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Policy Forum

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Control of antibiotic resistance in China must not be delayed: The current state of resistance and policy suggestions for the government, medical facilities, and patients

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Summary **Antibiotics are medicines used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medicines. Antibiotic resistance is rising to dangerously high levels in all parts of the world, leading to higher medical costs, prolonged hospital stays, and increased mortality. In the European Union alone, drug-resistant bacteria are estimated to cause 25,000 deaths and cost more than US$1.5 billion every year in healthcare expenses and productivity losses. The problems of antibiotic misuse and antibiotic resistance are quite serious in China. In 2015, results of a study by the State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences indicated that the total antibiotic usage in China in 2013 was approximately 162,000 tons, including human use (48%) and use in animals (52%). This amount accounted for about half of the antibiotic usage worldwide. The per-capita use of antibiotics in China is more than 5 times that in Europe and the United States. These data mean that China is one of the world's leading countries with serious problems in terms of antibiotic misuse and antibiotic resistance. The current article analyzes the current state and harms of antibiotic misuse and causes of antibiotic resistance in China. The Government needs to pay close attention to the issue of antibiotic resistance in China and formulate a strategy at the national level. Thus, the following suggestions are offered: *i*) The Chinese Government should implement policies that promote antibiotic research and development; *ii*) Medical facilities in China should create multidisciplinary teams (MDTs) and encourage early action by MDTs to control the spread of multi-drug-resistant bacteria (MDRB); *iii*) An intervention in the form of health education should target patients and accompanying family members (AFM) in China. In other words, antibiotic resistance is not a personal problem but an urgent public health problem. Without urgent action, China is heading for a post-antibiotic era in which common infections and minor injuries can once again kill. Therefore, the aforementioned proposals have been offered with the hope that policy suggestions help to limit the phenomenon of antibiotic misuse and antibiotic resistance in China.**

Keywords: Antibiotic resistance, antibiotic misuse, China

1. Introduction

The early 20th century was a time of ground-breaking scientific progress. One major advance was the development of penicillin and other antibiotics that have prevented thousands and even millions of people from dying of bacterial infections. The ability to treat diseases played a key part in increasing life expectancy and improving human health, but diminished ability to combat infections may lead to an acceleration of antimicrobial resistance the 21st century (*1*).

Antibiotics are medicines that are used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use

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of these medicines (*2*). Antimicrobial resistance has been detected in all parts of the world and is one of the greatest challenges to global public health today. Although antimicrobial resistance is a natural phenomenon, it is also being caused by misuse of antimicrobials, inadequate or inexistent programs for infection prevention and control (IPC), poor-quality medicines, limited laboratory capacity, inadequate surveillance, and insufficient regulation of the use of antimicrobials (*3*).

Antibiotic resistance is rising to dangerously high levels in all parts of the world. New mechanisms of resistance emerge and spread globally every day, threatening our ability to treat common infectious diseases. A growing list of infections – such as pneumonia, tuberculosis, blood poisoning, and gonorrhea – are becoming harder, and sometimes impossible, to treat as antibiotics become less effective (*4*). In addition, antibiotic resistance leads to higher medical costs, prolonged hospital stays, and increased mortality. In the European Union alone, drug-resistant bacteria are estimated to cause 25,000 deaths and cost more than US$1.5 billion every year in healthcare expenses and productivity losses (*5*).

The problems of antibiotic misuse and antibiotic resistance are quite serious in China. Yang Zhiyin, the chairman of the behavioral medicine branch of the Chinese Medical Association, noted that about 200,000 people died due to adverse drug reactions, 40% of which were due to antibiotic misuse. In addition, antibiotic use per capita is about 138 grams in China but only 13 grams in the US. These data mean that China is one of the world's leading countries with a serious problem of antibiotic misuse (*6*). Without urgent action, China is heading for a post-antibiotic era in which common infections and minor injuries can once again kill. This article describes the current state of antibiotic resistance in China and it describes the international experience of the World Health Organization (WHO) in order to put forward policy suggestions for the Chinese Government, medical facilities, and patients and their family members.

2. Antibiotic resistance in China

The total use of antibiotics in China tends to be "unknown" because there is no authoritative source of data at the national level. In 2015, however, the results of a study by the State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences indicated that the total antibiotic usage in China in 2013 was approximately 162,000 tons, including human use (48%) and use in animals (52%). This amount accounts for about half of the antibiotic usage worldwide. The per-capita usage of antibiotics in China is more than 5 times that in Europe and the United States (*7*). Thus, the Government needs to pay close attention to the issue of antibiotic resistance in China and formulate a strategy at the national level.

2.1. *Current state of antibiotic misuse in China*

2.1.1. *Irrational use in clinical settings*

Because of information asymmetry in medicine, physicians tend to be the indirect selectors and purchasers and of drugs, largely influencing the rational use of antibiotics through their prescribing behavior. However, irrational use of antibiotics in clinic settings is a major factor for antibiotic misuse in China. This phenomenon is apparent in the following three aspects.

i) *Incorrect drug selection*. Physicians cannot prescribe antibiotics "where indicated" since most physicians prescribe based on their own experiences, and a study has reported that pathogens are detected in inpatients at a rate of 16.5% (*8*). In addition, the commercialization of medicine has led some physicians to use expensive drugs out of self-interest. Some physicians tend to prescribe new or imported drugs to avoid complications, but these drugs may have difficulty producing the desired effect and they can more easily produce antibiotic resistance. In addition, a study found that 93.7% of all infusions given to outpatients were given to children and that 97.3% of all infusions given to children included antibiotics (*9*).

ii) *Incorrect drug dosing*. There are many phenomena and problems with incorrect dosage when antibiotics are used in clinical practice, such as irrational dosing, courses, and drug combinations. Data from the World Health Organization (WHO) indicated that antibiotic utilization was as high as 80% in inpatients and 95% in surgical departments, which far exceeded the international standards of 30% and 22-25% in the West (*10*). In addition, a retrospective study of antibiotic use in 1,688 patients indicated that antibiotic utilization was 74.76% (*11*). However, an antibiotic susceptibility test was performed for 6.09% of those patients, 38.04% of patients lacked definite symptoms or had mild symptoms, 31.31% of patients received more than two antibiotics, 9.09% of patients received antibiotics for a prolonged period, 8.08% of patients frequently received different antibiotics, and 7.40% of patients received an improper combination of antibiotics.

iii) *Poor monitoring of prophylaxis*. At present, antibiotics are too often used for perioperative prophylaxis in different departments of medical facilities. For insurance purposes, antibiotics have become an essential medicine in surgery. A study of 1,235 patients in a hospital in Guangdong found that the rate of antimicrobial use was 100% in Surgery, Gynecology, and Ophthalmology (*12*).

2.1.2. *Irrational antibiotic use by patients*

Patients are not merely a victim of antibiotic misuse

but are also responsible for the acceleration of antibiotic resistance. A WHO multi-country survey revealed widespread public misunderstanding about antibiotic resistance (*13*). One thousand and two online interviews of Chinese yielded the following findings: *i*) 57% of respondents reported taking antibiotics within the past 6 months; 74% say they were prescribed or provided the antibiotic by a doctor or nurse; 5% say they purchased them on the Internet, *ii*) More than half (53%) of respondents wrongly believed that they should stop taking antibiotics when they felt better, rather than taking the full course as directed, *iii*) 61% of respondents thought, incorrectly, that colds and flu can be treated by antibiotics, *iv*) Two-thirds (67%) of respondents were familiar with the term "antibiotic resistance" and three quarters (75%) said it is "one of the biggest problems in the world," and *v*) 83% of respondents said that farmers should give fewer antibiotics to animals – the highest proportion of any country in the survey.

2.1.3. *Irrational antibiotic use in animal husbandry*

Annually, 97,000 tons of antibiotics are used in animal husbandry in China, accounting for 46.1% of all antibiotics used per year. Thirty percent of these antibiotics were used as feed supplements and 10% were used to treat diseases in livestock. A study has revealed that antibiotic metabolites are found in 50% of the animal foods and dairy products in Chinese supermarkets, including prohibited drugs such as chloramphenicol, tetracyclines, sulfonamides, and nitrofurans (*14*). In addition, a study by the East China University of Science and Technology in 2014 indicated that there are 68 antibiotics in surface water in China (*15*).

2.2. *Harms of antibiotic misuse in China*

2.2.1. *The creation of multi-drug resistant bacteria (MDRB)*

Antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi, and parasites change in ways that render the medications used to cure the infections they cause ineffective. When the microorganisms become resistant to most antimicrobials, they are often referred to as "superbugs." This is a major concern because a resistant infection may kill, it can spread to others, and it imposes huge costs on individuals and society (*16*).

The 2015 *Report on Monitoring of Bacterial Drug Resistance* in China indicated that a total of 2,400,786 strains of bacteria were detected from October 2014 to September 2105 (*17*). These strains included 695,066 strains of Gram-positive bacteria (28.9%) and 1,705,720 strains of Gram-negative bacteria (71.1%). The top ten bacteria that were detected are listed in Figure 1.

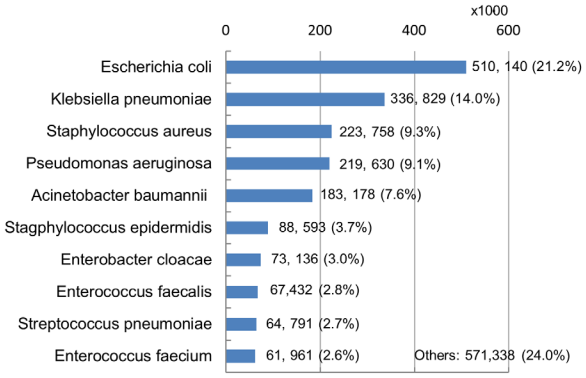

Figure 1. Bacterial drug resistance monitoring repott of China in 2015.

Antimicrobial resistance is the broader term for the resistance of different types of microorganisms to antibacterials, antivirals, antiparasitics, and antifungals. Antimicrobial resistance has become a leading public health problem. Ciprofloxacin was efficacious and caused few adverse reactions 20 years ago, but it is now inefficacious in more than 60% of patients (*18*). In addition, a study from UK has found that failure to find ways to cope with multi-drug resistant bacteria (MDRB) will result in the deaths of 10 million people and loss of US$ 100 trillion worldwide. Data have revealed that 80,000 people have died due to antimicrobial resistance in China (*19*). If this situation persists, it will lead to the death of 1 million people in China in 2050.

2.2.2. *Causing nosocomial infection (NI)*

A nosocomial infection (NI) is an "infection that is acquired in the hospital and becomes evident after hospital discharge" and that "was not present neither was incubating at the moment of patient admission at a hospital" (*20,21*). Several studies have investigated the mortality rate in patients developing a postoperative infection in General Surgery, and they noted a mortality rate of 7.5% in patients with a single-pathogen nosocomial infection and a mortality rate of 17.1% in patients with a multiple-pathogen mixed infection (*22*). The occurrence of an NI is a serious threat to the patient's health and also increases the burden of medical care on the patient's family (*23,24*).

2.2.3. *Difficulties of innovative drug research and development*

Although new antibiotics have emerged, antibiotic development lags behind the pace at which bacteria develop drug resistance; development of a new antibiotic takes 10 years while bacteria develop resistance in only 2 years (*25*). Since the 1980s, no new antibiotics have been discovered or synthesized except daptomycin. According to the WHO, there are some new antibiotics

Table 1. Steps at all levels of society to reduce the spread of drug resistance according to the WHO

Positions	Actions
General public	• Preventing infections by regularly washing hands, practicing good food hygiene, avoiding close contact with sick people and keeping vaccinations up to date. • Only using antibiotics when prescribed by a certified health professional. • Always taking the full prescription. • Never using left-over antibiotics. • Never sharing antibiotics with others.
Health workers and pharmacists	• Preventing infections by ensuring hands, instruments and environment are clean. • Keeping patients' vaccinations up to date. • When a bacterial infection is suspected, perform bacterial cultures and testing to confirm. • Only prescribing and dispensing antibiotics when they are truly needed. • Prescribing and dispensing the right antibiotic at the right dose for the right duration.
Policymakers	• Having a robust national action plan to tackle antibiotic resistance. • Improving surveillance of antibiotic-resistant infections. • Strengthening infection prevention and control measures. • Regulating and promoting the appropriate use of quality medicines. • Making information on the impact of antibiotic resistance available. • Rewarding the development of new treatment options, vaccines and diagnostics.
The agricultural sector	• Ensure that antibiotics given to animals - including food-producing and companion animals - are only used to treat infectious diseases and under veterinary supervision. • Vaccinate animals to reduce the need for antibiotics and develop alternatives to the use of antibiotics in plants. • Promote and apply good practices at all steps of production and processing of foods from animal and plant sources. • Adopt sustainable systems with improved hygiene, biosecurity and stress-free handling of animals. • Implement international standards for the responsible use of antibiotics, set out by OIE, FAO and WHO.
The healthcare industry	• Investing in new antibiotics, vaccines, and diagnostics.

in development, but none of these are expected to be effective against the most dangerous forms of antibiotic-resistant bacteria.

2.3. *Reasons for antibiotic misuse and antibiotic resistance in China*

2.3.1. *Inadequate government oversight and a lack of policy incentives for new drug research and development*

Although the Ministry of Health of China has issued standards and regulations stipulating limited use of antibiotics in medical facilities, the Government failed to control the sales of antibiotics, causing a "market boom" in antibiotics. Despite the serious state of research into new antibiotics, the Government has not legislated clear and definite incentives for new antibiotic research and development.

2.3.2. *Improper dosing by physicians*

Improper dosing by physicians in China is mainly due to: *i*) physicians relying on their own experiences, *ii*) a low level of expertise; *iii*) the need to protect at-risk patients, and *iv*) a dearth of clinical pharmacists. A study of drug selection by physicians indicated that 35% of physicians selected a drug based on their own experiences and 80% selected a drug without being sure of its type or whether its use was sanctioned by the government (*26*).

2.3.3. *Patient factors*

Many patients tend to misuse antibiotics by taking them on their own and failing to comply with instructions because of their lack of medical knowledge and misconceptions. A study found that 23% patients took medication on their own over the past year and 25.1% failed to follow a doctor's advice.

3. Policy suggestions for the Chinese Government

3.1. *International experience of the WHO*

In countries where antibiotics can be bought without a prescription, the emergence and spread of resistance is worse according to the WHO. Similarly, antibiotics are often over-prescribed by health workers and over-used by the public in countries without standard treatment guidelines.

Given the ease and frequency with which people now travel, antibiotic resistance is a global problem, requiring efforts from all nations. Antibiotic resistance is accelerated by the misuse and overuse of antibiotics, as well as poor infection prevention and control. Steps can be taken at all levels of society to reduce the impact and limit the spread of resistance (Table 1).

3.2. *Government: Policy incentives for new drug development*

The Government of China should adopt policy incentives to promote antibiotic research and development. A model could be the *Generating Antibiotic Incentives Now Act* (GAIN Act). In the US, the Senate examined and adopted the *Food and Drug Administration (FDA) Safety and Innovation Act*, which featured the GAIN Act to encourage pharmaceutical companies and biotechnology firms to research and development innovative antibiotics. The Act provides that qualified infectious disease products (QIDP) can qualify for incentives, which include *i*) fast-tracking in the research stage, *ii*) priority review in the approval stage, and *iii*) patent exclusivity for an additional five years post-marketing. In addition, the GAIN Act requires the FDA to release at least 3 guidelines on clinical research into antibacterials every year and timely updates. Using these incentives, the FDA has awarded 28 QIDP qualifications, and 4 have (Dalvanc, Sivextro, Orbactiv, and Zerbaxa) have been approved by FDA and are now publicly available (*27*).

In addition to the GAIN Act, the FDA relaxed the requirements for clinical trials of antibiotics but specified a narrow range in order to prevent their misuse. In addition, the US has also established a plan for antibiotic research and development, which means that the Government provides financing for companies and it established a fund for loans to small and medium-sized enterprises (*28*).

In the value chain of drug research and development, scientific, funding, and review obstacles can all influence innovation by pharmaceutical companies and research institutes, while policy incentives can mitigate the effects of those obstacles to some extent. Therefore, the Chinese Government should implement policies that promote antibiotic research and development.

3.3. *Medical facilities: A multidisciplinary team (MDT) model*

An MDT is a group composed of members from different healthcare professions with varied but complimentary experience, qualifications, and skills that contribute to the achievement of an organization's specific objectives. An MDT approach is usually used in cancer care and has been widely accepted throughout much of Europe and the US (*29*). An MDT improves coordination and communication among and decision-making by team members and could help to reduce NIs caused by MDRB.

A growing number of hospitals in China have created MDTs for diagnosis and management of care. However, most of these teams provide specific consultations, so they have difficulty serving all departments at the same time. If a patient has a disease that is treated by a different department, a transfer would cause increased bureaucracy and a financial burden for the patient. Hence, medical facilities in China should create MDTs and encourage early action by MDTs to control the spread of MDRB (*30*).

3.4. *Patients and accompanying family members (AFM): Intervention in the form of health education*

Due to insufficient nursing personnel, the ratio of doctors to nurses of China was 1:1.04 in 2014, which is far below the standard of developed countries (*31*). For the most part, nurses provide treatment, basic nursing, and perform administrative work in China, so they have difficulty meeting the emotional and livelihood needs of patients. The presence of family members accompanying patients (accompanying family members, or AFM) tends to be a phenomenon specific to China, and an intervention in the form of health education is a key factor for MDRB control in China (*32*).

Health education for inpatients and AFM should: *i*) Instruct patients in the correct way to cough, *ii*) encourage patients to become mobile as early as possible, *iii*) instruct the patient's family on turning the patient over and the correct way to tap the patient on the back, *iv*) instruct the patient in assisting with elimination, and *v*) encourage the patient to drink more water to replenish bodily fluids and prevent dry phlegm.

Given the current state of AFM in China, the following points should be observed. *i*) Rules and regulations should be devised to govern AFM, *ii*) a unified management system should be created for professional caregivers, and *iii*) one option for AFM could be to supplement the care provided by professional caregivers.

4. Conclusion

In summary, this article has described the accelerated development of antimicrobial resistance around the world. The problems of antibiotic misuse and antibiotic resistance are quite serious in China. The current state of resistance and its harms and the causes of antibiotic misuse in China have been described in detail. Policy suggestions for the Chinese Government, medical facilities, and patients and their family members have been offered in the hopes that these efforts will limit the phenomenon of antibiotic misuse and antibiotic resistance in China.

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Review

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Atypical pathogen infection in community-acquired pneumonia

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Summary **Community-acquired pneumonia (CAP) is a world wide cause of morbidity and mortality. The etiology of CAP is different between countries and changes over time. With the increasing incidence, atypical pathogens are attracting more and more attention all over the world. In many countries, atypical pathogens are one of the main pathogens of CAP, and even could be the most prevalent etiology in China. Atypical pathogen infections can cause multi-system complications, which leads to a worse prognosis. Although still controversial, empirical antibiotic coverage of atypical pathogens in CAP may improve outcomes, shorten length of hospitalization, reduce mortality and lower total hospitalization costs. The macrolide resistance rate of atypical pathogens, especially *Mycoplasma Pneumoniae* (*M. Pneumoniae*) is high, so fluoroquinolones or tetracyclines should be considered as alternative therapy.**

Keywords: Atypical pathogen, community-acquired pneumonia (CAP), macrolide-resistant, empirical atypical coverage

1. Introduction

Community-acquired pneumonia (CAP) is one of the common diseases that pose a threat to human health. A few CAP inpatients develop severe community-acquired pneumonia (SCAP) and require intensive care unit (ICU) treatment. Due to frequent complications and a long hospitalization period, mortality among these patients is high (*1-3*). More than 2 million children under age 5 are killed by pneumonia every year world wide, more than AIDS, malaria, and measles combined (*4*). According to statistics based on a survey conducted by 122 research centers from 35 countries with 4300 patients, the incidence of pneumonia caused by atypical pathogens is high, with a detectable rate over 20% (*5*). In recent years, faced with aging society, increasing damaging factors to the immune system, changing nature of pathogens and rising antibiotic resistance, the treatment of CAP now encounters many new problems. Some scholars believe that atypical respiratory pathogens like the *Mycoplasma Pneumoniae* (*M. Pneumoniae)* and *Chlamydophila pneumoniae* (*C. Pneumoniae*) will replace *Streptococcus pneumoniae* as the most common pathogens for CAP (*6*).

Despite the absence of the earliest documentation of atypical pneumonia, the disease gradually became known in the 1920s and 1930s *via* various reports and papers at the time (*7-9*). The term atypical pneumonia can be interpreted in a sense that the pneumonia is caused by atypical pathogens or the patients present atypical clinical symptoms. Using a broader definition, atypical pathogens include all pathogens other than typical bacteria, *e.g.*, *Mycoplasma*, *Chlamydophila*, *Legionella*, *Rickett's organism*, *Coxiella*, *Bacillus tularense*, *Leptospira*, fungi, and various viruses (*10*). In a narrower sense, atypical pathogens causing pneumonia mainly include *M. Pneumoniae*, *C. Pneumoniae*, and *Legionella Pneumophila* (*L. Pneumophila*). Sometimes, *Rickettsia* and *Chlamydia psittaci* are also considered as atypical pathogens.

2. Clinical diagnosis of CAP

CAP due to *M. Pneumoniae* and *C. Pneumoniae* are usually seen in younger patients without comorbidity and has a mild clinical course. (*11,12*), while most pneumonia patients due to *L. Pneumophila* need to be treated in the ICU (*13,14*). The clinical symptoms of atypical pathogen CAP can be misleading, for the

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patients might have atypical symptoms like muscle pain, weakness, dry cough and so on (*15*).

CAP caused by *M. Pneumoniae* and *C. Pneumoniae* have similar clinical symptoms: generally no distinctive characteristics of normal bacteria infection; highly concentrated in the family; coughing lasting for over 5 days without sputum and no acute deterioration; normal or slight elevation of WBC; and procalcitonin level, ≤ 0.1 μg per liter. *L. Pneumophila* pneumonia has similar clinical symptoms compared to common bacterial pneumonia: super acute cause accompanied with septic shock, and lack of upper respiratory symptoms. It can also present acute deterioration of initial upper respiratory illnesses, which reminds clinicians of co-infection of virus and bacteria; white-cell count, > 15,000 or ≤ 6,000 cells per cubic millimeter; dense segmental or lobar consolidation, and procalcitonin level, ≥ 0.25 μg per liter (*16*).

L. Pneumophila pneumonia usually presents extrapulmonary symptoms: neurological symptoms like headache, drowsiness, disordered consciousness; cardiovascular abnormalities like relative infrequent pulse; gastrointestinal symptoms like nausea, vomiting, abdominal pain and liver dysfunction in the early phase like transient slightly increased aminotransferase; kidney damage like microscopic hematuria, moderate increase in creatinine; damage in the lung can be rales, pleural effussion, but the chest X-rays lack specificity.

Instead of consolidation in the lung, CAP caused by *M. Pneumoniae* can be mainly small airway infection, causing pulmonary interstitial change, which is hardly detectable in X-rays and presents as "tree-in-bud" in chest CT (*17*). In the high resolution chest CT, we may see lobule centricity nodules, bronchial wall thickening, lobular or period of distribution of ground glass and consolidation shadows, inclined to one side or both sides patchy distribution, also can be diffuse distribution. Chest CT of *C. Pneumoniae* pneumonia mainly presents as consolidation shadow, ground glass shadow, and patchy fuzzy shadow, which is consistent with the scope of bronchitis. It can also present centrilobular nodules, and "tree-in-bud" mixed with ground glass shadow and consolidation shadow, but rarely as the main observation.

Several diagnostic methods detect atypical pathogens, including: isolation, complement fixation, serologic testing, and molecular-based detection assays (*18,19*). Each of these methods has limitations. Isolation is considered to be the "gold standard", but it is tedious and time consuming, requires expertise, and yields inconsistent results. Antigen detection and serological tests are the most commonly applied technologies but have inadequate sensitivity and specificity. The sensitivity is only 31.8% with single IgM antibody testing to diagnose *M. Pneumoniae* pneumonia. When diagnosing *C. Pneumoniae* pneumonia, the sensitivity of adult IgA or IgG antibody tests is 78%, with specificity of 21-91% (*20*). Because of the delay in antibody generation, serological testing is not qualified for early diagnosis of the disease but is of great significance for epidemiology studies. Urinary antigen detection is recommended for the early diagnosis of *L. Pneumophila* pneumonia, but with the limitation of only detecting serotype 1. The molecular detection technology on the other hand could offer high sensitivity and specificity with fast speeds and high volumes, making it a promising alternative. Morozumi, *et al*. (*21*), using real-time PCR assays, determined 429 clinical specimens, and the sensitivities and specificities of *M. Pneumoniae* wee 100% and 95.4% respectively, compared with the results of conventional culture tests. The whole process from DNA extraction to analysis was finished in less than 2 hours, the limit of detection was 5 copies for *M. Pneumoniae*, 3 copies for *C. Pneumoniae*, and 2 copies for *L. Pneumophila*. So this can give great help to clinicians for rapid identification of the loads of atypical pathogens. In terms of *C. Pneumoniae*, standard procedures for testing, specimens and treatment are still missing and the impact on testing results is yet to be seen. Meanwhile, the PCR approach is overly complicated and very demanding for personnel and equipment, and therefore is not generally applied in labs.

3. Prevalence of atypical pneumonia

In Table 1, according to CAPO that is based on 4,337 patients: the atypical pathogen detectable rates in North America, Europe, Latin America and Asia/Africa are 22%, 28%, 21% and 20% respectively (*22*). However, different countries and regions have different atypical detectable rates. A CAP epidemic survey (*23*) that enrolled 3,523 CAP patients (15% outpatients and 85% inpatients) from November 1996 to July 2008 shows that 1,463 patients are etiology positive. The survey indicates that *Streptococcus pneumoniae* is the main cause of CAP in Europe with 42% of the detectable rate. Atypical pathogens and mixed infections are also significant causes with detectable rates standing at 18% and 14% respectively. Also in Spain, Alberto Capelastegui and his colleagues discovered a 50% detectable rate in a prospective study (*24*). Atypical pathogens were significantly more frequent among outpatients (67%), than among inpatients(30.6%). A study in Chile that included 356 patients showed that *Streptococcus pneumoniae* and viruses are the most common pathogens, with atypical pathogens accounting for 22% of the infections (*25*). Two studies in Netherlands found that *Streptococcus pneumoniae* was the main cause of CAP, with 25% and 22% of detectable rates. But there were inconsistent detectable rates between the two studies in terms of atypical pathogens (9% and 20%) (*26,27*). Whereas a study in the north of Israel shows the detectable rate of

Table 1. Studies of the prevalence of atypical pneumonia in different countries and regions

Authors	Country	Population	Main findings
Arnold FW, *et al*. (*22*)	21 countries (region: North America, Europe, Latin America, Asia/Africa.)	4,337 patients, from 21 countries, Sep. 1996 – Apr. 2004.	The incidence of CAP due to atypical pathogens was 22, 28, 21, and 20% in North America, Europe, Latin America, Asia/Africa, respectively.
Cillóniz C, *et al*. (*23*)	Spain	3,523 patients attending the Hospital Clinic, Nov. 1996 – Jul. 2008.	The most frequent aetiology among outpatients was the atypical pathogen group (36%), and in patients treated on the ward atypical pathogen took up 16%.
Capelastegui A, *et al*. (*24*)	Spain	700 patients recruited from Galdakao Hospital, Apr. 2006 – Jun. 2007	Atypical pathogens were significantly more frequent among outpatients (67%), while 30.6% among inpatients.
Luchsinger V, *et al*. (*25*)	Chile	356 patients in two hospitals, Feb. 2005 – Dec. 2007.	Streptococcus pneumoniae and RSV were the most common aetiology, while The incidence of CAP due to atypical pathogens was about 22%.
Spoorenberg S. *et al*. (*26*)	The Netherlands	505 patients admitted to the St. Antonius Hospital or the Gelderse Vallei Hospital, 2004 – 2010.	The incidence of CAP due to atypical pathogens was about 9% among inpatients.
Gageldonk-Lafeber ABV, *et al*. (*27*)	The Netherlands	339 patients from the Jeroen Bosch Hospital (JBH), Nov. 2007 – Jan. 2010.	Infection with atypical acteria was detected in 69 (20%) of the patients.
Fahmi S, *et al*. (*28*)	Israel	126 patients and 24 controls, conducted at HaEmek Medical Center, Afula, Nov. 2006 – Aug. 2007.	Atypical bacteria was found in 66 (52.4%), and co-infection was very frequent.
Liu YN, *et al*. (*29*)	China	665 adult patients at 12centers in 7 Chinese cities, Dec. 2003 – Nov.2004.	*M. Pneumoniae* was the most prevalent aetiology (126/610, 20.7%). Atypical pathogens were identified in 62/195 (31.8%) patients carrying bacterial pathogens.
Tao LL, *et al*. (*30*)	China	593 patients at 36 centers in 22 cities of 16 provinces, Jun. 2004 – Aug. 2005.	*M. Pneumoniae* was the most prevalent aetiology (38.9%) , and the incidence of CAP due to C. Pneumoniae and L. Pneumophila was 11.4% and 4.0%, respectively.
Chen K, *et al*. (*31*)	China	1,204 children patients, from Zhongda Hospital, Nanjing, Aug. 2011 – Aug. 2013.	*M. Pneumoniae* was the most predominant pathogen(40.78%), and the incidence of CAP due to *C. Pneumoniae* and *L. Pneumophila* was 0.91% and 0.33%, respectively.
Diego V, *et al*. (*32*)	Spain	3,934 non-immunosuppressed hospitalized patients of CAP admitted toHospital Universitari de Bellvitge, Feb. 1995 – Dec. 20 10.	214 (5.4%) had *L. Pneumophila* pneumonia.
Francisco A, *et al*. (*33*)	Spain	104 adult patients with severe CAP in four hospitals, Jan. 2005 – Jun. 2006.	An etiologic agent was identified in 62 patients (59.6%), with the second frequent being L. Pneumophila (8.6%), followed by *M. Pneumoniae* (6%), *C. Pneumoniae* (4%).

atypical pathogens is 52.4% (*C. Pneumoniae* 20.6%, *M. Pneumoniae* 18.3%, L pneumoniae 7.1% and others) (*28*). A large epidemiological survey from China in 2006 showed different results compared to that of the European countries, with atypical pathogens being the leading cause of CAP in China. *M. Pneumoniae* was the most prevalent etiology (20.7%), followed by *Streptococcus pneumoniae* (10.3%) (*29*). Co-infections took a great part of community respiratory infections, most of which was co-infection with bacteria and atypical pathogens. In another 2 national CAP surveys in China (*30*), *M. pneumoniae* infection had become the most common cause of CAP among adults, with rates of 20.7% and 38.9% respectively, far exceeding the rates of *Streptococcus pneumoniae* (10.3% and 14.8%). Keping Chen, *et al*. (*31*) reported that the most predominant pathogen was *M. Pneumoniae*, with a positive percentage of 40.78% and *M. Pneumoniae* was significantly associated with seasons, and was most common in the late summer and autumn.

L. Pneumophila is a relatively frequent causative pathogen among hospitalized patients with CAP and is associated with high mortality. A 15-year study (*32*) showed that among 3,934 non-immunosuppressed hospitalized patients with CAP, 214 (5.4%) had *L. Pneumophila* pneumonia, and 38 (17.8%) patients required ICU admission, and the inhospital case-fatality rate was 6.1% (13 of 214patients). In a clinical study from Santiago, Chile, a total of 104 patients with severe CAP were observed from 2005 to 2006. All the patients required ICU admission, of whom an etiologic agent was identified in 62 patients (59.6%), top 7 were as follows: *Streptococcus pneumoniae* (26%), *L. Pneumophila* (8.6%), *M. Pneumoniae* (6%), *C. Pneumoniae* (4%), Gram-negative bacillus (3%), influenza A virus (3%), and *Staphylococcus aureus* (3%). *L. Pneumophila* is the second etiologic agent in SCAP, after *Streptococcus pneumoniae*. Global mortality at 28 days in severe CAP was 25% and that of *L. Pneumophila* was 33.3% (three of nine cases), but the difference was not significant with non-Legionella severe CAP mortality (33% *vs* 24.5%) (*33*). There is a relatively high incidence of *L. Pneumophila* in global CAP, particularly in the United States (14%) (*12*) and Spain (12.5%) (*34*). Even in Asia, the incidence is as high as 6.6% (*32*).

4. The prognosis of patients with pneumonia due to atypical pathogen infection

As said before, pneumonia due to atypical pathogen infection is often mild or moderate, but when it turns into severe pneumonia, the outcome is usually fatal. A retrospective study showed that, acute respiratory distress syndrome (ARDS) developed in 6 of 11 pneumonia patients due to *C. Pneumoniae* infection, the mortality in the group of APACHE II ≥ 12 was 83%, and 100% in the group of CURB-65 ≥ 2 (*35*). Multi-lobar involvement, should be identified earlier. A study (*36*), conducted in Europe with a group of average age 66-year-old patients with pneumonia, showed that elderly patients with *L. Pneumophila* infection had a worse prognosis. The study reported that the general mortality was as high as 23%. Of those who died, five (83%) had UK community-acquired *L. Pneumophila*.

4.1. *Atypical pathogen infection can cause extrapulmonary complications, which leads to a worse prognosis*

Atypical pathogen infection can cause extrapulmonary complications, such as damage to heart, liver, kidney, blood system and mucous membrane. Sometimes, the infection appears to cause more severe disease with multisystem dysfunction. In the respiratory system, the complications can be exacerbation of chronic obstructive pulmonary disease (COPD), inducing bronchial asthma, developing to ARDS, increasing the risk of lung cancer etc. In the main causes of acute exacerbation of COPD (AECOPD), atypical pathogens account for 5-10%, mainly *M. Pneumoniae* and *C. Pneumoniae*, followed by *L. Pneumophila*. As many as 14% of patients with AECOPD are associated with *M. Pneumoniae* infection, and 5.0-8.9% with *C. Pneumoniae* infection (*37*). Infection with *C. Pneumoniae* may interact with allergic inflammation to increase asthma symptoms (*38,39*). *L. Pneumophila* pneumonia is more likely to develop to ARDS, compared to other pathogens (*33*). Although still controversial, *C. Pneumoniae* infection may be associated with lung cancer, and *C. Pneumoniae* infection may be a potential risk factor for lung cancer (*40-43*). Complications in the cardiovascular system can be as follows: inducing coronary artery disease, myocardial infarction, unstable angina, atherosclerosis and cerebral infarction. A study from China found that compared with healthy persons, the *C. Pneumoniae* infections in CAD patients were detected more, with a positive rate of 81.3% (104/128) to 46.3% (37/80), and the incidence rate of myocardial infarction or more than double vessel lesions was significantly higher in the *C. Pneumoniae* infection group (*44*). Another study showed that there was a positive correlation between azithromycin treatment and secondary prevention of CAD (*45*). A meta-analysis (*46*) indicated that *C. Pneumoniae* infection was significantly associated with an increased risk of cerebral infarction. There are other extrapulmonary complications, such as hepatic function insufficiency, and septic shock. Huong Ple T *et al*. (*47*) found that severe-atypical CAP presented at a significant rate in Vietnamese children (45.12%). The factors significantly associated with severe-atypical CAP were age, co-infection with typical bacteria, co-infection with respiratory viruses, respiratory/cardiac system malformation and neonatal pneumonia.

4.2. *Increasing resistance is an important factor for prognosis*

The wide application of antibiotics promoted atypical pathogens to change in form, structure, and metabolism, which increases the difficulty of antibiotic treatment. In Japan, the macrolide resistance rate of *M. Pneumoniae* increased every year among children, and the resistance rate was as high as 30.6% (37/121) in 2006 (*48*). Also the macrolide resistance rates were 3.0% in Germany (*49*), 9.8%in France (*50*). A report from China in 2010 indicated that the resistance rate of 67 *M. Pneumoniae* isolates from 356 ambulatory adult and adolescent patients with respiratory tract infection was 69% (46 of 67) (*51*). All 46 macrolide-resistant strains harbored point mutations in the 23S ribosomal RNA gene. In addition, it was also found that mutations in L4 and L22 were not responsible for macrolide resistance. Patients infected with macrolide-

resistant *M. Pneumoniae* required a significantly longer duration of antibiotic therapy and had a longer time of resolution of fever. Moxifloxacin or levofloxacin was the most common alternative therapy. 2013, Principi, *et al*. (*52*) reported that, in comparison with patients with susceptible strains treated with macrolide, most subjects with macrolide-resistant *M. Pneumoniae* have more persistent signs and symptoms that, in some cases, have led the attending physician to replace the macrolide with tetracycline or fluoroquinolone in order to obtain a more rapid clinical result. Another study showed that, the incidence of extrapulmonary complications in the macrolide-resistant (MR) group was significantly higher than that in the macrolide-sensitive (MS) group, such as liver function abnormalities, myocarditis, rash, encephalitis and so on. Moreover, the radiological findings were more serious in the MR group than in the MS group (*53*).

Thus, the interaction of drug resistance and complications, led to serious clinical symptoms, long durations, and worse prognosis.

5. Antibiotic treatment for atypical pneumonia

For the empirical treatment of CAP, it's recommended to consider the coverage of atypical pathogen with different guidelines (*54-57*). But, there are controversial results for atypical pathogen coverage treatment. A meta-analysis indicated that empirical antibiotic coverage of atypical pathogens in hospitalized patients with community-acquired pneumonia showed no benefit of survival or clinical efficacy in this synthesis of randomized trials (*58*). In contrast, a population-based, multicenter, retrospective cohort study in China got opposite results (*59*). The study was conducted from June 2010 to May 2011, and 827 CAP patients were enrolled. It indicated that the all-causes mortality was much lower in the atypical pathogen coverage (APC) group than in the non-APC group (0.9% *vs*. 4.9%, respectively). And clinical improvement at 72 h (87.7% *vs*. 85.0%) and the clinical cure rate (91.1% *vs*. 88.3%) were more favorable in the APC group, but with no significant difference. Moreover, the APC group had a shorter mean length of stay (10.2 days *vs*. 11.6 days). In addition, the mean total hospitalization costs for the APC group were markedly lower (US$ 1,172.7 *vs*. US$ 1,510.7).

In China, there is a significantly higher macrolide resistant rate for *M. Pneumoniae*, 71.4% for erythromycin and 60.4% for azithromycin, respectively, and no fluoroquinolone-resistant or tetracycline-resistant strains were observed (*60*). Compared with macrolide, patients of *L. Pneumophila* pneumoinia treated with fluoroquinolone tend to have shorter durations of fever, shorter hospitalization time, fewer complications and so on. In the CAP guidelines of many countries, fluoroquinolone is the priority selection for atypical pathogens. The infection group of Chinese Thoracic Society recommended that (*15*), based on current studies, if the patients get no better with macrolide treatment for 72 hours, clinicians should consider the possibility of macrolide-resistant *M. Pneumoniae*, and change to fluoroquinolones or tetracyclines. Moxifloxacin or levofloxacin was the most common alternative therapy.

6. Conclusion

Though the etiology of CAP is different between countries and changes over time, atypical pathogens were playing an important role in CAP all over the world. In China, atypical pathogens, such as *M. Pneumoniae*, *C. Pneumoniae*, *L. Pneumophila*, are part of the main causes, and *M. Pneumoniae* was the most prevalent pathogen. Atypical pathogen infections often cause mild or moderate pneumonia, but *L. Pneumophila* or co-infection with bacteria can lead to severe pneumonia and high mortality. Though still controversial, considering highly prevalent atypical pathogens, especially *M. Pneumoniae*, empirical antibiotic coverage of atypical pathogens is recommended, and it can improve the outcomes, shorten the length of hospitalization, reduce the mortality and lower total hospitalization costs. Macrolide resistance rate was high, but no quinolone-resistant *M. Pneumoniae* strain was found. So, if the patients get no better with macrolide treatment for 72 hours, fluoroquinolones or tetracyclines should be considered for alternative therapy. In China, it would be moxifloxacin or levofloxacin.

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Review

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Nosocomial infection and its molecular mechanisms of antibiotic resistance

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Summary **Nosocomial infection is a kind of infection, which is spread in various hospital environments, and leads to many serious diseases (*e.g.* pneumonia, urinary tract infection, gastroenteritis, and puerperal fever), and causes higher mortality than community-acquired infection. Bacteria are predominant among all the nosocomial infection-associated pathogens, thus a large number of antibiotics, such as aminoglycosides, penicillins, cephalosporins, and carbapenems, are adopted in clinical treatment. However, in recent years antibiotic resistance quickly spreads worldwide and causes a critical threat to public health. The predominant bacteria include Methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Acinetobacter baumannii*. In these bacteria, resistance emerged from antibiotic resistant genes and many of those can be exchanged between bacteria. With technical advances, molecular mechanisms of resistance have been gradually unveiled. In this review, recent advances in knowledge about mechanisms by which (*i*) bacteria hydrolyze antibiotics (*e.g.* extended spectrum β-lactamases, (*ii*) AmpC β-lactamases, carbapenemases), (*iii*) avoid antibiotic targeting (*e.g.* mutated vanA and *mecA* genes), (*iv*) prevent antibiotic permeation (*e.g.* porin deficiency), or (*v*) excrete intracellular antibiotics (*e.g.* active efflux pump) are summarized.**

Keywords: Hospital-acquired infection, mutations, PBP2a, SCC*mec*, OprD, MexEF-OprN.

1. Introduction

Nosocomial infection, also known as hospital-acquired infection, is a kind of infection, which is contracted from the environment or staff of a healthcare facility (*1*). It can be spread in various hospital environments, including nursing homes, wards, operating rooms, or other clinical settings. Infection happens in the clinical setting through a large number of pathways. In addition to contaminated equipment, bedding articles, or aerosols, staff also can spread infection (*2*). An epidemiological investigation implemented by WHO in fifty five hospitals of fourteen countries from four WHO Regions (Europe, Eastern Mediterranean, South-East Asia and Western Pacific) revealed an average of 8.7% of hospital patients had a nosocomial infection. At any time, over 1.4 million people worldwide suffer from infectious complications acquired in hospitals (*3*). The morbidities of nosocomial infection were reported from hospitals in the European, Eastern Mediterranean, South-East Asia and Western Pacific as 7.7, 11.8, 10.0, and 9.0% respectively (*4*). Nosocomial infections could lead to functional disability and mental stress of patients. In addition, nosocomial infections are also one of the leading causes of death (*5*).

In hospitals, patients are exposed to a diversity of microbes. Many different bacteria, viruses, fungi and parasites may lead to nosocomial infections (*6*). Most recently hospital-acquired infections are caused by common bacteria that usually lead to no or milder disease compared to in-patients such as, *Staphylococcus aureus*, *enterococci*, *Pseudomonas spp.* and E*nterobacteriaceae* (*7*). After being infected, patients commonly receive antibiotics. Through selection and

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exchange of genetic resistant elements, antibiotics boost the emergence of multi-drug resistant strains. Bacteria which are sensitive to the antibiotics are suppressed or killed, while resistant strains survive and may become endemic and burst out in the hospital (*8,9*). Based on previous research, the major mechanisms of antibiotic resistance include extended spectrum β-lactamases (ESBLs), AmpC β-lactamases, carbapenemases, staphylococcal cassette chromosome *mec* (SCC*mec*), VanA ligase, porin deficiency, and active efflux pumps (Figure 1). The above mechanisms will be introduced in the following sections.

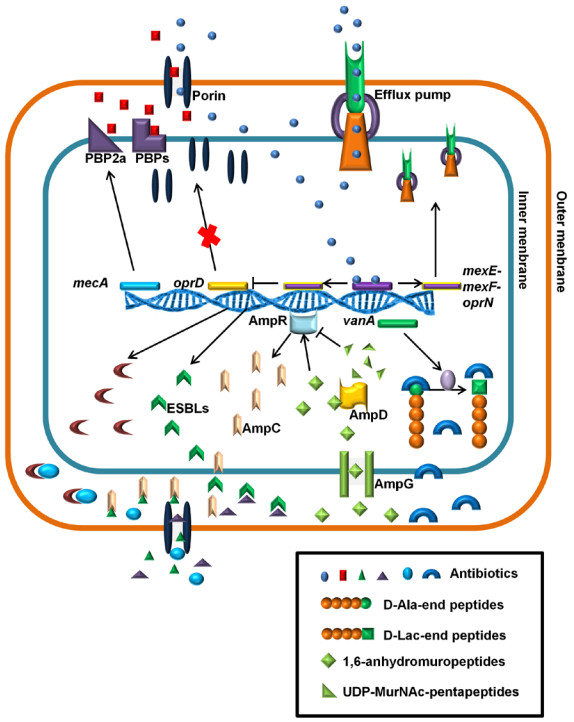

Figure 1. Molecular mechanisms of antibiotic resistance

2. Hydrolyzing antibiotics I: ESBLs

The emergence of third-generation cephalosporins in clinical treatment in the early 1980s was reported as a significant breakthrough to antagonize β-lactamase-mediated antibiotic resistance. Soon after, the first research into plasmid-encoded-lactamases which are able to hydrolyze extended-spectrum cephalosporins was reported in 1983 (*10*). The genes, which encode β-lactamases much similar to SHV-1, TEM-1, and TEM-2, exhibited mutations of single nucleotides and were soon discovered to have the ability to acquire resistance to extended-spectrum cephalosporins (Table 1) (*11,12*). For now,various ESBLs contained in Gram-negative bacteria such as *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* have proved to be capable of resistance to most of β-lactam antibiotics. Because ESBLs-producing bacteria are able to hydrolyze a large number of β-lactam antibiotics, the utility of those antibiotics for infections caused by such bacteria is reduced. Moreover, the plasmids containing the genes that encode ESBLs usually also contain genes that cause resistance to aminoglycosides and trimethioprim/sulfamethoxazole. There have been more and more reports of plasmid-induced attenuation in susceptibility to aminoglycosides, often being associated with plasmid-

Table 1. Resistance-related β-lactamases

β-lactamases	Families	Targets	Susceptibility (to clavulanic acid)	Classes	*Ref.*
Extended spectrum β-lactamases (ESBLs)	TEM family and SHV family	Targets of broad-spectrumβ-lactamases(Aminopenicillins,benzylpenicillin,carboxypenicillins, narrow-spectrum-cephalosporins), oxyimino-cephalosporins,monobactam	++++	A	(*19-27*)
	CTX-M family	Targets of broad-spectrumβ-lactamases, cefepime	++++	A	(*28-30*)
	XOA family	Same as above	+	D	(*21,31,32*)
	Others (PER-1, VEB family, GES family, IBC-2, BES-1, IBC-1, SFO-1, and TLA-1)	Same as TEM family and SHV family	++++	A	(*33, 34*)
AmpC	FOX family, CMY family, LAT family, DHA family, and MOX family	Targets of broad-spectrumβ-lactamases, cephamycins	0	C	(*35-40*)
Carbapenemase	IMP family and VIM family	Targets of broad-spectrumβ-lactamases,cephamycins,carbapenems	0	B	(*41-44*)
	KPC family	Same as above	+++	A	(*41-44*)
	OXA family	Same as above	+	D	(*41,42,45*)

induced cephalosporin resistance (*13,14*). Even when plasmid-mediated attenuation of susceptibility of quinolone y is absent, there also is an obvious correlation between quinolone resistance and ESBLs production (*15-17*). The reason for such correlation is not yet understood.

2.1. *TEM family*

Mutations of single nucleotides at many sites in genes coding TEM-1β-lactamases can be achieved in the laboratory with complete activity (*18*). Those mutations, which change the ESBLs phenotype transform the configuration of the active site of the enzyme, and allow interaction between active site and oxyimino-β-lactams (*18-20*). Exposing the active site to β-lactam substrates also leads to susceptibility of the ESBLs to β-lactamase inhibitors, such as clavulanic acid. More than one hundred and thirty members of the TEM family are now recognized, and their diversity supplies a useful pathway to trace the transmission of individual resistance genes (*21,22*).

2.2. *SHV family*

SHV-1 coincides in 68% of its amino acids sequence with TEM-1 and shares its molecular structure (23). Like the TEM family, members of the SHV family have certain amino acid mutations at the active site. More than fifty members of the SHV family recently have been identified through unique combinations of amino acid replacements (*24*). The SHV family recently has been shown in surveys of resistant strains in Europe and America (*25,26*). SHV-5 and SHV-12 are prevalent among the members of the SHV family (*27*).

2.3. *CTX-M family*

Another family of ESBLs not a member of the TEM or SHV families was named CTX-M to emphasize its greater activity against cefotaxime compared to ceftazidime. More than forty members of CTX-M are currently known (*28*). Belying their name, some hydrolyze ceftazidime more rapidly than they do cefotaxime. CTX-M-14, CTX-M-3, and CTX-M-2 are the most widespread (*29,30*).

2.4. *OXA family*

Twelve members of the OXA family have recently been discovered *(21*). They were found mainly in *P. aeruginosa* in clinical samples from France and Turkey (*31*). Major members of the OXA family are relatively resistant to clavulanic acid-induced inhibition. Some of them have resistance mainly to ceftazidime, but OXA-17 shows stronger resistance to cefotaxime and cefepime than to ceftazidime (*32*).

2.5. *Other members of ESBLs*

Other members of ESBLs are rare and have been discovered predominantly in *P. aeruginosa* and in some small areas: PER-1 was found in France, Italy, and Turkey; VEB family was found Southeast Asia; and GES family and IBC-2 were found in South Africa, France, and Greece (*33*). A part of these ESBLs were discovered in *Enterobacteriaceae* as well, but other rare ESBLs, such as BES-1, IBC-1, SFO-1, and TLA-1, have been discovered only in *Enterobacteriaceae* (*34*).

3. Hydrolyzing antibiotics II: AmpC β-lactamases

AmpC β-lactamases, which are usually induced by β-lactams, are expressed in many Gram-negative bacteria. Mutations in relevant genes lead to increasing expression levels and promote the emergence of cephalosporin resistance in *Enterobacter cloacae* (*35*). The AmpC β-lactamases in *E. coli* are present at a low expression level and the AmpC-encoded gene is deficient in the chromosome of *klebsiella* and *salmonella* strains. However, plasmid-expressed AmpC β-lactamases can endow those bacteria with similar resistance as *Enterobacter cloacae* mentioned above. Until recently, more than twenty AmpC β-lactamases have been found expressed by plasmids (*36*).

As shown in Figure 1, The ampC-related regulatory pathway includes the following three elements: (*i*) AmpG which is a permease on the inner membrane; (*ii*) AmpD which is an amidase in cytoplasm; and (*iii*) AmpR, a transcription factor, is a member of the LysR family, a group of regulatory proteins (*37*). These three elements are necessary for expression of AmpC β-lactamases in both *Enterobacteriaceae* and *P. aeruginosa* (*38*). In the regular process of cell wall recycling, 1,6-anhydromuropeptides are disassembled from the cell wall and transferred into the cytoplasm by AmpG permease. The 1,6-anhydromuropeptides are cut by AmpD protein to produce tripeptides, which are subsequently transformed into UDP-MurNAc-pentapeptides. UDPMurNAc-pentapeptides couple with AmpR proteins combining in the intergenic region between *ampR* and *ampC*, and generating a structure that inhibits activation of *ampC*. Low expression levels of AmpC are generated, and the β-lactamase localizes to the periplasmic space. When β-lactams, such as cefoxitin and imipenem, permeate the outer membrane of bacteria, they reach the periplasmic space, and combine with target penicillin binding proteins (PBPs). The amount of 1,6-anhydromuropeptides increases, and AmpD is unable to efficiently deal with the high levels of cell wall pieces. The anhydro-MurNAc-peptides substitute UDP-MurNAc-pentapeptides binding to AmpR, leading to a structural change of the enzyme. AmpR is changed into a role of transcriptional promoter, AmpC is produced at higher levels, and concentration of AmpC rises in the

periplasmic space. When the concentration of β-lactam decreases below its "alarm level" the amount of anhydro-MurNAc-peptides in cytoplasm also decreases, and AmpD's ability to efficiently cut these peptides is restored. In another case, mutations of nucleotides in genes leads to deficiency of AmpD or down-regulates expression of ampD damage in the process of wall fragment recycling and leads to increased concentration of anhydro-MurNAc-peptides in the cytoplasm. As a result, the combining of anhydro-MurNAc-peptides to AmpR makes AmpR "locked" in a structure as transcriptional activator of ampC, and produces high levels of AmpC β-lactamases (*37,39,40*).

4. Hydrolyzing antibiotics III: Carbapenemases

Carbapenemases are a kind of β-lactamase with various hydrolytic abilities. They have been identified to have ability to damage penicillins, cephalosporins, and carbapenems. Bacteria generating the carbapenemases, which resist these antibiotics by breaking β-lactams, frequently lead to serious nosocomial infections. Carbapenemases belong to the A, B, and D molecular class of β-lactamases (*41*). Class A and D β-lactamases have a mechanism of serine-based hydrolysis, while class B β-lactamases are metallo-β-lactamases which have zinc in their active site (*42*). The carbapenemases group of class A contains members of the KPC, NMC, IMI, SME, and GES families. Among these families, the KPC carbapenemases are the most predominant, usually existing on plasmids in *Klebsiella pneumoniae* (*43,44*). The carbapenemases group of class D contains OXA β-lactamases usually found in *Acinetobacter baumannii*. The metallo-β-lactamases were first found in *Pseudomonas aeruginosa* strains, but at present, there is an increasing worldwide emergency of this class of β-lactamases in the *Enterobacteriaceae* (*45*).

5. Avoid antibiotics targeting I: *mecA* mutation

SCC*mec* is a mobile genetic element of *Staphylococcus* bacterial strains. This genetic sequence contains the *mecA* gene, which codes for resistant proteins to the antibiotic methicillin, and is the only known way for *Staphylococcus* species to spread the gene in the wild by horizontal gene transfer. *mecA* leads to resistance to methicillin and other β-lactam antibiotics. After *mecA* is absorbed into bacteria, it is inserted into the *S. aureus* chromosome (*46,47*). *mecA* produces penicillin-binding protein 2a (PBP2a), which is much different from former penicillin-binding proteins because mutations have changed its conformation to make it hard to bind methicillin or other β-lactam antibiotics to its active site. Thus, PBP2a can continuously promote the transpeptidation required for peptidoglycan cross-linking to perform cell wall synthesis in the presence of antibiotics. As a result of the incapability of PBP2a to combine with β-lactam moieties, activation of *mecA* promotes resistance to all other β-lactam antibiotics including methicillin (*48*). *mecA* is controlled by regulatory genes *mecI* and *mecR1*. MecI often combines with the *mecA* promoter and plays an inhibitor role (*49*). In the existence of β-lactam antibiotics, MecR1 promotes a signaling transduction pathway that causes activation of *mecA* (*50*). This activation is initiated by MecR1-induced cleavage of MecI, which decreases MecI inhibition. *mecA* is also regulated by two co-repressors BlaI and BlaR1. *blaI* and *blaR1* are homologous to *mecI* and *mecR1*, respectively, and usually play a role as regulators of *blaZ* which leads to penicillin resistance (*51,52*). The nucleotide sequences recognized by MecI and BlaI are the same, thus BlaI can also combine with the *mecA* operator to inhibit activation of *mecA* (*53*).

6. Avoid antibiotics targeting II: *vanA* mutation

Glycopeptides repress cell wall synthesis in Gram-positive bacteria by combining with the C-terminal D-Ala-D-Ala of the pentapeptide precursors of peptidoglycan, further blocking the reactions of transglycosylation and transpeptidation (*54*). Recently, glycopeptide-resistant *enterococci* have spread throughout the whole world. So far, seven types of resistant elements (VanA, -B, -C, -D, -E, -G, and -L) in enterococci have been discovered and they have seven corresponding operons (*vanA*, *-B*, *-C*, *-D*, *-E*, *-G*, and *-L*) which play roles of synthesis of a novel combining site (peptidoglycan precursors terminating in D-Ala-D-lactate in VanA, -B, and -D type or D-Ala-D-serine in VanC, -E, -G, and -L type) leading to a decreased affinity to glycopeptides and substitution of the normal precursors ending in D-Ala-D-Ala (*55-57*).

A two-component regulatory system VanR-VanS controls vancomycin resistance in vancomycin-resistant *enterococci* (VRE) and vancomycin-resistant *Staphylococcus aureus* (VRSA) (*58*). VanS is a membrane-related sensor for vancomycin which regulates the phosphorylation of VanR. VanR is a transcriptional activator of the operon which encodes VanH, VanA and VanX. VanH is a dehydrogenase which converts pyruvate to D-Lac, and VanA is a ligase which combines D-Ala and D-Lac by creating an ester bond between them. Vancomycin can only combine with D-Ala- D-Ala but not to D-Ala-D-Lac, and thus vancomycin resistance appears. VanX is a dipeptidase which cleaves the normal peptidoglycan component D-Ala-D-Ala that prevents it from leading to vancomycin sensitivity. VanY is a D,D-carboxypeptidase that cuts the end D-Ala residue of the peptidoglycan if substitution of D-Ala-D-Ala by VanX is not thorough. Thus, D-Ala-D-Lac substitutes for the normal D-Ala-D-Ala in peptidoglycan synthesis resulting in vancomycin resistance (*59-61*).

7. Prevent antibiotics permeation: *oprD* mutation and porin deficiency

The outer membrane of Gram-negative bacteria has a semi-permeable barricade which decreases the import of antibiotics, and the outer membrane of *P. aeruginosa* is only 8% as permeable as that of *Escherichia coli* (*62*). However, for surviving, *P. aeruginosa* has to allow import of nutrients through the outer membrane, and this is achieved through a system of water-filled protein channels named porins. DNA sequencing of the *P. aeruginosa* genome has recognized one hundred and thirty known or supposed outer membrane proteins, with sixty four of these outer membrane proteins classified into three families of porins (*62*). These porins play a significant physiological role in the transport of sugars, amino acids, and phosphates, and so on (*63,64*). Some hydrophilic antibiotics, such as β-lactams, aminoglycosides, tetracyclines, and some fluoroquinolones, have been shown to pass through the outer membrane porins (*65-68*). Thus, deficiency of porins can diminish susceptibility of *P. aeruginosa* to some antibiotics.

OprD porin-mediated resistance contains mechanisms that down-regulate the transcriptional level of the *oprD* gene and/or mutations which replace the translational production of a normal porin. At the transcriptional level of *oprD*, disturbing mechanisms contain (*i*) breakdown of the *oprD* promoter, (*ii*) terminating the transcription of *oprD* prematurely, (*iii*) co-regulation with trace metal ion resistance, (*iv*) salicylate-induced decrease, and (*v*) down-regulated transcriptional expression by coregulation with the active efflux pump encoded by mexEF-oprN. The oprD promoter breakdown appears as a result of deletions or insertions in the upstream region of oprD. It was reported that a deletion containing the putative promoter and initiation codon blocked transcription of *oprD* (*69-72*). Based on previous research, IS1394 and an ISPa16-like insertion element have been proposed as an upstream region of the *oprD* in imipenem-resistant strains of *P. aeruginosa* showing down-regulated *oprD* expression (*73,74*).

8. Excreted intracellular antibiotics: mexEF-oprN and actived efflux pump

On the one hand, the deficiency of porins such as OprD is an effective obstacle for antibiotic import into the cell, on the other hand, a decrease in antibiotic concentration can also be realized *via* export through membrane-located efflux pumps. Efflux pumps have been classified into five superfamilies (*75,76*). The superfamilies contain (*i*) the ATP-binding cassette (ABC) superfamily, (*ii*) the small multidrug resistance superfamily, (*iii*) the major facilitator superfamily, (*iv*) the resistance-nodulation-division (RND) superfamily, and (*v*) the multidrug and toxic compound extrusion superfamily.

One of the most important regulatory mechianisms is the coincident overproduction of the MexEF-OprN efflux pump and downregulation/upregulation of the OprD porin (*77*). In wild *P. aeruginosa*, MexT is silenced owing to either the existence of repressing mutations or the deficiency of a secondary effecter (*78*). As a result, expression of *mexEF-oprN* stays at a low level, and expression of *oprD* stays at a basal level providing a proper amount of OprD in the outer membrane sufficient for normal cellular intake (*79*). In *nfxC*-type mutants, MexT becomes active *via* a mutation in *mexT*. The activated MexT protein up-regulates transcription of *mexEF-oprN* causing overexpression of the efflux operon and overproduction of the MexEF-OprN efflux pump. At the same time, MexT down-regulates *oprD* at the transcriptional and translational levels, leading to a decreased amount of *OprD* (*80*). On the other hand, loss of MexS, a supposed oxidoreductase/dehydrogenase, has been thought to lead to formation of secondary metabolites which may serve as effecters for MexT (*77*). These effecters could combine with MexT, change the structure of the regulatory protein, and alter MexT into an activating situation. As a result, MexT can up-regulate the expression of *mexEF-oprN* and down-regulate the expression of *oprD*, similar to the mechanism mentioned above. There also is a third mechanism. Loss of the universal regulatory protein MvaT is also associated with the positive regulation of the *mexEF-oprN* operon (*81*). The mechanism of MvaT-related regulation has not been discovered, but it works independent of MexT and MexS. In contrast to the MexT- and MexS-related regulatory mechanisms, loss of MvaT leads to a positive regulation of both *mexEF-oprN* and *oprD* expression.

9. Conclusion

The capacity of bacteria to evolve resistance to antibiotics has long been realized, but our knowledge about the tremendous variety of molecular mechanisms has been enriched enormously most recently. Technology advances in genomics, proteomics, and structural biology have analyzed many of the molecular mechanisms promoting resistance and will continuously provide more and more intensive explanations. Based on these newest discoveries, the development of novel antibiotics, which can resist or grant knowledge of resistance mechanisms will be accelerated. For speeding up development of new antibiotics, academic institutions and pharmaceutical companies should make joint efforts in the future.

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Mini-Review

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Controversy and progress for treatment of acute cholangitis after Tokyo Guidelines (TG13)

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Summary **Tokyo Guideline 2013 (TG13) is an international guideline for the diagnosis, classification and treatment of acute cholangitis. Progress and controversy for the two years after TG13 was summarized. Endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography (MRCP) are both effective imaging tests for common bile duct (CBD) stones. More factors *e.g.* obesity may be involved in severity assessment. Initiation of broad-spectrum antibiotics addressing the typical Gram-negative enteric bacteria spectrum and early biliary drainage are the mainstay therapeutic options. Early laparoscopic exploration is also an option for stone-related nonsevere acute cholangitis besides endoscopic retrograde cholangial or percutaneous transhepatic cholangial drainage. Surgical biliary drainage should be avoided in severe cholangitis.**

Keywords: Acute cholangitis, Tokyo guideline 2013 (TG13), endoscopic retrograde cholangiography (ERC), percutaneous transhepatic cholangiography (PTC), laparoscopic common bile duct exploration (LCBDE)

1. Introduction

Gallstone disease estimates are about 20% in European and Northern American populations. Common bile duct (CBD) stones are estimated to be in 10-20% of individuals with symptomatic gallstone disease (*1*).

Acute bacterial cholangitis is a common surgical emergency in the spectrum of acute biliary infection with high mortality rates. Thus, there is a need for straightforward diagnostic evaluation and immediate treatment initiation. With this background, Tokyo Guideline derived from international meetings in 2007 (*2*) and updated in 2013 (*3*) was published for the diagnosis, classification and treatment of acute cholangitis. Here, we summarize some progress and controversial issues after Tokyo Guideline 2013 (TG13).

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2. Imaging diagnosis

There are various modalities available for imaging of the biliary tract. The most powerful is endoscopic ultrasound (EUS) and magnetic resonance cholangiopancreatography (MRCP). Trans-abdominal ultrasound still has its role as a first imaging test in initial evaluation due to its wide availability. In the emergency ward, despite its low sensitivity in CBD stone detection ranges from 25 to 63% (*4*). Yet, ultrasound has high diagnostic accuracy in the demonstration of biliary dilatation. It has to be kept in mind that a definition of biliary dilatation is not enough, although a normal bile duct diameter should be less than 8 mm (*5*). MRCP has an accuracy of detecting CBD stones surpassing 90%, though there is a clinically significant weakness in the detection of small stones (*6*). EUS is a minimally invasive endoscopic procedure superior to endoscopic retrograde cholangiography (ERC) in detecting malignant causes of cholestasis and is at least equal to ERC in terms of stone detection (*7*). EUS-guided ERC has also been reported and may become a rational option (8, 9). Computed tomography has its clinical value above all in unstable patients with high suspicion of underlying malignancy or suspicion of hepatic abscesses.

In a meta-analysis of a pooled data set of 301 patients, the aggregated sensitivities of EUS and MRCP for CBD stone detection were 93 and 85%, whereas their specificities were 96 and 93%, respectively (*10*).

3. Severity assessment

There is a wide spectrum of disease courses in acute bacterial cholangitis, ranging from self-limiting to life-threatening with the need to tailor treatment accordingly. An estimated 70% of patients respond to medical treatment comprising supportive and antimicrobial therapy (*11*). Criteria for severity assessment in the TG13 definition of acute bacterial cholangitis are as follows: grade III (severe): presence of organ dysfunction; grade II (moderate): risk of increased severity without early biliary drainage; and grade I (mild) (*12*). However, the prospective validation needs to be further studied. G. Paul Wright (*13*) reported a study using TG13 in a US population. They found that obesity was still a risk factor for the development of acute cholangitis. It was related to severity assessment. So in the future, more factors may be involved for severity assessment.

4. Treatment

Treatment is directed at the two main pathophysiologic components of acute cholangitis, biliary infection and obstruction. Patients with severe disease indicators or significant comorbidities are to be admitted to the intensive care unit.

4.1. *Antibiotic Treatment*

Most pathogens relevant to cholangitis initiation and perpetuation are derived from gastrointestinal microbiota including Gram-negative enteric bacteria and enterococci. Administration of antibiotic agents should be initiated empirically as early as possible in any patient with a clinical suspicion of cholangitis. If there are signs of septic shock as outlined in the Surviving Sepsis Campaign guidelines, antibiotics should be administrated < 1 hour, otherwise < 4 hours for definitive diagnostic studies, and in any event before drainage procedures are performed (*14*).

The TG13 working group has issued expert opinion based recommendations concerning antibiotic usage in acute bacterial cholangitis (*15*). The importance of the quality of biliary drainage is highlighted by another study demonstrating that, in the setting of successful ERC drainage, the clinical results were the same after 3 versus 5 days of antibiotic treatment (*16*). In contrast, in the presence of residual stones or ongoing biliary obstruction, antimicrobial treatment should be extended until the resolution of the anatomical alteration.

Concerning bile cultures, which have been reported to be positive in the range of 59-93%, TG13 recommends acquisition of bile samples for microbial testing at the beginning of any drainage procedure (*15*). In contrast, the rate of positive blood cultures in the cholangitis population is about 21-71%. Since the results of blood cultures usually do not affect clinical management and outcomes, routine blood cultures remain a matter of controversy.

There are laboratory and clinical data showing that antimicrobial agents secreting into bile had a better effect. Thus theoretically, biliary secreted antibiotics may be better than non-secreted antimicrobial compounds (*17-19*).

4.2. *Endoscopic and surgical Treatment*

Previously, before effective biliary drainage procedures and more potent antibiotic agents became available, the mortality of acute severe cholangitis nearly approached 100% with conservative treatment, however it has now declined to 2.7-10% (*20*), highlighting the need for the removal of biliary obstruction as the source of ongoing infection in acute bacterial cholangitis. Biliary drainage can be achieved in multitude ways, *e.g*. ERC, percutaneous transhepatic cholangiography (PTC), EUS-guided drainage, or surgical drainage. There are various endoscopic transpapillary options available, including biliary stent or nasobiliary drain placement above the obstruction site, all of which have appropriate indications corresponding to disease severity and clinical context (*21*). Stenting has an equal effectiveness compared to nasobiliary drainage; however, it is associated with improved patient comfort, while the nasobiliary tube has the potential advantage of repeated bile aspiration for microbiologic analysis, flushing, and cholangiographic evaluation.

Overall, endoscopic sphincterotomy and stone extraction have been reported to be successful in more than 90% of cases, with adverse event rates close to 5% and mortality rates < 1% (*22*). After failure of primary wire-guided biliary cannulation, sphincterotomy or percutaneous transhepatic drainage procedures, may become necessary. However, the complication rates for these more advanced techniques are much higher than for standard procedures (*23*). In few centers, EUS-guided biliary drainage has been introduced as a viable alternative after failed ERC access (*24,25*). Although this approach requires further standardization and clinical trial validation.

The management of biliary stones is still being debated. Considering the success rates of stone clearance from the CBD, both procedures namely laparoscopic CBD exploration and ERC were similar (91.7 *vs*. 88.1%) (*26*). However, Koc *et al*. (*27*) quoted that the success rate of the laparoscopic common bile duct exploration (LCBDE) + laparoscopic cholecystectomy (LC) group were 96.5%, which was higher than that in the ERC + LC group (94.4%). The overall success

Table 1. Studies comparing early LCBDE and delayed LCBDE

Study (year, reference)	Study type	Subject group	Surgery-related complications	Death	Length of stay (days)	Death
2015 (36)	retrospective	Early	7/32	0	13.34	0
	case-control	Delayed	8/41	0	18.32	0
2014 (38)	retrospective	Early	5/94	2/94	3	2/94
	case-control	Delayed	8/121	0	3	0
2008 (39)	prospective	Early	6/33	1/33	/	1/33
	case-control	Delayed	5/48	0	/	0
2005 (40)	retrospective	Early	1/20	0	2	0
	case-control	Delayed	1/24	0	2	0

The results showed that the complication rate between the early and delayed LCBDE didn't have statistical significance ($p > 0.05$ in the four studies respectively). There were 3 deaths in the early LCBDE group of two studies. The 3 patients were all older than 80 years old and all suffered from severe acute cholangitis. We could deem that early LCBDE was suitable for mild and moderate acute cholangitis, but not for severe acute cholangitis because of the high mortality rate.

rate was 88.1% in the LCBDE group and 79.8% in the ECR group ($p = 0.20$) (*26*). The two procedures for uncomplicated gallstones and CBD stones had nearly similar complication rates, which were 7 and 11.1% ($p > 0.05$), respectively. Noble *et al*. (*28*) described enhanced attainment rates for laparoscopic versus endoscopic management even for higher-risk patients in complications and hospital stay. Difficulty with cannulation and impacted stones represent the common causes of failure with ERC procedures (*28,29*). The main reason for unsuccessful clearance following ERC was impacted stones in 13.1% of the patients (*26*). LC after ERCP is recognized to be troublesome with a higher post complication rate and much more likelihood of conversion to an open approach as a result of inflammation and fibrosis in and around Calot's triangle (*30,31*). When compared to one-stage laparoscopic CBD exploration, it was found that LC could be very difficult in 33.4% of the patients primarily due to severe adhesions in the ERP group (*26*).

Additionally, ERC-caused dysfunction of the Oddi sphincter can be permanent and leads to damage of the sphincter barrier, which impedes duodenobiliary reflux (*32*). Duodenal reflux into the bile duct is associated with a high rate of bacteria colonization following sphincterotomy, which is one of the important mechanisms of biliary duct stone formation. Ding *et al*. (*33*) supported this mechanism and reported a higher rate of recurrence of CBD stones (9.47% *vs*. 2.06%) in the ERC + LC group vs. LCBDE group at long-time follow-up approximately up to 3 years. It is essential to deliberate the long-term follow up in order to validate the effectiveness, not only early stone clearance rates. Some studies showed that the duration of postoperative hospital stay in the LCBDE + LC was significantly shorter than that in the ERC + LC group (*26,34,35*).

In the past few years, comparison between open surgery and laparoscopic surgery were performed (*37*). The results showed that the prognosis was similar for acute cholangitis, but the length of hospital stay (LOS), total cost, and complication rate in the laparoscopic group were lower although the average operation time was longer than open surgery.

As laparoscopic skill develops, average operation time was shorter year after year. Some studies (*36,38-40*) comparing early LCBDE with delayed LCBDE (> 72 h from the onset) were performed (Table 1). The result showed that the complication rate between the early and delayed LCBDE didn't have statistical significance ($p > 0.05$ in the four studies respectively). However, there were 3 deaths in the early LCBDE group while no deaths occurred in the delayed LCBDE group. The 3 patients were all older than 80 years old, and all suffered from severe acute cholangitis. We can deem from the 4 studies that early LCBDE was suitable for mild and moderate acute cholangitis, but not for severe acute cholangitis because of the high mortality rate. However, there were still a few experts trying to perform emergency LCBDE for severe acute cholangitis (*38,39*). Some even performed primary closure of the common bile duct in emergency LCBDE (*39*). Though, it was quite debatable.

5. Conclusion

EUS and MRCP were both effective imaging tests for CBD stones. More factors *e.g*. obesity may be involved for severity assessment. Initiation of broad-spectrum antibiotics addressing the typical Gram-negative enteric bacteria spectrum and early biliary drainage are the therapeutic mainstay options. Early LCBDE is also an option for stone-related non severe acute cholangitis.

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Original Article

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Workload, burnout, and medical mistakes among physicians in China: A cross-sectional study

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Summary **The purpose of this study is to determine the prevalence of burnout among different grade hospitals and to examine if a relation exists between burnout and medical mistakes. A multi-center cross-sectional survey was conducted. Physicians were interviewed in hospitals from 10 provinces in China. Burnout was measured using the Chinese version of the Maslach Burnout Inventory-General Survey. Overall, 1,537 physicians were included in this study. Of these, 76.9% reported some burnout symptoms or serious burnout symptoms and 54.8% reported committing medical mistakes during the last year. 39.6%, 50.0%, and 59.5% of the respondents in primary, secondary, and tertiary hospitals respectively reported having made mistakes over the course of the previous year. Multivariate analysis demonstrated that being female was protective against medical mistakes (OR = 0.72, 95% CI: 0.58-0.89), whereas physician-reported 60 or more work hours per week (OR = 1.65, 95% CI: 1.22-2.22), and physicians who reported serious burnout (OR = 2.28, 95% CI: 1.63-3.17) were independently associated with higher incidence of medical mistakes. In conclusion, Chinese physicians reported high workloads, high rates of burnout and high medical mistakes. Physicians in tertiary hospitals were especially overworked and suffered the most serious burnout. Longer work hours per week, and burnout were the independent risk factors for medical mistakes.**

Keywords: Workload, burnout, medical mistakes, physicians, cross-sectional study

1. Introduction

China is facing great pressure in providing quality care for patients in a landscape where available healthcare resources are limited. However, resource waste is not uncommon in the Chinese healthcare system. According to the 2012 Health Statistical Report made by Ministry of Health in China, bed utilization rate was 58.9% in primary hospitals while the rate was 104.5% in tertiary hospitals. One of the reasons why bed utilization rates are low in primary hospitals is that some patients do not trust the community or township hospital (*1,2*), resulting in everyday overcrowding in Chinese secondary or tertiary hospitals with relatively few patients seeking treatment in primary hospitals. The gap between healthcare demand and supply has caused Chinese healthcare providers in the secondary and tertiary setting to become overworked (*3*), and leads to an intensive relationship between physicians and patients. The last decade has been witness to a number of doctors in China being killed by their patients.

One common consequence of long hours is physician burnout. This term was originally coined by Freudenberger and Richelson based on psychoanalytical case studies, with burnout manifesting itself as extreme physical and emotional exhaustion, often unrecognized by the individual (*4*). Maslach (*5,6*) provided a more detailed account of burnout syndrome based upon qualitative and quantitative research, suggesting that the components of burnout are exhaustion, cynicism, and inefficacy.

During the last two decades, several studies have

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characterized the prevalence of healthcare provider burnout and its associated risk factors (*7-9*). However, few studies have examined the impact of physician burnout on healthcare practice and no studies have examined differential burnout rates by hospital characteristics. The purpose of this study is to reveal the severity of burnout among physicians in different grades of hospitals in China, and the impact physician burnout has on the occurrence of medical mistakes.

2. Methods

The Institutional Review Board (IRB) of West China Hospital in Sichuan University approved this study. As the survey was anonymous it is impossible to adopt written informed consent. Oral informed consent was approved by the IRB and obtained from each subject. An informed consent form was presented on the cover of the questionnaire. Once a questionnaire was completed we consider that the participant has orally agreed to participate in the survey.

2.1. *Study design and participants*

A multi-center cross-sectional survey was conducted in China from November to December 2013. Physicians in hospitals from 10 provinces (Sichuan, Chongqing, Gansu, Guizhou, Guangdong, Shanxi, Hu'nan, Zhejiang, Yunnan, and Ningxia) were selected. Overall 12 tertiary, 9 secondary, and 25 primary hospitals were included in this study. For any secondary or tertiary hospital, we required selecting physicians from at least 10 clinical departments and no less than 10 persons in each age group (across a total of 4 age groups). For primary hospitals, all on-duty physicians were included in the survey.

2.2. *Measures*

Workload was measured by two indicators. The first was work hours per week, and the second was number of daily service patients. Medical mistakes include self-report of any of the following: *i*) patient was harmed, *ii*) medication errors, *iii*) treatment delayed, and *iv*) incomplete or incorrect item in the patient's record.

Burnout was measured with the Chinese version of Maslach Burnout Inventory-General Scale (MBI-GS) (*10*) which includes 15 items for measuring the three dimensions of burnout: exhaustion (5 items), cynicism (4 items), and reduced professional efficacy (6 items). The 6 items of reduced professional efficacy were reverse scored (Supplement Table 1). The following equation was used to produce the weighted sum score of the scale (*11*):

$$0.40 \times exhaustion + 0.30 \times cynicism + 0.30 \times reduced\ professional\ efficacy$$

Physician participants were then divided into three groups on the basis of their syndrome score (range 0-6). Group 1: No burnout symptoms (scores 0-1.49); group 2: Some burnout symptoms (scores 1.50-3.49), and group 3: Serious burnout symptoms (scores 3.50-6). The categorization means that the symptoms in group 1 were experienced on average a few times per year or never, in group 2 a few times per month or nearly weekly, and in group 3 several times per week or daily.

2.3. *Data Collection*

We took several precautions to address potential sources of bias and ensure the quality of this study. When developing the questionnaire, we organized an inter-disciplinary group to review the questions in order to ensure the items were easily understandable. The self-reported questionnaire with instructions was developed to collect information on demographic characteristics, workload, burnout, and medical mistakes of each participant. Investigators at each center received critical training prior to the formal investigation, and were responsible for the completeness of the questionnaires. Participant physicians were ineligible if he/she was an intern. Informed consent was obtained from each subject. The eligible questionnaire should have been completed with no errors of logic, otherwise it was excluded.

2.4. *Statistical Analysis*

Missing data, if it existed, would be described as the number and proportion. We did not use imputation technique for addressing the missing data because participants may differ dramatically in characteristics given our experience. Frequencies, percentages, means and standard deviations were calculated for descriptive data, ANOVA were used to evaluate differences in continuous variables, and Chi-square tests were used to test for significance in categorical data. Forward stepwise multivariate logistic regression was used to identify risk factors for medical mistakes. A p-value of < 0.05 was considered to be statistically significant. Data were analyzed using SPSS version 17.0 (SPSS Inc, Chicago, Ill).

3. Results

One thousand eight hundred questionnaires were issued in total, and 1,607 were collected (response rate, 89.3%). The reason for non-responders was that they were too busy to fill out the questionnaires. Of the 1,607 respondent questionnaires, 70 did not meet the eligible criteria and were excluded. Overall, 1,537 physicians were included in this study. Of them, 57.3% were male, 73.4% were married, 14.7% reported either some college or lower education. Of the physicians who responded, 46.9% were junior professional title;

Table 1. Physician's characteristics among different grade hospitals in China

Items	Primary (n = 192)	Secondary (n = 354)	Tertiary (n = 991)	p
Age (mean ± SD)	36.5 ± 9.9	35.9 ± 9.4	35.5 ± 8.0	0.299
Age group, n (%)				0.057
< 30	50 (26.0)	90 (25.4)	252 (25.4)	
30-39	81 (42.2)	161 (45.5)	439 (44.3)	
40-49	35 (18.2)	69 (19.5)	232 (23.4)	
≥ 50	26 (13.5)	34 (9.6)	68 (6.9)	
Gender, n (%)				0.025
Male	104 (54.2)	189 (53.4)	603 (60.8)	
Female	88 (45.8)	165 (46.6)	388 (39.2)	
Marriage, n (%)				0.250
Unmarried/divorced/widowed	42 (21.9)	93 (26.3)	274 (27.6)	
Married	150 (78.1)	261 (73.7)	717 (72.4)	
Education, n (%)				< 0.001
High school or lower	33 (17.2)	13 (3.7)	7 (0.7)	
Some college	90 (46.9)	59 (16.7)	25 (2.5)	
Bachelor's degree	67 (34.9)	260 (73.4)	455 (45.9)	
Master's degree or higher	2 (1.0)	22 (6.2)	504 (50.9)	
Professional title, n (%)				< 0.001
Junior	135 (70.3)	169 (47.7)	417 (42.1)	
Middle	47 (24.5)	126 (35.6)	296 (29.9)	
Senior	10 (5.2)	59 (16.7)	278 (28.0)	

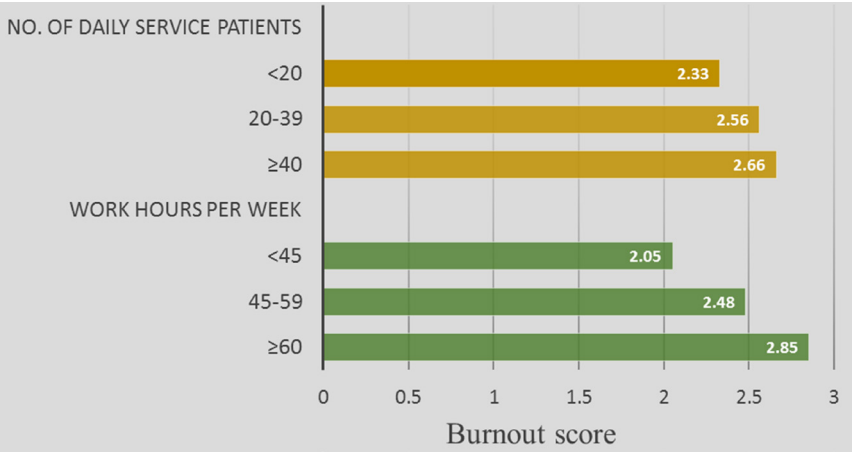

Figure 1. Burnout score distribution in number of daily service patients and work hours per week.

and their mean (SD) age was 35.8 (8.6) years (range, 20-73 years). More detailed information on participants' characteristics among the different grade hospitals in China has been presented in Table 1.

Overall, 76.9% of all physicians reported either some burnout symptoms or serious burnout and 54.8% of physicians reported committing medical mistakes over the course of the previous year. The average work hours per week (mean ± SD) were 54.1 ± 10.7 and the average number of daily service patients (mean ± SD) was 27.8 ± 25.1. In addition, Figure 1 revealed that work hours per week and number of patients for daily service were positively correlated with burnout score (both $p < 0.001$).

Table 2 shows physician reported workload, burnout, and mistakes among different hospital grades in China. The average work hours per week (mean ± SD) were 46.6 ± 9.1, 52.2 ± 11.5, and 56.2 ± 9.9 ($p < 0.001$), and the average number of daily service patients (mean ± SD) were 15.3 ± 13.2, 22.8 ± 18.3, and 32.1 ± 27.7 ($p < 0.001$) in primary, secondary, and tertiary hospitals, respectively. When categorized for work hours per week, the percentage of no less than 60 work hours a week average for a physician in a tertiary hospital (31.8%) was the highest; and when grouped with the patient number for daily service, that was no less than a 40 patient number a day average for a physician in a tertiary hospital (29.8%) also appeared the greatest workload. Meanwhile, proportions of physicians with some burnout symptoms were 52.6%, 59.0%, and 56.0%; and those of physicians with serious burnout were 10.9%, 17.3%, and 23.7% ($p < 0.001$) in primary, secondary, and tertiary hospitals, respectively. Similarly, the burnout score was highest in tertiary hospitals (2.6 ± 1.1), followed by secondary (2.4 ± 1.1) and primary (2.0 ± 1.2) hospitals. In regard

Table 2. Physician's workload, burnout, and mistakes among different grade hospitals in China

Items	Primary (n = 192)	Secondary (n = 354)	Tertiary (n = 991)	p^*
Workload				
Work hours per week (mean ± SD)	46.6 ± 9.1	52.2 ± 11.5	56.2 ± 9.9	< 0.001
Work hour group per week, n (%)				< 0.001
< 45	139 (72.4)	132 (37.3)	206 (20.8)	
45-59	37 (19.5)	148 (41.7)	470 (47.4)	
≥ 60	16 (8.1)	74 (21.0)	315 (31.8)	
Patient number of daily service (mean ± SD)	15.3 ± 13.2	22.8 ± 18.3	32.1 ± 27.7	< 0.001
Patient number group of daily service, n (%)				< 0.001
< 20	132 (68.5)	176 (49.7)	367 (37.0)	
20-39	46 (23.9)	114 (32.2)	329 (33.2)	
≥ 40	14 (7.6)	64 (18.1)	295 (29.8)	
Burnout score (mean ± SD)	2.0 ± 1.2	2.4 ± 1.1	2.6 ± 1.1	< 0.001
Burnout category, n (%)				< 0.001
No burnout	70 (36.5)	84 (23.7)	201 (20.3)	
Some burnout symptoms	101 (52.6)	209 (59.0)	555 (56.0)	
Serious burnout	21 (10.9)	61 (17.3)	235 (23.7)	
Mistakes, n (%)				< 0.001
No	116 (60.4)	177 (50.0)	401 (40.5)	
Yes	76 (39.6)	177 (50.0)	590 (59.5)	
Mistakes by subtypes, n (%)				
Patient was harmed	21 (10.9)	35 (9.9)	121 (12.2)	0.484
Medication errors	5 (2.6)	18 (5.1)	77 (7.8)	0.014
Treatment delayed	23 (12.0)	38 (10.7)	155 (15.6)	0.050
Incomplete or incorrect item in patient's records	64 (33.3)	163 (46.0)	530 (53.5)	< 0.001

*The p value means the statistical difference among the three grade hospitals.

to self-reported medical mistakes, 39.6%, 50.0%, and 59.5% of the respondents in primary, secondary, and tertiary hospitals reported having made mistakes over the course of the last year. For subtypes of mistakes, incomplete or incorrect items in patient's records are the most common mistakes (occurrence rates were 33.3%, 46.0%, and 53.5% in primary, secondary, and tertiary hospitals, respectively, $p < 0.001$). Meanwhile, the proportion of medication errors and delayed treatments were higher in tertiary hospitals ($p = 0.014$ and 0.050, separately).

The results of univariate analysis for risk factors associated with medical mistakes are shown in Table 3. Medical mistakes were significantly lower among females (OR = 0.68, 95% CI: 0.55-0.83) and higher among those with a Bachelor's degree (OR = 2.43, 95% CI: 1.35-4.38) and Master's degree or higher (OR = 2.68, 95% CI: 1.48-4.85), those with longer work hours per week, those who reported suffering some burnout symptoms (OR = 1.57, 95% CI: 1.23-2.02), those reporting suffering serious burnout symptoms (OR = 2.79, 95% CI: 2.03-3.83), and those who worked at secondary hospitals (OR = 2.25, 95% CI: 1.63-3.08) and tertiary hospitals (OR = 1.53, 95% CI: 1.07 -2.18).

In the multivariate analysis, being female was protective against medical mistakes (OR = 0.72, 95% CI: 0.58- 0.89), whereas physician -reported 45 to 59 work hours per week (OR = 1.40, 95% CI: 1.08-1.81), physcician-reported 60 or more work hours per week (OR = 1.65, 95% CI: 1.22-2.22), physicians who reported some burnout symptoms (OR = 1.46, 95% CI: 1.13-1.89) and physicians who reported serious burnout (OR = 2.28, 95% CI: 1.63-3.17) and physician who worked at secondary hospitals (OR = 1.58, 95% CI: 1.12-2.23) were independently associated with a higher incidence of medical mistakes (Table 3).

4. Discussion

Physician burnout is not only a critical issue involving physician health, but also that of patient safety. This multicenter cross sectional survey revealed that being male, longer work hours per week, and increased burnout symptoms were all risk factors for medical mistakes. Meanwhile, doctors in tertiary hospitals were most overworked, suffered the most serious burnout, and made the most mistakes, while those in primary hospitals were least overworked, with least burnout symptoms, and made the least mistakes. However, overall, the absolute workload, prevalence of burnout, and medical mistakes among physicians in China were consistently high.

The heavy workload of hospital physicians is a major problem for the Chinese health care system. This study showed that physicians' mean work hours per week were 54.1, similar to another report (*12*). However, the mean work hours reflected the fact that many doctors work long hours each day, and this could potentially impact many components of healthcare such as patient satisfaction, outcomes of disease, prescribing practice, physician satisfaction, and risk of malpractice claims (*13*). Unfortunately, most decision makers and hospital administrators are unaware or have potentially ignored the impact of over-worked healthcare

Table 3. Risk factors of medical mistakes by logistic stepwise regression

Items	Univariate analysis			Adjusted analysis		
	OR	95% CI	*p*	OR	95% CI	*p*
Gender						
Male						
Female	0.68	0.55-0.83	< 0.001	0.72	0.58-0.89	0.003
Age (year)						
< 30						
30-39	1.06	0.83-1.36	0.647			
40-49	0.93	0.70-1.25	0.648			
≥ 50	0.68	0.46-1.01	0.056			
Marital status						
Unmarried/divorced/widowed						
Married	0.91	0.73-1.15	0.439			
Education						
High school or lower						
Some college	1.81	0.95-3.45	0.069			
Bachelor's degree	2.43	1.35-4.38	0.003			
Master's degree or higher	2.68	1.48-4.85	0.001			
Professional title						
Junior						
Middle	1.03	0.82-1.31	0.750			
Senior	0.93	0.72-1.21	0.621			
Work hours per week						
< 45						
45-59	1.70	1.35-2.16	< 0.001	1.40	1.08-1.81	0.010
≥ 60	2.29	1.75-3.00	< 0.001	1.65	1.22-2.22	0.001
No. of daily service patients						
< 20						
20-39	1.10	0.87-1.39	0.411			
≥ 40	0.98	0.75-1.26	0.867			
Burnout						
No burnout						
Some burnout symptoms	1.57	1.23-2.02	< 0.001	1.46	1.13-1.89	0.004
Serious burnout	2.79	2.03-3.83	< 0.001	2.28	1.63-3.17	< 0.001
Hospital level						
Primary						
Secondary	2.25	1.63-3.08	< 0.001	1.58	1.12-2.23	0.010
Tertiary	1.53	1.07-2.18	0.020	1.19	0.82-1.74	0.345

Abbreviations: CI, confidence interval; OR, odds ratio.

providers in China, which has potentially contributed to highly intense doctor-patient relationships and might partially explain why attacks on medical personnel by dissatisfied patients has become more common in China (*14*).

Meanwhile, the number of patients one doctor can manage is another concern. Our research revealed that the average number of patients managed per shift was 27.8. Currently, there are no standard recommendations that address the daily maximum number of patients one doctor should manage per shift. Using data from the online physician network QuantiaMD, Johns Hopkins researchers found physicians said they could safely see 15 patients per shift (*15*). Healthcare providers and administrators need to keep in mind that the goal of healthcare is not just to see patients, but to make the right decisions and management.

Burnout is also common among physicians internationally. In the United States, it is estimated 30% to 40% of clinicians experienced burnout (*16*). Shanafelt and colleagues (*17*) reported that more than 75% of their study participants met the criteria for burnout. Another review presented that there are reports of burnout in 25-60% of doctors (*18*). This empirical study found that overall 76.9% of participants suffered from some burnout symptoms or serious burnout symptoms, demonstrating that burnout among Chinese doctors is an area of concern.

Medical errors or mistakes are another common concern internationally. The IOM revealed that up to 98 000 patients die each year in the hospital as a result of preventable medical errors (*19*). Many aspects of patient care may be compromised by burnout. Physicians who have burnout are more likely to report making recent medical errors or mistakes, and burnout has been associated with reduced patient satisfaction with medical care and patient adherence to treatment plans (*16,20-23*). However, few empirical studies provide solid evidence for the relationship between burnout and medical errors. Our paper revealed that over half of physicians admitted that they had made mistakes over the course of the last year, and

the logistic regression analysis showed that being male, working greater than 45 hours per week, and burnout symptoms were independently associated with increased physician-reported mistakes. The above evidence suggests that medical facility decision makers should consider interventions to reduce clinician workload and associated burnout for the safety of their patients and physicians.

Some of the findings deserve to be discussed here. Logistic regression revealed that medical mistakes were significantly lower among females, which possibly relates to an important female characteristic-carefulness. Though univariate analysis showed that medical mistakes were higher among those with a Bachelor's degree and Master's degree or higher, multivariate analysis denied the relation. Another interesting finding is that after multivariate analysis, physicians who worked at a tertiary hospital were not indicated as an independent risk factor associated with higher incidence of medical mistakes while those who worked at a secondary hospital were identified as a risk factor. Although the physician working at a tertiary hospital showed the highest average workload and burnout symptoms, heavy workload and serious burnout do not necessarily result in medical mistakes. Furthermore, this finding was also potentially associated with the fact that in China tertiary hospitals usually have a higher management capability which could prevent the occurrence of mistakes.

Though several studies have examined physician burnout, no previous work has analyzed the reported burnout prevalence among different hospital levels. A significant finding of this paper is that there were statistical differences in workload, burnout, and mistakes among primary, secondary, and tertiary hospitals in China. The bigger or more advanced hospitals were found to be associated with increased workload, reported burnout, and reported mistakes. There are several factors that might contribute to this finding. On the one hand, China does not have a policy to encourage patients to initially see a doctor in community clinics or a primary hospital, which means a patient can choose any level hospital in which to seek care. Many people in China do not trust the care quality in community clinics or primary hospitals (*1,2*). In some cases, self-treatment is still the first choice for patients, especially in remote rural areas. If self-treatment fails, then a patient might then choose to go to the best hospital (usually a tertiary hospital), if it is accessible and affordable, to see a doctor. These factors have led to shorter available management time for patients at larger hospitals, which can result in inadequate physician-patient communication. The current doctor-patient relationship in China is highly volatile. Doctors have been abused, injured, and even murdered by patients or relatives of patients in hospitals and clinics across the country (*14*). Evidence showed that about 80% of the physical attacks occurred in tertiary hospitals (*24*).

One limitation of this study was that the medical mistakes were self-reported, which might lead to unreliable findings. However, participants are more likely to underestimate rather than overestimate the number of mistakes they made in the past year. Another limitation is that the study design was not random and consisted of a convenient sample which could potentially impact the findings of this paper in that participants were biased in their responses by their inclusion in the study. Nevertheless, our study is a multi-center design and we developed sampling requirements before implementing the formal investigation and thus believe the sample in this paper should be a fair representation of the general population of Chinese physicians.

Currently, patient-centered care is the primary goal of each hospital. The reality of this model will require healthcare providers taking more time to communicate with patients. However, our findings revealed that more work hours were associated with an increase in the incidence of medical mistakes. Therefore, reducing individual clinician work hours is an urgent issue in China. To date, no regulations have been implemented or formally called for regarding the capping of physician work hours in China. Considering the large healthcare demand in China, it will be difficult to take measures to reduce physician workload and burnout within a single hospital. Policy makers and health insurance companies should work in collaboration with hospitals and healthcare provider professional groups to determine optimal workloads, and develop some national guidelines or policies to encourage patients to see doctors in different level hospitals according to their own illness severity. Doing so could finally promote quality of care, prevent burnout, and achieve patient safety.

To better manage physicians, it should be born in mind that heavy workload could be a risk factor for burnout, and both heavy workload and burnout could result in medical mistakes.

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Supplement Data

Supplement Table 1. Original scores of MBI-GS 15 items in different grade hospitals in China (mean ± SD)

Items	Primary (*n* = 192)	Secondary (*n* = 354)	Tertiary (*n* = 991)	Total (*n* = 1,537)
1. I feel emotionally drained from my work	2.50 ± 1.68	3.26 ± 1.65	3.66 ± 1.59	3.42 ± 1.66
2. I feel used up at the end of the day	2.46 ± 1.77	3.50 ± 1.69	3.93 ± 1.59	3.64 ± 1.71
3. I feel tired when I get up in the morning and have to face another day at work	2.45 ± 1.82	3.23 ± 1.79	3.63 ± 1.77	3.38 ± 1.82
4. Working with people all day is a real strain for me	2.37 ± 1.79	3.09 ± 1.81	3.57 ± 1.73	3.31 ± 1.80
5. I feel burned out from my work	1.70 ± 1.79	2.24 ± 1.78	2.63 ± 1.81	2.42 ± 1.82
6. I have become more callous toward work since I took this job	1.83 ± 1.87	2.20 ± 1.76	2.51 ± 1.83	2.35 ± 1.84
7. I have become less enthusiastic about my work	1.60 ± 1.67	2.10 ± 1.81	2.42 ± 1.82	2.25 ± 1.82
8. I doubt the significance of my work	1.63 ± 1.79	2.10 ± 1.86	2.24 ± 1.89	2.13 ± 1.88
9. I have become more and more indifferent in the contribution of my job	1.49 ± 1.68	2.09 ± 1.93	2.31 ± 1.99	2.16 ± 1.96
10. I deal effectively with the problems of clients	3.77 ± 2.11	4.29 ± 1.88	4.50 ± 1.57	4.36 ± 1.73
11. I feel that I am contributing to my company	4.00 ± 2.11	4.32 ± 1.87	4.31 ± 1.81	4.27 ± 1.87
12. In my opinion, I am good at my job	4.06 ± 2.11	4.34 ± 1.79	4.41 ± 1.61	4.35 ± 1.72
13. I feel very happy when I accomplish some tasks of my job	4.00 ± 2.14	4.24 ± 1.83	4.33 ± 1.62	4.27 ± 1.74
14. I have accomplished many worthwhile things in this job	3.71 ± 2.13	4.20 ± 1.82	4.28 ± 1.60	4.19 ± 1.73
15. I am confident that I can accomplish all tasks effectively	4.04 ± 2.05	4.48 ± 1.65	4.35 ± 1.54	4.34 ± 1.64

Items 1 to 5 reflect exhaustion, 6 to 9 refer to cynicism, and 10 to 15 relate to reduced professional efficacy.

Original Article

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Salivary melatonin levels and sleep-wake rhythms in pregnant women with hypertensive and glucose metabolic disorders: A prospective analysis

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Summary **In preeclampsia and gestational diabetes, the sympathetic nerves are activated, leading to disrupted sleep. Melatonin, which transmits information to regulate the sleep-wake rhythm and other such biorhythms, has been implicated in insulin resistance, antioxidant behaviors, and metabolic syndrome. In addition, its reduced secretion increases the risk of hypertension and diabetes. The aim of this study was to elucidate the features of melatonin secretion, sleep quality, and sleep-wake rhythms in pregnant women with complications. Fifty-eight pregnant women with pregnancy complications (hypertensive or glucose metabolic disorders) and 40 healthy pregnant women completed questionnaires, including sleep logs and the Pittsburgh Sleep Quality Index (PSQI), during the second to third trimesters. Their salivary melatonin levels were also measured. Pregnant women with complications had significantly lower morning ($p < 0.001$), daytime ($p < 0.01$), evening ($p < 0.001$), night ($p < 0.01$), daily mean ($p < 0.001$), peak ($p < 0.001$), and bottom ($p < 0.01$) melatonin values than healthy pregnant women. Pregnant women with complications also had significantly smaller melatonin amplitudes than healthy pregnant women ($p < 0.001$). Among pregnant women with complications, the duration ($p < 0.05$) and frequency ($p < 0.01$) of wake after sleep-onset were significantly greater in the poor sleep group than in the favorable sleep group which was divided by PSQI cutoff value. Pregnant women with hypertensive or glucose metabolic disorder complications had smaller circadian variation in salivary melatonin secretion, and their values were lower throughout the day than healthy pregnant women.**

Keywords: Melatonin, preeclampsia, gestational hypertension, gestational diabetes mellitus, sleep-wake rhythm, sleep quality

1. Introduction

The incidence of pregnancy at an advanced maternal age is continuously increasing; however, it is associated with an increased risk of pregnancy-induced hypertension (*1*) and an increased incidence of gestational diabetes mellitus (GDM) (*2*). Under the conditions of preeclampsia (*3,4*) and GDM (*5*), the sympathetic nerves are activated. Moreover, patients with preeclampsia often experience sleep disruptions due to an increased frequency of movement during rapid eye movement sleep (*6*) and those with diabetes tend to have difficulty falling asleep (*7*).

Exposure to light at night due to an irregular sleep schedule increases the incidence of elevated blood pressure (*8*) and pregnancy complications (*9*); therefore, a regular sleep rhythm and quality of sleep are crucial issues during pregnancy. Melatonin, a neuroendocrine hormone secreted from the pineal gland (*10*), reaches maximum secretion levels 3-5 h after usual sleep-

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onset (*11*) and decreases after awakening or upon light exposure (*12*). Melatonin is thus a humoral factor that transmits information to maintain biorhythms, such as the sleep-wake rhythm (*13*). Inhibited melatonin secretion in adult humans has also been associated with the incidence of obesity, metabolic syndrome (*14*), insulin resistance (*15*), and antioxidant activity (*16*). Experiments with rats have shown that melatonin stimulates insulin output and decreases free fatty acid synthesis (*17*). In addition, a large-scale survey of adult women demonstrated an association between low melatonin production and hypertension risk (*18*). Furthermore, the risk of developing diabetes has been reported to be higher among adults when melatonin secretion is low (*19*). However, no previous studies have assessed the association between melatonin secretion and the sleep-wake rhythm in pregnant women with certain complications. Thus, the aim of this study was to elucidate the features of melatonin secretion, sleep quality, and the sleep-wake rhythm in pregnant women with hypertensive or glucose metabolic disorders.

2. Methods

2.1. *Subjects and diagnostic criteria*

This study included 58 pregnant women who were diagnosed with a pregnancy-related complication (hypertensive or glucose metabolic disorder) in the second trimester (n = 35; week 16 day 0 to week 27 day 6) or third trimester (n = 23; week 28 day 0 to week 40 day 6). Patients were diagnosed using specified diagnostic criteria by a hospital obstetrician (*20,21*). All patients received prenatal checkups between February 2009 and April 2012 at a university hospital in the Saitama prefecture and provided informed consent to participate in the present study. Among the 35 women who were diagnosed in the second trimester and participated in the present study, 20 were continuously surveyed until the third trimester. Forty healthy pregnant women in the third trimester (week 28 day 0 to week 40 day 6), who received prenatal checkups between August 2006 and April 2008 at a hospital in Osaka Prefecture, were included as controls and each also provided informed consent to participate in the present study.

The diagnostic criteria for hypertensive disorders included conditions of preeclampsia, gestational hypertension, and chronic hypertension. Glucose metabolic disorders included diabetes mellitus and gestational diabetes mellitus (GDM), which was diagnosed according to the criteria revised in 2010.

2.2. *Survey methods*

Although the onset of pregnancy complications typically occur in the third trimester, the survey was initiated in the second trimester because severe cases develop from the second trimester and often result in delivery before the third trimester. Surveys were administered to the healthy control subjects in the third trimester. All participants who gave informed consent for the study filled out a self-administered questionnaire and took saliva samples themselves at home, and sent these to us by mail. They were required to complete a questionnaire describing their characteristics and lifestyles, the Pittsburgh Sleep Quality Index (PSQI), and sleep logs. Saliva samples were collected for measuring melatonin levels. Information on diagnoses and treatment courses were collected from medical records, as was medical information relating to the type of delivery, gestational weeks, birth weight, delivery abnormalities, and postpartum course.

2.3. *Sleep logs*

Sleep logs are used as a form of auxiliary diagnosis in the field of psychiatry and enable ascertainment of life rhythms (*22*). In the present study, sleep logs, in which the subjects filled in their sleep time every 30 min, were recorded daily for 1 week. Using a sleep-wake rhythm analysis program for Windows Ver. 3.0 (IAC, Tokyo, Japan), we analyzed sleep parameters that included total sleep time (total sleeping hours per day), nocturnal sleep time (sleeping hours per night), the duration of wake after sleep-onset [waking hours in the night (WASO)], the frequency of WASO, longest sleep time (LST), and sleep-onset time for LST.

2.4. *Pittsburgh Sleep Quality Index (PSQI)*

PSQI is a self-administered questionnaire (Likert scale of 0 to 3) developed by the University of Pittsburgh, Department of Psychiatry (Pittsburg, PA, USA) for assessing sleep quality (*23*). A Japanese language edition of PSQI was created by Doi *et al*. (*24*). The following seven components were scored: sleep quality (C1), sleep latency (C2), sleep duration (C3), habitual sleep efficiency (C4, the proportion of hours spent asleep in bed), sleep disturbance (C5), the use of sleeping medication (C6), and daytime dysfunction (C7). These elements were scored from 0 to 3, and the scores were added together to calculate a total PSQI global score (PSQIG, 0-21). For all items, the higher the score, the more sleep was determined to have been disrupted. The cut-off point for the total score was 5.5 points (*23,24*). Patients with a PSQIG < 5.5 points were included in the favorable sleep group, and those with a PSQIG ≥ 5.5 points were included in the poor sleep group.

2.5. *Measurement of melatonin concentration*

Saliva was collected at home 4 times daily for 3 days: before breakfast (6-8 a.m.), lunch (11 a.m.-1 p.m.),

dinner (6-8 p.m.), and before going to sleep. At least 1.0 mL of saliva was collected in a 1.5-mL Safe-Lock Tube (Eppendorf, Hamburg, Germany) and immediately frozen at -20°C. Diurnal variation, acrophase (time of peak), and the amplitude of melatonin levels were analyzed. The melatonin concentration of the supernatant of the thawed and centrifuged (1500 rpm, 15 min, 4°C) saliva was measured using a radioimmunoassay, as described by Samejima *et al*. (*25*), in which the measurement technique described by Miles *et al*. was modified (*26*). Measurements were made in duplicate using the following procedure. Melatonin antibodies (UK Code No. AB/S/01; Stockgrand, Ltd., Surrey, UK) were diluted 6000-fold with 0.1 M tricine (pH 7.5) (Sigma-Aldrich Corp., St. Louis, MO, USA) buffered with 0.9% NaCl and 0.1% gelatin (Sigma-Aldrich Japan K.K., Tokyo, Japan). For a standard solution of melatonin, melatonin powder (M5250-1G, Code No. M5259; Sigma-Aldrich Corp.) was used. For the radioimmunoassay, [^{3}H]-melatonin (GE Healthcare Bio-Sciences, Tokyo, Japan) was used with charcoal (242276-250G activated charcoal DARCO; Sigma-Aldrich Corp.), DextranT-70 (Extrasynthese SA, Genay, France), and scintillation cocktail (Hionic-Fluor; PerkinElmer, Inc., Waltham, MA, USA). All procedures were performed in duplicate. The intra- and inter-assay coefficients of variation in the experiments were 3.5% and 10.7 %, respectively.

2.6. *Statistical analysis*

We used an unpaired t-test to compare mean values between the two groups, a paired t-test to compare mean values for continuous cases from the second to third trimester, the χ^2 test to compare frequency, one-way analysis of variance to confirm circadian variation in the two groups, two-way analysis of variance to compare circadian variation, and Pearson's correlation coefficient to analyze relationships between each of the sleep indicators. All statistical analyses were performed using SPSS J for Windows ver. 22.0 (IBM-SPSS, Inc., Chicago, IL, USA).

2.7. *Ethical Considerations*

The study protocol was approved by the ethics committees of the Osaka University Graduate School of Medicine, Osaka, Japan and the Saitama Medical University, Saitama, Japan.

3. Results

3.1. *Subject attributes*

The ages (mean 35.1 ± 4.2SD), gestational weeks at delivery (mean 38.0 ± 1.3SD), infant weight (mean 2898 ± 434SD), complications (Hypertensive disorder 31, Glucose metabolism disorder 19, both 8), treatments, and outcomes upon delivery of the study subjects are shown in Table 1.

3.2. *Circadian variation in salivary melatonin concentration*

For both pregnant women with complications in either the second or third trimester and healthy pregnant women, melatonin levels were the highest at night, decreased throughout the early morning, and were the lowest during the day, demonstrating significant daily fluctuations ($p < 0.001$) (Figure 1). Pregnant women with complications had significantly lower morning ($p < 0.001$), daytime ($p < 0.01$), evening ($p < 0.001$), night-time ($p < 0.01$), daily mean ($p < 0.001$), peak ($p < 0.001$), and bottom ($p < 0.01$) melatonin levels than healthy pregnant women (Figure 1). The amplitude, or the difference between the highest and lowest melatonin values in one day, was significantly smaller for pregnant women with complications than that for healthy pregnant women ($p < 0.001$). Among pregnant women with complications, the times of peak ($p < 0.05$) and bottom ($p < 0.05$) melatonin levels were significantly later in the third trimester than those in the second trimester (Table 2).

3.3. *Sleep parameters in pregnant women*

There were no significant differences in the analyzed sleep parameters between pregnant women with complications and healthy pregnant women in the third trimester. However, pregnant women with complications had significantly later sleep-onset times ($p < 0.01$), shorter LST ($p < 0.01$), and shorter nocturnal sleep times ($p < 0.01$) in the third trimester than those in the second trimester (Table 3).

3.4. *Comparison of sleep indicators according to PSQI*

Among the pregnant women with complications, 15 (42.9%) were in the poor sleep group (PSQIG score ≥ 5.5) in the second trimester and 23 (53.5%) were in the poor sleep group in the third trimester, while 20 women (50.0%) in the control group were in the poor sleep group during the third trimester. No significant difference in the frequency of poor sleep group and the mean value by PSQI, was observed between pregnant women with complications and healthy pregnant woman in the third trimester. Among pregnant women with complications, the duration and frequency of WASO of the sleep logs were significantly greater in the PSQI poor sleep group than those in the favorable sleep quality group in the second trimester ($p < 0.05$ and $p < 0.01$, respectively). In the third trimester, sleep-onset times were significantly later in the poor sleep group than those in the favorable sleep quality group ($p < 0.05$).

Table 1. Demographic characteristics

Items	Pregnant women with complications (n = 58)					Healthy pregnant women (n = 40)				
	Mean	SD	Range	n	%	Mean	SD	Range	n	%
Age (years)	35.1	4.2	27-45			33.3	4.3	27-43		
Height (cm)	157.4	6.3	142-170			-				
Non-pregnant weight (kg)	66.8	17.1	40.0-106.6			-				
Non-pregnant BMI (%)	26.8	6.2	18.4-41.7			-				
Weight increase (kg)	6.8	5.2	-6.8 to 18.0			-				
Infant weight (g)	2898.3	434.0	1855-4168			3048.2	403.6	2235-3880		
Gestational week (weeks)	38.0	1.3	34-40			39.0	1.5	35-42		
Employment										
Yes				22	37.9				15	37.5
No				34	58.6				25	62.5
Unknown				2	3.4					
Parity										
Primipara				32	55.2				34	85.0
Multipara				26	44.8				6	15.0
Complications										
Hypertensive disorder										
Preeclampsia				5	8.6				-	
GH				5	8.6				-	
HT				21	36.2				-	
Glucose metabolism disorder										
GDM				19	32.9				-	
Both										
HT + GDM				6	10.3				-	
GH + GDM				1	1.7				-	
GH + DM				1	1.7				-	
Treatment during pregnancy										
Yes				41	70.7				-	
No				17	29.3				-	
Type of treatment										
Dietary advice				7	17.1				-	
Oral medicine				18	43.9				-	
Infusion or injections				16	39.0				-	
Delivery type										
Normal delivery				25	43.1				26	65.0
Caesarean section				29	50.0				13	32.5
Forceps delivery				4	6.9				0	0.0
Unknown									1	2.5
Postpartum treatment										
Yes				25	43.1				-	
No				33	56.9				-	

SD, standard deviation; BMI, body mass index; GH, gestational hypertension; HT, chronic hypertension; GDM, gestational diabetes mellitus; DM, diabetes mellitus

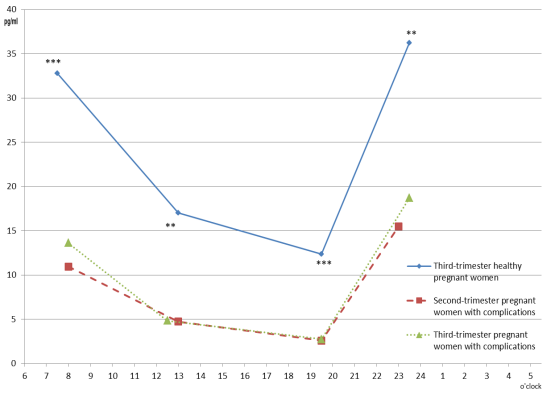

Figure 1. Circadian changes in salivary melatonin levels. Salivary melatonin levels in pregnant women with complications were significantly lower than those in healthy pregnant women at all times (morning, daytime, evening, and nighttime). $^{***}p < 0.001$, $^{**}p < 0.01$

3.5. *Correlation between melatonin values and sleep indicators*

PSQI sleep efficiency (C4) for healthy pregnant women in the third trimester showed a negative correlation with night-time (r = -0.388, $p < 0.05$), peak (r = -0.324, $p < 0.05$), and amplitude (r = -0.324, $p < 0.05$) melatonin values. However, in pregnant women with complications, these correlations were not observed in either the second or third trimester.

4. Discussion

This is the first study to analyze long-term melatonin secretion, sleep-wake rhythms, and sleep quality in pregnant women with hypertensive or glucose

Table 2. Melatonin indicators

Items	Second-trimester pregnant women with complications (n = 35)		Third-trimester pregnant women with complications (n = 43)		Third-trimester healthy pregnant women (n = 40)		p-value
	Mean	SE	Mean	SE	Mean	SE	
Daily mean melatonin (pg/mL)[†]	8.4	1.2	10.2	1.4	23.8	2.6	< 0.001
Peak value (pg/mL)[†]	20.1	2.8	22.5	2.9	46.4	4.4	< 0.001
Bottom value (pg/mL)[†]	1.7	0.4	2.4	0.5	7.3	1.3	< 0.01
Amplitude (pg/mL)[†]	18.4	2.6	20.0	2.6	39.2	3.8	< 0.001
Peak value time (o'clock)[‡]	24.2	0.9	27.0	0.7	25.6	1.0	< 0.05
Bottom value time (o'clock)[‡]	15.6	0.7	17.7	0.6	16.9	0.7	< 0.05

[†]Comparison between third-trimester pregnant women with complications and healthy pregnant women using the unpaired t-test. [‡]Comparison between second- and third-trimester pregnant women with complications using the unpaired t-test. SE, standard error.

Table 3. Sleep log indicators

Items	Second-trimester pregnant women with complications (n = 35)		Third-trimester pregnant women with complications (n = 43)		p-value	Third-trimester healthy pregnant women (n = 40)	
	Mean	SE	Mean	SE		Mean	SE
Total sleep time (h)	8.1	0.2	7.6	0.2	NS	7.8	0.2
Nighttime sleep time (h)	7.2	0.2	6.5	0.2	< 0.01	6.8	0.2
Duration of wake after sleep-onset (min)	20.4	4.6	25.2	5.8	NS	25.4	6.9
Frequency of WASO (times)	0.2	0.0	0.3	0.1	NS	0.3	0.1
Longest sleep time (h)	7.1	0.2	6.2	0.2	< 0.01	6.6	0.2
Sleep-onset time for LST (o'clock)	23.7	0.2	24.5	0.2	< 0.01	24.4	0.2

Comparison between the second- and third-trimester pregnant women with complications using the unpaired t-test. Comparison between the third-trimester pregnant women with complications and healthy pregnant women using the unpaired t-test. SE, standard error; WASO, waking hours after sleep onset; LST, longest sleep time; NS, not significant

metabolic disorders from the second to third trimester.

We measured salivary melatonin levels as a physiological indicator of sleep. Serum melatonin levels fluctuate throughout the day and peak at night (*27*). In the present study, we measured melatonin using saliva samples rather than blood samples because the salivary melatonin assay measured biologically active melatonin, since only free melatonin passes through the parotid membrane. Moreover, the collection of saliva is easier and less invasive than blood sampling at home.

Our study first elucidated that pregnant women with hypertensive or glucose metabolic disorder complications had lower melatonin secretion throughout the day (morning, daytime, evening, and night-time) than healthy pregnant women in both the second and third trimesters. Similar results were obtained with daily mean, peak, and bottom melatonin levels, indicating that pregnant women with complications have inhibited melatonin secretion throughout the day by measuring circadian variation in salivary melatonin secretion four times a day.

Melatonin is a humoral factor that controls the circadian rhythm of living organisms over approximately 24 hour cycles including the sleep-wake cycle, with high levels of melatonin observed after entering sleep at night. Insulin concentration, which adjusts blood glucose levels and blood pressure varies during this 24-hour cycle, with low levels reached at night. There are three types of melatonin receptors, comprising MT1, MT2 and MT3 receptors. When MT2 receptors bind with melatonin, this results in adjustment to the biological clock. There are also melatonin receptors present in the beta cells of the pancreas. MT2 receptors in beta cells of the pancreas are involved in insulin secretion adjustment as they block cyclic adenosine monophosphate (cAMP), an important metabolism signal that controls insulin secretion, and induce the formation of cyclic guanosine monophosphate (cGMP) (*28*).

However, genetic mutation in the MT2 receptors prevents them from responding to melatonin, thereby blocking insulin release from beta cells in the pancreas and increasing risk for type 2 diabetes. This has been confirmed by genetic analysis of approximately 12,000 subjects (*29*). Blood pressure exhibits diurnal variation over a 24-hour cycle mainly due to autonomic nervous action. Various factors are related to hypertension. These are thought to include, in addition to excessive sodium intake and autonomic nervous irregularity (*30*), disturbance of the biological clock and decreased melatonin secretion (*31*). Melatonin, for which high levels are observed at night during sleep, causes vasodilation, suppression of the sympathetic nervous system and hypotension (*32*). Melatonin increases cytoplasmic calcium and nitric monoxide in endothelial cells, thereby increasing vasodilation lowering the serum norepinephrine level. Therefore, low levels

of melatonin may be an onset factor for pregnancy complications, which exhibit pathophysiology that is similar to type 2 diabetes and hypertension (*33*).

Melatonin reportedly protects cellular apoptosis in the placenta by acting as an antioxidant (*34*) and a free radical scavenger (*35*). Melatonin also inhibits the vasospasticity of the umbilical artery (*36*), and when administered, possibly prevents the risk of preeclampsia (*37*). These findings suggest that the risk of preeclampsia is higher when melatonin secretion is decreased. Meanwhile, when melatonin secretion is reduced, the action of insulin receptors is negatively affected in adults (*38*), thereby increasing insulin resistance in non-pregnant women (*15*). Therefore, there is an increased risk of diabetes when melatonin secretion is reduced, which presumably also increases the risk of gestational diabetes in pregnant women.

In the present study, we observed later times of peak and bottom melatonin values in the third trimester compared with those in the second trimester; melatonin secretion rhythm lagged as a result of the significant delay in third trimester sleep-onset times. The amplitude in salivary melatonin secretion was decreased in pregnant women with complications in the third trimester in the present study and was likely because melatonin decreases after awaking, upon light exposure (*12*), or when sleep is shallow (*39*), and the sleep state often becomes shallow by polysomnography in the third trimester (*40*).

Healthy pregnant women with greater sleep efficiency had longer sleep times at night and greater melatonin peak values and amplitude. However, this trend was not observed in pregnant women with complications, as this group demonstrated shorter nocturnal sleep times and LST in the third trimester. This may have been because the sympathetic nervous system is dominant in women with gestational diabetes and preeclampsia (*4,30*), meaning that secretion of the sleep-inducing substance melatonin at night, when the parasympathetic nervous system becomes dominant, was lower in pregnant women with complications than in healthy pregnant women in this study. This meant that they had poorer quality sleep and that healthy sleep efficiency and nighttime sleeping were impaired. Poor sleep efficiency, a PSQI component, due to arousal during sleep or difficulty falling asleep resulted in decreased melatonin secretion. However, melatonin secretion in pregnant women has been reported to correlate with melatonin secretion in cord blood (*41*) and to impact the production of fetal melatonin receptors through the placental stage (*42*), suggesting that melatonin secretion in pregnant women impacts the sleep-wake rhythm in infants after birth.

Therefore, promoting ample melatonin secretion by adjusting the sleep-wake rhythm during pregnancy and improving sleep quality is important not only to prevent hypertensive and glucose metabolic disorders but also to develop the sleep-wake rhythm in the fetus. Enhancing melatonin secretion requires strategies to increase sleep efficiency. For example, we recommend the following to improve sleep efficiency: darkening the room early at night (*12*), bathing before bed because body temperature and melatonin secretion are related (*43*), going to the toilet before bed to reduce WASO caused by frequent urination (*40*), going to bed early to increase sleeping hours (*44*), and maintaining a regular daily life rhythm.

There were certain limitations to the present study that should be addressed. First, we could not clarify the features of melatonin secretion associated with each diagnosis (hypertension and diabetes) because of the small number of subjects. In addition, the present study did not include matched controls because the control group only included healthy pregnant women in the third trimester.

5. Conclusion

Pregnant women with hypertensive or glucose metabolic disorders had smaller circadian variation in salivary melatonin secretion, and their melatonin values were lower throughout the day than healthy pregnant women. Moreover, pregnant women with these complications exhibited shorter nocturnal sleep times and LST. Overall, the sleep quality of both pregnant women with complications and healthy pregnant women was worse compared with that of non-pregnant women.

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Original Article

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Efficacy and safety of antiretroviral regimens including raltegravir to treat HIV-infected patients with hemophilia

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Summary **When treating HIV-infected patients with hemophilia, adverse drug reactions and interactions and the effect of treatment on bleeding disorders must be considered. Raltegravir is the first HIV integrase inhibitor, but its use in patients with hemophilia is rarely reported. Nine HIV-positive patients with hemophilia were retrospectively studied with a focus on the virological response, changes in the CD4 count, the tendency to bleed, and the response to replacement therapy before and after raltegravir-based antiretroviral therapy (ART). The nine patients were highly treatment-experienced patients and they received raltegravir-based ART for at least nine months. The patients had their own reasons for changing to raltegravir-based ART. During treatment, the CD4 count increased progressively in four patients, with a median absolute increase of 233 cells/mm^3, while the count stabilized in the remaining five patients. Two previous recipients of lopinavir/ritonavir (LPV/r) who failed to respond to lamivudine (3TC) + zidovudine (ZDV) + efavirenz (EFV) had a viral rebound. Genotyping indicated multidrug resistance to nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). A pattern of resistance to raltegravir was evident, including the primary mutation N155H and the secondary mutation T97A. In the two patients, the tendency to bleed decreased markedly and monthly usage of clotting factor VIII decreased significantly decreased. In the remaining seven patients, the viral load remained < 40 copies/mL, there was no evidence of an increased tendency to bleed, and no evidence of changes in the response to replacement therapy. All of the patients had a stable condition with no signs of disease progression and no serious adverse reactions. Results indicated that Raltegravir-based therapy offered a safe and well-tolerated option for HIV-positive patients with hemophilia.**

Keywords: Raltegravir, hemophilia, HIV, treatment, efficacy, safety

1. Introduction

Antiviral treatment for HIV-positive patients with hemophilia presents numerous problems in terms of safety and effectiveness. Most of these patients have been infected with the hepatitis C virus (HCV) *via* blood or sex, and HCV causes varying degrees of liver damage, They would receive PEG-IFN treatment plus ribavirin, HIV-positive patients with hemophilia are more prone to osteoporosis (*1*) and abnormal lipid metabolism (*2*) than patients with an HIV infection alone, and their tendency to bleed varies. When antiretroviral therapy (ART) is administered, adverse drug reactions and interactions and the effect of that therapy on bleeding disorders must be considered.

The emergence of new drugs targeting new sites during the HIV replication cycle has led to tremendous changes in therapy to manage HIV over the past few years (*3*). Raltegravir is the first HIV integrase inhibitor. Raltegravir was originally approved for the treatment of multidrug-resistant HIV and raltegravir has been shown to be generally safe and well-tolerated (*4*). However,

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the use of raltegravir in patients with hemophilia is rarely reported. The current study analyzed the safety and efficacy of raltegravir-based ART in patients with hemophilia.

2. Materials and Methods

2.1. *Patient population and data collection*

The use of raltegravir-based ART was retrospectively analyzed in HIV-positive patients with hemophilia. All of the patients examined in this study have a documented history of HIV infection (over 10 years) and tested positive for anti-HCV antibodies and/or HCV RNA. Each patient had received ART before and was switched to raltegravir-based ART for different medical reasons for at least 9 months. All of the patients were instructed to take a 400-mg tablet of raltegravir twice daily at approximately 12-hour intervals, regardless of whether a meal was scheduled or not.

Clinical data on the patients were obtained from their original records using a data abstraction sheet, and medical charts and laboratory findings were retrospectively reviewed before and during treatment with raltegravir. The specific focus of this study was on virological response and changes in the CD4 count from the baseline. Clinical status was assessed based on clinical data and efficacy was assessed based on virological and immunological outcomes.

Safety data were collected for all patients once raltegravir was initiated. Clinicians were asked to assess the potential relationship between treatment and adverse events. If adverse events were deemed to be associated with the drug, they were recorded. Moreover, the use of coagulation factor VIII was calculated to assess the tendency to bleed and the response to replacement therapy.

2.2. *Analysis of drug resistance*

Negative plasma viremia was defined as plasma HIV RNA below 40 copies/mL, which was the detection limit of the HIV RNA assay. Virological response was defined as two consecutive measurements of HIV RNA < 40 copies/mL at least one week apart, and virologic failure was confirmed by a rebound in HIV RNA > 40 copies/mL. After treatment failure, the genotype of the HIV was determined.

Polymerase Chain Reaction and Sequencing HIV RNA was extracted from plasma as previously described by Boom *et al*. (*5*), followed by a reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR of fragments of the pol gene encoding the protease (PR) enzyme, the reverse transcriptase (RT) enzyme, and the integrase (IN) enzyme, as described below. One-tube RT-PCR was performed in accordance with the manufacturer's recommendations (One Step RT-PCR; TaKaRa). After running fragments on an agarose gel, DNA fragments of the expected size were isolated and gel was extracted using the QIA quick spin gel extraction kit (QIAGEN, Valencia, CA). The forward and reverse strands of the amplified products were sequenced.

To amplify the pol gene fragments encoding the PR, RT, and IN (HXB2 positions 2068-5221, 3154 base pairs [bp]), the primers Pol-1e (5'- TGG AAA TGT GGA(G) AAG(A) GAA(G) GGA C-3' , HXB2 positions 2029-2050) and Pol-x (5'- CCT GTA TGC AG(A) A(C)C CCC AAT ATG TT-3', HXB2 positions 5241-5265) were used for 1-tube RT-PCR, followed by nested PCR using the primers Pol-3 (5'-ACT GAG AGA CAG GCT AA TTT AGG GA-3′HXB2 positions 2068-2095) and Pol-4e (5'- CTC CTA GTG GGA TRT GTA CTT CYG ARC TTA-3' HXB2 positions 5221-5192).

To determine the resistance mutations associated with drugs, the pol gene sequences were entered in the Stanford DB database (*http://hivdb.stanford.edu*). This program provides analysis of the sequences of the PR, RT, and IN genes of HIV in comparison to existing published sequences of these genes.

2.3. *Measurement of the CD4 count*

EDTA-anticoagulated blood samples were subjected to flow cytometry (BD Company, USA, CYTOMICS-FC500) at the Shanghai Public Health Clinical Center (SHAPHC) to determine the CD4 count. In this study, the baseline CD4 count was defined as the last count measured up to one month before raltegravir-based ART began.

2.4. *Ethical considerations*

The study was approved by the Ethics Committee of the SHAPHC. Signed informed consent was obtained from all participants in accordance with this study's protocol.

3. Results

3.1. *Clinical information on patients*

In this study, information on nine HIV-positive patients with hemophilia was examined at the SHAPHC. All of the patients received ART and all were regularly followed-up.

Changes in treatment regimens and patient baseline characteristics are shown in Table 1. All of the patients had a stable condition, as indicated by a viral load < 40 copies/mL, a CD4 count > 200 cells/mm^3, and co-infection with HCV. None of the patients had cirrhosis or decompensated liver disease. Seven patients had received PEG-IFN plus ribavirin therapy and had achieved a sustained virologic response (SVR), the remaining two patients being treated. Information on

Table 1. Baseline characteristics of the 9 patients examined in this study

Patient No.	HIV RNA* (copies/mL)	CD4+ cell count before*/after (cells/mm^3)	HCVRNA* (copies/mL)	Anti-HCV*	ALT* (U/L)	Cr* (mmol/L)	TG* (mmol/L)
1	< 40	442/447	< 1,000	+	24	61	1.46
2	< 40	514/723	< 1,000	+	65	86	1.16
3	< 40	396/653	< 1,000	+	28	70	2.87
4	< 40	388/655	< 1,000	+	14	56.7	3.19
5	< 40	244/266	< 1,000	+	52	63	1.24
6	< 40	512/627	1.14e6	+	41	78	0.67
7	< 40	433/467	< 1,000	+	82	112	5.41
8	< 40	541/555	2.06e7	+	50	71	1.77
9	< 40	766/729	< 1,000	+	16	44	1.86

*Upon introduction of raltegravir-based antiretroviral therapy.

Table 2. Information on prior antiretroviral therapy and regimens including raltegravir

Patient No.	Age (years)	ART (before)	Reason	ART (including RAL)	Follow-up (months)	Degree of hemophilia
1	56	EFV+3TC+ZDV	nightmares, severe anemia	RAL+3TC+TDF	24	severe
2	61	EFV+3TC+D4T	depression, insomnia, numbness	RAL+3TC+TDF	20	severe
3	38	EFV+3TC+TDF	severe osteoporosis	RAL+ 3TC+ EFV	14	severe
4	46	LPV/r+3TC+TDF	bleeding from the psoas	RAL+3TC+TDF	12	severe
5	35	LPV/r+3TC+TDF	bleeding from the knee	RAL+3TC+TDF	12	severe
6	52	EFV+3TC+ZDV	depression	RAL+3TC+TDF	12	severe
7	41	EFV+3TC+ZDV	insomnia, anxiety	RAL+3TC+ZDV	14	severe
8	44	EFV+3TC+ZDV	insomnia, , dizziness	RAL+3TC+ZDV	14	severe
9	27	LPV/r+3TC+TDF	intracranial hemorrhage	RAL+3TC+TDF	9	severe

Note: ART, antiretroviral therapy; EFV, efavirenz; 3TC, lamivudine; ZDV, zidovudine; D4T, stavudine; TDF, tenofovir; LPV/r, lopinavir/ritonavir; RAL, raltegravir.

prior ART and raltegravir-based ART is shown in Table 2. Three patients switched treatment due to severe spontaneous bleeding or an increased tendency to bleed and a lower response to replacement therapy for LPV/r, five patients had adverse reactions to EFV, such as depression, insomnia, or nightmares, and one patient had severe osteoporosis due to tenofovir (TDF). The duration of raltegravir treatment ranged from 9 to 24 months (mean: 14.6 months). The most commonly used antiretrovirals in optimum background treatment were 3TC, TDF, and EFV.

3.2. *The efficacy of and adverse reactions to raltegravir-based ART*

During treatment, none of the patients died, none developed AIDS-related opportunistic infections, AIDS-related tumors, or clinical symptoms of immune deficiency, and none had a lower CD4 count. In contrast, four patients had a progressive increase in their CD4 count, with an average absolute increase of 233 cells/mm^3 (mean: 212 cells, range: 115-267 cells). In the other five patients, the CD4 count stabilized at the previous level (Table 1). Moreover, seven patients had a sustained viral load below 40 copies/mL after the introduction of raltegravir. However, two patients had a rebound in HIV. Both had been treated with 3TC + ZDV + EFV and both exhibited virologic failure, so the treatment was changed to 3TC + TDF + LPV/r. The subsequent load of HIV RNA was below 40 copies/mL. Due to severe bleeding and the lower response to replacement treatment the treatment regimens were changed to 3TC + TDF + raltegravir, but virologic failure occurred again, as indicated by a viral load of 2,640 copies/mL at 3 months, 11,600 copies/mL at 6 months and 22,000 copies/mL at 9 months in one patient, in the other patient, the viral load was 6,800 copies/mL 12 months after the introduction of raltegravir.

Anti-HCV therapy failed in the Patient 8 within one year of treatment, and the HCV RNA load in that patient remained at about 10^5copies/mL. In contrast, a SVR was achieved in Patient 6.

Adverse reactions that are commonly associated with raltegravir include diarrhea, nausea, and headaches. Overall, there were no serious adverse events and no adverse reactions associated with raltegravir except for temporary joint pain and muscle soreness in two patients. There was no worsening of lipodystrophy or liver function (data not shown). After the change from LPV/r to raltegravir, three patients had less of a tendency to bleed and the response to coagulation factor VIII therapy improved significantly. Monthly usage of coagulation factor VIII by each patient also decreased significantly (data not shown). In addition, there was no increase in the frequency of bleeding or any change in the efficacy of replacement therapy with clotting factors in the other six patients.

Table 3. Antiretroviral regimens and respective integrase mutations in the two patients on a regimen including RAL

Patient	Therapeutic regimen	Viral load	Exposure time to RAL (m)	Primary mutation	Secondary mutation	Additional mutation
1	ZDV+3TC+EFV	rebound				S119R, K156N, T125S
	3TC+TDF+ LPV/r	< 40copies/mL				
	3TC+TDF+RAL	22,000 copies/mL	9	N155H	T97A	S119R,K156N
2	ZDV+3TC+EFV	rebound				S119R, K156N
	3TC+TDF+ LPV/r	< 40 copis/mL				
	3TC+TDF+RAL	6,800 copies/mL	12	N155H	T97A	S119R,K156N

Note: ZDV, zidovudine; 3TC, lamivudine; EFV, efavirenz; TDF, tenofovir; LPV/r, lopinavir/ritonavir; RAL, raltegravir.

3.3. *Analysis of drug resistance*

Resistance mutation sites were examined in two patients after virologic failure of the first-line drug and raltegravir-based ART. The virus was resistant to NRTIs (resistance mutations: A62V, D67N, K70R, V75I, M184V, and K219E) and NNRTIs (resistance mutations: V90I, K103EK, and G190Q). Major or minor resistance mutations to protease inhibitors (PIs) and primary or secondary resistance mutations to integrase inhibitors (INIs) were not noted, but the additional mutations S119R, K156N, and T125S were noted.

After resistance to raltegravir-based ART was noted, the NRTI resistance mutations A62V, D67N, T69N, K70R, V75I, M184V, and K219E and the NNRTI resistance mutations V90I and G190Q were detected. In contrast, the mutation K103EK was no longer evident. Analysis of raltegravir resistance profiles revealed a pattern of mutation, including a primary mutation, N155H, and a secondary mutation, T97A. The primary mutation N155H and the secondary mutation T97A were accompanied by additional mutations, S119R and K156N, in two patients (Table 3).

4. Discussion

Raltegravir is a first-in-class HIV-1 integrase inhibitor that is safe and efficacious for both initial treatment and treatment of treatment-experienced patients with HIV-1 infection. The antiviral mechanism of raltegravir is its inhibition of the integration of viral DNA into the host cell nucleus (*6*).

Although the current sample size was small and cannot accurately yield results for all patients, HIV-positive patients with hemophilia had a stable or increased CD4 count, effective antiviral efficacy, and a stable clinical status during raltegravir-based ART.

PI might cause a higher risk of bleeding in patients with hemophilia. Of the nine current patients, three had a significant tendency to bleed, severe spontaneous bleeding, and a reduced response to replacement therapy with an ART regimen including LPV/r, so that therapy was discontinued. After the change to raltegravir-based ART, the patients had less tendency to bleed and a significantly improved response to coagulation factor VIII treatment. The monthly use of factor VIII decreased substantially.

Two patients had a virological rebound after raltegravir-based ART, so the virus infecting these patients presumably had resistance mutations. The virus was resistant to NRTIs and NNRTIs but sensitive to PI. No primary or secondary mutations were noted but additional mutations were noted when strains that were resistant to first-line drugs were genotyped. The additional mutations are associated with resistance to several INIs, both in vitro and vivo, but whether they are associated with clinical resistance to raltegravir is unclear (*7-9*).

Primary mutations in the catalytic domain of integrase reduced the susceptibility of HIV to raltegravir through three dependent pathways: Q148R/K/H, N155H, and Y143R/C. Secondary mutations can further increase the extent of raltegravir resistance and improve viral fitness in some cases. Analysis of resistance mutations to raltegravir revealed a pattern of mutation, including a primary mutation, N155H, and a secondary mutation, T97A, in two patients.

Several studies have indicated that a few drug-resistant HIV-1 strains present at the baseline, detectable by highly sensitive genotyping, might play an important role in the occurrence of virologic failure in patients treated with PIs or RT inhibitors (*10,11*). However, the link between baseline mutations and future raltegravir resistance has not been confirmed. A potential limitation of the current study is that population sequencing was used. Primary resistance mutations could be detected through use of a more sensitive method. Secondary and additional mutations are detected more frequently in baseline samples from therapy-naive and treatment-experienced patients (*12-14*). The frequency of all detected mutations was < 1% of the viral population, but the frequency of variation was similar in patients that responded to raltegravir and patients that did not respond to raltegravir, suggesting that these low-frequency resistance mutations do not significantly result in treatment failure. However, a point worth noting is that more secondary mutations were found at the baseline in the patients who failed to respond to treatment than in patients who responded to treatment, although the difference in the frequency of mutations was not statistically significant. Secondary mutations at the baseline were associated only with the appearance

of primary resistance mutations and served to identify patterns of mutations. Pre-existing minor mutations associated with resistance to raltegravir can appear in a large proportion of the viral population under drug selection pressure in a small subset of patients who do not respond to treatment.

The viral load was < 40 copies/mL and the virus was resistant to NRTIs and NNRTIs in two patients. Additional mutations in the virus were present before raltegravir-based ART began, and antiviral treatment of those two patients indicated that additional mutations may be related to raltegravir resistance or pre-existing mutations associated with resistance to raltegravir may appear in the viral population under drug selection pressure. To understand this issue, a longitudinal follow-up of a large number of patients treated with raltegravir needs to be conducted using highly sensitive methods.

Raltegravir-based ART was generally well-tolerated, and transaminase levels, kidney function, and TG levels were not affected by raltegravir administration. Only two patients suffered muscle aches and joint pain clearly related to raltegravir. In addition, the frequency of bleeding and bleeding patterns did not change and the response to replacement therapy did not decrease in patients who had not received LPV/r before.

Conclusion: Raltegravir-based therapy is safe, it causes few adverse reactions, it is well-tolerated, and it is an effective option for initial treatment of HIV-positive patients with hemophilia. However, sequencing of drug resistance genes is required when using raltegravir as salvage therapy, so raltegravir should be used in combination with other effective drugs.

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Original Article

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Allograft inflammatory factor-1 in the pathogenesis of bleomycin-induced acute lung injury

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Summary **Allograft inflammatory factor-1 (AIF-1) is a protein expressed by macrophages infiltrating the area around the coronary arteries of rats with an ectopic cardiac allograft. Some studies have shown that expression of AIF-1 increased in a mouse model of trinitrobenzene sulfonic acid-induced acute colitis and in acute cellular rejection of human cardiac allografts. These results suggest that AIF-1 is related to acute inflammation. The current study used bleomycin-induced acute lung injury to analyze the expression of AIF-1 and to examine its function in acute lung injury. Results showed that AIF-1 was significantly expressed in lung macrophages and increased in bronchoalveolar lavage fluid from mice with bleomycin-induced acute lung injury in comparison to control mice. Recombinant AIF-1 increased the production of IL-6 and TNF-α from RAW264.7 (a mouse macrophage cell line) and primary lung fibroblasts, and it also increased the production of KC (CXCL1) from lung fibroblasts. These results suggest that AIF-1 plays an important role in the mechanism underlying acute lung injury.**

Keywords: Allograft inflammatory factor 1, AIF-1, acute lung injury, IL-6, TNF-α, KC

1. Introduction

Acute lung injury (ALI) and its severe manifestation, acute respiratory distress syndrome (ARDS), are life-threatening inflammatory conditions that involve acute respiratory failure (*1,2*). Lung injury causes the release of pro-inflammatory cytokines and the recruitment of neutrophils in lung tissue (*3-6*). Activation of cytokines and infiltrating neutrophils damage the alveolar-capillary barrier and lead to subsequent pulmonary fibrosis (*7-9*). Despite recent advances in studies of the pathogenesis of ALI and ARDS, the molecular mechanism that initiates ALI and ARDS has not been elucidated in detail.

Allograft inflammatory factor 1 (AIF-1) is a 17-kDa Ca^{2+}-binding EF-hand intracellular protein that is encoded by the HLA class III genomic region (*10-12*). AIF-1 was originally cloned from activated macrophages in atherosclerotic allogenic heart grafts undergoing chronic immune rejection in a rat (*13*). Although the expression of AIF-1 has been noted in various tissues such as the testes, spleen, lymph nodes, lungs, thymus, and synovium, the detailed role of AIF-1 remains unclear (*10,14-16*).

The expression of AIF-1 increased two-fold in acutely rejected liver allografts in comparison to accepted liver grafts (*17*). Studies have shown that expression of AIF-1 increased in a model of trinitrobenzene sulfonic acid-induced acute colitis and in acute cellular rejection of cardiac allografts (*18,19*). *In vitro*, CRL-2192 (a rat macrophage cell line) expressed a certain level of endogenous AIF-1, and this expression was enhanced with the pro-inflammatory cytokines IL-1β and TNF-α (*20*). AIF-1-transfected CRL-2192 cells had enhanced levels of monocyte chemoattractant protein 1 and enhanced cell migration (*20*). AIF-1-transfected RAW 264.7 cells had enhanced production of the inflammatory cytokines IL-6 and IL-12 as a result of stimulation with a lipopolysaccharide (*21*). These

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findings suggest that AIF-1 plays a crucial role in the mechanism of inflammation. The present study used a model of bleomycin-induced lung injury to examine the expression and function of AIF-1 in ALI.

2. Materials and Methods

2.1. *Bleomycin-induced ALI*

C57BL/6 mice (8-10 weeks of age) were purchased from Shimizu Laboratory Supplies Co., Ltd. Mice were anesthetized by intraperitoneal administration of pentobarbital. Thirty μL of bleomycin hydrochloride (Nippon Kayaku Co, Tokyo, Japan) solution containing 2.15 U/kg of bleomycin dissolved in sterile saline was injected directly into the trachea using a 0.9-mm feeding needle (KN-348 Natsume Seisakusho Co., Tokyo, Japan). Control mice received the same volume of sterile saline alone. Mice were sacrificed on Day 1 by intraperitoneal administration of excess pentobarbital (120-150 mg/kg). Mice were euthanized if their body weight fell below 80% of that measured at the baseline. The study was approved by the Animal Research Committee, Graduate School of Medical Science, Kyoto Prefectural University of Medicine.

2.2. *Histology*

The lungs were excised on Day 1 after intratracheal administration of bleomycin and immediately fixed with 4% paraformaldehyde. The samples were then embedded in paraffin and stained with hematoxylin and eosin (H&E) method. Images were acquired using a DMBA210 microscope (Shimadzu Rika, Tokyo, Japan) equipped with Motic Images Plus2.2s software (Shimadzu Rika).

2.3. *Immunohistochemical analysis*

The lungs were excised on Day 1 after intratracheal administration of bleomycin and immediately fixed with 4% paraformaldehyde. Paraffin-embedded sections were deparaffinized in xylol and rehydrated through a graded series of ethanol solutions. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 30 min. Sections were stained with anti-AIF-1 antibody (Sigma) (1:150), anti-F4/80 antibody (Serotec) (1:100) or normal goat IgG isotype control overnight followed by MAX-PO (Nitirei Bioscience) for 30 min. Finally, sections were stained with diaminobenzidine (Nitirei Bioscience). All sections were counterstained with hematoxylin for 2 min.

2.4. *Bronchoalveolar lavage fluid (BALF)*

BALF analyses were performed on Day 1 after intratracheal administration of bleomycin as previously described (*22*). Briefly, the lungs and trachea were exposed and a 20-gauge intravenous catheter was inserted into the trachea. A total of 1 mL of PBS was instilled three times and withdrawn from the lungs *via* an intratracheal cannula. More than 95% of the fluid was recovered as BALF, which was then centrifuged at 1,000 rpm for 5 min at 4°C. The supernatant was collected and stored at -80°C for use in ELISA. The levels of AIF-1, IL-6, TNF-α, and KC in the BALF were measured using commercial ELISA kits (an AIF-1 ELISA kit; USCN Lifescience, an IL-6 ELISA kit and a TNF-α ELISA kit; eBioscience, an KC ELISA kit; R&D Systems), according to the manufacturer's instructions. The optical density was measured at 450 nm using a SoftMaxPro40 plate reader. Each measurement was determined in three separate experiments on BALF. The total number of cells in BALF was counted using a Fuchs-Rosenthal hemocytometer (ERMA Inc., Tokyo, Japan). The BALF solution was placed in a cytospin (Cytospin 2; Shandon Inc., Pittsburgh, PA, USA), centrifuged at 700 rpm for 10 minutes, and stained with Diff-Quick (Sysmex, Kobe, Japan). The number of total cells, neutrophils, and macrophages were counted. At least 200 cells per slide were evaluated on the basis of morphological criteria using a light microscope.

2.5. *Isolation of primary lung fibroblasts*

Primary lung fibroblasts were isolated from C57BL/6 mice and cultured as previously described (*23,24*). Briefly, normal C57BL/6 mice (8-10 weeks-of-age) were sacrificed by intraperitoneal administration of excess pentobarbital and lung tissue was promptly collected, washed with phosphate buffered saline (PBS), and cut into approximately 2.0-mm^3 blocks. The blocks were seeded onto the bottom of a culture flask containing RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and the fibroblasts were cultured at 37°C in a 5% CO_2 incubator. The medium was changed every 2-3 days. Lung fibroblasts from three to five passages were used for all experiments.

2.6. *Cell culture*

A mouse macrophage cell line, RAW 264.7, was obtained from the RIKEN cell bank (Tsukuba, Japan). RAW264.7 cells were seeded in 12-well plates (5×10^5 cells/well) and primary lung fibroblasts were seeded in 12-well plates (2×10^5 cells/well) in RPMI-1640 supplemented with 10% FBS for 24 h. Media were replaced with serum-free RPMI-1640 and the cells were serum-starved overnight. Next, the cells were stimulated for 24 h by addition of RPMI-1640 supplemented with various concentrations of recombinant AIF-1 (CUSABIO, endotoxin removed) or a lipopolysaccharide (LPS) (Sigma). The cell culture supernatant was collected and stored at -80°C until required. The levels of IL-6, TNF-α

and KC in the cell culture supernatant were measured using commercial ELISA kits. The optical density was measured at 450 nm using a SoftMaxPro40 plate reader. Each measurement was determined in three separate experiments.

2.7. *Quantitative real-time RT-PCR analyses*

On Day 1 after intratracheal administration of saline or bleomycin, cells were seeded at a concentration of 1 × 10^5 cells/well in 24-well plates and cells were incubated in RPMI-1640 supplemented with 10% FBS for 24 h. The next day, the medium was washed with PBS three times and only cells adhering to the bottom of plates (alveolar macrophages) were collected. Total RNA was obtained from the macrophages with an RNeasy Plus Mini Kit (Qiagen). Quantitative real time RT-PCR was performed on StepOne™ & StepOnePlus™ Real-Time PCR Systems (Life Technologies) using the Thunderbird Probe qPCR Mix (TOYOBO). The following TaqMan expression assays were used: Mm00479862_g1 (AIF-1), Mm99999915_g1 (GAPDH). Results in duplicate were normalized to GAPDH expression. Data are expressed as the mean -fold change relative to control samples.

2.8. *Statistical analysis*

Results are expressed as either the mean ± SEM or the median as appropriate. All values were analyzed with a Mann-Whitney *U* test. p values < 0.05 were considered statistically significant.

3. Results

3.1. *Inflammatory cells increased in lung tissue and BALF from mice with bleomycin-induced ALI*

To estimate the degree of inflammation in lung tissue after treatment with saline or bleomycin, tissue samples were stained with H&E. Lung tissue from mice exposed to bleomycin on Day 1 displayed severe inflammation in comparison to tissue from the control group (Figures 1A-1D). BALF from mice exposed to bleomycin showed that the total cell number and the number of neutrophils and macrophages on Day 1 increased in comparison to numbers in the control group (Figure 1E).

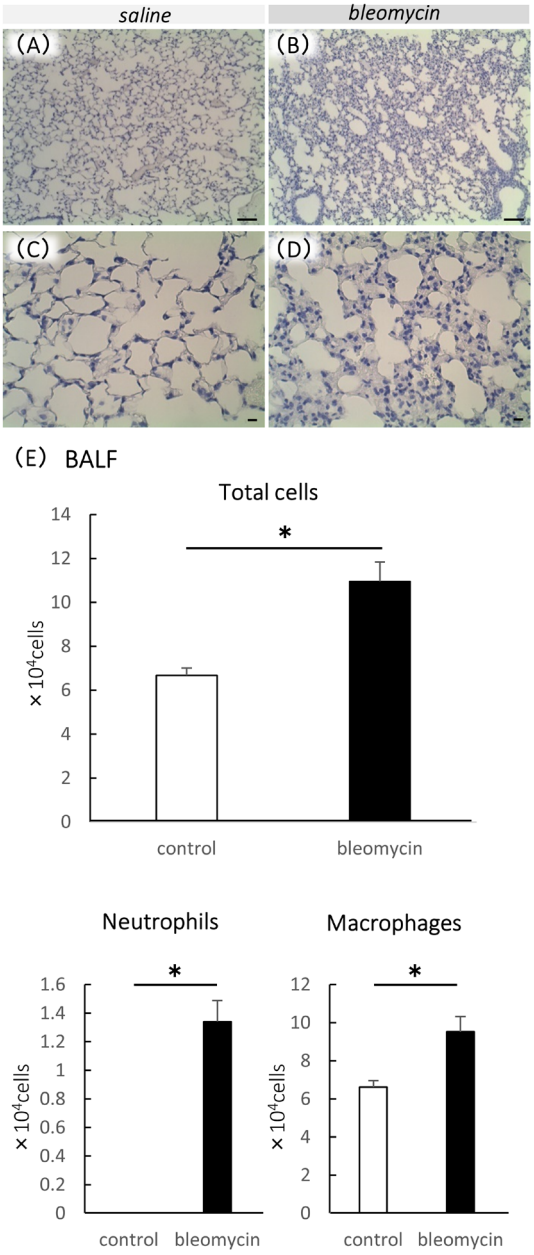

Figure 1. Infiltration of inflammatory cells in lung tissue and BALF from mice with bleomycin-induced acute lung injury. (A-D) H&E staining. **(E)** Cell number in BALF (n = 4 mice per group). **(A**, **C)** Lung tissue from control on Day 1. **(B**, **D)** Lung tissue from mice with bleomycin-induced acute lung injury on Day 1. **(E)** Total cell number, the number of neutrophils and macrophages in BALF on Day 1. **(A**, **B)** magnification, ×100; Scale bars, 100 μm. **(C**, **D)** magnification, ×400; Scale bars, 10 μm. Each bar represents the mean ± SE. $^*p < 0.05$.

3.2. *AIF-1 is expressed in lung tissue from mice with bleomycin-induced ALI*

To estimate the expression of AIF-1 in lung tissue on Day 1 after treatment with saline or bleomycin, samples were stained with an anti-AIF-1 Ab or anti-F4/80 Ab. Cells expressing AIF-1 were not evident in lung tissue after treatment with saline (Figures 2A and 2E). In contrast, invasive cells (arrow head) that were mostly round with round nuclei and that expressed AIF-1 were evident in lung tissue after treatment with bleomycin (Figures 2B and 2F). Invasive macrophages (arrow head) that were mostly round with round nuclei and that expressed F4/80 were evident in lung tissue after treatment with bleomycin (Figures 2D and 2H). These results indicate that AIF-1 was expressed in invasive macrophages. Immunostaining of lung tissue with normal IgG was completely negative after treatment with bleomycin (Figures 2C and 2G).

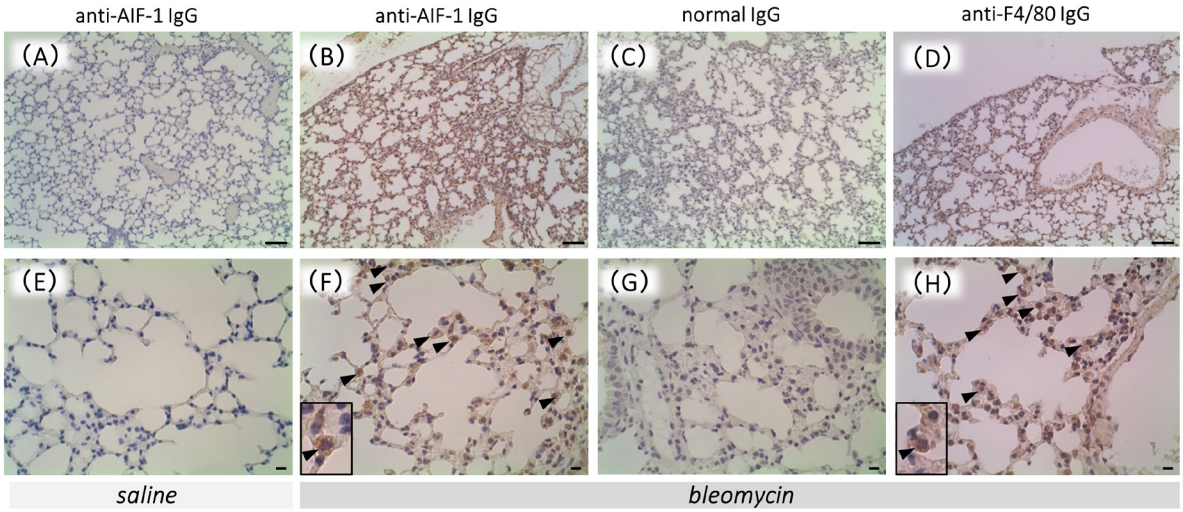

Figure 2. Expression of AIF-1 in lung tissue from mice with bleomycin-induced acute lung injury. (A-H) Immunohistochemical analysis. **(A**, **E)** Lung tissue on Day 1 after treatment with saline. **(B-D**, **F-H)** Lung tissue on Day 1 after treatment with bleomycin. **(A**, **E)** Immunostaining with an anti-AIF-1 antibody. **(B**, **F)** Immunostaining with an anti-AIF-1 antibody (brown) (arrow head). **(C**, **G)** Immunostaining with normal IgG. **(D**, **H)** Immunostaining with an anti-F4/80 antibody (brown) (arrow head). **(A-D)** magnification, ×100; Scale bars, 100 μm. **(E-H)** magnification, ×400; Scale bars, 10 μm.

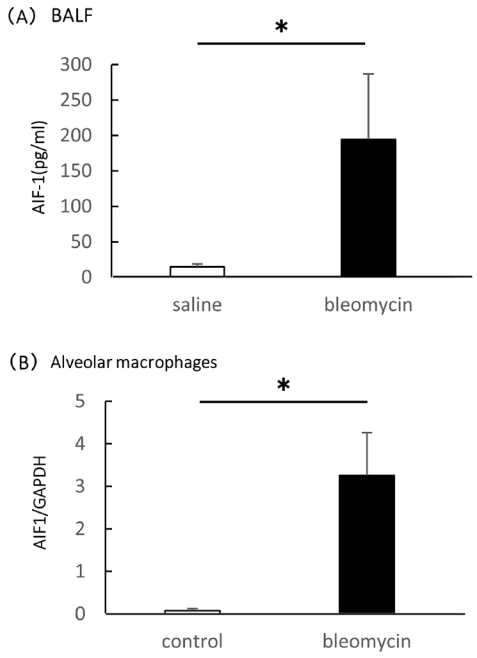

Figure 3. Expression of AIF-1 in BALF and alveolar macrophages in BALF from mice with bleomycin-induced acute lung injury. (A) AIF-1 ELISA ($n = 4$ mice per group). **(B)** AIF-1 real-time PCR ($n = 4$ mice per group). **(A)** AIF-1 level in BALF on Day 1. **(B)** The level of AIF-1 mRNA in alveolar macrophages in BALF on Day 1. Each bar represents the mean ± SE. *$p < 0.05$

3.3. *AIF-1 is expressed in BALF and alveolar macrophages in BALF from mice with bleomycin-induced ALI*

To examine the expression of AIF-1 in BALF on Day 1 after treatment with saline or bleomycin, the concentration of AIF-1 was measured using ELISA. In BALF, the level of AIF-1 expression was significantly increased in comparison to that in the control group (Figure 3A). The expression of AIF-1 was examined in alveolar macrophages from BALF. Real-time PCR analysis indicated that the level of AIF-1 mRNA was significantly increased in alveolar macrophages from BALF on Day 1 after intratracheal administration of bleomycin in comparison to administration of saline (Figure 3B).

3.4. *AIF-1 stimulates RAW264.7 cells and lung fibroblasts to secrete IL-6 and TNF-α*

To estimate the levels of inflammatory cytokines in BALF after treatment with saline or bleomycin, the levels of IL-6 and TNF-α were measured. BALF from mice exposed to bleomycin on Day 1 displayed significantly increased levels of IL-6 and TNF-α in comparison to levels in the control group (Figures 3A and 4B). The effect of recombinant AIF-1 on cytokine secretion by RAW264.7 cells and lung fibroblasts was examined *in vitro*. Cultured RAW264.7 cells and lung fibroblasts were stimulated for 24 h with various concentrations of recombinant AIF-1 or LPS. The levels of IL-6 and TNF-α secreted by AIF-1 (10 ng/mL-1 μg/mL)-treated RAW264.7 cells were significantly higher than those secreted by control cells, and this effect was dose-dependent (Figures 4C and 4D). The levels of IL-6 and TNF-α secreted by AIF-1-treated lung fibroblasts were also significantly higher than those secreted by control cells, and this effect was also dose-dependent (Figures 4E and 4F).

3.5. *AIF-1 stimulates lung fibroblasts to secrete KC*

The number of neutrophils in BALF increased on Day 1 after mice were exposed to bleomycin (Figure 1E). The

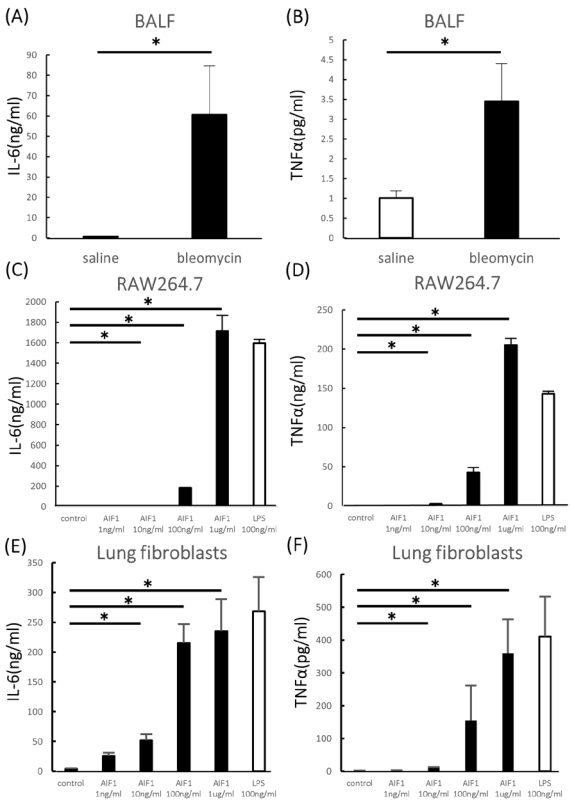

Figure 4. The levels of IL-6 and TNF-α in BALF from mice with bleomycin-induced acute lung injury and the levels of IL-6 and TNF-α secreted from RAW264.7 cells and lung fibroblasts stimulated by recombinant AIF-1. (A, B) IL-6 and TNF-α levels in BALF on Day 1 after intratracheal administration of saline or bleomycin. (n = 4 mice per group). **(C-F)** IL-6 and TNF-α levels in supernatant (n = 5 mice per group).The levels of IL-6 and TNF-α in the supernatant from cultured RAW264.7 cells and lung fibroblasts were measured after cells were exposed to various concentrations of rAIF-1 for 24 h. Each bar represents the mean ± SE. $^*p < 0.05$.

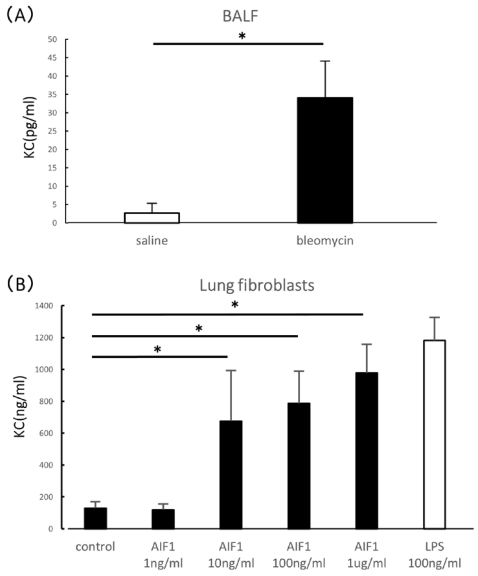

Figure 5. The level of KC in BALF from mice with bleomycin-induced acute lung injury and the level of KC secreted from lung fibroblasts stimulated by recombinant AIF-1. (A) KC level in BALF (n = 4 mice per group). BALF analyses were performed on Day 1 after intratracheal administration of bleomycin. **(B)** KC level in supernatant (n = 5 mice per group). The concentration of KC in the supernatant from cultured lung fibroblasts was measured after cells were exposed to various concentrations of rAIF-1 for 24 h. Each bar represents the mean ± SE. $^*p < 0.05$.

concentration of KC, which is involved in chemotaxis and cell activation of neutrophils, was measured next. BALF from mice exposed to bleomycin on Day 1 had a significantly increased level of KC in comparison to control cells (Figure 5A). The effect of recombinant AIF-1 on chemokine secretion by lung fibroblasts was examined *in vitro*. Cultured lung fibroblasts were stimulated for 24 h with various concentrations of recombinant AIF-1 or LPS. The level of KC secreted by AIF-1 (10 ng/mL-1 μg/mL)-treated lung fibroblasts was significantly higher than that secreted by control cells, and this effect was dose-dependent (Figure 5B). Recombinant AIF-1 did not stimulate the secretion of KC from RAW264.7 cells (data not shown).

4. Discussion

The current study has shown that AIF-1 was expressed in lung macrophages and increased in BALF from mice with bleomycin-induced ALI. This study also showed that recombinant AIF-1 stimulated the secretion of IL-6 and TNF-α in RAW264.7 cells and lung fibroblasts and it stimulated the secretion of KC in lung fibroblasts. Taken together, these findings suggest that AIF-1 plays an important role in the mechanism underlying the etiology of ALI.

AIF-1 was expressed in macrophages in lung tissue with bleomycin-induced ALI. Evidence of AIF-1 expression in macrophages coincides with previous studies that detected AIF-1 in macrophages in a model of atherosclerotic allogenic heart grafts or in human macrophage-like cell lines (*13,25*). AIF-1 was also expressed in BALF from mice with bleomycin-induced ALI. These results suggest that AIF-1 was produced by lung macrophages in bleomycin-induced ALI and that AIF-1 may play a role in the mechanism of inflammation.

Bleomycin induces inflammatory changes in lung tissue with increased expression of pro-inflammatory cytokines in a short period of time (*26*). In the current study, levels of IL-6 and TNF-α were significantly increased in BALF from mice exposed to bleomycin in comparison to the control group on Day 1. Both IL-6 and TNF-α are known to be crucial pro-inflammatory cytokines that have variable biological effects on inflammation, immune responses, cell differentiation and proliferation, hematopoiesis, and oncogenesis (*27-29*). The current study also indicated that recombinant AIF-1 stimulates the production of IL-6 and TNF-α from a macrophage cell line (RAW264.7) and lung fibroblasts *in vitro*. Although the biological function of lung macrophages has not been completely elucidated and additional research is needed to clarify the role that AIF-

1 plays, the current results suggest that AIF-1 is produced by lung macrophages and that lung macrophages might produce IL-6 and TNF-α as autocrines.

A study has reported that fibroproliferative activity is present in the early phase in lungs of patients with ARDS (*30*). Histology has also indicated that fibroproliferation is present in the early phase in the lungs of patients with ARDS (*31,32*). As indicated above, lung fibroblasts are thought to play an important role in ALI. Previous studies by the current authors demonstrated that recombinant AIF-1 can stimulate the secretion of IL-6 in human synoviocytes and normal human dermal fibroblasts (*16,33*). However, lung fibroblasts secreted TNF-α in addition to IL-6 when stimulated with recombinant AIF-1. These results suggest that AIF-1 produced by lung macrophages contributes to the development of bleomycin-induced ALI by inducing the production of IL-6 and TNF-α by stimulating lung macrophages in an autocrinal manner and by stimulating lung fibroblasts in a paracrinal manner.

Another interesting finding from the current study is that the expression of KC and the number of neutrophils were significantly increased in BALF from mice exposed to bleomycin in comparison to the control group. KC (CXCL1) has been proposed as a functional homologue of human IL-8 and is associated with neutrophil recruitment and inflammation (*34*). The level of IL-8 in BALF is reported to be significantly higher in patients who subsequently develop ARDS than in patient who do not develop ARDS (*35,36*). Ma *et al*. showed that the level of KC in BALF was elevated on Day 1 after intratracheal administration of bleomycin (*37*). These results coincide with the current finding. Moreover, the current study also indicated that recombinant AIF-1 stimulated the secretion of KC in lung fibroblasts. These results suggest that AIF-1 contributes to the recruitment of neutrophils by inducing the production of KC by lung fibroblasts in bleomycin-induced ALI.

In conclusion, the current study showed that AIF-1 was overexpressed in lung macrophages and increased in BALF from mice with bleomycin-induced ALI. AIF-1 can stimulate the secretion of IL-6 and TNF-α in macrophages and lung fibroblasts. This study also indicated that KC, a chemokine that induces neutrophil recruitment and inflammation, is secreted in lung fibroblasts stimulated with AIF-1. These findings suggest that AIF-1 plays a critical role in the mechanism underlying the etiology of ALI.

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Original Article

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Multifarious effects of 17-β-estradiol on apolipoprotein E receptors gene expression during osteoblast differentiation *in vitro*

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Summary **Apolipoprotein E (ApoE) regulated bone metabolism in mice might mediate uptake of lipid particles into target cells such as osteoblasts *via* receptor-mediated endocytosis by apoE receptors, which includes the low-density lipoprotein receptor (LDLR) family and heparan sulfate proteoglycans (HSPGs). There is no report regarding the expression of ApoE receptors mRNA induced by estrogen during osteoblast differentiation *in vitro*. Primary osteoblasts were collected from the calvaria of newborn mice and were subjected to osteoblast mineralization culture with serial concentrations of 17-β-estradiol (E2) *in vitro*. RNA was isolated at days 0, 5 and 25 of differentiation. Real-time PCR was conducted to analyze apoE receptors mRNA levels. We found that most LDLR family members genes were induced during osteoblast differentiation *in vitro*. The effect of E2 on apoE receptors gene expression during osteoblast differentiation was multifarious. The most noted members of the LDLR family involved in the maintenance of bone metabolism were LRP5, LRP6, LRP4, and Apoer2. LRP6 was up-regulated, while LRP5, LRP4, and Apoer2 were down-regulated by E2. Given that LRP6 is required for early stages of differentiation, we speculate E2 promotes osteoblast differentiation mainly in the early stage.**

Keywords: 17-β-estradiol,Apolipoprotein E receptors, Low-density Lipoprotein Receptors Family, Heparan sulfate proteoglycans, Osteoblast differentiation, Reproductive endocrine metabolic network

1. Introduction

Osteoblasts, the bone-forming cells, arise from multipotential mesenchymal stem cells (MSC), which are capable of giving rise to a number of cell lineages, such as adipocytes, myoblasts, or chondrocytes (*1*). When maintained under suitable culture conditions, they form bone-like nodules that represent the end product of proliferation and differentiation of relatively rare osteoprogenitor cells present in the starting cell population.

When exposed to osteogenic differentiation medium supplemented with 17-β-estradiol (E2), MSCs increase the expression of bone morphogenetic protein (BMP) and osteocalcin, and significantly increase the deposition of calcium (*2,3*). E2 also stimulates the expression of osteogenic genes for alkaline phosphatase (ALP) and type I collagen by MSCs (*4*). Regarding the role of estrogens in the osteogenic differentiation of MSCs, there is evidence that E2 supports growth and differentiation mostly through estrogen receptor α (ERα) (*5*). These observations suggest that estrogen could profoundly affect osteoblast physiology. Estrogen promotes bone health in part by reducing osteoblast apoptosis due to

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activation of the extracellular signal-regulated kinase (ERK) signaling pathway and down-regulation of c-Jun N-terminal kinase (JNK), which alters activity of a number of transcription factors (*6-8*).

Lipoproteins function as plasma carriers that transport lipids and lipophilic vitamins, which have been shown to influence bone metabolism, between peripheral blood and tissues (*9*). Cellular lipoprotein uptake is dependent on the interaction of their protein moieties, such as apolipoprotein E (ApoE), a 34 kDa glycoprotein, which plays a central role in lipoprotein metabolism, with endocytotic cell surface lipoprotein receptors. Over the last few decades, numerous studies have confirmed apoE regulated bone metabolism in mice (*10-13*), but the mechanism is still undefined. One possible molecular explanation was provided by a series of experiments that characterized the role of apoE in the uptake of triglyceride-rich lipoproteins (TRL) and TRL-associated vitamin K into osteoblasts (*10*).

ApoE mediates uptake of these particles into target cells such as osteoblasts *via* receptor-mediated endocytosis by the apoE receptors, which are the low-density lipoprotein receptor (LDLR) family and heparan sulfate proteoglycans (HSPGs) (*14-16*). ApoE has a strong affinity for and is the main ligand for members of the LDLR family. The LDLR family is a highly conserved receptor family with diverse functions in cellular physiology (shown in Table 1) (*17-32*). LDLR is the prototype of the entire family, and members of this family are structurally and functionally related to it. The other core members of the LDLR family include the very-low-density lipoprotein receptors (VLDLR), apolipoprotein E receptor 2 (Apoer2), low-density lipoprotein receptor-related proteins (LRPs), and megalin (*17*).

Genome-wide expression analysis had been conducted to identify genes regulated during osteoblastic differentiation. The results showed that among the LDLR family, megalin was up-regulated, while LRP1 and the LDLR were down-regulated (*33*). However, the previous report only screened very few members of the LDLR family during osteoblastic differentiation. Binding of estrogens to the receptors in the nucleus stimulates transcription of target genes resulting from direct interactions of the receptor proteins with DNA or from interactions with other transcription factors (*34*). However, there is no report regarding the expression of ApoE receptors mRNA induced by estrogen during osteoblast differentiation *in vitro*. Thus, the current study sought to observe the regulation of the LDLR family gene expression by E2 during this process.

2. Materials and Methods

2.1. *Chemicals and reagents*

Serum-free and phenol red-free minimal essential medium (α-MEM) was obtained from Gibco-BRL (Gaithersburg, MD, USA). The Penicillin-streptomycin was purchased from the Beyotime Institute of Biotechnology (Shanghai, China). Collagenase, E2, ascorbic acid, β-glycerophosphate disodium salt hydrate and dexamethasone were purchased from Sigma-Aldrich Co (Saint Louis, MO, USA). Dispase was obtained from Hoffmann-La Roche Ltd (Basel, Schweiz). RNAiso Plus, PrimeScript RT reagent kit and SYBR Premix Ex Taq II reagent kit were purchased from TaKaRa Biotechnology (Otsu, Japan).

2.2. *Mice*

C57Bl/6 mice, 8-weeks-old, with a body mass between 20 and 30 g were purchased from the Laboratory Animal Facility of Chinese Academy of Sciences (Shanghai, China), and habituated to the housing conditions for 3 days. Afterwards, they were housed four (two male and two female) per cage on a reversed 12 hours light and 12 hours dark cycle. Food and water were available *ad libitum* at room temperature. Newborn mice were used to isolate primary osteoblasts. The housing and handling of experimental animals were performed in accordance with the guidelines of the Chinese Council for Animal Care.

2.3. *Primary osteoblast isolation*

Osteoblasts were collected from the calvarium of newborn mice separately at two days as follows (*35*). Skull bones were extracted and digested (five times, 10 min each time) in α-MEM containing 0.1% collagenase and 0.2% dispase. Supernatant from the first 10-min digestion was discarded. Cells obtained from the remainder of the digestions were pooled and 5×10^5 cells were seeded into serum-free and phenol red-free α-MEM containing 10 units/mL penicillin and 10 μg/mL streptomycin in 6-well culture plates until they reached 80% confluence.

2.4. *Osteoblast mineralization culture and E2 treatment in vitro*

The osteogenic differentiation medium consisted of serum-free and phenol red-free α-MEM, 20 mM ascorbic acid, 1 M β-glycerophosphate disodium salt hydrate and 1 mM dexamethasone (*36*). For osteoblast mineralization culture, we treated the 80% confluent primary osteoblasts with the osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) (*37*) or saline for 0 d, 5 d and 25 d (*10*), respectively.

2.5. *RNA isolation and quantitative real-time reverse transcription PCR*

After stimulation, cells were pooled, total RNA was isolated and purified separately using the RNAiso

Table 1. Introduction of LDLR family members

Receptors	Tissue expression	Ligands	Functions
LDLR (*17,23*)	Liver, brain, heart, intestine, kidney, muscle, adrenal, lung, placenta, ovary, testis, bone	Apolipoprotein B, apolipoprotein E, low-density lipoproteins	Lipoprotein/cholesterol uptake
VLDLR (*17,23*)	Brain, heart, kidney, muscle, adipose, adrenal, lung, placenta, ovary, testis, bone	Apolipoprotein E, Reelin, lipoprotein lipase, tissue factor pathway inhibitor	Regulation of neuronal migration during embryonic development (predominantly cerebellum)
LRP1 (*19,23*)	Liver, lung, brain, bone	Apolipoprotein E, chylomicron remnants, a2-macroglobulin, amyloid precursor protein, protease/ protease inhibitor complexes, lipoprotein lipase, hepatic lipase, sphingolipid activator protein, Factor VIIa/tissue factor pathway inhibitor, plasminogen activators/plasminogen activator inhibitor-1, Factor XIa, Factor VIIIa, MMP9, MMP13, pregnancy zone protein, complement C3, C1-inhibitor, antithrombin III, heparin cofactor II, a1-antitrypsin, thrombospondin 1 and 2, Pseudomonas exotoxin A, rhinovirus, lactoferrin, heat shock protein 96, HIV tat protein	Lipoprotein and protease uptake, modulation of APP processing, protecting the vasculature, modulation of intracellular signaling, synaptic transmission?
LRP1B (*21,23*)	Brain, kidney, uterus	Unknown	Putative tumor suppressor gene
Megalin (*17,22-24*)	Kidney, lung, placenta, ovary	Apolipoprotein B, apolipoprotein E, apolipoprotein J, apolipoprotein H, albumin, cubilin, plasminogen activators/plasminogen activator inhibitor-1, parathyroid hormone, retinol binding protein, vitamin D binding protein	Embryonic renal development, vitamin homeostasis, renotubular reabsorption of proteins, regulation of thyroid and parathyroid functions, promoting morphogen signaling, embryonic cholesterol homeostasis?
LRP3 (*25*)	Muscle, ovary.	Unknown	Unknown
LRP4 (*18,26-28*)	Muscle, bone	Agrin, dickkopf-1, sclerostin	Participate in Agrin-LRP4-MuSK signaling pathway, involved in Wnt and bone morphogenetic protein signaling pathways
LRP5 (*21,31*)	Liver, heart, intestine, kidney, muscle, pancreas, lung, bone	Wnt proteins, dickkopf proteins (?)	Regulation of bone formation and ocular embryonic development, presumably as Wntcoreceptor
LRP6 (*21,31*)	Liver, heart, intestine, kidney, muscle, pancreas, lung, bone	Wnt proteins, dickkopf proteins	Wnt signal transduction, generation of caudal paraxial mesoderm, mid- and hindbrain development, anteroposterior and dorsoventral patterning of the developing limbs
Apoer2 (*17,23,32*)	Brain, placenta, ovary, testis	Apolipoprotein E, Reelin	Regulation of neuronal migration during embryonic development (predominantly hippocampus and neocortex), positive regulator of Wnt/β-catenin signaling
LRP10 (*30*)	Brain, muscle, heart	Unknown	Inhibiting the canonical Wnt/β-catenin signaling pathway
sorLA/LRP11 (*17,23*)	Liver, brain, adrenal, ovary, testis	Apolipoprotein E, head activator peptide	Head regeneration in hydra, presumably function in neurodevelopment
LRP12 (*29*)	Human heart muscle	Unknown	Activated protein C kinase 1, muscle integrin binding protein, and SMAD anchor for receptor activation

Plus according to the provided protocol. The reverse transcription reaction was performed according to the protocol from the PrimeScript RT reagent kit. Afterwards, mRNA expression was determined *via* quantitative real-time PCR using SYBR Premix Ex Taqreagent kit on Applied BiosystemsInc 7900 HT (Waltham, MA, USA) in a final volume of 50 μL according to the manufacturer's instructions. The

Table 2. Sequences of the primers for low-density lipoprotein receptors family and β-actin

LDLR	FP*	5'-ACTGGTTGCCCTCCTTGTC-3'
	RP**	5'-GCTCGTCCTCTGTGGTCTTC-3'
VLDLR	FP	5'-GCCATCACATCCTGACTGAA-3'
	RP	5'-CCCAAGAAACCAGCAACATT-3'
LRP1	FP	5'-ATGCCAATGAGACCGTATGC-3'
	RP	5'-GGCTGAGGGAGATGTTGATG-3'
LRP1B	FP	5'-CGAGAGGATGACTGTGGTGA-3'
	RP	5'-AGTGCCATTTGTTGCTGATG-3'
Megalin	FP	5'-CTGGTGAGGAAAGGAGTTGG-3'
	RP	5'-AAACGGACCCACAAATGAAG-3'
LRP3	FP	5'-CATTTCTACCCTGCCTCTGC-3'
	RP	5'-CTCGTCACTCCACCCTCTTC-3'
LRP4	FP	5'-ATCCTCCGTGCCAACCTTA-3'
	RP	5'-GTCCCAGAGTCGGTCCAGTA-3'
LRP5	FP	5'-CTGTGCTGATGGGTCTGATG-3'
	RP	5'-TGACGAAGAGGGAGAGGATG-3'
LRP6	FP	5'-TGACGCACAGGCTACTCAAC-3'
	RP	5'-CCACCAGATAAAGACGCACA-3'
Apoer2	FP	5'-ATTTGTTTGGTCGTCGGTTC-3'
	RP	5'-TCCCTGTGGTCTCTGGAAAG-3'
LRP10	FP	5'-GCTGTGATGGGATTGATGC-3'
	RP	5'-GTCCTCCAAGGTGAGATTGC-3'
sorLA /LRP11	FP	5'-CACGCCATTGTCCTTTATGA-3'
	RP	5'-CGGAGTCAGTCACAGTCAGC-3'
LRP12	FP	5'-GCTGGGTCCGCTTTACACTA-3'
	RP	5'-ATCGTCGTCTTCTCGTCCAC-3'
Sdc2	FP	5'-GACAACCACAGCCACTCCAT-3'
	RP	5'-ATGCCTCCAACTCCTCCTCT-3'
HSPG2	FP	5'-TGGTGCCTCACTGTCAAACT-3'
	RP	5'-GATGGTATGTGGTCGGTGTG-3'
β-actin	FP	5'-CCTCTATGCCAACACAGT-3'
	RP	5'-AGCCACCAATCCACACAG-3'

* FP, Forward Primer; ** RP, Reverse Primer.

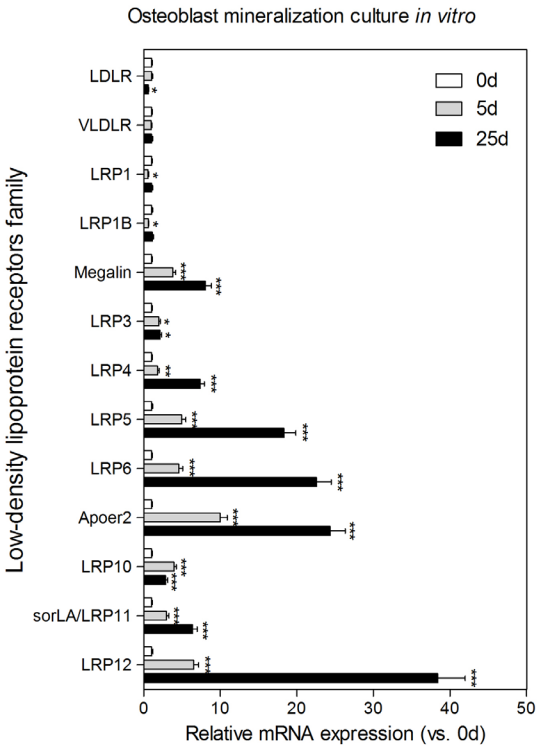

Figure 1. Most members of the LDLR family genes were induced during osteoblast differentiation. Primary mouse calvarial osteoblasts treated with osteogenic differentiation medium were pooled at days 0, 5, and 25 of differentiation. Then, we isolated total RNA from each sample to identify the apolipoprotein genes whose expression was induced during osteoblast mineralization. The LDLR family genes mRNA levels at days 5 and 25 of differentiation relative to day 0 of differentiation. $^{*}p < 0.05$, $^{***}p < 0.001$.

corresponding primers used are listed in Table 2. Values of mRNA expression were normalized to those of the house keeping gene β-actin. All real-time PCR experiments were performed in triplicate.

2.6. *Statistical analysis*

All values are presented as the mean ± S.D. Statistically significant differences were assessed by one-way ANOVA followed by Tukey's test. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. *Most of members of the LDLR family genes were induced during osteoblast differentiation*

We pooled the primary mouse calvarial osteoblasts at days 0, 5, and 25 of differentiation (*10*). Then, we isolated total RNA from each sample to identify the *LDLR* family genes whose expression was induced during osteoblast differentiation. β-actin was used as a control for standard gene expression. Most members of the *LDLR* family genes, such as, megalin, *LRP3*, *LRP4*, *LRP5*, *LRP6*, *Apoer2*, *LRP10*, *LRP11* and *LRP12*, were induced during osteoblast differentiation (Figure 1, $p < 0.05$, $p < 0.01$, $p < 0.001$). Besides, *LRP1* and *LRP1B* gene expression at 5 day of differentiation was down-regulated compared to day 0 of differentiation (Figure 1, $p < 0.05$). *LDLR* gene expression at 25 days of differentiation was down-regulated compared to 0 day of differentiation (Figure 1, $p < 0.05$). There was no significant change in the expression of VLDLR between days 0, 5, 25 of differentiation (Figure1, $p > 0.05$).

3.2. *Regulation of LDLR family core members by E2 during osteoblast differentiation*

As described above, the core members among LDLR family include LDLR,VLDLR, LRP1, LRP1B, LRP4, LRP5, LRP6, megalin, Apoer2 and sorLA/LRP11, which are the confirmed ApoE receptors (*16,38,39*). In the current study, we found multifarious effects of E2 on *LDLR* family genes expression during osteoblast differentiation.

3.2.1. *VLDLR,megalin and LRP6 were up-regulated by E2 during osteoblast differentiation in a dose dependent manner*

There was no significant change in expression of

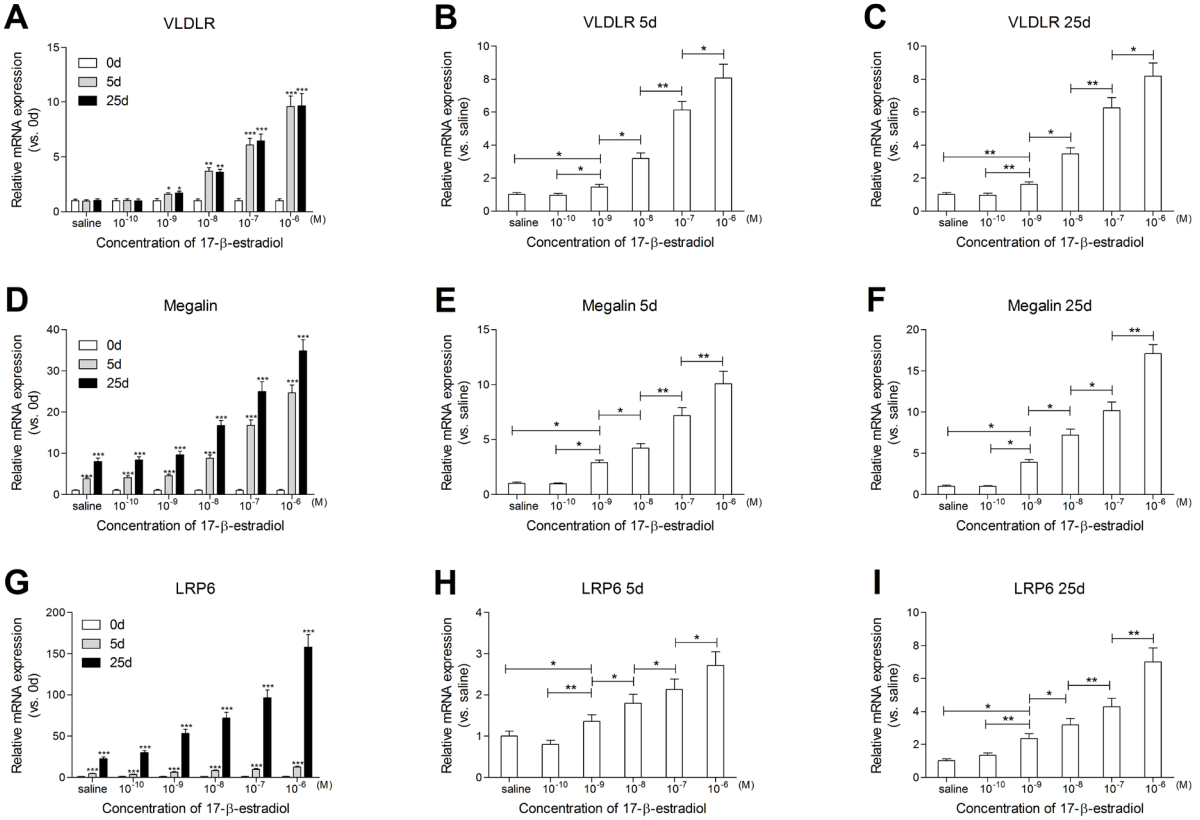

Figure 2. VLDLR, megalin and LRP6 were up-regulated by E2 during osteoblast differentiation in a dose dependent manner. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G)** VLDLR,megalin and LRP6 mRNA levels relative to it at the osteoblasts treated with saline for 0d. **(B**, **E**, **H)** VLDLR,megalin and LRP6 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I)** VLDLR, megalin and LRP6 mRNA levels relative to treatment with saline at day 25 of differentiation.$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

VLDLR in the saline and 10^{-10} M E2 treated groups at days 5 and 25 of differentiation when compared with day 0 of differentiation (Figure 2A, $p > 0.05$). When the concentration of E2 was elevated, expression of VLDLR at days 5 and 25 of differentiation was increased and superior to it at day 0 of differentiation (Figure 2A, $p < 0.05$, $p < 0.01$, $p < 0.001$). Whether in the saline group or in E2 treated groups, expression of megalin and LRP6 at days 5 and 25 of differentiation was increased compared to day 0 of differentiation (Figures 2D and 2G, $p < 0.001$).Moreover, at day 5 of differentiation and day 25 of differentiation, the mRNA expression of VLDLR, megalin and LRP6 were up-regulated by E2 in a dose dependent manner (Figures 2B, 2C, 2E, 2F, 2H, and 2I, $p < 0.05$, $p < 0.01$).

3.2.2. *LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 during osteoblast differentiation in a dose dependent manner*

Expression of LRP4, LRP5 and sorLA/LRP11 at days 5 and 25 of differentiation were increased compared to day 0 of differentiation in the saline group, 10^{-10} M E2 treated group and 10^{-9} E2 treated group (Figures 3A, 3D, and 3J, $p < 0.05$, $p < 0.01$, $p < 0.001$). There was no significant change in expression of LRP4, LRP5 and sorLA/LRP11 in the 10^{-8} M E2 treated group at day 5 of differentiation when compared with day 0 of differentiation (Figures 3A, 3D, and 3J, $p > 0.05$). In the 10^{-7} M E2 treated group and 10^{-6} M E2 treated group, mRNA levels of LRP4, LRP5 and sorLA/LRP11 at day 5 of differentiation were inferior to day 0 of differentiation (Figures 3A, 3D, and 3J, $p < 0.05$, $p < 0.01$). However, mRNA levels of LRP4 and LRP5 in the 10^{-8} M E2 treated group, 10^{-7} M E2 treated group and 10^{-6} M E2 treated group at day 25 of differentiation were still superior to day 0 of differentiation (Figures 3A and 3D, $p < 0.001$). The mRNA levels of sorLA/LRP11 in the 10^{-8} M E2 treated group and 10^{-7} M E2 treated group at day 25 of differentiation were superior to day 0 of differentiation, while in the 10^{-6} M E2 treated group the mRNA levels of sorLA/LRP11 showed no significant difference between day 0 and 25 of differentiation (Figure 3J, $p > 0.05$).

Expression of Apoer2 at day 5 and 25 of differentiation were increased compared to day 0 of differentiation in saline group and E2 treated groups except the 10^{-6} M E2 treated group (Figure 3G, $p < 0.01$, $p < 0.001$). There was no significant change in expression of Apoer2 in the 10^{-6} M E2 treated group at day 5 of differentiation when compared with day 0 of

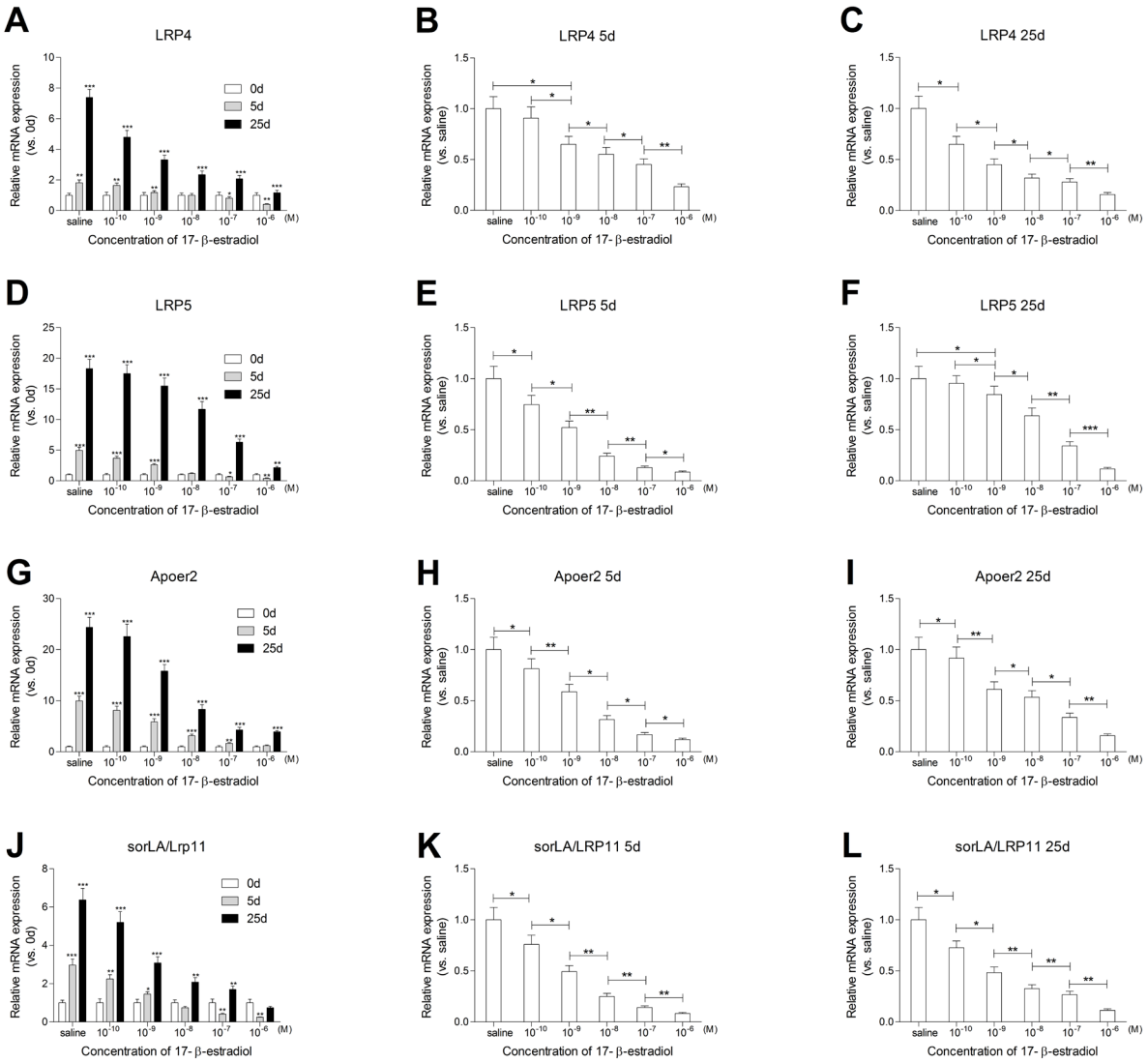

Figure 3. LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 during osteoblast differentiation in a dose dependent manner. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G**, **J)** LRP4, LRP5, Apoer2 and sorLA/LRP11 mRNA levels relative to osteoblasts treated with saline for 0d. **(B**, **E**, **H**, **K)** LRP4, LRP5, Apoer2 and sorLA/LRP11 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I**, **L)** LRP4, LRP5, Apoer2 and sorLA/LRP11 mRNA levels relative to treatment with saline at day 25 of differentiation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

differentiation (Figure 3G, $p > 0.05$), but the mRNA level of Apoer2 in the 10^{-6} M E2 treated group was still superior to it at day 0 of differentiation (Figure 3G, $p < 0.001$).

Moreover, at day 5 of differentiation and day 25 of differentiation, the mRNA expression of LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 in a dose dependent manner (Figures 3B, 3C, 3E, 3F, 3H, 3I, 3K, and 3L, $p < 0.05$, $p < 0.01$).

3.2.3. *Expression of LDLR, LRP1 and LRP1B were not affected by E2 during osteoblast differentiation*

There was no significant change in expression of LDLR in the saline and E2 treated groups at day 5 of differentiation when compared with day 0 of differentiation (Figure 4A, $p > 0.05$) but at day 25 of differentiation, mRNA levels of LDLR were decreased and inferior to day 0 of differentiation either in saline group or in E2 treated groups (Figure 4A, $p < 0.05$). Interestingly, mRNA levels of LRP1 and LRP1B at day 5 of differentiation were inferior to it day 0 of differentiation either in saline group or in E2 treated groups (Figures 4D and 4G, $p < 0.05$). However, at day 25 of differentiation, there was no significant change in expression of LRP1 and LRP1B in the saline and E2 treated groups when compared with day 0 of differentiation (Figures 4D and 4G, $p > 0.05$). Moreover, E2 did not affect the expression of *LDLR*, *LRP1* and *LRP1B* genes either at day 5 of differentiation or day 25 of differentiation (Figures 4B, 4C, 4E, 4F, 4H, and 4I, $p > 0.05$).

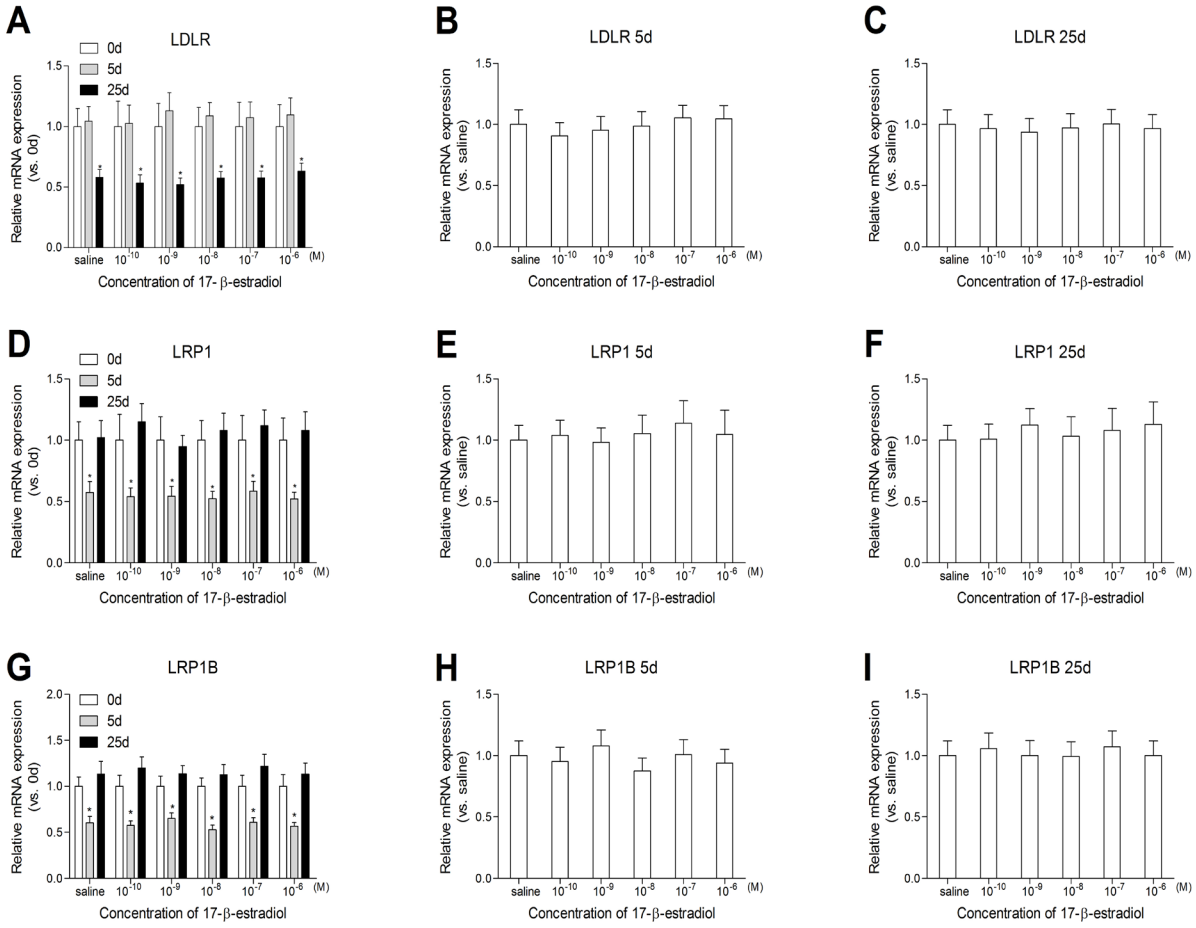

Figure 4. Expression of LDLR, LRP1 and LRP1B were not affected by E2 during osteoblast differentiation. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G)** LDLR, LRP1 and LRP1B mRNA levels relative to osteoblasts treated with saline for 0d. **(B**, **E**, **H)** LDLR, LRP1 and LRP1B mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I)** LDLR, LRP1 and LRP1B mRNA levels relative to treatment with saline at day 25 of differentiation.*$p < 0.05$.

3.3. *Regulation of other novel members in LDLR family by E2 during osteoblast differentiation*

There are several novel members in the LDLR family including LRP3, LRP10 and LRP12 (*25,29,40*), which are uncertain if they are receptors for apoE. Interestingly, either in the saline group or in E2 treated group, *LRP3*, *LRP10* and *LRP12* genes were all induced at day 5 and 25 of differentiation (Figures 5A, 5D, and 5G, $p < 0.05$, $p < 0.01$, $p < 0.001$). However, at day 5 of differentiation and day 25 of differentiation, E2 did not affect *LRP3* gene expression (Figures 5B and 5C, $p > 0.05$), it up-regulated *LRP10* gene expression in a dose dependent manner (Figures 5E and 5F, $p < 0.05$, $p < 0.01$), and down-regulated *LRP12* gene expression in a dose dependent manner (Figures 5H and 5I, $p < 0.05$, $p < 0.01$).

3.4. *Regulation of syndecan 2 (Sdc2) and HSPG2 by E2 during osteoblast differentiation*

Given HSPGs are receptors for apoE, Sdc2 and HSPG2 have been suggested to be involved in skeleton formation (*41,42*), and thus we analyzed the regulation of Sdc2 and HSPG2 by E2 during osteoblast differentiation as well. Our results showed that either in the saline group or in E2 treated group, both *Sdc2* and *HSPG2* genes were induced at day 5 and 25 of differentiation (Figures 6A and 6D, $p < 0.05$, $p < 0.01$, $p < 0.001$). Moreover, E2 up-regulated *Sdc2* gene expression in a dose dependent manner (Figures 6B and 6C, $p < 0.05$, $p < 0.01$), but did not affect *HSPG2* gene expression (Figures 6E and 6F, $p > 0.05$), either at day 5 of differentiation or day 25 of differentiation.

4. Discussion

The process of osteoblast differentiation has been subdivided into three developmental stages: proliferation, extracellular matrix synthesis and maturation, and mineralization, each with characteristic changes in gene expression (*43*). Many independent studies of gene expression patterns during osteoblast differentiation have been described (*10,33,44,45*), in which apoE was the

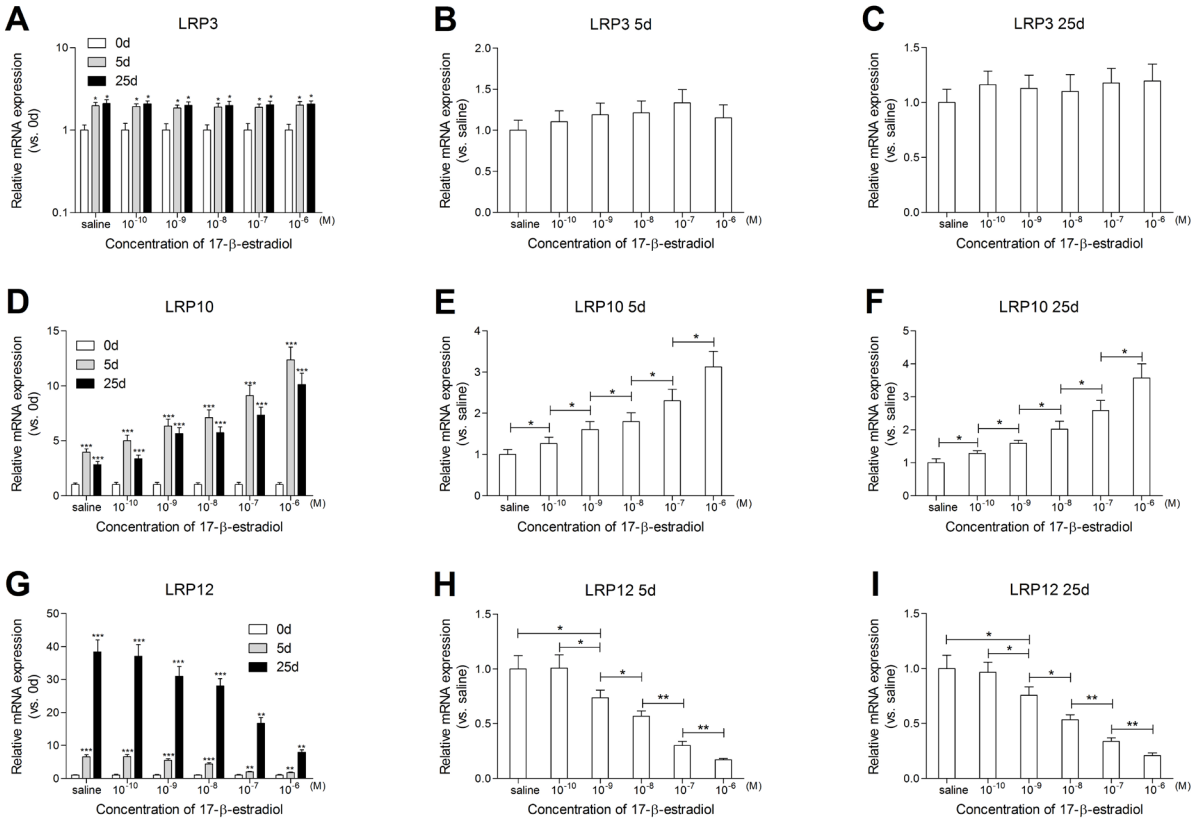

Figure 5. Regulation of other novel members in LDLR family by E2 during osteoblast differentiation. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0d, 5d and 25d, respectively. **(A**, **D**, **G)** LRP3, LRP10 and LRP12 mRNA levels relative to osteoblasts treated with saline for 0 d. **(B**, **E**, **H)** LRP3, LRP10 and LRP12 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F**, **I)** LRP3, LRP10 and LRP12 mRNA levels relative to treatment with saline at day 25 of differentiation.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

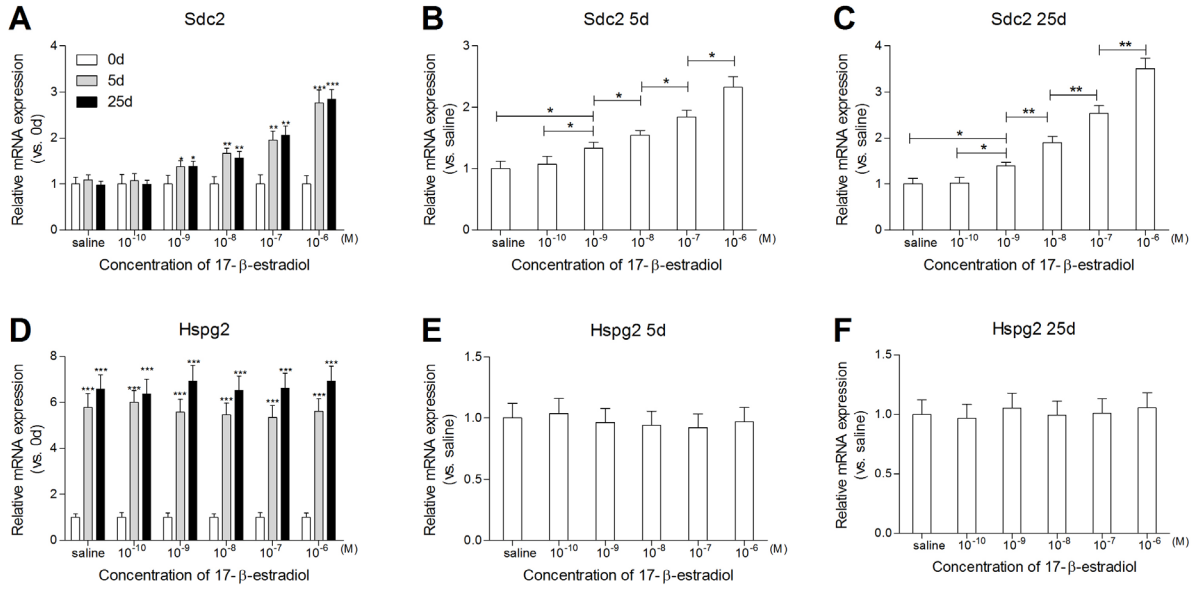

Figure 6. Regulation of Sdc2 and HSPG2 by E2 during osteoblast differentiation. Primary osteoblasts were treated with osteogenic differentiation medium containing serial concentrations of E2 (10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) or saline for 0 d, 5 d and 25 d, respectively. **(A**, **D)** Sdc2 and HSPG2 mRNA levels relative to osteoblasts treated with saline for 0d. **(B**, **E)** Sdc2 and HSPG2 mRNA levels relative to treatment with saline at day 5 of differentiation. **(C**, **F)** Sdc2 and HSPG2 mRNA levels relative to treatment with saline at day 25 of differentiation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

only apolipoprotein strongly induced during this process (*10,33*). ApoE regulated bone metabolism in mice is possible due to uptake of lipid and lipid soluble vitamins such as vitamin K into osteoblasts (*10*). It is a common pattern of receptor-mediated endocytosis by the apoE receptors including the LDLR family and HSPGs, for apoE mediated uptake of lipid particles into target cells such as osteoblasts (*14-16*). Generally, apoE mediates the interaction of apoE-containing lipoproteins and lipid complexes with the LDLR family. Interaction with HSPGs appears to attract and sequester apoE-containing lipoproteins at cell surfaces as well as to facilitate their interaction with the LDLR family (*16,46*).

Among LDLR family, confirmed ApoE receptors include: LDLR, VLDLR, LRP1, LRP1B, LRP4, LRP5, LRP6, megalin, Apoer2 and sorLA/LRP11 (*16,38,39*). Some of these receptors, such as LDLR and LRP1, influence ApoE levels (*47,48*). Others, such as Apoer2, VLDLR and LRP5/LRP6, play an important role in cellular development and are involved in signaling pathways like neural signaling and Wnt signaling (*38,49*). As we know, osteoblasts are derived from mesenchymal stem cells (MSCs). Wnt canonical signaling plays an important role in osteoblast differentiation both during embryogenesis and in adult life (*50*). However, there are several novel members in the LDLR family such as, LRP3, LRP10 and LRP12 (*25,29,40*), which are uncertain if they are receptors for apoE.

Traditionally, members of the LDLR family have been regarded as cell surface endocytosis receptors that function in delivering their ligands to lysosomes for degradation and providing essential nutrients for cellular functions (*17,51*). However, recent studies suggest that members of the LDLR family may participate in several signal transduction pathways including the regulation of mitogen-activated protein kinases, cell adhesion, vesicle trafficking, neurotransmission, and neuronal migration (*52,53*). About twenty years ago, causal mutations in the *LRP5* gene were identified to be involved in two rare bone disorders, which were related to the Wnt/β-catenin pathway (*54-56*). A number of reports were exploded to highlight the role of the Wnt/β-catenin pathway in the regulation of bone homeostasis (*57-59*). Recently, the most highlighted members of the LDLR family involved in the maintenance of bone metabolism were LRP5, LRP6, LRP4, and Apoer2 (*26,60*). Interestingly, results in the current study showed that all of these four members were induced during osteoblast differentiation (Figure 1).

The current study also observed the regulation of apoE receptors mRNA expression by E2 during osteoblast differentiation *in vitro*. We found multifarious effects of E2 on apoE receptors genes expression during osteoblast differentiation. Among certain apoE receptors, VLDLR, megalin and LRP6 were up-regulated by E2 during osteoblast differentiation in a dose dependent manner (Figure 2), whereas LRP4, LRP5, Apoer2 and sorLA/LRP11 were down-regulated by E2 during osteoblast differentiation in a dose dependent manner (Figure 3). Expression of LDLR, LRP1 and LRP1B were not affected by E2 during osteoblast differentiation in a dose dependent manner (Figure 4).

LRP5 and LRP6, sharing around 71% homology at the nucleotide level, are structurally related proteins and consist of co-receptors with the frizzled family of 7 transmembrane spanning proteins (*61*). Wnts bind to these receptors resulting in a series of downstream intracelullar events (*56*). Although both LRP5 and LRP6 are needed for normal bone development, they have distinct roles as well. LRP5 and LRP6 control osteoblast differentiation at different stages respectively. LRP5 is involved in late stages of differentiation, while LRP6 is required for early stages of differentiation (*31*).

Both LRP4 and Apoer2 are identified as novel receptors involved in bone metabolism. LRP4 is a novel receptor binding to osteoblast expressed dickkopf-1 (Dkk1) and sclerostin, plays a physiological role in the regulation of bone growth and turnover likely through Wnt and BMP signaling pathways (*28,62*). Apoer2 has been shown as a positive factor of the canonical Wnt signaling pathway, increasing Wnt-induced transcriptional responses, promoting Wnt-induced β-catenin accumulation, and controlling osteoblast differentiation (*32*).

LDLR, VLDLR and LRP1 are the main endocytic receptors recognizing apoE-containing lipoproteins (*51*). Both of them are expressed in most tissues. LDLR is ubiquitously expressed and is a key receptor for maintaining cholesterol homeostasis in mammals (*51*). In contrast with LDLR which is widely distributed, VLDLR is not expressed in liver (*63*). Osteoblasts exhibit high levels of protein expression of LRP1 and LDLR, but VLDLR is expressed to a lower degree (*64*). No reports about LDLR affects on osteoblast physiology exist so far. However, Okayasu M *et al*. found impaired osteoclastogenesis and increased bone mass in *Ldlr*$^{-/-}$ mice because of a defect in osteoclastic cell-cell fusion, and this change was accompanied by decreases in bone resorption parameters, with no changes in bone formation parameters (*65*). As a receptor for removal of apoE-rich chylomicron remnants, LRP1 plays a predominant role among the LDLR family members in vitamin K1 uptake through chylomicron remnants endocytosis in human osteoblasts (*64*).

Named megalin because of its huge molecular structure, and is a member of the LDLR family also called LRP2 that is abundantly expressed in different epithelial cell types (*66*). Megalin is involved in embryonic renal development, including vitamin D homeostasis, sex hormone signaling, and holoprosencephaly (*51,67-69*). Severe vitamin D deficiency and bone disease were shown in *megalin*$^{-/-}$ mice due to being unable to retrieve the steroid from the glomerular filtrate (*70*). However, Wang C *et al*. reported that polymorphisms of the LRP2

Table 3. Alternations of apolipoprotein E receptors expressions

Receptors	During osteoblast differentiation		Regulation by 17-β-estradiol
	5d *vs*. 0d	25d *vs*. 0d	
LDLR	No significant difference	Decreased	No significant difference
VLDLR	No significant difference	No significant difference	Increased
LRP1	Decreased	No significant difference	No significant difference
LRP1B	Decreased	No significant difference	No significant difference
Megalin	Increased	Increased	Increased
LRP3	Increased	Increased	No significant difference
LRP4	Increased	Increased	Decreased
LRP5	Increased	Increased	Decreased
LRP6	Increased	Increased	Increased
Apoer2	Increased	Increased	Decreased
LRP10	Increased	Increased	Increased
sorLA/LRP11	Increased	Increased	Decreased
LRP12	Increased	Increased	Decreased
Sdc2	No significant difference	No significant difference	Increased
HSPG2	Increased	Increased	No significant difference

gene were not a major factor that contributes to the peak BMD variation in the Chinese population (*71*).

Besides, among the novel LDLR family members, E2 did not affect *LRP3* gene expression, up-regulated *LRP10* gene expression in a dose dependent manner, and down-regulated *LRP12* gene expression in a dose dependent manner (Figure 5). Both of them are novel members found in recent decades (*25,29*), thus no report about these members relative to bone metabolism has been shown.

HSPGs are composed of a core protein to which heparan sulfate (HS) side-chains are covalently linked and occur in the extracellular matrix and on cell surfaces, while HS is a linear polysaccharide found in all animal tissues. HSPGs bind to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes (*72,73*), such as bone and organ formation (*74*). A report concluded that there are 15 members in the HSPGs. Among these members, Sdc2 and HSPG2 have been suggested to be involved in skeleton formation (*41,42*).

In the current study, E2 up-regulated *Sdc2* gene expression in a dose dependent manner, but did not affect *HSPG2* gene expression (Figure 6). Members of the fibroblast growth factor (FGF) family appear to play major roles during skeletal development and postnatal osteogenesis, HSPGs are cell surface transmembrane proteins that interact with and promote the binding and signaling of FGFs (*42*). Sdc2 is abundant in putative precursor cells of hard and connective tissue, and its expression is high in prechondrogenic cells, decreases in differentiating chondrocytes, and persists in the perichondrium and periosteum at the onset of osteogenesis (*42*). HSPG2 is abundant in the extracellular matrix of cartilage and the lacunocanalicular space of adult bones, and deficiency in HSPG2 during bone development enhances osteogenesis and decreases quality of adult bone in mice (*41*).

Sex steroid hormones act on their target cells by binding to members of the nuclear hormone receptor superfamily: estrogens bind to estrogen receptor (ER) α or ERβ, and androgens bind to the androgen receptor (AR) (*75*). Mice with deletion of ERα in MSC showed decreased periosteal bone formation due to decreased canonical Wnt signaling pathway (*76*). In our study, LRP5, LRP6, LRP4, and Apoer2, which are involved in the Wnt signaling pathway, presented different effects with E2. During osteoblast differentiation, LRP6 was up-regulated by E2 in a dose dependent manner, while LRP4, LRP5 and Apoer2 were down-regulated by E2 in a dose dependent manner (Figure 3). Given that LRP6 is required for early stages of differentiation (*31*), we speculate E2 promotes osteoblast differentiation mainly in the early stage. Moreover, reports about the other members relative to osteoblast physiology are rare. Thus, further investigation is needed to clarify whether these molecules are involved in osteoblast differentiation and related mechanisms.

In conclusion, the current study showed that most members of the *LDLR* family genes were induced during osteoblast differentiation *in vitro*, and the effect of E2 on apoE receptors genes expression was multifarious during this process (shown in Table 3). Among the apoE receptors, LRP6 was up-regulated by E2 in a dose dependent manner during osteoblast differentiation. Given LRP6 is required for early stages of differentiation, we speculate E2 promotes osteoblast differentiation mainly in the early stage.

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Original Article

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Lentivirus-mediated knockdown of CEP55 suppresses cell proliferation of breast cancer cells

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Summary **Centrosomal protein 55 (CEP55), as a microtubule-bundling protein, plays an important role in cell cycle regulation. CEP55 has been recognized recently in several human cancers. In this study, we first observed that the mRNA level of CEP55 is commonly up-regulated in breast cancer compared with their normal counterparts as demonstrated by data derived from Oncomine database. To further evaluate the functional role of CEP55 in breast cancer cells. Expression of CEP55 was efficiently knocked down using lentivirus-mediated RNA interference in human breast cancer cell line ZR-75-30, as evidenced by quantitative real-time PCR (qRT-PCR) and Western blot analysis. Further investigations revealed that CEP55 knockdown significantly inhibited cell proliferation and colony formation. Moreover, flow cytometer analysis indicated knockdown of CEP55 induced cell cycle arrested at G0/G1 phase and cell apoptosis. These findings suggest that CEP55 plays a crucial role in promoting breast cancer cell proliferation and it might be a potential therapeutic target in breast cancer.**

Keywords: Breast cancer, CEP55, RNA interference, cell proliferation

1. Introduction

Breast cancer is recognized as one of the most frequently diagnosed cancers in women in the worldwide (*1*), whose main characteristic is the uncontrollably proliferative ability of cancer cells (*2*). Although significant progress has been made in surgical techniques and early detection for breast cancer in recent years, higher recurrence and mortality rate remain to be major obstacles in the effective therapeutics for breast cancer (*3*). The environmental factors and genetic factors have been proved to be closely associated with breast cancer (*4*). However, the deep molecular mechanism underlying the growth and development of breast cancer remains elusive. Therefore, it is urgently needed to identify novel therapeutic targets, which might provide fundamental information for future new therapy for breast cancer.

Centrosomal protein 55 (CEP55) has been identified as a microtubule-bundling protein required for cytokinesis. It is located to the mitotic spindle during prometaphase and metaphase and recruited into the midbody during cytokinesis (*5,6*). In addition, CEP55 is a key regulator of cytokinesis essential for the midbody structure and vesicle trafficking (*6*). Accumulating evidences have shown CEP55 is highly expressed in several cancers including human colon cancer and lung cancer (*7-10*). Interestingly, CEP55 has been demonstrated to be involved in tumorigenesis of breast cancer (*11-13*). However, the functional role of CEP55 in breast cancer is still largely unknown. As our best knowledge, regulation of cell cycle is closely related with the occurrence and development of tumor (*14*). Cyclins are regulatory proteins involved in cell cycle process, which is associated with CEP55 expression levels (*7*). Related study indicates that knockdown of CEP55 induces cytokinesis failure and generates genomic instability, even down regulates tumor suppressor genes and activates oncogenes (*5*), which suggests CEP55 expression plays a tight role in cell division occurrence. Considering CEP55 is implicated as an oncogene in human lung cancer (*15*), it might be supposed to be involved in human breast cancer.

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In this study, we first determined the expression pattern of CEP55 in Oncomine database. Furthermore, we investigated the biological function of CEP55 in breast cancer cell growth. Our results might help to gain better understanding of the role of CEP55 in breast cancer growth and development.

2. Materials and Methods

2.1. *Analysis of Oncomine cancer gene microarray database*

To evaluate the expression level of CEP55 in breast cancer, publicly available Oncomine cancer microarray database (*www.oncomine.com*) was used to examine the expression of CEP55 in cancer tissue and determine the association of CEP55 with breast cancer outcomes. Richardson Breast 2 (*16*), Sorlie Breast 2 (*17*) and Ma Breast 4 (*18*) Ductal breast carcinoma were used to compare CEP55 expression levels between cancer and normal tissues. Ma Breast 4 (*18*), Karnoub Breast (*19*), Curtis Breast (*20*) and The Cancer Genome Atlas (TCGA, *http://tcga-data.nci.nih.gov/tcga/*) invasive breast carcinoma gene expression data were also used to compare CEP55 expression levels between cancer and normal tissues. The gene expression of CEP55 was compared between breast cancer tissues with normal breast tissues according to the standard procedures as previously described (*21*).

2.2. *Cell culture*

Human breast cancer cell lines ZR-75-30 and embryonic kidney cell line 293T (HEK293T) were purchased from the Cell Bank of Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). ZR-75-30 and HEK293T were cultured in RPMI-1640 (Hyclone SH30809.01B, Logan, UT, USA) and Dulbecco's modified Eagle's medium (DMEM, Hyclone SH30243.01B), respectively, supplemented with 10% fetal bovine serum (FBS, Biowest, Kansas City, MO, USA). The two lines were maintained in atmosphere of 5% CO_2 at 37°C.

2.3. *Construction of recombinant lentivirus and cell infection*

According the sequence of CEP55 (NM_001127182.1 and NM_018131.4) downloaded from the GEO database, two short hairpin RNAs (shRNA), including sequence 1 (5'-GCAGCGGGAAGTCTATGTAAAC TCGAGTTTACATAGACTTCCCGCTGCTTTTT-3') and sequence 2 (5'-CCCAAGTGCAATATACAGTAT CTCGAGATACTGTATATTGCACTTGGGTTTTT-3') were designed to knock down CEP55 expression. A scrambled shRNA (shCon, 5'- GCGGAGGGTTTGAA AGAATATCTCGAGATATTCTTTCAAACCCTCCGC TTTTTT-3') was used as control. Then these stem-loop-stem oligos were synthesized and inserted into pFH-L vector (Shanghai Hollybio, China) containing a green fluorescent protein (GFP) gene as a reporter gene. The recombined pFH-L vector was triple transfected into 80% confluent HEK293T cells with packing plasmids pVSVG-I and pCMVΔR8.92 (Shanghai Hollybio, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For lentivirus infection, ZR-72-30 cells were seeded into six-well plates and transfected with two CEP55 shRNAs and control shRNA (shCon), respectively and then correspondingly divided into four groups: CEP55 knockdown cells (KD-1 and KD-2), negative control cells (shCon) and blank control (Con). After 5 day infection, the infection efficiency was determined by counting the numbers of GFP-expressing cells under a fluorescence microscope (DMI4000B, Leica Microsystems, Germany).

2.4. *Knockdown efficiency determination*

To investigate the knockdown efficiency of CEP55, we determined the mRNA and protein levels of CEP55 in ZR-72-30 cells using quantitative real-time PCR (qRT-PCR) and Western blot analysis, respectively.

For qRT-PCR analysis, total RNA was extracted from each group cell with TRIZOL Reagent (Invitrogen, Grand island, NY, USA) according to the manufacturer's instructions. Reverse transcription was performed using SuperScript II RT 200 U/Ml (Invitrogen, Carlsbad, CA, USA). The β-actin was applied as an endogenous control. Primers were listed as follows: CEP55 (forward: 5'-AGCAGCAAGAAGAACAAACAAGGG-3' and reverse: 5'-AGTGACTAATGGCTCTGTGATGGC-3') and β-actin (forward: 5'-GTGGACATCCGCAAAGAC-3' and reverse: 5'-AAAGGGTGTAACGCAACTA-3'). The qRT-PCR reaction system contained 10 μL 2 × SYBR premix ex taq, 0.5 μL primers (2.5 μM), 5 μL cDNA and 4.5 μL ddH_2O. The qRT-PCR analysis was performed in triplicated on BioRad Connet Real-Time PCR platform (BioRad, Hercules, CA, USA) using the following reaction procedure: initial denaturation at 95°C for 1 min followed by 40 cycles (denaturation at 95°C for 5 s and annealing extension at 60°C for 20 s). The $2^{-\Delta\Delta Ct}$ method was used to normalize the expression of CEP55 mRNA to the expression of the β-actin.

For Western blot analysis, total protein of each group cell was extracted using ice-cold protein lysis buffer (10 mM EDTA, 100 mM Tris-Hcl (pH 6.8), 4% SDS and 10% Glycine). The protein concentration was determined by BCA protein assay. Total 30 μg proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Blots were blocked and then probed with rabbit anti-CEP55 (1:1,000 dilution, Proteintech Group, Inc,

no: 23891-1-AP) and rabbit anti-GAPDH (1:500,000 dilution, Proteintech Group, Inc, no: 10494-1-AP) overnight. After washing, the blots were incubated with HRP-conjugated goat anti-rabbit (1:5,000, Santa Cruz, SC-2054) as a second antibody for 20 min at room temperature. Blots were visualized by enhanced chemiluminescence (Millipore, Billerica, MA).

2.5. *Methylthiazol tetrazolium (MTT) assay*

After infection for 72 h, ZR-75-30 cells from different groups were seeded in a 96-well plate at a density of 3000 cells per well. MTT solution was added to the wells at different time points (1, 2, 3, 4 and 5 days) followed by incubation at 37°C for 4 h after lentivirus infection. Acidic isopropanol (10% SDS, 5% isopropanol and 0.01 mol/L HCl) was then added to stop the reaction. The optical density was measured with an enzyme-linked immunosorbent assay reader (Bio-Rad) at a wavelength of 595 nm.

2.6. *Colony formation assay*

After infection for 72 h, ZR-75-30 cells from different groups were plated into six-well plates and cultured for 6 days to form natural monolayer colony. Cells were fixed with methanol and then stained with 4% crystals purple according to previously description (*22*). Cell colonies (more than 50 cells per colony) were imaged and counted under the fluorescence microscopy.

2.7. *Flow cytometry assay*

After 6 day infection, ZR-75-30 cells from different groups were reseeded on 6-cm dishes and cultured for 6 days. For cell cycle analysis, cells were fixed in 70% ethanol and kept at 4°C for 30 minutes. The suspension was filtered and stained with propidium iodide at 4°C in the dark. The cells were then analyzed by FAC Scan (FACS) flow cytometer (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instruction. For cell apoptosis analysis, cells were dual stained with an Annexin V-APC and 7-AAD Apoptosis Detection kit (Keygen, Nanjing, China) according to the manufacturer's instruction. Cell apoptosis profiles were determined using FlowJo software (TreeStar, San Carlos, CA, USA).

2.8. *Statistical analysis*

All statistical analyses were performed using SPSS 19.0 software and GraphPad Prism 5.0. Data were expressed as mean ± standard deviation (SD) of three independent experiments. The difference between CEP55 knockdown group and non-knockdown group was evaluated using the Student's *t*-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. *CEP55 mRNA expression was up-regulated in breast cancer*

We analyzed six independent microarray datasets from Oncomine database for CEP55 expression. As shown in Figure 1, the comparison showed the expression level of CEP55 was significantly up-regulated in various breast cancer types, compared to normal tissues ($p < 0.05$).

3.2. *Lentivirus-mediated RNA interference markedly suppressed CEP55 expression*

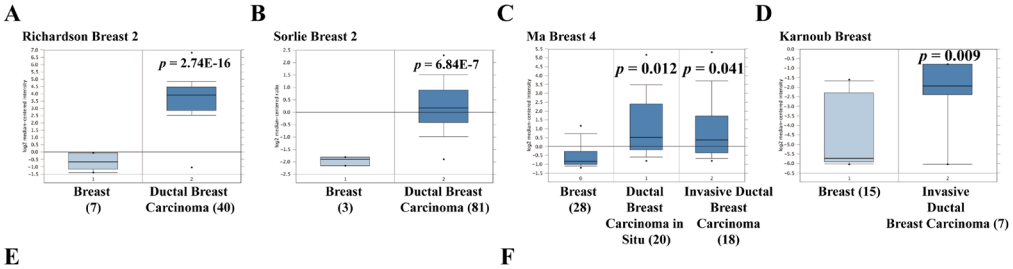
To investigate the role of CEP55 in breast cancer cells, two specific CEP55-targeting shRNA were used to infect ZR-75-30 cells. As shown in Figure 2A, efficiency of lentivirus infection was over 80%, as evidenced by GFP expression in ZR-75-30 cells from different groups. Furthermore, the efficiency of CEP55 knockdown was determined by qRT-PCR and Western blot analysis. As depicted in Figure 2B, endogenous CEP55 mRNA of ZR-75-30 cells was significantly reduced in KD-1 and KD-2 group ($p < 0.001$) compared with that in shCon and Con groups. Consistent with the results of qRT-PCR, the protein expression of CEP55 of ZR-75-30 cells in KD-1 and KD-2 group was noticeably depleted (Figure 2C). Collectively, the lentivirus system constructed could efficiently suppress CEP55 expression in breast cancer cells.

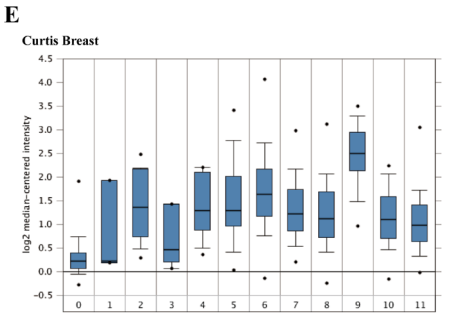
3.3. *Depletion of CEP55 obviously inhibited the proliferation of breast cancer cells*

To elucidate whether the knockdown of CEP55 related with the malignant phenotype of ZR-75-30 cells, MTT assay was performed to examine the cell proliferation in ZR-75-30 cells from different groups. As shown in Figure 3A, the proliferation rate of KD-1 and KD-2 cells was significantly decreased compared with the shCon and Con groups ($p < 0.001$), while there was no significant difference between shCon and Con groups. In addition, we tested the effect of CEP55 knockdown on the growth of ZR-75-30 cells by colony formation assay (Figure 3B). According to the result of monolayer culture, the number of surviving colonies of KD-1 was obviously reduced and the size of single colony was apparently fewer than that in shCon and Con groups (Figure 3C, $p < 0.001$). Taken together, CEP55 might be indispensable for ZR-75-30 cell proliferation.

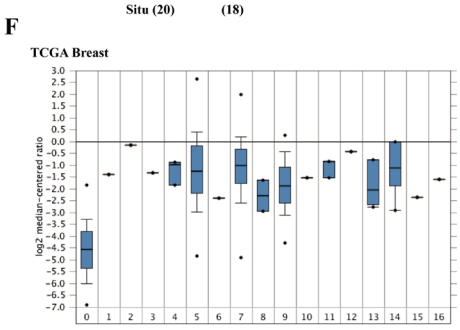
3.4. *Knockdown of CEP55 induced cell cycle arrest and apoptosis*

To further investigate the mechanism underlying CEP55 knockdown suppressed cell growth, the cell cycle distribution and apoptosis were detected in ZR-





No.	Subsets of breast tissues	Number of tissues	*P* value
0	Breast	144	-
1	Benign Breast Neoplasm	3	-
2	Breast Carcinoma	14	2.36E-5
3	Breast Phyllodes Tumor	5	-
4	Ductal Breast Carcinoma in Situ	10	-
5	Invasive Breast Carcinoma	21	3.90E-6
6	Invasive Ductal Breast Carcinoma	1556	2.30E-120
7	Invasive Ductal and Invasive Lobular Breast Carcinoma	90	5.78E-27
8	Invasive Lobular Breast Carcinoma	148	-
9	Medullary Breast Carcinoma	32	7.42E-19
10	Mucinous Breast Carcinoma	46	-
11	Tubular Breast Carcinoma	67	-



No.	Subsets of breast tissues	Number of tissues	*P* value
0	Breast	61	-
1	Apocrine Breast Carcinoma	1	-
2	Breast Large Cell Neuroendocrine Carcinoma	1	-
3	Ductal Breast Carcinoma	1	-
4	Intraductal Cribriform Breast Adenocarcinoma	3	-
5	Invasive Breast Carcinoma	76	9.18E-34
6	Invasive Cribriform Breast Carcinoma	1	-
7	Invasive Ductal Breast Carcinoma	392	9.88E-40
8	Invasive Ductal and Lobular Carcinoma	3	-
9	Invasive Lobular Breast Carcinoma	36	1.54E-19
10	Invasive Papillary Breast Carcinoma	1	-
11	Male Breast Carcinoma	3	1.05E-5
12	Metaplastic Breast Carcinoma	1	-
13	Mixed Lobular and Ductal Breast Carcinoma	7	2.17E-6
14	Mucinous Breast Carcinoma	4	-
15	Papillary Breast Carcinoma	1	-
16	Pleomorphic Breast Carcinoma	1	-

Figure 1. Microarray data extracted from Oncomine database. The expression of CEP55 gene obtained from **(A)** Richardson Breast 2 dataset, **(B)** Sorlie Breast 2 dataset, **(C)** Ma Breast 4 dataset, **(D)** Karnoub Breast dataset, **(E)** Curitis Breast dataset and **(F)** TCGA Breast dataset, respectively is shown as histograms.

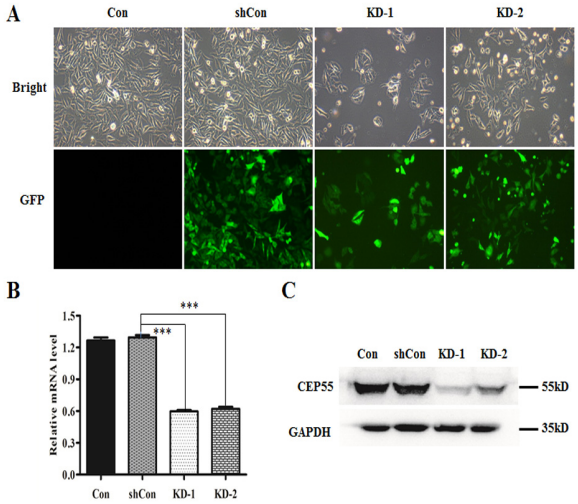


Figure 2. Knockdown of CEP55 expression by lentivirus-mediated shRNA in breast cancer cells, ZR-75-30. (A) Images recorded cells under bright and fluorescence microscopes after lentivirus infection at a magnification of ×100. Knockdown efficiency of CEP55 was determined by **(B)** qRT-PCR and **(C)** Western blot analysis. ***$p < 0.001$, compared to shCon and Con.

75-30 cells after lentivirus infection. As shown in Figure 4A, cells in three groups (Con, shCon and KD-1) were stained by PI and analyzed by FACS. Statistical analysis indicated that the percentage of cells in G0/G1 was significantly increased in the KD-1 group (81.02 ± 0.24%), compared with shCon (68.38 ± 0.26%) and Con (67.98 ± 0.37%) groups, accompanied by a decrease in the S phase (Figure 4B, $p < 0.001$). Additionally, more cells were obviously accumulated in the sub-G1 phase representing early apoptotic cells (Figure 4C, $p < 0.001$). As depicted in Figure 5A, Annexin V-APC vs 7-AAD plots from the gated cells showed the populations corresponding to viable (Annexin V-/7-AAD-), necrotic (Annexin V-/7-AAD+), early apoptotic (Annexin V+/7-AAD-) and late apoptotic (Annexin V+/7-AAD+) cells. Statistical analysis indicated both early apoptotic cells and late apoptotic cells were significantly increased in the KD-1 group, in contrast to shCon and Con groups (Figure 5B, $p < 0.001$). These results suggested that knockdown of CEP55 induced cell cycle arrest in G0/G1 phase and cell apoptosis.

4. Discussion

Human breast cancer is one of the most leading causes of diseases worldwide (*23*). What's more, it is the most common malignancy in female, leading to approximately

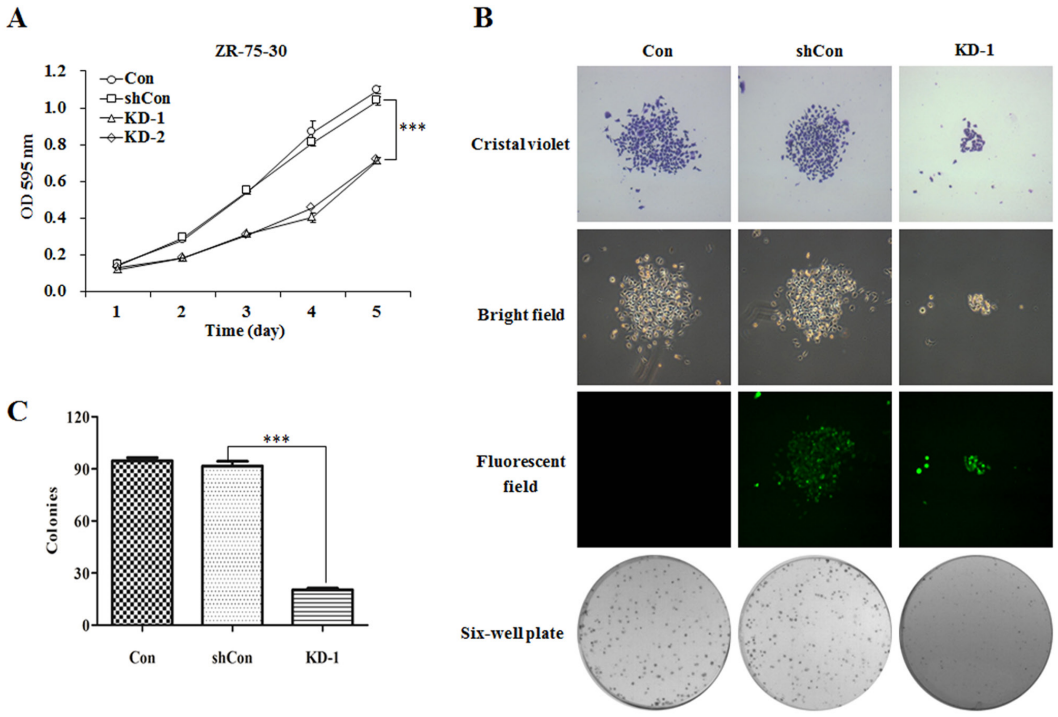


Figure 3. Knockdown of CEP55 suppressed the proliferation and colony formation capacity of ZR-75-30 cells. (A) Growth curves of ZR-75-30 cells with four different treatments (Con, shCon, KD-1 and KD-2) determined by MTT analysis. **(B)** Representative microscopic images of colonies stained by crystal violet and full vision of six-well plate under a microscope. **(C)** Statistical analysis of the colonies numbers in all three groups (Con, shCon, KD-1). ***$p < 0.001$, compared to shCon and Con.

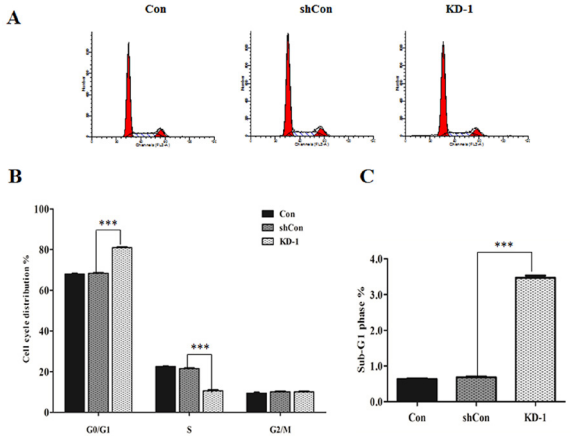


Figure 4. Effect of CEP55 silencing on cell cycle distribution of ZR-75-30 cells. (A) Cell cycle distribution of ZR-75-30 cells with three different treatments (Con, shCon, KD-1) was analyzed by flow cytometry using PI staining. **(B)** Statistical analysis of the percentages of ZR-75-30 cells in G0/G1, S and G2/M phases. **(C)** Statistical analysis of the percentage of ZR-75-30 cells in the sub-G1 phase. ***$p < 0.001$, compared to shCon and Con.

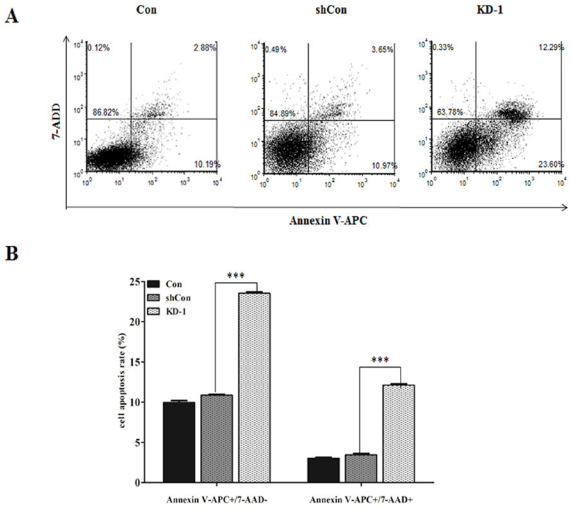


Figure 5. Effect of CEP55 silencing on apoptosis of ZR-75-30 cells. (A) Apoptosis of cells infected with lentivirus after Annexin-V/7-AAD staining. **(B)** Apoptotic cells including early-stage (Annexin V+/7-AAD-) and late-stage (Annexin V+/7-AAD+) apoptosis. ***$p < 0.001$, compared to shCon and Con.

40,000 deaths per year (*24*). More recently, CEP55 was found to be overexpressed in various kinds of tumor progression, including colon carcinoma (*25*) and gastric specimens (*15*). In this study, the data extracted from Oncomine database suggested CEP55 gene expression was significantly increased in breast cancer tissues compared to that in normal tissues. To further explore the role of CEP55 in breast cancer. Two different CEP55 shRNAs were designed to specifically block the endogenous expression of CEP55 in human breast cancer cell ZR-75-30. Further analysis found decreased CEP55 expression significantly inhibited cell growth and proliferation ability and induced cell cycle arrest and apoptosis.

Centrosome acts an essential component of cell cycle progression (*26*). It has been reported that the centrosome-associated proteins correlate with many kinds of diseases, including carcinoma (*27*). CEP55, as

member of the centrosome-associated protein family, is located in the centrosome during the interphase, dissociates from the centrosome in the M phase and condenses to the midbody during cytokinesis (*5*), which suggest that CEP55 plays a crucial role in cell cycle progression. In addition, previous studies have indicated that CEP55 plays an essential role in G2/M phase in hepatocellular carcinoma (*7*) and lung cancer cells (*15*). These studies are different from our results, which might be ascribed to the different cancers and cell lines. Flow cytometry also showed a noticeable accumulation of cells in the sub-G1 phase after CEP55 knockdown. Furthermore, Annexin V-APC/7-AAD double staining demonstrated that knockdown of CEP55 increased the apoptotic cells. Related reporter has indicated that CEP55 peptides might be useful as part of a therapeutic strategy for therapy-resistant breast cancer patients (*13*). Therefore, we could infer that the growth inhibition by CEP55 silencing in breast cancer was probably due to the induction of centrosome-related apoptosis.

In summary, this study demonstrated the crucial role of CEP55 in promoting breast cancer cell proliferation *in vitro*. However, it is necessary to investigate the specific mechanism through which CEP55 regulates the growth of breast cancer and further confirm whether or not CEP55 could act as a therapeutic target for breast cancer.

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Brief Report

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Effect of Shufeng Jiedu capsules as a broad-spectrum antibacterial

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Summary **This study sought to investigate the broad-spectrum antibacterial action of an alternative medicine, Shufeng Jiedu capsules (SFJDC). Antibacterial testing was performed to determine whether SFJDC had broad-spectrum antibacterial action *in vitro*, and testing was performed to verify whether SFJDC prevented death due to a *Streptococcus* or *Staphylococcus aureus* infection in mice. Results of antibacterial testing suggested that SFJDC are a broad-spectrum antibacterial and that SFJDC are superior to Lianhua Qingwen capsules as a broad-spectrum antibacterial. Results of testing revealed that SFJDC lowered the mortality rate, it reduced mortality, it increased average survival time, and it increased the lifespan of mice dying due to a *Staphylococcus aureus* or *Streptococcus* infection. Thus, SFJDC could become a complement to broad-spectrum antimicrobials in clinical settings.**

Keywords: Shufeng Jiedu capsules, broad-spectrum antibacterial, *Staphylococcus aureus*, *Streptococcus*

1. Introduction

Antibiotics are a class of drugs used to inhibit bacterial growth or kill bacteria. They can inhibit or kill bacteria by blocking the synthesis of bacterial cell walls, increasing bacterial membrane permeability, inhibiting protein synthesis, or blocking replication and transcription of bacterial DNA. Antibiotics come in various types, such as aminoglycosides, quinolones, macrolides, and β-lactams. Wide-spectrum antibiotics can strongly inhibit most Gram-negative bacteria as well as Gram-positive bacteria, and they can inhibit *Rickettsia*, *Spirochetes*, and some *Protozoa* spp. However, the long-term overuse of broad-spectrum antibiotics results in many dangers: drug resistance, side effects, and an imbalance in intestinal flora (*1-7*). With extensive use of broad-spectrum antibiotics in clinical settings, resistance to antibiotics has increased, *e.g.* extended spectrum β-lactamases are able to hydrolyze penicillins, carbapenems, and amoxicillin. Thus, there is an urgent need for an alternative in clinical settings.

Shufeng Jiedu capsules (SFJDC) are a traditional Chinese medicine that is mainly used to treat upper respiratory tract infections such as the flu, swelling and pain in the throat, mumps, and strep throat (*8*). In China, SFJDC have been listed as a drug to combat avian influenza after years of clinical observations. This product is approved as a traditional Chinese medicine to "reduce heat and remove toxins" (*qingwen jiedu*) and "drain the lungs and eliminate heat" (*xuanfei xiere*). SFJDC have previously been shown to be active in the experimental model used in the present study. Lianhua Qingwen capsules (LHQWC) are another traditional Chinese medicine used to treat upper respiratory tract infections, and this medicine has antimicrobial activity according to previous studies (*8,9*), so it served as a positive control in the current study. A recent study by the current authors indicated that SFJDC have some effect on a bacterial infection with few adverse reactions and good tolerability, but their broader antibacterial activity has not been known. Thus, the current study sought to explore the broad-spectrum antimicrobial action of SFJDC.

2. Materials and Methods

2.1. *Test substance*

All investigations were performed with a single batch (No.150602) of SFJDC kindly provided by the

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manufacturer Jiren Pharmaceutical (Anhui, China). In accordance with a SFJDC dose of 6.24 g/day for adults (per 60 kg body weight), equivalent doses on the basis of allometric means in mice would be about 1.1 g/kg/day. Based on this calculation, doses in animal experiments correspond to approximately half, one, or two times the average equivalent dose for a human adult. Hence, mice received doses of 0.55, 1.1, or 2.2 g/kg/day. All treatments were administered by gavage. SFJDC were suspended in distilled water and administered to mice in a volume of 0.2 mL/10 g.

2.2. *Reference drug*

LHQWC (batch No. A1409089) produced by Shijiazhuang Yiling Pharmaceutical (Hebei, China) served as a reference drug. In accordance with the dose in humans (4.2 g/60 kg/day), an equivalent oral dose on the basis of the allometric mean was selected for mice (0.77 g/kg/day).

Amoxicillin capsules (batch No. 50505005) produced by Zhuhai United Laboratories (Guangdong, China) also served as a reference drug. This product is a broad-spectrum antibiotic. In accordance with the dose in humans (2.0 g/60 kg/day), an equivalent oral dose on the basis of the allometric mean was selected for mice (0.363 g/kg/day).

2.3. *Bacteria*

Staphylococcus aureus (26003, 361), a *Staphylococcus* sp. (26101), *Staphylococcus epidermidis* (Standard strain, 108), *Streptococcus* spp. (10, 12), *Escherichia coli* (44113, 117, 178), *Pseudomonas aeruginosa* (209, 210, 211), *Streptococcus pneumonia* (31001, 163), a *Proteus* sp. (29108), *Neisseria gonorrhoeae* (29106), and *Candida albicans* (standard strain) were acquired from Chinese Pharmaceutical and Biological Products and were stored at -80°C until use.

2.4. *Animals*

Specific-pathogen-free CD-1 (ICR, male and female, 18-20 g) mice were all purchased from Charles River (Beijing, China). All animal work was performed under the guidelines of the China Academy of Chinese Medical Sciences and was approved by the China Academy of Chinese Medical Sciences Committee.

2.5. *Broad-spectrum antibacterial testing in vitro*

S. aureus (26003, 361), a *Staphylococcus* sp. (26101), *Staphylococcus epidermidis* (Standard strain, 108), *Streptococcus* spp. (10, 12), *E. coli* (44113, 117, 178), *P. aeruginosa* (209, 210, 211), *S. pneumonia* (31001, 163), a *Proteus* sp. (29108), *N. gonorrhoeae* (29106), and *C. albicans* (Standard strain) were each cultured for 24 h with nutrient broth at 37°C, and then the turbidity was adjusted to three hundred million bacteria per milliliter with nutrient broth according to Maxwell turbidimetry. All of the bacterial solutions were further diluted a thousand times with nutrient broth and 10 μL was added to 96-well plates. Fifty, 25, 12.5, 6.25, 3.125, 1.5625, and 0.87125 mg/mL of SFJDC and LHQWC were each added to the bacterial solution and cultured for 24 h at 37°C. Afterwards, the bacteria in solution were compared with normal bacteria.

2.6. *Mouse model of death due to an S. aureus infection*

A total of 120 mice were randomly distributed according to body weight into a model control group, a LHQWC group, an amoxicillin group (2.75 mL/kg), and three SFJDC groups (0.55, 1.10, and 2.20 g/kg). Drugs were orally administered daily for 3 days at a volume of 0.2 mL/10 g. Animals in the model control group were administered 0.2 mL/10g of distilled water under the same conditions. Beginning on day 3, the mice were treated with intraperitoneally injected with 0.2 mL *S. aureus*. The day after infection, the number of deaths in each group was recorded for 7 consecutive days.

2.7. *Mouse model of death due to a Streptococcus infection*

A total of 120 mice were randomly distributed according to body weight into a model control group, a LHQWC group, an amoxicillin group (2.75 mL/kg), and three SFJDC groups (0.55, 1.10, and 2.20 g/kg). Drugs were orally administered daily for 3 days at a volume of 0.2 mL/10g. Animals in the model control group were administered 0.2 mL/10g of distilled water under the same conditions. Beginning on day 3, the mice were intraperitoneally injected with 0.2 mL of a *Streptococcus* sp. The day after infection, the number of deaths in each group was recorded for 7 consecutive days.

2.8. *Statistical analysis*

Data were analyzed using SPSS software. A χ^2 test was used to statistically analyze the reduction in mortality, and a *t*-test was used to statistically analyze the average survival time. A $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. *Effect of SFJDC as a broad-spectrum antibacterial in vitro*

A clear broth indicated that a drug effectively inhibited bacteria, and the minimum inhibitory concentration of SFJDC and LHQWC for a bacterium indicated the antibacterial action of the drug. Results indicated

that SFJDC were a broad-spectrum antibacterial that inhibited all 18 species of bacteria. SFJDC were more effective at killing *S. aureus* and *Staphylococcus*, and SFJDC were superior to LHQWC as a broad-spectrum antibacterial (Tables 1-3).

3.2. *Prevention of death from an S. aureus infection with SFJDC in mice*

As shown in Table 4, the *S. aureus* control group had a mortality rate of 95% and an average survival time of 1.4 day. Compared to the control group, the 3 groups receiving SFJDC had a lower mortality rate of 70%, 75%, and 80%, respectively. The 3 groups receiving SFJDC had a reduction in mortality of 26.32%, 21.05%, and 15.79%, respectively. The group receiving 0.55g/kg of SFJDC had a significantly increased average survival time ($p < 0.01$). The 3 groups receiving SFJDC had a significant increase in lifespan of 107.14%, 82.14%, and 71.43%, respectively.

3.3. *Preventing death due to a Streptococcus infection with SFJDC in mice*

As shown in Table 5, the *Streptococcus* control group had a mortality rate of 70% and an average survival

Table 1. The anti-bacterial effects of SFJDC *in vitro*

Name of bacterium	Bacterium No.	SFJDC (mg/mL)							Control
		50	25	12.5	6.25	3.125	1.5625	0.87125	
Staphylococcus aureus	26003	-	-	-	-	-	-	+	+
Staphylococcus aureus	361	-	-	-	-	-	+	+	+
Staphylococcus sp.	26101	-	-	-	-	-	-	+	+
Staphylococcus epidermidis	Standard strain	-	-	-	-	-	+	+	+
Staphylococcus epidermidis	108	-	-	-	-	-	+	+	+
Streptococcus sp.	10	-	-	-	+	+	+	+	+
Streptococcus sp.	12	-	-	+	+	+	+	+	+
Escherichia coli	44113	-	+	+	+	+	+	+	+
Escherichia coli	117	-	+	+	+	+	+	+	+
Escherichia coli	178	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa	209	-	-	+	+	+	+	+	+
Pseudomonas aeruginosa	210	-	-	-	+	+	+	+	+
Pseudomonas aeruginosa	211	-	+	+	+	+	+	+	+
Streptococcus pneumonia	31001	-	-	-	-	-	+	+	+
Streptococcus pneumonia	163	-	-	+	+	+	+	+	+
Proteus sp.	29108	-	+	+	+	+	+	+	+
Neisseria gonorrhoeae	29106	-	-	-	-	+	+	+	+
Candida albicans	Standard strain	-	-	-	+	+	+	+	+

"-": inhibitory effect; "+": no inhibitory effect.

Table 2. The anti-bacterial effects of LHQWC *in vitro*

Name of bacterium	Bacterium No.	LHQWC (mg/mL)							Control
		50	25	12.5	6.25	3.125	1.5625	0.87125	
Staphylococcus aureus	26003	-	-	+	+	+	+	+	+
Staphylococcus aureus	361	-	-	+	+	+	+	+	+
Staphylococcus sp.	26101	-	-	+	+	+	+	+	+
Staphylococcus epidermidis	Standard strain	-	-	+	+	+	+	+	+
Staphylococcus epidermidis	108	-	-	+	+	+	+	+	+
Streptococcus sp.	10	-	-	+	+	+	+	+	+
Streptococcus sp.	12	-	+	+	+	+	+	+	+
Escherichia coli	44113	-	+	+	+	+	+	+	+
Escherichia coli	117	-	+	+	+	+	+	+	+
Escherichia coli	178	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa	209	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa	210	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa	211	-	+	+	+	+	+	+	+
Streptococcus pneumonia	31001	-	-	+	+	+	+	+	+
Streptococcus pneumonia	163	-	+	+	+	+	+	+	+
Proteus sp.	29108	-	+	+	+	+	+	+	+
Neisseria gonorrhoeae	29106	-	-	-	+	+	+	+	+
Candida albicans	Standard strain	-	-	+	+	+	+	+	+

"-": inhibitory effect; "+": no inhibitory effect.

time of 3.2 days. Compared to the control group, the 3 groups receiving SFJDC had a lower mortality rate of 20%, 30%, and 40%, respectively. The groups receiving 0.55 or 1.1 g/kg of SFJDC had a significant reduction in mortality ($p < 0.01$) of 71.43%, 57.14%, and 42.86%, respectively. The groups receiving 0.55 or 1.1 g/kg of SFJDC had a significantly increased average survival time ($p < 0.01$). The 3 groups receiving SFJDC had a significant increase in lifespan of 84.38%, 68.75%, and 51.56%, respectively.

Based on Gram staining, bacteria were divided into two categories, Gram-positive bacteria and Gram-negative bacteria. Most pyogenic bacteria are Gram-positive bacteria that can produce exotoxins that make people sick, while most intestinal bacteria are Gram-negative bacteria that produce endotoxins that make people sick. A broad-spectrum antibiotic can strongly inhibit most Gram-negative bacteria as well as Gram-positive bacteria, and it can inhibit *Rickettsia*, *Spirochetes*, and some *Protozoa* spp. The current study examined the broad-spectrum antimicrobial activity of SFJDC by antibacterial testing *in vitro* and *in vivo*. *In vitro* testing was done with six genera of Gram-positive bacteria, *i.e. S. aureus* (26003, 361), a *Staphylococcus* sp. (26101), *S. epidermidis* (Standard strain, 108), *Streptococcus* spp. (10, 12), *S. pneumonia* (31001, 163) and *C. albicans* (Standard strain), and four genera of Gram-negative bacteria, *i.e. E. coli* (44113, 117, 178), *P. aeruginosa* (209, 210, 211), a *Proteus* sp. (29108), and *N. gonorrhoeae* (29106). Results indicated that SFJDC are a broad-spectrum antibacterial and that SFJDC inhibited all 18 species of bacteria, but a larger dose of SFJDC was needed to kill Gram-negative bacteria than Gram-positive bacteria. This suggests that SFJDC are more effective at killing Gram-positive bacteria. SFJDC were most effective at killing *S. aureus* and *Staphylococcus*, and SFJDC was superior to LHQWC as a broad-spectrum antibacterial.

S. aureus is the number one cause of hospitalization and surgery for children, and the leading cause of bacteraemia in people > 65 years of age (*10,11*). A recent review (*12*) has noted alarming rates of *S. aureus* infections /100,000 population/y. Over the past few decades, bacteremia rates have been 20-38%, and this rate jumps to > 100 for people over the age of 70. *S. aureus* is the pathogen that is most frequently involved in combined infections (*13-16*). The current study investigated the ability of SFJDC to prevent death from an *S. aureus* infection in mice. Compared

Table 3. The minimum inhibitory concentration (MIC) of SFJDC for different bacteria

Name of bacterium	Bacterium No.	MIC (mg/mL)	
		SFJDC	LHQWC
Staphylococcus aureus	26003	1.5625	25.0000
Staphylococcus aureus	361	3.1250	25.0000
Staphylococcus sp.	26101	1.5625	25.0000
Staphylococcus epidermidis	Standard strain	3.1250	25.0000
Staphylococcus epidermidis	108	3.1250	25.0000
Streptococcus sp.	10	12.5000	25.0000
Streptococcus sp.	12	25.0000	50.0000
Escherichia coli	44113	50.0000	50.0000
Escherichia coli	117	50.0000	50.0000
Escherichia coli	178	50.0000	50.0000
Pseudomonas aeruginosa	209	25.0000	50.0000
Pseudomonas aeruginosa	210	12.5000	50.0000
Pseudomonas aeruginosa	211	50.0000	50.0000
Streptococcus pneumonia	31001	3.1250	25.0000
Streptococcus pneumonia	163	25.0000	50.0000
Proteus sp.	29108	50.0000	50.0000
Neisseria gonorrhoeae	29106	6.2500	12.5000
Candida albicans	Standard strain	12.5000	25.0000

Table 4. Prevention of death due to a *Staphylococcus aureus* infection with SFJDC in mice

Groups	Dose (g/kg)	Number of animals	Mortality rate (%)	Reduction in mortality (%)	Average survival time (d)	Increase in lifespan (%)
Control	-	20	95	-	1.40 ± 1.35	-
LHQWC	0.77	20	80	15.79	2.30 ± 2.43	64.29
Amoxicillin	0.363	20	10	89.47**	6.40 ± 1.85**	357.14
SFJDC	0.55	20	70	26.32	2.90 ± 2.77**	107.14
SFJDC	1.1	20	75	21.05	2.55 ± 2.65	82.14
SFJDC	2.2	20	80	15.79	2.40 ± 2.46	71.43

**$p < 0.01$ compared to control.

Table 5. Preventing death due to a *Streptococcus* infection with SFJDC in mice

Groups	Dose (g/kg)	Number of animals	Mortality rate (%)	Reduction in mortality (%)	Average survival time (d)	Increase in lifespan (%)
Control	-	20	70	-	3.20 ± 2 .63	-
LHQWC	0.77	20	35	50	5.10 ± 2.69**	59.38
Amoxicillin	0.363	20	0	100**	7.00 ± 0.00**	118.75
SFJDC	0.55	20	20	71.43**	5.90 ± 2.27**	84.38
SFJDC	1.1	20	30	57.14**	5.40 ± 2.52**	68.75
SFJDC	2.2	20	40	42.86	4.85 ± 2.72	51.56

**$p < 0.01$ compared to control.

to the control group, the 3 groups receiving SFJDC had a lower mortality rate and reduced mortality. The group receiving 0.55 g/kg of SFJDC had a significantly increased average survival time ($p < 0.01$) and a significantly increased lifespan. Findings suggest that SFJDC prevented death due to an *S. aureus* infection in mice. This study also investigated the ability of SFJDC to prevent death due to a *Streptococcus* infection in mice. Compared to the control group, the 3 groups receiving SFJDC had a lower mortality rate. The groups receiving 0.55 or 1.1 g/kg of SFJDC had a significantly reduced mortality ($p < 0.01$). The groups receiving 0.55 or 1.1 g/kg of SFJDC had a significantly increased average survival time ($p < 0.01$), and all 3 groups receiving SFJDC had a significantly increased lifespan. These findings suggest that SFJDC could become an alternative to broad-spectrum antimicrobials in clinical settings.

Results of the current study indicated that the antibacterial action of SFJDC is inversely correlated with the dose. This finding may be related to the absorption of a traditional Chinese medicine. Previous studies found that absorbance of traditional Chinese medicines was inversely correlated with the dose in a certain range, but the reason for this phenomenon has yet to be determined.

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Brief Report

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Recombinant expression, purification and crystallographic studies of the mature form of human mitochondrial aspartate aminotransferase

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Summary **Mitochondrial aspartate aminotransferase (mAspAT) was recognized as a moonlighting enzyme because it has not only aminotransferase activity but also a high-affinity long-chain fatty acids (LCFA) binding site. This enzyme plays a key role in amino acid metabolism, biosynthesis of kynurenic acid and transport of the LCFA. Therefore, it is important to study the structure-function relationships of human mAspAT protein. In this work, the mature form of human mAspAT was expressed to a high level in *Escherichia coli* periplasmic space using pET-22b vector, purified by a combination of immobilized metal-affinity chromatography and cation exchange chromatography. Optimal activity of the enzyme occurred at a temperature of 47.5ºC and a pH of 8.5. Crystals of human mAspAT were grown using the hanging-drop vapour diffusion method at 277K with 0.1 M HEPES pH 6.8 and 25%(v/v) Jeffamine® ED-2001 pH 6.8. The crystals diffracted to 2.99 Å and belonged to the space group *P*1 with the unit-cell parameters *a* =56.7, *b* = 76.1, *c* = 94.2 Å, *α* =78.0, β =85.6, *γ* = 78.4º. Elucidation of mAspAT structure can provide a molecular basis towards understanding catalysis mechanism and substrate binding site of enzyme.**

Keywords: Aspartate aminotransferase (AspAT), plasma membrane fatty acid binding protein (FABPpm), kynurenine aminotransferase-IV, crystallization, moonlighting protein

1. Introduction

Aspartate aminotransferase (AspAT, EC 2.6.1.1) is present as two homologous, genetically independent isozymes in animal cells, one located in the cytoplasm (cAspAT) and the other in mitochondria (mAspAT) (*1,2*). mAspAT was recognized as a moonlighting enzyme because it was found to have two or more different functions. Similar to cAspAT, mAspAT catalyzes the reversible reaction of L-aspartate and α-ketoglutarate (α-KG) into oxaloacetate and L-glutamate *via* a ping-pong mechanism, with pyridoxal 5'-phosphate (PLP) as an essential cofactor (*3*). Glutamine not only provides a carbon source to fuel the tricarboxylic acid (TCA) cycle *via* α-KG, but also provides nitrogen for the synthesis of nonessential amino acids and nucleotides, *i.e.*, purines, pyrimidines, alanine, serine, aspartate, ornithine, glycine, cysteine, arginine, asparagine, and proline (*4,5*). Therefore, mAspAT is one of the key enzymes that links amino acid metabolism to carbohydrate metabolism through catalysis of the reversible transamination reaction.

Different to cAspAT, mammalian mAspAT is also recognized as kynurenine aminotransferase-IV because this enzyme is capable of catalyzing the irreversible transamination of kynurenine to produce kynurenic acid (KYNA) and plays a role in the biosynthesis of KYNA in rat, mouse and human brains (*6-8*). KYNA is an endogenous antagonist of *N*-methyl-D-aspartate and α7-nicotinic acetylcholine receptors (*9,10*). In addition, KYNA is identified as an endogenous ligand for an orphan G-protein-coupled receptor (*11*). Abnormal concentration of KYNA in brain tissue has been observed in patients with mental and neurological disorders,

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including the Huntington's disease, Alzheimer's disease, and schizophrenia (*12*). Therefore, mAspAT can be envisioned to be a valid molecular target for the treatment of these neurological diseases.

Additionally, mAspAT is recognized as plasma membrane fatty acid binding protein (FABPpm) because this enzyme has a high affinity for long-chain fatty acids (LCFA) and is a key enzyme involved in the transport of the saturated LCFA and unsaturated LCFA (*13,14*). Accordingly, it is important to study the structure-function relationships of mAspAT protein because it possesses both enzymatic catalytic activity and LCFA binding activity. To date, AspATs have been purified from many sources and X-ray structures have been determined for those from *Escherichia coli* (*15*), cytosolic yeast cytoplasm (*16*), pig cytoplasm (*17*), chicken mitochondria (*18*), and chicken cytoplasm (*19*). However, the crystal structure of the human mAspAT has not been solved.

Human mAspAT contains 4 cysteine residues forming two disulfide bonds. In addition, the overexpression of mAspAT is toxic to the growth of host cells as mAspAT is a key metabolic enzyme in amino acid metabolism. This study aimed to generate and purify recombinant human mAspAT protein in a high level that is sufficient for characterization analysis and further structural studies. We report here the expression, purification and characterization of human mAspAT expression in *E. coli* periplasmic space using the plasmid pET-22b. Moreover, the crystallization and preliminary X-ray analysis of mAspAT protein were also performed.

2. Materials and Methods

2.1. *Materials*

The pET-22b (+) vector and *E. coli* strain BL21 (DE3) were obtained from Novagen (Beijing, China). pMD18-T Simple Vector, *E. coli* strain JM 109, T4 DNA ligase, *Ex* Taq DNA polymerase, *Noc* I and *Xho* I restriction enzymes were purchased from Takara Biotechnology (Dalian, China). The Amicon Ultra centrifugal filter (3 kDa) used for filtration of the cell culture medium was obtained from Millipore Corporation (Bedford, MA, USA). The AST (SGOT) Reagent Kit used for monitoring the activity of mAspAT was purchased from BIO QUANT (San Diego, CA, USA). Crystallization Screening kits were purchased from Hampton Research (Oklahoma City, OK, USA). All primers were synthesized by Takara Biotechnology.

2.2. *Construct of expression vector*

The nucleotide sequence encoding for the mature form of human mAspAT (GenBank accession no. M22632.1) was artificially synthesized by Taihe Biotechnology Co., Ltd. (Beijing, China). *Nco* I and *Xho* I restriction sites were added to the N-terminus and C-terminus of the mAspAT sequence by PCR, respectively, using the forward primer F1 5'-CATGCCATGGCAGAGCCAG CTCCTGGTGGA-3' (*Nco* I site is underlined) and the reverse primer R1 5'-CCGCTCGAGCTTGGTGACCT GGTGAATGGCAT-3' (*Xho* I is underlined). The PCR product was digested with *Nco* I-*Xho* I, and cloned into the *Nco* I-*Xho* I site of the pMD18-T simple vector and then transformed into *E. coli* strain JM 109. Nucleic acid sequences of the cloning DNA fragment were confirmed by DNA sequencing (BigDye™ Kit, Applied Biosystems, USA) and ABI PRISM™ 3730XL DNA Analyzer, according to the recommended protocols. The target DNA fragment was further subcloned in the same site of pET-22b (+) vector, resulting in pET22b-hmAspAT. The resulting vector was then transformed into chemically competent *E. coli* strain BL21 (DE3) by heat shock for protein expression.

2.3. *Expression of human mAspAT*

The single colony of *E. coli* BL21 (DE3) harboring the expression vector in 30 mL of Luria-Bertani (LB) medium containing 100 μg/mL ampicillin, and then cultivated at 37ºC until the optical density (OD_{600}) reached 0.6. The cells were harvested by centrifugation at 4,000 × g for 10 min, and resuspended in 3 L fresh LB medium containing 100 μg/mL ampicillin. Subsequently, protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 h at 16ºC. The cells were harvested by centrifugation at 8,000 × g for 15 min and washed with buffer A (20 mM NaH_2PO_4, 0.5 M NaCl, pH 7.4). Approximated 45 g (wet weight) cells were obtained from 3 L culture. After centrifugation, the cell pellets was resuspended in 40 mL (for 1 L culture) ice-cold extraction buffer A, and lysed by ultrasonication at ice-cold temperature using an UP400S instrument (Dr. Hielscher GmbH, Stuttgart, Germany). The cell lysis was centrifuged at 12,000 rpm for 15 min to separate soluble (supernatant) and precipitated (pellet) fractions.

2.4. *Purification of human mAspAT*

The resulting supernatant was filtered with a 0.22 μm syringe filter and then loaded onto a 5 mL HisTrap™ FF crude column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with buffer A. After washing the column with buffer A containing 50 mM imidazole, the target protein was eluted with buffer B (20 mM NaH_2PO_4, 0.5 M NaCl, 300 mM imidazole, pH 7.4). The buffer was exchanged with 20 mM Tris buffer (pH 7.5) containing 20 mM NaCl using a 5 mL HiTrap™ desalting column (GE Healthcare). The desalted sample was loaded onto a 1 mL SP Sepharose™ FF column (GE Healthcare). After washing, the column

was eluted with a linear gradient of NaCl from 20 to 500 mM in Tris buffer (pH 7.5) at a flow rate of 1 mL/min. Fractions with enzyme activity were pooled and the buffer was exchanged for 20 mM Tris (pH 7.0) containing 20 mM NaCl by using a 5 mL HiTrap™ desalting column (GE Healthcare), and then the protein was concentrated to a final concentration of 5 mg/mL with a 3 kDa cut-off concentrator (Millipore). During purification, the activity of mAspAT was monitored using the AST (SGOT) Reagent Kit (BQ Kits). In this method, a diazonium salt was used which selectively reacted with the oxalacetate to produce a color complex that was measured photometrically. According to the protocol, reaction mixture including enzyme, substrate, assay buffer and assay developer was incubated at 37°C for 60 min, and OD_{450} was then measured. One unit of enzyme is defined as the amount of enzyme which generates 1.0 μmol of glutamate per minute at 37°C. The purity of the eluted protein was analyzed by SDS–PAGE and found to be > 95%.

2.5. *SDS-PAGE and Western blot analysis*

SDS-PAGE analysis was performed using 12% resolving gel and 5% stacking gel. The protein bands were visualized by Coomassie brilliant blue R-250 and then analyzed by image-density analysis software (Gel-Pro, USA). Soluble fractions of cell lysates after IPTG induction were subjected to SDS-PAGE and transferred to polyvinylidene difluoridemembrane (Millipore). The membranes were blocked with 5% defatted milk at room temperature for 60 min and then incubated with anti-his tag mouse monoclonal antibody (1 μg/mL, Millipore) at 4ºC overnight. The membranes were washed twice with PBS buffer and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (1:5,000, Millipore) at room temperature for 90 min. Finally, the membranes were washed five times with PBS buffer, and a 3′-diaminobenzidine kit was used for color development (Sigma-Aldrich, St. Louis, Mo, USA).

2.6. *Crystallization and X-ray diffraction of human mAspAT*

Initial crystallization screening was carried out in 24-well tissue-culture plates at 277 K by the hanging-drop vapor diffusion method using commercially available Index Screen, Crystal Screen, Crystal Screen 2 and PEG/Ion Screen (Hampton Research). Crystals were grown in a mixture containing 1 μL of protein (5 mg/mL in 20 mM Tris pH 7.0, 20 mM NaCl) and 1 μL of reservoir solution and were equilibrated against 400 μL reservoir solution. Crystallization conditions were optimized based on the initial screening.

Single crystals were soaked for several minutes in a reservoir solution containing 25%(v/v) glycerol and were then flash-cooled in liquid nitrogen. All X-ray diffraction data were collected on the ESRF beamline BL-17U at the Shanghai Synchrotron Radiation Facility in China and were processed using the HKL-2000 package (*20*). Diffraction data were collected at a wavelength of 0.98 Å, an oscillation angle of 1º, an exposure time of 1 s per image and a crystal-to-detector distance of 250 mm.

3. Results and Discussion

Human mAspAT is encoded by nuclear gene and synthesized in the cytoplasm as precursor protein containing N-terminal presequences of 30 amino acid residues in length (*21*). After completion of translation, human mAspAT is imported into mitochondria matrix *via* several apparently discrete steps including proteolytic cleavage of the presequence with processing proteases and assembly into mature protein (*22,23*). Therefore, the mature form of human is post-translationally imported into the mitochondrial matrix and lack 30 amino acid residues from the N-terminus compared with the precursor (*24*). The recombinant expression results indicated that the precursor form of mAspAT was found to express as inclusion body in *E. coli* expression system, however, the mature could be found in the supernatant of the bacterial homogenate (*25*). Therefore the mature form of human mAspAT was chosen to study the recombinant expression and crystallization of protein.

Human mAspAT is a toxic protein to host cell and also contains 4 cysteine residues forming two disulphide bonds. In order to obtain the large amounts of soluble mature mAspAT in *E. coli* for the crystallization study, we introduced the use of pET-22b plasmid as the expression vector. The pET-22b vector possessed an N-terminal *pel*B secretion signal under the control of the strong bacteriophage T7 promoter, which directed the recombinant protein to the *E. coli* periplasmic space (*26*). The periplasm of *E. coli* contains the disulfide oxidoreductases and isomerases, which is an oxidizing environment and can facilitate formation of disulfide bonds that are always required for correct protein folding (*27,28*). Therefore the periplasm of *E. coli* was expected to be an ideal compartment for expression of human mAspAT.

The plasmid pET22b- hmAspAT was then transformed into *E. coli* BL21 (DE3). When the culture was propagated at 37°C until OD_{600} reached 0.6, the expression of the fusion protein was induced with 1.0 mM of IPTG, and then the culture was grown for an additional 20 h at 16°C. After lysis, the supernatant was analyzed by Western blotting using anti-his tag mouse antibody. Western blot analysis confirmed that the recombinant human mAspAT was present in the soluble fraction of the cell lysate after induction with IPTG (Figure 1A).

Recombinant human mAspAT was purified by a combination of immobilized metal-affinity chromatography and cation exchange chromatography. The purified protein was resolved as a single band in the SDS-PAGE gel with a molecular mass of about 43 kDa, indicating that these two chromatographic steps were very effective (Figure 1B, lane 3). The final yield of pure protein was approximately 10 mg from 1 L of expression culture, allowing the preparation of approximately 2 mL of a 5.0 mg/mL protein sample for crystallization assays. After two chromatography steps, the specific activity and overall recovery of purified mAspAT were 36.3 U/mg and 19.4%, respectively (Table 1). The purification of mAspAT protein is summarized in Table 1.

The optimal pH for enzyme activity was determined to be pH 8.5 (Figure 2A), similar to that (pH 8.0) of AspAT from *Bacillus subtilis* (*29*). Actually, the enzyme activity over the pH range 8.0-10.0 was more than 60% of the maximum activity (Figure 2A). Moreover, human mAspAT showed optimal activity at 47.5°C and maintained over 80% activity at temperatures from 40 to 60°C, indicating a certain degree of thermostability in this temperature range (Figure 2B). The recombinant human mAspAT tended to have relatively high activity and stability in alkaline environments, similar to that of the AspATs from *B. subtilis* and *B. circulans* (*29,30*). Thus the recombinant human mAspAT had optimal

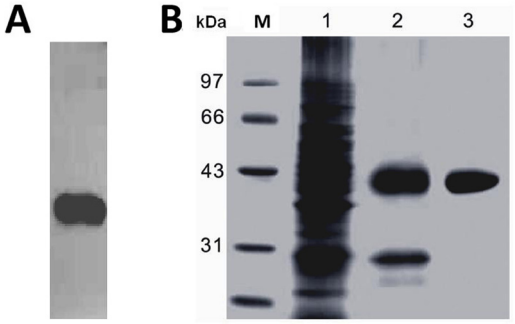

Figure 1. Quality assessment of human mAspAT expression and purification. (A) Western blot analysis of human mAspAT expression using an anti-his tag mouse monoclonal antibody. Lane 1, soluble fraction of the cell lysate after induction with IPTG. **(B)** SDS–PAGE analysis of human mAspAT samples during protein purification. Lane M, protein marker; lane 1, soluble fraction after cell lysis; lane 2, elution fractions from the HisTrap™ FF crude column; lane 3, elution fractions from the SP Sepharose™ FF column.

Table 1. Purification efficiency of recombinant human mAspAT

Purification step	Specific activity (U/mg)	Purification (fold)	Yield (%)
Lysate supernatant	0.574	1	100
His Trap	12.5	8.42	21.9
SP Sepharose	36.3	26.8	19.4

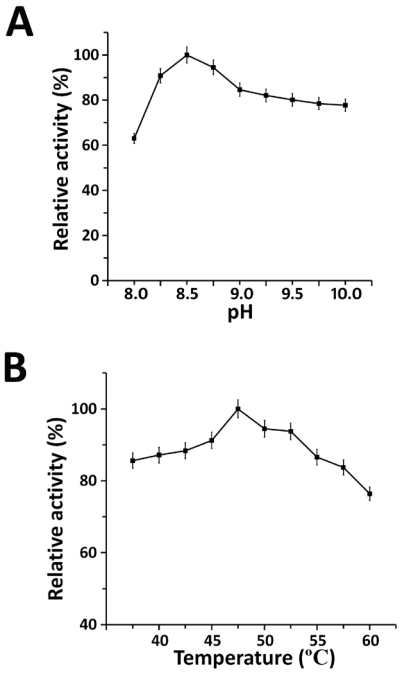

Figure 2. Effects of pH and temperature on activity of purified recombinant human mAspAT. Enzyme activity was determined at different pHs **(A)** and temperatures **(B)** using the AST (SGOT) Reagent Kit.

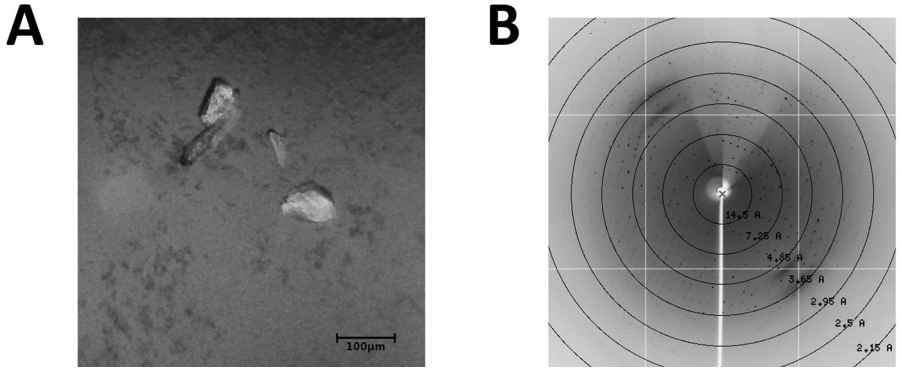

Figure 3. Crystals and X-ray diffraction image of recombinant human mAspAT. (A) Crystals grown in 0.1 M HEPES pH 6.8 and 25%(v/v) Jeffamine® ED-2001 pH 6.8 at 277 K. **(B)** X-ray diffraction image of human mAspAT.

Table 2. Data collection and processing

Diffraction source	BL-17U, ESRF
Wavelength (Å)	0.98
Temperature (K)	100
Crystal-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	*P*1
a, *b*, *c* (Å)	56.7, 76.1, 94.2
α, *β*, *γ* (°)	78.0, 85.6, 78.4
Resolution range (Å)	2.99
Total No. of reflections	578579
No. of unique reflections	30161
Completeness (%)	97.2 (97.1)
Average I/σ (I)	4.3 (2.0)
R_{merge} (%)*	0.083 (0.265)
R_{meas} (%)**	0.117 (0.375)

* $R_{merge} = \Sigma_{hkl} \Sigma_i | I_i(hkl) - \langle I(hkl) \rangle | / \Sigma_{hkl} \Sigma_i I_i(hkl)$.
** $R_{meas} = \Sigma_{hkl} \{N(hkl) / [N(hkl)-1]\} \frac{1}{2} \Sigma_i | I_i(hkl) - \langle I(hkl) \rangle | / \Sigma_{hkl} \Sigma_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of the $N(hkl)$ observations Ii(hkl) of each unique reflection *hkl* after scaling.

activity at pH 8.5 and 47.5°C.

Initial crystallization screening yielded a crystallization hit under the condition No. 39 of the Index Screen consisting of 0.1 M HEPES pH 7.0 and 30%(v/v) Jeffamine® ED-2001 pH 7.0. This condition was subsequently optimized by testing 231 new crystallization combinations with varying pH values from 6.5 to 7.5 using HEPES buffer and Jeffamine® ED-2001 concentrations from 20 to 40%(v/v). Finally, we obtained an optimal composition for the reservoir solution of 0.1 M HEPES pH 6.8 and 25%(v/v) Jeffamine® ED-2001 pH 6.8 (Figure 3A).

The human mAspAT crystal data were collected to a resolution of 2.99 Å (Figure 3B) and processed using the HKL-2000 package (*20*). The crystals belonged to the space group *P*1, with unit cell parameters *a* =56.7, *b* = 76.1, *c* = 94.2 Å, *α* =78.0, *β* =85.6, and *γ* =78.4º. The space group of cytosolic AspATs from chicken (*19*), pig heart (*17*), and *Saccharomyces cerevisiae* (*16*) is $P2_12_12_1$, while the space group for AspAT crystals from *E. coli* is $P2_1$ (*15*). Data collection statistics are summarized in Table 2.

In conclusion, we constructed the expression construct for human mAspAT and expressed it as an active enzyme in *E. coli* periplasmic space. Moreover, the recombinant human mAspAT was purified and its some biochemical properties were also examined. The crystals of mAspAT protein have been obtained by the hanging-drop vapor-diffusion method and an X-ray diffraction data set was collected from a single crystal to 2.99 Å resolution. This study may provide a method useful for the recombinant preparation of other cytotoxic proteins. The structure of mAspAT protein will provide insight into the structure reactivity relationships of human mAspAT and its substrates. The human mAspAT structure will be useful for screening of its inhibitors and may also have implications for future therapeutic approaches.

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RETRACTED: A systematic review and meta-analysis of feasibility, safety and efficacy of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) versus two-stage hepatectomy (TSH).

On January 4, 2016, the authors of the article entitled "A systematic review and meta-analysis of feasibility, safety and efficacy of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) versus two-stage hepatectomy (TSH)" (PMID: 26559020) contacted the Editor-in-Chief of Bioscience Trends regarding an oversight in their methodology. ALPPS is a surgical procedure that was first introduced in 2012. In its early stages, the procedure had several names, and a handful of researchers referred to ALPPS as "in situ liver transection with portal vein ligation." However, this term was not included as a search term in the article comparing ALPPS and TSH. Once the authors became aware of this oversight, they contacted the Editor-in-Chief of Bioscience Trends to retract their paper. The Editor-in-Chief thoroughly examined the article and made the decision to retract the paper as the authors requested. The reason for this retraction is because the described meta-analysis lacked certain key search terms. The article is meaningful, but this oversight in methodology might undermine the findings of the meta-analysis.

Jianjun GAO, Managing Editor

Reference

Sun ZP, Tang W, Sakamoto Y, Hasegawa K, Kokudo N A systematic review and meta-analysis of feasibility, safety and efficacy of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) versus two-stage hepatectomy (TSH). *BioScience Trends. 2015 ;9(5):284-288.*

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Guide for Authors

1. Scope of Articles

BioScience Trends is an international peer-reviewed journal. BioScience Trends devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of 100 references. Mini reviews are also accepted.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 2,000 words in length (excluding references).

Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references).

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in BioScience Trends in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references.

3. Editorial Policies

Ethics: BioScience Trends requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to BioScience Trends, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter signed by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit http://www.biosciencetrends.com/downcentre.php (Download Centre).

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Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in BioScience Trends.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in BioScience Trends and need assistance before submitting a manuscript. Authors can visit this organization directly at http://www.iacmhr.com/iac-eso/support.php?lang=en. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

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

interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

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

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

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Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

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

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al.* Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. Examples are given below:

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Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

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

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

Example 4 (Sample web page reference):
Ministry of Health, Labour and Welfare of Japan. Dietary reference intakes for Japanese. *http://www.mhlw.go.jp/houdou/2004/11/h1122-2a.html* (accessed June 14, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that the symbols and numbers appeared in the figures should be clear. Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays. All color figures will be reproduced in full color in the online edition of the journal at no cost to authors.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g.* mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide www.bipm.org/en/si/ for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and BioScience Trends accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g.*, Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to BioScience Trends for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to BioScience Trends online at http://www.biosciencetrends.com. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at office@biosciencetrends.com.

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author via e-mail. Corrections must be returned to the editor (proof-editing@biosciencetrends.com) within 3 working days.

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