal migration guidance, and variously controlled by the different regulatory modules described here. It will be most interesting to see whether the observed co-evolution of a suite of mitochondrial genes with PGRMC1 (*8*) is related to any role of PGRMC1 in directing mitochondrial function, especially the changes in mitochondrial function that are associated with progression from single celled oocyte and zygote through embryogenesis towards a multicellular orgnanism (*38*). These reflect the changes in mitochondrial function required in the evolutionary progression from protist eukaryotes to metazoans as considered in this paper.

### 4.4. *Is the PGRMC1/Sigma 2 Receptor function related to multicellularity?*

The Sigma 2 Receptor (S2R) is an unidentified receptor activity that binds a large number of hydrophobic S2R ligands associated with neural disorders and cancer (*39*). Sigma receptors were originally described in 1976 as a subtype of the opiate receptor, based on the properties of (±)-SKF-10,047 (N-allylnormetazocine) and the structurally related analogues morphine and ketazocine. This led to the classification of three opiate receptor subtypes, μ for morphine, κ for ketazocine, and σ for (±)-SKF-10,047. It was subsequently found that the (-) stereoisomer of SKF-10,047 bound to the μ and κ opiate receptors. However the (+) isomer bound to an unknown non-opiate receptor, which became known as the σ (Sigma) receptor. Various ligands were found to bind this enigmatic receptor, some of which revealed that there were two distinct proteins with σ receptor activity, which became known as σ1 (S1) and σ2 (S2) receptors (reviewed by (*39*)). The sigma 1 receptor (S1R) was cloned in 1996, and found to have low homology with a sterol isomerase (*40*). It has been reported to be involved in lipid transport, the regulation of cholesterol-rich lipid raft microdomain formation at the plasma membrane, and the metabolism of cholesterol-containing cytoplasmic lipid droplets (*41*). S1R possesses protein chaperone function, where it provides an ER luminal hydrophobic binding site which binds to and stabilizes certain proteins, and which is located at cholesterol-rich ER regions, including mitochondria-associated ER membranes and the ER-cytoplasmic membrane interface (*42*). Like PGRMC1 (*12*), S1R is also associated with Insig-1, where S1R is implicated in the ER-associated degradation of proteins in a possibly sterol-dependent manner (*42,43*).

The identity of S2R remains unknown, however radio-ligand studies have shown that its activity was upregulated 10-fold in proliferating compared to quiescent cancer cells. In addition, certain S2R ligands bound their receptor at the cell surface, translocated to the mitochondrion, and killed tumor cells *via* both apoptotic and nonapoptotic mechanisms (*39,44*), which indicates an essential function for the S2R in some cancers. S2R ligands were internalized by both phenyl arsine oxide inhibitable receptor-mediated endocytosis, as well as *via* undefined non-inhibitable mechanism such as passive diffusion or non-receptor-mediated endocytosis (*45*).

In 2011 PGRMC1 was identified by Mach and colleagues as being part of a protein complex with S2R (*46*). A photoaffinity-labeled S2R ligand was cross linked to PGRMC1, indicating that PGRMC1 must be present with intimate proximity in an S2R-containing protein complex (*46*). In several cell types, but not all, PGRMC1 is required for S2R activity. This led to some confusion as to the possible identity of S2R and PGRMC1 (for review: (*5*)), which remains formally unresolved. However S2R is probably a separate 18 kDa protein unrelated to PGRMC1 (*5*).

S2R activity is vitally important for many cancer cells (*39*), and is associated with cancer stem cell properties related to proliferative status and survival (*47-49*). It is conceivable that this PGRMC1-S2R system, that regulates differentiation, proliferative, and survival decisions, reflects a remnant of the ancestral control of replication of the primitive single celled eukaryote. Thereby, the requirement of cancer and cancer stem cells for S2R activity may represent an ancestral S2R unicellular replication licensing function, which is modulated by PGRMC1-like proteins in metazoans that must impose strict restraints on proliferative activity.

If so, the PGRMC1 Y180 and/or the Y139 SH2 target motifs are probably involved since these originated with the appearance of multicellularity. The verification or falsification of this prediction must await the identification of the 18 kDa PGRMC1-associated protein which contains the S2R activity (for review: (*5*)).

## 5. Conclusions

Signalling and regulatory motifs on the PGRMC1 protein are shown here to have evolved along with animals during the metazoan radiation. This study portrays PGRMC1 as a cornerstone protein with functions central to eukaryotic biology and the origin of multicellular animals, that is potentially able to dramatically alter eukaryotic cell biology because of the deep evolutionary dependence of multiple cell functions upon MAPR proteins. If PGRMC1 regulates functions operating at a foundational cornerstone level of cell biology, then those alterations could have wide ranging pleiotropic effects relating to the strictures of multicellular life. Just as Archimedes thought to move the world with a fulcrum and a sufficiently large lever, so PGRMC1 phosphorylation could exert either tremendous metaphorical leverage, or occupy a cellular fulcrum with roots in the Precambrian. Obviously, such pleiotropic properties could be highly problematic for healthy biology when usurped by pathological processes such as cancer (*5,20*) or Alzheimer's disease (*50*). This raises the spectre that it is perhaps not the expression level of PGRMC1 which is most important to disease, but its state of modification. Since practically nothing is known about PGRMC1 modifying enzymes, this highlights an area which requires urgent investigation.

## Acknowledgements

This work has received no direct Australian competitive grant support since 2008. This work was supported by Charles Sturt University (CSU) School of Biomedical Sciences (SBMS) Compact grant A541-900-xxx-40513, SBMS support A534-900-xxx-41066, and CSU Competitive grant A102-900-xxx-40002, all to MAC. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This publication reflects only the author's views. The funder is not liable for any use that may be made of the information herein.

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*(Received January 10, 2017; Revised February 21, 2017; Accepted February 22, 2017)*

## Supplemental Data

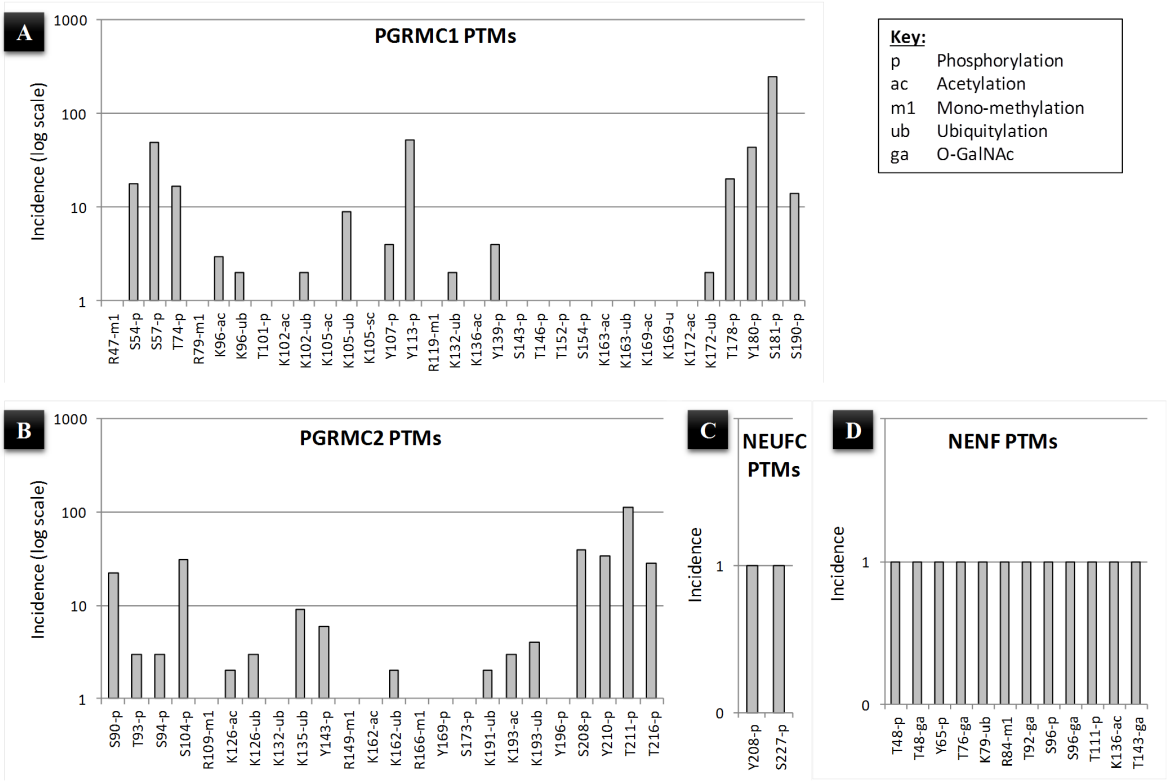


**Figure S1. Post-translational modifications documented in the Phosphosite data base for human MAPR proteins PGRMC1 (A), PGRMC1 (B), Neuferricin (C) and Neudesin (D).**

Original Article

DOI: 10.5582/bst.2016.01229

# Androgen receptor gene CAG repeat polymorphism and ovarian cancer risk: A meta-analysis

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**Summary** **Ovarian cancer is one of the common gynecological malignancies worldwide. It is usually diagnosed at a later stage, thus missing the best opportunity for treatment. Despite the advancement of ovarian cancer treatment, the prognosis is still poor. Androgen receptor (AR) may play a role in ovarian carcinogenesis. Previous studies regarding the association between AR CAG repeat length and ovarian cancer risk reported inconsistent results. Therefore, we conducted a meta-analysis to evaluate the association between AR CAG repeat length and ovarian cancer risk following the MOOSE guidelines. PubMed, Web of Science, EBSCO and other databases were searched up to September 15th 2016. Case control studies with clear definition of CAG repeat length and detailed genotype information were included. Two authors independently reviewed and extracted data. Pooled analysis and subgroup analysis stratified by ethnicity were performed for different genetic models. Begg's funnel plot and Egger's test were performed for publication bias estimation. Overall, there was no association between the AR CAG repeat polymorphism and ovarian cancer risk. However, short CAG repeat polymorphism was associated with increased ovarian cancer risk in African Americans and Chinese under the dominant model, whereas a reverse association was observed in Caucasians and Italians under the other three models. Our study results should be interpreted with caution. Further well-designed epidemiological and functional studies are needed to elucidate the role of AR in ovarian carcinogenesis.**

***Keywords:*** Androgen receptor (AR), CAG polymorphism, ovarian cancer risk, meta-analysis

## 1. Introduction

Ovarian cancer is one of the common gynecological malignancies among women worldwide (*1*). It is the second most commonly diagnosed gynecological malignancies and second leading cause of death from gynecological malignancies. In 2012, there were about 238,700 incident cases of and 151,900 deaths due to ovarian cancer (*1*). The etiology of ovarian cancer has not been well elucidated, although previous researches have demonstrated that several factors, including family history, diet, obesity, inflammation, use of estrogen and hormone-replacement therapy, reproductive factors such as null-parity, early age at menarche, late age at menopause and oral contraceptive use, and genetic susceptibility may contribute to ovarian cancer development (*2*).

Epidemiologic and biological data have suggested that androgens and androgen receptor (AR) may play a role in the occurrence of ovarian cancer (*3,4*). The AR is

Released online in J-STAGE as advance publication February 28, 2017.

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a ligand-dependent transcriptional factor mediating the actions of testosterone and dihydrotesterone (*5*). Mapped to X chromosome (q11.2-12), the *AR* gene includes eight exons. In exon 1 is a trinucleotide cytosine, adenine, guanine (CAG) repeat, which encodes a polyglutamine tract with varying lengths (*5*). It is reported that different ethnicities have different CAG repeat lengths, with the shortest being reported in African-Americans (mean,20; range,10-29) and the longest in Mexican-Americans (mean,25; range,16-32) (*6*). Studies have shown that CAG repeat lengths were associated with the risks of different cancer types in various populations, such as breast cancer, prostate cancer and colorectal cancer (*7-9*). In terms of ovarian cancer, some studies have shown that long CAG repeat allele was associated with increased ovarian cancer risk (*10,11*), while other studies have reported an inverse association between CAG repeat length and ovarian cancer risk (*12,13*). Furthermore, several studies suggested no relationship between CAG repeat length and ovarian cancer (*14-17*). These conflicting results may be explained by ethnically diverse populations and different sample sizes in each publication. To the best of our knowledge, so far no meta-analysis has been conducted to investigate the association between *AR* CAG repeat polymorphism and the risk of ovarian cancer, as well as genetic heterogeneity across different ethnic groups. Therefore, we performed the present meta-analysis to evaluate the association between *AR* CAG repeat polymorphism and ovarian cancer risk following the Meta-analyses of Observational Studies in Epidemiology (MOOSE) guidelines (Supplementary Table S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=8*).

## 2. Materials and Methods

### 2.1. *Literature Searches*

Studies in English were searched in PubMed, Web of Science, EBSCO and Cancer Genetic Markers of Susceptibility (CGEMS), and reports in Chinese were searched in China National Knowledge Infrastructure (CNKI), the Database of Chinese Scientific and Technical Periodicals (VIP) and the China Biology Medical Literature database (CBM), from the earliest date up to September 15$^{th}$ 2016. The search terms included ("androgen receptor" or the gene abbreviation "*AR*") and ["CAG" or "(CAG)n" or "polymorphism" or "short tandem repeat"] and ("ovarian cancer" or "ovarian carcinoma" or "ovarian neoplasms"). Titles and abstracts of the search results were first screened, and full texts of promising articles were retrieved and evaluated in detail. References from identified articles and reviews were also examined. If the full text of an article or detailed information was not available online, we proceeded to contact the corresponding author of the article by e-mail.

### 2.2. *Evaluation criteria*

The following criteria were applied to select studies for inclusion in the meta-analysis: *i*) articles about *AR* CAG polymorphism and ovarian cancer risk, *ii*) clear definition of CAG_S (shorter allele), CAG_L (longer allele) and detailed genotype information, *iii*) case control studies, *iv*) if multiple publications for a single study were reported, only the latest publication with the most complete or updated data was selected. Studies did not report an adequate description of the epidemiological design, statistical analysis, or separate analyses for *AR* CAG repeat in relation to ovarian cancer risk were excluded. Case series were also excluded.

### 2.3. *Data extraction*

Data were extracted by two authors (Yang D. and Yan D.) independently, and any differences were resolved by consensus after discussion. The following information was extracted from each study: first author, population (ethnicity of participants), year of publication, sample size, and genotype counts for cases and controls.

### 2.4. *Statistical analysis*

The association between *AR* CAG repeat polymorphism and ovarian cancer risk was evaluated by odds ratios (ORs) and their 95% confidence intervals (CIs), and the ORs were calculated for the allele genetic model, additive genetic model, dominant genetic model, and recessive genetic model, respectively. The choice of using fixed or random effects model was determined by the results of the between-study heterogeneity test, which was measured using the $Q$ test and $I^2$ statistic. If the test result was $I^2 \geq 50\%$ or $P_Q < 0.1$, indicating the presence of heterogeneity, the random effect model was selected; otherwise, the fixed-effects model was chosen (*18*). Subgroup analysis was performed based on the ethnicity. Begg's funnel plot and Egger's test were conducted to estimate the possible publication bias (*19*). All statistical analyses were performed using Review Manager 5.3 (The Cochrane Collaboration, Oxford, UK) and Stata 12.0 (Stata-Corp, College Station, Texas, USA).

## 3. Results

### 3.1. *Study Characteristics*

The initial search retrieved 18 potentially relevant publications (17 published in English and 1 in Chinese). Ten of these publications were excluded according to the evaluation criteria: 6 publications were not case-control studies (*20-25*), and another 4 did not provide detailed genotype or allele distribution data (*26-29*). Finally, 8 case-control studies containing 6613 cases and 7041

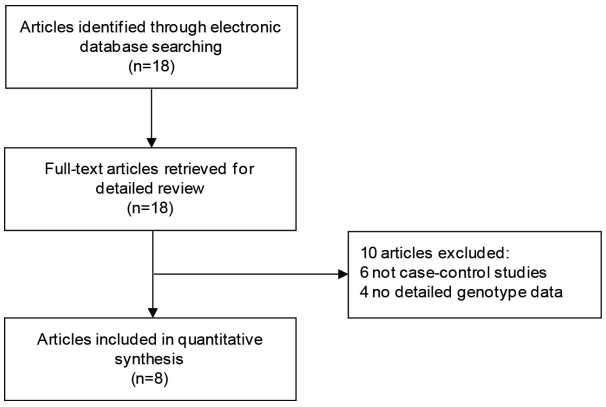


**Figure 1. Flow chart of the study selection process.**

controls were included in the current meta-analysis (*10-17*). A flow chart of study selection process was shown in Figure 1, and the baseline characteristics of all included studies were presented in Table 1.

3.2. *Overall analysis*

The pooled analyses of the association of *AR* CAG repeat polymorphism with ovarian cancer risk were shown in Figure 2 to Figure 5. In this study, CAG_S is referred to repeat length ≤ 21 for Chinese, Caucasians and Italians, while for African Americans CAG_S is referred to repeat length < 16. The results suggested that *AR* CAG repeat polymorphism was not associated with ovarian cancer risk under the allele, additive, dominant and recessive models (for L allele versus S allele: OR = 1.06, 95%CI = 0.87-1.31, $P = 0.56$; $I^2 = 76\%$ and $P_Q = 0.0009$ for heterogeneity; for LL versus SS: OR = 1.23, 95%CI = 0.88-1.72, $P = 0.23$; $I^2 = 62\%$ and $P_Q = 0.02$ for heterogeneity; for SL+LL versus SS: OR = 0.91, 95%CI = 0.72-1.15, $P = 0.45$; $I^2 = 83\%$ and $P_Q < 0.00001$ for heterogeneity; for LL versus SL+SS: OR = 1.16, 95%CI = 0.84-1.59, $P = 0.36$; $I^2 = 73\%$ and $P_Q = 0.003$ for heterogeneity). The existence of study heterogeneity is found in all models.

3.3. *Subgroup analysis*

The results of subgroup analysis showed significant positive associations of long CAG repeat allele with ovarian cancer risk among Caucasians (L allele versus S allele: OR = 1.12, 95%CI = 1.02-1.23, $P = 0.02$; $I^2 = 0\%$ and $P_Q = 0.88$ for heterogeneity) and Italians (L allele versus S allele: OR = 1.45, 95%CI = 1.03-1.23, $P = 0.03$; $I^2 = 19\%$ and $P_Q = 0.27$ for heterogeneity) under the allele model, but a significantly decreased ovarian cancer risk was found among African Americans with long CAG repeat allele (OR = 0.42, 95%CI = 0.26-0.68, $P = 0.0004$) under the allele model. The details were presented in Figure 2.

The subgroup analysis of the additive model of *AR*

**Table 1. Characteristics of studies of androgen receptor gene CAG polymorphism and ovarian cancer susceptibility**

| Author | Population | Year | Short SS[a] | | Any long SL and LL | | Case | | | Control | | | OR(95%CI) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Case | Control | Case | Control | SS | SL | LL | SS | SL | LL | Allele | Additive | Dominant | Recessive |
| Spurdle *et al. (16)* | Australian Caucasians | 2000 | 75 | 128 | 244 | 425 | 75 | 149 | 95 | 128 | 281 | 144 | 1.07 (0.88,1.30) | 1.13 (0.77,1.65) | 0.98 (0.71,1.36) | 1.20 (0.89,1.64) |
| Menin *et al. (14)* | Italians | 2001 | 18 | 32 | 32 | 69 | 18 | 20 | 12 | 32 | 57 | 12 | 1.17 (0.72,1.91) | 1.78 (0.66,4.77) | 0.82 (0.40,1.68) | 2.34 (0.97,5.68) |
| Santarosa *et al. (11)* | Italians | 2002 | 27 | 35 | 94 | 65 | 27 | 57 | 37 | 35 | 47 | 18 | 1.66 (1.14,2.43) | 2.66 (1.25,5.67) | 1.87 (1.04,3.39) | 2.01 (1.06,3.81) |
| Terry *et al. (10)* | American Caucasians | 2005 | 212 | 249 | 693 | 727 | 212 | 432 | 261 | 249 | 488 | 239 | 1.14 (1.00,1.29) | 1.28 (1.00,1.65) | 1.12 (0.91,1.38) | 1.25 (1.02,1.53) |
| Schildkraut *et al. (15)* | American Caucasians | 2007 | 163 | 198 | 321 | 324 | 163 | 237 | 84 | 198 | 240 | 84 | 1.12 (0.94,1.34) | 1.21 (0.84,1.75) | 1.20 (0.93,1.56) | 1.09 (0.79,1.53) |
| | African-Americans | | 11 | 5 | 88 | 136 | 11 | 28 | 60 | 5 | 25 | 111 | 0.42 (0.26,0.68) | 0.25 (0.08,0.74) | 0.29 (0.10,0.88) | 0.42 (0.24,0.74) |
| Liu *et al. (17)* | Chinese | 2011 | 2 | 4 | 38 | 44 | | | | | | | | | 1.73 (0.30,9.96) | |
| Zhu *et al. (12)* | Chinese | 2016 | 673 | 509 | 1127 | 1291 | | | | | | | | | 0.66 (0.57,0.76) | |
| Meng *et al. (13)* | Chinese | 2016 | 1048 | 818 | 1747 | 1982 | | | | | | | | | 0.69 (0.62,0.77) | |

[a]CAG_S is referred to repeat length ≤ 21 for Chinese, Caucasians and Italians, while for African Americans CAG_S is referred to repeat length < 16.

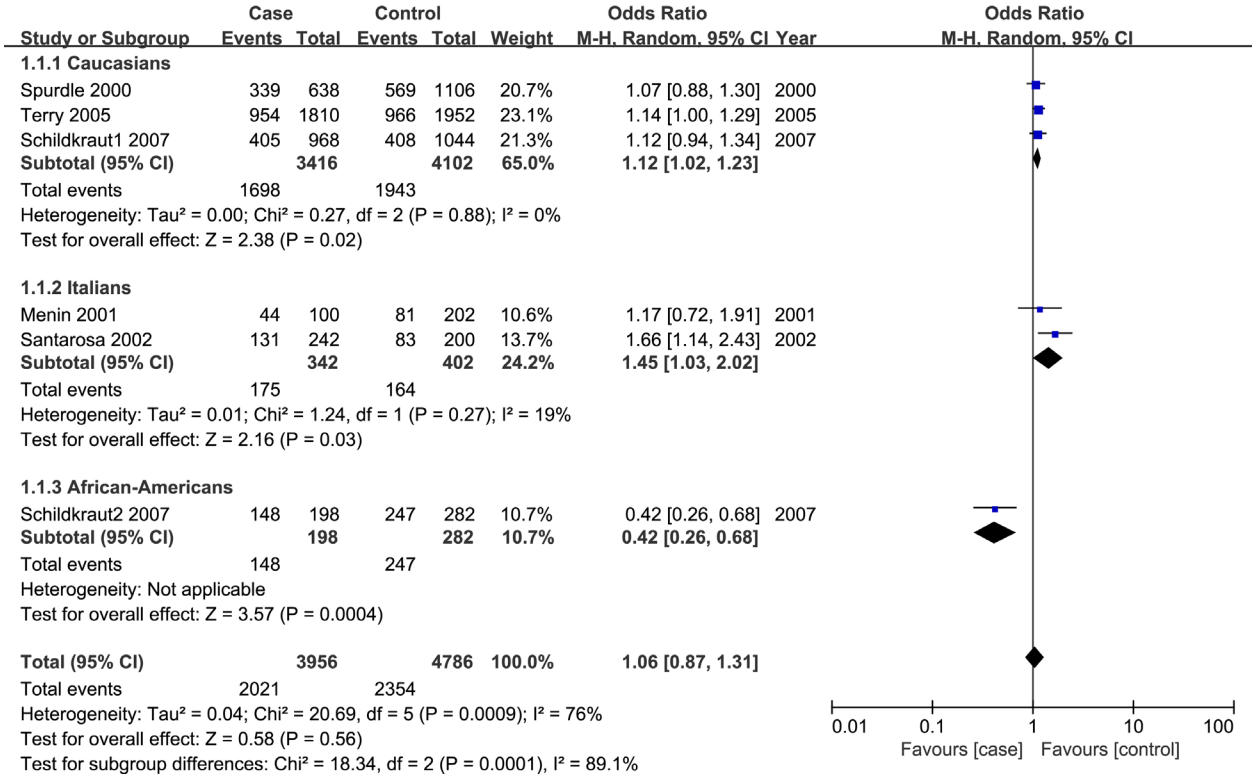


**Figure 2. Forest plot of the association between the *AR* CAG repeat polymorphism and ovarian cancer risk under the allele model. Each study is shown by an OR and the 95%CI.**

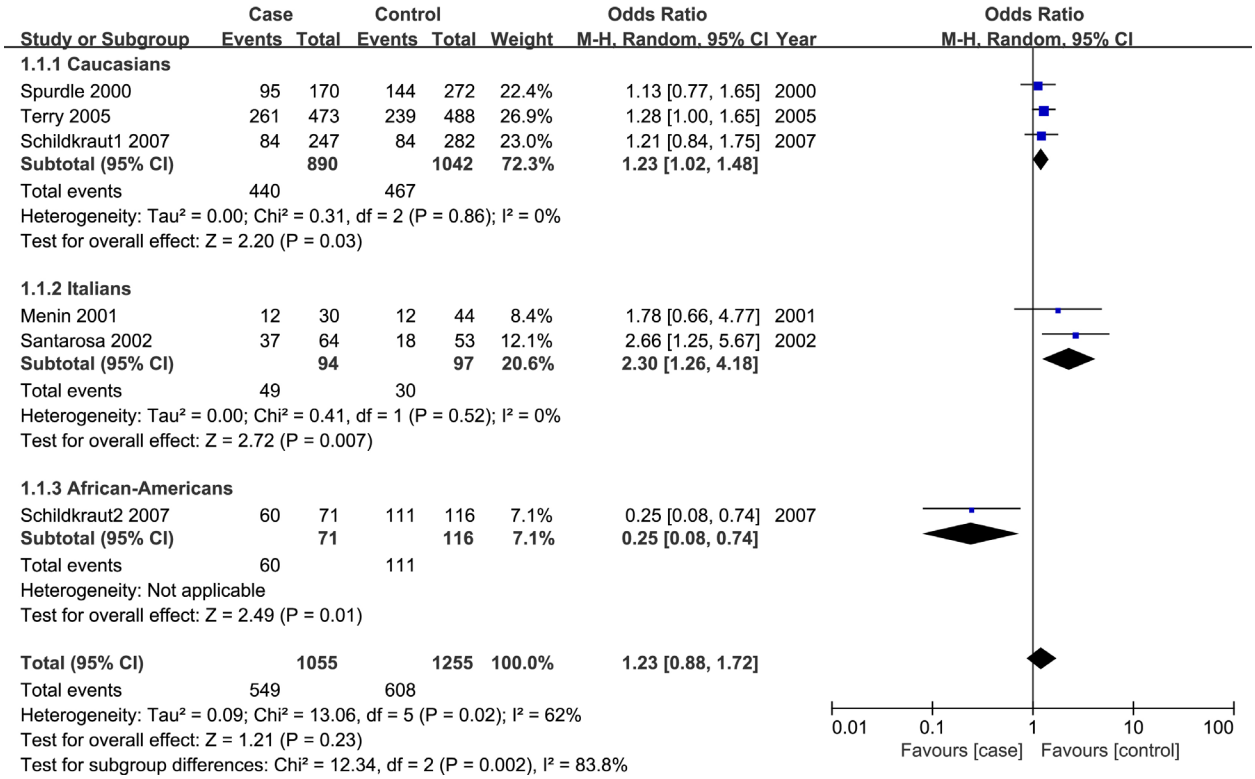


**Figure 3. Forest plot of the association between the *AR* CAG repeat polymorphism and ovarian cancer risk under additive model. Each study is shown by an OR and the 95%CI.**

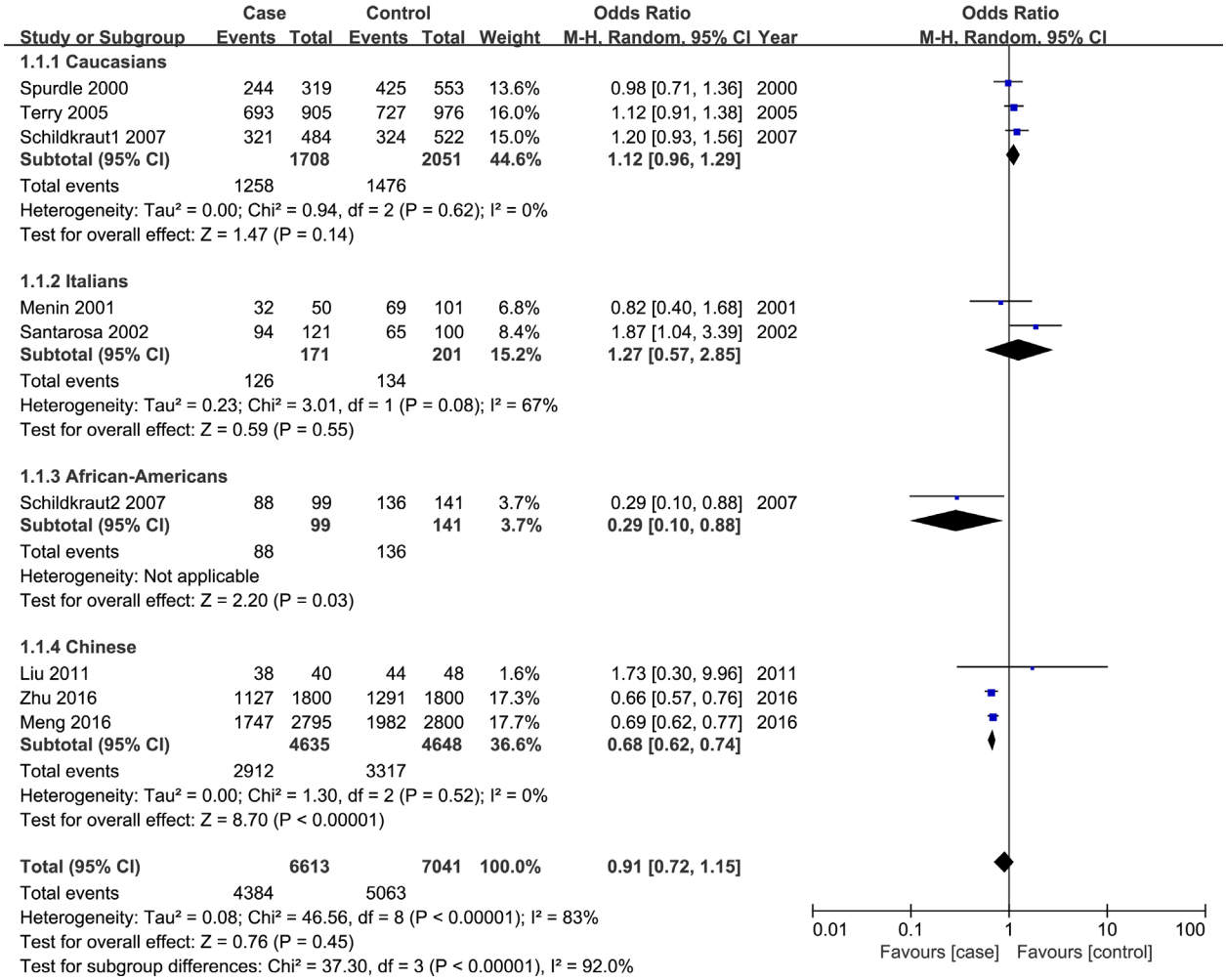


**Figure 4. Forest plot of the association between the *AR* CAG repeat polymorphism and ovarian cancer risk under dominant model. Each study is shown by an OR and the 95%CI.**

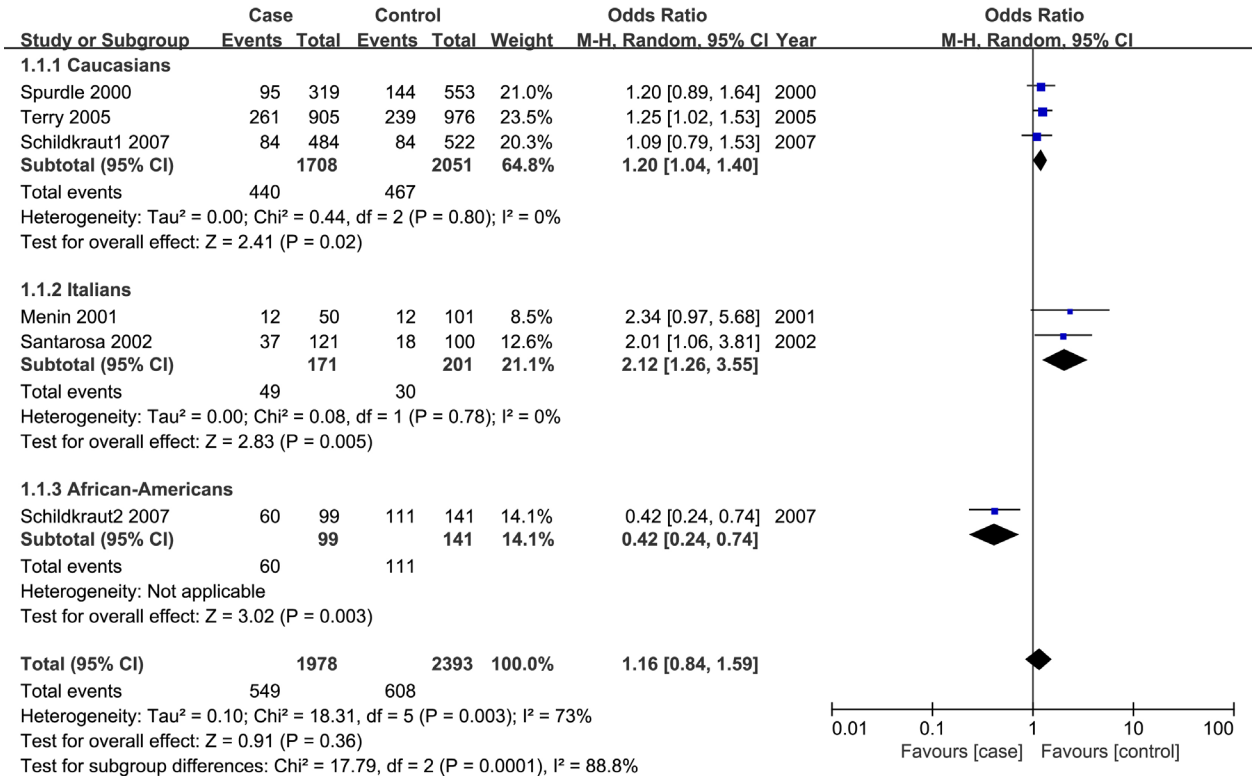


**Figure 5. Forest plot of the association between the *AR* CAG repeat polymorphism and ovarian cancer risk under recessive model. Each study is shown by an OR and the 95%CI.**

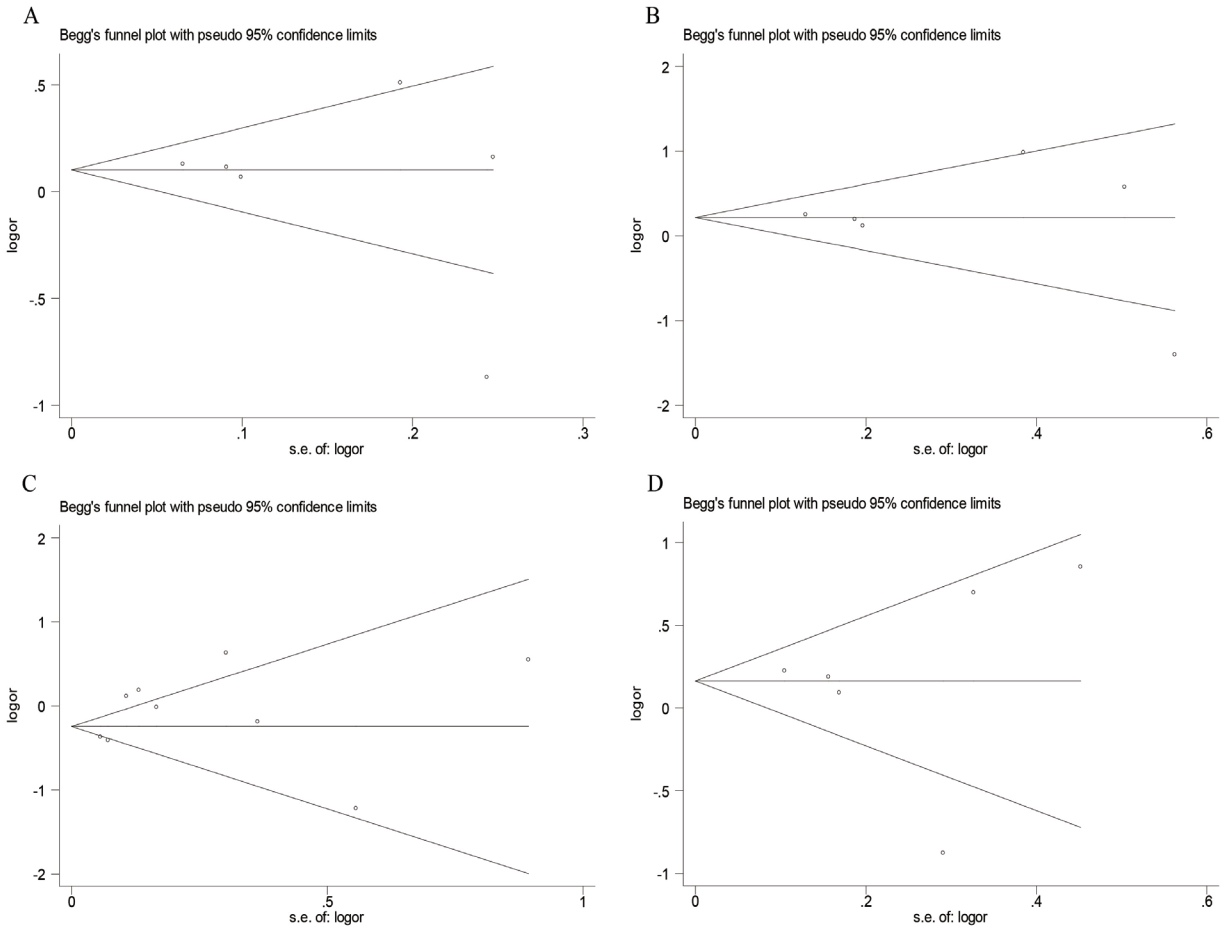


**Figure 6. Begg's funnel plot analysis to detect publication bias. Each point represents a separate study for the indicated association. (A)**, allele model; **(B)**, additive model; **(C)**, dominant model; **(D)**, recessive model.

CAG repeat polymorphism was shown in Figure 3. For the additive model, significantly increased ovarian cancer risk was found among Caucasians and Italians with long CAG repeat allele (LL versus SS: OR = 1.23, 95%CI = 1.02-1.48, $P$ = 0.03; $I^2$ = 0% and $P_Q$ = 0.86 for heterogeneity; OR = 2.30, 95%CI = 1.26-4.18, $P$ = 0.007; $I^2$ = 0% and $P_Q$ = 0.52 for heterogeneity), and a significant negative association among African Americans with long CAG repeat allele (LL versus SS: OR = 0.25, 95%CI = 0.08-0.74, $P$ = 0.01).

For the dominant model of *AR* CAG repeat polymorphism, there was a significant negative association of long CAG repeat allele and ovarian cancer risk among African Americans and Chinese (SL+LL versus SS: OR = 0.29, 95%CI = 0.10-0.88, $P$ = 0.03; OR = 0.68, 95%CI = 0.62-0.74, $P$ < 0.00001; $I^2$ = 0% and $P_Q$ = 0.52 for heterogeneity). No significant association was found among Caucasians and Italians (OR=1.12, 95%CI = 0.96-1.29, $P$ = 0.14; $I^2$ = 0% and $P_Q$ = 0.62 for heterogeneity; OR = 1.27, 95%CI = 0.57-2.85, $P$ = 0.55; $I^2$ = 67% and $P_Q$ = 0.08 for heterogeneity). The details were shown in Figure 4.

The results of subgroup analysis showed that a significant positive association of long CAG repeat allele with ovarian cancer risk among Caucasians and Italians under the recessive model (OR = 1.20, 95%CI = 1.04-1.40, $P$ = 0.02; $I^2$ = 0% and $P_Q$ = 0.80 for heterogeneity; OR = 2.12, 95%CI = 1.26-3.55, $P$ = 0.005; $I^2$ = 0% and $P_Q$ = 0.78 for heterogeneity), and a significant negative association was found among African Americans (OR = 0.42, 95%CI = 0.24-0.74, $P$ = 0.003). The details were shown in Figure 5. We did not calculated the association of CAG repeat polymorphism with ovarian cancer risk among Chinese under the allele, additive and recessive model due to the lack of detailed allele information in these models.

### 3.4. *Publication bias*

Begg's funnel plot did not indicate evidence of publication bias in the pooled analyses of the association between AR CAG repeat polymorphism and ovarian cancer risk under the allele, additive, dominant and recessive models (Figure 6). Egger's test also suggested no obvious publication bias in overall models ($P$ = 0.586 for L allele versus S allele; $P$ = 0.787 for LL versus SS; $P$ = 0.225 for SL+LL versus SS; $P$ = 0.960 for LL versus SL+SS).

## 4. Discussion

The present meta-analysis, including 6613 cases and 7401 controls from 8 case control studies, evaluated the association between the *AR* CAG repeat polymorphism and ovarian cancer risk. Our overall analysis results showed no association between *AR* CAG repeat polymorphism and ovarian cancer risk. However, in subgroup analysis stratifying by ethnic groups, CAG_L was significantly associated with increased ovarian cancer risk among Caucasians and Italians under the allele model, additive model, and recessive model. In contrary, a negative association was observed of the CAG_L and ovarian cancer risk among African Americans under all models (allele, additive, dominant, and recessive models). In addition, a negative association was shown between CAG_L and ovarian cancer risk among Chinese under the dominant model. Furthermore, no obvious publication bias was detected in the pooled analyses of the association of *AR* CAG repeat polymorphism with ovarian cancer risk under the allele, additive, dominant and recessive models, suggesting that the result was relatively stable.

Worldwide, ovarian cancer is the seventh most common and the eighth leading cause of cancer death in females (*1*). Despite the advances in ovarian cancer treatment, the five-year survival rate is still below 45% (*30*). Although epidemiological studies have identified a number of ovarian cancer risk factors, the etiology of ovarian carcinogenesis is far from clear. Host genetic susceptibility plays an important role in ovarian cancer development. Mutations in genes such as *BRCA1*, *BRCA2*, *BRIP1* and *RAD51*, as well as more than 20 low-risk susceptibility loci located in *CHEK2*, *WNT4*, *TERT* and *ABO* have been suggested to contribute to ovarian cancer risk (*31-33*). The *AR* gene, more than 90 kb long, codes for a protein which functions as a steroid-hormone activated transcription factor. The receptor dissociates from accessory proteins upon binding the hormone ligand, then translocates into the nucleus, dimerizes, and further stimulates transcription of androgen responsive genes. The protein contains 3 main functional domains: the N-terminal domain, DNA-binding domain, and androgen-binding domain. There are 2 polymorphic trinucleotide repeat segments in the N-terminal transactivation domain of the AR protein. The exon 1 of *AR* gene contains a polymorphic CAG repeat, and the length of CAG repeats ranges from 6 to 39 among people of different ethnicity (*6*). The abnormal range of CAG repeat length is usually associated with the risk of developing different cancer types including ovarian cancer (*7-11*). However, previous studies of the association between *AR* CAG repeat polymorphism and ovarian cancer risk have shown inconsistent results (*10-17*). In this meta-analysis, we performed a comprehensive evaluation of the relationship between *AR* CAG repeat polymorphism and ovarian cancer risk under the allele, additive, dominant and recessive models.

*AR* CAG repeat lengths vary among different ethnicities, and African Americans have shorter CAG repeat lengths than Caucasians and Italians (*6*). The association between CAG repeat length and cancer risk has been studied extensively in recent years. A study conducted in Brazil has reported that shorter CAG repeat length was associated with lower disease-free survival and higher risk of recurrence or metastasis in head and neck cancer among the general population (*34*). A meta-analysis revealed that long (> 22) CAG repeat length was a protective factor against breast cancer risk under the dominant model (*35*). However, studies from Taiwan showed the association between CAG repeat length and the risk of hepatocellular carcinoma (HCC) was sex dependent. Shorter CAG repeat length was associated with increased risk of HCC in men, but was associated with less susceptibility in women (*36,37*), suggesting different mechanisms are involved in the HCC development regarding men and women. The shorter CAG repeat length is associated with an increased risk of hyperandrogenic manifestations including hirsutism, annovulation, and acne in women and baldness and prostatic hyperplasia in men, perhaps because shorter length may facilitate chronic androgen stimulation which can result in enhanced proliferative activity (*5,13*). Compared to healthy women, patients with ovarian cancer have high levels of circulating androgen before the disease diagnosis, and ARs are usually detected in most ovarian cancer patients (38). A study about the association of *AR* gene polymorphism and polycystic ovary syndrome (PCOS) revealed that shorter CAG repeat length was associated with the higher risk of PCOS (*39*). Moreover, women with PCOS under 54 years of age had an increased risk of developing ovarian cancer (OR = 2.52, 95%CI = 1.08-5.89) (*40*), suggesting that abnormal CAG repeat length might contribute to ovarian cancer through inducing PCOS. Interestingly, our meta-analysis suggest longer CAG repeat length was associated with increased ovarian cancer risk among Caucasians and Italian women, but was protective among African Americans and Chinese. Our results need to be interpreted with caution, since only a relatively small number of available studies have been included.

There are some limitations, which are common in the meta-analysis of genetic polymorphism and disease risk. First, as mentioned above, our meta-analysis only involved eight studies including two studies in American Caucasians, one study in Australian Caucasians, two studies in Italians, three studies in Chinese and only one in African-Americans. Moreover, detailed allele information was insufficient in the studies of Chinese. The number of study in each ethnic population is limited and the conclusion is perhaps partial for lacking enough evidences to estimate the association between

CAG repeat length and ovarian cancer risk. Second, the existence of heterogeneity in overall analyses may affect the accuracy of results. Heterogeneity is often caused by different environmental and ethnic background of population enrolled in each study, and it is inevitable in pooled analysis of included studies. Third, the etiology of ovarian cancer is complicated, including genetic and environmental factors, and their complex interactions. Lack of information of other physiological or environmental factors such as diet, obesity, inflammation status, and use of estrogen and hormone-replacement therapy has prevented us from further evaluating the association between the CAG repeat polymorphism and ovarian cancer risk.

In summary, our meta-analysis suggested that there was no association between the *AR* CAG repeat polymorphism and ovarian cancer risk in overall populations. The short CAG repeat polymorphism was associated with increased ovarian cancer risk in African Americans and Chinese under the dominant model. Whereas the long CAG repeat polymorphism was associated with increased ovarian cancer risk in Caucasians and Italians under the allele, additive and recessive models. Our study results suggest the association between *AR* CAG repeat polymorphism and ovarian cancer risk may differ by different ethnic groups. However, only a few studies are available to be included in this meta-analysis, therefore our study results need to be interpreted with caution. Future well-designed epidemiological studies with adequate sample size and appropriately chosen controls among different ethnic groups especially minority groups should be performed to more accurately estimate the association between CAG repeat polymorphism and ovarian cancer risk. Furthermore, functional studies are needed to elucidate the exact mechanism of *AR* gene in ovarian cancer so as to provide more information for effective prevention and treatment strategies in specific and to improve women's health in general.

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*(Received December 11, 2016; Revised February 16, 2017; Accepted February 19, 2017)*

Original Article

DOI: 10.5582/bst.2017.01029

# Comparison of the docetaxel concentration in human plasma measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS) and a nanoparticle immunoassay and clinical applications of that assay

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**Summary** **To determine the feasibility of using a nanoparticle immunoassay for clinical therapeutic drug monitoring (TDM) of docetaxel concentrations, a sensitive and simple method of liquid chromatography-tandem mass spectrometry (LC-MS/MS) was established to measure the docetaxel concentration in human plasma and the results of LC-MS/MS and the immunoassay were compared. Docetaxel and paclitaxel (the internal standard, or IS) in human plasma were extracted through protein precipitation, separated on a Diamonsil C18 column (150 mm × 4.6 mm, 5 μm), ionized with positive ions, and detected with LC-MS/MS in multi-reaction monitoring (MRM) mode. Plasma samples from 248 cancer patients were assayed with LC-MS/MS and a nanoparticle immunoassay. Data from the samples were analyzed with the statistical software SPSS and the software MedCalc. Results indicated that the calibration curve of the validated method of LC-MS/MS was linear over the range of 10-2,000 ng/mL, with an lowest limit of quantitation (LLOQ) of 10 ng/mL, and the intra- and inter-day precision and accuracy were both < ± 15%. Comparison of the two methods indicated that results of the LC-MS/MS were closely related to those of the nanoparticle immunoassay, with a correlation coefficient (R) of 0.965 and acceptable 95% confidence intervals (CI) of – 231.7-331.1 ng/mL. Overall, the established method of LC-MC/MS and the nanoparticle immunoassay were both suitable for measurement of the docetaxel concentration in human plasma, and the immunoassay was far more cost-effective and better at clinical TDM of docetaxel in clinical practice.**

***Keywords:*** LC-MS/MS, nanoparticle immunoassay, docetaxel, therapeutic drug monitoring

## 1. Introduction

Docetaxel (Taxotere) is a widely used antitumor agent of the taxoid family with broad activity against a variety of solid tumors, such as breast cancer, non-small cell lung cancer (NSCLC), hormone refractory prostate cancer, gastric adenocarcinoma, and squamous cell carcinoma of the head and neck (*1-3*). However, the use of docetaxel may be limited by its narrow therapeutic range and unpredictable interindividual variability, which would induce hematologic toxicity and undesirable effects. The interindividual variability of the drug's pharmacokinetics (PK) and thus drug exposure mainly contributes to its unpredictable toxicity (*4*).

The optimal dosage of or regimen for docetaxel was 60-100 mg/m$^2$ administered intravenously every 3 weeks based on body surface area (BSA). However, BSA-based dosing can cause docetaxel exposure to vary among patients as much as 10-fold (*5*). Population PK analysis indicated that docetaxel exposure was related to α1-acid glycoprotein (AAG) levels, hepatic function,

Released online in J-STAGE as advance publication April 17, 2017.

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age, and BSA (*6-8*), and the variability of PK may induce severe toxicities, including neutropenia, anemia, diarrhea, asthenia, alopecia, and nausea, even at the therapeutic dosage (*9*). Moreover, studies have found that the area under the plasma concentration versus time curve (AUC), a parameter for docetaxel exposure, is associated with hematological toxicity and can predict grade 4 neutropenia (*10*). Therefore, PK-guided dosing of docetaxel may be beneficial because it ensures its antitumor efficacy and it minimizes the incidence of severe toxicities during therapy.

Chromatography (*e.g*. liquid chromatography coupled with mass spectroscopy (*11-13*) or UV detection (*14,15*)) has generally been used to measure the concentration of docetaxel or its metabolites in human plasma or serum. These techniques are more specific and sensitive, but the expensive equipment and the complicated protocol make them ill-suited to routine measurement of docetaxel, and these drawbacks may hinder clinical TDM of docetaxel. A nanoparticle immunoassay based on turbidimetry and monoclonal antibodies that compete with docetaxel has been developed and preliminarily verified to be suitable for clinical TDM of docetaxel (*16*). Therefore, an alternate immunoassay that is simple, rapid, and cost-effective would allow routine monitoring of docetaxel.

Since 2014, a nanoparticle immunoassay performed with an automated biochemistry analyzer has been in clinical use at this Hospital. To provide further evidence that the nanoparticle immunoassay and its corresponding commercial version are suitable for measuring the docetaxel concentration in human plasma, a method of liquid chromatography-tandem mass spectrometry (LC-MS/MS) was established as a "gold standard" and both methods were compared.

## 2. Materials and Methods

### 2.1. *Reagents and equipment*

Docetaxel (Lot: 100666-201002, purity: 98.0%), was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Paclitaxel (Lot: 100382-201102, purity: 99.6%) was obtained from the National Institute for Food and Drug Control (Beijing, China) and served as the internal standard (IS). Pure water (Lot: 20151102) was obtained from the Hangzhou Wahaha Group Co., Ltd. (Hangzhou, China). Methanol (Lot: 0000118131) and acetonitrile (Lot: 0000059829) were from J.T. Baker (USA), and both were chromatography grade. A Diamonsil $C_{18}$ column was from Dikma Technologies (Beijing, China). The MyDocetaxel™ (Lot: 1504030D) reagent kit and quantity control kit (Lot: 1509040C) were both from Jiangsu Changxing Medical Technology Co., Ltd. (Jiangsu, China).

An Agilent 1200 series HPLC system, equipped with a G1312B duplex pump, G1316B thermostatted column compartment, G1367C auto-sampler, G1379B vacuum degasser, and an Agilent 6410 Triple Quadruple mass spectrometer with electrospray ionization (ESI), were obtained from Agilent Technology. A DiRui CS600-B biochemical analyzer was provided by Changchun Medical Technologies Co., Ltd.

### 2.2. *Plasma samples*

Two hundred and forty-eight plasma samples were collected from patients receiving docetaxel-based regimens at Qilu Hospital of Shandong University between October 2014 and May 2016. Two blood samples were collected from each patient in EDTA-anticoagulant tubes, one at the end of infusion, and the other 1 hour after the infusion. After centrifugation, the plasma was separated and stored at -80°C for further analysis. This study was conducted in accordance with the Declaration of Helsinki and patient consent was obtained.

### 2.3. *LC-MS/MS assay*

#### 2.3.1. *Conditions for chromatography and mass spectrometry*

Separation of docetaxel and the IS from plasma was achieved on a Diamonsil $C_{18}$ column (150 mm × 4.6 mm, 5 μm) at 30°C with a thermostatted column oven. The mobile phase was 0.1% formic acid:acetonitrile (40:60, v/v) with a flow rate of 0.6 mL/min. Mass spectrometry was performed in the positive ion MRM mode, with an ion transition of m/z 830.5→550.4 for docetaxel and 876.4→308.2 for the IS, respectively. Other parameters for mass spectrometry were: a spray gas flow of 9 L/min, a spray gas (nitrogen) temperature of 350°C, a capillary voltage of 4,000 V, and a nebulizer pressure of 40 psi. The fragment voltage was 100 V for docetaxel and 120 V for the IS, the collision energy was 23 eV for docetaxel and 32 eV for the IS, and the EMV was 200 V.

#### 2.3.2. *Preparation of stock solutions, working solutions, calibration samples, and quality control samples*

Primary stock solutions of 1 mg/mL of docetaxel and the IS were separately prepared in methanol. Primary stock solutions were diluted with the mobile phase to yield standard working solutions of docetaxel (0.1, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 μg/mL). The IS was dissolved in the mobile phase to yield 4 μg/mL of a working solution. All solutions were stored at 4°C and equilibrated to room temperature before use.

Calibration samples and quality control (QC) samples were prepared by spiking blank plasma with a given volume of different working solutions. The calibration samples consisted of seven nonzero concentrations of

docetaxel: 10, 50, 100, 250, 500, 100, and 2,000 ng/mL. QC samples of docetaxel for the lowest limit of quantitation (LLOQ), a low level of QC (L), a middle level of QC (M), and a high level of QC (H) were 10, 25, 200, and 1,600 ng/mL, respectively.

2.3.3. *Sample preparation*

Two hundred μL of a plasma sample was mixed with 10 μL of IS (4 μg/mL) and vortexed for 0.5 min. Six hundred μL of methanol was added and the mixture was vortexed again for 3 min, followed by centrifugation at 10,800 rpm for 5 min. The supernatant was transferred and 10 μL was injected for analysis.

2.3.4. *Validation of the two methods*

The established method of LC-MS/MS for measurement of the docetaxel concentration in human plasma was validated in accordance with FDA guidelines, including specificity, matrix effects, linearity, recovery, precision, accuracy, and stability.

*Specificity*. The specificity of the method was evaluated by comparing chromatograms for six different lots of blank human plasma to identify the potential interference of endogenous substances in peak regions for docetaxel and the IS according to HPLC.

*Matrix effect and Recovery*. Six different human blank plasma samples were extracted and spiked with the analyte at high and low QC levels and with the IS. The areas of corresponding peaks were compared to areas of peaks produced by standard solutions, and the peak area ratio was defined as the matrix effect. The mean overall recovery of the analyte and the IS was determined based on the ratio of the peak area (extracted plasma standards/plasma samples after extraction). The analyte was measured at high and low QC levels in six different blank plasma samples and extracted as described above. Recovery of the IS was determined at 200 ng/mL with the method described above.

*Calibration curve and LLOQ*. Calibration curves were plotted with seven concentrations and each was plotted three times. Calibration curves were typically described by the equation $y = ax + b$, where y corresponds to the peak-area ratio of the analyte to the IS, and x represents the plasma concentration of the analyte. The linearity of the calibration curve was assessed by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$). LLOQ was also evaluated based on accuracy and precision.

*Accuracy and Precision.* The intra-day accuracy and precision were estimated by analyzing the docetaxel concentration at four levels, *i.e*. LLOQ (10 ng/mL), L (25 ng/mL), M (200 ng/mL), and H (1,600 ng/mL), in blank plasma within one day, and the inter-day precision was determined by analyzing samples of the four QC levels on three consecutive days.

*Stability*. Stability was studied using the L and H levels of QC in five samples stored or processed under different conditions, *i.e*. storage at -20°C for 1, 7, or 28 days, freezing (-20°C) and thawing (24 ± 2°C) for one or two cycles, leaving extracted samples to stand on the bench top for 4 h, and leaving samples in the LC-MS/MS auto-sampler for 6 h at room temperature.

2.4. *Nanoparticle immunoassay*

The principle of and protocol for a nanoparticle immunoassay of docetaxel were previously described in detail (*16*). Briefly, this assay was based on a competitive assay format using a selective docetaxel monoclonal antibody. Six different concentration calibrators (0, 75, 150, 300, 600, and 1,000 ng/mL) were used to generate the calibration curve, and three QC standards (115, 225, and 800 ng/mL) accompanied the sample test. A plasma sample was first added to reagent 1, and a reaction was started by adding reagent 2. Photometric detection was performed at 600 nm. The difference in absorbance was determined and the concentration was calculated from the calibration curve. An automated biochemical analyzer was used to measure the concentration of the analyte. The MyDocetaxel kit consists of reaction reagents (reagent 1 and reagent 2), six calibrator concentrations, and three control concentrations. Samples were processed according to the manufacturer's instructions.

2.5. *Statistics*

Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using regression analysis. The statistical software SPSS was used to evaluate the correlation between concentrations measured with the two methods and the software MedCalc was used to draw a Bland-Altman plot, which helped to reveal differences and the extent of differences in measurements, any systematic bias, and possible outliers.

## 3. Results

3.1. *Validation of LC-MS/MS*

*Specificity*. The specificity of LC-MS/MS was evaluated by comparing chromatograms as shown in Figures 1 and 2. The full-scan and product ion mass spectrum of docetaxel and the IS are shown in Figure 1. Typical MRM chromatograms are shown in Figure 2, where A is the blank plasma, B is the docetaxel standard and the IS, C is blank plasma spiked with docetaxel and the IS, and D is a patient plasma sample (Patient No. 10) spiked with the IS. There was no endogenous interference with measurement of the docetaxel concentration in the blank or human plasma sample.

*Matrix effects and Recovery*. The recovery of

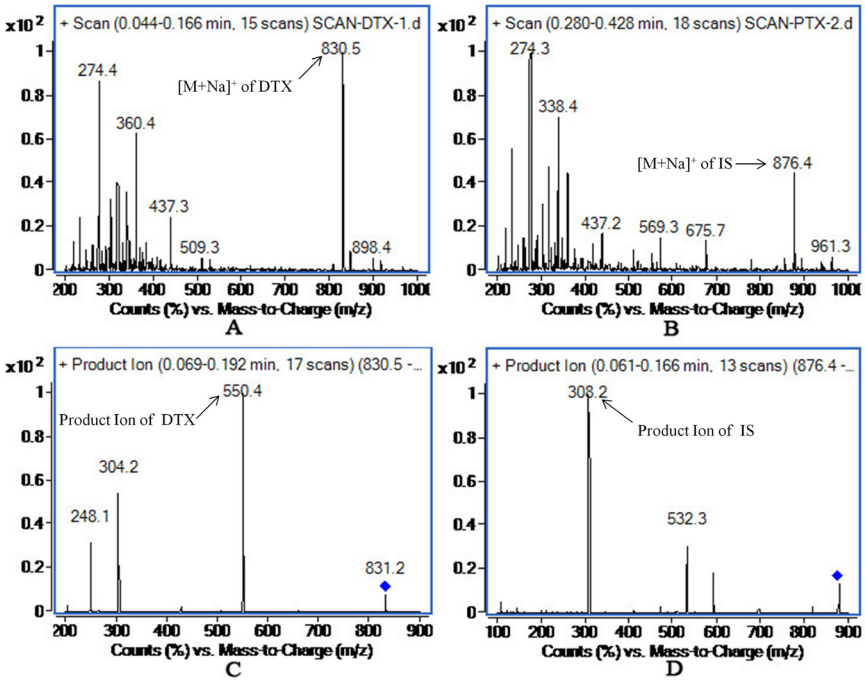


**Figure 1. Full-scan and product ion mass spectrum of docetaxel (A, C) and the IS (B, D).**

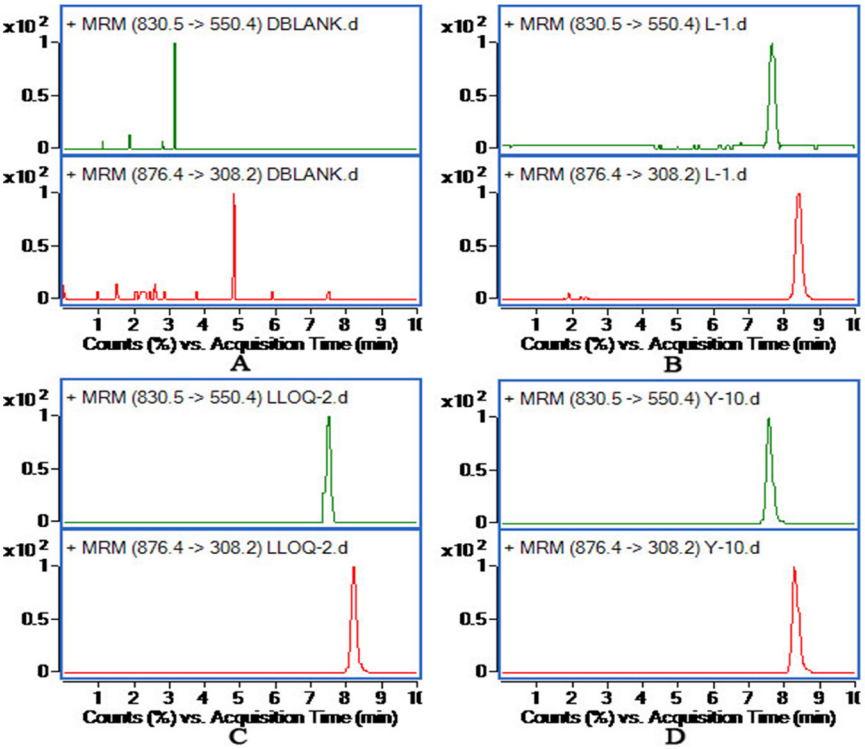


**Figure 2. Typical MRM chromatograms of docetaxel and the IS. A**: Blank plasma; **B**: Docetaxel and the IS (both were 10 ng/mL); **C**: LLOQ; blank plasma spiked with docetaxel (10 ng/mL) and the IS (100 ng/mL); **D**: Plasma from Patient No. 10 after treatment with docetaxel was mixed with a standard solution of the IS.

docetaxel and the IS was acceptable and reproducible. No matrix effects were observed. The mean recovery and matrix effects were 95.76% and 112.57% for docetaxel and 99.41% and 107.18% for IS, and the RSD was less than 15%, indicating that blank plasma samples were free from interference by endogenous substances, so docetaxel could be quantified.

*Calibration curve and LLOQ*. The calibration curve for docetaxel was linear over the concentration range with the regression equation (weight = $1/X^2$), as indicated by y = 0.0039x + 0.0025 and a correlation coefficient ($r^2$) of 0.9986. LLOQ was defined as the lowest concentration on the standard calibration curve with acceptable repeatability and recovery and at least

**Table 1. The intra- and inter-day accuracy and precision of docetaxel levels in human plasma ($n$ = 5)**

| Concentration (ng/mL) | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|
| | Mean ± SD | Accuracy% | RSD% | Mean ± SD | Accuracy% | RSD% |
| 10 | 9.84 ± 0.48 | 98.4 | 4.92 | 9.51 ± 0.77 | 95.1 | 8.12 |
| 25 | 24.10 ± 1.42 | 96.4 | 5.88 | 24.69 ± 1.75 | 98.7 | 7.08 |
| 200 | 196.49 ± 8.67 | 98.2 | 4.41 | 198.74 ± 21.07 | 99.4 | 10.60 |
| 1600 | 1,530.68 ± 44.03 | 95.7 | 2.88 | 1,603.7 ± 191.29 | 100.2 | 11.90 |

**Table 2. Stability of docetaxel in human plasma ($n$ = 5)**

| Stability study | L (25 ng/mL) | | | H (1,600 ng/mL) | | |
|---|---|---|---|---|---|---|
| | Mean ± SD | Accuracy% | RSD% | Mean ± SD | Accuracy% | RSD% |
| post-extraction (4 h) | 23.98 ± 1.70 | 95.9 | 7.09 | 1,475.89 ± 109.36 | 92.2 | 7.41 |
| in auto sampler (6 h) | 23.33 ± 1.96 | 93.3 | 8.40 | 1,706.96 ± 118.50 | 106.7 | 6.94 |
| freeze-thaw (one cycle) | 21.91 ± 3.22 | 87.7 | 14.70 | 1,390.07 ± 44.06 | 86.9 | 3.17 |
| freeze-thaw (two cycles) | 27.54 ± 1.51 | 110.2 | 5.47 | 1,548.27 ± 60.99 | 96.8 | 3.94 |
| frozen (1 day) | 24.68 ± 1.42 | 98.7 | 5.76 | 1,594.85 ± 65.09 | 99.7 | 4.08 |
| frozen (7 days) | 24.22 ± 0.92 | 96.9 | 3.78 | 1,668.02 ± 89.58 | 104.3 | 5.37 |
| frozen (28 days) | 25.43 ± 2.33 | 101.7 | 9.19 | 1,558.87 ± 160.08 | 97.4 | 10.30 |

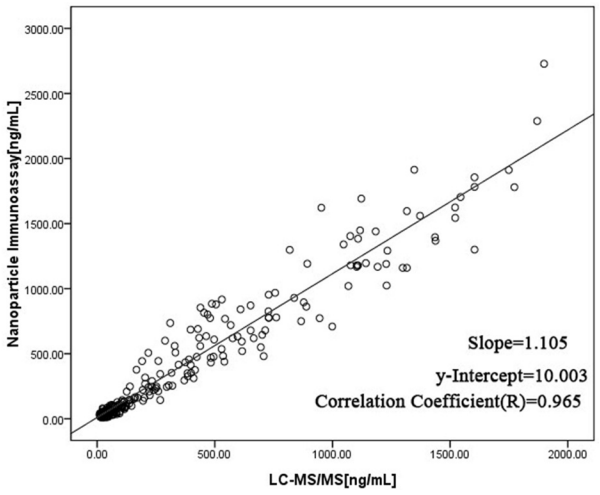


**Figure 3. Correlation between the docetaxel concentration measured in plasma samples from cancer patients ($n$ = 248) with a nanoparticle immunoassay and LC-MS/MS.**

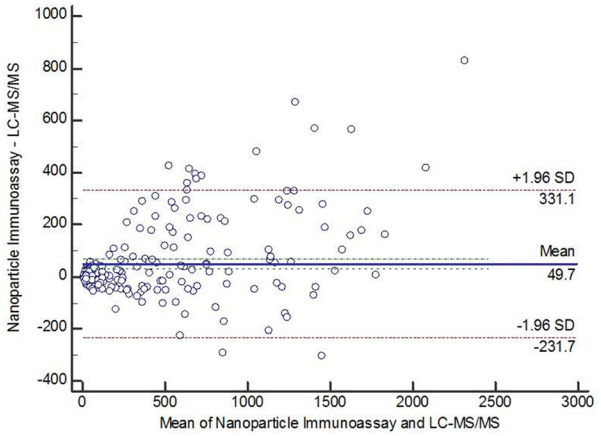


**Figure 4. Bland-Altman plot of the mean docetaxel concentration measured with a nanoparticle immunoassay and LC-MS/MS.**

10 times the response of the blank at the baseline.

*Accuracy and Precision*. Accuracy and precision results are as shown in Table 1. The results were within the accepted limits and therefore the assay was accurate and precise.

*Stability*. Results of a study of the stability of docetaxel in human plasma are shown in Table 2. Data indicated that docetaxel was stable in plasma under the indicated conditions.

### 3.2. *Methods comparison*

LC-MS/MS and the nanoparticle immunoassay were compared using 248 human plasma samples obtained from patients receiving docetaxel-based therapy commonly used in China. Both methods can be used to measure the docetaxel concentration in plasma samples. The docetaxel concentration ranged from 10.23-1,899.17 ng/mL according to the validated method of LC-MS/MS, with a mean of 378.1 ng/mL. The docetaxel concentration ranged from 9-2,728 ng/mL according to the nanoparticle immunoassay, with a mean of 427.7 ng/mL. Results of Deming regression analysis revealed a slope of 1.105 (95% CI of 1.067 to 1.142) with a y intercept of 10.003 ng/mL (95% CI of – 12.095 to 32.101 ng/mL), a standard error estimate of 135.7, and a correlation coefficient of 0.965 (Figure 3).

In addition, a plot of the differences between the two assays with respect to their mean concentration indicated that their results were similar, although slight differences were noted. The Bland-Altman plot for docetaxel is shown in Figure 4. As is evident from the plot, the 95% CI was – 231.7-331.1 ng/mL, and most results were in the 95% CI, though results from 14 samples fell outside the 95% CI. The mean bias was positive (49.7 ng/mL).

## 4. Discussion

While the chromatography conditions for the current study were being determined, various combinations of the mobile phase were tested to achieve good separation from the IS, a better peak, a high response, and a short retention time. Moreover, the mobile phase was evaluated to enhance MS sensitivity and minimize matrix effects. All of the mobile phases were combined with ammonium acetate (5 mM), formic acid at 0.1% (v/v), or both. A mobile phase of 0.1% formic acid:acetonitrile (40:60, v/v) was optimal. The column temperature and flow rate parameters were studied to provide fast and reliable separation, and the best results were obtained when the column temperature was 30°C (versus 25, 35, or 40°C) and the flow rate was 0.6 mL/min (versus 0.5 or 0.8 mL/min). Under these conditions, retention times of docetaxel and the IS were consistent and reproducible. A major advantage of the immunoassay is that it involves extraction through protein precipitation, which is easier and more consistent than liquid-liquid extraction (*11,13*) and solid phase extraction (*17-19*). This simple sample pretreatment allowed measurement of the docetaxel concentration and it had acceptable matrix effects.

Personalized medicine is facilitated through TDM of the concentration of a drug or its active metabolites in biological samples, allowing adjustment of the drug dosage to improve its efficacy and minimize its toxicity. Studies have indicated that monitoring the exposure to some anticancer drugs helps to reduce drug-related toxicity and improve therapeutic efficacy (*20-22*). However, clinical TDM of docetaxel has been limited, possibly due to the lack of cost-effective tools to monitor drug concentrations in plasma. A recent study has indicated that the AUC, a parameter for docetaxel exposure, is associated with the drug's therapeutic efficacy and toxicity (*23*). Measuring the docetaxel concentration to calculate the AUC can help with clinical TDM of docetaxel-based chemotherapies.

Plasma samples were collected from cancer patients at this Hospital and the two methods of measuring the docetaxel concentration were compared. Results indicated that the results of the two methods were closely correlated. Thus, the MyDocetaxel nanoparticle immunoassay can be used to measure the docetaxel concentration in plasma samples. The mean concentration measured with the immunoassay was slightly higher than that measured with LC-MS/MS and differed from the concentration reported in a study by Cline *et al*. (*16*). This difference may be due to the fact that docetaxel concentrations in the samples used in the previous study were much higher than those in the current study. Therefore, more clinical samples from patients need to be compared to obtain more definitive results.

The LLOQ of the validated method of LC-MS/MS was 10 ng/mL, which was lower than that of the MyDocetaxel nanoparticle immunoassay (52 ng/mL). The specificity and sensitivity of LC-MS/MS may allow more accurate measurement of the docetaxel concentration in cancer patients treated with a lower dose. The MyDocetaxel nanoparticle immunoassay yielded a docetaxel concentration below the LLOQ (52 ng/mL), but this result may be not accurate and it may partially account for any lack of correlation.

Docetaxel is predominantly metabolized in the liver by the hepatic cytochrome P450 (CYP) 3A isoforms CYP3A4 and CYP3A5 (*8,9*). Docetaxel metabolites include M1, M2, M3, and M4 and degradation products 7-epi-docetaxel and 10-deacetylbaccatin. Antibodies in the immunoassay cross-reacted more or less with metabolites as docetaxel degraded (*16*), which may account for the difference in the mean concentration measured with the nanoparticle immunoassay and LC-MS/MS, resulting in a positive bias of 49.7 ng/mL.

In conclusion, both the established method of LC-MS/MS and the commercial MyDocetaxel nanoparticle immunoassay were accurate, precise, and suitable for measurement of the docetaxel concentration in human plasma. Results of the two methods were closely correlated in the range of 10 to 2,000 ng/mL. Since the nanoparticle immunoassay was more convenient, had a higher throughput, and was more cost-effective, it is a better tool for TDM of the docetaxel concentration and can provide an experimental basis for individualized therapy in routine clinical practice.

## Acknowledgements

This study was supported by the Major National Science and Technology Project (2012ZX09303-016-003).

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*(Received February 8, 2017; Revised March 2, 2017; Accepted March 13, 2017)*

Original Article

DOI: 10.5582/bst.2016.01233

# Relationship between thromboelastography and long-term ischemic events as gauged by the response to clopidogrel in patients undergoing elective percutaneous coronary intervention

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**Summary** **Ischemic events after percutaneous coronary intervention (PCI) remain a major concern for patients with coronary heart disease (CHD). The aim of the current study was to investigate whether thromboelastography (TEG) was a satisfactory technique to measure platelet function *in vitro* in order to improve risk stratification and the individual response to antiplatelet therapy. The diagnostic and prognostic utility of the maximum amplitude of adenosine diphosphate induced platelet-fibrin clots ($MA_{ADP}$) was measured with TEG in 759 patients undergoing elective PCI. A 600-mg dose of clopidogrel was taken more than 12 h before surgery in addition to a maintenance dose of aspirin 100 mg/day and clopidogrel 75 mg/day for 2 y. Platelet-fibrin clot strength was also measured in this study. An $MA_{ADP}$ > 34 mm significantly predicted ischemic events after PCI, as indicated by an area under the curve (AUC) of 0.79 (95% CI: 0.72-0.87, $P < 0.05$) according to receiver operating characteristic (ROC) curve analysis. The multivariate Cox proportional hazards model identified $MA_{ADP}$ > 34 mm and an FBG level > 7.0 mmol/L as significant independent predictors of first ischemic events at the 2-year time point ($P < 0.05$). With adequate clopidogrel pretreatment, patients who underwent elective PCI and who experienced ischemic events could be diagnosed with a certain $MA_{ADP}$ according to TEG. TEG could be a good tool to measure platelet function.**

***Keywords:*** Antiplatelet, clopidogrel, thromboelastography (TEG), percutaneous coronary intervention (PCI)

## 1. Introduction

Ischemic events usually occur in patients who undergo percutaneous coronary intervention (PCI). Whether those events occur mainly depends on the activation of platelets and the generation of thrombin, and both processes are mediated by a variety of agonists (*1,2*). Dual antiplatelet treatment with clopidogrel and aspirin to suppress platelet reactivity (PR) has proven to be an efficacious therapy and can be used to prevent ischemic events in patients with coronary heart disease (CHD) after PCI (*1,3,4*). Thromboelastography (TEG) has been performed in order to monitor and alleviate ischemic events, and TEG platelet mapping can adeptly assess the response of circulating platelets to both aspirin and clopidogrel in whole blood, as a previous study by the current authors indicated (*5*). A TEG analysis can provide a large amount of information on the overall likelihood of thrombosis and the reaction to antiplatelet therapy. In TEG, an oscillating cup and a rotating pin suspended in a blood sample are connected by blood clots. Blood clot strength is measured using the proportional amplitude of the rotating pin. The maximum amplitude (MA) is the maximum clot strength, which defined as the parameter MA (*2,6*). Thus far, TEG is the only point-of-care testing (POCT) of platelet function that has been approved by China's Food and Drug Administration (FDA). However, the relationship between TEG and ischemic events in patients undergoing PCI has yet to be determined.

A previous study by the current authors indicated a definite relationship between gender and clopidogrel

Released online in J-STAGE as advance publication March 19, 2017.

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resistance in patients after PCI (*5*). The aim of the current study was to investigate a potential cut-off value for the maximum amplitude of adenosine diphosphate-induced platelet-fibrin clots ($MA_{ADP}$) measured with TEG and to examine its predictive and prognostic value with regard to the occurrence of long-term ischemic events in Chinese patients.

## 2. Materials and Methods

### 2.1. *Patients*

Seven hundred and fifty-nine consecutive patients who underwent elective PCI from January 2014 to February 2015were enrolled in a prospective observational study. All patients over the age of 18 had been administered aspirin in a dosage of 100 mg/day for at least 7 days. Exclusion criteria included a history of bleeding, an acute myocardial infarct (AMI) up to 48 h before enrolment, and use of glycoprotein (GP) IIb/IIIa inhibitors prior to the procedure. All of the patients were pre-treated with aspirin and a 600-mg dose of clopidogrel more than 12 h prior to the procedure, followed by a maintenance dose of aspirin 100 mg/day and clopidogrel 75 mg/day for 2 y. This study was approved by the Institutional Review Board of Shanghai Chest Hospital (7563893).

### 2.2. *Blood sampling and measurement of platelet-fibrin clot strength*

Blood samples were obtained 18 to 24 hours after PCI. After the procedure, blood was drawn by venipuncture into vacutainer tubes (Becton-Dickinson, NJ, USA) with 3.8% trisodium citrate and USP lithium heparin (for TEG assay). The vacutainer blood tube was filled with blood and gently inverted three to five times in order to ensure that the sample was completely mixed with the anticoagulant.

Platelet-fibrin clot strength was measured with the TEG Hemostasis System (Haemoscope Corporation, IL, USA) according to the manufacturer's instructions. Quantitative and qualitative values for the physical properties of clots were obtained with the automated analytical software TEG Hemostasis Analyzer (Haemoscope Corporation, IL, USA) according to the manufacturer's instructions.

Heparinized blood (360 μL) was rapidly added dropwise to a heparinase-coated cup and analyzed with TEG to detect the maximum amplitude of thrombin-induced clot strength ($MA_{Thrombin}$). The mixed blood (340 μL) was added dropwise to a noncoated cup containing activator F and reptilase to generate a blood cross-linked clot without thrombin generation or platelet stimulation ($MA_{Fibrin}$). The last sample (340 μL) of heparinized blood was added dropwise to a nonheparinase-coated cup containing activator F and adenosine diphosphate-induced platelet-fibrin (ADP) or arachidonic acid (AA) to induce a blood-crosslinked clot with platelet activation ($MA_{ADP}$ or $MA_{AA}$).

### 2.3. *Definitions and clinical follow-up*

Platelet aggregation induced by ADP was defined as $\%Aggregation = [(MA_{ADP}-MA_{Fibrin})/(MA_{Thrombin}-MA_{Fibrin})] \times 100\%$, and this value was calculated with software. The cut-off point for high on-treatment platelet reactivity (HPR) was expressed as ≥ 70% ADP-induced platelet aggregation with 2 μmol of ADP as measured with TEG (*6*).

Patients were followed for 24 months during hospitalization depending on the occurrence of adverse events. Patients that complied with antiplatelet medication were contacted by phone or by the clinic and an appointment was made for the postoperative follow-up. Endpoints included stent thrombosis, cardiac death, ischemic stroke, unplanned revascularization, and myocardial infarction. Stent thrombosis was determined using the definition of the Academic Research Consortium (*7*). Cardiac death included a death due to any cardiovascular cause. Myocardial ischemia correlated with the overexpression of troponin I was regarded as myocardial infarction (*8*).

### 2.4. *Statistical analysis*

Statistical analysis was performed with SPSS version 21.0 (SPSS, Inc., IL, USA). Categorical variables were expressed as *n* (%) and continuous variables were expressed as the mean ± standard deviation (SD). The chi-square test and Student's *t*-test were respectively used to compare categorical variables and continuous variables among groups. Receiver operating characteristic (ROC) curve analysis was performed to determine the diagnostic accuracy of $MA_{ADP}$ in relation to ischemic events. Demographic and procedural variables were included in a multivariate Cox proportional hazards model to identify prognostic factors. $P < 0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. *Patients and demographic characteristics*

Seven hundred and fifty-nine patients who underwent non-emergent PCI were enrolled and administered aspirin and clopidogrel therapy. One hundred and eighty-two of these patients were diagnosed with unstable angina and the remainder were diagnosed with stable angina. The baseline characteristics of patients are shown in Table 1. The average age was (66.0 ± 9.6) years old and 72% of the patients were men. Fifty-eight patients (7.6%) experienced an ischemic event in the 2 years after elective PCI. In short, patients who

**Table 1. Baseline characteristics**

| Demographic characteristics | Total ($n$ = 759) | Ischemic group ($n$ = 58) | Non-ischemic group ($n$ = 701) | $P$ |
|---|---|---|---|---|
| Age (yrs) | 66.0 ± 9.6 | 66.8 ± 9.2 | 65.5 ± 9.8 | 0.653 |
| Gender | | | | |
| Male, $n$ (%) | 547 (72) | 33 (57) | 514 (73) | 0.001 |
| Female, $n$ (%) | 212 (28) | 25 (43) | 184 (27) | |
| BMI (kg/m$^2$) | 25 ± 4 | 25 ± 5 | 25 ± 4 | 0.986 |
| Risk factors | | | | |
| Smoking (%) | 76 (10) | 4 (7) | 72 (10) | 0.610 |
| Hypertension (%) | 539 (71) | 48 (82) | 491 (70) | 0.519 |
| Diabetes (%) | 197 (26) | 21 (36) | 176 (25) | 0.485 |
| Hyperlipidemia (%) | 30 (4) | 4 (7) | 26 (4) | 0.364 |
| Prior PCI (%) | 175 (23) | 16 (27) | 149 (21) | 0.718 |
| Prior MI (%) | 99 (13) | 16 (27) | 83 (12) | 0.172 |
| Diagnosis | | | | |
| SA (%) | 577 (76) | 51 (88) | 526 (75) | 0.469 |
| UA (%) | 182 (24) | 7 (12) | 175 (25) | |
| Laboratory data | | | | |
| eGFR (mL/min/1.73 m$^2$) | 88.1 ± 39.7 | 80.9 ± 39.6 | 88.4 ± 39.7 | 0.540 |
| Platelets (×10$^9$/L) | 193.5 ± 67.5 | 214.3 ± 65.3 | 193.1 ± 67.6 | 0.590 |
| FBG (mmol/L) | 6.9 ± 2.9 | 8.7 ± 3.4 | 6.8 ± 2.9 | 0.040 |
| CRP (mmol/L) | 4.2 ± 12.3 | 18.1 ± 32.3 | 3.7 ± 11.1 | 0.326 |
| TC (mmol/L) | 4.3 ± 1.1 | 4.6 ± 2.0 | 4.3 ± 1.1 | 0.609 |
| TG (mmol/L) | 2.0 ± 1.5 | 2.9 ± 3.3 | 2.0 ± 1.3 | 0.498 |
| LDL (mmol/L) | 2.5 ± 0.9 | 2.6 ± 1.8 | 2.5 ± 0.9 | 0.880 |
| HDL (mmol/L) | 1.0 ± 0.3 | 0.9 ± 0.1 | 1.0 ± 0.3 | 0.480 |
| CK-mb (ng/mL) | 2.7 ± 9.3 | 1.7 ± 0.7 | 2.8 ± 9.4 | 0.795 |
| TnI (ng/mL) | 0.4 ± 2.5 | 0.1 ± 0.1 | 0.5 ± 2.6 | 0.641 |

BMI: Body mass index; PCI: Percutaneous coronary intervention; MI: Myocardial infarction; SA: Stable angina pectoris; UA: Unstable angina pectoris; eGFR: Estimated glomerular filtration rate; FBG: Fasting blood glucose; CRP: C reactive protein; TC: Total cholesterol; TG: Triglycerides; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; CK-MB: Creatine kinase MB; TnI: Troponin I.

**Table 2. Parameters for platelet function**

| Items | Ischemic group | Non-ischemic group | $P$ |
|---|---|---|---|
| $MA_{AA}$ | 62.0 ± 9.0 | 60.4 ± 6.8 | 0.669 |
| $MA_{ADP}$ | 40.8 ± 10.1 | 26.7 ± 13.7 | < 0.001 |
| AA% | 88.8 ± 41.9 | 81.0 ± 23.1 | 0.298 |
| ADP% | 81.1 ± 32.4 | 71.4 ± 28.5 | 0.271 |

$MA_{AA}$: Maximum amplitude of arachidonic acid-induced platelet-fibrin clot strength; $MA_{ADP}$: Maximum amplitude of ADP-induced platelet-fibrin clot strength; AA%: Inhibition of platelet aggregation induced by arachidonic acid; ADP%: Inhibition of platelet aggregation induced by adenosine diphosphate.

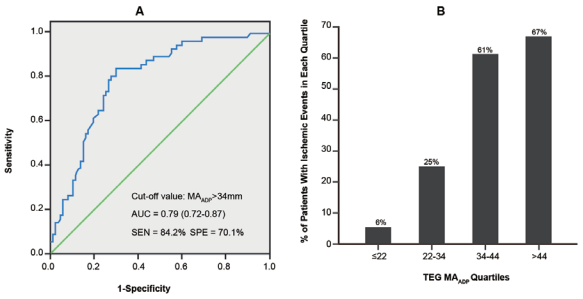


**Figure 1. ROC curve and quartile analyses of MAADP values. (A)** The receiver operating characteristic curve for MAADP. The cut-off value for MAADP is 34 mm. The diagnostic accuracy of MAADP for ischemic events is 0.79 (0.72-0.87), with a sensitivity of 84.2% and a specificity of 70.1%. **(B)** The observed frequency of patients with ischemic events in each quartile of MAADP values is shown in the figure.

experienced an ischemic evens were more often female (43% *vs*. 27%, $P < 0.05$) and had a higher level of fasting blood glucose (FBG) compared to patients in the non-ischemic group ((8.7 ± 3.4) mmol/L *vs*. (6.8 ± 2.9) mmol/L, $P < 0.05$).

### 3.2. *Association between platelet aggregation and ischemic events*

Blood samples of all 759 patients were analyzed with the TEG system. The prevalence of HPR was 36% according to TEG ($n$ = 273). As shown in Table 2, post-treatment platelet AA-induced aggregation according to TEG did not differ markedly between the two groups ($P > 0.05$) (88.8 ± 41.9% in the ischemic group *vs*. 81.0 ± 23.1% in the non-ischemic group). Post-treatment platelet ADP-induced aggregation according to TEG was not correlated with ischemic events ($P > 0.05$) (81.1 ± 32.4% in the ischemic group *vs*. 71.4 ± 28.5% in the non-ischemic group). However, patients who experienced ischemic events had significantly greater ADP-induced platelet-fibrin clot strength ($MA_{ADP}$) than did patients who did not experience ischemic events (40.8 ± 10.1 mm *vs*. 26.7 ± 13.7 mm, $P < 0.05$). ROC analysis, which assessed the association between $MA_{ADP}$ and ischemic events, indicated that $MA_{ADP}$ had good predictive significance, resulting in an area under the curve (AUC) of 0.79 (95% CI: 0.72-0.87, $P < 0.05$, Figure 1A).

Quartile analysis of the $MA_{ADP}$ consistently indicated that the incidence of ischemic events increased with higher quartiles (Figure 1B). Sixty-one percent of patients in the third quartile (an $MA_{ADP}$ of 34-44 mm) experienced ischemic events and 67% of patients in the fourth quartile (an $MA_{ADP}$ > 44mm) experienced ischemic events. Independent predictors of long-term ischemic events were assessed with a multivariate Cox proportional hazards model. $MA_{ADP}$, being female, and FBG were independently and dramatically related to ischemic events, with a respective risk ratio of 8.9 (95% CI: 4.2-18.3), 5.3 (95% CI: 2.7-11.1), and 2.2 (95% CI: 1.1-3.9) ($P < 0.05$, Table 3).

## 4. Discussion

Patients undergoing PCI usually suffer from ischemic events, and the standard therapy for these patients has been the combined use of aspirin and clopidogrel (*4*). The PREPARE-POST-STENTING study first indicated a relationship between $MA_{ADP}$ and the occurrence of ischemic events in 2005 (*2*). Light transmittance aggregometry was used to determine platelet reactivity. Several studies have used TEG to predict platelet reactivity and ischemic events after PCI (*2,9*). The current study hypothesized that the $MA_{ADP}$ according to TEG would provide supererogatory information for post-stenting risk assessment. Results indicated that an $MA_{ADP}$ greater than 34 mm according to TEG suggested a higher risk of ischemic events. However, Gurbel *et al*. reported that an $MA_{ADP}$ greater than 47 mm according to TEG significantly predicted long-term ischemic events in contrast to other measurements (*9*). Race is known to be an independent predictor of survival of coronary disease and individual differences in platelet reactivity are known to be heritable; several studies have found a lower frequency of epistaxis in Asians and African Americans than Caucasians (*10,11*).

Multifactorial processes such as clinical, demographic, and hemostatic components influence ischemic events, and especially those after PCI (*12*). The current results suggested that only gender and the level of FBG differed significantly between ischemic and non-ischemic groups. Interestingly, the current results based on a Cox model indicated that female patients had a much higher risk of experiencing ischemic events than did male patients, and a previous study yielded a similar finding. Several studies have indicated that HPR is more prevalent in females than males because of a stronger platelet aggregation reaction to platelet agonist stimulation (*13-15*). This might be caused by a high of clotting at the baseline, the effects of female hormone, or a diminished reaction to clopidogrel in female patients.

MA parameters according to TEG can detect the maximum amplitude of platelet aggregation and fibrin-platelet binding by GP IIb/IIIa (*9*). Circulating platelets are variably inhibited by platelet inhibitors. The $MA_{ADP}$ indicated the level of platelet reactivity induced by ADP. According to the current results, $MA_{ADP}$ may accurately predict the long-term ischemic events after PCI based on platelet inhibition by ADP. However, there has been reluctance to monitor antiplatelet therapy over the long term. There are several reasons for this reluctance, such as the introduction of artifacts due to laboratory methods, incomplete reflection of the actual thrombotic process *in vivo*, and failure to unequivocally establish a causal relationship between test results and the occurrence of thrombotic events. Over the past few years, knowledge of platelet receptor physiology has improved significantly and more patient-friendly assays of platelet function have been introduced. However, a large-scale prospective trial has yet to yield definitive evidence indicating that improved antiplatelet therapy based on a platelet function test actually helps patients. At present, TEG is a preferable technique to identify patients at risk of experiencing ischemic events, but the effectiveness of this technique needs to be assessed further in large-scale trials in the future. Thrombotic events occurred at a relatively low rate in previous prospective trials, and larger samples are needed to assess the potential for TEG to provide personalized treatment for patients with CHD (*16*).

That said, the current study had several limitations. First, this subject was a prospective study that was not randomized, and findings still need to be verified in larger randomized clinical trials. Second, the clopidogrel loading dose used in this study may result in different levels of antiplatelet action in different patients.

In summary, the quantitative measurement of $MA_{ADP}$ with TEG allows more individualized antiplatelet treatment to prevent ischemic events, and this approach may help to improve predictive accuracy and facilitate personalized antiplatelet treatment.

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*(Received December 14, 2016; Revised January 16, 2017; Accepted February 21, 2017)*

Original Article

DOI: 10.5582/bst.2016.01228

# Loss of SETD2, but not H3K36me3, correlates with aggressive clinicopathological features of clear cell renal cell carcinoma patients

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**Summary** **Recent studies facilitated by DNA sequencing identified the histone modifying gene *SETD2* as the second most frequent mutant gene in sporadic clear cell renal cell carcinoma (ccRCC) patients. SETD2 functions as a tumor suppressor in ccRCC. However, its clinical association and biological functions are not fully delineated. The aim of this study is to evaluate the clinical significance of SETD2 in ccRCC patients. SETD2 and its canonical histone modification product H3K36me3 were analyzed by immunohistochemistry (IHC) in 155 ccRCC patients from two independent cohorts retrospectively. Both SETD2 and H3K36me3 were heterogeneously stained and down-regulated in ccRCC tissues, compared with normal controls. The SETD2 protein deficiency rate was 34.07%, which is much higher than the reported *SETD2* gene inactive mutation rate. Furthermore, low SETD2 protein expression, but not H3K36me3 expression, was associated with the aggressive phenotype of ccRCC patients. In addition, cox multivariate analysis identified low SETD2 protein expression as an independent prognostic factor for overall survival of ccRCC patients. Consistently, using RNA-Seq data of ccRCC patients from The Cancer Genome Atlas, we validated our findings that low SETD2 mRNA expression is significantly associated with the aggressive phenotypes, and predicted a worse outcome for ccRCC patients. In conclusion, our study demonstrated a massive down-regulation of SETD2 protein in ccRCC, and identified SETD2 protein, but not H3K36me3, as an independent good prognostic marker, which warrants further study focusing on the non-methyltransferase role of SETD2 in kidney tumor biology.**

***Keywords:*** SET domain-containing protein 2, clear cell renal cell carcinoma, H3K36me3, prognosis

Released online in J-STAGE as advance publication March 6, 2017.

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## 1. Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common tumor originating from adult kidney, and is the most malignant subtype of all genitourinary tumors (*1*). Although the early diagnosis of kidney cancer has dramatically improved with the advancement of ultrasound scanning, the prognosis for advanced ccRCC remains poor, and the recurrence and mortality rate of ccRCC have been constantly rising (*2*).

The Von Hippel-Lindau (VHL) gene inactivation by mutation or methylation has been reported in up to 91% of patients with sporadic ccRCC (*3*). Loss of VHL protein, pVHL, leads to abnormal activation of the HIF pathway, which contributes to renal carcinogenesis (*4*). However, VHL gene mutation alone is insufficient to initiate renal cancer, suggesting additional gene mutations are required (*5-7*). Based on well-established VHL gene mutant status, systematic sequencing in recent studies have identified recurrent mutations in a number of genes located near the VHL gene in sporadic ccRCC (*8*), including PBRM1, SETD2, BAP1, KDM6A and JARID1c (*9*). Among which changes SETD2 mutations occur in 3-12% of sporadic ccRCC patients (*5,10*). However, according to our previous studies, we found a much higher SETD2 protein deficiency rate. We believe it is much more likely that the SETD2 mutation rate is underestimated, due to the heterogeneous nature of ccRCC.

SETD2 protein is a histone modifier, which is responsible for trimethylation of lysine-36 of histone H3, H3K36me3 (*11*). H3K36me3 is known to be associated with transcription activation as well as elongation (*9,11*). It is reported that SETD2 mutations have been found to be associated with an advanced clinical stage and poor prognosis in patients with primary ccRCC (*12,13*), which implied that SETD2 is a tumor suppressor. However, the SETD2 protein deficiency rate, its downstream targets and the underlying mechanisms of SETD2 in ccRCC remain unclear.

In this study, we use immunohistochemical staining (IHC) to retrospectively evaluate the association between SETD2, and its main functional product H3K36me3 expression levels and the clinicopathological features in patients with primary or metastatic ccRCC in two independent cohorts. Sequencing data and clinical information from the TCGA ccRCC project were extracted and analyzed to validate our findings.

## 2. Materials and Methods

### 2.1. *Patients and samples*

The study involved a total of 155 formalin-fixed, paraffin-embedded tissue samples, including 20 normal renal tissues and 135 ccRCC tissues, which were pathologically diagnosed and underwent radical nephrectomy in Qilu Hospital of Shandong University or Weihai Municipal Hospital from 2005 to 2013. Before sample collection for research purposes, patients' consent and approval from the Ethics Committee of Qilu Hospital and Weihai Municipal Hospital were obtained. For the purpose of survival analysis, more cases with advanced disease were enrolled. Those cases with a family history in first-degree relatives as judged by questioning at the time of admission for surgery were excluded. Patients who received adjuvant therapy, chemotherapy or radiotherapy were excluded from this study. The median age at diagnosis for the 135 subjects was 55 years (23-78 years). Of the 135 cases, 12.6% ($n$ = 17) had lymph node metastasis, 9.6% ($n$ = 13) had renal vein invasion and 10.4% ($n$ = 14) had distant metastasis. Median follow-up time was 76 months (15-129 months). 18 subjects had experienced recurrence by the time of the last follow-up and 9 patients died of ccRCC. Patients' clinicopathological parameters were obtained from the patients' medical records in their hospitals and summarized in Table 1.

### 2.2. *Immunohistochemistry (IHC)*

For the IHC staining, 5-μm-thick sections, which contained representative histology of the ccRCC were prepared. Tumor adjacent normal renal tissues were used as controls. The sections were deparaffinized, followed by quenching the endogenous peroxidase activity using 3% hydrogen peroxide. Sections were autoclaved in 10 μM citrate buffer (pH 6.0) for 2 min for antigen retrival. 5% albumin was used to block non-specific binding. The primary antibody used in this study was anti-SETD2 antibody (1:150; ab184190, Abcam, Cambridge, MA, USA) and anti-H3K36me3 antibody (1:150; ab9050, Abcam, Cambridge, MA, USA). The sections were incubated in the primary antibody diluted solution at 4°C in a humidified chamber. After washing with PBS, sections were incubated with secondary antibody at 37℃ for 30 min. The immunoreactions were visualized using DAB Horseradish Peroxidase Color Development Kit (P0202, Beyotime, Haimen, China), and then counterstained with hematoxylin.

### 2.3. *Evaluation of IHC staining*

Immunoreactive activities were evaluated by two investigators, without clinical or pathological information of the slides. SETD2 and H3K36me3 expression were scored as follows: the extension of staining-positive tumor cells (0, 0-10%; 1, 11-30%; 2, 31-60%; 3, > 61%) and the staining intensities (0, no staining; 1, weak staining in nucleus; 2, moderate staining in nucleus; and 3, strong staining in nucleus). The immunoreactive scores were defined as the sum of extension and intensity. Cases with a score of ≥ 5 were defined as high expression, and cases of < 5 were defined as low expression. Moreover, for SETD2 gene status evaluation, it is defined as SETD2 protein deficiency if the extension of SETD2-staining-positive tumor cells is less than 60%, or the tumor cells are weakly stained or not stained.

### 2.4. *RNA-Seq data set*

The SETD2 expression profiles of ccRCC containing

25 normal renal tissues, 541 primary ccRCC tumors and the corresponding clinical information were acquired from The Cancer Genome Atlas Research Network (TCGA, *http://cancergenome.nih.nov*). Reads were aligned using STAR version 2.4.2 and gene expression was represented as FPKM (Fragments Per Kilobase of transcript per Million mapped reads).

2.5. *Statistical analysis*

Multiple group statistical comparisons were analyzed by one-way ANOVA, followed by post hoc test. Associations between the SETD2 expression levels and the clinicopathological parameters were analyzed with a chi-square test. Survival analysis was performed with Kaplan-Meier curves and log rank test. Multivariate Cox regression model was used to identify the independent prognostic factors. $p$ value less than 0.05 was recognized as statistically significant. All analyses were performed using SPSS 18.0. software (SPSS Inc., Chicago, IL, USA).

## 3. Results

3.1. *The expression of SETD2 in ccRCC tissues*

SETD2 and its functional product H3K36me3 protein expression levels were tested in 135 cases of ccRCC FFPE specimens and 20 tumor adjacent normal renal tissues by an immunohistochemical approach. As shown in Figure 1, SETD2 and H3K36me3 restricted expression in the nucleus of the normal renal tubular cells in a homogeneous pattern, while they were heterogeneously stained in the nucleus of cancer cells. SETD2 was positively stained in all (20/20) normal renal tissues, however, the SETD2 protein deficiency rate in ccRCC tissues was 34.07% (46/135), based on the IHC staining evaluation. H3K36me3, SETD2's canonical protein product, showed a similar expression pattern with SETD2. However, using the same IHC evaluation method, no association between SETD2 and H3K36me3 expression levels was observed (Table 1).

3.2. *The association between SETD2 expression and the clinicopathological variables of ccRCC patients*

*SETD2* gene inactive mutation is a major event along with the kidney cancer formation and progression. The association of its protein expression with the clinicopathological parameters of ccRCC patients was evaluated. As shown in Table 1, low SETD2 expression levels significantly correlated with higher primary tumor stage ($p$ = 0.024), distant metastasis ($p$ = 0.0001), higher Fuhrman Nuclear Grade ($p$ = 0.018),

**Table 1. Associations between patient characteristics and SETD2&H3K36me3 expression**

| Variables | Number. of patients | SETD2 expression | | | H3K36me3 expression | | |
|---|---|---|---|---|---|---|---|
| | | Low ($n$ = 72) | High ($n$ = 63) | $p$ value | Low ($n$ = 72) | High ($n$ = 63) | $p$ value |
| Sex | | | | | | | |
| Male | 105 | 58 | 47 | 0.407 | 48 | 57 | 0.29 |
| Female | 30 | 14 | 16 | | 17 | 13 | |
| Age | | | | | | | |
| ≤ 55 | 62 | 31 | 31 | 0.474 | 30 | 32 | 0.959 |
| > 55 | 73 | 41 | 32 | | 35 | 38 | |
| p T stage | | | | | | | |
| T1-2 | 99 | 47 | 52 | 0.024 | 44 | 55 | 0.153 |
| T3-4 | 36 | 25 | 11 | | 21 | 15 | |
| Lymph node metastasis | | | | | | | |
| N0 | 116 | 58 | 58 | 0.112 | 57 | 59 | 0.286 |
| N1 | 17 | 12 | 5 | | 6 | 11 | |
| Distant metastasis | | | | | | | |
| M0 | 120 | 58 | 62 | 0.001 | 54 | 66 | 0.061 |
| M1 | 14 | 13 | 1 | | 10 | 4 | |
| Fuhrman nuclear grade | | | | | | | |
| G1-2 | 79 | 36 | 43 | 0.018 | 38 | 41 | 0.944 |
| G3-4 | 41 | 28 | 13 | | 20 | 21 | |
| Clincial stage | | | | | | | |
| I | 66 | 26 | 40 | 0.001 | 32 | 34 | 0.09 |
| II | 25 | 16 | 9 | | 7 | 18 | |
| III | 23 | 11 | 12 | | 12 | 11 | |
| IV | 20 | 18 | 2 | | 13 | 7 | |
| Renal vein invasion | | | | | | | |
| None | 119 | 60 | 59 | 0.069 | 58 | 61 | 0.859 |
| Invasion | 13 | 10 | 3 | | 6 | 7 | |
| H3K36me3 expression | | | | | | | |
| Low | 65 | 40 | 25 | 0.066 | | | |
| High | 70 | 32 | 38 | | | | |

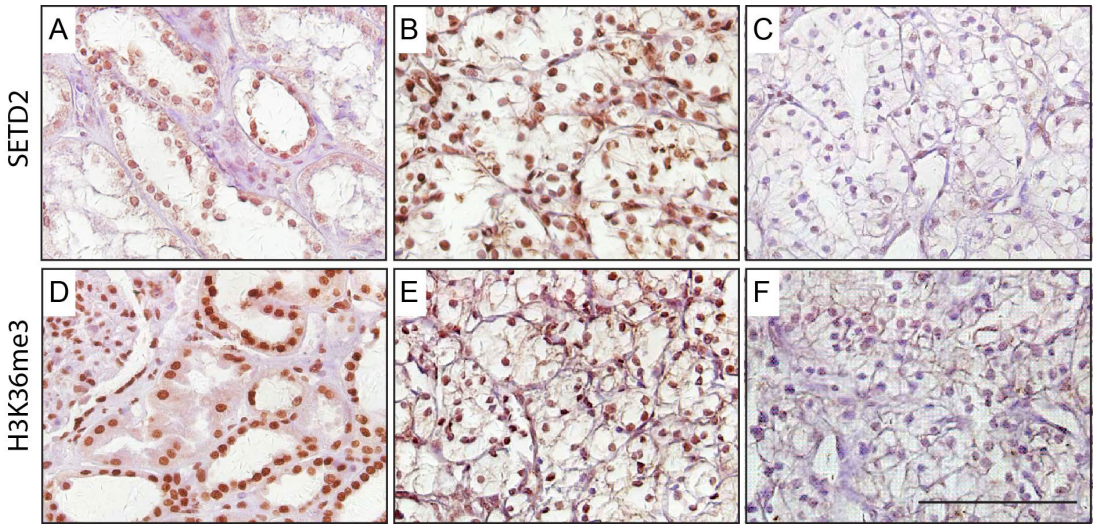


**Figure 1. Immunohistochemical staining of SETD2 and H3K36me3 in ccRCC samples. (A,B)** High SETD2 expression in ccRCC tumor tissues and adjacent normal renal tissues, which mainly localized in the nucleus. **(C)** Low SETD2 expression in ccRCC tissues. **(D,E)** High H3K36me3 expression in ccRCC tumor tissues and adjacent normal renal tisssues, with a similar expression pattern of SETD2 expression. **(F)** Low H3K36me3 expression in ccRCC tissues. Original magnification 200×; insert bar = 100 μm.

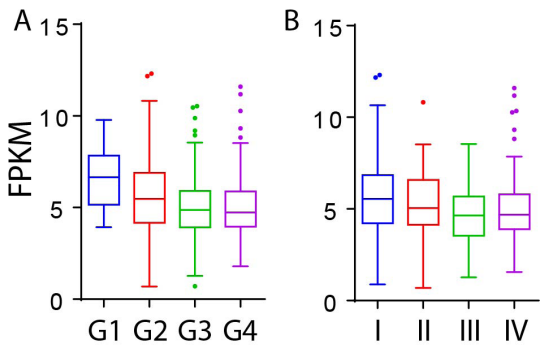


**Figure 2. The clinical association of SETD2 mRNA expression in TCGA ccRCC RNA-Seq database (*n* = 541). (A)** The low expression of SETD2 mRNA significantly associated with advanced Fuhrman Nuclear Grade ($p = 0.0015$, one-way ANOVA). **(B)** The low expression of SETD2 mRNA predicts advanced clinical stage ($p = 0.0004$, one-way ANOVA).

and advanced clinical stage ($p = 0.001$). There was no significant correlation of the SETD2 expression with gender, age or lymph node metastasis (all $p > 0.05$; Table 1). Of note, though a trend was observed between low SETD2 expression and renal vein invasion ($p = 0.069$), it failed to reach statistical significance, which may be due to the small sample size. On contrast, no significant association between H3K36me3 expression and the clinicopathological variables mentioned above was observed. In parallel, using an alternative approach to evaluate the clinical significance of SETD2 expression, TCGA ccRCC RNA-Seq data and the corresponding clinical information was extracted. Consistently, low SETD2 mRNA expression was significantly associated with high Fuhrman Nuclear Grade ($p = 0.0015$, Figure 2 A) and advanced clinical stage ($p = 0.0004$, Figure 2B).

### 3.3. *SETD2 expression and the postoperative survival of ccRCC patients*

Kaplan-Meier analysis compared by log rank test was used for survival analysis. As shown in Figure 3 A and B, low SETD2 expression was significantly associated with better overall survival of ccRCC patients ($p < 0.0001$, HR = 0.1143). However, H3K36me3 expression failed to show any correlation with patients' outcome. Consistently, survival analysis using the TCGA ccRCC RNA-Seq data indicates that low SETD2 mRNA expression was significantly correlated with Overall Survival ($p = 0.0044$, HR = 0.6493) and Progression-Free Survival ($p = 0.0030$, HR = 0.5796) of ccRCC patients (Figure 3 C and D). In addition, multivariate analysis by a Cox regression hazard model identified lymph node metastasis ($p = 0.0004$), distant metastasis ($p = 0.0003$), Fuhrman Nuclear Grade ($p = 0.0319$), advanced clinical stage ($p = 0.0277$), and SETD2 protein expression ($p = 0.0004$) as independent prognostic factors (Table 2).

## 4. Discussion

Recent sequencing efforts identified novel recurrent mutations on chromosome 3p21 in ccRCC, including SETD2, BAP1, PBRM1, and KDM6A, which are responsible for modifying histones and chromatin remodeling (*14*) and play an important role in kidney cancer formation and progression (*5,10,15,16*). Previous studies indicated that SETD2 mutation occurred in 3-12% of sporadic ccRCC patients, which ranked second of the most frequent gene mutations in ccRCC next to VHL gene mutation (*17*). In our present study, IHC staining revealed that SETD2 was

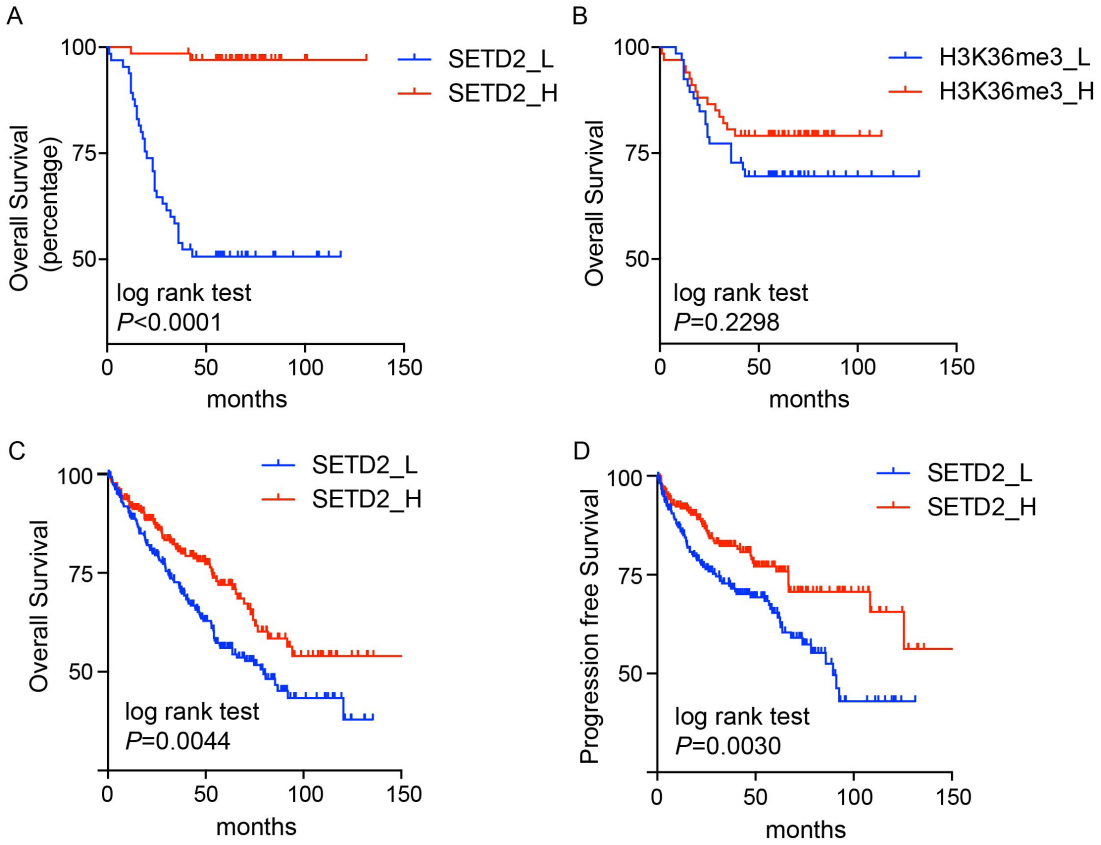


**Figure 3. Kaplan-Meier curve analysis for survival based on SETD2 or H3K36me3 expression in Qilu-Weihai cohort (*n* = 135, A and B) or in TCGA ccRCC cohort (*n* = 541, C and D). (A)** The high SETD2 protein expression predicts better overall survival ($p < 0.0001$). **(B)** No significant difference was observed between ccRCC patients with high or low H3K36me3 expression in terms of overall survival. The high expression of SETD2 mRNA predicts better Overall Survival ($p = 0.0044$, **C**) and Progression-free Survival ($p = 0.0030$, **D**). All *p* values calculated with log rank test.

**Table 2. Multivariate analysis indicates SETD2 protein expression as an independent predictor for ccRCC patients overall survival (*n* = 135)**

| Items | *p* value | Hazard Ratio | 95% confidence interval | |
|---|---|---|---|---|
| sex | 0.2234 | 2.076 | 0.641 | 6.727 |
| age | 0.0586 | 0.402 | 0.156 | 1.034 |
| T | 0.7632 | 1.162 | 0.437 | 3.095 |
| N | 0.0004 | 8.590 | 2.601 | 28.375 |
| M | 0.0003 | 8.747 | 2.678 | 28.564 |
| Fuhrman Nuclear Grade | 0.0319 | 2.825 | 1.094 | 7.295 |
| Clinical Stage | 0.0277 | 4.821 | 1.189 | 19.552 |
| Renal vein invasion | 0.4640 | 1.408 | 0.564 | 3.515 |
| SETD2 expression | 0.0004 | 0.045 | 0.008 | 0.249 |
| H3K36me3 expression | 0.8018 | 0.902 | 0.403 | 2.017 |

positively expressed in the cell nucleus of all normal renal tubular tissues. However, its expression was lost in part of the cancer tissues. If we define the SETD2 protein deficiency as the SETD2-postitive rate is less than 60% of block tumor cells, or the tumor cells are weakly stained or not stained, then the SETD2 protein deficiency rate was 34.07%, determined by IHC, which was much higher than the *SETD2* gene mutation rates of 3-12%. There may be two reasons. First of all, ccRCC exhibited a heterogeneity nature, which easily could cause sampling bias. To date, most *SETD2* gene mutation status was based on multiple kinds of sequencing, which could only reflect the gene status of very circumscribed cancer lesions and potentially underestimate the mutation rates (*13,18-21*). In comparison, IHC could detect the protein expression of a whole block section, which is more objective. Second, the *SETD2* gene inactive mutation may not be the only reason leading to its protein deficiency, alternative ways exist, such as miR-106b-5p, which could target SETD2 and inactivate its function in ccRCC (*22*). Considering the important role of SETD2 protein in kidney cancer formation, we recommend further study focusing on the mechanism of SETD2 protein deficiency.

Numerous studies revealed that chromatin remodeling plays an important role in cancer development (*5,8*). SETD2 protein is responsible for H3K36me3, which may lead to chromatin accessibility changes (*23,24*) and hinder mismatch repair (MMR) (*25*), and eventually cause RNA processing defects and promote oncogenesis (*26*). Previous studies support SETD2's role as a tumor suppressor in ccRCC (*27*). In our present study, low SETD2 expression was found to be associated with an aggressive phenotype of ccRCC, and was identified as an independent prognostic factor in two independent ccRCC cohorts. Consistently, using the RNA-Seq data from the TCGA ccRCC cohort, we also found a significant association between SETD2 mRNA expression and the advanced clinical features of ccRCC patients.

Considering the close connection of H3K36me3 to SETD2, here we assessed H3K36me3 expression using IHC side by side. Previous study found a correlation coefficient between SETD2 and H3K36me3 in non-metastatic ccRCC specimens (*28*). In contrast, our study only observed a trend of H3K36me3-SETD2 correlation in advanced ccRCC patients, which failed to reach statistical significance. Consistently, a high-resolution profiling of H3K36me3 in metastatic ccRCC found that H3K36me3 is not significantly affected by monoallelic loss of SETD2 (*26*). Subsequently, we evaluated the association of H3K36me3 expression with the clinicopathologic parameters independently, and found no significant associations, except for a trend of low H3K36me3 towards distant metastasis. Consistently, Thai *et al*. from the Mayo Clinic found that H3K36me3-negative tumors had a worse outcome only in a low-risk SSIGN (stage, size, grade and necrosis) group, but not in a high-risk SSIGN group (*29*). In our study, for survival analysis, more cases at an advanced clinical stage were enrolled, which resemble the high-risk SSIGN group in the Mayo cohort. The above findings suggest a less important role for H3K36me3 in the progression of ccRCC at advanced stages. Considering the complex fact that besides SETD2, H3K36 demethylases, such as the JHDM3/JMJD2 family, were also important during the regulation of H3K36me3 levels, there is no need to be fussy about the controversy of the correlation of SETD2 and H3K36me3 expression (*28*). Further studies focusing on both methytransferase and demethylase of H3K36me3 are warranted to figure out the dynamic regulation of H3K36me3 during kidney cancer initiation and progression.

In conclusion, our study demonstrated that SETD2 protein was massively down-regulated in ccRCC, besides a SETD2 inactive mutation, multiple reasons contribute to its protein deficiency. Moreover, out study identified SETD2 protein, but not H3K36me3, as a good independent prognostic marker, and encourage further study to investigate its non-H3K36me3 role in kidney tumor biology.

**Acknowledgements**

This study was supported by the National Natural Science Foundation of China (No. 81372335) and the Youth Foundation of the Second Hospital of Shandong University (No. Y2015010039).

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*(Received December 11, 2016; Revised January 25, 2017; Accepted February 14, 2017)*

Original Article

DOI: 10.5582/bst.2016.01205

# Role of the pretreatment $^{18}$F-fluorodeoxyglucose positron emission tomography maximal standardized uptake value in predicting outcomes of colon liver metastases and that value's association with Beclin-1 expression

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**Summary** **The current study sought to evaluate the predictive and prognostic performance of the maximum standardized uptake value ($SUV_{max}$) prior to treatment in 43 patients with colon cancer and unresectable liver metastases. Patients with colon cancer who underwent $^{18}$F-FDG-PET/computed tomography (CT) scans for staging before the start of first-line 5-fluorouracil-based chemotherapy were retrospectively analyzed. Expression of Beclin-1 in cancer cells was evaluated in primary tumors using immunohistochemical staining. The pretreatment $SUV_{max}$ for liver metastases was not able to predict progression-free survival but was significantly associated with poorer overall survival, with a hazard ratio of 2.05 (95 % CI, 1.016-4.155). Moreover, a negative correlation was noted between $SUV_{max}$ and expression of a marker of autophagy – Beclin-1 (rho = -0.42, $p$ = 0.006). This suggests that the pretreatment $SUV_{max}$ in $^{18}$F-FDG PET/CT is a useful tool to help predict survival outcome in patients with colon cancer and unresectable liver metastases and may significantly distinguish between patients with low and high levels of Beclin-1 expression (AUC = 0.809, 95% CI: 0.670-0.948, $p$ = 0.001).**

***Keywords:*** Maximum standardized uptake value ($SUV_{max}$), Beclin-1, colon cancer

## 1. Introduction

Colorectal cancer (CRC) is the most commonly diagnosed gastrointestinal cancer worldwide, with more than 1.2 million new cases and 600,000 deaths annually (*1*). The liver is identified as the most common site for the hematogenous spread of metastatic neoplasms (*2,3*). These metastases are mainly a result of colorectal cancer (*4*). About 25% of patients are diagnosed with metastases initially and up to 50% of all patients with CRC will develop metastases, which leads to the high mortality rates reported for CRC. The 5-year survival rate for patients with CRC is close to 60% (*5*). Correct diagnosis, staging, and restaging are crucial to providing these patients with optimal therapeutic options.

Over the past few decades, 18-fluorodeoxyglucose

Released online in J-STAGE as advance publication February 28, 2017.

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positron emission tomography ($^{18}$F-FDG PET/CT) has become an increasingly key component in the clinical management of liver metastases (*6*). Positron emission tomography (PET) with 2-deoxy-2-[fluorine-18] fluoro-D-glucose ($^{18}$F-FDG), an analogue of glucose, provides valuable functional information based on the increased glucose uptake and glycolysis of cancer cells and depicts metabolic abnormalities before morphological changes occur. $^{18}$F-FDG PET/CT acquires PET and CT data during the same imaging session and allows for precise anatomical localization of the lesions detected on the $^{18}$F-FDG PET scan. The glucose analogue $^{18}$FDG has become the most often used PET tracer in oncology and it is widely used to visualize abnormal glucose metabolism (*7*). In CRC, $^{18}$F-FDG PET/CT plays a key role in recurrent disease detection as well as in the assessment of residual post-therapy masses, the localization of recurrence in patients with an unexplained elevation of carcinoembryonic antigen in the serum, and the staging of patients before surgical resection of local recurrence and distant metastasis (*5,8*). Currently, the standardized uptake value (SUV) is a quantitative method commonly used in PET. In comparison to other quantitative approaches, the SUV is clinically appealing because of its simplicity and high reproducibility, thanks to the use of modern computer software (*9*). The SUV also plays an important role in the evaluation of patient responses to therapies (*7,10*). Nevertheless, there is still debate as to the exact role that $^{18}$F-FDG PET/CT can play in identifying prognostic factors for survival in patients with CRC and unresectable liver metastases (*11*).

Autophagy is the process of recycling of long-lived proteins and damaged organelles, and this process which is induced in tumor cells mainly to continue surviving (*12*). Various stress factors, such as hypoxia, an acidic environment, and starvation can intensify autophagic activity in tumor cells (*13*). Studies have reported that Beclin-1 is an essential marker of autophagy and that it plays different roles in several types of cancer (*12,14*). Recently, several studies suggested that oncogenes and autophagy play important roles in regulating the shift to aerobic glycolysis in cancer cells in order to promote cell survival (*15,16*). Mammalian cells have a complicated network that interconnects different signaling pathways to regulate autophagy, and autophagy can be induced or inhibited by glucose (*17*).

The aim of the current retrospective study was to evaluate the predictive and prognostic performance of pretreatment $SUV_{max}$ in 43 patients with colon cancer and unresectable liver metastases. Because $^{18}$F-FDG PET/CT has been rarely used to examine protein expression, the goal here was to determine if Beclin-1 expression and $SUV_{max}$ were associated.

## 2. Materials and Methods

### 2.1. *Patients and treatment selection*

All procedures were approved by the Scientific Research Ethics Committee at "Prof. Dr. Paraskev Stoyanov" Medical University, Varna. Patients who underwent $^{18}$F-FDG PET/CT as a part of their standard diagnostic workup for colon liver metastases were identified retrospectively and sequentially from PET/CT studies performed between January 1, 2012 and December 31, 2015. The inclusion criteria were (*a*) pathologically confirmed colon cancer and (*b*) multiple liver metastases (> 3 tumors) that were unresectable (largest size ≥ 5 cm) according to PET/CT. The exclusion criteria were (*a*) metastases at other site of at the time of PET/CT, (*b*) uncontrolled diabetes.

All patients were treated with first-line chemotherapy (CT) in the form of FOLFOX (leucovorin, fluorouracil (5-FU), and oxaliplatin) or FOLFIRI (leucovorin, fluorouracil (5-FU), and irinotecan) with or without bevacizumab/panitumumab until progression of the disease. Patients received a minimum of 3 months of treatment.

### 2.2. *Analysis of KRAS mutations*

Tissue sections were microscopically examined for adequacy using hematoxylin and eosin staining and were manually macrodissected. DNA was extracted from paraffin-embedded formalin-fixed tumor tissue and the Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation status was determined using a allele-specific real time polymerase chain reaction - based assay (AMOY Dx KRAS Seven Mutations Detection Kit, Amoy Diagnostics Co., Ltd., China) in the National Genetic Laboratory, Sofia.

### 2.3. *Immunohistochemical staining*

Specimens of primary colon adenocarcinoma were obtained from 43 patients in Clinical Pathology, "St. Marina" University Hospital. The diagnosis was microscopically confirmed by a pathologist. All hematoxylin eosin-stained specimens from the 43 patients with colon cancer contained cancerous tissues. Five-micrometer sections were cut from the paraffin blocks and placed on glass slides. Sections were deparaffinized with xylene and dehydrated in a graded series of ethanol to deionized water. Antigen retrieval was performed in pre-heated EnVision FLEX Target Retrieval Solution (working solution) in PT Link tanks and slides were incubated for 30 minutes at 97°C in medium with a pH of 9. After cooling, the slides were placed in diluted, room-temperature FLEX Wash Buffer (20×) for 1-5 minutes. Sections were stained using

the FLEX protocol in Dako Autostainer/Autostainer Plus. Samples were tested with recombinant rabbit polyclonal antibody to Beclin-1 (ABCAM's RabMab technology ab62557). The antibody (AntiBeclin-1, diluted 1:400) was incubated for 20 minutes. Levels of Beclin-1 expression were detected using the UltraVision detection system Anti-polyvalent, HRP/DAB. The reaction was developed with the appropriate substrate-chromogen (DAB, Diaminobenzidine) reagent. Counterstaining was done using Mayer's hematoxylin for the evaluation of immunostaining. Ten random high-power fields were examined under ×400 magnification for each patient. Digital images were obtained using the Leica Aperio ScanScope AT2 device (Aperio Technologies, Vista, CA) and further analyses of the scanned images were performed with ImageScope V12.1.0.5029 (Aperio).

### 2.4. *H-score assessment*

Two independent pathologists with no prior knowledge of the clinical data scored all immunohistochemically stained specimens for Beclin-1 based on the staining intensity and the percentage of positively stained tumor cells. Staining intensity was classified into 4 grades: 0 (pale yellow or no staining), 1 (yellow), 2 (deep yellow), and 3 (brown) (*18*). The percentage of positively stained tumor cells was scored in 4 grades: 0 (0-10%), 1 (10-25%), 2 (25-50%), and 3 (50-100%). The intensity and percentage of positively stained tumor cells were scored after counting at least 10 high-power fields at 400×. Mean H-scores were calculated as follows: [(Intensity reader 1 × Percentage reader 1) + (Intensity reader 2 × Percentage reader 2)]/2. The median value was used to divide patients into 2 groups, those with low levesl of expression (< median) and those with high levels of expression (≥ median) (Figure 1).

### 2.5. *$^{18}$F-FDG PET/CT image acquisition and analysis*

An $^{18}$F-FDG PET/CT image was obtained using a PET/CT scanner (PHILIPS Gemini TF) consisting a dedicated lutetium orthosilicate full-ring PET scanner and a 16-slice CT scanner. Standard patient preparations included at least 6 hours of fasting and a serum glucose level of less than 120 mg/dL before $^{18}$F-FDG administration. PET/CT imaging was performed 60 minutes after intravenous injection of $^{18}$F-FDG. Sixty minutes after $^{18}$F-FDG administration, a low-dose CT (50 mAs, 120 kV) scan covering the area from the skull to the proximal thighs was performed for the purpose of attenuation correction and precise anatomical localization. Afterwards, an emission scan was performed in three-dimensional mode with an emission scan time of 39 mm/sec. PET data were obtained using a high-resolution whole-body scanner with an axial field of view of 57.6 cm. The average total PET/CT examination time was 20 minutes. SUVs were calculated using the concentration of FDG in the volume of interest as was measured with PET. This concentration was divided by the injected dose and multiplied by body weight as a normalization factor. Following decay and scatter correction, PET images were reconstructed iteratively with attenuation correction and reoriented in axial, sagittal, and coronal slices. The row action maximum-likelihood algorithm was used for three-dimensional reconstruction. An $SUV_{max}$ greater than the median (7.3) was denoted as a high $SUV_{max}$, and an $SUV_{max}$ equal to or lower than the median (7.3) was denoted as a low $SUV_{max}$.

### 2.6. *Statistical design and analysis*

Descriptive statistics were used. Categorical features were summarized with frequencies and percentages. Statistical analysis included 43 patients treated with chemotherapy (CT) alone or CT/targeted therapy. The predictive and prognostic performance of $SUV_{max}$ prior to treatment was evaluated in 43 patients with colon cancer and unresectable liver metastases. The Mann-Whitney *U* test, $\chi^2$ test, and Spearman correlation were used to compare and identify correlations between the pretreatment $SUV_{max}$ and clinicopathological characteristics such as gender, age, and level of Beclin-1 expression. The specificity and sensitivity with which $SUV_{max}$ distinguished between patients with low levels of Beclin-1 expression and patients with high levels of expression were evaluated using receiver operating curve (ROC) analysis. The diagnostic accuracy of biomarkers was also determined by obtaining the largest possible area under the curve (AUC) in ROC analysis. Progression-free survival (PFS) was defined as the time from assignment of treatment until disease progression. Overall survival (OS) was defined as the interval between diagnosis of the disease and death or the date of last follow-up. Survival curves were estimated using the Kaplan-Meier method and differences were assessed using the log-rank test. Hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were calculated using Cox regression models. Two-tailed *p*-values < 0.05 were considered significant.

## 3. Results

### 3.1. *Clinical and pathological features*

The current study retrospectively analyzed 43 patients with confirmed unresectable liver metastatic colon cancer that was stage IV, as defined by the American Joint Committee on Cancer (AJCC), 7$^{th}$ edition. The patients' Eastern Cooperative Oncology Group (ECOG) performance status was < 2. Chemotherapy

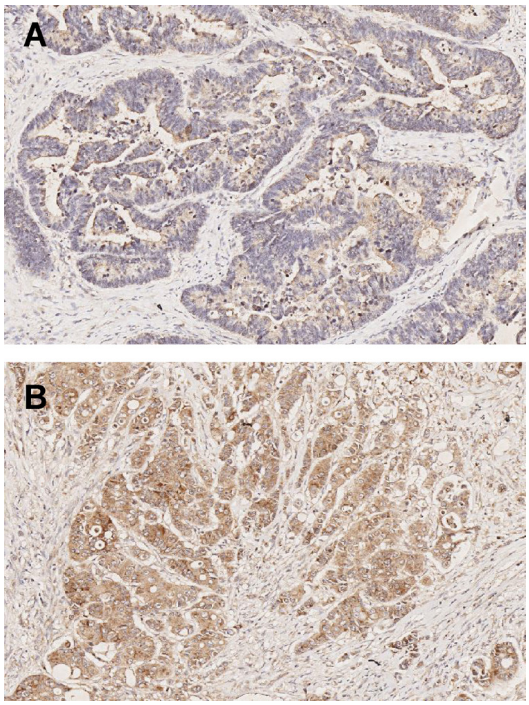


**Figure 1. Immunohistochemical staining of Beclin-1 in human colon carcinomas. (A)** Patient with a low level of expression **(B)** Patient with a high level of expression.

**Table 1. Clinical and pathological patient characteristics at the baseline by $SUV_{max}$ levels**

| $SUV_{max}$ | Low | High | *p*-value |
|---|---|---|---|
| Age at diagnosis | 63.1 ± 9.0 | 66. 1± 9.3 | 0.4 |
| Distribution by sex, % | Females, 40.9 | Females, 36.4 | 0.6 |
| | Males, 59.1 | Males, 63.6 | |
| KRAS mutation status, % | RAS M+, 50.0 | RAS M+, 40.9 | 0.3 |

was administered at "St. Marina" University Hospital. The mean age of the patients was 64.9 ± 9.3; 58.2% ($n$ = 25) were men, and 41.8% ($n$ = 18) were women. No significant difference in the pretreatment $SUV_{max}$ was noted in men and women. No correlation between patient age and the pretreatment $SUV_{max}$ was noted. Eighteen patients (41.8%) had KRAS mutations. Patients with KRAS mutations ($n$ = 18) had a pretreatment SUVmax that did not differ significantly from that of patients ($n$ = 25) with wild-type KRAS (WT).

Disease imaging with $^{18}$F-FDG PET/CT was performed at the baseline and tumor response was assessed (by PET/CT or CT) at regular intervals – every 3 months for all cycles of 5-FU-based chemotherapy ± targeted therapy until progression of the disease. Clinical and pathological patient characteristics at the baseline by $SUV_{max}$ are summarized in Table 1.

### 3.2. *Predictive and prognostic performance of the $SUV_{max}$ prior to treatment in patients with colon cancer and unresectable liver metastases*

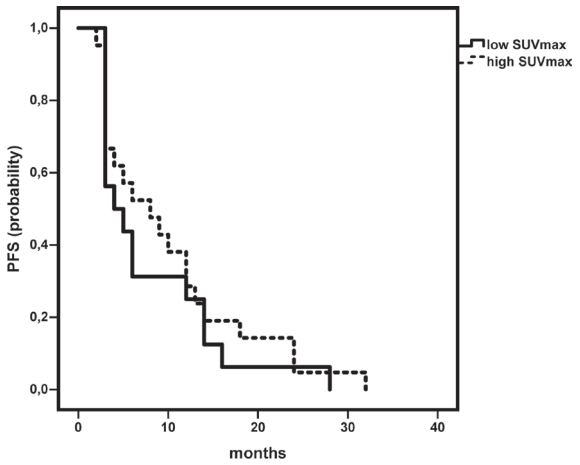


**Figure 2. Comparison of progression-free survival (PFS) by pretreatment $SUV_{max}$.** Kaplan-Meier estimates of PFS in patients with colon cancer with liver metastases by pretreatment $SUV_{max}$ (categorized as high or low compared to the median). There was no difference in the mean PFS between groups with a high (mean 10.04 months 95% CI, 6.45-13.64) and a low $SUV_{max}$ (mean 7.87 months 95% CI, 4.38-11.3).

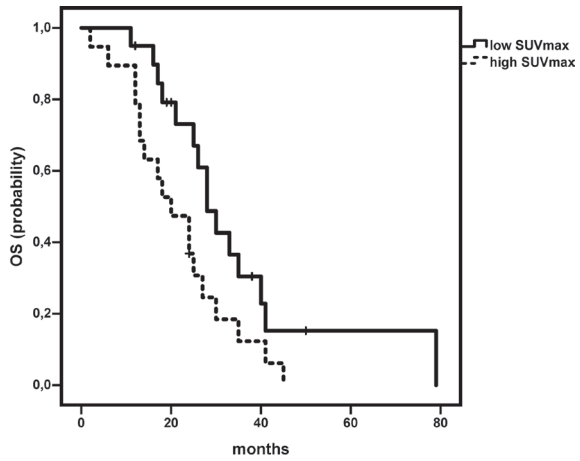


**Figure 3. Comparison of overall survival (OS) by pretreatment $SUV_{max}$.** Kaplan-Meier estimates of OS in patients with colon cancer with inoperable liver metastases by pretreatment $SUV_{max}$ (categorized as high or low compared to the median). There was a difference in the mean OS between groups with a high and a low $SUV_{max}$ (log rank test $p$ = 0.03, high $SUV_{max}$, mean: 21.6 months (95% CI, 16.3-27.2) *vs*. low $SUV_{max}$, mean: 29.3 months (95% CI, 22.8-36.6)).

Patients with a high $SUV_{max}$ prior to treatment had no significant difference in PFS compared to those with low values (mean 10.04 months *vs*. 7.87 months) (Figure 2). Patients with a high $SUV_{max}$ prior to treatment had significant difference in OS compared to those with low values (mean 21.6 months *vs*. 29.3 months) (Figure 3). Univariate analysis indicated that a high $SUV_{max}$ on $^{18}$F-FDG PET/CT prior to treatment was significantly associated with a poorer OS, with a HR of 2.05 (95% CI, 1.016-4.155, $p$ = 0.04). However, that association was not apparent in multivariate analysis (Table 2).

### 3.3. *Correlation between Beclin-1 and $SUV_{max}$ and KRAS status*

**Table 2. Results of univariate and multivariate Cox proportional regression analysis to predict overall survival**

| Variable | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | Hazard ratio | 95% CI | *p*-value | Hazard ratio | 95% CI | *p*-value |
| Age | 1.02 | 0.98-1.06 | 0.18 | 1.006 | 0.96-1.05 | 0.78 |
| Gender | 1.18 | 0.57-2.45 | 0.65 | 1.32 | 0.54-3.23 | 0.53 |
| KRAS status | 2.17 | 0.96-4.68 | 0.08 | 2.87 | 0.83-7.38 | 0.13 |
| $SUV_{max}$ | 2.05 | 1.02-4.15 | 0.04 | 2.41 | 0.87-6.66 | 0.09 |
| Beclin-1 expression | 1.25 | 0.57-2.74 | 0.56 | 1.33 | 0.52-3.42 | 0.55 |
| Chemotherapy ± Targeted therapy | 0.87 | 0.42-1.82 | 0.72 | 0.42 | 0.12-1.5 | 0.17 |

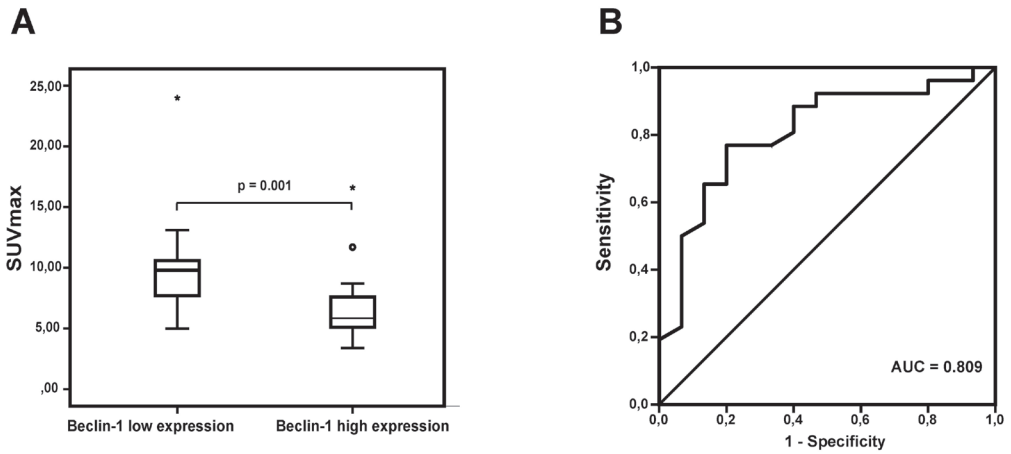


**Figure 4. Association between expression of Beclin-1 and $SUV_{max}$. (A)** A bar graph depicting the $SUV_{max}$ in patients with low and high levels of Beclin-1 expression. The Mann-Whitney U test was used to detect significant differences in $SUV_{max}$ in both groups. Two-tailed p-values < 0.05 were considered to be significant. **(B)** Receiver operating curve (ROC) analysis where $SUV_{max}$ was used to differentiate between patients with low and high levels of Beclin-1 expression. The diagnostic accuracy of $SUV_{max}$ was determined by obtaining the largest possible area under the curve (AUC) in ROC analysis.

Spearman's correlation analysis revealed that the coefficient of correlation between levels of Beclin-1 expression and $SUV_{max}$ was -0.42 ($p$ = 0.006) (Supplemental Data. Figure S1). Patients with low levels of Beclin-1 expression had a significantly higher SUVmax in comparison to patients with high levels of expression (mean 10.01 ± 4.4 *vs*. 6.6 ± 2.7, $p$ = 0.001) (Figure 4A). Expression of Beclin-1 was not related to KRAS status, age, or gender. In addition, ROC analysis revealed that at the optimal cut-off values $SUV_{max}$ distinguished between patients with low and high levels of Beclin-1 expression (AUC = 0.809, 95% CI: 0.670-0.948, $p$ = 0.001) with a sensitivity of 76.9% and a specificity of 80% (Figure 4B).

### 3.4. *Effects of Beclin-1 expression on PFS and OS*

Patients with low levels of Beclin-1 expression in the primary tumor tended to have a longer PFS compared to those with high levels of expression (log rank test $p$ = 0.06). Patients with low levels of Beclin-1 expression tended to have a greater therapeutic benefit in terms of PFS (HR 1.89 95% CI, 0.89 to 3.97, $p$ = 0.09) than patients with high levels of Beclin-1 expression had. Patients with low levels of Beclin-1 expression in the primary tumor had no significant difference in OS compared to those with high levels of expression (Table 2).

## 4. Discussion

PET/CT is an imaging technique that is widely used to stage colorectal liver metastases and to assess the metabolic response to chemotherapy. PET/CT findings can prove crucial to clinical decision-making. The current retrospective study sought to evaluate the predictive and prognostic performance of the $SUV_{max}$ prior to treatment in 43 patients with colon cancer and inoperable liver metastases. The current results suggest that a high $SUV_{max}$ prior to treatment may be a key marker of poor survival in patients with unresectable liver metastases. Moreover, results revealed a possible association between Beclin-1 – an essential marker of autophagy – and $SUV_{max}$.

Several $^{18}$F-FDG PET studies with small samples have suggested the prognostic value of metabolic imaging, but overall patient survival has not been reported as an indicator for treatment response (*7*). A small study of 25 patients with CRC examined the correlation between the 2-year PFS and $^{18}$F-FDG PET metabolic response as determined by criteria of the European Organization for Research and Treatment of

Cancer (*19*). In that study, a post-therapeutic change in $SUV_{max}$ of 2.0 or less in the lesion with the highest $^{18}$F-FDG uptake was a strong predictor of PFS. Another study has indicated that the degree of tumor uptake of $^{18}$F-FDG, as measured with SUV, serves as an independent prognostic factor in liver metastases (*20*). Other studies, however, found no association between $SUV_{max}$ and patient prognosis (*21,22*).

One study has reported that the baseline $^{18}$F-FDG-PET predicts the probability of an objective response but not the probability of a metabolic response, and the study also reported that complete metabolic responders had a significantly better PFS (*23*). In addition, other studies have reported that a high $^{18}$F-FDG uptake $SUV_{max}$ at follow-up and a high level of standardized added metabolic activity (SAM) are significant prognostic factors for PFS as well as OS (*24,25*). These findings provide further support for the prognostic power of $^{18}$F-FDG PET imaging in assessing the treatment response of patients with metastatic CRC; these findings also highlight the importance of a PET-guided treatment algorithm in the management of these patients. While a high $SUV_{max}$ at follow-up has been reported to be an adverse prognostic factor, the $\Delta SUV_{max}$ between the baseline and follow-up had no prognostic value. In contrast, the reduction in the total metabolic tumor burden as determined with ΔSAM was significantly related to PFS and OS (*11,25*). There is probably more than one reason for this discrepancy. On one hand, $SUV_{max}$ is more likely to vary as a result of several factors such as image noise and resolution, reconstruction methods, and the sensitivity of the scanner. On other hand, $SUV_{max}$ does not provide complete depiction of the treatment response to therapy since it indicates only the metabolic activity per gram of tissue in one voxel and does not take into consideration the total tumor metabolic load (*11*).

The current data suggest that intense glucose metabolism in liver metastases indicates tumor aggressiveness and can be used as a negative prognostic marker. However, there are large discrepancies between the cutoff values used to differentiate between high SUV and low SUV PET values. The wide range of SUV thresholds reported in studies could be the result of several factors such as institutional differences in techniques, the heterogeneity of the sample, and variance in PET scanners and protocols for data acquisition. Thus, some studies report that categorization with a wide range of SUVs resulted in significantly discriminative log-rank probability values (*26*). These findings imply that the relationship between SUV and prognosis could be gradual rather than a threshold-based one.

The use of $^{18}$F-FDG PET/CT is relatively uncommon in investigations of gene expression. Only a few studies have found an association between $^{18}$F-FDG uptake and KRAS mutation (*27,28*). The current results confirm the findings of a recent clinical study that reported finding no association between KRAS mutations and $^{18}$F-FDG uptake in patients with metastatic colon cancer (*29*). A number of other studies have also indicated that autophagy is upregulated in KRAS-driven cancers (*30,31*). Different studies have cited Beclin-1 as a potential marker in monitoring the prognosis in CRC, with greatly divergent results regarding its function (*32-34*). The current results suggest that patients with colon cancer and low levels of Beclin-1 expression also have a high $SUV_{max}$, and this value may in turn be associated with a longer PFS. $SUV_{max}$ is a favorable prognostic indicator of OS in patients with colon cancer (*11*), and the current data suggest that high levels of Beclin-1 expression correlate with a low $SUV_{max}$. ROC analysis revealed that $SUV_{max}$ can be used to distinguish between tumors with low and high levels of Beclin-1 expression. This study is, to the extent known, the first to evaluate the relationship between Beclin-1 and $SUV_{max}$ on PET/CT imaging. Nevertheless, the role of autophagy in the metastasis and prognosis of human CRC is still poorly understood. Future studies may further evaluate the potential association between expression of Beclin-1 and $SUV_{max}$.

Although the statistical power of the current findings is limited by the small sample, this study has verified that patients with a high $SUV_{max}$ prior to treatment should be considered to have a higher risk of death. The current results suggest that pretreatment $SUV_{max}$ on $^{18}$F-FDG PET/CT is a useful tool to help predict survival outcome in patients with colon cancer and unresectable liver metastases and may significantly distinguish between patients with low and high levels of Beclin-1 expression. This may allow an earlier and more intensive approach to standard therapy in order to improve clinical outcomes or to assist in identifying patients who are eligible for clinical trials of a new molecularly targeted therapy for CRC.

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*(Received November 3, 2016; Revised January 23, 2017; Accepted January 24, 2017)*

## Supplemental Data

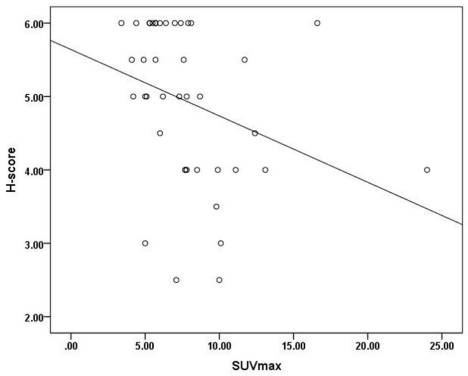


**Figure S1. Correlation between expression of Beclin-1 and $SUV_{max}$.**

Brief Report

DOI: 10.5582/bst.2016.01218

# The periplasmic sensing domain of *Pseudomonas fluorescens* chemotactic transducer of amino acids type B (CtaB): Cloning, refolding, purification, crystallization, and X-ray crystallographic analysis

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**Summary** ***Pseudomonas fluorescens* is a plant growth promoting rhizobacterium that provides nutrients for growth and induces systemic resistance against plant diseases. It has been linked with a number of human diseases including nosocomial infections and bacterial cystitis. Chemotactic motility of *P. fluorescens* towards root exudates plays a crucial role in establishing a symbiotic relationship with host plants. The *P. fluorescens* chemotactic transducer of amino acids type B (CtaB) mediates chemotaxis towards amino acids. As a step towards elucidation of the structural basis of ligand recognition by CtaB, we have produced crystals of its recombinant sensory domain and performed their X-ray diffraction analysis. The periplasmic sensory domain of CtaB has been expressed, purified, and crystallized by the hanging-drop vapor diffusion method using ammonium sulfate as a precipitating agent. A complete data set was collected to 2.2 Å resolution using cryocooling conditions and synchrotron radiation. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 34.5, b = 108.9, c = 134.6 Å. Calculation of the Matthews coefficient and the self-rotation function using this data set suggested that the asymmetric unit contains a protein dimer. Detailed structural analysis of CtaB would be an important step towards understanding the molecular mechanism underpinning the recognition of environmental signals and transmission of the signals to the inside of the cell.**

***Keywords:*** Chemotaxis, receptor, sensing domain, symbiosis

## 1. Introduction

*Pseudomonas fluorescens* and other fluorescent Pseudomonads belong to the group of plant growth promoting rhizobacteria (PGPR) that form a symbiotic relationship with host plants (*1,2*). PGPR strains exhibit beneficial effects on plants by fixing nitrogen, producing siderophores and solubilizing essential elements in soil (*1,2*). In addition, they exert indirect beneficial effects by preventing growth or activity of phytopathogens and inducing systemic resistance against plant diseases (*2*). They produce different types of secondary metabolites including fungicides and hydrogen cyanide, which protect roots against pathogens (*3*). Furthermore, some strains of *P. fluorescens* participate in biodegradation of xenobiotic compounds and bioremediation of heavy metals (*3,4*).

*P. fluorescens* is considered to be an opportunistic pathogen for humans (*5*). It has been linked with a number of human diseases including nosocomial infections and bacterial cystitis. In addition, *P. fluorescens* has been isolated from a large number of respiratory specimens taken from hospital patients,

Released online in J-STAGE as advance publication February 28, 2017.

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although its association with pulmonary infections is not well understood (*5*). Furthermore, Sutton CL *et al*. (*5*) reported that more than half of the patients with Crohn's disease develop antibodies against *P. fluorescens*. *P. fluorescens* can also cause blood transfusion-related bacteraemia and catheter-associated bacteraemia amongst the immunosuppressed patients (*6*). Nosocomial outbreaks of bacteraemia due to *P. fluorescens* have been reported (*5*). However, the information on pathogenesis mechanism of *P. fluorescens* is very limited.

Flagella-mediated motility and chemotaxis of *P. fluorescens* towards different nutrients present in root exudates or the rhizosphere play a crucial role in establishing a symbiotic relationship with plants (*2,7-9*). Previous mutagenesis studies demonstrated that chemotaxis is important for the root-tip colonization by *P. fluorescens* (*9-11*). Furthermore, motility and chemotaxis are important virulence factors of many pathogenic bacteria, and it is likely that they play an important role in *P. fluorescens* pathogenesis in humans. The environmental chemical signals are sensed by bacterial membrane-embedded methyl-accepting chemotaxis protein (MCP) receptors (*12*). Upon binding of the signal molecule, MCPs trigger a chemotactic signaling cascade and control bacterial movement towards or away from chemoattractants and repellents, respectively (*12*).

Of the 37 putative MCPs identified in the genome of *P. fluorescens* Pf0-1 to date, ligands are known for only seven. The MCPs termed chemotactic transducers of amino acids (CtaB, CtaB, and CtaC) sense amino acids as attractants (*13*). MCPs Pfl01_3768 and Pfl01_0728 were identified as receptors for L-malate, succinate, and fumarate (*14*). Finally, the chemoreceptor for 2-nitrobenzoate NbaY was shown to be involved in the metabolism of aromatic compounds (*15*).

The periplasmic sensing domain of CtaB has been shown to recognize a broad range of amino acids (16 in total) (*13*). The structural basis of how CtaB recognizes its ligands and transmits the signal across the membrane in response to ligand binding is yet to be determined. Data on bacterial receptors that are structurally and functionally homologous to CtaB is limited. The presence of the conserved consensus motif DXXX(R/K)XWYXXA (*16*) and the Cache (calcium channels and chemotaxis receptors) motif (residues 107-185) (*17*) in the amino acid sequence of CtaB allows us to putatively assign it to the family of receptor proteins with a periplasmic tandem Per-Arnt-Sim (PAS) sensing domain (PTPSD) that recognises amino acids directly. The crystal structures of two different PTPSDs with specificity to amino acids have been recently reported, providing first insights into the structural basis of their ligand specificity. Analysis of PTPSD of *Campylobacter jejuni* Tlp3 in complex with isoleucine (PDB code 4xmr) revealed a strongly hydrophobic pocket accommodating the aliphatic side chain of the ligand, consistent with Tlp3's preference for isoleucine and, likely, other branched amino acids such as valine and leucine (*16*). The crystal structure of PTPSD of *V. cholerae* Mcp37 has been reported in complex with alanine and serine (PDB codes 3c8c and 5ave (*18*)). CtaB PTPSD shares 25 and 29% sequence identity with PTPSDs of Tlp3 and Mcp37, respectively. This protein provides an example of a PTPSD-type receptor with an extremely broad substrate specificity. To elucidate the structural basis of the CtaB's ligand promiscuity, we have initiated X-ray crystallographic studies on recombinant CtaB PTPSD. Here, we report its cloning, refolding, purification and crystallization together with the analysis of the diffraction data.

## 2. Materials and Methods

### 2.1. *Cloning and overexpression of CtaB PTPSD as inclusion bodies (IBs)*

The membrane topology and the boundaries the periplasmic sensing domain of CtaB (CtaB PTPSD, residues 32-272) from *P. fluorescens* Pf0-1 (UniProt ID Q3KK38) were predicted by TOPCONS server (*http://topcons.net/*) (*19*) (Figure 1). The sequence encoding CtaB PTPSD was codon optimized for expression in *Escherichia coli*, synthesized and ligated into the pET151/D-TOPO vector (Invitrogen) by Genscript to generate an expression vector that harbors an N-terminal His6 tag followed by a TEV protease cleavage site. The expression vector was introduced into *E. coli* BL21 (DE3) (Novagen) and cells were grown in Luria-Bertani medium supplemented with 50 µg/mL ampicillin to an $OD_{600}$ of 0.6 at 310 K. Overexpression of CtaB PTPSD was induced with 0.5 mM isopropyl-b-D-1-thiogalactopyranoside (Thermo Scientific) and growth was continued for 3.5 h at 210 K. The cells were harvested by centrifugation at 6,000 g for 15 min at 277 K. The cells were resuspended in buffer *A* (10 mM Tris-HCl buffer pH 8.0 and 200 mM NaCl), lyzed by sonication and centrifuged at 10,000 g for 30 min at 277 K. SDS-PAGE gel electrophoresis of clarified supernatant and pellet confirmed that CtaB PTPSD expressed in inclusion bodies (IBs).

### 2.2. *Solubilization of IBs, protein refolding and purification*

Purification of CtaB PTPSD from IBs was performed following the procedure described earlier with some modifications (*20*). Briefly, IBs were washed two times with buffer *B* (10 mM Tris-HCl pH 8.0, 0.2 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich), 1% Triton X-100 (Sigma-Aldrich)) and once in buffer *C* (10 mM Tris-HCl pH 8.0, 0.2 mM PMSF), and centrifuged at 10,000 g for 30 min to purify IBs. The IBs were then solubilized in buffer *D* (10 mM Tris/

HCl pH 8.0, 8 M urea (Amresco), 10 mM dithiothreitol (DTT, Sigma-Aldrich), 0.2 mM PMSF) under gentle stirring for 30 min at 277 K. The protein solution was then clarified by centrifugation at 30,000 g for 30 min at 277 K. Protein concentration was determined using the Bradford assay (*21*). CtaB PTPSD was refolded by diluting 100 mg denatured protein into 250 mL buffer *E* (3 M urea, 10 mM Tris-HCl pH 8.0, 0.4 M L-arginine monohydrochloride) followed by a 48 h incubation at 227 K with continuous mixing. The sample was then dialyzed against 7 L buffer *A* for overnight at 277 K. NaCl and imidazole were then added to the protein solution to final concentrations of 500 and 15 mM, respectively. The protein sample was then loaded onto a 5 mL HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with buffer *F* (10 mM Tris-HCl pH 8.0, 500 mM NaCl, 15 mM imidazole). The column was washed with 20 column volumes of buffer *F* containing 20 mM imidazole to remove unbound proteins, and the protein was eluted with buffer *F* supplemented with 500 mM imidazole. The $His_6$-tag was removed by overnight incubation with a $His_6$-TEV protease at 277 K while dialyzing the sample against buffer *G* [50 mM Tris-HCl pH 8.0, 2 mM dithiothreitol, 200 mM NaCl, 1% (*v*/*v*) glycerol]. NaCl and imidazole were then added to the sample to final concentrations of 500 and 15 mM, respectively. The TEV protease and the uncleaved protein were removed on a HiTrap Chelating HP column. The flowthrough was concentrated to 2 mL in an Amicon Ultracel 10 kDa cutoff concentrator and purified further by passing through a Superdex 200 HiLoad 26/60 gel-filtration column (GE Healthcare) equilibrated with buffer *A*. The protein purity was estimated to be greater than 95% by the SDS-PAGE analysis (Figure 2). The oligomeric state of $VfcA^{peri}$ was calculated using a calibration plot of log MW versus the retention volume [$V_{retention}$ (mL) = 549.3 – 73.9 × logMW] available at the EMBL Protein Expression and Purification Core Facility website (*http://www.embl.de/pepcore/pepcore_services/protein_purification/chromatography/hiload26-60_superdex200/index.html*).

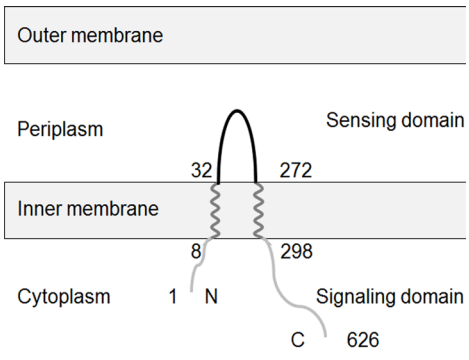


**Figure 1. The predicted membrane topology of *P. fluorescens* CtaB and the boundaries of the periplasmic sensory domain CtaB PTPSD characterized in this study.**

2.3. *Crystallization*

Prior to crystallization, the protein sample was concentrated to 10 mg/mL, centrifuged for 20 min at 13,000 g and transferred into a clean tube. The preliminary crystallization screening was carried out by the hanging-drop vapour-diffusion method using an automated Phoenix crystallization robot (Art Robbins Instruments). Commercial crystallization screens (Crystal Screen, Index Screen HT, The JCSG Screen and PEG/Ion Screen HT (Hampton Research, Laguna Niguel, CA) were used. Crystals appeared after one day in the condition No. 54 of The JCSG Screen consisting of 0.2 M zinc acetate, 20% (*w*/*v*) polyethylene glycol (PEG) 3000 and 0.1 M imidazole pH 8.0. Refinement to improve the quality of the crystals (Figure 3) resulted in the final optimized condition that contained 14% (*w*/*v*) PEG 3000, 0.15 M zinc acetate sulfate and 0.1 M imidazole pH 7.5.

2.4. *Data collection and processing*

Prior to data collection, crystals were briefly soaked in a cryoprotectant solution consisting of 0.18 M zinc acetate, 20% (*w*/*v*) PEG 3000, 0.1 M imidazole pH 7.5, 10 % (*v*/*v*) glycerol, and cryocooled by plunging in liquid nitrogen. An X-ray diffraction data set was collected from a single crystal on the MX1 beamline of the Australian Synchrotron (AS). A total of 420 images (Figure 4) were collected using a 0.5º oscillation width. The data were processed and scaled using *iMosflm* (*22*) and *AIMLESS* from the *CCP4* suite (*23*). The statistics of data collection and processing are summarized in Table 1.

## 3. Results and Discussion

Recombinant *P. fluorescens* CtaB PTPSD was expressed

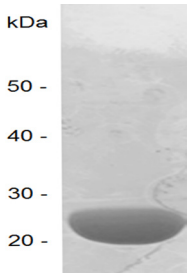


**Figure 2. Coomassie Blue-stained 15% SDS-PAGE gel of recombinant CtaB PTPSD.**

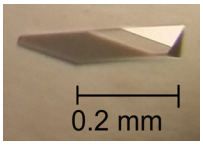


**Figure 3. A putative crystal of CtaB PTPSD.**

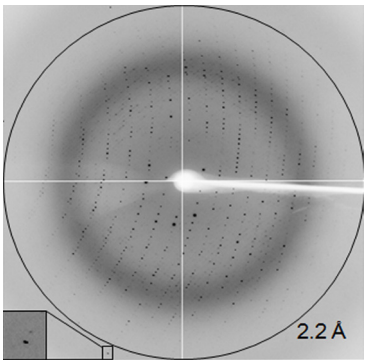


**Figure 4. A representative 0.5° oscillation image of the data collected using an ADSC Quantum 210r CCD detector on the MX1 station of the Australian Synchrotron, Victoria, Australia.** A magnified rectangle shows diffraction spots beyond 2.2 Å resolution.

**Table 1. Data collection and processing**

| | |
|---|---|
| Diffraction source | MX1 beamline, Australian Synchrotron |
| Wavelength (Å) | 1.0 |
| Temperature (K) | 100 |
| Detector | ADSC Quantum 210r CCD |
| Rotation range per image (°) | 0.5 |
| Total rotation range (°) | 420 |
| Exposure time per image (s) | 1 |
| Space group | $P2_12_12_1$ |
| *a*, *b*, *c* (Å) | 34.5, 108.9, 134.6 |
| α, β, γ (°) | 90, 90, 90 |
| Mosaicity (°) | 0.6 |
| Resolution range (Å) | 35.0-2.2 (2.3-2.2) |
| Total No. of reflections | 216,640 (31,679) |
| No. of unique reflections | 26,718 (3,862) |
| Completeness (%) | 100 (100) |
| Redundancy | 8.1 (8.2) |
| [*I/σ(I)*] | 16.2 (4.9) |
| $R_{pim.}$ | 0.027 (0.157) |
| Overall B factor from Wilson plot (Å$^2$) | 41.4 |

Values for the outer shell are given in parentheses.

with a cleavable N-terminal His6-tag from the pET151/D-TOPO plasmid in *E. coli* BL21 (DE3) upon induction of T7 polymerase. The protein was found in inclusion bodies (IBs). It was refolded from IBs and purified to > 95% electrophoretic homogeneity based on Coomassie Blue staining of SDS-PAGE gels (Figure 2). The protein migrated as a single band on SDS-PAGE with a molecular weight of ~25 kDa. This value was close to molecular weight (26.7 kDa) calculated from the amino acid sequence. When subjected to size-exclusion chromatography, the protein eluted as a single peak with a retention volume of 225 mL corresponding to an approximate molecular weight of 24.5 kDa, which suggested that *P. fluorescens* CtaB PTPSD is a monomer in solution under the tested buffer conditions.

An X-ray diffraction data set was collected from a cryo-cooled crystal of CtaB PTPSD to 2.2 Å using the AS facility (Figure 4). Processing of the diffraction data using the autoindexing routine in *iMosflm* and the analysis of systematic absences implemented in

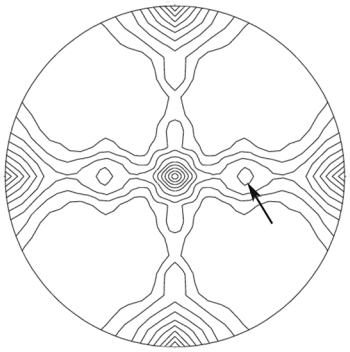

**Figure 5. The self-rotation function for CtaB PTPSD (κ = 180°).** The noncrystallographic twofold axis is marked by an arrow.

*AIMLESS* suggested that the crystals have the $P2_12_12_1$ symmetry, with unit cell parameters a = 34.5, b = 108.9, c = 134.6 Å. The average *I/σ(I)* value was 16.2 for all reflections (resolution range 35.0-2.2 Å) and 4.9 in the highest resolution shell (2.3-2.2 Å). A total of 216,640 measurements were made of 26,718 independent reflections. Data processing gave a $R_{pim}$ of 0.027 for intensities (0.157 in the resolution shell 2.3-2.2 Å), and these data were 99.9% complete (100% completeness in the highest resolution shell).

Under the assumption that there are two molecules of CtaB PTPSD in the asymmetric unit, the calculated Matthews coefficient (*24*) was 2.64 Å$^3$ Da$^{-1}$ and the corresponding solvent content was approximately 53%. Analysis of the self-rotation function computed using *POLARRFN* (*23*) with diffraction data in the resolution range 30-6 Å$^3$ and an integration radius of 16 Å revealed the presence of a twofold symmetry axis (κ = 180°) represented by a peak at (φ = 44.7°, ω = 0°) with a height of 4σ (Figure 5). Together, this analysis suggests that the CtaB PTPSD crystals contain two molecules per unit cell. Phasing by molecular replacement has not been possible due to low sequence similarity with the known structures deposited in the RCSB PDB database. A search for heavy-atom derivatives with the aim to solve the structure using multiple isomorphous replacement and/or multi-wavelength anomalous dispersion methods is in progress.

We have previously observed that expression of periplasmic sensory domains of bacterial MCP receptors in *E. coli* often results in their deposition predominantly in inclusion bodies (*16,20,25-27*). The recombinant ligand sensing domain of *P. fluorescens* CtaB is another example of a molecule of this type that required extraction from IBs and refolding. We succeeded in producing folded protein and high-quality crystals by following the refolding procedure that we have recently developed (*25,26*). The purified protein was monomeric in solution, in line with previous studies that showed PTPSDs from other receptors to be also monomeric in solution (*16,20,25-27*).

X-ray crystallographic analysis of CtaB PTPSD in complex with various amino acid ligands is expected to provide an explanation of the structural basis behind the broad ligand specificity of this receptor. We note that the crystal structure of PTPSD of another 'promiscuous' amino acid MCP receptor, *V. cholerae* Mcp37, has been recently reported (*18*). However, only the crystal complexes of that protein with alanine and serine have been characterised, which makes it difficult to predict how larger amino acids can fit into its relatively small ligand-binding pocket.

The results presented here are important because they lay the foundation for future systematic structural studies that will be able to address the fundamental biological question of how this receptor, and similar receptors in other important bacteria, sense environmental cues, how they transduce the signal across the membrane and thus control bacterial movement.

**Acknowledgements**

Part of this research was undertaken on the MX1 beamline of the AS, Victoria, Australia. We thank the AS staff for their assistance with data collection. We are also grateful to Dr. Danuta Maksel and Dr. Robyn Gray at the Monash University Protein Crystallography Unit for assistance with the robotic crystallization trials.

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*(Received November 22, 2016; Revised December 20, 2016; Accepted January 16, 2017)*

Brief Report

DOI: 10.5582/bst.2017.01019

# Simultaneous resection for colorectal cancer with synchronous liver metastases is a safe procedure: Outcomes at a single center in Turkey

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**Summary** **The optimal surgical strategy for treating colorectal cancer with synchronous liver metastases is subject to debate. The current study sought to evaluate the outcomes of simultaneous colorectal cancer and liver metastases resection in a single center. Prospectively collected data on all patients with synchronous colorectal liver metastases who underwent simultaneous resection with curative intent were analyzed retrospectively. Patient outcomes were compared depending on the primary tumor location and type of liver resection (major or minor). Between January 2005 and August 2016, 108 patients underwent simultaneous resection of primary colorectal cancer and liver metastases. The tumor was localized to the right side of the colon in 24 patients (22%), to the left side in 40 (37%), and to the rectum in 44 (41%). Perioperative mortality occurred in 3 patients (3%). Postoperative complications were noted in 32 patients (30%), and most of these complications (75%) were grade 1 to 3 according to the Clavien-Dindo classification. Neither perioperative mortality nor the rate of postoperative complications after simultaneous resection differed among patients with cancer of the right side of the colon, those with cancer of the left side of the colon, and those with rectal cancer (4%, 2.5%, and 2%, respectively, $p = 0.89$) and (17%, 33%, and 34%, respectively; $p = 0.29$)]. The 5-year overall survival of the entire sample was 54% and the 3-year overall survival was 67 %. In conclusion, simultaneous resection for primary colorectal cancer and liver metastases is a safe procedure and can be performed without excess morbidity in carefully selected patients regardless of the location of the primary tumor and type of hepatectomy.**

***Keywords:*** Liver metastases, colorectal cancer, synchronous, simultaneous resection

## 1. Introduction

Colorectal cancer remains a major global health problem, as evinced by the fact that it is the third most common malignancy and a leading cause of cancer-related death (*1*). The most common metastatic site is the liver (*2*). Synchronous colorectal liver metastases are defined as liver metastases detected at or before diagnosis or surgery of the primary tumor (*3*). Colorectal cancer with synchronous liver metastases is found in approximately 20-25% of patients at the time of diagnosis (*4,5*). Nevertheless, surgical resection of all tumor sites is considered the only curative therapy for long-term survival from colorectal liver metastases (CRLM) (*6,7*). Several large case series from tertiary centers have reported 5-year survival rates of 21-58% (*6,8-10*). There are several options for the treatment of resectable synchronous liver metastases, including a staged, liver-first approach and simultaneous resection. The traditional surgical strategy for resectable synchronous CRLM is a two-stage approach that includes colorectal cancer resection followed by chemotherapy and a delayed hepatic resection of a CRLM. This approach might result in the progression

Released online in J-STAGE as advance publication February 17, 2017.

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of liver disease in the interval from colorectal resection to hepatic resection and preclude resection of CRLM (*11*). Recently, a reverse staged approach has been used in order to address the hepatic tumour burden first (liver-first approach) and to prevent any delays in liver-directed and systemic therapies (*12,13*). Simultaneous resection removes all tumour burdens in one operation and it permits the prompt commencement of adjuvant chemotherapy. Furthermore, it avoids the hepatotoxic side effects of neoadjuvant chemotherapy, which means that simultaneous resection is a safer operation with lesser risk of post-operative liver failure and complications (*14-17*). However, some medical facilities do not recommend simultaneous resection if simultaneous resection is needed to treat rectal cancer or in patients undergoing extensive liver resection because it may increase the risk of perioperative complications (*14*). Although several studies have demonstrated the safety of simultaneous liver resection for CRLM, these studies did not evaluate the outcomes of simultaneous liver resection depending on the primary tumor location and type of liver resection (major or minor). Most studies have reported morbidity and mortality rates stratified only by the extent of hepatic resection as major or minor hepatectomy but they have consolidated all forms of colorectal resection into a singular entity (*18-20*). However, current evidence suggests that the morbidity of different forms of colorectal resection varies based on the type and location of resection as well as the use of intestinal diversion (*21,22*). One could reasonably expect the type of colorectal resections to influence the morbidity of synchronous resections as well.

Although several studies have demonstrated the safety of simultaneous liver resection for CRLM, studies on simultaneous liver resection depending on the primary tumor location and type of liver resection remain limited and the outcomes are still subject to debate. Therefore, the current study sought to evaluate the outcomes of simultaneous colorectal cancer and liver metastases resection in one center. This study also compared the outcomes for patients in the study cohort depending on the primary tumor location and type of liver resection (major or minor).

## 2. Materials and Methods

### 2.1. *Study design & setting*

The study was designed as a retrospective cohort study. Prospectively collected data on all patients with synchronous CRLM who underwent simultaneous colorectal and liver resection with curative intent at the Marmara University School of Medicine, Pendik Training & Research Hospital from 2005 to 2016 were analyzed retrospectively. This study was approved by the Research Ethics Committee of Marmara University, and all patients signed a written informed consent form before participation in this study.

### 2.2. *Preoperative evaluations*

All patients were preoperatively staged using computed tomographic (CT) scans and magnetic resonance imaging. Positron emission tomography (PET) was used in selected patients as necessary.

### 2.3. *Inclusion and exclusion criteria*

Patients with synchronous CRLM who underwent resection with curative intent with or without (neo) adjuvant therapy were included in this study. The decision to perform simultaneous resection was made *via* a team approach including surgeons, medical oncologists, and radiologists specializing in hepatobiliary diseases. Patients who fulfilled the following criteria underwent simultaneous resection: no unresectable extrahepatic metastases, adequate predicted volume and function of the hepatic remnant post-resection, no comorbidities such as cardiovascular or pulmonary disease, and American Society of Anesthesiologists (ASA) status > III. The total number of hepatic metastases, their location unilaterally or bilaterally, and the existence of extrahepatic metastases were not considered exclusion criteria. However simultaneous resection was not performed in patients with comorbidities or unresectable extrahepatic metastases, patients who needed emergency surgery (*i.e.* bleeding, perforation, or obstruction), patients with abnormal liver parenchyma (hepatosteatosis, fibrosis, or cirrhosis), and patients who did not consent to undergo simultaneous resection.

### 2.4. *Clinical data*

Information on age, gender, the ASA status, histological diagnosis, number, maximum size, and distribution of liver metastases before surgery, the surgical procedure, pathological TNM stage, (neo)adjuvant treatment, postoperative mortality, and morbidities such as anastomosis leakage, bleeding, pulmonary complications, liver failure, and reoperation was obtained from medical records. In patients who received pre-operative chemotherapy, first-line chemotherapy was 5-fluorouracil along with oxaliplatin or irinotecan. This chemotherapy was associated with either VEGF (bevacizumab) or EGFR (cetuximab)-targeted therapy in accordance with the RAS mutation status. Chemotherapy response was evaluated every 2-3 months. Recurrences and distant metastases were also documented during follow-up. Recurrence, whether loco-regional or distant, was confirmed histologically or clinically (a palpable mass or tumor that may be associated with clinical deterioration identified on

imaging studies and verified with increased serum CEA level).

2.5. *Surgical procedure*

All patients underwent open surgery and standard regional lymphadenectomy for the primary tumor with tumor-free surgical and circumferential margins. Intraoperative ultrasonography of the liver was routinely performed to detect unidentified liver metastases and to assess the potential for resection. Parenchymal transection was performed using Péan forceps under intermittent total hepatic inflow vascular clamping for 15 min at 5-min intervals. Major hepatectomy was defined as the resection of three or more Couinaud's segments. Perioperative mortality was defined as death within 30 days of simultaneous resection. Surgical morbidity was stratified as recommended by Clavien and Dindo (*23*).

2.6. *Statistical analyses*

Survival curves were created using the Kaplan-Meier product-limit method and compared using the log-rank test. One-way ANOVA, a Kruskal Wallis test, and a chi square test were used to compare groups. Statistical significance was defined as $p < 0.05$. A software program (SAS version 8; SAS Institute Inc., Cary, NC) was used for statistical analyses.

## 3. Results and Discussion

Between January 2005 and August 2016, a total of 108 patients with synchronous CRLM underwent simultaneous colorectal and liver resection at this facility. Demographic data, histopathological characteristics, and clinical characteristics for patients are shown in Table 1. The median age of the patients was 62 years (range: 56-71 years); 58 of the patients (54%) were male. All patients were diagnosed as having an adenocarcinoma. The majority had primary tumor metastasis to lymph nodes (76%) and multiple liver metastases (69%). Major liver resection was performed on 41 patients (38%). The tumor was localized to the right side of the colon in 24 patients (22%), to the left side in 40 (37%), and to the rectum in 44 (41%). Perioperative mortality occurred in only 3 patients (3%). Postoperative complications were noted in 32 patients (30%), most of these complications (75%) were grade 1 to 3 according to the Clavien-Dindo classification (*23*).

The 5-year overall survival of the entire sample was 54% and the 3-year overall survival was 67% (Figure 1 and 2). Neither perioperative mortality nor the rate of postoperative complications after simultaneous resection differed among patients with cancer of the right side of the colon, those with cancer of the left side of the colon, and those with rectal cancer [(4%, 2.5% and 2%, respectively, $p = 0.89$) and (17%, 33% and 34%, respectively; $p = 0.29$)] (Table 2).

This study, which included 108 patients with synchronous CRLM, found that postoperative complications and perioperative mortality did not differ among patients with cancer of the right side of the colon, those with cancer of the left side of the colon, and those with rectal cancer or by the type of liver resection (minor or major).

The debate over whether or not to perform simultaneous liver resection for CRLM has changed over the past decade; in light of improvements in surgical techniques and postoperative care, simultaneous resection for synchronous CRLM has been performed more often (*16,17,18,24*), and simultaneous resection has become the treatment of choice in many medical facilities. In fact, the data as a whole strongly suggest

**Table 1. Demographic data on and clinical and histopathological characteristics of the study cohort**

| Items | *n* = 108 | % |
|---|---|---|
| Gender | | |
| Female | 50 | 46.3 |
| Male | 58 | 53.7 |
| N stage | | |
| N0 | 26 | 24.0 |
| N+ | 82 | 75.9 |
| Number of lymph nodes invaded by the primary tumor | | |
| No | 26 | 24.0 |
| Yes | 82 | 75.9 |
| Surgical margin | | |
| R0 | 98 | 90.7 |
| R1 | 10 | 9.2 |
| ASA status | | |
| ASA I | 14 | 12.9 |
| ASA II | 71 | 65.7 |
| ASA III | 23 | 21.3 |
| Type of liver resection | | |
| Minor rejection | 67 | 62.0 |
| Major resection | 41 | 37.9 |
| Number of liver metastases | | |
| Single | 34 | 31.4 |
| Multiple | 74 | 68.5 |
| Extrahepatic metastases | | |
| No | 92 | 85.1 |
| Yes | 16 | 14.8 |
| Neoadjuvant chemotherapy | | |
| No | 63 | 64.2 |
| Yes | 35 | 35.7 |
| Distribution of liver metastases | | |
| Bilobar | 49 | 48.0 |
| Unilobar | 53 | 51.9 |
| Postoperative complications | | |
| No | 76 | 70.3 |
| Yes | 32 | 29.6 |
| Clavien-Dindo Grade | | |
| Grade I | 2 | 6.4 |
| Grade II | 10 | 32.2 |
| Grade III | 12 | 38.7 |
| Grade IV | 5 | 16.1 |
| Grade V | 2 | 6.4 |
| Survival | | |
| Death | 48 | 44.4 |
| Alive | 60 | 55.5 |
| Perioperative mortality | | |
| No | 105 | 97.2 |
| Yes | 3 | 2.7 |

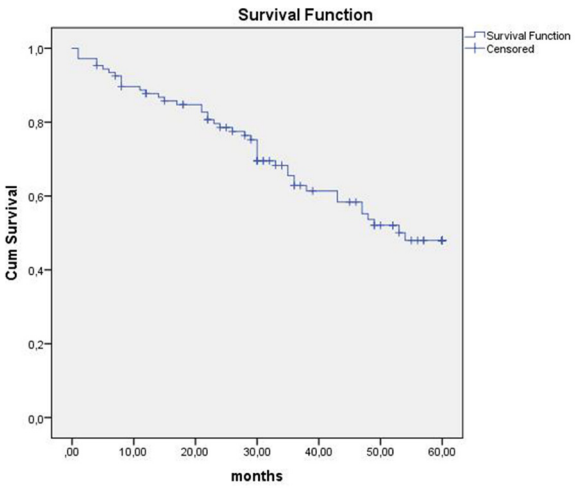


**Figure 1. Five-year overall survival of the entire cohort.** The 5-year overall survival of the entire cohort was 54%.

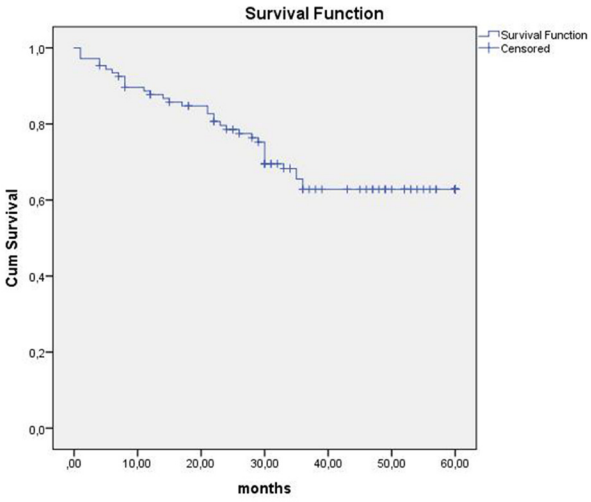


**Figure 2. Three-year overall survival of the entire sample.** The 3-year overall survival of the entire sample was 67%.

that simultaneous resection of synchronous CRLM at the time of resection of the primary colorectal lesion is both safe and as effective as staged surgery (*16,25*). However, an optimal strategy depending on the location of the primary tumor and the safety of major liver resection, especially in cases of primary rectal cancer with liver metastases, is still a matter of debate in many medical facilities.

The current study fills that gap by focusing on this issue. This study has also identified the need for studies related to the simultaneous resection of synchronous CRLM to be conducted.

Major limitations of this study were the small sample size, the differences in treatment procedures, and the retrospective design of the study.

The benefits of staged resection in terms of perioperative complications and oncological outcomes are still subject to debate when compared with simultaneous resection, but the patient must undergo two major operations, resulting in a longer hospital stay and greater hospital costs (*26*). Over the last decade, the mortality and morbidity rates of colorectal and liver surgery have decreased because of advances in surgical devices, surgical techniques, ablation techniques, anesthetic techniques, and postoperative care (*27*). Moreover, channeling these procedures to medical facilities with a high volume and to research facilities where they are performed by surgeons with advanced specialized training and a supporting institutional infrastructure has contributed to the improved safety of major hepatic resection (*28*). Consequently, synchronous resection of stage IV colorectal cancer has become widely accepted. In the current authors' institution, the treatment of choice for colorectal cancer and synchronous liver metastases is simultaneous resection, even if a low anterior resection and/or major hepatectomy is required. Simultaneous resection has demonstrated significant advantages in terms of lower postoperative complication rates, a shorter hospital stay, and a notable decrease in the global costs of treatment. This is also due to the elimination of a second surgical procedure needed to treat metastases (*17,25,29*). As noted here, 43% of patients with rectal cancer underwent simultaneous major liver resection and did not have a higher incidence of complications (or a substantial morbidity in comparison to patients with primary cancer elsewhere in the colon cancer who underwent simultaneous major liver resection).

Many studies have noted that patients who underwent a simultaneous approach had a significantly shorter total length of stay in the hospital and lower costs than those underwent a staged approach had (*17,25*). There were no statistically differences in the hospital stays of the 3 groups of patients in the current study.

There is a consensus among surgeons that liver resection should be an R0 resection as much as possible, while an R1 resection has potentially poor oncologic outcomes including a higher rate of recurrence. Numerous previous reports have found that R1 resection results in a lower disease-free survival and worse overall survival than R0 does (*8,30-32*). In the current study, R1 resection was performed at a similar rate among the 3 groups: 12.5% for patients with cancer of the right side of the colon, 12.5% for those with cancer of the left side of the colon, and 4.5% for those with rectal cancer. These rates are low in comparison to the range (5-46%) reported in other studies (*8,30,31,33-37*). Preoperative chemotherapy is widely used in the early treatment of metastatic disease to improve patient eligibility for surgical resection and to decrease the rate of recurrence after surgery (*38*). However, there is no evidence that preoperative chemotherapy significantly improves the overall survival compared to surgery alone (*39*). Preoperative chemotherapy may have the benefit of shrinking unresectable metastases and increasing the resectability of metastases that were

**Table 2. Comparison of outcomes among the study groups**

| Items | Patients with cancer of the right side of the colon, *n* = 24 (%) | Patients with cancer of the left side, *n* = 40 (%) | Patients with rectal cancer, *n* = 44 (%) | *p* |
|---|---|---|---|---|
| Age (years) | 63.21 ± 11.78 | 58.8 ± 11.75 | 63.8 ± 11.06 | 0.115 |
| Gender | | | | |
| Female | 15 (62.5) | 18 (45.0) | 17 (38.6) | 0.165 |
| Male | 9 (37.5) | 22 (55.0) | 27 (61.3) | |
| N stage | | | | |
| N0 | 6 (25.0) | 12 (30.0) | 8 (18.1) | 0.446 |
| N+ | 18 (75.0) | 28 (70.0) | 36 (81.8) | |
| Number of lymph nodes invaded by the primary tumor | | | | |
| No | 6 (25.0) | 12 (30.0) | 8 (18.1) | 0.446 |
| Yes | 18 (75.0) | 28 (70.0) | 36 (81.8) | |
| Surgical margin | | | | |
| R0 | 21 (87.5) | 35 (87.5) | 42 (95.4) | 0.375 |
| R1 | 3 (12.5) | 5 (12.5) | 2 (4.5) | |
| ASA status | | | | |
| ASA I | 4 (16.6) | 4 (10.0) | 6 (13.6) | 0.002 |
| ASA II | 8 (33.3) | 30 (75.0) | 33 (75.0) | |
| ASA III | 12 (50.0) | 6 (15.0) | 5 (11.3) | |
| Hospital stay (days) | 8.33 ± 3.07 | 8.85 ± 5.5 | 9.98 ± 6.47 | 0.759 |
| CEA (ng/mL) | 79.19 ± 137.67 | 44.69 ± 74.33 | 179.37 ± 534.37 | 0.239 |
| Number of lymph nodes invaded by the primary tumor | 4.44 ± 4.03 | 3.14 ± 3.19 | 4.69 ± 3.64 | 0.213 |
| Type of liver resection | | | | |
| Minor rejection | 17 (70.8) | 25 (62.5) | 25 (56.8) | 0.522 |
| Major resection | 7 (29.1) | 15 (37.5) | 19 (43.1) | |
| Number of liver metastases | | | | |
| Single | 12 (50.0) | 11 (27.5) | 11 (25.0) | 0.083 |
| Multiple | 12 (50.0) | 29 (72.5) | 33 (75.0) | |
| Extrahepatic metastases | | | | |
| No | 19 (79.1) | 37 (92.5) | 36 (81.8) | 0.249 |
| Yes | 5 (20.8) | 3 (7.5) | 8 (18.1) | |
| Neoadjuvant chemotherapy | | | | |
| No | 15 (75.0) | 29 (76.3) | 19 (47.5) | 0.016 |
| Yes | 5 (25.0) | 9 (23.6) | 21 (52.5) | |
| Distribution of liver metastases | | | | |
| Bilobar | 6 (28.5) | 20 (51.2) | 23 (54.7) | 0.128 |
| Unilobar | 15 (71.4) | 19 (48.7) | 19 (45.2) | |
| Postoperative complications | | | | |
| No | 20 (83.3) | 27 (67.5) | 29 (65.9) | 0.285 |
| Yes | 4 (16.6) | 13 (32.5) | 15 (34.0) | |
| Clavien-Dindo Grade | | | | |
| Grade I | 0 (0) | 0 (0) | 2 (14.2) | 0.545 |
| Grade II | 1 (25.0) | 5 (38.4) | 4 (28.5) | |
| Grade III | 2 (50.0) | 5 (38.4) | 5 (35.7) | |
| Grade IV | 0 (0) | 3 (23.0) | 2 (14.2) | |
| Grade V | 1 (25.0) | 0 (0) | 1 (7.1) | |
| Size of largest metastases (mm) | 28.71 ± 12.05 | 39.26 ± 25.46 | 38.5 ± 23.46 | 0.432 |
| Number of hepatic metastases | 2.33 ± 2.04 | 2.98 ± 1.99 | 3.86 ± 3.26 | 0.069 |
| Survival time (months) | 46.38 ± 29.9 | 30.43 ± 18.1 | 40.86 ± 25.63 | 0.072 |
| Survival | | | | |
| Death | 11 (45.8) | 19 (47.5) | 18 (40.9) | 0.822 |
| Alive | 13 (54.1) | 21 (52.5) | 26 (59.0) | |
| Perioperative mortality | | | | |
| No | 23 (95.8) | 39 (97.5) | 43 (97.7) | 0.894 |
| Yes | 1 (4.1) | 1 (2.5) | 1 (2.2) | |

originally unresectable while not affecting resectable metastases (*40*). In the current study, simultaneous resection was favored when the disease was initially resectable; nearly half of the patients with rectal cancer and a quarter of the patients with cancer of the right or left side of the colon with liver metastases received preoperative chemotherapy. The 5-year overall survival ranges from 40 to 60% with complete resection of liver disease (*10,41*) and the 5-year overall survival is 6% for patients treated non-surgically. Untreated CRLM has a poor prognosis with a median survival of 6-12 months (*42,43*). Synchronous CRLM may have less favorable cancer biology and be associated with lower expected survival compared to metachronous metastases (*44*). In the current study, the 5-year overall survival of the entire cohort was 54%, and the 5-year overall survival did not differ significantly among patients with cancer of the right side of the colon, those with cancer of the left side of the colon, and those with rectal cancer. Mortality in this current series was similar among the 3 groups, and it was also consistent with the mortality rate of 2% generally reported for rectal procedures alone (*45*) or for major liver resection alone (*46*).

Advances in modern technology and minimally invasive approaches including laparoscopic and robotic-assisted resection of colorectal cancer and synchronous

colorectal liver metastases have led to promising preliminary results by specialized and well-trained teams in selected instances. However, prospective and randomized trials are needed to define the oncological benefits and to ascertain the role of a one-stage minimally invasive approach for colorectal cancer with synchronous liver metastases (*47*). None of the current patients underwent laparoscopic or robotic surgery.

In a study using the ACS-NSQIP database, the overall rate of severe morbidity was 29% in all patients who underwent simultaneous resection (*48*). This figure is comparable to the morbidity rate (major and minor) in the current study. With simultaneous resection, morbidity was 34% for patients with rectal cancer, 32.5% for those with cancer of the left side of the colon, and 16.6% for those with cancer of the right side of the colon.

Some studies have claimed that simultaneous resection should be discouraged when the hepatectomy would be major or when complex rectal surgery is to be performed, in light of the significantly higher postoperative mortality and morbidity (*18*). However, recent studies have noted the safety of simultaneous resection of primary rectal cancer and liver metastasis (*13,49,50*). A recent study reported that synchronous treatment strategy could be considered when liver and colorectal surgeons agree on the safety of this approach (*49*). However, a well-designed prospective randomized trial of simultaneous resection of synchronous CRLM should be conducted in the future to assess the impact of the primary tumor location and type of hepatectomy.

## 4. Conclusion

Simultaneous resection of primary colorectal cancer and CRLM is a safe procedure and can be performed without excess morbidity in carefully selected patients regardless of the location of the primary tumor and type of hepatectomy.

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*(Received January 22, 2017; Revised February 1, 2017; Accepted February 2, 2017)*

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(*Revised February 2013*)

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