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New medical education reform in China: Towards healthy China 2030

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Summary

On July 11, 2017, the State Council of China issued a bold plan to revolutionize medical education and promote collaboration between medical education and practice. The cornerstone of the plan is training more qualified medical professionals to improve public healthcare on the path to Healthy China 2030. According to this plan, a "5+3" training system will be instituted to train medical professionals in China, and top medical colleges will be encouraged to recruit more students. However, given the less-than-ideal professional status of Chinese doctors, the frequent incidents of violence against them, long working hours and a heavy workload, and an unsatisfactory income, attracting personnel to work in medicine and health care has become a challenge. Prior to the end of 2016, there were 3.19 million practicing (assistant) physicians in China, amount to 2.31 per thousand population. The average workload of physicians was 7.3 outpatient visits per day and 2.6 beds per day, and these figures are much higher for physicians working in tertiary hospitals. Studies have found that 78% of physicians work more than 8 hours a day and 7% of physicians work more than 12 hours a day, but the average annual income of physicians in 2015 was 77,000 yuan (about \$12,360), in contrast to an average annual income of \$294,000 for physicians in the United States. Medical humanities education is also emphasized by the new medical education reform to foster the humanistic spirits of medical students in order to improve public healthcare in China. In the face of a mindset that "medical technology comes first" and growing expectations among the public, public education is needed to provide the public with a more comprehensive view by explaining the limitations of modern medicine since "medicine is not a panacea". Additional efforts should be undertaken by the Government, organizations, physicians, patients, and the public to create a virtuous cycle of healthcare in China.

Keywords: Healthcare, reform, medical education, medical humanities

1. Introduction

On July 11, 2017, the State Council of China issued a bold plan to revolutionize medical education and promote collaboration between medical education and practice (1). This was the first guideline issued by the State Council of China on medical education reform since 1949. The main goals of this reform are to make breakthroughs in the system of medical education management and

to foster the best medical personnel through incentives by 2020. Additional goals are to foster a better policy environment for reform and development of medical education, to standardize the training system for medical personnel, and to meet China's health needs by 2030 (2).

The cornerstone of the plan is to train more qualified medical professionals by reforming medical education in order to improve the public healthcare in China. In addition, medical humanities education is emphasized as part of medical education to promote the integration of humanities education and professional education and to foster the humanistic spirits of medical students in order to improve public healthcare in China (2). The goals are laudable, but many pressing issues must be addressed.

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2. "5+3" Training system vs. Dampened enthusiasm for the study of medicine

Prior to the end of 2016, there were 11.17 million health personnel in China, of which 3.19 million were practicing (assistant) physicians (3). However, only 51% of physicians have received undergraduate or postgraduate education, and most had not received standard training in clinical medicine (4). This led to substantial differences in clinical practice. Chinese medical personnel face challenges in terms of both education and professional training.

According to this new medical education reform, a "5+3" training system – a 5-year-program of undergraduate clinical medicine plus a 3-year residency training or a 3-year postgraduate program – will be instituted to train medical professionals in China. In order to increase the supply of quality professionals, top medical colleges are encouraged to recruit more s medical students of higher quality.

Due to the less-than-ideal professional status of Chinese doctors and frequent incidents of violence against them over the past five years (5-7), a growing number of outstanding young people are losing their enthusiasm to study medicine, and there is declining interest among medical graduates in pursuing careers in clinical practice in China. A study has indicated that Chinese universities have produced about 4,727,977 medical graduates over the past 10 years, but the total number of registered physicians in clinical practice increased by only about 752,233 (15.91%) (8).

Physicians' dissatisfaction with the practice of medicine will affect how the profession is viewed. In 2015, the Chinese Medical Doctors Association analyzed 9,524 responses to a survey, and 64.48% of physicians

responded that they would not encourage their child to become a physician in the future (9). Making matters worse, the sudden death of a 25-year-old resident in anesthesiology at Sir Run-Run Shaw Hospital, Zhejiang University School of Medicine on June 28, 2017 shocked the public (10). This was the fifth case of a sudden death of a young doctor in 2017, and it caused widespread concern about the grim situation for young doctors in China in terms of long working hours and a heavy workload.

According to data from the National Health and Family Planning Commission of China (11), there were 983,394 medical and health care facilities nationwide prior to the end of 2016. This included 29,140 hospitals, 926,518 primary health care facilities (community health care centers, township hospitals, *etc.*). There were 3.19 million practicing (assistant) physicians, amounting to 2.31 per thousand population (Table 1).

The workload of physicians in hospitals is considerable. Physicians dealt with 3.27 billion outpatient visits in 2016, with an average workload of 7.3 visits per day, and they dealt with 175.3 million inpatients, with an average workload of 2.6 beds per day (Table 2). In particular, physicians working in tertiary hospitals are responsible for 8.1 outpatient visits per day and 2.7 beds per day. Physicians at primary hospitals dealt with more outpatient visits. Physicians working at community health care centers had an average workload of 15.9 visits per day and physicians working at health clinics in towns and townships had a workload of 9.5 visits per day (Table 2).

More than 30,000 Chinese physicians were surveyed about their income in 2016, and results indicated that the average annual income of physicians in 2015 was 77,000 yuan (\$12,360) (12). In contrast, a study involving over

Table 1. The national number of hospitals and primary health care facilities, beds and their usage, and practicing (assistant) physicians in China in 2016*

Facilities	Number	Beds	Bed usage (%)	Average duration of hospitalization (days)
Hospitals	29,140	5,688,875	85.3	9.4
Public hospitals	12,708	4,455,238	91.0	9.6
Private hospitals	16,432	1,233,637	62.8	8.6
Hospitals				
Tertiary hospitals	2,232	2,213,718	98.8	10.1
Secondary hospitals	7,944	2,302,887	84.2	8.8
Primary hospitals	9,282	517,837	58.0	9.0
Primary health care facilities	926,518	1,441,940	—	—
Community health care centers	8,918	182,191	54.6	9.7
Community health care station	25,409	20,498	—	—
Township hospitals	36,795	1,223,891	60.6	6.4
Village clinic	638,763	—	—	—
Clinic (Infirmary)	201,408	154	—	—
Practicing (assistant) physicians (million)			3.19	
Practicing (assistant) physicians per thousand population			2.31	

*Data are from the National Health and Family Planning Commission of China (11).

Table 2. The national medical service workload and the workload of physicians in China in 2016*

Facilities	Outpatients (100 million visits)	Average workload posed by outpatients (visits)	Inpatients (million)	Average workload posed by inpatients (beds per day)
Hospitals	32.7	7.3	175.3	2.6
Public hospitals	28.5	7.6	147.5	2.6
Private hospitals	4.2	5.5	27.8	2.2
Hospitals				
Tertiary hospitals	16.3	8.1	76.9	2.7
Secondary hospitals	12.2	6.9	75.7	2.7
Primary hospitals	2.2	6.1	10.4	1.9
Primary health care facilities	43.7	—	41.7	—
Community health care centers	5.6	15.9	3.1	0.6
Township hospitals	10.8	9.5	38.0	1.6

*Data are from the National Health and Family Planning Commission of China (11).

19,200 physicians in the United States in 2017 indicated that the average annual full-time compensation for physicians providing patient care was \$294,000 (13). The per capita disposable income of urban residents in China is 21,966 yuan (\$3,527), and the income in cities such as Shanghai, Beijing, and Zhejiang is as high as 40,000-50,000 yuan (\$6,000-8,000) (12). In contrast, the physicians' income is not low. However, many physicians believe that their work is not adequately reflected in their income in light of their huge workload and work stress, the time and effort spent to receive their education, and the long-standing risks to their physical safety that they must face because of violence against health professionals. A point worth noting is that physicians are commonly overworked. According to one study, 78% of physicians work more than 8 hours a day and 7% of physicians work more than 12 hours a day (12).

In the face of these challenges, the Chinese Government is expected to devote greater attention to the following aspects as it reforms medical education: improving the mechanism for balancing the supply of and demand for medical training; attracting talents to engage in medical and health work; speeding up the establishment of mechanism for rationally reflecting the expertise and labor value of medical personnel, and improving the evaluation mechanism of physicians to enhance the occupation attraction.

3. Medical humanities education vs. Pressing issues in healthcare

Incentive policies, including the increase in income, and improved evaluations are greatly needed in order to guide career choices by young Chinese. Meanwhile, medical humanities education is also being emphasized. As indicated in this new medical education reform, medical humanities education should be provided during medical education to promote the integration of humanities education and professional education and to foster the humanistic spirits of medical students in order to improve healthcare in China.

Revival of the humanities in Chinese medical education is being given great expectation as a way to resolve predicaments in Chinese healthcare (14,15), and medical humanities education will be enhanced under the new plan to reform medical education. However, will medical humanities education actually resolve those problems? One cannot help but be somewhat circumspect.

Previous reform of medical education emphasized medical humanities in an effort to increase the professionalism of future physicians. Some universities have established institutes of medical humanities, such as the Institute of Medical Humanities of Peking University that was founded in 2008. Courses such as an online course entitled Introduction to Medical Humanities created at Fudan University in 2015 have benefited almost 500,000 students from 140 universities (16). However, healthcare in China still has many pressing issues, including the social phenomenon of mistrust between patients and physicians, violence against health professionals, commercialization of healthcare, and perverse incentives for medical professionals. The mindset of "medical technology comes first" and the rising expectations among the public compound the problem.

Moreover, the current courses in medical humanities were arbitrarily established due to a lack of organizational independence. Medical humanities is spread out amongst the disciplines of philosophy, sociology, political theory, education, and traditional Chinese medicine. Various problems like a shortage of instructors are obstacles that are delaying the integration of humanities into the medical curriculum at most medical universities in China.

4. "Medical technology comes first" vs. "Medicine is not a panacea"

Today, medical humanities education has placed greater emphasis on the doctor's sense of social responsibility in order to train medical students to develop sensitivity to,

empathy for, and understanding of the human condition. Nonetheless, a point worth mentioning is that in addition to the doctor's level of expertise and humanistic care, the present predicaments facing healthcare in China are caused by a variety of factors, including national health policy, medical costs, and patient circumstances. A wide range of legal, social, and financial efforts are also necessary.

In the face of a mindset that "medical technology comes first" and growing expectations among the public, public education is needed to provide the public with a more comprehensive view by explaining the limitations of modern medicine since "medicine is not a panacea".

The focus of new medical education reform in China is "to foster medical personnel with a high level of both expertise and humanistic spirits". Additional efforts should be undertaken by the Government, organizations, doctors, patients, and the public to create a virtuous cycle of healthcare in China.

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Intravenous polymyxins: Revival with puzzle

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Summary

With the increasing incidence of multi-drug resistant strains, especially carbapenem resistant strains, polymyxins (mainly colistin and polymyxin B) based regimens seem to be a revival as an effective treatment of last resort in these infections. Evidence from 47 clinical trials or case series we reviewed showed that polymyxins based regimens are effective and have less toxicity compared with previous trials. When used alone, the mortality of intravenous polymyxins ranged from 0% to 74.3%, clinical response (cure and improvement) rate was 7-82.1%, and microbiological eradication was 27.3-73.9%. The main reasons for the combination therapy are to get potential synergistic effects and to prevent the selection of heteroresistant strains. Several studies showed combination therapy seemed to be more effective than monotherapy, though a few doubts remain. Clinically, polymyxins can be used in combination with several antibiotics, such as carbapenem, sulbactam, tigecycline, fosfomycin, glycopeptide, rifampicin and so on, but the optimal combination regimen is yet to be confirmed. The optimal dose of polymyxins is also controversial. With the limited clinical evidence, it's suggested loading dose regimens may be more effective, but more attention should be paid to adverse effects. Although recommended in some studies, high dose polymyxins regimens with inconsistent clinical evidence need more trials to confirm. It is important to note that concerning dosing regimens, colistin and polymyxin B are not quite the same. In renal impaired patients polymyxin B should be prescribed without dosing adjustment. Risk of renal failure may increase in the following situations, such as the combination of intravenous colistin plus intravenous vancomycin, higher doses-colistin, and intravenous colistin combined with inhalational colistin. In conclusion, there're still controversies in combination regimens, dosing strategies and so on. Prospective trials of larger sample size are needed.

Keywords: Intravenous, polymyxins, colistin, polymyxin B

1. Introduction

Polymyxins are bactericidal drugs that exhibit their antibacterial activity by disrupting bacterial cell membranes, leading to cell lysis (1). It has been approved by the US food and drug administration (FDA) and has been available since 1959 for the treatment of infections caused by Gram-negative bacteria (2,3). There're two commercially available polymyxin antibiotics: polymyxin B and colistin (also known as polymyxin E). Colistin-containing products are

commercially available in two forms, colistin sulphate and the inactive prodrug colistin methanesulfonate (CMS). Colistin sulfate is for oral and topical use, while CMS is for parenteral and aerosol therapy; both forms may be given by inhalation (4-9). CMS is hydrolyzed to colistin, which is the base component responsible for its antibacterial activity (10), and is less toxic than colistin sulphate (11). While colistin is more stable than colistimethate in human plasma (12). CMS is much more commonly used internationally (e.g. North America, South America, Asia, Europe and Australia), whereas parenteral polymyxin B is mainly available in the USA, Brazil and Singapore (13).

Nosocomial infections caused by multidrug-resistant (MDR) or even pan-drug resistant (PDR) microorganisms, are common worldwide and have an increasing incidence (14). As multidrug-resistant (MDR) strains are now increasingly observed

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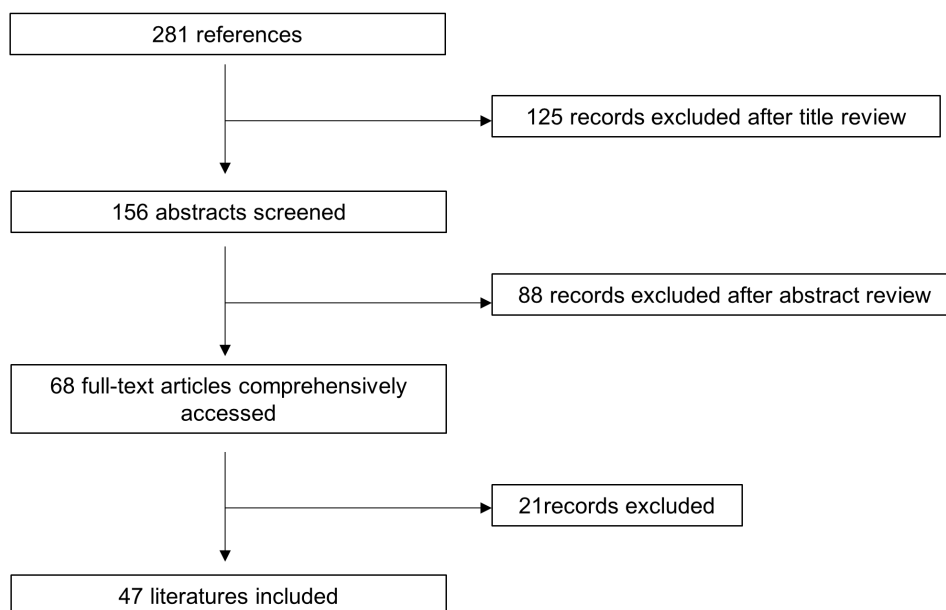


Figure 1. Flow chart demonstrating studies that were processed for inclusion.

worldwide, polymyxins are often considered as the treatment of last resort, for its favorable properties of rapid bacterial killing with a narrow spectrum of activity and an associated slow development of resistance (15). According to different type of infections, polymyxins can be used mainly in 3 routes: intravenous, inhalational (aerosolized) and intrathecal/intraventricular and sometimes more than one route is seen in one patient. Polymyxins can also be used alone or in combination with other antibiotics.

Because of systematic toxicities, polymyxins were not used often between the 1970s and 1990s, and the number of studies analyzing its use and pharmacology was minimal (15). As mentioned before, the lack of treatment options for MDR gram-negative bacilli (GNB) has led to the re-emergence of polymyxin as an antimicrobial therapy, but clinical data is still rare. We have tried to review evidence of intravenous polymyxins in clinical practice.

2. Data collection

We conducted a comprehensive literature search using the electronic PubMed database for relevant articles, without year or language restriction. We retrieved relevant articles using the following terms: "colistin" or "polymyxin E" or "polymyxin B", and "intravenous or systemic" as well as their combinations in terms of "case reports, clinical conference, clinical study, clinical trial, comparative study, controlled clinical trial, evaluation studies, editorial, letter, meta-analysis, multicenter study, observational study, pragmatic clinical trial, randomized controlled trial, review, or systematic reviews". The search was focused on studies that had been conducted in humans. The last search was

updated on October 2016 and is shown in Figure 1, out of a total of an initially identified 281 references, we listed 47 clinical trials or case series. Of them, there are 26 retrospective trials, 4 randomized controlled trials, 2 present observational studies, 13 prospective cohort trials, and 2 case series. There were 4 from USA, 1 from Argentina, 4 from Brazil, 2 from China (Taiwan), 6 from Greece, 1 from India, 1 from Israel, 6 from Italy, 2 from Korea, 1 from Maryland, 1 from Morocco, 1 from South Africa, 2 from Spain, 3 from Thailand, 11 from Turkey, and 1 from Vietnam (Table S1, Table S2, Table S3, <http://www.biosciencetrends.com/action/getSupplementalData.php?ID=13>).

3. Monotherapy or Combination Therapy, which is better?

As the most widely used route of administration, intravenous polymyxins are effective in most infection sites. The cure rates of colistin based regimens are reported to be 53.7-79.1% in GNB infections (16,17). A recently published 10-year case series indicated that CMS use increased, more than half of the patients were discharged alive, and no significant nephrotoxicity was observed in the 5603 patients prescribed CMS (along with other antibiotics) (18). It's reported that colistin based treatment in different kinds of infections due to GNB has a clinical response rate of 43.1-79% (19-22), and microbiological response rate of 66.7% (34/51) (19). Intravenous polymyxins therapy can be used either as monotherapy or in combination with other antibiotics.

3.1. Monotherapy

Several retrospective studies have investigated

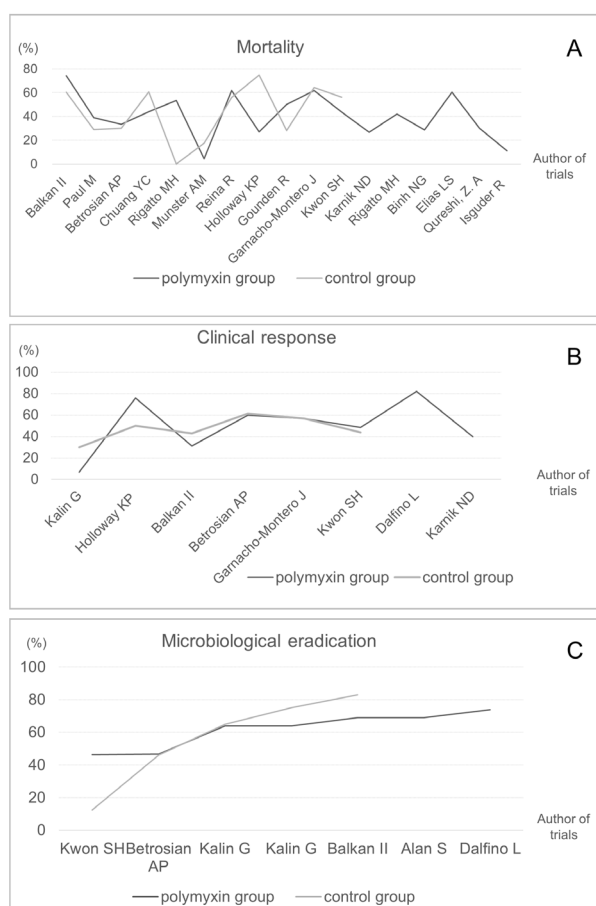


Figure 2. Outcome of monotherapy.

intravenous polymyxins alone in MDR GNB infections, most of which were pneumonias. It's indicated that the mortality ranged from 0% to 74.3% (4,21,23-37), clinical response (cure and improvement) rate 7-82.1%, and microbiological eradication 27.3-73.9% (24,33-38). In respect to the colistin only susceptible strains, it's reported that the mortality in hospitals of intravenous colistin monotherapy was 50% (16/32) (Figure 2), 11 patients died in Intensive Care Unit (ICU) (39), and clinical cure was obtained in 82.1% of infectious episodes (23/28) and bacteriological clearance was achieved in 73.9% (17/23) of the cured infectious episodes (38).

There have been several clinical studies evaluated polymyxins monotherapy versus other antibiotics. In an early prospective cohort study, it's indicated that colistin appeared to be as safe and as effective as other antimicrobials for treatment of sepsis caused by *A. baumannii* and *Pseudomonas aeruginosa* (*P. aeruginosa*) in critically ill patients (4). Balkan, II, *et al.* also found that there was no significant difference between colistin monotherapy and non-colistin based combinations in the treatment of MDR- *Acinetobacter* spp BSIs in terms of efficacy and 14-day mortality (37). But a larger sample size study discovered that colistin was less effective and more toxic than

β -lactam antibiotics (30). It's reported that polymyxin B treatment in the currently recommended dosage may be inferior to other drugs in the treatment of ventilator-associated pneumonia (VAP) and tracheobronchitis caused by organisms tested as susceptible *in vitro* to this agent (29).

Some studies demonstrated that comparing colistin with imipenem, there was no significant difference in the mortality rates of VAPs due to pan-drug-resistant (PDR) *A. baumannii* or *P. aeruginosa* (40), or MDR and XDR *A. baumannii* (32). Kwon SH *et al.* reported that in *A. baumannii* infection, microbiologically negative conversion rate was significantly higher in the colistin group than the tigecyclin group, but there was no statistically significant difference in mortality rate between the two groups during hospital stay (36). While another retrospective study, including 294 adults with MDR *A. baumannii* pneumonia, found an excess mortality of 16.7% in the tigecycline group (41). A small comparative clinical study evaluated colistin versus ampicillin-sulbactam for treatment of VAP due to MDR *A. baumannii* (42). This prospective study found no difference regarding clinical and microbiological outcomes in both of the studies. Also, there was no significant difference in ICU mortality between the colistin and tobramycin treatment in infections due to MDR *A. baumannii* (39). Holloway KP *et al.* found that clinical cure was observed in 22 of 29 (76%) patients of MDR *A. baumannii* infection treated with polymyxin B and 2 of 4 (50%) patients treated with doxycycline, microbiological cure was observed in 17 of 21 (81%) patients treated with polymyxin B and 2 of 3 (67%) patients treated with doxycycline (43).

3.2. Combination therapy

The main reasons for the combination therapy are to get potential synergic effects and to prevent the selection of heteroresistant strains (44). Heteroresistant strains can emerge in patients who receive colistin monotherapy (19). Qureshi ZA *et al.* found that all 19 patients initially infected with colistin-susceptible *A. baumannii* received therapy with intravenous CMS, inhaled CMS, or both, prior to isolation of colistin-resistant *A. baumannii* and the median interval between the isolation of the colistin-susceptible *A. baumannii* isolate and the colistin-resistant *A. baumannii* isolate was 20 days (range, 4-99). They also observed an all-cause mortality of 30% (6/20) at 30 days (45).

The effect of combination therapy in severe infections with MDR GNB was proved early (20). A study including 104 patients with carbapenem-resistant (CR) bacterial infection indicated polymyxin B combination therapy with all-cause mortality of 47% during hospitalization and 77% after 6 months (46). Is combination therapy better than monotherapy? What's the optimal combination for clinical practice?

3.2.1. Combination therapy vs. monotherapy

A retrospective study of 41 VAP patients from Korea compared colistin monotherapy (22 patients) and a combination of colistin and antibiotics (19) (35) (Figure 3). The study showed that there were no differences in outcome variables between the two groups, such as length of ICU stay, treatment success rate, ICU mortality and hospital mortality. Simsek F *et al.* found that colistin monotherapy and colistin combined therapy are likely to achieve similar treatment response rates with VAP and also in patients with BSI (19). However, there is inconsistent evidence. It's reported that in BSI due to XDR *A. baumannii*, the rates of complete response/cure and 14-day survival were relatively higher and microbiological eradication was significantly much higher in the combination group, and the in-hospital crude mortality rate was significantly lower in the combination group (47). Rigatto MH *et al.* also found that, in critically ill patients with XDR *A. baumannii* (83 cases) or *P. aeruginosa* infections, combination therapy with intravenous polymyxin B and antimicrobials lacking *in vitro* activity was independently associated with lower rates of 30-day mortality than polymyxin B monotherapy (42.4% vs. 67.6%) (48).

In order to obtain synergistic effects, antimicrobials used frequently with colistin include imipenem/meropenem and sulbactam (16,44). An early retrospective study indicated that the effectiveness of colistin monotherapy did not appear to be inferior to that of colistin-meropenem combination therapy for patients with MDR bacterial infections (27). Yilmaz GR *et al.* found that in VAP due to MDR or XDR *A. baumannii*, a clinical and microbiological response was better in the groups that received colistin alone and carbapenem-colistin combination when compared with the group that received sulbactam and colistin (31). Mortality rates were also found to be lower in these groups. However, there was no statistical difference between each group. No difference in clinical response was seen between the patients infected with MDR vs. XDR *A. baumannii*. Another study indicated that clinical response rates were 29.8% and 40 %, respectively and the bacteriological response rates were 72.3% and 85.7 % in colistin and the colistin/sulbactam combination therapy (34). Although, the difference was not statistically significant, clinical cure rates or bacteriological clearance rates were better in the combination group than colistin monotherapy.

A potent *in vitro* synergistic activity against MDR strains was observed when a glycopeptide (vancomycin or teicoplanin) was combined with colistin (49,50). The question is whether it is the same in clinical practice? Garnacho-Montero J *et al.* reported that, in critically ill patients with CR *A. baumannii* infections, clinical outcomes did not differ between 29 patients treated with colistin plus vancomycin and 28 patients treated with

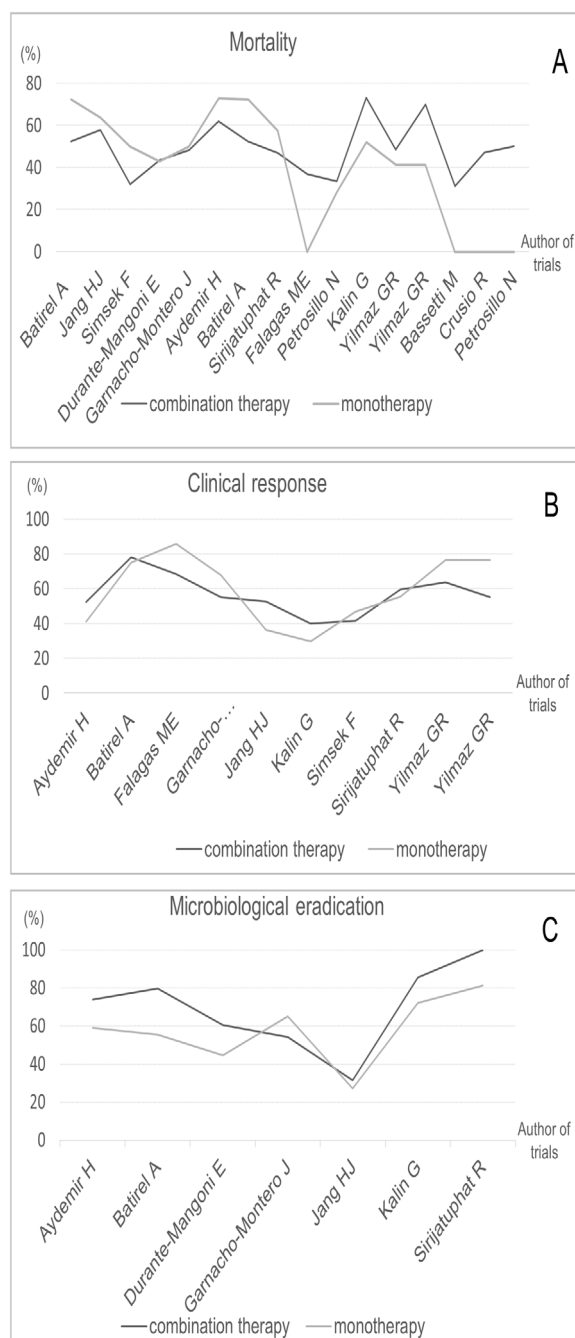


Figure 3. Outcome of combination therapy.

colistin alone (51). A later retrospective study with a larger sample size also found that patients that received combination therapy of colistin and glycopeptides didn't have better outcomes in days of ICU stay, days of hospital stay and 30-day mortality, than those treated with colistin alone (28). It's also indicated in the study that when this combination lasted ≥ 5 days, it was associated with a higher survival rate.

There're also some prospective studies comparing monotherapy and combination therapy. Aydemir H *et al.* conducted an open, prospective, randomized, single-center trial to compare the responses of colistin treatment alone with a combination of colistin and rifampicin

in the treatment of VAP caused by CR *A. baumannii* (52). It's reported that clinical, laboratory, radiological and microbiological response rates were better in the combination group, although these differences were not significant, and the time to microbiological clearance was significantly shorter in the colistin-rifampicin group. From another multicenter, a parallel, randomized, open-label trial from Italy found no difference for infection-related death and length of hospitalization between a colistin-rifampicin group and colistin monotherapy group in serious XDR *A. baumannii* infections (53), but the increased rate of *A. baumannii* eradication with combination treatment could still imply a clinical benefit. Then, a preliminary open-label randomized controlled study from Thailand found a significantly more favorable microbiological response, a trend toward more favorable clinical outcomes and lower mortality in a colistin-fosfomycin combination therapy group compared with colistin monotherapy group in CR *A. baumannii* infections (54).

3.2.2. Different combination therapies

3.2.2.1. Colistin-carbapenem vs. colistin-sulbactam

Carbapenem and sulbactam have been used frequently in combination with colistin, but clinical superiorities have not come to a conclusion. A case series reported (55) 80% (4/5) of the transplant recipients with XDR *A. baumannii* infections were treated successfully with colistin-carbapenem combination therapy based on positive interactions *in vitro* tests, while 91% (10/11) patients died in the combination treatment of colistin and other antibiotics. Batirel A *et al.* found that, for BSI patients due to XDR *A. baumannii*, colistin-carbapenem, colistin-sulbactam, and colistin with other agent combinations did not reveal significant differences with respect to 14-day survival and clinical or microbiological outcome before and after propensity score matching (47). A similar conclusion was drawn in XDR *A. baumannii* pneumonia (VAP and hospital-acquired pneumonia) by Khawcharoenporn T *et al.* (56). But as mentioned before, Yilmaz GR *et al.* also (31) reported that clinical and microbiological response was better in a colistin-carbapenem group than a colistin-sulbactam group in VAP due to MDR or XDR *A. baumannii*. Mortality rates were also found to be lower in a colistin-carbapenem group, although with no statistical difference.

Because all the inconsistent clinical evidence was from retrospective trails, prospective and well designed trials are needed.

3.2.2.2. Colistin-carbapenem vs. colistin-tigecycline

It's indicated that colistin and tigecycline were the two most active *in vitro* agents against CR *A. baumannii* and XDR *A. baumannii* (57,58), and *in vitro* comparative

studies of different antimicrobial combinations against CR *A. baumannii* have demonstrated colistin and carbapenem to be more consistently synergistic than colistin and tigecycline (58-60). But *in vitro* results are not directly applicable to clinical practice. Khawcharoenporn T *et al.* reported that in XDR *A. baumannii* pneumonia, the 28-day survival rate and mean length of hospital stay were not statistically different between a colistin-tigecycline group and a colistin-carbapenem group (56). While another prospective, observational, multicenter study found that an increased 14-day mortality was associated with colistin-tigecycline therapy given a tigecycline minimum inhibitory concentration greater than 2 mg/L, 50% of patients in the colistin-carbapenem group survived to hospital discharge compared with 31% of patients in the colistin-tigecycline group (61). Although the XDR *A. baumannii* strains in this study were potentially susceptible to colistin and tigecycline, resistant to carbapenem, the combination of colistin-carbapenem appeared to be more effective than colistin-tigecycline.

3.2.2.3. Colistin-fosfomycin vs. doripenem-fosfomycin

There is not a widely used regimen of colistin in combination with fosfomycin. Only a retrospective study from Thailand suggested an equivalency of regimens that contained high-dose, 4-h infusion of doripenem plus fosfomycin versus colistin plus fosfomycin for treatment of CR *P. aeruginosa* pneumonia with doripenem MICs of 4-8 mg/L. Both regimens were feasible, effective and well tolerated amongst patients with *P. aeruginosa* isolates of intermediate resistance to doripenem.

3.2.2.4. Colistin-glycopeptide

As mentioned before, when combined with colistin, glycopeptides (vancomycin or teicoplanin) showed *in vitro* synergistic activity against MDR strains. Petrosillo N *et al.* found no difference in 30-day mortality, in the 4 groups as follows: colistin alone, colistin-glycopeptide, colistin plus other anti-GNB drugs, colistin-glycopeptide plus other anti-GNB drugs (28). But it's also indicated in this study that the colistin-glycopeptide combination was a protective factor for mortality if administered for > 5 days.

3.2.2.5. Colistin-rifampicin

Synergy against MDR or XDR *A. baumannii* was shown in both *in vitro* (62-64) and experimental studies (65,66) when colistin was combined with rifampicin. By altering membrane permeability, colistin may facilitate rifampicin entry within the bacterial cell and therefore enhance its killing activity (67,68). Three uncontrolled clinical studies have assessed the safety and clinical efficacy of the colistin-rifampicin combination, showing very high

overall response rates (69-71).

Despite the lack of a control group and the limited number of patients, colistin in association with rifampicin appears to be effective in treating patients with infections caused by multidrug-resistant *A. baumannii*.

3.2.2.6. Colistin-vancomycin-meropenem

Colistin-based combinations, with or without the addition of carbapenems, have been considered the milestone of the treatment. A case series evaluated the role of vancomycin in addition to colistin-meropenem against multidrug resistant *A. baumannii* causing severe infections in a pediatric ICU (72). All 4 patients treated with a colistin-vancomycin-meropenem combination had a positive outcome with no infection relapses. Maybe it's an alternative to use when we come up with a poor clinical response.

4. Dosing, how to prescribe?

As we said at the beginning, polymyxins have been available for clinical use for more than 50 years, and have never been subjected to contemporary drug development procedures. As a result, there is very limited pharmacokinetic (PK) data available to guide appropriate dosage selection, especially in critically ill patients. However, dosing recommendations for colistin and polymyxin B have been updated significantly due to the relatively recent availability of assays to detect concentrations of both active and pro-drugs in serum and other biological sites (73,74). The optimal dose of polymyxins is controversial (75,76).

4.1. Loading Doses

Dosing recommendations derived from PK/PD data support the use of a loading dose of polymyxins in order to more rapidly achieve target serum concentrations (77-79). Toxicities associated with colistin do not appear to increase with use of a loading dose (38,46). Limited clinical evidence is available. In a preliminary study (38), critically ill patients with 28 infectious episodes, received a loading CMS dose of 9 MU, followed by a maintenance dose of 4.5 MU every 12 hours. Clinical cure was observed in 23 cases (82.1%). Acute kidney injury developed during 5 treatment courses (17.8%). A prospective observational cohort study included 104 patients with infections due to carbapenem-resistant Gram-negative bacteria. All patients were treated with a loading dose of 25,000 IU/kg of polymyxin B, followed by 25,000 IU/kg/day in divided doses, with dose adjustment for glomerular filtration rate (GFR) less than 50 mL/min. Clinical success was achieved in 50% and reinfection occurred in 25%. Treatment-related acute renal failure occurred in 14.4% (46). Karaïskos I *et al.* reported six post-neurosurgical ventriculitis and

meningitis cases caused by extensively drug-resistant *A. baumannii* (80). Three patients were given a loading colistin dose of 6 MU, but the 6 patients were treated in combination with intraventricular colistin, thus the benefit from loading colistin can't be confirmed. However, no nephrotoxicity was found in this study. In another retrospective study of XDR *A. baumannii* pneumonia, only 13 of 166 patients received an IV colistin regimen (300 mg colistin loading followed by 150 mg q12 h) (56), in addition to other antibiotics. All 13 patients presented acute kidney injury, with a median of 5 days, but renal function for all cases had improvement when IV colistin was switched to inhaled colistin. Binh NG *et al.* reported that among critically ill Vietnamese patients with low body weight, a loading dose adjusted according to total actual body weight, was associated with low nephrotoxicity (26).

Clinical outcome data comparing colistin dosing strategies with or without initial loading doses are not available. With the limited clinical evidence, it's suggested that loading dose regimens may be more effective, but more attention should be paid to the adverse effects, and comparing trials and prospective trials are needed to assess the clinical use of loading dose regimens.

4.2. Dosing strategies

The most common dose of colistin (given as CMS) for patients with normal renal function is 2.5 mg/kg, given intravenously every 12 h. However, data suggest that the current recommended dosing regimens may lead to serum levels of colistin that are less than the minimum inhibitory concentration (MIC) for *Acinetobacter* infections (81).

Kalin G *et al.* found that the clinical cure rate of colistin was 30 % in the normal-dose (2.5 mg/kg every 12 h (maximum 300 mg)) and low-dose (adjusted according to creatinine clearance) groups, whereas the rate was only 7% in the high-dose (2.5 mg/kg every 6 h (maximum 600 mg)) group (33). The bacteriological clearance rates were 64, 65, and 75% in the high-dose, normal-dose, and low-dose groups, respectively. There were no statistically significant differences in clinical cure rates or bacteriological clearance rates among the different dosage groups. A prospective observation study found that in severe infections due to COS gram-negative bacteria, the high-dose (4.5 MU every 12 hours), extended-interval CMS regimen has a high efficacy, without significant renal toxicity (38). It was indicated that a personalized dosing protocol of colistin was effective, with low nephrotoxicity, among critically ill Vietnamese patients with low body weight (26).

Almost all modern PK studies on polymyxins are for colistin that is administered parenterally as its inactive prodrug CMS (77,82). In contrast, polymyxin B is available for direct parenteral administration, that

is, as the antibacterial entity (83). Therefore, current PK findings for CMS/colistin cannot be extrapolated to polymyxin B. The current recommended dose of IV polymyxin B for patients with normal renal function is 1.5-2.5 mg/kg/day in two divided doses administered as a 1 h infusion (84) (Bedford Laboratories. Bedford, OH 44146: Bedford Laboratories; 2004. Polymyxin B for injection (package insert)). It's also reported that polymyxin B at doses ≥ 200 mg/day was associated with lower in-hospital mortality, but significantly higher risk of severe renal impairment (23). While Rigatto MH *et al.* found that the development of AKI was significantly associated with 30 day mortality: 52.3% (90 of 172) versus 41.6% (99 of 238), polymyxin B dose ≥ 150 mg/day was associated with a higher risk of developing any degree of AKI and renal failure regardless of patient weight, which was also an independent risk factor (25). This risk did not significantly increase with doses ≥ 200 mg/day.

In renal impairment, the elimination of CMS by the kidney would be decreased and a greater fraction of the administered dose would be converted to colistin. So CMS dosing should be adjusted in renal impaired patients according to estimated glomerular filtration rate or creatinine clearance (31,39), and polymyxin B used to be so (43). However, recent data suggest that polymyxin B does not require adjustment for renal dysfunction (78,85,86). Polymyxin B is eliminated mainly by nonrenal pathways, and the total body clearance appears to be relatively insensitive to renal function (86). Sandri AM *et al.* first demonstrated that total body weight is a patient characteristic that influences polymyxin B PK and that the total body clearance, and hence daily dose requirement of polymyxin B is not affected by renal function (78).

There is limited clinical evidence derived from several case reports for polymyxins use in patients receiving renal replacement therapy (RRT). It's observed that colistin levels during continuous venovenous hemodiafiltration (CVVHDF) was significantly lower (87). And it's also indicated that to achieve colistin plasma concentrations at the steady state considered adequate (2.5 mg/L), the colistin methanesulfonate sodium maintenance dose during CRRT had to be similar to or even higher than that used in patients with preserved kidney function (73,77). For patients receiving intermittent RRT, it's suggested to give an additional dose of colistin methanesulfonate sodium after dialysis (77,88), but no standardized dose recommendations are currently available for these long-lasting modalities of intermittent RRT. For end-stage renal disease (ESRD) patients receiving intermittent hemodialysis (HD), evidence from 10 patients indicated that HD should be conducted at the end of a dosing interval and a supplemental dose should be administered (89). A report of 8 continuous ambulatory peritoneal dialysis (CAPD) patients suggested that CMS doses should not be increased during

CAPD because clearance by CAPD was low for both CMS and formed colistin (90). Little is known about the effect of dialysis on the clearance of polymyxin B, and a case report of 2 patients indicated that the recommended polymyxin B doses should not be reduced for patients on continuous venovenous hemodialysis (CVVHD) (85).

So, concerning dosing regimens, colistin and polymyxin B are not quite the same. High dose polymyxins regimens with inconsistent clinical evidence need more trials to confirm. In renal impaired patients polymyxin B should be prescribed without dosing adjustment.

5. Toxicities: Worthy of attention but less common than we thought

The most common adverse effect of polymyxins is nephrotoxicity which is particularly more common in patients with high baseline creatinine at the initiation of treatment, while neurotoxicity, ranging in severity from reversible paresthias to respiratory failure, is a less common side effect (91). On the other hand, the reported frequency and severity of nephrotoxicity is lower as compared to the figures reported in 1970s (91). This maybe because of the more purified formulations of the drug as well as closer monitoring of patients.

5.1. Nephrotoxicity

The reported incidence of intravenous -colistin-related nephrotoxicity decreased from 36% in the 1960s to 14-19% in the 1990s, before rising to 24% (92) (Figure 4). Reported rates of nephrotoxicity vary widely from 0-59.6% of patients treated with polymyxins in *A. baumannii* (Table S1, <http://www.biosciencetrends.com/action/getSupplementalData.php?ID=13>). This

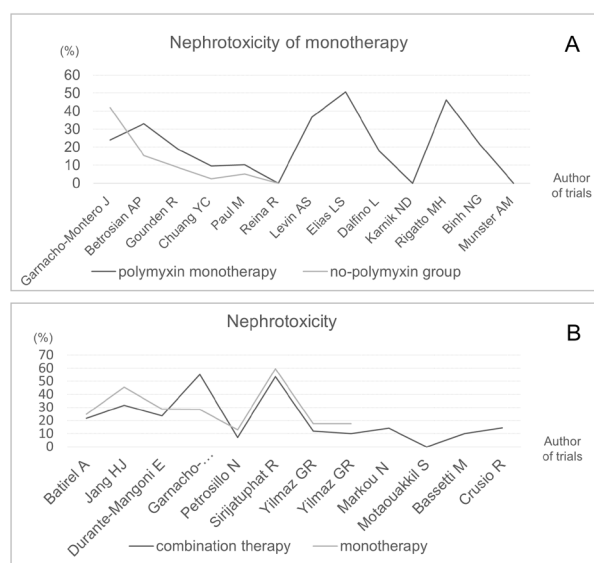


Figure 4. Nephrotoxicity of polymyxin therapy.

maybe because these data are generated from a number of small, non-comparative studies or case series, with heterogeneous patient populations, and varying dosing schemes. It's also because different definitions of nephrotoxicity, such as AKIN (Acute Kidney Injury Network) criteria (51,93), RIFLE (33,34,47,53,54), KIDIGO (56), and other criteria (19,36,42). So it's difficult to compare nephrotoxicity between trials.

It has been reported that the nephrotoxicity of colistin was not significantly different compared to tobramycin (39), imipenem (32) or ampicillin/sulbactam (42), while the colistin-based treatment had a significantly higher nephrotoxicity than tigecycline-based treatment (36,41).

No difference was found between IV colistin monotherapy or combination therapy with rifampicin (53), glycopeptides (28), fosfomycin (54), carbapenem or sulbactam (31,47). But the combination of intravenous colistin plus intravenous vancomycin is associated with an increased risk of renal failure (51).

Higher doses-colistin were reported increasing the nephrotoxicity risk (33), while Kalin G *et al.* reported in another study and found that nephrotoxicity rate was higher (6 of 15 patients, 40%) in patients who received a higher dose than a standard dose (15 of 56 patients, 26.8%) and adjusted dose (1 of 18 patients, 5.6%), but with no statistical difference (34).

There was no significant clinical adverse effect for inhalational colistin (94-96), but in addition to intravenous colistin, the results were conflicted. Kalin G *et al.* suggested that inhalational colistin increased the nephrotoxicity risk, however, a recent study found no difference in nephrotoxicity risk with additional inhalational colistin therapy (33).

5.2. Neurotoxicity

In the past, the most frequently experienced neurological adverse effects were paresthesias that occurred in approximately 27% and 7.3% of patients receiving intravenous and intramuscular colistimethate sodium, respectively (97,98). But recently performed studies are not in agreement with the previously reported data. Several studies indicated that no neurological side effects were noted (52,54,71,99) with intravenous or inhaled colistin. While two studies noted very low incidence of neurotoxicity, 3 (0.14%) patients (47), and 1 (0.99%) patient (53), respectively. Neurotoxicity was observed in 2 (6%) patients who received intravenous polymyxin B (43).

6. Use in children: Also seemed to be safe and effective

The incidence of MDR Gram-negative bacteria has been emerging as an agent for nosocomial infection of children (100). Some case reports indicated that intravenous colistin was effective and tolerable in children infected

by *A. baumannii* (99,101-103). It's has been reported in several trials that colistin is effective in the treatment of severe nosocomial infections caused by MDR Gram-negative bacteria and is generally well tolerated in pediatric patients, even after relatively long-term use (21,22). A similar result was found in a case series of 8 meningitis patients due to multidrug-resistant and pan-resistant *Acinetobacter spp.*, with a regimen of IV polymyxin plus intrathecal polymyxin. Moreover, colistin treatment in neonates has been reported (104,105). Alan S *et al.* even conducted a study investigating premature infants with nosocomial infections due to *A. baumannii* including MDR *A. baumannii*, with a recovery rate of 81% (17/21), and microbiological clearance of 69% (9/13) (93).

7. Conclusion

As carbapenem resistance is now increasing worldwide, polymyxins based regimens seem to be revived as an effective treatment of last resort. Although polymyxins have been used for over half century, there're still many issues to be confirmed. In spite of treatment success, persistence of bacterial growth and emerging resistance raises concern for long-term efficacy of polymyxin monotherapy. Though the clinical benefits have been subject to controversy, combination therapy is still recommended for two main reasons. First, to prevent the selection of heteroresistant strains, and second, to get potential synergic effects. There have been no greater side effects with combination therapy than monotherapy except for the colistin-vancomycin combination. The optimal dosing strategies and combination regimens are still controversial. Most of the data came from retrospective or small sample size prospective trials, so prospective trials of larger sample size are needed.

Acknowledgements

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APOBEC-mediated genomic alterations link immunity and viral infection during human papillomavirus-driven cervical carcinogenesis

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Summary

Cervical cancer is one of the most frequently diagnosed cancers and is a major cause of death from gynecologic cancers worldwide; the cancer burden from cervical cancer is especially heavy in less developed countries. Most cases of cervical cancer are caused by persistent infection with carcinogenic human papillomavirus (HPV) genotypes 16 and 18. Non-resolving inflammation caused by HPV infection provides a microenvironment that facilitates cancer development. Molecular alterations during the process of HPV-induced carcinogenesis are characterized by DNA methylation within the HPV genome, promoter hypermethylation of tumor suppressor genes in the host genome, as well as genomic instability caused by viral DNA integrating into the host genome. Catalytic polypeptide-like apolipoprotein B mRNA editing enzymes (APOBECs) normally function as part of the innate immune system. APOBEC expression is stimulated upon viral infection and plays an important role in HPV-induced cervical cancer. APOBECs catalyze the deamination of cytosine bases in nucleic acids, which leads to a conversion of target cytosine (C) to uracil (U) and consequently a change in the single-stranded DNA/RNA sequence. APOBEC proteins mediate the complex interactions between HPV and the host genome and link immunity and viral infection during HPV-driven carcinogenesis. Understanding the effects of APOBECs in HPV-induced cervical carcinogenesis will enable the development of better tools for HPV infection control and personalized prevention and treatment strategies.

Keywords: Cervical cancer, human papillomavirus, inflammation, APOBEC

1. Introduction

Cervical cancer is the fourth most common cancer among women worldwide (1). There were about

527,600 new cases of and 265,700 deaths due to cervical cancer in 2012 (1). About 85% of the cases and 87% of the deaths occurred in resource-poor countries, posing a significant public health burden in these regions (1,2). In China alone, there were an estimated 98,900 new cases of and 30,500 deaths attributed to cervical cancer in 2015, and these figures were higher than those for any other type of gynecologic tumor (2). Moreover, the incidence of cervical cancer among young Chinese women (≤ 30 years old) has been increasing by 2-3% each year (2). Approximately 80% of cases of cervical cancer involve squamous cell carcinoma, which develops through pre-malignant lesions known as cervical intraepithelial neoplasia (CIN) that range from grade I to III (3). Cervical adenocarcinomas accounts

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for 10-20% of cases, with less well-characterized preceding stages. Almost all cases of cervical cancer are caused by persistent infection with certain carcinogenic human papillomavirus (HPV) genotypes (4). HPVs are a large and diverse group of viruses, with new types continually being identified (5,6). There are at least 15 genotypes of carcinogenic HPV, and the two most prevalent and carcinogenic types are HPV types 16 and 18 (7). The half-life of an infection with a high-risk HPV type is estimated to be 8-10 months. Infection with HPV-16 has the longest duration, with an average persistence of 16 months (8). HPV-16 causes more than half of all cervical cancers, while HPV-18 is implicated in many cases of endocervical adenocarcinoma, which account for about 15-20% of all cervical cancers (9). In addition, HPV-16 and HPV-18 also cause a vast amount of HPV-related cancers at other anatomic sites including the cervix, penis, vulva, vagina, anus, and oropharynx (10).

Epidemiological studies in multiple populations have indicated that persistent infection with a high-risk HPV type consistently precedes the appearance of precancerous changes, which include CIN 3, severe dysplasia or dyskaryosis, and carcinoma *in situ* (4). A prospective study has indicated that infection with carcinogenic HPV types is required for the development, maintenance and progression of these precancerous changes to invasive cancer (4). There are four steps by which HPV causes cervical cancer: HPV transmission, viral persistence, progression to precancer, and invasion (4). However, backward steps such as clearance of HPV infection and regression of precancer to normality (4) also occur, indicating interaction between HPV and the host immune response. In most infected women, the infection spontaneously resolves, and the infection even resolves in women who are at the most sexually active ages with very frequent infection with HPV. Only a very small fraction (about 10%) of women will develop viral persistence, and only some of those who are chronically infected with a high-risk HPV type will have a high risk of infection progressing to neoplastic lesions (9). The average total time from infection with a carcinogenic HPV type to the occurrence of invasive cervical cancer is 25-30 years or longer (11). HPV infection is basically a sexually transmitted disease. The most significant risk factors associated with HPV infection are related to individual's sexual behavior: starting sexual activity at an early age, a large number of sexual partners, sexual contact with high-risk individuals, and HIV infection; in contrast, male circumcision and the strict use of condoms are factors that protect women from HPV transmission (6). The main determinants of an HPV infection progressing to precancer and cancer are: the viral type, a persistent infection according to repeated examinations, integration of viral DNA into the host genome, and methylation of the HPV genome (12). Although the

type-specific viral load per cellular unit appears to be associated with cervical cancer, a longitudinal study has not provided sufficient evidence that it can serve as a clinically useful predictor (4). However, HPV infection alone is not enough to trigger cervical cancer. Most women infected with HPV test negative within 2 years (4), while those with a persistent infection with high-risk HPV are at the greatest risk of developing cervical cancer, indicating that other viral factors or cellular events are required for progression from precancerous disease to cervical cancer.

The carcinogenic HPV types are evolutionarily related and belong to the genus *Alphapapillomavirus* with a small double-stranded circular DNA genome of approximately 8,000 pairs of bases. The HPV genome includes three regions: a long control region (LCR), an early region (E1, E2, E4, E5, E6, and E7), and a late region (L1 and L2). Eight genes are coded for by the HPV genome. The LCR involves in DNA replication, the early region regulates DNA replication and oncogenesis, and the late region produces viral capsule products (13). E6 and E7 are key oncoproteins with multiple cellular targets. During the process of HPV-induced carcinogenesis, E6 and E7 block apoptosis and deregulate the host cell growth cycle by binding to and interacting with p53 and retinoblastoma tumor suppression protein (pRB), respectively (14). In addition, these two proteins interact with various host cell targets, including those that regulate genomic instability (ATM, ATR and γ -tubulin), cell proliferation (E6AP, HDAC, P107, P130, and Cyclins), apoptosis (Caspase 8, BAX, BAK, IRF3, P600, and P48), and immortalization (TERT, MYC, and NFX123), thus interrupting multiple cellular pathways (14).

2. Alterations of the HPV genome and the host genome during the evolution of HPV as it induces carcinogenesis

DNA methylation within the HPV genome is one of the earliest and most common molecular alterations in multistep cervical carcinogenesis (15). Methylation of the HPV LCR and the *L1* gene increases with the severity of cervical dysplasia (15). A study has suggested that the status of HPV viral methylation may serve as a biomarker to help distinguish benign HPV infections from those that progress to precancer (15). An association between methylation of CpG sites in the HPV-16 *L1* gene and CIN2+ has been noted, suggesting that the detection of methylated viral DNA may distinguish CIN2+ from a high-risk HPV infection with no evidence of CIN2+ (15). Methylation profiles vary greatly among different genotypes. HPV-18 and HPV-45 are reported to exhibit a broader range and greater number of methylated CpG sites compared to HPV-16 and HPV-31 (12). Nevertheless, the association between viral methylation and precancer is a feature of

the four most important carcinogenic HPV types (HPV-16, 18, 31, and 45).

Parallel but distinct from HPV gene methylation, promoter hypermethylation of tumor suppressor genes in HPV-infected host cells is also an early and frequent epigenetic event (16). Promoter methylation in cervical precursors and invasive cancers has been noted for tumor suppressor genes in various cellular pathways, including genes involved in the cell cycle [*p16*, cyclin A1 (*CCNA1*), and the fragile histidine triad (*FHIT*)], cell adhesion [cell adhesion molecule 1 (*CADMI*) and E-cadherin (*CDH1*)], apoptosis (*DAPK*), cell signaling pathways [retinoic acid receptor- β 2 (*RAR β 2*) and Ras association domain family 1 isoform A (*RASSF1A*)], the Wnt/ β -catenin pathway [adenomatous polyposis coli (*APC*)], the p53 signaling pathway (p73), and DNA repair [*O*⁶-methylguanine-DNA methyltransferase (*MGMT*)](16). Moreover, methylation profiles differ for squamous cell carcinomas and adenocarcinomas. Studies have investigated the possibility that gene-specific hypermethylation profiles could serve as predictive biomarkers of cervical cancer risk, but their findings must be verified in prospective studies. In addition, folate is presumed to play a role in modulating the risk of cervical cancer by influencing promoter hypermethylation of tumor suppressor genes (17).

Moreover, HPV directly promotes genomic instability by integrating viral DNA into the host genome, which causes abnormal regulation of cell cycle control as well as epigenetic alterations that result in the silencing of tumor suppressor genes (e.g., *APM-1*, and *CASZ1*) and that result in overexpression of oncogenes (e.g., *MYC*) that facilitate the progression of cervical cancer (18-20). HPV integration into the host genome can be a driver mutation in cervical malignant transformation (21). A recent study reported that HPV-16 and/or HPV-18 integration into the host genome were associated with structural abnormalities and increased target gene expression (22). Both the integration rate and number of integration sites are reported to be higher in tissue from cervical carcinoma than in tissue from low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL), indicating a correlation between the level of HPV integration and the CIN grade (23). Based on these findings, the level of HPV integration could serve as a predictor of disease progression. The circular HPV genome is converted into a linear truncated DNA after integration (18). Breakpoints are distributed throughout the viral genome and are more likely to occur in *E1* than in *E2* but are less prone to occur in the LCR, while the viral *E6* gene may also be disrupted during certain events (21). Furthermore, significant enrichment of viral gene *E7*>*E4*>*E5*>*E6* reads has been noted among cervical tumor samples, which is consistent with the known biological roles of the HPV genes in carcinogenesis (24).

3. APOBEC links immunity and viral infection during the evolution of papillomavirus as it drives the development of cervical cancer

Catalytic polypeptide-like apolipoprotein B mRNA editing enzymes (APOBECs) are a group of enzymes that catalyze the deamination of cytosine bases in nucleic acids, causing the conversion of target cytosine (C) to uracil (U) and consequently a change in the single-stranded DNA/RNA sequence (25). There are at least 11 members of the APOBEC family, including activation-induced cytidine deaminase (AID), APOBEC1 (A1), APOBEC2 (A2), APOBEC3 (A3), APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3C (A3C), APOBEC3DE (A3DE), APOBEC3F (A3F), APOBEC3G (A3G), APOBEC3H (A3H), and APOBEC4 (A4) (26). The comprehensive biological functions and characteristics of the APOBEC family of proteins have been summarized elsewhere (25). APOBEC family members normally function as part of the innate immune system, which plays a key role in combating exogenous infection and especially viral infection. APOBEC expression is stimulated by viral infection *via* a complex network of innate immunity responses that involve components including Toll-like receptors, interferons, interleukins, and even the p53 protein (27). Previous studies have indicated that the APOBEC3 protein is a vital factor in HPV-driven carcinogenesis. APOBEC3 is presumed to play a role in inhibiting viral infection. APOBEC3A and APOBEC3C can enhance the ability of the human immune system to recognize HPV infection (28) while APOBEC3G can suppress the proliferation and invasion of cervical cancer cells (29).

3.1. Host immune response during HPV-induced chronic inflammation

The host immune response plays a vital part in infection with a high-risk HPV type. An effective immune response will facilitate spontaneous virus clearance, while a compromised one may facilitate the process of CIN progression and cervical carcinogenesis (30). Host immune responses, including humoral, cellular, and innate immunity, are considered to play important roles in the outcomes of HPV persistence and progression to cervical cancer. HPV may remain undetected for a very long time as infected cells evade the immune system. In addition, a persistent HPV infection stimulates immunotolerance of the host immune system and provides a microenvironment that facilitates further infection and progression of cervical lesions (30).

3.2. Effects of HPV viral infection on APOBEC expression

In HPV16 infection, levels of APOBEC3A and APOBEC3B mRNA are both reported to be up-regulated in low- and high-grade cervical lesions in

comparison to levels in normal tissues, and this up-regulation is possibly due to HPV16 oncoproteins E6/E7 (31). A previous study noted the expression of three genes of the APOBEC3A family, *hA3A*, *hA3B*, and *hA3H*, in skin keratinocytes, which are the main host cells for HPV infection (32). However, APOBEC3A expression was reported to be lower in cervical cancer tissues than in normal tissues (33). Changes in the level of APOBEC3A expression during different stages of cervical carcinogenesis suggest that APOBEC3A has antiviral and anticancer action. A study of precancerous lesions as well as cell lines found that APOBEC3A restricted HPV by editing HPV DNA (34). APOBEC3A can inhibit cervical cell proliferation, migration, and invasion and it can promote apoptosis depending on the level of cytidine deaminase activity (33). In addition, APOBEC3A restricts the functions of HPV16-E6, HPV16-E7 and HPV18-E6 through a cytidine deaminase-dependent mechanism, but it does not affect HPV18-E7. These findings indicate that APOBEC3A has antiviral and anticancer action by differentially inhibiting HPV E6 and E7 expression depending on the level of cytidine deaminase activity (33).

3.3. Effects of APOBEC on the HPV genome and the host genome

During the natural process of viral infection, the APOBEC3 protein may be involved in editing the HPV16 genome. APOBEC3 can gain access to the HPV genome during viral replication and induce C to U substitutions consistent with deaminase activity. Whether a viral infection triggers APOBEC3 mutagenesis is unclear, but a previous study detected APOBEC3 mutational signatures in the LCR of the HPV16 genome in CIN (34). A study noted a higher number of A/T mutations per sample in the *E2* gene in CIN3 compared to CIN1, suggesting that the APOBEC3 protein may induce clustered hypermutations in the LCR of the HPV16 genome (35). APOBEC3-induced mutations appear to accumulate with the progression of cervical lesions. Both C to T and G to A hypermutation patterns were detected in the coding strand of the *E2* gene of the HPV16 genome in CIN1 samples, while the C to T hypermutation was more prevalent in CIN3 samples (33). In cultured cervical keratinocytes, APOBEC3 subfamily proteins induce adenine/thymine clustered hypermutations in the *E2* gene of the HPV16 genome (36).

Besides targeting the viral DNA/RNA, the APOBEC family proteins also target host genomic DNA, generating enriched clusters of signature mutations in the genome (37). APOBEC family members can generate C→T mutations and deaminate cytosines in the host genome. Results from next-generation sequencing studies have suggested that APOBECs can cause base substitutions in tumor genomes. Clustered

mutations (termed kataegis) identified in breast cancer have a higher prevalence of the TCW motif (W refers to either A or T), which has more stringent APOBEC mutational specificity (38,39). Overexpression of the APOBEC3B protein is correlated with enrichment of the *APOBEC3B* mutation signature in the genomes of patients with cervical cancer (27). In-depth analysis of both whole-genome and exome sequencing data sets revealed a significant presence of APOBEC mutation patterns in cervical and head and neck cancers, both of which can be caused by HPV infection (27).

3.4. APOBEC-mediated cancer-driver mutations in papillomavirus-driven tumorigenesis

APOBEC may directly contribute to HPV-driven tumorigenesis by inducing mutations in putative cancer-driver genes. The APOBEC-mediated mutational process is reported to account for a large portion of major oncogenic *PIK3CA* mutations (helical domain mutations E542K and E545K) (40,41). Sequencing has fully characterized genomic alterations in HPV-associated cervical carcinomas (22,42). A study found that the APOBEC signature was significantly enriched up to 6-fold in most samples (150 out of 192) (22). In addition, the APOBEC mutation load is closely correlated with the total number of *PIK3CA* helical domain mutations per sample (22). Moreover, *PIK3CA* mutations often occur with mutations and deletions in *PTEN* (a well-established tumor suppressor gene), although not at a significantly higher rate (22). These findings highlight the potential role of APOBEC mutagenesis as a primary source of carcinogenic mutations in cervical cancer.

4. Conclusion

Chronic infection with HPV is one of the largest contributors to avoidable cancer deaths in China (2). The incidence of and the mortality rate for cervical cancer nationwide have significantly increased (2). A more worrying trend is that cervical cancer is occurring earlier and causing death in younger age groups in some developed urban areas (43). With the increasing prevalence of HPV infection especially in young women, combined with inadequate Papanicolaou (Pap) test screening and poor uptake of HPV vaccines, cervical cancer will continue to pose a huge public health burden to mainland China (2,44). Therefore, effective prevention and control strategies need to be identified. The standard treatment for cervical cancer is currently a combination of platinum-based chemotherapy and radiation, while few targeted therapies are available (41). Induction of cervical cancer by HPV represents an evolutionary process, which is modulated by viral, environmental, and host factors. The coexistence of HPV integration hotspots with scattered breakpoints and the strong tendency for HPV

to integrate into the functional regions of the human genome could be results of this evolutionary process. HPV may randomly integrate into the host genome based on genome accessibility from the beginning, but during the long-term course of carcinogenesis HPV may efficiently survey the human genome and select those integration sites that favor functional changes facilitating the malignant transformation of host cells (21). Key molecules and signaling pathways play crucial roles in milestone events during the carcinogenic process. Agents targeting special molecules and/or signaling pathways such as the PI3K signaling pathway may yield potential therapeutic benefits (22). The biological and/or immunological interactions between HPV and the host must be unraveled in order to develop better tools to control HPV infection and its malignant consequences. Moreover, revealing the genetic features of cervical cancer will enable personalized oncology, which promises to deliver tailored therapies to improve outcomes. Well-designed epidemiological studies must be conducted to sufficiently examine the importance of these events, to validate the potential of predictive markers and therapeutic agents, and to facilitate their subsequent use.

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The clinical management of hepatocellular carcinoma worldwide: A concise review and comparison of current guidelines from 2001 to 2017

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Summary

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the second leading cause of cancer-related mortality worldwide. In this review, we made a review on current guidelines published from January 2001 to June 2017 worldwide with a focus on the clinical management of HCC. The electronic databases MEDLINE, the Chinese SinoMed, and the Japanese CiNii were systematically searched. A total of 18 characteristic guidelines for HCC management were finally included, including 8 guidelines from Asia, 5 from Europe, and 5 from the United States of America (USA). If guidelines were published in multiple versions, the most recent update was included, and surveillance, diagnosis, and treatment were compared. The composition of and recommendations in current guidelines on HCC varied, so these guidelines were regrouped and diagnostic and treatment algorithms were summarized graphically to provide the latest information to clinicians. The diagnostic criteria were grouped into 2 categories of a "Size-based pathway" and a "Non-size-based pathway." The treatment criteria were divided into 4 categories: *i*) Criteria based on the Barcelona Clinic Liver Cancer staging system; *ii*) Criteria based on the modified Union of International Cancer Control staging system; *iii*) Criteria based on the Child-Pugh class of liver function; and *iv*) Criteria based on tumor resectability. Findings from comparison of current guidelines might help target and concentrate efforts to improve the clinical management of HCC. However, further studies are needed to improve the management and outcomes of HCC. More straightforward or refined guidelines would help guide doctors to make better decisions in the treatment of HCC in the future.

Keywords: Hepatocellular carcinoma, clinical guideline, surveillance, diagnosis, treatment

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, with > 500,000 new cases annually, and it is the second leading cause of cancer-related mortality worldwide (1-4). Over the past 2 decades, various studies have examined the clinical management of HCC (5-8), which has witnessed

remarkable improvements in treatment options and the emergence of new treatments involving certain combinations of drugs. However, the overall outcomes of HCC are still far from satisfactory. In accordance with a management model, guidelines are defined as "systematically developed statements to assist practitioner and patient decisions about appropriate healthcare for specific clinical circumstances" (9). If adequate guidelines are devised, they could: *i*) serve as a roadmap for clinicians to develop individualized decision-making algorithms; *ii*) improve the quality of care and patients' outcomes; and *iii*) support and influence regional or national authorities that allocate resources (7).

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Table 1. Eighteen characteristic guidelines for the clinical management of HCC worldwide

Area/Year	Guidelines	Drafted by	Aspects covered	Ref.
Asia				
2009	AOS Guideline	Asian Oncology Summit 2009	D&T+P+S	(11)
2009	Chinese Guideline*	Chinese Society of Liver Cancer, Chinese Society of Clinical Oncology, Chinese Society of Hepatology Liver Cancer Study Group	D&T+S	(12)
2010	APASL Guideline	Asian-Pacific Association for the Study of the Liver	D&T+E+P+S	(13)
2012	Saudi Guideline	Saudi Association for the Study of Liver Diseases and Transplantation; Saudi Oncology Society	D&T+E+P+S	(14)
2013	J-HCC Guideline	Group formed to establish "Guidelines for evidence-based clinical practice for the treatment of liver cancer"	D&T+E+P+S+F	(15)
2014	JSH Guideline	Japan Society of Hepatology	D&T+S	(16)
2014	Korean Guideline	Korean Liver Cancer Study Group and National Cancer Center	D&T+E+P	(17)
2014	INASL Guideline	The Indian National Association for Study of the Liver	D&T+E+P+S	(18)
Europe				
2003	BSG Guideline	British Society of Gastroenterology	D&T+E+S	(19)
2004	BASL Guideline	Belgian Association for the Study of the Liver	D&T+E+P+S	(20)
2009	GOIM Guideline	Italian Southern Oncological Group	D&T+E	(21)
2012	EASL Guideline	European Association for Study of Liver, European Organization for Research and Treatment of Cancer	D&T+E+P+S	(22)
2012	ESMO Guideline	European Society for Medical Oncology	D&T+E+P+S+F	(23)
American				
2007	ACS Guideline**	American College of Surgeons	D&T	(24)
2010	WGO Guideline	World Gastroenterology Organization	D&T+E+P+S	(25)
2010	NCI Guideline	United States National Cancer Institute	D&T+E	(26)
2011	AASLD Guideline	American Association for the Study of Liver Disease	D&T+S	(27)
2017	NCCN Guideline	National Comprehensive Cancer Network	D&T+E+S	(28)

*The National Health and Family Planning Commission (NHFPC) of China also issued the Standards for Diagnosis and Treatment of Primary Liver Cancer (2017) on June 26, 2017 (44), which was an updated version of standards that issued on 2011. **A review article published on J Am Coll Surg by the American College of Surgeons. D&T, diagnosis and treatment; E, epidemiology; P, prevention; S, surveillance; F, follow-up.

Since the year 2001 when the European Association for the Study of the Liver (EASL) issued their HCC guideline (10), at least 20 guidelines have been published or updated thus far, and each has its own advantages. Nonetheless, gaps in knowledge and areas of controversy regarding certain aspects of HCC management are evident and cannot be ignored.

In this review, we made a review on current guidelines published worldwide from 2001 to 2017 with a focus on the clinical management of HCC. Surveillance, diagnosis, and treatment in the characteristic guidelines were compared to provide the latest information to clinicians.

2. Characteristic guidelines for the clinical management of HCC

This review involved a systematically search of the electronic databases MEDLINE, the Chinese SinoMed (<http://www.sinomed.ac.cn/zh/>), and the Japanese CiNii (<http://ci.nii.ac.jp/>) for applicable results from January 2001 to June 2017. No language restriction was applied to the search strategy. Search terms (medical subject headings or keywords) included: "hepatocellular carcinoma," "guidelines/practice guidelines," "consensus," "liver cancer," and "liver carcinoma."

Inclusion criteria were as follows: *i*) credibility, as measured by whether the guidelines were widely cited by subsequent guidelines or other publications regarding the management of HCC after the original

guidelines were published; *ii*) influence, an indication that the guidelines were created with the support of government or academic/medical societies and that the guidelines attracted nationwide attention with respect to their implementation and the standard care for HCC; and *iii*) multifaceted, meaning that the guidelines included aspects of the diagnosis and treatment of HCC at a minimum. If the guidelines were published in multiple versions, the most recent update was analyzed. Furthermore, references listed in guidelines were manually searched for other potential sources. The title and abstract of retrieved studies were evaluated for relevance and compliance. If compliance was not clearly defined by the abstract, the full text was reviewed for further assessment.

In line with the criteria above, 18 guidelines that were published between 2001 and 2017 were identified for analysis, including 8 guidelines from Asia, 5 from Europe, and 5 from the United States of America (USA) (Table 1) (11-28). These 18 characteristic guidelines were examined with a focus on the clinical management of HCC, and surveillance, diagnosis, and treatment in those guidelines were compared.

3. High-risk population and surveillance of HCC

Identification of the risk factors for HCC and devising of appropriate methods for surveillance of the high-risk population are crucial to early diagnosis and a better

outcome. This process is usually divided into 3 parts: *i*) determining risk factors, *ii*) screening the population with risk factors for individuals who need to be monitored, and *iii*) devising the form of surveillance that yields the most benefit.

The current review found that 14 of the 18 guidelines clearly described risk factors and surveillance. Most of that information was common among the guidelines, but there were discrepancies among guidelines due to regional differences in disease and other variables. HCC has been proven to be linked to liver disease independently and its major risk factors can be divided into those that are cirrhosis-related and those that are non-cirrhosis-related. The former includes hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcoholic cirrhosis, genetic causes (hemochromatosis and tyrosinosis), nonalcoholic steatohepatitis, stage IV primary biliary cirrhosis, alpha one antitrypsin deficiency, and other causes of cirrhosis; the latter includes being an HBV carrier with a family history of HCC, being Asian and elderly (males ≥ 40 years and females ≥ 40 years), and being an African/North American black infected with hepatitis B (28,29). Among these risk factors, hepatitis B is the leading cause of HCC in Africa and East Asia while hepatitis C is the leading cause in Europe, Japan, and North America (30,31). As mentioned, cirrhosis caused by various etiologies is the strongest predictor of HCC, with an associated annual incidence of HCC of 1-6% (32,33).

HCC surveillance is cost-effective, and especially so for the high-risk population. A combination of ultrasound (US) and measurement of alpha-fetoprotein (AFP) are the most widely used and effective methods of detecting HCC worldwide (34,35). However, several studies indicated that AFP alone has limited and inconsistent sensitivity and specificity as a screening biomarker, and elevated levels of AFP may be found in < 20% of patients with early-stage HCC (36-38). AFP has been excluded from surveillance and/or diagnostic criteria in guidelines issued in some Western countries, such as the 2005 and 2011 versions of the American Association for the Study of Liver Diseases (AASLD) Guideline, the EASL Guideline, and the National Comprehensive Cancer Network (NCCN) Guideline (22,27,28,39).

In contrast, some expert panels consider AFP to be a good surveillance marker due to its wide utility in diagnostic settings, where it has been studied extensively (40), and its role in combination with US, which can significantly maximize early detection of HCC (41). Of the 18 guidelines that were reviewed here, 5 recommended US for screening with AFP while 6 suggested US alone. The usefulness of other biomarkers, including the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3) and des-gamma-carboxy prothrombin (DCP), has been studied (42,43). Use of these biomarkers in HCC surveillance is recommended by the J-HCC Guideline and the JSH Guideline (12,14). Use of DCP as

a serum biomarker was also mentioned by the Standards for Diagnosis and Treatment of Primary Liver Cancer published by the National Health and Family Planning Commission (NHFPC) of China on June 26, 2017 (44).

The ideal surveillance interval should be evaluated from the perspective of cost-effectiveness by considering the clinical status and available resources. Generally, the surveillance interval is 6 to 12 months for the high-risk population according to the guidelines. A prospective cohort study found that patients with HBV had a better survival with a surveillance interval of 6 months than with 12 months (45). However, other studies have found no significant differences in survival or the rate of HCC detection with intervals of 6 and 12 months (46,47). Of the 18 guidelines that were reviewed here, 8 tended to recommend a surveillance interval of 6 months and 2 recommended an interval of 6 to 12 months.

The definition and description of the high-risk population varied according to the guidelines. According to the J-HCC Guideline and the JSH Guideline, individuals with a high risk of developing HCC who need to be surveilled are classified as the very-high-risk population or the high-risk population. The very-high-risk population includes: *i*) individuals with hepatitis B-related liver cirrhosis and *ii*) individuals with hepatitis C-related liver cirrhosis. The surveillance protocol for those individuals is a US and measurement of tumor markers (AFP/DCP/AFP-L3) every 3-4 months, or dynamic CT/MRI every 6-12 months for patients with cirrhosis or obesity who cannot readily undergo US. The high-risk population includes: *i*) individuals with chronic hepatitis B, *ii*) individuals with chronic hepatitis C, and *iii*) individuals with liver cirrhosis (causes other than HBV or HCV). The recommended form of surveillance is a US and measurement of tumor markers every 6 months.

The NCCN Guideline, INASL Guideline, and EASL Guideline classified patients who are at risk of developing HCC into a cirrhosis group and a non-cirrhosis group. The INASL Guideline and EASL Guideline also took liver function (Child-Pugh) into consideration for the cirrhosis group. Those 2 guidelines stress that patients on the waiting list for liver transplantation (LT), regardless of their liver function status, should be screened for HCC in order to detect tumor progression (whether it exceeds conventional criteria) and to help define prioritize transplantation. The NCCN Guideline did not recommend surveilling the non-cirrhosis group for chronic HCV with advanced fibrosis, but the INASL Guideline and EASL Guideline do recommend surveilling that group. Similarly, the Saudi Guideline suggests surveillance of all cirrhotic patients, but it also stated that there was insufficient evidence to advise surveillance for chronic hepatitis C without cirrhosis. The WGO Guideline divided the criteria for HCC screening into 3 parts: hepatitis B carriers, cirrhosis not due to hepatitis B, and general patients. General

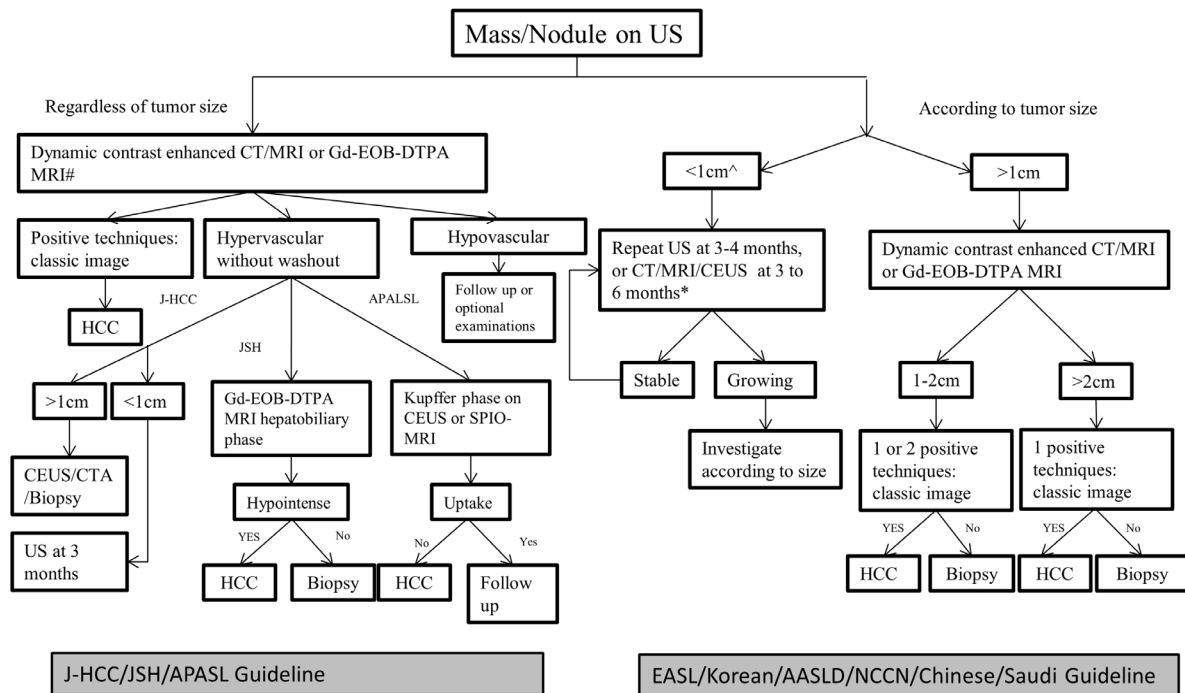


Figure 1. The diagnostic algorithm for hepatocellular carcinoma in current guidelines. # Gd-EOB-DTPA MRI recommended for first-line surveillance and diagnosis of HCC by JSH Guideline. ^ Continuously rising serum AFP level with hepatitis activity under control with two positive techniques of HCC radiological hallmarks can make a diagnosis of HCC by Korean Guideline. * CT/MRI/CEUS at 3 to 6 months by NCCN Guideline; US at 3-4 months by EASL/AASLD Guideline.

patients referred to patients who were previously eligible for HCC screening and included cirrhotic patients who were successfully treated for chronic viral hepatitis. The AASLD guideline grouped together patients who would benefit from surveillance and patients in whom there was no evidence of a benefit from surveillance. The remaining guidelines did not divide the population who needed to be surveilled into smaller groups.

Obviously, there are regional differences in epidemiology that might change with time. For example, the importance of HBV as a cause of HCC is declining, meanwhile the importance of non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) as risk factors for HCC are on a rise (48). Future guidelines should pay close attention to these changes and each country should devise its own method of HCC surveillance depending on local epidemiology. The current comparison of guidelines could help organizations devise a meaningful and easily understood form of surveillance.

4. Diagnostic criteria for HCC according to characteristic guidelines worldwide

The diagnosis of HCC is generally based on a combination of clinical and laboratory features, as well as radiographic and histopathologic presentation. The diagnostic algorithms in the 18 guidelines that were reviewed here have been summarized in a flowchart (Figure 1). Although there were differences among the

guidelines, the final diagnosis of HCC was based on imaging techniques or biopsy.

If US reveals a nodule or mass in an at-risk individual, there are mainly 2 pathways for diagnosis of HCC according to current guidelines. These 2 categories were simply designated the "Size-based pathway" and the "Non-size-based pathway."

4.1. Size-based pathway for HCC diagnosis

The "Size-based pathway" for diagnosis of HCC starts with tumor size (larger or smaller than 1 cm). HCC nodules with a small diameter are difficult to distinguish from cirrhotic nodules, and a previous study found that most nodules with a diameter < 1 cm were not HCC nodules (49). This is the main reason why the AASLD Guideline and EASL Guideline recommend a close follow-up of those patients by repeating US every 3 or 4 months. The NCCN Guideline recommends at least a contrast-enhanced CT, MRI, or CEUS every 3 to 6 months. Kim *et al.* argued that hyper-intensity on both T2 and diffusion-weighted images is helpful in the diagnosis of hypervascular HCC nodules smaller than 1 cm in diameter (50). The Korean Guideline established stricter criteria for diagnosis of HCC nodules < 1 cm. Nodule size according to 2 or more imaging modalities is a typical hallmark of HCC in combination with elevated serum AFP and absence of hepatitis activity (17). The technique that first detected nodules should be performed again 3 to 6 months later. If the nodules have remained

the same size, a close follow-up should be performed. Otherwise, special attention should be paid to the growing nodule size.

Liver nodules larger than 1 cm in size should be evaluated with dynamic contrast-enhanced CT/MRI or Gd-EOB-DTPA MRI. Evidence of one or more radiological hallmarks of HCC, arterial hypervascularity, and venous/late-phase washout are considered indicative of HCC. A non-biopsy diagnosis based on a nodule size > 1 cm has been updated several times. According to the 2002 version of the EASL Guideline, a positive imaging finding plus AFP levels > 400 ng/mL can result in a diagnosis of HCC when nodules > 2 cm (22). In 2005, the AASLD Guideline excluded AFP from the diagnostic algorithm and recommended radiological hallmarks according to 2 imaging techniques to diagnose HCC nodules between 1 and 2 cm in size. For nodules > 2 cm, a hallmark detected by 1 imaging technique would be sufficient (39). The 2010 version of the AASLD Guideline updated criteria as: an imaging technique revealing a radiological hallmark of HCC is sufficient for diagnosis of tumors 1-2 cm in diameter (19). However, the Chinese Guideline still include AFP $\geq$ 400 for 1 month or $\geq$ 200 for 2 months as a diagnostic criterion for nodules 1-2 cm in size (12).

Needle biopsy of a suspicious liver lesion could guide management for patients who do not exhibit a classic imaging presentation and serology, although it is not recommended generally because of the possibility of tumor dissemination outside the liver. The incidence of needle-tract tumor seeding following biopsy of a HCC is 2.7% overall, or 0.9% per year (51). Moreover, the NCCN Guideline stresses that a negative biopsy result does not rule out HCC if a nodule or mass has increased in size.

4.2. Non-size-based pathway for HCC diagnosis

In the "Non-size-based pathway," patients will be scheduled for dynamic imaging regardless of tumor size. All of the guidelines indicate that a definitive diagnosis of HCC can be made when dynamic CT/MRI reveals intense arterial uptake followed by a "washout" of contrast. Moreover, the updated JSH Guideline includes Gd-EOB-DTPA MRI (gadoteric acid disodium, a liver-specific contrast agent) as a tool for first-line surveillance and diagnosis of HCC (12). This new contrast agent is specifically absorbed by normal hepatocytes, resulting in contrast enhancement. Therefore, HCC nodules lacking normal hepatocytes are hypo-intense, and this difference can help distinguish tumors from non-tumorous ("normal") nodules (52,53).

When an advanced imaging technique reveals only hypervascularity with no washout, the diagnostic algorithms differ among the guidelines that were reviewed here. Recommendations in the J-HCC Guideline (15) depend on tumor size. If the tumor

diameter is larger than 1 cm, other optional examinations should be performed, including Gd-EOB-DTPA-MRI, SPIO-MRI, CEUS, CTA, and biopsy. A 3-month follow-up is recommended for patients with a tumor < 1 cm in diameter and elevated levels of tumor markers while dynamic CT/MR is recommended for a larger tumor. In the JSH Guideline, a tumor that is hypo-intense during the hepatobiliary phase of GD-EOB-DTPA-MRI can be diagnosed as HCC provided that cavernous hemangioma is first ruled out by other modalities (16). A biopsy is necessary if the tumor is iso-intense or hyper-intense in the hepatobiliary phase. According to the APASL Guideline, a lesion can be diagnosed as HCC when high SPIO-enhanced MRI signals or a defect in the Kupffer phase of Sonazoid-enhanced US is evident (13). However, the APASL Guideline only recommends a close follow-up instead of a biopsy for patients with intense uptake in SPIO-MRI or CEUS.

There is still a lack of a broad consensus on the most appropriate diagnostic algorithm to use when initial dynamic CT/MRI reveals a hypo-vascular mass in the arterial phase. The updated J-HCC Guideline published in 2013 suggested that an optional examination should be undergone by patients with a tumor larger than 1.5 cm and it suggested a follow-up of 3 months for those with a tumor smaller than 1.5 cm (15). The JSH Guideline stresses presentation in the hepatobiliary phase of GD-EOB-DTPA-MRI. If hypo-intensity is present, Sonazoid CEUS is recommended; otherwise, follow-up should be continued (16). The APASL Guideline tended to recommend SPIO-enhanced MRI or Sonazoid CEUS for those patients (13). A close follow-up was recommended in the event of a negative imaging finding.

5. Treatment criteria for HCC according to characteristic guidelines worldwide

The treatment algorithm for HCC is constantly changing as the criteria for hepatic resection expand, locoregional therapies advance, novel targeted systemic therapies are introduced, techniques for internal and external radiation therapy improve, and the possibility of receiving a transplant increase. However, long-term outcomes of HCC depend on both the medical complexity of HCC (involving multiple confounding factors: tumor heterogeneity, liver function and performance status) as well as the choice of an appropriate treatment, posing a challenge for both patients and clinicians.

An important aim of guidelines is to feature up-to-date, specific, quality evidence to help clinicians select the most appropriate treatment. Recently updated guidelines include those by the NCCN (2017), Korea (2014), JSH (2014), INASL (2014), J-HCC (2013), ESMO (2012), EASL (2012), and Saudi Arabia (2012). The treatment algorithms in these 8 updated guidelines and in the 10 other guidelines can be grouped into the following 4 categories: *i*) Criteria based on the Barcelona

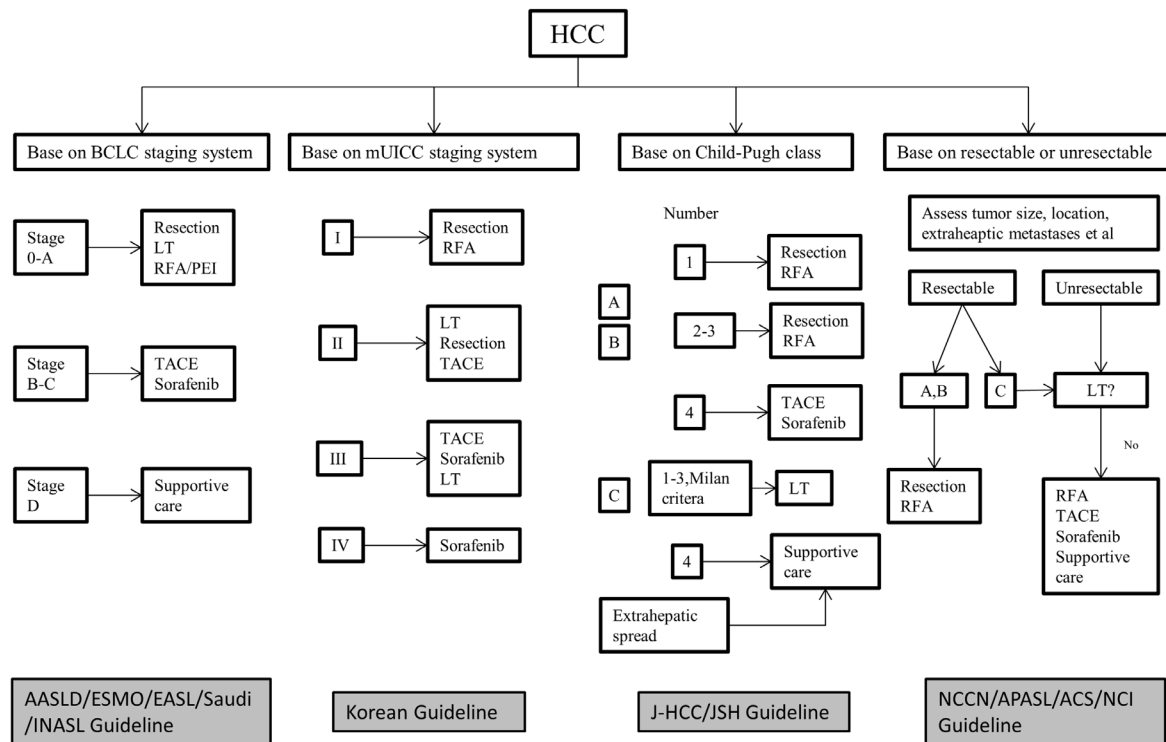


Figure 2. The treatment algorithm for hepatocellular carcinoma in current guidelines. BCLC staging system: Barcelona Clinic Liver Cancer staging system; mUICC staging system: modified Union of International Cancer Control staging system.

Clinic Liver Cancer (BCLC) staging system; *ii*) Criteria based on the modified Union of International Cancer Control (mUICC) staging system; *iii*) Criteria based on the Child-Pugh class of liver function; and *iv*) Criteria based on tumor resectability (resectable or unresectable) (Figure 2).

5.1. Treatment criteria based on the BCLC staging system

The BCLC staging system takes tumor stage, liver function, and physical status into account, and this system had been widely adopted for HCC staging and treatment (54). Moreover, the BCLC staging system is the only staging system that assigns treatment strategies based on specific prognostic subclasses, an approach that has proven effective (55). The spectrum of treatment options with curative intent may be a subject of some controversy, but it generally consists of liver resection, LT, and ablation. Patients with stage 0 or stage A liver cancer may have a 5-year survival rate of 40-70% after treatment with curative intent. Liver resection still remains the mainstay of HCC treatment in non-cirrhotic patients or in selected cirrhotic patients with a single lesion. The AASLD Guideline repeatedly stresses the usefulness of measuring portal pressure in predicting patient outcomes and optimizing patient selection for liver resection; this usefulness of this index has also been verified in Japan (56). The AASLD Guideline also indicated that patients with portal hypertension

or multiple lesions could receive a survival benefit from resection. The algorithm in the ESMO Guideline excluded hypertension and it expanded the criteria for clinical decision-making with regard to resection (23). The EASL Guideline added a recommendation of anatomical resection, which should be performed wherever feasible particularly for patients with a tumor of 2 to 5 cm in size (57).

LT is indicated for patients with BCLC stage A cancer meeting the Milan criterion (solitary HCC nodule < 5 cm in size or fewer than 3 nodules, none larger than 3 cm in diameter). Patients with cancer meeting the Milan criterion had a 5-year overall survival rate of 65-78% after LT, which is why this criterion was integrated into the BCLC staging system (58). This strict criterion also has certain limitations. According to the ESMO Guideline, LT is ruled out for patients with cancer meeting the Milan criterion and poor liver function (Child-Pugh class C), who would be classed as BCLC stage D. The University of California San Francisco (UCSF) criterion extends beyond the Milan criterion, and the UCSF criterion results in comparable outcomes according to the INASL Guideline (59). On the whole, primary recommendations for LT have remained the same.

Percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA) are the most widely used forms of ablative treatment. They are considered the standard treatment for HCC that is BCLC 0-A stages and that is not amenable to surgery. Recent studies

have found that RFA or PEI, as first-line treatment, can yield similar outcomes to surgical resection when tumors are smaller than 2 cm in size and BCLC stage 0 (60,61). While according to INASL Guideline, patients with stages 0 were only recommended to receive ablation when they were not potential candidates for LT. Substantial evidence is required to verify the effectiveness of ablation as a first-line treatment for very early HCC.

Transarterial chemoembolization (TACE) is the primary treatment option for BCLC stage B HCC (62). The current guidelines reviewed here recommend TACE at about the same level as they did previously. Recent studies have found transarterial radioembolization (TARE) might outperform TACE in terms of tumor downstaging, and its combined use with Yttrium-90 microspheres may result in an encouraging outcome in terms of survival (63,64). Thus, TARE with Yttrium 90 could be considered as an alternative to TACE, particularly in cases of HCC and portal vein thrombosis.

Molecularly targeted therapy with sorafenib is indicated when BCLC stage C HCC or BCLC stage B HCC progresses after TACE. Two widely cited RCTs have revealed that sorafenib can serve as a first-line treatment in patients with HCC who still have liver function but who can no longer be treated with other more effective therapies (65,66). Recent studies on sorafenib have reported safety data and its efficacy in prolonging survival (67-69).

Patients in the terminal stage (BCLC stage D) should receive the best supportive care. External beam radiation therapy has only been tested in non-controlled studies. The INASL Guideline contends that it cannot recommend radiation therapy for management of HCC until its effectiveness is verified in clinical trials.

5.2. Criteria based on the mUICC staging system

The Korean Guideline adopted the mUICC as a primary staging system. Its recommendations for first-line treatment are based on mUICC staging system, but its algorithm only applied to patients with Child-Pugh class A HCC, no portal hypertension, and an Eastern Cooperative Oncology Group (ECOG) performance status of 0-1. The basic criteria of the mUICC staging system include: *i*) the number of tumors, *ii*) the diameter of the largest tumor, and *iii*) vascular or bile duct invasion. The best treatment option for a stage I tumor (single/ ≤ 2 cm/VI-) is resection or RFA. There are 3 options for Stage II cancer: *i*) resection or RFA (tumor size ≤ 3 cm) is recommended for treatment of stage IIa cancer (single/ > 2 cm/VI-); *ii*) LT (for cancer meeting the Milan criterion) is the first option for treatment of stage IIb cancer (multiple/ ≤ 2 cm/VI-), and TACE or RFA is an alternative when there are more than 3 nodules; and *iii*) stage IIc cancer (single/ ≤ 2 cm/VI+) is amenable to TACE. The mainstay for treatment of

stage III cancer is TACE or sorafenib. However, LT must be taken into account when cancer meets the Milan criterion. Sorafenib is better suited to treatment of a stage IV tumor. The Korean Guideline also added that external beam radiation therapy could be useful in alleviating symptoms caused by primary HCC or metastases.

5.3. Criteria based on the Child-Pugh class of liver function

An algorithm based on the Child-Pugh class of liver function is utilized in Japan. The class is based on 3 factors: liver function, the number of tumors, and tumor size. Before a Child-Pugh class is assigned, whether extrahepatic spread is present is first determined. If extrahepatic spread is present, chemotherapy is the treatment of choice for Child-Pugh class A cancer. Palliative care is recommended for patients with decreased liver function. Undoubtedly, liver resection has been the first option for a solitary tumor that is Child-Pugh class A/B. According to the 2013 version of J-HCC Guideline (15), RFA is also recommended for tumor < 3 cm. For patients with 2 to 3 tumor nodules, resection or RFA/TACE is recommended depending on their size. For patients with more than 4 tumor nodules, TACE is first recommended, but JSH Guideline contends that resection can sometimes be performed, and ablation is sometimes performed in combination with TACE.

LT is recommended for patients younger than 65 with cancer meeting the Milan criterion, even if they have class C liver function according to the Child-Pugh score.

5.4. Criteria based on tumor resectability (resectable or unresectable)

Treatment algorithms in the NCCN Guideline (2017), APASL Guideline (2010), NCI Guideline (2010), and ACS Guideline (2007) are based on tumor resectability. Initially, tumor resectability should be evaluated based on parameters like liver function, the presence of portal hypertension, tumor location, and the presence of extrahepatic metastases. If a tumor is resectable, resection or RFA (tumor with small diameter) is recommended. LT should also be considered for patients with cancer that is Child-Pugh class C. LT has become the first-line treatment for patients with unresectable tumors that nonetheless meet the Milan or United Network for Organ Sharing (UNOS) criteria. If those patients are not optimal candidates for transplantation, the choice of locoregional therapy, sorafenib, or supportive care depends on individual circumstances (including tumor location, liver function, and institutional capabilities). Moreover, the NCCN Guideline added that transplantation can be considered or recommended for those patients who initially failed to meet the Milan criterion but who received successful downstaging therapy.

6. Conclusion

In this article, we made a review on current 18 characteristic guidelines for HCC management published worldwide between 2001 and 2017, including 8 guidelines from Asia, 5 from Europe, and 5 from the US. This work compared those guidelines in terms of surveillance, diagnosis, and treatment with a focus on the clinical management of HCC. The composition of and recommendations in current guidelines on HCC varied, so these guidelines were regrouped and diagnostic and treatment algorithms were summarized graphically to provide the latest information to clinicians.

Over the past few decades, HCC has changed from an almost universal death sentence to a cancer that can be prevented, detected at an early stage, and effectively treated, but HCC is still the third leading cause of cancer-related mortality worldwide, and the leading cause of death among patients with chronic liver disease. Findings from this comparison of current guidelines might help target and concentrate efforts to improve the clinical management of HCC. However, further studies are needed to improve the management and outcomes of HCC. More straightforward or refined guidelines would help guide doctors to make better decisions in the treatment of HCC in the future.

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Surgical management for bile duct injury

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Summary

The management of bile duct injury (BDI) remains a considerable challenge in hepatobiliary surgery. BDI is mainly iatrogenic, and mostly occurs in cholecystectomy. Laparoscopic cholecystectomy (LC) has been performed widely, however, the incidence of BDI associated with LC increases 2-3 times compared to that in open cholecystectomy (OC). BDI also occurs in robotic cholecystectomy. In China, the evidence-based Practice Guideline for Diagnosis and Treatment of BDI was published by the Biliary Surgery Group of Surgery Branch of Chinese Medical Association, with the purpose of reducing the incidence of BDI as well as promoting its optimal diagnosis and treatment. Surgery remains the mainstay of treatment for BDI and traumatic bile duct stricture. The definitive repair involves a series of procedures including exposing the proximal and distal bile duct, anastomotic bile duct tissue preparation, minimally invasive tissue anastomoses, and so on. Successful management is a surgical challenge requiring great specialized experience and precise surgical skill. The application of precision biliary surgery is recommended for promoting standardized management of BDI.

Keywords: Bile duct injury, traumatic bile duct stricture, surgical repair, guideline

1. Introduction

The management of bile duct injury (BDI) remains a considerable challenge in hepatobiliary surgery. Since 1905, Mayo *et al.* first reported the use of choledochoduodenostomy to repair two cases of BDI associated with cholecystectomy (1). Biliary surgeons worldwide have been committed to the prevention and treatment of BDI. BDI would not only lead to exceedingly morbid complications including biliary fistula, jaundice, and bile duct stenosis affecting the patient's long-term prognosis, but also increase the unnecessary medical burden (2,3). While long-term impact on patients is associated with a significant decrease in Quality of Life, loss of productivity in both paid and unpaid work and high rates of disability benefits use (2,4). BDI mostly occurs in cholecystectomy

(5-8). The incidence of BDI associated with open cholecystectomy (OC) is 0.125-0.3% (9-12), but the rate is up to 0.4-0.6% for cases that underwent laparoscopic cholecystectomy (LC) (13-16), as well as increasing complexity (17). With advances in technology, single-incision laparoscopic cholecystectomy and robotic cholecystectomy have been performed, but the incidence of BDI was also reported (18,19), and more rigorous training in biliary surgery may be needed (20,21).

The definitive repair surgery remains the mainstay of treatment for BDI and traumatic biliary stricture (22). However, even in a high volume biliary surgery center with extensive experience, the incidence of stricture after repair surgery of BDI still reaches 10-20% (23,24). Only 1/3-1/2 of BDI can be diagnosed in time, more than 70% of BDI is initially repaired by surgeons who do not specialize in such repair surgery (25,26). Non-definitive surgical exploration and the implementation of definitive repair surgery with inappropriate timing are ubiquitous (12,27). Moreover, delayed referral to a specialist center increases morbidity (28). In China, the evidence-based Practice Guideline for Diagnosis and Treatment of BDI was published by the Biliary Surgery Group of Surgery Branch of Chinese Medical Association, with the purpose of reducing the incidence

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Table 1. Key points for preoperative accurate assessment of bile duct injury (BDI)

- The location of bile duct injury or stenosis.
- The degree and length of bile duct loss or stenosis; Proximal bile duct with or without expansion and the expansive degree.
- Left and right hepatic duct are connected or unconnected.
- Right posterior hepatic duct with or without injury.
- Whether or not combined with vascular injury.
- Whether or not develops secondary bile leakage, abdominal infection, liver abscess, sclerosing cholangitis, biliary cirrhosis, *etc.*

of BDI as well as promoting its optimal diagnosis and treatment (29). How to improve the success rate of BDI repair, and reduce the recurrence rate of restenosis after repair is a considerable challenge in hepatobiliary surgery.

Evidence has shown that the success of definitive repair surgery on BDI relies on preoperative accurate assessment of the type of injury, selection of appropriate surgical procedures, reasonable repair methods, and the application of precision biliary surgery (30).

2. Preoperative accurate assessment

Surgeons should accurately assess all the details associated with BDI before performing a definitive repair surgery (Table 1). Based on the comprehensive assessment of the detailed information, the type of BDI can be determined (29,31). It is the basis for the development of rational treatment strategies and the selection of appropriate surgical procedures to ensure success of repair surgery.

When performing repair of BDI, location and extent of injury are clear in most cases. However, in the presence of severe inflammation or fibrous scar in the hepatic hilar region, inexperienced surgeons may not be able to correctly identify the site of injury, followed by blind suture in the Calots triangle or simply placing the T tube under the injured site. Even if location of the injury is clear, surgeons may also make a miscarriage of justice during surgery and follow the wrong treatment, such as taking the damaged right posterior segmental duct as the aberrant bile duct and make a ligation. Besides, the exact location of the injury is often difficult to determine if the pancreatic segmental bile duct is damaged during bile duct exploration or the choledochopancreato-duodenal junction is damaged during endoscopic sphincterotomy (EST) with Oddi. Although in most cases, location of the BDI is single, there may be multiple injuries, especially with traumatic bile duct injury. In these complex conditions, surgeons should pay attention to the comprehensive information of intraoperative cholangiography, bile ductal blue staining or water injection test, intraoperative choledochoscopy and other measures, then accurately determine the details of BDI.

A complete imaging of the bile duct should be obtained by radiographic examination before a definitive repair of BDI. Available methods include percutaneous transhepatic cholangiography (PTC) (32), endoscopic

retrograde cholangiopancreatography (ERCP), fistulography, computed tomography (CT), magnetic resonance cholangiopancreatography (MRCP) (33), *etc.* Indications for these inspection techniques are described in detail in the Practice Guideline for Diagnosis and Treatment of BDI published by Biliary Surgery Group of Surgery Branch of Chinese Medical Association (29). Surgeons should choose appropriate means of examination based on the combined information of patient's condition and the local medical conditions, and should not perform exploratory surgery instead of preoperative anatomical imaging assessment.

3. Appropriate timing for repair

The localized inflammatory state is one of the major determinants of the prognosis of definitive repair surgery. The ideal repair or reconstruction procedure should be carried out without inflammation (34). Based on this principle, intraoperative BDI are suggested to be repaired immediately by experienced biliary surgery specialists (35). If it cannot be performed with the support of specialists, patients should be treated with drainage and referred to specialist hospitals for early repair (36,37). For BDI detected soon after surgery, such as injury without local inflammation can be performed with primary repair (38). In cases with abdominal infection, biliary peritonitis, vascular injury, or other complicated conditions, delayed repair should be performed after the measures of controlling bile leakage and infection and improving the patient's general condition (39,40). Although the early idea holds that the timing of delayed repair should be at least 3 months away from the injury, current evidence suggests that definitive repair surgery may be performed 4-6 weeks after local inflammation and infection are effectively controlled (41,42).

4. Optimal surgical procedure for repair

Surgical procedures for definitive repair of BDI include duct-to-duct choledochostomy, bile duct jejunum Roux-en-Y anastomosis, hepatectomy, and so on (13,43,44). The optimal surgical procedure for repair should be determined by clinicians based on analysis of BDI type, biliary obstruction duration, previous biliary repair surgery history, degree of liver damage, and the patient's general condition (Table 2).

There are several classifications for BDI (45-47). We proposed a new classification of BDI. The classification

Table 2. The injury sites and repair modes of bile duct injury (BDI)

Injury types	Injury sites	Times of injury diagnosis	Repair principle	Repair modes
Type I	Pancreatic segmental bile duct and choledocho-pancreato-duodenal junction	Intraoperative or early postoperative stage	Primary repair and effective drainage	i) Simple BDI: Kocher incision, Repair the injury under direct vision + T-tube drainage of common bile duct ii) Severe injury: Transection of common bile duct + choledochojejunostomy
		Late or infected cases	Staged operation	First stage: Bile and pancreatic juice and intestinal fluid diversion Second stage: choledochojejunostomy and / or gastrointestinal anastomosis
Types II1 and II2	Extrahepatic bile duct and conjunctive region of bile duct	Intraoperative or early postoperative stage	"must-be" repaired or reconstructed	i) Slight laceration can be used for simple suture ii) Combined tissue defect, but proximal distal tension free, duct-to-duct anastomosis is suggested. iii) Large tissue defect or serious damage, difficult to repair, choledochojejunostomy could be the choice.
		Late or merge infected cases	The same as above.	See above.
Type II3	First branches of hepatic duct	Intraoperative or early postoperative stage	"should be" repaired or reconstructed	i) Slight laceration can be used for simple suture ii) Combined tissue defect, but proximal distal tension free, duct-to-duct anastomosis is suggested iii) Large tissue defect or serious damage, difficult to repair, choledochojejunostomy could be the performed.
		Late stage or obvious infection.		When chronic liver abscess or secondary diffused hepatolithiasis and segment atrophy, the resection of affected bile duct and affected tributary sectionectomy plus choledochojejunostomy is advised.
Type II4	Secondary braches of hepatic duct	Intraoperative or early postoperative stage	Consideration of reparation or reconstruction by condition.	i) For difficult reparation but with sufficient compensatory liver function, ligation of the injured bile duct. ii) For insufficient functional remnant liver, chose duct-to duct anastomosis or choledochojejunostomy
		Late or merge infected cases		i) For those asymptomatic isolated injuries, close follow-up. ii) Combined with bile leakage or infection, hepatectomy.
Type III	Upper branches of tertiary hepatic duct	Intraoperative or early postoperative stage	Without consideration of reparation or reconstruction	i) Ligation or suture of injured bile duct.
		Late or merge infected cases		ii) When bile leakage, PTCD or endoscopic stent placement. iii) Asymptomatic, localized biliary stricture, close follow-up.

includes type I involving pancreatic segmental bile duct and choledocho-pancreato-duodenal junction, types II1 and II2 involving extrahepatic bile duct and conjunctive region of bile duct, type II3 involving first branches of hepatic duct, type II4 involving secondary braches of hepatic duct, type III involving the upper branches of tertiary hepatic duct, and so on (29).

5. Application of precision biliary surgery

5.1. The fundamental principle of definitive repair of BDI

The surgical procedure must follow the fundamental principle of "Anastomosis and reconstruction must

build upon healthy, non-ischemic, non-inflammation and non-scarred bile duct". Many repair failures are due to failure to follow the above fundamental principles. For example, the boundaries of ischemia and devitalization of bile duct tissue caused by thermal injury are often unclear at the early stage of injury. In cases where bile duct ischemia and inactivation planes are difficult to determine accurately, surgery may be erroneously performed on ischemic bile ducts, this error is the main cause of postoperative anastomotic leakage and short-term stenosis. The scar of the bile duct wall or surrounding tissue used to try to restore the continuity of the bile duct and intestinal tract will inevitably lead to surgery failure.

5.2. Exposing the proximal and distal bile duct

The exposure of anastomotic proximal and distal healthy bile duct is the first step in injury repair. For BDI found during surgery, it is generally easier to reveal and determine the proximal and distal bile duct. When performing a staged operation to repair biliary stricture, it is possible to forward to the hepatoduodenal ligament and hilar area by separation of the liver surface adhesion or track the extrahepatic bile duct along the previous drainage fistula. In high-level bile duct stenosis, especially cases with repeated repair surgery failure, a fibrous connective tissue scar will be formed in the hepatic hilar area due to chronic inflammation, and the proximal bile duct stumps are more hidden in the deep part of the hepatic portal. In such a condition, it is very difficult to find bile duct stumps in the hepatoduodenal ligament by a conventional approach. Surgeons are suggested to find the dilated proximal dilatation of the bile duct by the following approaches.

5.2.1. Approach through hilar plate

In the posterior edge of the liver IVb along the hilar transverse groove after cutting the hilar plate the surgeon can reach the top of the hepatic duct junction, which reveals the proximal bile duct.

5.2.2. Approach through upper portion of hepatic portal

If the severe scar in the hepatic hilar area cannot be clearly dissected and is difficult to expose the bile duct by the hilar plate approach, it is suggested to go through liver IVb posterior margin and superjacent to the transverse sulcus of the hepatic portal, and dissect deeply into the liver parenchyma until confluent with the hepatic duct, and then reveal the proximal bile duct.

5.2.3. Approach through fissure of umbilical vein

If the bile duct cannot be exposed from the front area of hepatic portal, the approach of cutting off the liver

bridge in the Rex nest after dissection of the umbilical vein plate, showing the portal vein sagittal and corners, and cut umbilical vein panel in the right rear would be performed to expose the left hepatic duct.

5.2.4. Approach through posterior portion of hepatic portal

If narrow proximal bile duct cannot be exposed by the approach of anterior portion and upper portion of hepatic portal, the approach of dissecting portal vein from posterolateral hepatic duodenal ligament would be performed, and anatomy along the anterior wall of the portal vein until the right hepatic pedicle. The enlarged right proximal hepatic duct is located above the anatomical plane.

For cases with high-level bile duct injury, the most prone to error of revealing the proximal hepatic duct is missing and must independently open the bile duct. The omission of branches of a bold bile duct may cause postoperative recurrence of cholangitis and patients are forced to undergo reoperation. Following the principle of "bile duct is three rather than two" will avoid missing the right posterior hepatic duct (48). For high bile duct stenosis, there are a number of independent openings in the intrahepatic bile duct, the percutaneous transhepatic biliary drainage (PTCD) can be performed preoperatively for all the openings of the intrahepatic bile duct, and find them intraoperatively under the guidance of a drainage tube (49).

5.3. Anastomotic bile duct tissue preparation

The appropriate pruning or plastic surgery is necessary to reveal the proximal and distal bile duct, which then can be used for anastomosis. Surgical and postoperative early bile duct injury repair should be performed after removing ischemic inactivated bile duct tissue, and choosing the healthy bile duct wall for anastomosis. When performing duct-to duct choledochostomy for traumatic bile duct stricture, the narrow stenosis and scarring of the bile duct tissue should be removed, and then perform anastomosis for a healthy bile duct with a good blood supply. The key to choledochojejunostomy is to establish an adequate caliber of anastomosis in a narrow proximal bile duct with normal mucosa. Therefore, it is appropriate to remove scar tissue on the stump of the bile duct after fully revealing the proximal bile duct.

For type III of bile duct stenosis, the incision of the proximal wall of the proximal extrahepatic bile duct is usually taken; if necessary, the incision would be extended to the left hepatic duct and performing lateral-lateral anastomosis on the bile duct and jejunum. In type III of bile duct stenosis, for cases where the left and right hepatic duct is still connected, it is suggested to first reveal the anterior wall of left hepatic duct, and

then extend the incision rightwards to the anterior of the right hepatic duct. For cases, where the left and right hepatic duct is cut off, it is suggested to remove the sclera tissue of bile duct stump after the incision of the left and right hepatic duct. Forming an anastomotic stoma by suture of the medial margin of left and right hepatic duct and performing anastomosis with the jejunum, or performing the anastomosis on both sides of the hepatic duct incision and the jejunum. For type II3 of bile duct stenosis, the approach of dissecting hepatic portal, separating hilar plate, then performing incision of left hepatic duct or right hepatic duct in the narrow proximal are recommended. For type II4 of bile duct stenosis, the full exposure and incision of the right side of the secondary hepatic duct often needs to dissect the right liver pedicle Glisson sheath of the anterior wall of the gallbladder plate and resect part of liver tissue in the basilar part of S5.

5.4. Minimally invasive tissue anastomoses

For any kind of repair and reconstruction procedures, the fine coincidence technique should be performed to restore the integrity of the bile duct and its continuity with the intestinal tract, and a non-invasive suture needle should be selected for intermittent or continuous mucosal-mucosal anastomosis. The anastomotic stoma should be tight to prevent postoperative bile leakage, and it should also be observed to avoid excessive tightness damaging the blood supply of anastomotic tissues. The principles of single-layer stitching, stitching needle pitch, uniform margins, appropriate density, moderate knotting strength, and anastomosis without tension should be followed. The 6-0 fine suture needle could be used for thin bile ducts with thin walls; the 6-0 or 5-0 fine suture needle could be used for "duct-to-duct" choledochostomy in delayed repair. When performing choledochojejunostomy, the 5-0 or 6-0 fine suture needle could be used according to the thickness of the bile duct wall. Both absorbable lines and non-absorbable sutures can be used, but leaving non-absorbable lines in the cavity should be avoided. The main purpose of placing biliary drainage after definitive biliary repair is not to maintain an anastomotic opening, but to provide postoperative biliary decompression to prevent bile leakage and provide access for subsequent angiographic or cholangioscopic treatments (50). Therefore, the conventional placement of bile duct drainage is not necessary. Only for cases with unsatisfied anastomosis, obvious inflammation in the bile duct wall, or intrahepatic bile duct stones, short-term drainage could be placed, with the drainage time generally not more than 3 months.

In conclusion, the management of BDI remains a considerable challenge in hepatobiliary surgery. The success of definitive repair depends on great specialized experience and precise surgical skill. The application of

precision biliary surgery is recommended for promoting standardized management of BDI.

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A narrative review of non-operative treatment, especially traditional Chinese medicine therapy, for lumbar intervertebral disc herniation

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Summary

Lumbar intervertebral disc herniation (LIDH), as the main contributor to low back pain and sciatica, imposes a heavy burden on both the individual and society. Non-operative treatment or conservative treatment has proven effective in alleviation of the symptoms of LIDH and are considered to be a first-line choice for most cases. Active lifestyle, physical therapy, complementary and alternative medicine therapy or Traditional Chinese medicine (TCM) therapy, and pharmacotherapy are routinely used as effective non-operative treatment for LIDH patients. However, how to choose one or several conservative treatments with higher efficacy, less side effects, minimal injury, and low cost is still a challenge for doctors and LIDH patients. Furthermore, there are some national characteristics for some conservative treatments in different countries, which bring difficulties for the widespread use of these methods. Here we initiated a search on the non-operative treatment especially TCM therapy for LIDH mainly using PubMed, Web of Science, China National Knowledge Internet (CNKI), and Chinese biomedicine database since the 1980s with no restriction of language. According to these related references, we gave a narrative review which emphasizes up-to-date knowledge regarding the effectiveness and safety of various conservative methods with special consideration for TCM therapy including acupuncture, autonomy, Chinese massage, and Chinese herbal medicines, for LIDH treatment. We hope this review will further contribute to an understanding of conservative treatment as an important choice for LIDH patients and provide useful information for the development of more effective conservative methods for LIDH treatment.

Keywords: Lumbar intervertebral disc herniation (LIDH), low back pain, sciatica, non-operative treatment, traditional Chinese medicine (TCM) therapy

1. Introduction

Lumbar intervertebral disc herniation (LIDH) is one of the most common spinal degenerative disorders affecting 1-3% of the general population (1). It is a pathological condition that is defined as a displacement of disc components (nucleus pulposus or annulus fibrosis) beyond the intervertebral disc space (2). LIDH is one of the most common causes of lower-back pain and sciatica. Its diagnosis can be confirmed by radiological examination. However, MRI or CT findings of herniated

disc are not always accompanied by clinical symptoms (3). As the main contributor to low back pain and sciatica, LIDH greatly affects people's work, daily lives and quality of life, even permanent neurologic deficit and lifelong incontinence due to cauda equina syndrome (4). In recent years, with the changes in human's work and lifestyle, the incidence of LIDH has gradually increased and the onset age has tended to be younger. It mainly occurs among working adults aged < 50 years and has become a worrying occupational health issue because it sometimes affects continuation or resumption of employment (5). Therefore, LIDH imposes a heavy burden on both the individual and society.

As is well known, the aims of intervention for LIDH is to relieve pain, increase mobility and function, improve quality of life, and minimize adverse effects of treatments. Currently, a variety of therapeutic

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interventions have been proposed for the treatment of LIDH, including non-operative treatments (or conservative treatments) and surgical options. The choice of treatment, conservative or surgical, usually depends on symptom severity. In some cases, the recommendation for immediate surgery is necessary because of severe neurological symptoms, such as cauda equina syndrome. It is reported that only 15-20% of LIDH patients require immediate operative intervention (Figure 1A) (1). In other cases, the choice may be less clear. Although surgical treatment is one of the most common options for LIDH patients, the efficacy of this procedure relative to non-operative care remains controversial (6). Compared with conservative therapy, surgical treatment provided faster relief from back pain symptoms in patients with LIDH, but did not show a benefit over conservative treatment in midterm and long-term follow-up. No noteworthy difference could be observed between the therapeutic outcomes of conservative and surgical treatment after a period of 2 years (7,8). Moreover, the most common cause of poor outcome following lumbar disc surgery is recurrent herniation. Recurrence has been noted in 5% to 15% of patients with surgically treated LIDH (9). Conservative treatments of LIDH have been reported to have unique advantages, with the clinical symptoms of most patients diminished or even completely gone within a few weeks (10). In 2013, in USA more than 1 million patients received an epidural steroid injection as a conservative treatment for LIDH, let alone those seeking other conservative treatment methods within USA and those outside USA (11). Hence, some non-operative treatments have proven effective in alleviation of LIDH symptoms and are considered to be a first-line choice for most cases, particularly in the initial 6 weeks of conservative management (12). As shown in Figure 1B, active lifestyle, physical therapy, complementary and alternative medicine options (e.g., acupuncture, acupotomy, Chinese massage, and Chinese herbal medicine), and pharmacotherapy (e.g., non-steroidal anti-inflammatory drugs (NSAIDs), muscle relaxants, systemic steroids, and steroid injections) are routinely used as effective non-operative treatments for LIDH patients (7,13). In all of these non-operative treatments, some complementary and alternative medicine therapies or Traditional Chinese medicine (TCM) therapies, such as acupuncture, acupotomy, Chinese massage, and Chinese herbal medicine, have particularly attracted more and more attention for LIDH patients. TCM, as an important component of complementary and alternative medicine, evolved over thousands of years with its own unique system of theories, diagnostics and therapies in Asian countries, especially China (14). In the world including Western countries, these TCM therapies have been increasingly used in the last few decades and have become well known for its significant role in preventing and treating various diseases including LIDH (15).

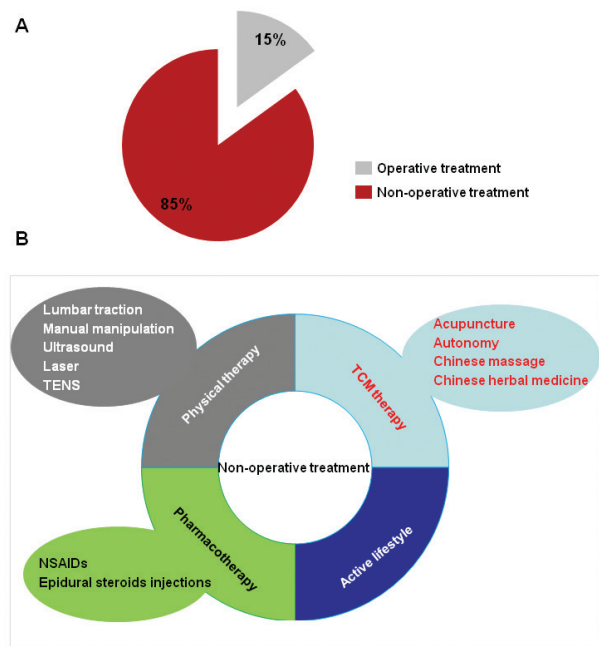


Figure 1. A variety of therapeutic interventions have been proposed for the treatment of LIDH, including non-operative treatment (or conservative treatment) and operative treatment. (A) The proportion of operative treatment and non-operative treatment. **(B)** Some routinely used effective non-operative treatments for patients with LIDH. (Abbreviation: NSAIDs: non-steroidal anti-inflammatory drugs, TCM: Traditional Chinese Medicine, TENS: transcutaneous electrical nerve stimulation.)

Therefore, a more detailed induction of conservative treatments for LIDH especially TCM therapies is necessary.

Currently, although conservative treatments are numerous, there is still a lack of satisfactory treatment for doctors and LIDH patients. Every method has some advantages and disadvantages. Moreover, there are some national characteristics for conservative treatment of LIDH in different countries, which brings difficulties for the widespread use of these methods. Therefore, how to choose one or several conservative methods with the characteristics of higher activity, less side effects, minimal injury, and low cost, has become the common goal for both doctors and LIDH patients. Here we will initiate a search of non-operative treatment especially TCM therapy for LIDH mainly using PubMed, Web of Science, China National Knowledge Internet (CNKI), and Chinese biomedicine database since 1980s with no restriction of language. According to these related references, we will give a narrative review, which emphasizes up-to-date knowledge regarding the effectiveness and safety of various conservative methods with special consideration of TCM therapy for LIDH treatment. We hope this review will further contribute to an understanding of conservative treatment as an important choice for LIDH patients and provide useful information for the development of more effective conservative methods for LIDH treatment.

2. How to choose: Rest in bed or stay active for acute low back pain and sciatica?

For over a century, bed rest has been considered to be the best solution for many musculoskeletal disorders. Traditionally, for LIDH patients with acute low back pain and sciatica, bed rest is one of the most basic symptomatic treatments and the basis for carrying out nonsurgical therapies. At the onset or acute phase of LIDH, 1-2 weeks of bed rest will be recommended for patients with severe pain (16). It is reported that the pressure on intervertebral discs is related to the patients' position and varies with body positions with the highest in sitting position and the lowest in recumbent position (17). When resting in bed, the pressure on intervertebral disc is reduced, which will be a benefit for removing tension of surrounding soft tissues, and restoring the biomechanical balance of the intervertebral disc? Rest in bed is also a benefit for improving local blood circulation, and eliminating inflammation and edema of surrounding soft tissues, which is better for nutrient supplies of the intervertebral disc. Furthermore, rest in bed is a benefit for the repair of damaged fiber ring and avoiding stimulation on spinal marrow or nerve root caused by movement (18).

In the clinic, patients with acute LIDH are usually advised to rest in bed absolutely to promote the restoration of damage to the intervertebral disc and slow the progression of intervertebral disc herniation (19). This recommendation is based on orthopedic teaching; however, it may directly affect the number of days lost from work or other activities. Therefore, how many days of bed rest are suitable for LIDH patients with acute low back pain? A randomized clinical trial conducted by Deyo *et al.* showed that there is no benefit of bed rest for seven days compared with bed rest for two days in terms of disability reduction (20). The 1994 clinical guidelines recommend that bed rest should be for short periods of 2-4 days, and they still advise activity limitation (21). More recently, the routine of bed rest has been challenged because of a lack of any evidence supporting its effectiveness. Vroomen *et al.* found that among patients with symptoms and signs of a lumbosacral radicular syndrome, bed rest is not a more effective therapy than no treatment (watchful waiting) (22). A systematic review conducted by Waddell *et al.* showed that bed rest is not an effective treatment for acute low back pain but may delay recovery. Advice to stay active and to continue ordinary activities results in a faster return to work, less chronic disability, and fewer recurrent problems (23). Moreover, two systematic reviews conducted by Hagen *et al.* and Hilde *et al.* showed that for nonspecific low back pain, there is strong evidence that advice to stay active rather than rest in bed results in less time missed from work, improved functional status, and less pain, while for patients with sciatica, there is no difference

in outcomes between staying active and resting in bed (24,25). Nowadays, advice to promote physical activity and discourage bed rest in patients with acute lumbar pain is implemented in many primary care guidelines (26). Therefore, the answer on how to choose for acute low-back pain and sciatica (rest in bed or stay active) is clearly, the older obsolete viewpoint of bed rest for LIDH patients should be discarded.

3. Some common TCM therapies for LIDH

Recently, TCM therapies have become increasingly popular and are used regularly by patients with chronic neurological disorders mainly including low back pain and sciatica. According to a 2004 survey, 43% of peripheral neuropathy patients use TCM therapies to manage symptoms, and many patients cited unsatisfactory pain control with standard medical treatment as the reason for selecting TCM (27). Most commonly used TCM therapies included acupuncture, acupotomy, Chinese massage, and Chinese herbal medicine.

3.1. Acupuncture

Acupuncture was developed in ancient China with pictographs dating from the Shang Dynasty (1600-1100 BC), which suggests that it was already practiced at that time. It has been used to heal various diseases and physiologic malfunctions in clinical practice for more than 2500 years in China. Acupuncture is based on the concepts of TCM in which health is seen as the result of the balance of energy called *qi* in the body. When this energy is imbalanced, health is compromised and disease occurs. It is believed that *qi* flows through channels or meridians and acupuncture is used to correct this imbalance via insertion of needles that stimulate skin spots located along the meridians (28). In recent years, acupuncture has become increasingly used as a complementary therapy in the Western world. Due to its efficacy, acupuncture has been recommended by the World Health Organization since 1980 as an effective alternative therapy for 43 different disorders including musculoskeletal pain (29). In 2002, the National Health Interview Survey found that 4.1% of the respondents reported using acupuncture in their lifetime, that 8 million of Americans had used this therapy (2 million in the previous year), and back pain was the most common reason for its use (34%) (30). Recently published National Institute for Health and Clinical Excellence (NICE) guidelines highlight the need for a course of acupuncture of up to 10 sessions over 12 weeks for patients with low back pain (31). In accordance with clinical practice guidelines from the USA, acupuncture for low back pain was weakly recommended in 2007, but moderate-quality evidence was reported in 2017, which revealed that acupuncture,

Table 1. Forty-nine most common used acupoints for LIDH

Meridians	Number	Acupoints (International common name)
Bladder Meridian	10	Geshu (BL17), Xiaochangshu (BL27), Shenshu (BL23), Dachangshu (BL25), Pangguangshu (BL28), Guanyuanshu (BL26), Weizhong (BL40), Zhibian (BL54), Chengshan (BL57), Kunlun (BL60)
Gallbladder Meridian	2	Huantiao (GB30), Yanglingquan (GB34)
Governor Vessel	2	Yaoyangguan (DU3), Shuigou (DU26)
Stomach Meridian	2	Zusanli (ST36), Juliao (ST3)
Spleen Meridian	2	Sanyinjiao (SP6), Xuehai (SP10)
Others	31	<i>Huatuo Jiaji</i> points (EX-B2), <i>Ashi</i> points (pressure points)

as a type of TCM therapy, has shown respectable efficacy and is broadly accepted internationally (32).

As acupuncture is increasingly being recommended worldwide, especially for treating low back pain, several hypotheses have been proposed to explain the analgesic effect of acupuncture. According to TCM theory, acupuncture possesses the effect of stimulating the circulation of blood and causing the muscles and joints to relax by stimulating acupuncture points. Li *et al.* investigated a universal rule on selecting acupoints in the treatment of LIDH by acupuncture in the recent 10 years (33). They indicated that there were 49 most common used acupoints for LIDH treatment by retrieving 173 references and using a hierarchical clustering statistical method. As shown in Table 1, 18 of these acupoints were mainly distributed in Bladder Meridian, Gallbladder Meridian, Governor Vessel, Stomach Meridian, and Spleen Meridian, and the rest were extra points (*Huatuo Jiaji*, EX-B2) and *Ashi* points. Modern studies indicated that the underlying mechanisms of acupuncture might include modulation of GABAergic neurons in the ventral tegmental area (VTA) through opioid receptors and suppression of dopamine (DA) release in the nucleus accumbens (34). Acupuncture also had the effect of activating A- δ fibers, which are involved in release of endorphins and are associated with an increase in levels of 5-hydroxytryptophan in the brain (35).

According to numerous recent studies, it is without doubt that acupuncture is an effective and safe treatment for relieving symptoms (*e.g.*, low back pain and sciatica) and improving function of LIDH. Lee *et al.* conducted a randomized controlled pilot trial on acupuncture for low back pain due to spondylolisthesis (36). Their findings indicated that acupuncture would be helpful in patients with spondylolisthesis as a safe conservative treatment without severe adverse events as can happen in some surgical procedures. Moreover, several systematic review and meta-analysis articles were conducted to assess the effectiveness of acupuncture for treating sciatica. Qin *et al.* found that the use of acupuncture may be more effective than NSAIDs (*e.g.*, ibuprofen, meloxicam, and diclofenac) and may enhance the effect of drugs for patients with sciatica (37). In addition, the warm needling therapy

and electro-acupuncture therapy, which are derived from conventional acupuncture, have been widely used and exhibited a significant effect on treatment of LIDH. Taken together, acupuncture as an adjunct to conventional therapy provides short-term clinically relevant improvements in pain and functional measures for the treatment of LIDH.

3.2. Autonomy

Acupotomy is a new TCM therapy invented by Prof. Hanzhang Zhu (1949 - 2006) in 1976, who is a famous professor of Beijing University of Traditional Chinese Medicine (38). According to TCM meridian theory and modern surgical principles, Prof. Zhu combined an acupuncture needle with a modern surgical scalpel, created a bladed needle or acupotomy which had a thick flat-head and a cylindrical body used as the main treatment tool. As the tool is a combination of an acupuncture needle and surgical scalpel, it is also named a "small needle knife". Acupotomy is a closed lysis therapy, which converts open surgery to closed surgery, thus reducing risk, time, and cost. It can reach lesions deep inside the body and performs proper procedures like cutting and peeling. It can strip adhesions, release contractures, clear blockages, and is characterized by smaller wounds, fewer complications, higher safety, lower cost, and significant treatment efficiency (39). Because of these advantages, in clinical practice, acupotomy is widely applied to treat chronic soft tissue injury and bone hyperplasia including LIDH, cervical spondylosis, knee osteoarthritis (KOA) and so on.

In recent years, acupotomy has gradually become popular with LIDH patients and some related studies have shown the efficacy of acupotomy for the treatment of patients with low back pain and sciatica caused by LIDH. To evaluate the effectiveness and safety of acupotomy for treating LIDH, Mu *et al.* conducted a systematic review and meta-analysis which included 13 randomized controlled clinical trials (RCTs) and clinical control trials (CCTs) and 667 LIDH patients (40). They found that acupotomy exhibited more significant effects on relieving symptoms and improving symptoms than other therapies. Its mechanisms might involve recovering

the kinetic state of soft tissues from peeling adhesions, removing attached tissues, and reducing pressure on the nerves of LIDH patients (41). As chronic adhesion is resolved and contractures are released, tissues of lumbar intervertebral discs are free to move during activity with normal local function and pain resolved. Also, surrounding blood circulation of tissues of lumbar intervertebral discs can be improved.

Currently, acupotomy is not only widely used in China to treat LIDH, but also gradually popular with LIDH patients of other countries, especially Korea. Yuk *et al.* conducted a clinical study including 437 patients to evaluate the effect of acupotomy in patients with degenerative lumbar spine stenosis (42). They found that the verbal numeric rating scale (VNRS) and the Oswestry disability index (ODI) scores of patients were significantly decreased, which means that acupotomy as a treatment for spinal stenosis has a significant effect on pain relief and functional recovery. Kim *et al.* found that acupotomy possesses a potential effect on recovering the kinetic state of soft tissue, and on reducing low back pain and radiating pain of patients suffering from LIDH (41). All these results provide evidence that acupotomy is effective for relieving pain and improving quality of life in patients with LIDH.

Taken together, acupotomy as an adjunct to conventional therapy provides a better and safe effect for treating LIDH. However, currently there are still not enough high grade evidence recommendations for acupotomy. In future studies, larger sample sizes and longer prospective randomized clinical trials are needed, and comparisons between acupotomy and other LIDH therapies must be implemented.

3.3. Chinese massage

Chinese massage (referred to as Tuina in China, Chuna in Korea, and Shiatsu in Japan) is one of the most popular complementary and alternative therapies, which has been practiced in China for over 2000 years. It involves a wide range of technical manipulations conducted by a practitioner's finger, hand, elbow, knee, or foot applied to muscle or soft tissue at specific body locations (43). Moreover, sometimes Tuina is conducted according to the principles of acupuncture including the use of acupoints or along specific meridians. There are six main styles of physical Tuina therapy, including wobbling, pushing, vibrating, squeezing, knocking and articular moving, of which squeezing involves pressing, pinching, kneading, grasping and rubbing. Constant softness and penetration under consistent intensities, frequencies and manipulation durations are applied to all these styles of Tuina (44). Previously, Chinese massage was mainly associated with pain relief. At present, it is a well-respected treatment modality known to be helpful and safe for a wide range of conditions including arthritis, anxiety, sleep problems, pain

management and injury repair (45). For these reasons, Chinese massage is rapidly gaining international favor and is widely accepted as a complementary and alternative medicine therapy in the world.

Although Chinese massage is helpful for patients suffering from varied pathological states, it has been shown to be particularly effective for disorders of musculoskeletal origin including LIDH. Some related studies have shown the efficacy of Chinese massage for the treatment of patients with low back pain and sciatica caused by LIDH. Dr. Long conducted a study, which enrolled 82 LIDH patients who were definitely diagnosed by CT scanning and treated with Chinese massage (46). He found that of 82 cases, 54 cases (65.8%) were cured, 26 cases were improved, and 2 cases failed. Cherkin *et al.* conducted a review of the evidence for the effectiveness, safety, and cost of massage therapy for acute and chronic back pain through three RCTs (47). They indicated that Chinese massage was effective for persistent back pain and might reduce the costs of care after an initial course of therapy. Yang *et al.* conducted a single center, two-arm, open-label RCT, which was a comparative effectiveness study of Chinese massage and conventional analgesics (ibuprofen) for pain relief and function recovery in patients with chronic low back pain (43). The results showed that Chinese massage was more effective for relieving pain and improving function. Moreover, the pain relief effects of Tuina might be associated with elevated pain thresholds and reduced AUC of C-fiber-evoked field potentials of the ipsilateral and contralateral nerves (44).

Because of the increased use of Chinese massage in the world, its safety and quality has gradually been paid more and more attention. Yin *et al.* conducted a systematic review to evaluate the adverse events of massage therapy in pain-related conditions (48). They indicated that disc herniation, soft tissue trauma, neurologic compromise, spinal cord injury, and dissection of the vertebral arteries were the main complications of massage, and spinal manipulation in massage has repeatedly been associated with serious adverse events especially, but the incidence of such events is low. However, although such serious adverse events associated with massage in general are few, for the practitioners to minimize massage adverse events, not only adequate training in biomedical knowledge are needed but also safe practice guidelines are required.

Taken together, Chinese massage as an adjunct to conventional therapy provides better and safe effects for treating LIDH. However, currently there are still not enough high grade evidence recommendations for Chinese massage. In the future, well designed and methodologically rigorous studies will be needed for collection of valuable, high-quality data to evaluate the efficacy of Chinese massage, and will contribute to providing a solid foundation for the clinical treatment of LIDH.

3.4. Chinese herbal medicine

Chinese herbal medicine has been used in the treatment of various disorders including LIDH for thousands of years in China, Japan, and other Asian countries. In terms of TCM theory, LIDH is known as "*Bi-Zheng*" and is usually caused by blood stasis and *qi* stagnation, cold-dampness, or deficiencies in liver and kidney function (49). Therefore, some Chinese herbal medicines such as Duo-Huo (*Radix Angelicae Pubescentis*), Dang-Gui (*Radix Angelicae Sinensis*), Huang-Qi (*Radix Astragali*), Du-Zhong (*Cortex Eucommiae*), and Niu-Xi (*Radix Achyranthis Bidentatae*), with the following efficacy are commonly used to treat conditions like LIDH: promoting blood circulation and relieving pain, nourishing the liver and kidney, strengthening muscle and bone, promoting blood circulation and clearing collaterals, dispelling wind and dampness, and invigorating *qi* (50). Modern pharmacological studies have shown that these Chinese herbs are chosen due to their known analgesic, anti-inflammatory, antispasmodic, and carminative effects (51). Moreover, some Chinese herbal formulations, which contain the above single herbs such as Duhuoji sheng Tang and Buyanghuanwu Tang are also widely used in the treatment of LIDH. In recent years of clinical practice, several Chinese herbs and Chinese herbal formulations have been found to have potentially beneficial effects on relieving pain and improving function of LIDH patients. Therefore, the pharmacology of the Chinese herbal medicines most commonly used as an adjuvant treatment in LIDH therapy must be understood by some doctors and other health care providers.

Recently, the effectiveness of Chinese herbal medicine for treating LIDH has been reported widely. To evaluate the efficacy of Chinese herbal medicine for LIDH, a systematic review of randomized controlled trials was conducted by Luo *et al.* (49). They found that both Duhuoji sheng Tang and Buyanghuanwu Tang are the most commonly used Chinese herbal formulations for LIDH treatment. They also found herbs that promote blood circulation, such as Dang-Gui (*Radix Angelicae Sinensis*), Ru-Xiang (*Olibanum*), Mo-Yao (*Myrrh*), and Chuan-Xiong (*Rhizoma Chuanxiong*), have beneficial effects for LIDH treatment. In addition, Qi-tonifying herbs, such as Huang-Qi (*Radix Astragali*), Du-Zhong (*Cortex Eucommiae*), and Niu-Xi (*Radix Achyranthis Bidentatae*), which improve kidney function or strengthen bones, are also effective. A brief outline on the pharmacology of these most commonly used Chinese herbal formulations and herbs is presented below (Table 2 and Table 3).

Duhuoji sheng Tang, set up by Simiao Sun (a famous physician in the Tang Dynasty), is a famous traditional herbal formulation that has long been used to treat LIDH, osteoarthritis, osteoporosis, cervical spondylosis and so on (50). It contains 15 herbs including Duo-Huo (*Radix*

Angelicae Pubescentis), Dang-Gui (*Radix Angelicae Sinensis*), Chuan-Xiong (*Rhizoma Chuanxiong*), Du-Zhong (*Cortex Eucommiae*), Niu-Xi (*Radix Achyranthis Bidentatae*), and so on. Duhuoji sheng Tang has become the most frequently prescribed herbal formulation in Taiwan for treating diseases of the musculoskeletal system and connective tissues (52,53). Currently, much of the pharmacological research has shown that Duhuoji sheng Tang has potent anti-inflammation, immunomodulatory, analgesia, and inhibition of platelet aggregation properties (54). Using Duhuoji sheng Tang alone or combined with other therapies can effectively improve pain, leg-raising height and other clinical symptoms of patients with prolapse of lumbar intervertebral discs (50).

Buyanghuanwu Tang, set up by Qingren Wang (a famous physician in the Qing Dynasty), is a popular Traditional Chinese Medicine comprised of seven commonly used Chinese herbal drugs: Huang-Qi (*Radix Astragali*), Dang-Gui (*Radix Angelicae Sinensis*), Chi-Shao (*Radix Paeoniae Rubra*), Chuan-Xiong (*Rhizoma Chuanxiong*), Hong-Hua (*Flos Carthami*), Tao-Ren (*Semen Persicae*), and Di-Long (*Pheretima*) (55). There are nine main bioactive components, *i.e.*, astragaloside I, astragaloside II, astragaloside IV, formononetin, ononin, calycosin, calycosin-7-O- β -d-glucoside, ligustilide and paeoniflorin in Buyanghuanwu Tang extract (56). This traditional herbal formulation has been widely used in Chinese clinical practice for treatment and prevention of ischemic cardio-cerebral vascular diseases and stroke-induced disability for thousands of years. According to the traditional Chinese medical literature, it is used to enhance blood circulation and activate energy (*qi*) flow through energy meridians (57). Currently, much of the pharmacological research has shown that Buyanghuanwu Tang has potent effects on regulating inflammation, apoptosis, angiogenesis and blood coagulation, and neurogenesis and nervous system development (58). Therefore, Buyanghuanwu Tang is commonly used to treat LIDH accompanied with spinal cord injuries and other nervous lesions. Using Buyanghuanwu Tang alone or combined with other therapies can effectively improve sciatica and low back pain, and enhance the quality of life of LIDH patients. Dr. Kang reported that Buyanghuanwu Tang exhibited significant effects in improving syndromes of patients with lumbar vertebral canal stenosis (59). Moreover, Buyanghuanwu Tang could effectively promote recovery of low limb numbness in patients with lumbar surgery (60).

Angelicae Sinensis Radix (Dang-Gui in Chinese, Dong Quai in English, Toki in Japanese, or Tanggwi in Korea), is the root of *Angelica sinensis* (Oliv.) Diel and has been used for thousands of years worldwide. Since, it has both properties of nourishing blood and promoting blood circulation and removing blood stasis, it is usually used for tonifying, replenishing, and invigorating blood as well as relieving pain,

Table 2. Traditional herbal formulations commonly used in LIDH treatment

Names	Composition	Biological activity	Evidence on treating LIDH	Ref.
Duhuojisheng Tang	Includes 15 herbs: Duo-Huo (<i>Radix Angelicae Pubescentis</i>), Dang-Gui (<i>Radix Angelicae Sinensis</i>), Chuan-Xiong (<i>Rhizoma Chuanxiong</i>), Du-Zhong (<i>Cortex Eucommiae</i>), Niu-Xi (<i>Radix Achyranthis Bidentatae</i>), Xi-Xin (<i>Asarum</i>), Qin-Jiao (<i>Radix Gentianae Macrophyllae</i>), Fu-Ling (<i>Poria cocos</i> Wolf), Rou-Gui (<i>Cassia Bark</i>), Fang-Feng (<i>Radix Saposhnikoviae</i>), Ren-Sen (<i>Panax ginseng</i>), Gan-Cao (<i>Glycyrrhiza uralensis</i>), Di-Huang (<i>Rehmannia glutinosa</i>), Sang-Ji-Sheng (<i>Chinese Taxillus Twig</i>), and Bai-Shao (<i>Radix Paeoniae Alba</i>)	Anti-inflammation, immunomodulatory, analgesia, and inhibition of platelet aggregation	Clinical: Effectively improving pain, leg-raising height and other clinical symptoms of patients with prolapse of lumbar intervertebral disc	50-54
Buyanghuanwu Tang	Includes 7 herbs: Huang-Qi (<i>Radix Astragali</i>), Dang-Gui (<i>Radix Angelicae Sinensis</i>), Chi-Shao (<i>Radix Paeoniae Rubra</i>), Chuan-Xiong (<i>Rhizoma Chuanxiong</i>), Hong-Hua (<i>Flos Carthami</i>), Tao-Ren (<i>Semen Persicae</i>), and Di-Long (<i>Pheretima</i>)	Regulating inflammation, apoptosis, angiogenesis and blood coagulation, and neurogenesis and nervous system development	Clinical: Effectively improving syndromes of patients with lumbar vertebral canal stenosis; Effectively promote recovery of low limbs numbness in patients with lumbar surgery	55-60

Table 3. Single herbs commonly used in LIDH treatment

Common name	Other names	Latin name	Major active ingredients	Biological activity	Evidence on treating LIDH	Ref.
Angelicae Sinensis Radix	Dang-Gui, Dong Quai, Toki, or Tanggwi	<i>Angelica sinensis</i> (Oliv.) Diel	Polysaccharides, ligustilide and ferulic	Anti-inflammation, anti-fibrosis, antispasmodic activity, anti-oxidation, and neuro-protection, as well as cardio- and cerebrovascular protective functions	Clinical: Effectively improve pain and enhance life quality of LIDH patients	14,50, 59-62
Radix Astragali	Huang-Qi, or Milk vetch	<i>Astragalus membranaceus</i> Bge. Var. <i>mongholicus</i>	Isoflavonoids, triterpenoid saponins, polysaccharides, amino butyric acids and various trace elements	Antioxidant, antitumour, hepatoprotective, anti-diabetic, antimicrobial, antiviral and immune enhancement activities	Clinical: Effectively improve the syndromes of low limbs numbness in patients with lumbar vertebral canal stenosis	14,50, 59,60, 63,64
Cortex Eucommiae	Du-Zhong	<i>Eucommia ulmoides</i> Oliv.	Lignans, iridoids, flavonoids, polysaccharides, terpenes, and proteins	Blood pressure reduction, blood lipid regulation, cardiovascular protection, anti-obesity, anti-inflammation, anti-virus, enhancement of immunologic function, resistance against senility and anti-fatigue	Preclinical: Promoting the proliferation of osteoblasts and improving bone mineral density of rats Clinical: Effectively improving low back pain and lumbar vertebral canal stenosis of LIDH patients	

lubricating the intestines, and treating female irregular menstruation and amenorrhea (14). Over 70 compounds have been identified from Dang-Gui, including polysaccharides, ligustilide and ferulic. Scientific reports on crude extracts and pure compounds and formulations of Dang-Gui revealed a wide range of pharmacological activities, including anti-inflammation, anti-fibrosis, antispasmodic activity, anti-oxidation, and neuro-protection, as well as cardio- and cerebrovascular protective functions (61). Some reports indicated that crude extracts or formulations of Dang-Gui could effectively improve pain and enhance life quality of LIDH patients (50,59,60,62). Dr. Yu indicated that Dang-Gui injection exhibited significant effects for treating the third lumbar transverse process syndrome with effectively improving pain and enhancing the

quality of life (62).

Radix Astragali (Huang-Qi in Chinese), isolated from the dried root of *Astragalus membranaceus* Bge. Var. *mongholicus*, is one of the most famous and frequently used herbal medicines and healthy food supplements used as a tonic. It has been used for over 2000 years in TCM prescriptions for the treatment of animal bites and poisons, wounds and burns, nephritis, diabetes, albuminuria, hypertension, cirrhosis, and various cancers (14,63). Chemical constituent investigations indicated that it contains several bioactive constituents including, isoflavonoids, triterpenoid saponins, polysaccharides, amino butyric acids and various trace elements (14). Modern pharmacological studies have shown that Huang-Qi and its active constituents possess antioxidant, antitumour,

hepatoprotective, anti-diabetic, antimicrobial, antiviral and immune enhancement activities (63). Recently, some reports have indicated that crude extracts or formulations of Huang-Qi could effectively improve pain and enhance life quality of LIDH patients (50,59,60,64). Jiang *et al.* found that large doses of Huang-Qi could effectively improve the syndromes of low limb numbness in patients with lumbar vertebral canal stenosis (64).

Cortex Eucommiae (Du-Zhong in Chinese), the bark of *Eucommia ulmoides* Oliv., has been traditionally used to treat many diseases in China in the form of tonics, analgesics, and sedatives for more than 2000 years (55). The natural products identified from Du-Zhong include lignans, iridoids, flavonoids, polysaccharides, terpenes, and proteins. Modern pharmacological studies have showed that Du-Zhong has some effects like blood pressure reduction, blood lipid regulation, cardiovascular protection, anti-obesity, anti-inflammation, anti-virus, enhancement of immunologic function, resistance against senility and anti-fatigue (65). In the clinic, it is mainly used to treat hypertension, lumbar diseases, and obstetrical and gynecological disease. Recently, some reports have indicated that crude extracts or formulations of Du-Zhong could effectively improve pain and enhance quality of life of LIDH patients (50,59,60). Du-Zhong exhibited significant effects for promoting the proliferation of osteoblasts and improving bone mineral density (66). Furthermore, some Chinese herbal formulations of Du-Zhong exhibited significant effects on treating LIDH. Fu *et al.* reported that a Chinese herbal formulation of Du-Zhong (Du-Zhong-Qiang-Yao-Tang) could more effectively improve low back pain and lumbar vertebral canal stenosis of LIDH patients compared to Mecobalamine (67).

Taken together, as Chinese herbal medicine has become popular in the world, more and more LIDH patients seek it as an alternative therapy. However, from the current clinical studies, Chinese herbal medicine is not the preferred therapy in the treatment of LIDH. Chinese herbal medicine is usually combined with other therapies for treating LIDH. In addition, although these Chinese herbal formulations and herbs are commonly prescribed by traditional Chinese physicians for LIDH treatment in the clinic, there are few clinical studies published currently in English. Thus, more rigorous trials are needed to confirm the efficacy of these Chinese herbal medicines for LIDH therapy in the future.

4. Pharmacotherapy

Pharmacotherapy, as one of most important conservative treatments, is widely used to improve symptoms of LIDH patients, of which drugs with anti-inflammatory and neurotrophic effects are commonly used. A randomized clinical trial enrolling

patients from 13 multidisciplinary spine clinics in 11 US states was conducted by Weinstein *et al.* (6). They indicated that when LIDH patients chose non-operative treatments, about 61% patients received anti-inflammatory medications (NSAIDs, cyclooxygenase 2 inhibitors, or oral steroids), 46% received opiates, and more than 50% received injections (*e.g.*, epidural steroids). As pharmacotherapy appears so important in LIDH treatment, the efficacy and side effects of these commonly used therapies especially oral NSAIDs and epidural steroids injections must be understood by some doctors and other health care providers.

NSAIDs are the most widely used over-the-counter drugs as well as the most prescribed class of drugs for a variety of conditions including pains, rheumatoid arthritis, osteoarthritis, musculoskeletal disorders, and other comorbid conditions (68). Millions of people suffer from pain resulting in the prolonged use of NSAIDs being common. Diclofenac, Ibuprofen, and Meloxicam are popular NSAIDs widely used in the clinic. They are reported to exhibit a significant effect on improving acute low back pain and sciatica caused by LIDH. Valat *et al.* reported Meloxicam and Diclofenac were equivalent in relieving the acute pain associated with osteoarthritis of the lumbar spine, however, Meloxicam was much better tolerated (69). Toroudi *et al.* found that Ibuprofen showed an analgesic effect to alleviate post-discectomy surgery pain in patients with LIDH (70). However, a systematic review conducted by Vroomen *et al.* indicated that compared with placebo, NSAIDs might be no more effective at improving global pain at 5 to 30 days in people with sciatic pain caused by LIDH (low-quality evidence) (71). In addition, NSAIDs also have undesirable side effects including ulcers, bleeding, kidney failure, and increased risk of heart attack and stroke (72). Therefore, although NSAIDs might be more effective in the acute stage of LIDH, it should be cautious for patients welfare to choose NSAIDs in the treatment of pain especially sciatic pain caused by LIDH.

Epidural steroid injections as a nonoperative management are commonly utilized to treat radicular pain due to LIDH especially in western countries (71). It may modulate the inflammatory cells, cytokines, or other pain mediators associated with lumbar disc herniation-related pain (73). Compared with no epidural steroids, epidural steroid injections may be more effective at improving limb pain and increasing patients' satisfaction at 2 weeks, but may be no more effective after more than 2 weeks in people with disc herniation. Moreover, it may be no more effective in the longer term at improving disability, as measured by the Roland Morris Disability Questionnaire and ODI scores, or functional outcomes such as straight leg raising and lumbar flexion (3). However, there is considerable controversy about the clinical efficacy of epidural steroid injections in the management of LIDH.

Radcliff *et al.* reported that patients with LIDH treated with epidural steroid injections had no improvement in short or long-term outcomes compared with patients who were not treated with epidural steroid injections (73). Given these data, we concluded that more studies are necessary to establish the value of epidural steroid injections for symptomatic LIDH.

5. Physical therapy

Physical therapy is widely used to treat patients with musculoskeletal disorders including LIDH. There are a vast variety of techniques that are commonly used by physical therapists in the treatment of low back pain and sciatica caused by LIDH. Some of the therapies include, but are certainly not limited to, education, exercise, lumbar traction, manual manipulation, application of heat, cryotherapy, ultrasound, laser, and transcutaneous electrical nerve stimulation (TENS) (74). For LIDH patients, it is a most common conservative treatment received during the first six weeks. According to a Spine Patient Outcomes Research Trial (SPORT) conducted by Weinstein *et al.* at 13 sites across the United States, about forty-four percent of LIDH patients received active physical therapy during the trial (6). Fritz *et al.* indicated that many patients with lumbar spinal stenosis pursuing conservative management receiving physical therapy, using physical therapy was associated with reduced likelihood of patients receiving surgery within 1 year (75). Studies of physical therapy for acute low back pain and sciatica caused by LIDH are heterogeneous because the intervention method differs. Therefore, it is difficult to assess one method of physical treatment, which would seem definitively to be superior to another. Individualized education during physical therapy, particularly when LIDH patients are focused on fear avoidance and staying active, appears to be helpful (76). Although traction is widely used by physiotherapists for treating LIDH, there is strong evidence that traction, either alone or in combination with other treatments, has little or no impact on pain intensity, functional status, global improvement and return to work among people with low back pain and sciatica (77). Various types of exercises have been used in the management of low back pain. For example, William's flexion exercise, and McKenzie extension exercise are commonly used in treatment of low back pain. Mulligan Sustained Natural Apophyseal Glides (SNAGs) as one of most important manual therapy treatments is also widely used by physiotherapists to treat this condition. A randomized control trial was conducted by Waqqar *et al.* to determine the effects of McKenzie extension exercise versus Mulligan SNAGs for chronic mechanical low back pain (78). They indicated that McKenzie extension exercises program is clinically slightly more effective in the management of pain and disability as compared with Mulligan

SNAGs, while Mulligan SNAGs are more effective in the improvement of lumbar ROM as compared with Mechanize EEP in the management of CMLBP. However, there is still little evidence from randomized controlled trials to support their use. In the future, more studies are necessary to establish the value of physical therapy for symptomatic LIDH.

6. Keeping active lifestyle for patients with LIDH in remission stage

According to the progress of LIDH, it is usually divided into three stages, namely acute stage, recovery stage and remission stage (79). In general, during the acute stage and recovery stage of LIDH, the main principle is improving blood circulation and relieving nerve root edema and inflammatory reaction, while in the remission stage, LIDH patients are advised to insist on an active lifestyle and functional training in their daily life and work to keep the stability of spinal biodynamics as far as possible to reduce recurrence of LIDH. Therefore, the above methods including TCM therapies, pharmacotherapy, and physical therapies are commonly used treatments in the acute stage and recovery stage of LIDH, and it is critical to keep an active lifestyle in the remission stage of LIDH. Moreover, LIDH is a multifaceted progressive irreversible condition and an inevitable part of aging with complex etiology. Although genetic influences are more dominant, the occupational and mechanical influences still persist as a major risk factor (80). Therefore, it should be better for LIDH patients to keep an active lifestyle in their daily life and work. Otherwise, some symptoms of LIDH like low back pain and sciatica will be triggered and aggravated. To reduce the incidence and recurrence rate of LIDH, the following notes should be advised repeatedly by doctors: (i) Don't bend over for a long time. (ii) Don't sit for a long time. (iii) Please pay attention to a warm waist. (iv) Please sleep on a hard bed. (v) Please eat more foods with higher calcium content such as milk, bean curd, sesame paste, earthnut, kelp, laver, and dried small shrimp.

7. Conclusion

LIDH is a common spinal disorder that usually favorably responds to conservative treatment. Here we give a narrative review on non-operative treatments such as TCM therapies including acupuncture, autonomy, Chinese massage, and Chinese herbal medicines, pharmacotherapy including NSAIDs and epidural steroid injections, physical therapy and keeping an active lifestyle, which are commonly used as nonsurgical management in clinics for patients with LIDH. Because all of these therapies possess their own advantages and disadvantages, we cannot make any conclusion that one method of non-operative treatment would seem

definitively to be superior to another, and little evidence is available to define optimal nonsurgical management. Nevertheless, these nonsurgical managements are widely used alone or in combination especially TCM therapies. Due to the convenient, safe, effective and less expensive characteristics of these TCM therapies, it should be a benefit for patients and society. However, currently there are still not enough high grade evidence recommendations for TCM therapies. In the future, well designed and methodologically rigorous studies will be needed for collection of valuable, high-quality data to evaluate the efficacy of non-operative treatments especially TCM therapies, and so will contribute to provide a solid foundation for the clinical treatment of LIDH.

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Prevalence of metabolically obese but normal weight (MONW) and metabolically healthy but obese (MHO) in Chinese Beijing urban subjects

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Summary

The aim of this study was to assess the prevalence of metabolic syndrome (MetS) in non-obese adults (body mass index (BMI) < 25 kg/m²) and the prevalence of obese adults (body mass index (BMI) ≥ 25 kg/m²) without MetS in Chinese Beijing urban subjects. A cross-sectional study was conducted and the subjects who came to the hospital to receive a health examination were enrolled randomly. Regardless of age stratification, men have a higher prevalence of MetS than women. Among the urban Beijing population, prevalence of metabolically obese but normal weight (MONW) is lower than metabolically healthy but obese (MHO) regardless of gender. Except for the underweight group, participants exhibit significant differences between MetS and non-MetS subgroups in all tested variables in normal weight and overweight groups, whereas MONW and MHO participants exhibit significant differences in all variables except for creatinine (CR), aspartate aminotransferase (AST), uric acid (UAC) and high-density lipoprotein cholesterol (HDL-C). Women tend to have a higher MONW prevalence but lower MHO prevalence than men. Accordingly, MetS happens more frequently among those 40-59 yr. Besides, sex, age, WC, SBP, DBP, ALT, FG, UAC, TG, HDL-C and LDL-C are risk factors for MetS after multivariate adjustment. In conclusion, the prevalence of MONW is lower than MHO regardless of gender. Women tend to have a higher MONW prevalence but lower MHO prevalence than men.

Keywords: Metabolic syndrome, metabolically obese but normal weight (MONW), metabolically healthy but obese (MHO), prevalence

1. Introduction

Metabolic syndrome (MetS), also named as syndrome X or the insulin resistance syndrome, has existed as a public health issue for almost eight decades (1). Recently, with the change in dietary structure and the pervasiveness of diabetes and obesity, more people have been identified with MetS. In the United States, around 35% of adults and 50% of the elderly people (more than

60 years old) were estimated to have MetS during 2003 and 2012 (2). In China, the condition is similar to the United States and many other countries and areas (3-5). A series of studies have shown a relatively higher prevalence of MetS in urban areas than in rural areas nationwide. Until 2014, the MetS prevalence was much higher than that of decades ago (6).

The explicit definition of MetS was raised two decades ago by different organizations such as World Health Organization (WHO), the European Group for the Study of Insulin Resistance (EGSIR) *etc.* Although different definitions agree on the major essential categorical components like glucose intolerance and insulin resistance, small conflicts still exist (7). For example, both the definitions from WHO and EGSIR considered obesity or central obesity, while the American Association of Endocrinology suggests that obesity or

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central obesity should not be included in the identification process of MetS, because people with normal weight may also be insulin resistant. In 2004, experts from International Diabetes Federation (IDF) pointed out that the criteria of obesity used for MetS should be different in Asian areas compared to the western world since different areas may have diverse overweight incidences (8). This advice suggests that researchers should take geographical and racial differences into consideration when a MetS condition needs to be identified.

Metabolically obese but normal weight (MONW) and metabolically healthy but obese (MHO) are common and reveal different treatment effects in the clinic, notable benefits can be gained through a more comprehensive understanding of the prevalence of this subgroup population. MONW refers to the people who have normal body mass index but are related to increased levels of triglycerides, high blood pressure and the other characteristics of MetS (9). Among the population 20-40 years old, MONW may happen at the probability of 10-18% with a high incidence of cardiovascular diseases (10). To the contrary, individuals with MHO may reveal an unhealthy appearance, but they are usually metabolically healthy and have lower risk of cardiovascular diseases. Studies about the prevalence of MONW and MHO have been performed in western populations (2); however, research on Asian populations are limited. We herein collected a large amount of relevant data of the Beijing urban population to comprehensively acknowledge local prevalence of MONW and MHO.

2. Materials and Methods

2.1. Study population

A total of 22,376 subjects (13,748 men, 8,628 women, age from 18 to 85) were enrolled in this study from the health examination center of Beijing Hospital during January 2010 and December 2010. The enrolled subjects were randomly selected from all the individuals who came to the center for health examinations. Analyses of risk factors closely correlated with MetS was restricted to individuals who had a complete physical and biochemical measurements ($n = 5,556$). We attempted to study the detailed biochemical characteristics within the MetS groups so we only included MetS patients with complete physical and biochemical measurements. The reason for unfinished ones is that they did not have the relevant physical and biochemical measurements examinations. All participants signed informed consent, and the protocol was approved by China Health Statistics Center.

2.2. Physical examination and biochemical analysis

Physical examination and laboratory tests were

performed as previously described (11,12). Their blood pressure (BP) was measured three times and the average was used for analysis. BP was measured when the subject was in a seated position using a manual mercury sphygmomanometer. Weight and height of every individual were measured three times respectively during the physical examination. Weight was measured to the minimum 100 g with light clothing and without shoes, and height was measured to the minimum 1 mm without shoes. Waist circumference (WC) was measured to the minimum 1 cm at the navel level, and calculated as the average of one measurement after inspiration and one after expiration.

Overnight fasting blood samples were collected and analyzed using a Hitachi Modular DPE system (Roche Diagnostics, Penzberg, Germany). The plasma glucose level was measured using the hexokinase enzymatic method. Concentrations of biochemical molecules were analyzed using an auto-analyzer (Model 747-200, Roche-Hitachi).

2.3. Definition of MetS, MONW, MHO, MHNW and MOO

The criteria we used to diagnose MetS were modified from those of the International Diabetes Federation (IDF) and the World Health organization-Asia Pacific region guideline, as having three or more of (1) WC ≥ 90 cm for male and ≥ 80 cm for female; (2) triglycerides (TG) concentration ≥ 150 mg/dL; (3) high-density lipoprotein cholesterol (HDL-C) concentration < 40 mg/dL for male and < 50 mg/dL for female or taking anti-hyperlipidemic medications; (4) BP $\geq 130/85$ mmHg or taking antihypertensive medications; (5) fasting plasma glucose >100 mg/dL or taking anti-diabetic medications, insulin or oral agents.

BMI was calculated as the ratio of weight in kilograms and the square of height in meters. According to WHO definitions, overweight was defined as BMI ≥ 25 kg/m². According to the relation between metabolically obesity and weight, participants of this study were divided into four categories: metabolically obese but normal weight (MONW), metabolically healthy and normal weight (MHNW), metabolically healthy but obese (MHO) and metabolically obese and obese (MOO).

2.4. Statistical analysis

This study was designed to provide relatively precise estimates of the urban Beijing population of the prevalence of MetS on gender by different age and BMI groups. Prevalence estimates were calculated for the overall urban Beijing population by 3 age groups or 3 BMI groups. Besides, the prevalence estimates of MONW and MHO were calculated for men and women. Prevalence estimates between groups were compared

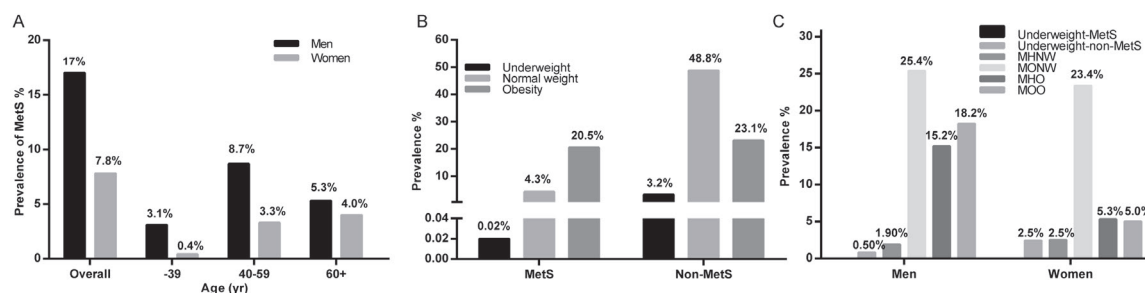


Figure 1. The prevalence of metabolic syndrome (MetS). A. The prevalence of MetS on gender by different age groups. Overall, Men had a much higher MetS prevalence than women did. Men had their highest MetS prevalence in their 40s and 50s but had their lowest MetS prevalence under 39. Women had the highest MetS prevalence in the > 60 group but the lowest MetS prevalence under 39. B. The prevalence of MetS on BMI. Overall, most of the population was normal weight with no MetS, and normal weight with MetS (MONW) had higher prevalence than obesity with non-MetS (MHO) did. C. The prevalence of MetS on BMI and gender. Underweight people did not show MetS. Among the men and women, MONW demonstrated a higher prevalence than MHO.

Table 1. Prevalence statistics of metabolic syndrome

Age (yr)	Men			Women		
	Total	MetS	Prevalence rate (%)	Total	MetS	Prevalence rate (%)
≤ 39	4267	685	16.0 (3.1)	2888	99	3.4 (0.4)
40-59	5871	1951	33.2 (8.7)	3829	738	19.3 (3.3)
≥ 60	3609	1177	32.6 (5.3)	1912	906	47.4 (4.0)
Total	13747	3813	27.8 (17.0)	8629	1743	20.2 (7.8)

MetS: metabolic syndrome.

using χ^2 test. Statistical significance was met with a two-tailed $P < 0.05$. Statistical analyses were done using SPSS 20.0 (IBM, Armonk, NY, USA). Categorical variables were presented as numbers and percentages. Continuous variables were presented as mean $\pm$ standard deviation (SD). After checking for the normality, continuous variables were analyzed using the Student's t -test. The differences between subgroups were analyzed using Student's t -test. The risk factor analysis was done using a univariate and multivariate logistic regression method.

3. Results

3.1. The prevalence of MetS among the participants

Approximately 24.8% of the participants have MetS (27.8% of the males and 20.2% of the females have MetS). Overall, men had a much higher MetS prevalence than women did.

In males, the prevalence of MetS was 3.1% among the ≤ 39 yrs, 8.7% among the 40-59 yrs and 5.3% among the ≥ 60 yrs. Men had their highest MetS prevalence in their 40s and 50s but had their lowest MetS prevalence under 39 (Figure 1A, Table 1).

In females, the prevalence of MetS was 0.4% among the ≤ 39 yrs, 3.3% among the 40-59 yrs and 4.0% among the ≥ 60 yrs (Figure 1A, Table 1). Women had the highest MetS prevalence in the > 60 group but the lowest MetS prevalence under 39 (Figure 1A).

3.2. Baseline characteristics of the participants

The baseline characteristics of the study populations are presented in Table 2. The enrolled 22376 subjects consisted of 13,748 males (61.4%) and 8,628 females (38.5%). 3.3% of the participants were underweight, 53.1% were normal weight and 43.6% were obese. Data for the 5556 MetS with complete physical and biochemical measurements are presented in Table S1 (<http://www.biosciencetrends.com/action/getSupplementalData.php?ID=11>). The prevalence of non-MetS is 160-, 11.35- and 1.13-fold of that of MetS in underweight, normal weight and the obesity groups, respectively, suggesting that the obese participants have a higher risk of getting MetS. In addition, the distal blood pressure (DBP), TG, uric acid (UAC) and alanine aminotransferase (ALT) differed significantly between the non-MetS subgroup and the MetS subgroup within the underweight group. However, in the normal weight group, all variables except for gender and UAC differed substantially between the non-MetS and the MetS subgroups. Besides, in the overweight group, despite of gender, age, height, weight/height (W/H) ratio and creatinine (CR), all variables exhibited significant differences between the non-MetS subgroup and the MetS subgroup. Furthermore, when comparing the MONW and MHO subgroups, gender, age, HDL-C, UAC, CR and aspartate aminotransferase (AST) did not show a difference but the rest of the variables did, indicating that 'the rest of the variables' could possibly

Table 2. Baseline characteristics of the subjects on BMI and MetS

Variables	Total	Underweight (<i>n</i> = 731, 3.3%)		Normal weight (<i>n</i> = 11884, 53.1%)		Obesity (<i>n</i> = 9761, 43.6%)		<i>P</i> [#]
		Non-MetS	MetS	<i>P</i>	Non-MetS (MHNW)	MetS (MONW)	<i>P</i>	
N (%)	22376 (100%)	727 (3.2%)	4 (0.02%)		10916 (48.8%)	968 (4.3%)		
Men (N, %)	13748 (61.4%)	184 (99.5%)	1 (0.5%)		5684 (93.2%)	415 (6.8%)		
Women (N, %)	8628 (38.6%)	543 (99.4%)	3 (0.6%)		5232 (90.4%)	553 (9.6%)		
Age (years)	50.6 ± 14.6	41.6 ± 18.5	64.0 ± 18.8		46.6 ± 15.8	61.3 ± 14.0		**
BMI (kg/m ²)	24.5 ± 3.5	17.5 ± 0.9	17.6 ± 1.0		22.2 ± 1.7	23.6 ± 1.3		**
Height (cm)	166.8 ± 8.0	164.3 ± 7.1	159.6 ± 5.4		166.0 ± 7.7	164.1 ± 9.2		**
WC (cm)	84.7 ± 10.3	67.9 ± 5.9	86.0 ± 6.3		78.6 ± 7.1	87.9 ± 5.4		**
W/H ratio	0.5 ± 0.1	0.4 ± 0.03	0.5 ± 0.03		0.5 ± 0.04	0.5 ± 0.03		**
SBP (mmHg)	122.8 ± 19.0	111.5 ± 16.6	132.8 ± 18.3		117.9 ± 16.0	134.0 ± 19.0		**
DBP (mmHg)	77.0 ± 9.0	70.9 ± 7.3	75.3 ± 13.1	*	74.5 ± 8.1	78.5 ± 8.8		**
FG (mmol/L)	5.4 ± 1.2	4.9 ± 0.6	5.4 ± 0.6		5.0 ± 1.0	6.0 ± 1.7		**
TC (mmol/L)	5.3 ± 1.0	5.0 ± 1.0	5.7 ± 1.3		5.2 ± 1.0	5.6 ± 1.1		**
TG (mmol/L)	1.7 ± 1.4	0.9 ± 0.5	2.6 ± 1.3	**	1.3 ± 0.9	2.2 ± 1.4		**
HDL-C (mmol/L)	1.4 ± 0.3	1.7 ± 0.3	1.3 ± 0.3		1.5 ± 0.3	1.2 ± 0.3		**
LDL-C (mmol/L)	2.9 ± 0.7	2.4 ± 0.6	3.1 ± 0.6		2.7 ± 0.7	3.1 ± 0.8		*
UAC(umol/L)	307.7 ± 88.1	237.1 ± 64.3	356.3 ± 133.2	*	281.7 ± 82.1	312.8 ± 84.2		**
ALT (U/L)	23.9 ± 18.2	14.7 ± 7.9	18.3 ± 16.6	*	19.7 ± 14.4	23.4 ± 15.3		**
AST(U/L)	24.2 ± 9.8	21.5 ± 5.4	23.0 ± 3.4		22.8 ± 10.2	25.0 ± 8.9		*
SUN (mmol/L)	4.9 ± 1.3	4.4 ± 1.2	4.1 ± 1.2		4.8 ± 1.3	5.2 ± 1.5		*
CR (umol/L)	72.3 ± 15.2	63.3 ± 13.0	60.5 ± 9.0		70.0 ± 14.8	70.0 ± 17.5		*

⁰ MetS: metabolic syndrome; non-MetS, non-metabolic syndrome.

¹ Data are mean ± SD or number (percentage, %).

² Underweight < 18.5 kg/m²; Normal weight: 18.5 ≤ BMI < 25 kg/m²; Obesity: BMI ≥ 25 kg/m².

³ BMI, body mass index; WC, waist circumference; W/H, weight/ height ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FG, fasting glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; UAC, uric acid; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; SUN, serum urea nitrogen; CR, creatinine.

⁴ *P*: difference of each risk factor between the Non-MetS and MetS subgroups.

⁵ *P*[#]: difference of each risk factor between the MONW and MHO subgroups.

^{6a} *P* < 0.05 and ^{6b} *P* < 0.01.

Table 3. Baseline characteristics of the subjects by gender, and BMI among the normal-weight

Variables	MHNW		MONW		P^1	P^2	P^3	P^4
	Men	Women	Men	Women				
N (%)	5684 (25.4%)	5232 (23.4%)	415 (1.90%)	553 (2.5%)				
BMI (kg/m ²)	22.72 ± 1.6	21.66 ± 1.7	23.87 ± 1.1	23.33 ± 1.4	**	**	**	**
Height (cm)	170.85 ± 6.1	160.60 ± 5.4	172.01 ± 6.3	158.18 ± 56.0		**	**	
WC (cm)	82.58 ± 5.5	74.23 ± 6.0	92.671 ± 2.7	84.31 ± 4.0	**	**	**	**
W/H ratio	0.48 ± 0.03	0.46 ± 0.04	0.54 ± 0.02	0.53 ± 0.03	**	**	**	**
SBP (mmHg)	121.79 ± 16.0	113.73 ± 15.0	134.47 ± 19.0	133.60 ± 19.0	**	**	**	
DBP (mmHg)	76.28 ± 8.1	72.57 ± 7.7	79.82 ± 9.3	77.50 ± 8.2	**		*	*
FG (mmol/L)	5.33 ± 1.2	5.02 ± 0.6	6.11 ± 1.6	5.87 ± 1.7	**	**	**	
TC (mmol/L)	5.16 ± 1.0	5.17 ± 1.0	5.40 ± 1.1	5.72 ± 1.1		**	**	*
TG (mmol/L)	1.48 ± 1.1	1.13 ± 0.6	2.31 ± 1.7	2.14 ± 1.0	**	**	**	**
HDL-C (mmol/L)	1.34 ± 0.3	1.58 ± 0.3	1.16 ± 0.3	1.28 ± 0.3	*	**	**	
LDL-C (mmol/L)	2.80 ± 0.7	2.63 ± 0.7	2.98 ± 0.7	3.12 ± 0.8		*		
UAC(umol/l)	329.48 ± 72.1	229.75 ± 56.9	355.36 ± 80.6	280.79 ± 72.0	*	**	**	*
ALT (U/L)	22.78 ± 16.3	16.33 ± 11.1	26.63 ± 16.4	20.98 ± 14.0		**	**	*
AST(U/L)	23.81 ± 12.3	21.60 ± 6.9	25.59 ± 9.4	24.57 ± 8.6		**	**	
SUN (mmol/L)	5.17 ± 1.3	4.33 ± 1.1	5.46 ± 1.4	4.92 ± 1.5	*	**	**	
CR (umol/L)	79.96 ± 12.0	59.05 ± 8.6	80.56 ± 15.4	62.12 ± 14.6	**	**	**	*

⁰ MHNW: metabolically healthy and normal weight; MONW: metabolically obese but normal weight.

¹ Normal weight: 18.5 ≤ BMI < 25 kg/m².

² BMI, body mass index; WC, waist circumference; W/H; weight/ height ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FG, fasting glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; UAC, uric acid; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; SUN, serum urea nitrogen; CR, creatinine.

³ P^1 represents the difference of each risk factor for men between MHNW and MONW subgroups.

⁴ P^2 represents the difference of each risk factor for women between MHNW and MONW subgroups.

⁵ P^3 represents the difference of each risk factor between men and women in MHNW subgroup.

⁶ P^4 represents the difference of each risk factor between men and women in MONW subgroup.

⁷ * P < 0.05 and ** P < 0.01.

be the risk factors for the imbalance of metabolism and body weight. Overall, the prevalence of non-MetS is higher than MetS (Figure 1B), and women tend to have lower prevalence of MHNW, MONW, MHO and MOO (Figure 1C). Biochemical factors together with BMI index could be significant indicators of MetS.

3.3. Characteristics by gender and BMI among the normal-weight subjects

BMI, WC, W/H, SBP, DBP, FG, TG, HDL-C, UAC, SUN and CR are significantly different for men in the MHNW group from those in the MONW group. Whereas, among women, all variables except for DBP differ in the MHNW group and MONW group. In the MHNW group, men and women have significantly different levels of all variables except for LDL-C. On the other hand, men and women have significantly different levels of BMI, WC, W/H, DBP, TC, TG, UAC, ALT and CR in the MONW group (Table 3). It is worth mentioning that MONW participants showed higher mean values for all physical examination and laboratory test variables except for HDL-C compared to MHNW participants (MONW participants have lower HDL-C levels than MHNW counterparts). Details of underweight and obesity groups can be found in Table S2 (<http://www.biosciencetrends.com/action/getSupplementalData.php?ID=11>). Data for the 5556 MetS in normal weight, underweight and obesity

groups with complete physical and biochemical measurements are presented in Table S3 (<http://www.biosciencetrends.com/action/getSupplementalData.php?ID=11>).

3.4. Characteristics of subjects by age among the non-MetS

Overall, MONW participants had the highest prevalence in 60 + yrs women (28.29%) and men (11.32%). MHO participants had a prevalence peak in the ≤ 39 yrs age group (men, 69.15% and women, 76.69%) (Figure 2). The characteristics of study subjects stratified by age among non-MetS are presented in Table 4. In three age groups (< 40 yrs, 40-59 yrs and > 60 yrs), the overall prevalence of non-MetS was 37.9%, 41.67% and 20.43%, respectively. BMI, FG, HDL-C, LDL-C, ALT, SUN, CR and UAC are significantly different between men and women in each age group. Height, WC, SBP, TC, TG and AST are significantly different between men and women in ≤ 39 yrs group and 40-59 yrs group. Detailed information in MetS and non-MetS groups across all age groups are shown in Table S4 (<http://www.biosciencetrends.com/action/getSupplementalData.php?ID=11>). Data for the 5556 MetS with complete physical and biochemical measurements across three age groups are presented in Table S5 (<http://www.biosciencetrends.com/action/getSupplementalData.php?ID=11>).

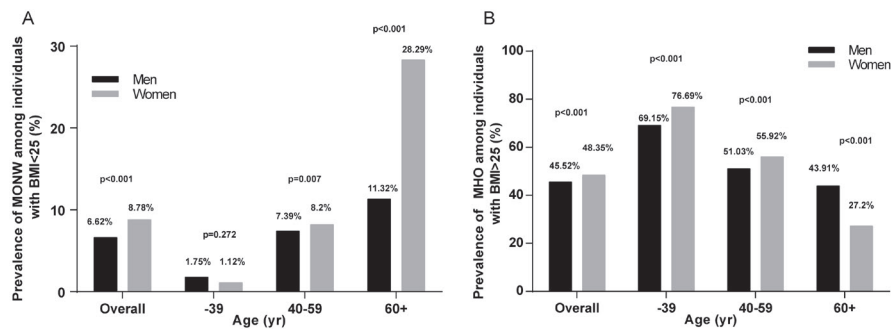


Figure 2. The prevalence of MONW and MHO. A. The prevalence of MONW among those with BMI < 25 kg/m² on age. Generally, women showed higher prevalence of MONW among the non-obese than men did. Both men and women had the highest prevalence of MONW in their 60s and over. Both men and women showed the lowest prevalence in the < 39 yrs group. **B.** The prevalence of MHO among the obese population by age. Overall, women demonstrated slightly higher prevalence of MHO than men did. Both male and female showed the highest prevalence in the <39 yrs group and the lowest prevalence in the > 60 yrs group.

Table 4. Baseline characteristics of the subjects by gender and age among the non-MetS

Variables	≤ 39 yrs		P	40-59 yrs		P	≥ 60 yrs		P
	Men	Women		Men	Women		Men	Women	
N (%)	3586 (21.32%)	2789 (16.58%)		3918 (23.29%)	3091 (18.38%)		2430 (14.45%)	1006 (5.98%)	
BMI (kg/m ²)	24.3 ± 3.0	21.1 ± 2.7	**	24.9 ± 2.6	22.9 ± 2.8	**	23.7 ± 2.8	22.9 ± 3.1	**
Height (cm)	173.1 ± 5.8	162.1 ± 5.3	**	171.1 ± 5.4	160.5 ± 5.0	**	167.1 ± 5.8	156.0 ± 5.7	**
WC (cm)	84.9 ± 7.5	72.4 ± 7.0	*	87.0 ± 6.8	76.8 ± 7.1	*	85.5 ± 7.8	78.7 ± 8.1	**
W/H ratio	0.5 ± 0.04	0.5 ± 0.04		0.5 ± 0.04	0.5 ± 0.1		0.5 ± 0.1	0.5 ± 0.1	**
SBP (mmHg)	117.4 ± 11.1	108.2 ± 10.4	*	120.5 ± 13.6	115.1 ± 24.5	*	133.3 ± 18.4	128.9 ± 18.8	**
DBP (mmHg)	76.1 ± 7.3	71.0 ± 67.0		78.7 ± 8.4	73.9 ± 8.0		76.8 ± 8.8	74.5 ± 7.8	**
FG (mmol/L)	5.0 ± 0.6	4.8 ± 0.4	**	5.4 ± 1.1	5.1 ± 0.6	**	5.7 ± 1.5	5.3 ± 0.8	**
TC (mmol/L)	5.0 ± 0.9	4.7 ± 0.8	**	5.3 ± 0.9	5.4 ± 1.0	**	5.3 ± 1.0	5.9 ± 1.0	**
TG (mmol/L)	1.5 ± 1.2	0.9 ± 0.4	**	1.7 ± 1.1	1.2 ± 0.6	**	1.4 ± 0.7	1.5 ± 0.7	**
HDL-C (mmol/L)	1.3 ± 0.3	1.6 ± 0.3	**	1.3 ± 0.3	1.6 ± 0.3	**	1.4 ± 0.3	1.6 ± 0.3	**
LDL-C (mmol/L)	2.8 ± 0.7	2.3 ± 0.6	**	3.0 ± 0.7	2.8 ± 0.7	**	2.8 ± 0.7	3.0 ± 0.7	**
UAC (umol/L)	338.6 ± 70.2	223.6 ± 51.4	**	335.4 ± 68.8	232.2 ± 56.1	**	340.2 ± 82.5	263.8 ± 71.9	**
ALT (U/L)	28.0 ± 21.7	14.7 ± 9.4	**	26.4 ± 19.0	18.3 ± 12.7	**	19.86 ± 11.62	17.45 ± 9.34	*
AST (U/L)	24.4 ± 9.1	20.4 ± 5.4	**	24.9 ± 13.6	22.5 ± 7.6	**	23.66 ± 7.69	23.31 ± 6.78	**
SUN (mmol/L)	4.9 ± 1.1	4.0 ± 0.9	**	5.1 ± 1.1	4.5 ± 1.1	**	5.64 ± 1.43	5.16 ± 1.19	**
CR (umol/L)	79.31 ± 9.3	57.49 ± 7.34	**	79.31 ± 10.28	59.13 ± 8.33	**	82.56 ± 15.78	63.75 ± 11.45	**

¹ Data are mean ± SD or number (percentage, %).

² Non-MetS, non-metabolic syndrome; BMI, body mass index; WC, waist circumference; W/H, weight/ height ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FG, fasting glucose; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; UAC, uric acid; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; SUN, serum urea nitrogen; CR, creatinine.

³ P represents the difference of each risk factor for Men and Women in Non-MetS subgroups

⁴ *P < 0.05 and **P < 0.01.

3.5. Risk factor analysis of MetS

Logistic regression analysis was done to analyze the risk factors of MetS. The results are shown in Table 5. The results demonstrate that sex, age, WC, SBP, DBP, ALT, FG, UAC, TG, HDL-C and LDL-C are all very significant risk factors for MetS.

4. Discussion

Our results revealed that not only obese but non-obese individuals in Beijing urban population also had metabolism-associated disorders. MONW is less common than MHO among all participants regardless of gender. Males had higher MHO incidence but lower MONW incidence than females. Age is associated with

other variables. Males aged between 40-59 yrs had the highest MetS prevalence compared to those aged above 60 yrs or under 39 yrs. Among females, the highest MetS prevalence is present after age 60, which may be due to postmenopausal status (13,14).

The worldwide MetS prevalence among adults ranges from 10% to 55%, depending on the ethnic group, urbanization, lifestyle and diagnostic criteria (15-18). For instance, in China, it was estimated that the overall prevalence of MetS in adults was 11.1% in 1991 and 26.1% in 2004. The changes of MetS prevalence across the last two decades suggest that the prevalence of MetS has become higher with the development of urbanization. From another study in 2011, Uyghur ethnic group has a higher prevalence of MetS than the Han group because of high intake of animal fats, proteins

Table 5. Univariate and multivariate logistic regression analysis of MetS

Variables	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Sex	1.516 (1.422,1.617)	< 0.001	0.053 (0.044,0.065)	< 0.001
Age (yrs)	1.037 (1.035,1.039)	< 0.001	1.024 (1.019,1.029)	< 0.001
WC (cm)	1.199 (1.193,1.206)	< 0.001	1.245 (1.229,1.260)	< 0.001
Height (cm)	1.012 (1.008,1.016)	< 0.001	1.003 (0.991,1.015)	0.616
Weight	1.066 (1.063,1.069)	< 0.001	0.998 (0.990,1.007)	0.676
BMI (kg/m ²)	1.549 (1.528,1.570)	< 0.001	1.023 (0.988,1.059)	0.207
SBP (mmHg)	1.050 (1.048,1.052)	< 0.001	1.025 (1.021,1.029)	< 0.001
DBP (mmHg)	1.082 (1.078,1.086)	< 0.001	1.034 (1.026,1.042)	< 0.001
ALT (U/L)	1.026 (1.024,1.028)	< 0.001	1.004 (1.000,1.008)	0.041
AST (U/L)	1.046 (1.042,1.050)	< 0.001	1.005 (0.997,1.014)	0.186
FG (mmol/L)	2.043 (1.968,2.120)	< 0.001	1.455 (1.392,1.521)	< 0.001
SUN (mmol/L)	1.242 (1.213,1.271)	< 0.001	0.967 (0.925,1.010)	0.129
CR (umol/L)	1.013 (1.011,1.015)	< 0.001	1.002 (0.997,1.007)	0.39
UAC(umol/L)	1.007 (1.006,1.007)	< 0.001	1.002 (1.001,1.002)	< 0.001
TG (mmol/L)	2.522 (2.431,2.616)	< 0.001	1.618 (1.538,1.702)	< 0.001
HDL-C (mmol/L)	0.032 (0.028,0.037)	< 0.001	0.051 (0.040,0.065)	< 0.001
LDL-C (mmol/L)	1.781 (1.702,1.863)	< 0.001	1.181 (1.101,1.267)	< 0.001

BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FG, fasting glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; UAC, uric acid; ALT, Alanine aminotransferase; AST, aspartate aminotransferase; SUN, serum urea nitrogen; CR, creatinine.

and salts and less exercise. Besides, the prevalence of MetS in Caucasians was higher than that in Asia (19). These studies confirmed that ethnic and life styles could contribute to the differences of MetS prevalence. We aimed to study the prevalence of MHO and MONW among the population in Beijing city only to comprehend the understanding of the current situation of MetS and obesity. In this study, we found that sex, age, WC, SBP, DBP, ALT, FG, UAC, TG, HDL-C and LDL-C are all very significant risk factors for MetS. With increase in age, the medial layer of the vessel wall appears gradually degenerated, the middle collagen content increases and the elastic layer fractures. With long-term hypertension, the structural change is more obvious and intensified, because the large artery stiffness exacerbates with aging, flexibility also declines with a high risk of vascular disease. In addition, our results showed that males between 40-59 yrs were prone to have MetS whereas females above 60 yrs were more likely to have MetS, suggesting that MetS is a serious public health burden affecting people in Beijing.

Having known the high prevalence of MONW and MHO among the Beijing urban population, we wanted to see which factors are closely correlated with MetS. Physical and blood examinations were both done to uncover the correlation between MetS and variables. UAC is the product of purine metabolism in humans and MetS individuals often have high UAC levels; however, the association of UAC levels and the prevalence of MetS remains contradictory (20-23). Little information on its association with MetS in Chinese population is available. Consistent with the previous finding, Pearson's correlation analyses in this

work suggests that hyperuricemia is more correlated with MetS in males than in females (24). Our findings also suggested that UAC and TG could be a risk factor for MetS. Previous studies showed that high TG level was associated with hyperuricemia (24,25). The possible explanation is that TG could promote the production of UAC and the synthesis of ribose-5-phosphate to phosphoribosyl pyrophosphate (PPRP) (26). The UAC level was found closely associated with MetS incidence among the Beijing urban population; however, the precise mechanism underlying the association of UAC with MetS has not been elucidated and further studies are needed.

Some epidemiological research has shown that ALT and AST with MetS risk factors BMI, DBP, TG, HDL-C, LDL-C and UAC (27,28). Previous studies suggest that high ALT levels are related to MetS and obesity in Japanese (27), Chinese (29) and Korean adolescents (30). The reason might be that ALT and AST are involved in fat accumulation in the liver and are closely correlated with fatty liver disease (27,31,32). Intriguingly, we also found that ALT was more closely associated with MetS than AST among our subjects; however, the underlying mechanism explaining this finding remains to be further explored.

There are limitations to the present study that warrant further research. First of all, the study population only consists of the Beijing urban population, so it cannot represent the prevalence of MetS in Beijing suburban areas or other provinces. Secondly, this study did not include the influence of ethnic or lifestyles on MetS prevalence, which have been indicated to have effects on MetS prevalence.

In summary, we conducted this cross-sectional

study to explore the prevalence of MONW and MHO among the Beijing urban population. The definition of MONW, MHO and MetS were made properly, therefore our findings could provide useful information for comprehension of the current situation of obesity and MetS. The prevalence of overweight and MetS in Beijing urban adults is dramatically high. Our findings provided useful information for the projection of future trends and developing national strategies and programs to address the challenges from the growing obesity and MetS.

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The effect of DHEA on apoptosis and cohesin levels in oocytes in aged mice

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Summary

Female fertility declines with age as the number of ovarian follicles decreases and aneuploidy increases. Degradation of the cohesin complex might be responsible for age-related aneuploidy. Dehydroepiandrosterone (DHEA) can improve the ovarian reserve and reduce the rate of aneuploidy, but the relationship between DHEA and cohesin levels in oocytes is still unknown. The aim of the current study was to evaluate the effect of the supplement DHEA on ovarian function, including the number of follicles and cohesin levels in oocytes. C57BL/6J mice at 3 weeks, 6 weeks, 12 weeks, 6 months, and 10 months of age were used to obtain a systematic view into follicle apoptosis and cohesin levels in oocytes. Nine-month-old C57BL/6J mice were administered saline ($n = 5$), 17 β -estradiol (100 μ g/kg per day, $n = 5$), or DHEA (5mg/Kg per day, $n = 5$). After 4 weeks, aged mice were weighed and sacrificed, and ovarian tissue samples were prepared. Anti-VASA staining and HE staining were used to count the number of follicles. Anti- γ H2AX staining and TUNEL were used to measure follicle apoptosis and immunofluorescent staining was used to detect the levels of three oocyte cohesin subunits: REC8, SMC1 β , and SMC3. Administration of the supplements 17 β -estradiol and DHEA to aged mice increased the number of primordial and primary follicles and decreased the age-related apoptosis of follicles. Levels of the cohesin subunits REC8 and SMC1 β declined with age, but DHEA and 17 β -estradiol tended to delay that decline. The supplement DHEA increased the number of primordial and primary follicles in aged mice by inhibiting follicle apoptosis and tended to delay the decrease in cohesin levels in oocytes.

Keywords: Dehydroepiandrosterone (DHEA), apoptosis, cohesin, oocyte, mice

1. Introduction

As society has advanced, more and more women have received an advanced education and are working than

before. The age at which women bear children has been postponed as a result, and later childbearing can be affected by the issue of the decline in female fertility. Female fertility declines with age (1,2), and women of advanced maternal age have a greater incidence of miscarriages and embryo aneuploidy (3-5). The decline in fertility is due not to uterine aging but to ovarian aging, which is the process by which both the quantity and quality of oocytes decrease with age (6-8).

The number of oocytes decreases from when a woman was still a fetus. Without regeneration, the number of oocytes drops from 7×10^6 to $< 1,000$ during

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menopause (9). In an adult woman, only a small proportion of oocytes are recruited during ovulation and enter meiosis from their original quiescent status; instead, more oocytes undergo apoptosis and are eliminated from the ovaries, leading to a decrease in the number of oocytes and eventually ovarian aging (10). Apoptosis is age-related and Hansen devised a model of reproductive aging in which the loss of oocytes and follicles gradually accelerates in women at the age of 37 (11). Oocyte maturation is also a process of folliculogenesis. The underlying mechanism of oocyte apoptosis and follicular atresia is still unclear, but numerous studies and different therapies have been designed to promote follicle growth and prevent follicular atresia, thus alleviating the loss of ovarian function due to the aging process (12).

The increased rate of aneuploidy in oocytes is considered to be a distinct manifestation of a decline in oocyte quality. Aneuploidy of oocytes is also one of the major causes of infertility, spontaneous abortions, and genetic diseases (13,14). The production of an oocyte involves a process called meiosis, which is an error-prone process in which a tiny mistake can result in aneuploidy (15). Previous studies have focused on several hypotheses of age-related aneuploidy including abnormal homologous recombination (16) and defective spindle assembly (17). However, recent studies have speculated that cohesin in chromosomes might be the main cause.

The cohesin complex is a ring-structured complex containing four subunits, and SMC1 β , SMC3, REC8, and STAG3 are the specific proteins in mice (18). Cohesin complexes are loaded on chromosomes during the replication of DNA in oocytes, with little to no turnover after loading (19). Cohesin complexes have multiple functions in cell life including chromosome segregation, DNA repair, and gene expression, and their most important function is to maintain the cohesion of chromosomes during mitosis and meiosis, which means that these complexes could be the chief culprits of age-related aneuploidy. Cohesin-specific subunit knockout mice have a high rate of aneuploidy, and that rate increases greatly with age (20). Different cohesin subunits and their functions have been summarized in Table 1. In senescence-accelerated mice (SAM), immunostaining of REC8 between the chromosome arms and centromeres in aged oocytes was either absent or disrupted (21). In oocytes of women in their 40s, levels of the cohesin complex subunits REC8 and SMC1 β decreased substantially in comparison to levels in women age 20 (22). In the process of maternal aging, ovarian function changes markedly in line with the loss of cohesin complexes in oocytes. However, the specific mechanism of the decrease in cohesin is still unknown.

Dehydroepiandrosterone (DHEA) is a type of androgenic steroid that is mainly produced in the adrenal cortex (23). The androgenic activity of DHEA is relatively weak and is estimated to be only 10% of

that of testosterone. DHEA is a precursor to sex steroids including testosterone and estradiol. The level of DHEA and dehydroepiandrosterone sulfate (DHEAS) declines with age, reaching its nadir at 80 years of age. This suggests that DHEA might be involved in the ovarian aging process. Moreover, the development of follicles requires androgen-based hormonal support and the production of DHEA could also be affected by the number of follicles, which makes the relationship of DHEA and ovary functions more complicated (24).

DHEA supplementation has also been used in clinical therapy to improve the fertility outcomes in *in-vitro* fertilization (IVF) treatment. DHEA was first used in an experiment by Casson *et al.* involving IVF (25). The supplement DHEA improved embryo quality and increased the chance of pregnancy in women with a poor ovarian reserve (26). When follicular growth was detected with transvaginal ultrasound after administration of DHEA, an increased antral follicle count (27) and more retrieved follicles and a greater number of follicles >17 mm were noted (28). A recent study suggested that DHEA reduces oocyte aneuploidy (29), thus improving outcomes for IVF patients. DHEA has been widely used around the world, but its effect on ovarian function and the mechanism of that action are still unclear. Whether DHEA could affect the quantity and quality of oocytes and how it would do so are still topics of speculation.

The aim of this study was to ascertain the effect of DHEA on ovary function, including the quantity and quality of oocytes in naturally aging mice. The number of follicles and follicular atresia were detected after administration to show how the supplement DHEA affected folliculogenesis. Detecting the levels of oocyte cohesin complexes would reveal whether DHEA affects oocyte quality. As a preliminary investigation of how DHEA combats ovarian aging, the current study might provide new insight into the effect that the supplement DHEA has on ovarian function and the mechanism of that action.

2. Materials and Methods

2.1. Animals

In total, 40 female C57BL/6J mice were purchased from the Laboratory Animal Facility of Chinese Academy of Science (Shanghai, China) and raised in the Shanghai Key Laboratory of Female Reproductive Endocrine-related Diseases, China. Mice were given a fresh supply of sterile drinking water and food and exposed to a 12-h day and 12-h night photoperiod during the experiment. This study was approved by the animal ethics committee of the Obstetrics and Gynecology Hospital of Fudan University. To facilitate a systemic study of the ovarian aging process, five mice were sacrificed at the ages of 3 weeks, 6 weeks, 3 months, 6 months, and 10 months. Ovaries were collected for subsequent examination.

Table 1. Properties and functions of cohesin complexes in mammals

Category	Name	Molecular Weight (kDa)	Gene location in human	Principal function in meiosis/mitosis	Related human disease	Effect in knock-out mice	Changes with age in humans	Changes with age in mice
Structural maintenance of chromosomes (SMC)	SMC1 α	121.1	Xp11.22	Mitotic cohesion, G2/M cell cycle, restructuring the DNA double helix	CdLS, Cancer, Epileptic encephalopathy, Acute myeloid leukemia	-	No SD	No SD
	SMC1 β *	116.1	22q13.31	Sister chromatid cohesion, assembly of axial elements during prophase, synapsis, recombination, chromosome movement	-	Males and females sterile	Decreases	Decreases
	SMC3	121.7	10q25.2	Sister chromatid cohesion, restructuring the DNA double helix	CdLS, Cancer, PCOS, AML in Down Syndrome infants, AML	Lethal to embryos (E14.5)	No SD	No SD
Stromalin	STAG1 (SA1)	122.1	-	Telomere replication, telomere cohesion	Cancer	Lethal to embryos (E11.5)	-	-
	STAG2 (SA2)	123.1	Xq25	Centromere cohesion, sister chromatid cohesion, repair of kinetochore-microtubule attachments	Cancer	-	-	-
Kleisin	STAG3 (SA3)*	122.5	7q22.1	Sister chromatid cohesion, centromeric cohesion, chromosome pairing and synapsis, DNA repair, progression of meiosis, axis formation	Male infertility, Premature failure, Williams-Beuren syndrome	Lethal to embryos (E11.5)	No SD	No SD
	RAD21	64.3	8q24.11	Axis formation, synaptonemal complex maintenance, monopolar attachment of sister kinetochores	CdLS, Cancer, Acute myeloid leukemia	-	No SD	No SD
	RAD21L*	55.6	17q21.31	Normal clustering of pericentromeric heterochromatin, axis formation, SC maintenance, DNA repair	-	Males infertile. Females are fertile but experience age-dependent sterility	-	-
	REC8*	54.7	14q12	Sister chromatid cohesion, centromeric cohesion, axis formation	Cancer, Male infertility	Males and females sterile.	Decreases	Decreases

* stands for meiosis-specific cohesin subunits.

2.2. Agent administration

Nine-month-old female C57BL/6 mice were used in this study and corresponded to women around 40 years of age (30). Fifteen mice were randomly divided into three groups: the control group, the E2 group, and the DHEA group. All of the mice were intragastrically administered the corresponding agent. The control group mice were administered saline ($n = 5$). Mice in the E2 group were administered 17β -estradiol (100 $\mu\text{g/kg}$ per day, $n = 5$) as a positive control (31). The DHEA group was administered DHEA (5mg/Kg per day, $n = 5$) (32). The dosages of the two hormones were calculated according to the dosages used in postmenopausal hormone therapy (33). After 4 weeks, all mice were sacrificed in order to collect blood and ovarian tissue samples.

2.3. Serum hormone measurements

Blood samples were collected from each mouse *via* cardiac puncture prior to sacrifice. After centrifugation, the serum samples were prepared to measure the concentration of AMH. EIA kits for AMH were used according to the manufacturer's protocol. The experiment was repeated three times.

2.4. Histological sample preparation and staining

Ovarian tissues were fixed overnight in 4% paraformaldehyde and then washed and stored in 70% ethanol at 4°C for embedding in paraffin. Eight-micrometer sections were processed for further use. Slides were stained with hematoxylin and eosin (HE) and then scanned using the Olympus Scanner (Tokyo, Japan). After staining, the follicles were classified into four stages: primordial, primary, secondary, and antral. The classifications of follicles was according to the features of granulosa cells (GCs) surrounding oocytes as described by Gougeon (34). Primordial follicles consisted of primordial oocytes surrounded by a single layer of GCs. When the single surrounding layer of GCs became cuboidal, these follicles were classified as primary follicles. Follicles were classified as secondary based on the presence of a visible follicular antrum. When several layers of cuboidal GCs without a visible atrium surrounded oocytes, the follicles were categorized as secondary follicles. When an antral cavity with follicular fluid appeared around oocytes, the follicles were categorized as antral follicles. Despite the size differences in each group, the total number of follicles in each ovary was manually counted by two independent researchers. At least three slides per mouse were used to analyze the number of follicles in each group. Data are expressed as the average number of follicles per ovary.

2.5. TUNEL staining

The atresia of follicles was detected using TdT-mediated dUTP nick-end labeling (TUNEL). The In Situ Death Detection Kit was purchased from POD, Roche Molecular Biochemicals, Mannheim, Germany. TUNEL was performed according to the manufacturer's instructions. Briefly, the prepared ovarian tissues were incubated with reaction mixtures including biotinylated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h. After incubation with a converter-POD solution for 30 min at 37°C, DAB was added. At least 30 slides from 5 mice in each group were stained with TUNEL to obtain data on follicular atresia. Follicles were considered TUNEL-positive if either the oocyte alone, the oocyte and GCs, or oocytes were negative but > 50% of GCs were positive (35). The percentage of TUNEL-positive follicles was manually calculated by two independent blinded examiners using the Olympus Scanner.

2.6. Immunohistochemistry

Vasa primary antibodies were purchased from Merck Millipore, Massachusetts, USA. $\gamma\text{H}_2\text{AX}$ primary antibodies were purchased from Cell Signaling Technology, Boston, USA. The immunohistochemical staining steps were strictly performed with standard protocols. Briefly, the prepared slides were washed three times and then stained with two primary antibodies: anti-VASA, and anti- $\gamma\text{H}_2\text{AX}$ antibody. After incubation with the primary antibodies overnight, the slides were washed with PBS three times and then incubated with biotinylated-conjugated secondary antibody (Zymed Laboratories-Invitrogen, San Francisco, CA, USA) for 15 minutes at room temperature. Follicles were considered $\gamma\text{H}_2\text{AX}$ -positive if the oocyte or > 50% of GCs were positive (36). Images were obtained using the Olympus Scanner. At least six slides per mouse were used to analyze the number of follicles that were positive for different types of staining from 5 mice in each group. $\gamma\text{H}_2\text{AX}$ -positive follicles and VASA-positive cells were counted manually and confirmed by at least two blinded researchers. Data are expressed as the average number of positive cells or follicles per ovary.

2.7. Immunofluorescent staining

Since the density of the cohesin subunit STAG3 changes little in the ovarian aging process (22), the current study only detected the levels of three cohesin subunits: REC8, SMC1 β , and SMC3. Ovarian slides were blocked and then incubated with primary and secondary antibodies as described (37). Briefly, slides were first incubated with blocking buffer (5% normal donkey serum and 5% normal goat serum in PBS), and then with the primary antibodies diluted in PBS at 4°C overnight at the following dilutions: rabbit anti-REC8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1,000;

mouse anti-SMC1 β (Rockland Antibodies & Assays, Limerick, PA, United States) and SMC3 (Cell Signaling Technology, Boston, USA). The slides were then washed in PBS three times and incubated with the secondary antibodies Alexa 594-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated goat anti-guinea-pig IgG (Invitrogen) or aminomethylcoumarin acetate-conjugated donkey anti guinea-pig IgG (Jackson Immuno Research, West Grove, PA, USA) at room temperature for 30 minutes. Finally, the slides were washed three times in PBS.

The slides were observed with a confocal laser microscope (LSM510, Zeiss, Oberkonchen, Germany) and images were captured with the LSM510 microscope. Densitometric analysis of the oocyte immunofluorescence signals was performed using Image-Pro Plus 6.0. The green signal intensity (REC8, SMC1 β , and SMC3) was defined as described in a previous study, except that DAPI was used as a control (22).

2.8. Statistical analysis

All data are expressed as the mean $\pm$ SD. Variance was analyzed with SPSS database using one-way analysis of variance (ANOVA) or the Student's *t* test. *P* < 0.05 was considered to be statistically significant.

3. Results

3.1. The number of follicles decreased with age, but the supplement DHEA increased the number of follicles in aged mice

In the aging process, female fertility in rodents and humans declines, and the quantity and quality of follicles serve as indices of this process. The current study detected the number of follicles in female C57BL/6J mice of different ages and then used VASA and HE staining to measure changes in the number of follicles in aged mice that were administered different agents for 4 weeks.

VASA protein is an oocyte-specific marker. VASA staining helped with follicle counting while VASA staining together with HE staining provides more objective results than HE staining alone. In ovarian sections from 3-week-old mice, the number of vasa-positive cells was as high as 90, but the number decreased rapidly with age from 3 weeks to 10 months (Figure 1a and Table 2). The supplements DHEA and 17 β -estradiol increased the number of vasa-positive cells in aged mice (Figure 1b and Table 3).

HE staining is used to distinguish different stages of follicles. The number of primordial follicles from mice declined rapidly with age from 3 weeks to 10 months (Figure 2a and Table 2). The number of primary follicles increased from 3 weeks to 3 months. The number of primary follicles peaked at 3 months and then sharply

decreased from 3 months to 10 months. The number of secondary follicles also decreased markedly from 3 weeks to 3 months and plateaued at 10 months (Figure 2a and Table 2). The number of antral follicles increased with age from 3 weeks to 6 weeks and plateaued at 10 months (Figure 2a and Table 2). The supplements DHEA and 17 β -estradiol increased the number of primordial and primary follicles in aged mice with little effect on follicles in other stages (secondary and antral) (Figure 2b and Table 3).

In order to compare the levels of follicular atresia in mice and the effect of DHEA on follicular atresia, ovarian sections were stained using TUNEL. The percentage of apoptotic follicles increased greatly with age from 3 weeks to 10 months (Figure 3a and Table 2). After the supplements DHEA and 17 β -estradiol were administered to aged mice for 4 weeks, the level of oocyte apoptosis was measured again. The level of oocyte apoptosis decreased significantly compared to the level in the control group (Figure 3b and Table 3).

γ H₂AX staining was used to detect damage to DNA where the Ser of H₂AX was phosphorylated, resulting in γ H₂AX (38). The percentage of γ H₂AX-positive follicles increased greatly with age from 3 weeks to 10 months (Figure 4a and Table 2). The supplements DHEA and 17 β -estradiol decreased the percentage of γ H₂AX-positive follicles in aged mice (Figure 4b and Table 3).

3.2. DHEA had little effect on the age-related decrease in cohesins in aged mice

In order to compare the cohesin levels in oocytes from mice of different ages, meiosis cohesin subunits REC8, SMC1 β , and SMC3 were detected from the dictate oocytes of mice of different ages, and the relative density of each protein was measured using immunofluorescence. After the supplement DHEA or 17 β -estradiol was administered for 4 weeks, the cohesin levels in oocytes from aged mice were measured again.

The density of the cohesin subunit REC8 decreased with age in mice. The density declined with age from 3 weeks to 10 months (Figure 5a and Table 3). Administration of E2 tended to delay the decrease in levels of REC8 in oocytes, but the levels did not differ significantly compared to those in the control group. Administration of DHEA had a similar effect on levels of REC8 in oocytes, and again the levels did not differ significantly (Figure 5b and Table 3). The cohesin subunit SMC1 β decreased with age in mice from 3 weeks to 10 months (Figure 6a and Table 3) and administration of DHEA and 17 β -estradiol to aged mice tended to delay that decrease (Figure 6b and Table 3). However, levels of SMC1 β did not differ significantly compared to those in the control group. In contrast, levels of SMC3 appeared to remain unchanged in the current study. No age-related changes in those were noted during the aging process (Figure 7a and Table 4), and DHEA and 17 β -estradiol

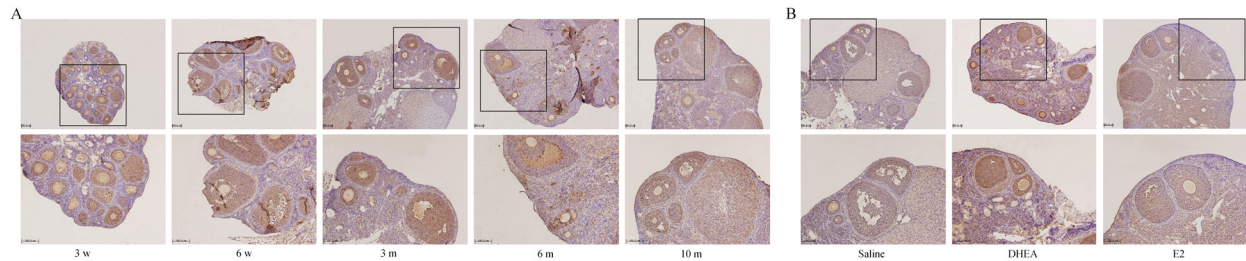


Figure 1. The elimination of oocytes in mice can be reversed by the supplement DHEA. Representative images of anti-VASA-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered saline, E2 (100 µg/kg per day), or DHEA (5 mg/Kg per day, $n = 5$) for four weeks (B). Bar = 100 µm.

Table 2. Number of follicles and follicular atresia in mice of different ages

Type of follicle	3 w	6 w	3 m	6 m	10 m
Vasa-positive cells	98.75 ± 1.053	79.67 ± 1.269*	63.08 ± 0.86578*	51.00 ± 0.7177*	46.33 ± 1.082*
Primordial Follicles	59.54 ± 1.233	48.85 ± 1.709*	30.15 ± 1.686*	19.69 ± 0.8799*	11.58 ± 1.264*
Primary Follicles	25.00 ± 1.638	35.00 ± 1.255*	48.08 ± 0.9806	30.50 ± 1.091*	16.25 ± 0.5383*
Secondary Follicles	17.17 ± 0.6945	11.75 ± 0.6867*	8.833 ± 0.4234	8.417 ± 0.3128	8.333 ± 0.6195
Antral Follicles	2.000 ± 0.4264	5.583 ± 0.4345*	5.917 ± 0.4516	6.750 ± 0.5919	7.250 ± 0.9545
% γ H ₂ AX-positive Follicles	21.49 ± 1.243	24.66 ± 0.5394*	42.07 ± 1.383*	49.82 ± 3.036*	61.16 ± 2.288*
%TUNEL-positive Follicles	11.25 ± 0.6593	16.79 ± 1.038*	31.83 ± 1.348*	39.03 ± 1.921*	45.59 ± 1.653*

Mice of different ages were sacrificed and ovarian slides were used to count the number of follicles and detect follicular atresia. Data are expressed as the mean ± standard deviation (SD). The P -value was determined with ANOVA. * $P < 0.05$, ** $P < 0.01$ compared to the previous one, respectively.

Table 3. Number of follicles and follicular atresia in aged mice administered different agents

Type of follicle	Saline	DHEA	E2
Vasa-positive cells	46.33 ± 1.082	49.58 ± 0.8021*	51.17 ± 0.8947*
Primordial Follicles	11.58 ± 1.264	14.77 ± 0.6994*	15.92 ± 0.7291*
Primary Follicles	16.25 ± 0.5383	18.42 ± 0.7732*	19.50 ± 1.270*
Secondary Follicles	8.333 ± 0.6195	8.583 ± 0.8744	10.17 ± 0.7470
Antral Follicles	7.250 ± 0.9545	6.333 ± 0.5412*	6.417 ± 0.3128*
% γ H ₂ AX-positive Follicles	61.16 ± 2.288	53.77 ± 1.424*	53.49 ± 2.275*
%TUNEL-positive Follicles	45.59 ± 1.653	40.77 ± 1.382*	40.18 ± 1.803*

Aged mice that were treated with saline, E2 (100 µg/kg per day), or DHEA (5 mg/Kg per day, $n = 5$) for four weeks were sacrificed and ovarian slides were used to count the number of follicles and detect follicular atresia. Data are expressed as the mean ± standard deviation (SD). The P -value was determined with ANOVA. * $P < 0.05$, ** $P < 0.01$ compared to the group administered saline.

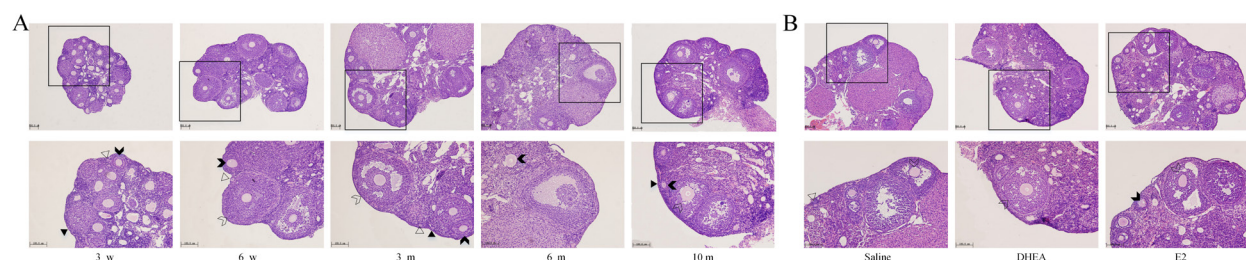


Figure 2. The number of follicles in different stages that were eliminated with age in mice. Representative images of HE-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered different agents for four weeks (B). Bar = 100 µm. "▲" represents primordial follicles, "△" represents primary follicles, "▲" represents secondary follicles, and "△" represents antral follicles.

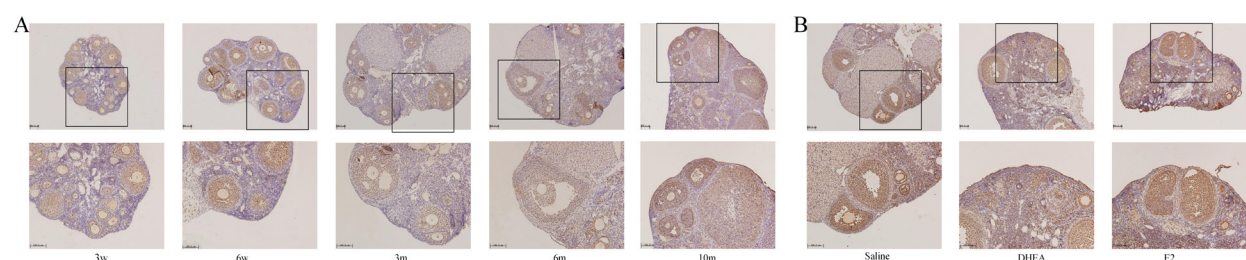


Figure 3. The increased apoptosis of oocytes in mice can be partly reversed by the supplement DHEA. Representative images of TUNEL-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered different agents for four weeks (B). Bar = 100 µm.

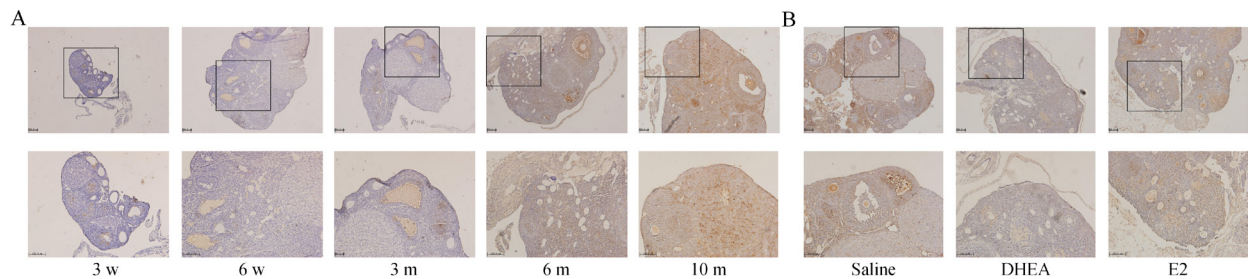


Figure 4. The increase in DNA double-strand breaks in oocytes in mice can be partly reversed by the supplements 17 β -estradiol and DHEA. Representative images of anti- γ H₂AX-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered different agents for four weeks (B). Bar = 100 μ m.

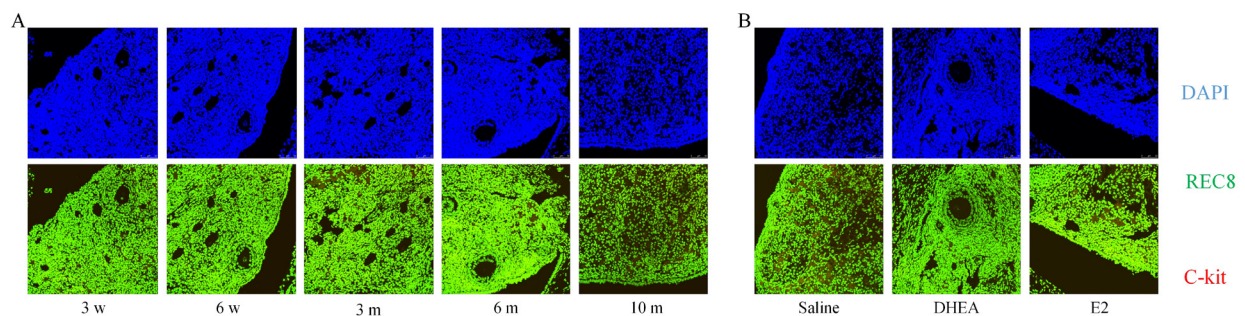


Figure 5. The decrease in levels of the cohesin complex subunit REC8 in mice can be partly delayed by the supplement DHEA. Representative images of immunofluorescence-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered different agents for four weeks (B). Bar = 50 μ m.

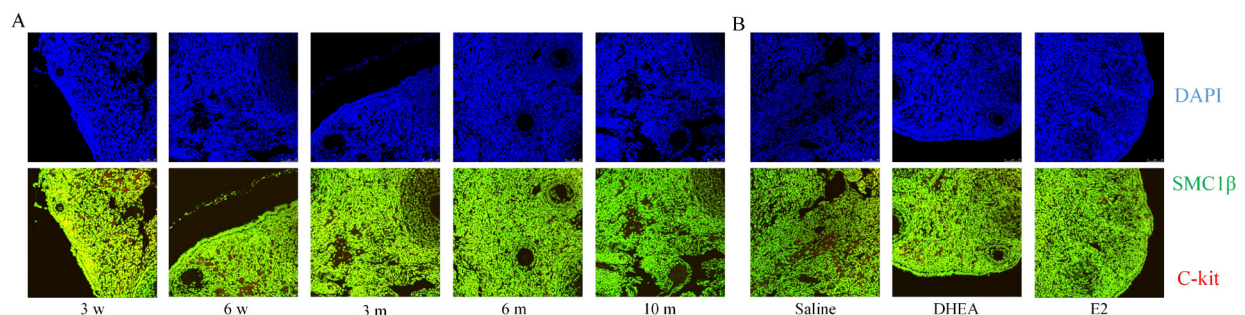


Figure 6. The decrease in cohesin complex subunits-SMC1 β in mice showed a trend to be delayed by the supplement DHEA. Representative images of immunofluorescence-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered different agents for four weeks (B). Bar = 50 μ m.

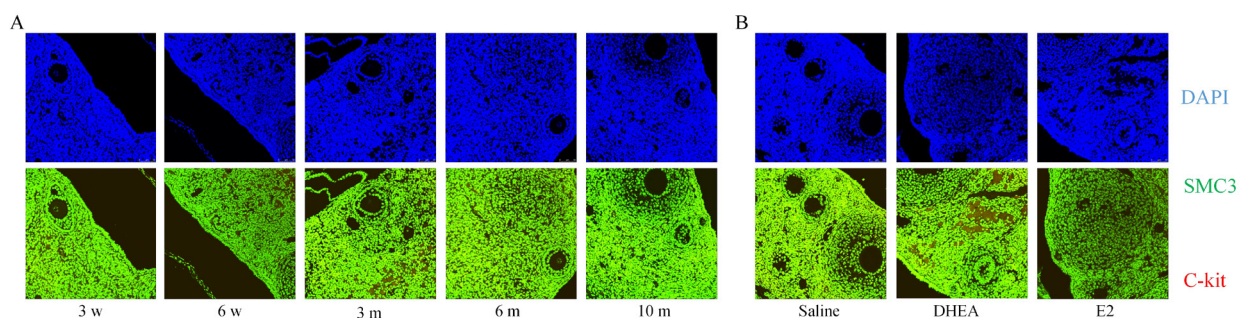


Figure 7. Levels of the cohesin complex subunit SMC3 in mice of different ages and different agents. Representative images of immunofluorescence-stained ovary sections from female C57BL/6J mice of different ages (A) and aged mice administered different agents for four weeks (B). Bar = 50 μ m.

Table 4. Relative density of cohesin subunits in mice of different ages

Cohesin subunit	3 w	6 w	3 m	6 m	10 m
REC8	0.7239 ± 0.05156	0.5501 ± 0.04139*	0.4045 ± 0.01736*	0.3226 ± 0.01668*	0.2660 ± 0.01933*
SMC1β	1.193 ± 0.06215	1.038 ± 0.03060*	0.9221 ± 0.04221*	0.7619 ± 0.03218*	0.6664 ± 0.02739*
SMC3	1.015 ± 0.03451	0.9433 ± 0.05124*	0.9305 ± 0.03390*	0.9246 ± 0.04650*	0.9145 ± 0.04117*

Mice of different ages were sacrificed and ovarian slides were used to detect the relative density of cohesin subunits. Data are expressed as the mean ± standard deviation (SD). The P-value was determined with ANOVA. * $P < 0.05$, ** $P < 0.01$ compared to the previous one, respectively.

Table 5. Relative density of cohesin subunits in aged mice administered different agents

Cohesin subunit	Saline	DHEA	E2
REC8	0.2660 ± 0.01933	0.3003 ± 0.02564	0.3187 ± 0.01981
SMC1β	0.6664 ± 0.027398	0.7402 ± 0.02528	0.7519 ± 0.03066
SMC3	0.9145 ± 0.04117	0.9275 ± 0.03268	0.9050 ± 0.02581

Aged mice that were administered saline, E2 (100 µg/kg per day), or DHEA (5 mg/Kg per day, $n = 5$) for four weeks were sacrificed and ovarian slides were used to detect the relative density of cohesin subunits. Data are expressed as the mean ± standard deviation (SD). The P-value was determined with ANOVA. * $P < 0.05$, ** $P < 0.01$ compared to the group administered saline.

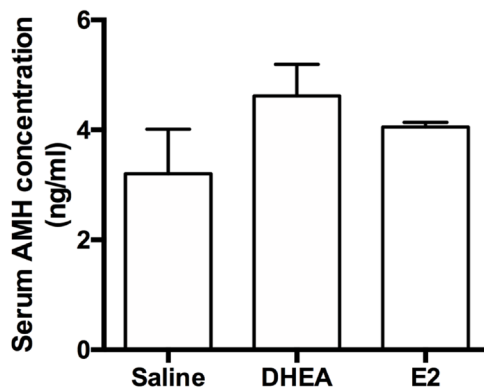


Figure 8. Administration of DHEA did not increase serum levels of AMH in aged mice. Aged mice were sacrificed after 4 weeks of administration, and sera were collected to determine concentrations of AMH. Steroid concentrations in sera were measured using an AMH enzyme immunoassay kit. Bars denote the mean ± standard deviation (SD). Agents are listed below the X-axis. The P-value was determined with ANOVA. * $P < 0.05$, ** $P < 0.01$.

had no effect on the density of SMC3 (Figure 7b and Table 5).

3.3. Administration of DHEA increased serum E2 but not AMH

DHEA is a precursor to sex steroids including testosterone and estradiol. Hence, the question was whether DHEA improved ovarian function directly or indirectly by increasing concentrations of estrogens. The administration of DHEA and E2 had no effect on AMH (Figure 8).

4. Discussion

Ovarian aging is a protracted process and more than 99.9% of follicles, including oocytes and GCs, are

programmed to die during this process (39). The current study indicated that DHEA and E2 increased the number of follicles and alleviated follicular atresia in aged mice. This study also provided a view into the changes in the number of follicles and follicular atresia in the aging process.

DHEA is a C19 androgenic steroid that has been used as a therapy in several areas, and especially in IVF. The use of DHEA in patients could improve embryo quality and live birth rates and thus increase the pregnancy rate in patients. The effect also seems to be time- and dose-dependent (40). Some clinical studies have also suggested that this effect could be achieved by reducing aneuploidy, increasing the antral follicle count, and improving follicular steroidogenesis (25,41,42). Although one study failed to obtain positive results (43), DHEA has consistently been a focus of research. These clinical trials may be less persuasive due to the number of patients and individual differences in different studies, and more studies have instead involved animal experiments.

Narkwichcean *et al.* (44) used a sheep model to explore effect and mechanism of the supplement DHEA *in vivo*. As the first *in vivo* study to explore the effect of DHEA on early follicle development, it suggested that DHEA could increase antral follicle population by increasing the rates of primordial follicle initiation and preantral follicle development. Rats that underwent a unilateral oophorectomy were also used as a DOR model and were administered DHEA in one study (45). The ovaries of these rats had a greater number of follicles in all stages, including primordial, primary, and growing (preantral and antral) stages, and a decreased rate of atresia after administration of the supplement DHEA for 45 days.

The current study used aged female C57BL/6J mice as a model to investigate the probable effect of DHEA

and its potential mechanism. In previous studies, DHEA was also used to induce PCOS-like syndromes in rodents. However, the dose of DHEA to induce PCOS in a mouse model is at least 30 mg/Kg (46), while the DHEA dose in the current study was only 5 mg/Kg (32). The supplement DHEA increased the number of follicles in aged mice, and particularly the number of primordial and primary follicles. This effect was presumably achieved by inhibiting follicular atresia (35). Anti- γ H₂AX staining indicated that atretic follicles were rescued by suppressing DNA double-strand breaks (36). DNA double-strand breaks play a major role in the ovarian aging process and the age-related decline in fertility. In some of the cells with damaged DNA, that DNA is repaired; if not, the cells undergo apoptosis (47). The current study suggested that DHEA decreases the extent of DSBs and it inhibits the apoptosis of oocytes, thus increasing the number of follicles.

The underlying mechanism of DHEA and folliculogenesis has been studied but is nonetheless still unknown. DHEA is a prohormone of androgen and may be beneficial for follicle maturation like androgen (48). Androgen receptors were seen in stroma and GCs in ovaries, and especially in primary follicles or other more advanced follicles. Excessive androgen levels may upregulate follicle formation, and Sen et al. contended that androgen signaling affected folliculogenesis *via* androgen receptors (49). They also found that androgen receptors in GCs and not in any other cells were the most important cells in this process. By stimulating the androgen receptors in GCs, androgen may promote preantral follicle development and could even prevent follicular atresia. DHEA treatment may increase androgen levels in GCs (50). As a result, DHEA may also promote preantral follicle growth and even rescue atretic follicles (51,52), suppressing the DNA double-strand breaks in follicles and thus inhibiting apoptosis (53).

The segregation of chromosomes is a process that requires high fidelity. The rate of a trisomy increases greatly from nearly 2% for women in their 20s to 35% for women in their 40s (13). A recent study has suggested that the deterioration of cohesins might be responsible for age-related aneuploidy (22). In mice, this phenomenon is thought to be related to the strain. Age-related aneuploidy and loss of cohesin subunits have been observed in C57BL-related strains (54,55), which led the current authors to choose C57/BL6J as a natural model for further study.

Several studies have noted a sharp decrease in cohesin levels in oocytes from aged mice in comparison to those in young mice (56,57). However, those studies failed to systemically reveal a specific relationship between age and cohesin levels in oocytes. The current study noted an almost linear decrease in cohesin levels in dictyate oocytes from mice of different ages, and the current study found that the supplement DHEA

partly alleviated the age-related decline in cohesin levels. To the extent known, this is the first report to systematically describe the age-related decrease in cohesin levels in oocytes and the effect of DHEA on cohesin levels. These findings may help to better understand the principle of cohesin deterioration in dictyate oocytes.

Cohesin complexes are a type of protein with a ring-like structure and are located at the centromeres of chromosomes as well as at the arms. The main function of cohesin complexes is to maintain the cohesion of chromosomes, so a lack of cohesion in chromosomes may be the main cause of oocyte aneuploidy. Unlike in mitosis, meiosis involves low or even minute levels of cohesin in oocytes. Cohesin complexes in oocytes are highly susceptible to changes in conditions (58) and undergo no turnover over the lifespan of females. Several *in vitro* studies have examined the mechanism of cohesin deterioration but failed to draw specific conclusions (59,60).

DHEA is an essential substrate in the process of steroidogenesis. Previous studies indicated that DHEA improved the outcomes of IVF by reducing aneuploidy and improving ovarian reserve (25,61). The current study found that administration of DHEA tended to delay the loss of cohesin in oocytes and thus reduce the rate of oocyte aneuploidy. The current study might provide other researchers with a new tack for the study of the underlying decline in cohesin levels in oocytes.

The use of DHEA in aged mice increased the quantity of oocytes and tended to improve the quality of oocytes, suggesting that the quality of oocytes might be influenced by cohesin levels. DHEA tended to delay the age-related loss of cohesins, perhaps by increasing the number of follicles. A previous study has also suggested that the oocyte pool might have been related to aneuploidy by affecting cohesion in chromosomes (62). The study in question also suggested a possible relationship between the oocyte pool and cohesin levels in oocytes. More studies of the effects of cohesin levels and *in vitro* studies need to be conducted to confirm this hypothesis.

The main limitation of the current study is that *in vivo* experiments were performed to reveal how DHEA combats ovarian aging. The mechanisms and pathways involved in this process were not fully investigated in this study. However, this study was a preliminary investigation of how DHEA combats ovarian aging, and the current findings may provide new insight into this subject.

The current study suggested that the supplement DHEA improved ovary function by increasing the number of follicles, inhibiting follicular atresia, and tending to delay the decrease in cohesin levels in oocytes in aged mice. The underlying mechanism and pathways involved in this process will be investigated further in the future.

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Blockage of cytosolic phospholipase A2 alpha by monoclonal antibody attenuates focal ischemic brain damage in mice

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Summary

The purposes of the current study were to investigate the effects of a monoclonal antibody (mAb) on cytosolic phospholipase A2 alpha (cPLA₂α) in mice with cerebral ischemia-reperfusion (IR) injury and to ascertain the potential mechanisms of those effects. This study evaluated whether the use of anti-cPLA₂α mAb could reduce stroke injury in a mouse model of cerebral IR injury. The expression/activity of cPLA₂α and cPLA₂α-derived proinflammatory lipid mediators such as prostaglandin E2 (PGE₂), leukotriene B4 (LTB₄), lysophosphatidylcholine (LPC), and free fatty acids (FFA) was assessed. This study also evaluated neurological deficits, motor function, pathological changes, apoptosis, and the area of infarction in the injured mice. Mice treated with anti-cPLA₂α mAb recovered neurological function and their condition improved, apoptosis in the brain decreased and infarct volume decreased, and expression of cPLA₂α, 5-LOX, COX-2, FFA, LPC, PGE₂, and LTB₄ was attenuated. Our findings indicate that cPLA₂α plays a key role in cerebral IR injury and that treatment with anti-cPLA₂α mAb after cerebral IR injury helps to reduce levels of proinflammatory cytokines, alleviate tissue damage, and reduce levels of deleterious lipid mediators. Thus, anti-cPLA₂α mAb treatment has the potential to treat ischemic brain damage.

Keywords: Cerebral ischemia-reperfusion (IR) injury, neurological deficit score (NDS), 5-lipoxygenase (5-LOX), cyclooxygenase-2 (COX-2)

1. Introduction

Cerebral ischemia is a result of a complex process, mainly including excitotoxicity, peri-infarct depolarization, inflammation, and apoptosis (1). The damage cascade is the recognized theory of the physiological and pathological mechanism of cerebral ischemia. Cerebral ischemia is associated with neuronal injury (2-4), where neuronal death occurs in the ischemic core as a result of failure to maintain membrane ion gradients in neurons, excitotoxicity due to elevated glutamate levels, and disruption of the blood-brain barrier (BBB). Oxidative stress and inflammatory

factors including lipoxygenases (LOXs), phospholipases (PLAs), and mitogen-activated protein kinases (MAPKs) are factors contributing to the pathology following cerebral ischemia. Several studies have found that group IVA cytosolic phospholipase A2 (cPLA₂α) is a key component in the pathway of stroke injury (3,5). cPLA₂α has been found to play a significant role in causing neurological injury following ischemic brain injury, and inhibition of cPLA₂α may reduce stroke injury (6). Preventing the overexpression of cPLA₂α may protect neuronal tissue from ischemic injury.

Phospholipases A2 (PLA₂) are key enzymes of phospholipid degradation and crucial to maintaining and determining membrane composition. A number of mammalian PLA₂ isotypes have been identified, and they are divided into three major subfamilies known as secretory PLA₂ (sPLA₂), cytosolic Ca²⁺-dependent PLA₂ (cPLA₂), and Ca²⁺-independent PLA₂ (iPLA₂). cPLA₂α is a member of the cPLA₂ class, and it has unique characteristics that include its preference for arachidonic acid (AA) at the sn-2 position of phospholipid substrate

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molecules (7). The enzyme catalyzes the hydrolysis of membrane phospholipids to release AA, which is subsequently metabolized into eicosanoids through cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) pathways (8,9). Metabolism of AA and the resulting eicosanoid products, including prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and 20-hydroxyeicosatetraenoic acid (20-HETE), have been implicated in the mechanism of injury (4). AA itself can cause BBB dysfunction and subsequent brain edema (10,11). cPLA2 α activity results in the production of proinflammatory lipid mediators, which play an important role in acute inflammatory responses and oxidative stress associated with neurological diseases, and blocking any of key pathways in the aa-metabolic network might inhibit progression of tissue injury. Several studies have suggested that cPLA2 α plays a key role in apoptosis and tissue injury (4,5,12,13).

The current study used monoclonal antibodies to study the effects of inhibiting the activity of cPLA2 α *in vivo* in a mouse model.

2. Materials and Methods

2.1. Materials

Anti-cPLA2 α mAb used in an animal model was originally prepared and provided by Detai Biologics Co., Ltd. The primary antibodies used for western blotting, such as cPLA2 α antibody, 5-LOX antibody, and β -actin antibody, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). COX-2 antibody was purchased from Sigma (St. Louis, MO, USA). The cPLA2 α Assay Kit was purchased from Cayman Chemicals (Interchim, Montlucon, France). TRIzol reagent was purchased from Invitrogen (USA). The PrimeScript Reverse Transcriptase Kit and the SYBR Premix Ex TaqTM II PCR Kit were purchased from Takara (Japan). Leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) ELISA kits were purchased from Cayman Chemical (Michigan, USA). The In Situ Cell Death Detection Kit was purchased from Roche (USA).

2.2. Animals and treatment

Male Swiss CD-1 mice (12-16 weeks, body weight = 25.57 ± 1.85 g) were used. All mice were housed in a temperature-controlled facility with a 12-h dark/light cycle and had free access to water and food except when otherwise specified. All procedures related to the care of animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The mice were randomly divided into a sham-operated group, a model group, a negative control (NC) group, and an mAb group. One hour before cerebral

ischemia-reperfusion (IR) injury, freshly prepared anti-cPLA2 α mAb (20 μ g) or class-matched control mAb (IgG2a) against keyhole limpet hemocyanin was intravenously administered to the mAb group and NC group. The sham-operated and model groups received only the vehicle solution. The mAb and vehicle solution were similarly administered every 24 h.

Mice were sacrificed at different time points after reperfusion. Some mice were anesthetized and perfused through the left ventricle with 0.01 M phosphate buffered saline. Brains were removed from the skull and prepared for examination. The rhinencephalon, cerebellum, and brain stem were removed, and the tissues from the coronal brain region were cut into slices (about 2 mm). Coronal sections of the brain were subjected to TTC staining, and 2 slices were fixed in 4% paraformaldehyde in PBS. The brain was directly removed from the remaining mice.

2.3. Focal cerebral ischemia in mice

Transient focal ischemia was induced using the intraluminal occlusion technique as described by Zhang J *et al* (6). Focal brain ischemia was produced by occlusion of the right middle cerebral artery (MCA) for 1 h, followed by reperfusion. Mice were anesthetized with 2% halothane in a mixture of 50% N₂O and 50% O₂ using a face mask. A filament (nylon monofilament 6/0, Sutures Aragó, Spain) was introduced (11 mm) through the external carotid artery to the level where the MCA branches out. Mice were allowed to recover from the anesthesia, and the mice were anesthetized again 5 min before reperfusion. The filament was carefully removed, and the cerebral blood flow was restored 1 h after MCA occlusion. Sham surgery was performed with a vertical cervical incision.

2.4. Determination of cerebral blood flow

In order to monitor the cerebral blood flow (CBF) in mice after surgery, a laser-Doppler flow probe was secured on the skull. The scalp was incised at the midline and the skull was exposed. The probes were affixed with glue to the skull surface 2 mm posterior and 3 mm lateral to the bregma and 1 mm caudal to the coronal suture on exposed temporal bone of the ipsilateral hemisphere. This location corresponds to the region supplied by the MCA that becomes severely ischemic upon occlusion (14). Occlusion of flow was indicated by a decrease in the laser-Doppler flow greater than 70% of the baseline. CBF signals from both hemispheres were simultaneously monitored throughout the surgery.

2.5. Evaluation of neurological deficits

Neurological deficits were evaluated using two

methods. Functional stroke injury was evaluated with the neurological deficit score (NDS) immediately after reperfusion and before sacrifice. Neurological impairment was assessed using an NDS between 0 and 4 points, where 0 points = no neurological deficits; 1 point = forelimb weakness; 2 points = circling to the affected side; 3 points = falling to the affected side; and 4 points = unable to walk spontaneously (15). Animals with a score of 1-3 points after reperfusion were used in the following experiment, and if necessary, were used to randomly supplement the number of laboratory animals. The mice were subsequently scored 12 h, 24 h, 48 h, and 72 h after reperfusion.

The rotarod test was used to analyze the motor coordination and resistance to fatigue of the mice. The initial rotating speed and accelerating speed of the rotarod machine (Med Associates) can be adjusted. The parameters used in this study were the latency time to the first fall and the number of falls in five minutes. Mice were trained at 2-20 rpm in the days prior to testing. Motor ability was tested 6 h, 12 h, 24 h, 48 h, and 72 h after surgery.

2.6. Determination of water content in the brain

Mice were sacrificed 24 h after reperfusion and their brains were removed and divided into ipsilateral and contralateral hemispheres. The brains were wrapped in tin foil and weighed and dried for 24 h at 100°C. Water content in the brain was calculated as (wet weight – dry weight) / wet weight × 100%.

2.7. Triphenyltetrazolium chloride (TTC) staining

The area of infarction was evaluated with TTC staining as described previously (16). Two-mm brain sections were incubated with 2% TTC at 37°C for 30 min with gentle shaking and then fixed with 10% formalin in PBS. The stained slices were photographed, and the size of the area of infarction was determined by subtracting the area of the non-infarcted ipsilateral hemisphere from that of the intact contralateral hemisphere. The percentage of the infarct volume was calculated by dividing the sum of the area from all sections of infarction by the total of that of the contralateral hemisphere to avoid the influence of tissue edema (17).

2.8. Histological examination

Fixed 2-mm brain sections were embedded in paraffin, sliced into 5-μm sections, and then dewaxed and stained with hematoxylin and eosin (H&E). The sections were histologically examined under a microscope.

2.9. Western blotting

Total proteins were extracted from frozen brain tissue (n

= 3 in each group). Brain tissue lysates were prepared as described previously (18). Briefly, brain tissue was homogenized and centrifuged, and the supernatant was collected. Bradford's method was used to determine the protein concentrations (19), and bovine serum albumin (BSA) standard protein was used as standard. Western blotting was performed using standard protocols. The proteins were separated with SDS-PAGE and then transferred and blocked. The primary antibodies were cPLA2α antibody, 5-LOX antibody, COX-2 antibody, and β-actin antibody. Protein bands were quantified using Quantity One software (Bio-Rad).

2.10. Measurement of PLA2 activity

In order to measure the inhibition of cPLA2α by anti-cPLA2α mAb, the cPLA2α Assay Kit was used to determine cPLA2α activity from protein extracts, as previously described (20,21). The results are expressed as μmol/min/μg of protein.

2.11. RNA extraction and qPCR

Total RNA extraction, RT-PCR, and real-time PCR were performed as described previously (22). Total RNA ($n = 3$ in each group) was extracted from tissues or cells using TRIzol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized using PrimeScript reverse transcriptase and oligo (dT). Quantitative real-time PCR was performed with the SYBR Premix Ex Taq™ II PCR kit according to the manufacturer's instructions. Relative levels of cPLA2α mRNA were calculated on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, CA, USA) using SYBR green and the DDCT method with β-actin as the endogenous reference gene amplified from the samples. The forward and reverse primers were as follows: cPLA2α, 5'-GGTGGGAGAGAAGAAAGAAGTC-3' and 5'-AGGGATTTTGGATTGTGCGACC-3'; COX-2, 5'-CCCTTCTGCCTGACACCTTTC-3' and 5'-AACCTTGCCAGCAATTTGC-3'; 5-LOX, 5'-TGCTGAGCGCAACAAGAAGA-3' and 5'-ACACCTGCCAGGCAGGA-3'; β-actin, 5'-GCTATGCTCTCCCTCACGCCAT-3' and 5'-TCACGCACGATTTCCCTCTCAG-3'. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method. β-actin served as an appropriate reference gene in this experiment.

2.12. Measurement of LTB4 and PGE2

The levels of leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) were examined using commercially available ELISA kits. The experiment need 50 μL serum samples from mice, and all operations were performed according to the manufacturer's instructions.

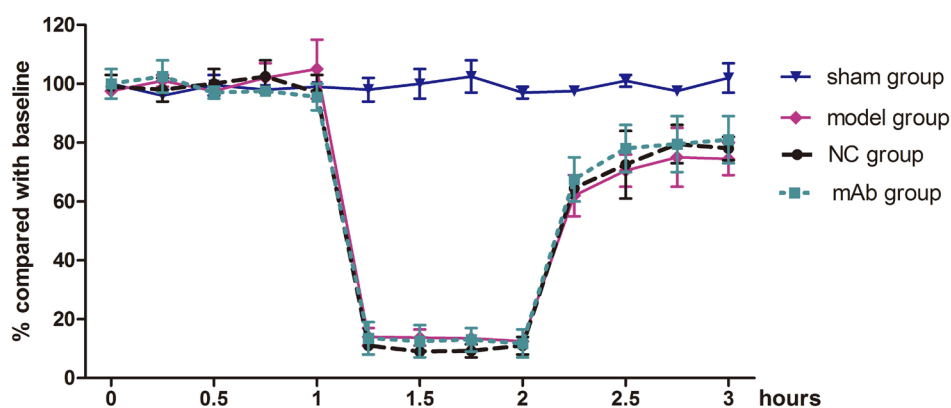


Figure 1. The effect of anti-cPLA2 α mAb on blood flow in the brains of model mice. Laser-Doppler flow measurement of cerebral blood flow was performed (as described in the Methods) before surgery (0-1 h), during the surgery (1-2 h), and after reperfusion (2-3 h). Results are the mean $\pm$ SD for 30 mice. Anti-cPLA2 α mAb treatment did not alter the relative volume of cerebral blood flow.

2.13. Determination of FFA and LPC

FFA and LPC were determined and quantified using HPLC. The needed lipids samples were extracted from brain tissue using the Folch method (23). FFA was determined and quantified using high-performance thin layer chromatography (HPTLC) plates (24). Quantification of LPC was performed using one-dimensional HPTLC as previously described (25).

2.14. TUNEL staining

To evaluate the degree of apoptosis, TUNEL staining was performed on brain sections using an In Situ Cell Death Detection Kit, POD (Roche). Tissues were prepared as paraffin sections and dewaxed before the TUNEL assay; all procedures were done in accordance with the manufacturer's instructions. Results were recorded using a fluorescence microscope equipped with AxioVision software. Four brains samples from each group were analyzed. At least 4 microscopic fields within the ischemic penumbra of each brain sample were photographed, and TUNEL-positive cells were counted in a double-blinded manner. An average of the TUNEL-positive cells counted from these areas was calculated to represent each brain section.

2.15. Statistical processing

All values are presented as the mean $\pm$ SD. Each value is the mean for each group from at least three separate experiments. Data were analyzed by comparing two groups using the Student's *t*-test and * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered to indicate significant differences.

3. Results

3.1. Effects of anti-cPLA2 α mAb on the expression and activity of cPLA2 α

The role of cPLA2 α in ischemic brain injury has been determined in cPLA2 α -knockout (cPLA2 α -/-) mice, which are partially protected from transient focal cerebral ischemia. The current study evaluated whether the use of anti-cPLA2 α mAb could reduce stroke injury in a mouse model of cerebral IR injury. In a separate group of mice, laser-Doppler flowmetry was initiated prior to treatment and continued until 1 h after reperfusion (Figure 1). The relative blood flow did not change as a result of anti-cPLA2 α mAb treatment during the period of observation (Figure 1). Anti-cPLA2 α mAb effectively decreased cPLA2 α expression in mice with ischemic damage 6 h, 12 h, 24 h, 48 h, and 72 h after surgery compared to expression in the NC group (Figure 2, $p < 0.05$, $n = 3$). cPLA2 α activity was measured in homogenates of ischemic (ipsilateral) hemispheres using an *in vitro* assay. cPLA2 α activity was reduced by anti-cPLA2 α mAb in comparison to the level of activity in the NC group and the model group 24 h after reperfusion (Figure 2D, $p < 0.05$, $n = 3$).

3.2. Effects of anti-cPLA2 α mAb on neurological function in animals after ischemic stroke

To study the effect of cPLA2 α on neurological function in ischemic stroke, NDS and rotarod tests were performed 2 h, 12 h, 24 h, 48 h, and 72 h after reperfusion. All of the animals had neurological injury 2 h after reperfusion, which suggests that the surgery was successful and would facilitate further analysis. Neurological deficits were evaluated again 12 h, 24 h, 48 h, and 72 h after reperfusion. The control mice had a higher NDS for up to 72 h; the score for the mAb group decreased in a time-dependent manner and was quite low after 72 h (Figure 3A). Neurological deficits did not differ significantly in animals that underwent surgery 6 h after surgery ($p > 0.05$). The NDS for the mAb group was significantly lower than that for the NC group and the model group 24 h, 48 h, and 72 h after a stroke ($p < 0.05$). Before surgery, the mice did not differ in their

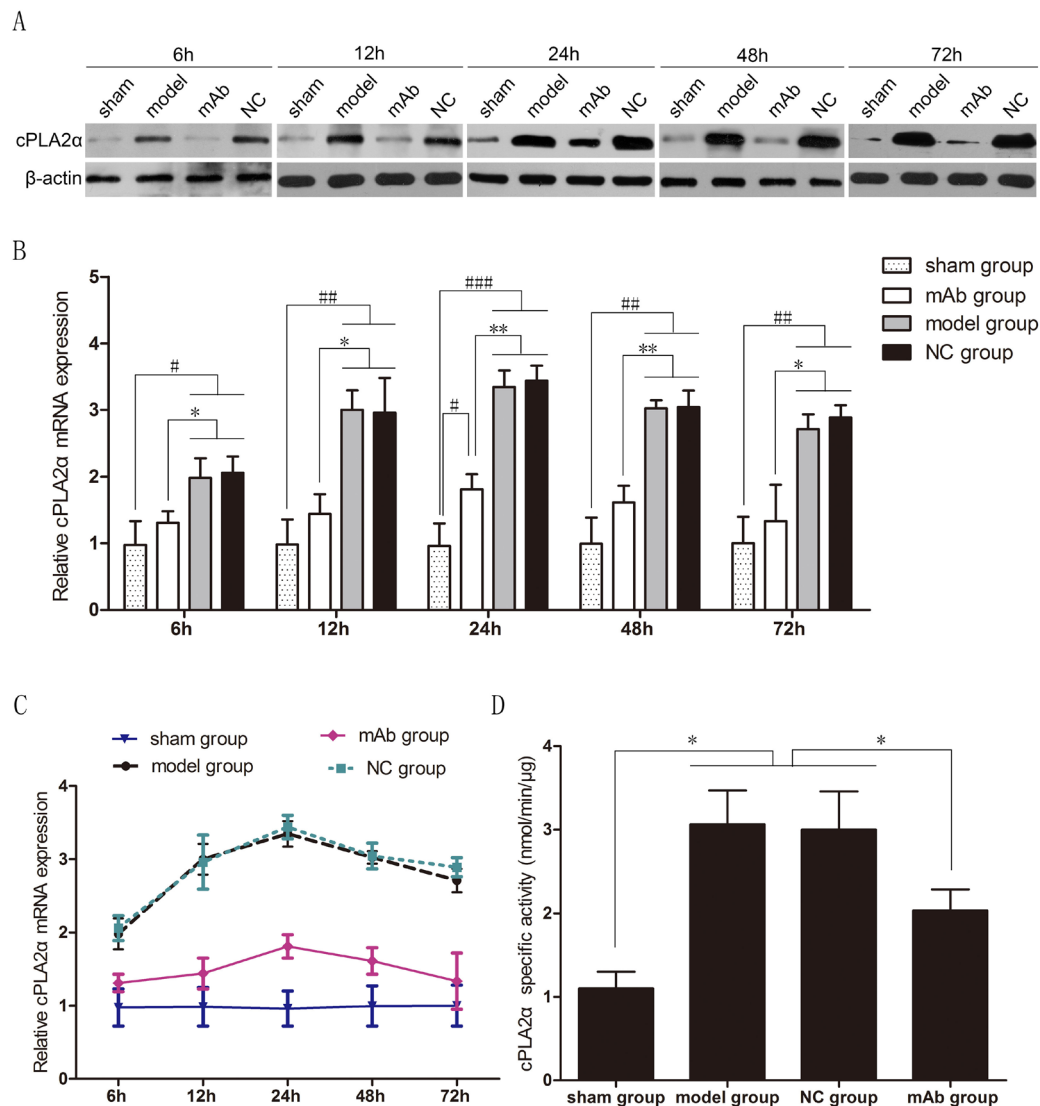


Figure 2. The effect of anti-cPLA2α mAb on the expression and activity of cPLA2α. (Panel A) A representative western blot of cPLA2α protein levels in whole brain lysates of mouse brains 6 h, 12 h, 24 h, 48 h, and 72 h after reperfusion following a stroke **(Panels B and C)**. Levels of cPLA2α mRNA expression 6 h, 12 h, 24 h, 48 h, and 72 h after reperfusion were detected with real-time PCR. Anti-cPLA2α mAb treatment effectively decreased cPLA2α expression in mice with ischemic damage 6 h, 12 h, 24 h, 48 h, and 72 h after surgery compared to the NC group and the model group. **(Panel D)** cPLA2α activity in mice with focal ischemic brain damage 24 h after reperfusion. cPLA2α activity was reduced by anti-cPLA2α mAb treatment in comparison to that in the NC group and the model group 24 h after reperfusion. Data are presented as the mean $\pm$ SD of three independent experiments. Statistically significant differences are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t*-test.

performance on the rotarod test (Figure 3B). However, 12 h after a stroke, the measured number of falls during the rotarod tests was significantly higher in mice that underwent surgery compared to sham-operated mice (Figure 3B, $p < 0.001$). Mice in the mAb group appeared to recover completely as assessed by the number of falls 72 h post-stroke, whereas the NC group and the model group still exhibited marked motor dysfunction at that time point. These data suggest that cPLA2α exacerbates stroke-induced motor dysfunction in mice.

3.3. Effects of anti-cPLA2α mAb on reducing ischemia-induced brain edema

Formalin-fixed, paraffin-embedded archival tissue The

wet-dry method was used to measure water content in the brain. The water content in ipsilateral hemispheres was calculated to be $77.5\% \pm 2.4\%$ in the sham-operated group compared to $85.9\% \pm 1.1\%$ in the model group and $85.9\% \pm 1.2\%$ in the NC group. The water content in the mAb group decreased significantly as a result of anti-cPLA2α mAb treatment (mAb vs. model, $82.8\% \pm 0.8\%$ vs. $85.9\% \pm 1.1\%$, $p < 0.05$; mAb vs. NC, $82.8\% \pm 0.8\%$ vs. $85.9\% \pm 1.2\%$, $p < 0.05$).

3.4. Effects of anti-cPLA2α mAb on reducing ischemia-induced brain infarct volume after reperfusion

Brain infarcts were measured 72 h after reperfusion using a standard laboratory volumetric analysis of

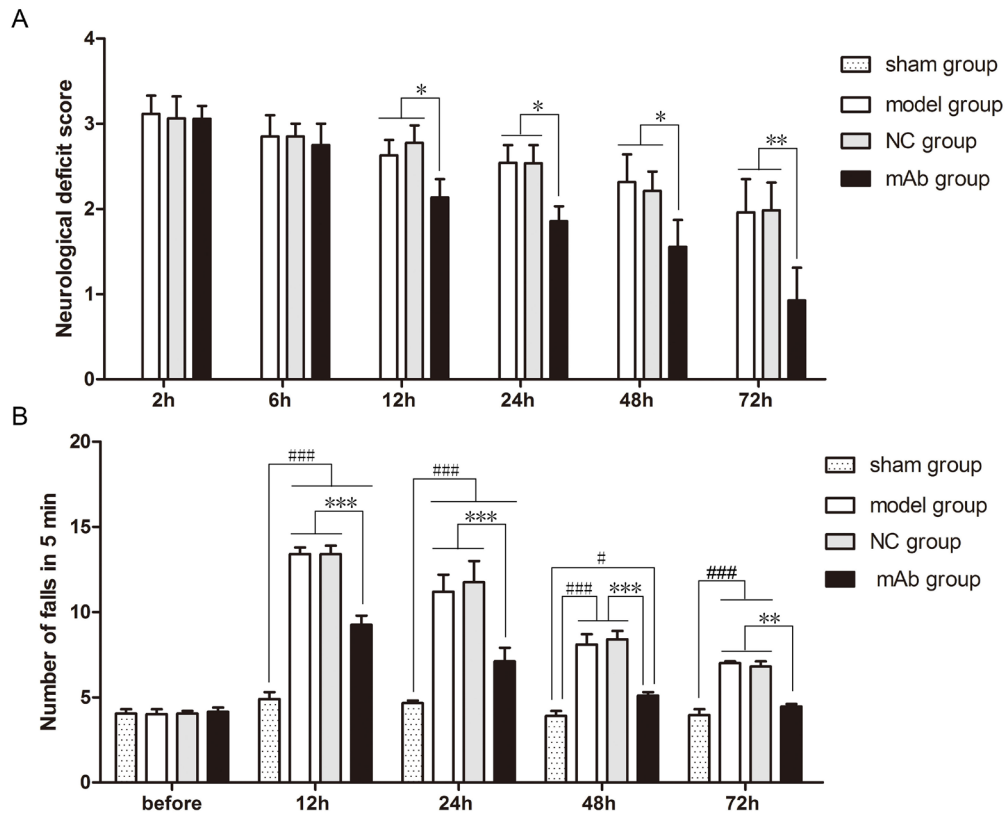


Figure 3. The effect of cPLA2 α on neurological function in ischemic stroke. (Panel A) Neurological deficit scores 2 h, 12 h, 24 h, 48 h, and 72 h after reperfusion. Neurological deficits did not differ significantly in animals that underwent surgery 6 h after surgery ($p > 0.05$). The neurological deficit score for the mAb group was significantly lower than that for the NC group or the model group 24 h, 48 h, and 72 h after a stroke ($p < 0.05$). **(Panel B)** The average number of falls in 5 min measured during a rotarod test. Before surgery, the mice did not differ in their performance on the rotarod test. However, 12 h after a stroke, the number of falls during the rotarod test was significantly higher for mice that underwent surgery compared to the number for sham-operated mice ($p < 0.001$). Mice in the mAb group appeared to recover completely as assessed by the number of falls at the subsequent time point after a stroke. Data are presented as the mean $\pm$ SD. Asterisks denote the following p -values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t -test. Experiments were performed in triplicate.

anterior and posterior views of coronal sections stained with TTC and corrected for swelling. No infarction was observed in the sham-operated group, and an extensive lesion was observed in both the model and NC groups. Anti-cPLA2 α mAb treatment effectively decreased cerebral injury with a significant reduction in infarct volume in the ischemic hemisphere in comparison to the NC group and the model group 72 h after reperfusion (Figure 4, $p < 0.01$, $n = 3$).

3.5. Effects of anti-cPLA2 α mAb on pathological changes in animals after ischemic stroke

Pathological changes were visually observed using HE staining. An examination revealed that the morphological structures of brain tissue in the sham-operated group were normal (Figure 5). In contrast, the morphological structures of brain tissue in the model group and NC group changed significantly; the tissues were loose and intercellular edema was visible. Similar results were observed in the mAb group: the morphological structures of brain tissue changed, but less markedly so (Figure 5). Such findings suggested that the treatment with anti-cPLA2 α mAb ameliorated pathological changes in

animals after ischemic stroke.

3.6. Effects of cPLA2 α on brain cell apoptosis in animals after ischemic stroke

TUNEL staining was performed to detect whether anti-cPLA2 α mAb treatment would decrease the number of apoptotic cells. The number of TUNEL-positive cells in brain tissue from injured mice increased significantly in comparison to the sham-operated group 6 h, 24 h, 48 h, and 72 h after reperfusion (Figure 6, $p < 0.001$, $n = 3$). The number of TUNEL-positive cells in the mAb group decreased in comparison to that in the NC group and the model group (Figure 6, $p < 0.01$, $n = 3$). Results indicated that anti-cPLA2 α mAb may ameliorate pathological changes by inhibiting apoptosis after surgery.

3.7. Effects of anti-cPLA2 α mAb on the expression of proinflammatory lipid mediators

cPLA2 α play an important role in catalyzing the hydrolysis of phospholipids in sn-2, generating FFA and lysophospholipids (26). To test whether anti-cPLA2 α

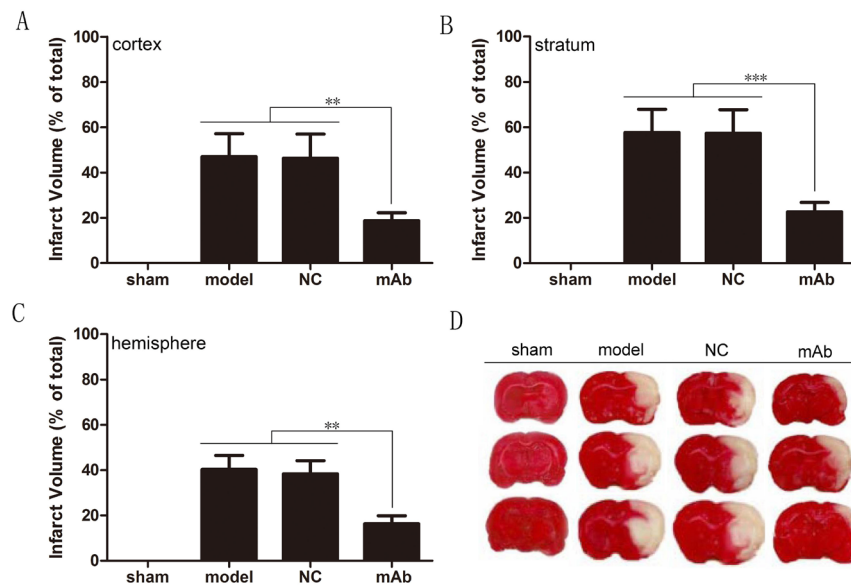


Figure 4. The effect of anti-cPLA2 α mAb on reducing ischemia-induced brain infarct volume 72 h after reperfusion. Continuous infusion with anti-cPLA2 α mAb reduces ischemia-induced brain infarct volume 72 h after reperfusion. Infarct volume ($\pm$ SD) in the striatum, cortex, and hemisphere was measured. Cortical and hemispheric infarct volumes were significantly smaller in the mAb group than those in the NC group or the model group. * $p < 0.05$, ** $p < 0.01$.

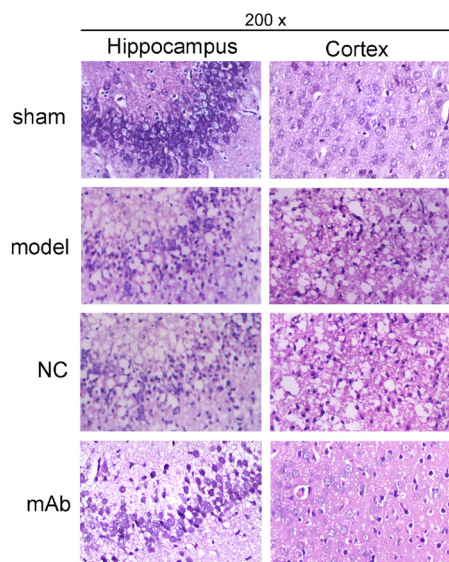


Figure 5. The effect of anti-cPLA2 α mAb on pathological changes in the brains of mice after surgery. Pathological changes in hippocampal and cortical tissues as a result of anti-cPLA2 α mAb treatment (magnification, $\times 200$). An examination revealed that the morphological structures of brain tissue in the sham-operated group were normal. The morphological structures of brain tissue in the model group and NC group changed significantly; the tissues were loose and intercellular edema was visible. The morphological structures of brain tissue in the mAb group also changed, but less markedly so in comparison to the model group and the NC group.

mAb decreased the levels of phospholipid degradation products, cPLA2-derived injurious lipid mediators and the expression of 5-LOX and COX-2 in brain tissue were assessed. Anti-cPLA2 α mAb treatment significantly reduced the levels of 5-LOX and COX-2 in brain tissue (Figure 7B and D, $p < 0.01$, $n = 3$). Anti-cPLA2 α mAb treatment decreased FFA and LPC compared to levels in the NC group and the model group (Figure 7E and

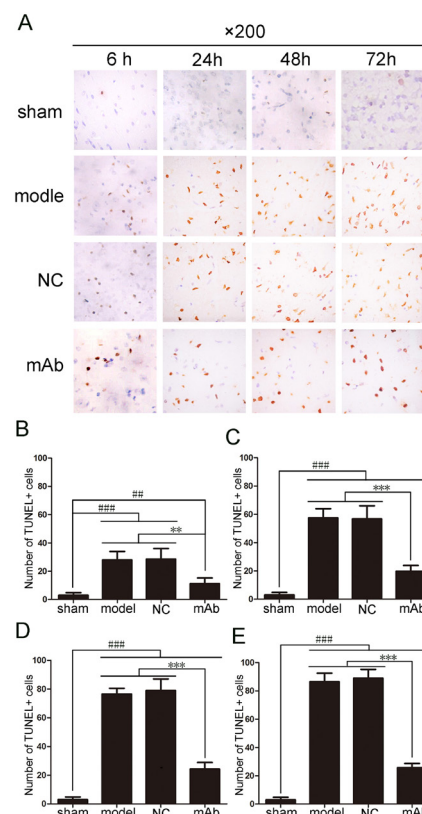


Figure 6. The effect of anti-cPLA2 α mAb on apoptosis in brain cells of mice after surgery. (Panels B, C, D, E) The number of TUNEL-positive cells was counted 6 h, 24 h, 48 h, and 72 h after reperfusion. The number of TUNEL-positive cells in the ischemia reperfusion group increased significantly compared to that in the sham-operated group 6 h, 24 h, 48 h, and 72 h after reperfusion. The number of TUNEL-positive cells in the mAb group was smaller than that in the NC group or the model group. Data are presented as the mean $\pm$ SD. Statistically significant differences are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mAb group vs. the NC group or model group; # $p < 0.05$, ### $p < 0.001$, sham-operated group vs. the model group, NC group, or mAb group.

G, $p < 0.05$, $n = 3$). The mAb group had significantly lower levels of PGE2 in serum than the NC group or the model group 24 h after surgery (Figure 7H, $p < 0.01$, $n =$

3). Similarly, the levels of LTB4 in serum were lower in the mAb group than those in the NC group or the model group (Figure 7F, $p < 0.001$, $n = 3$).

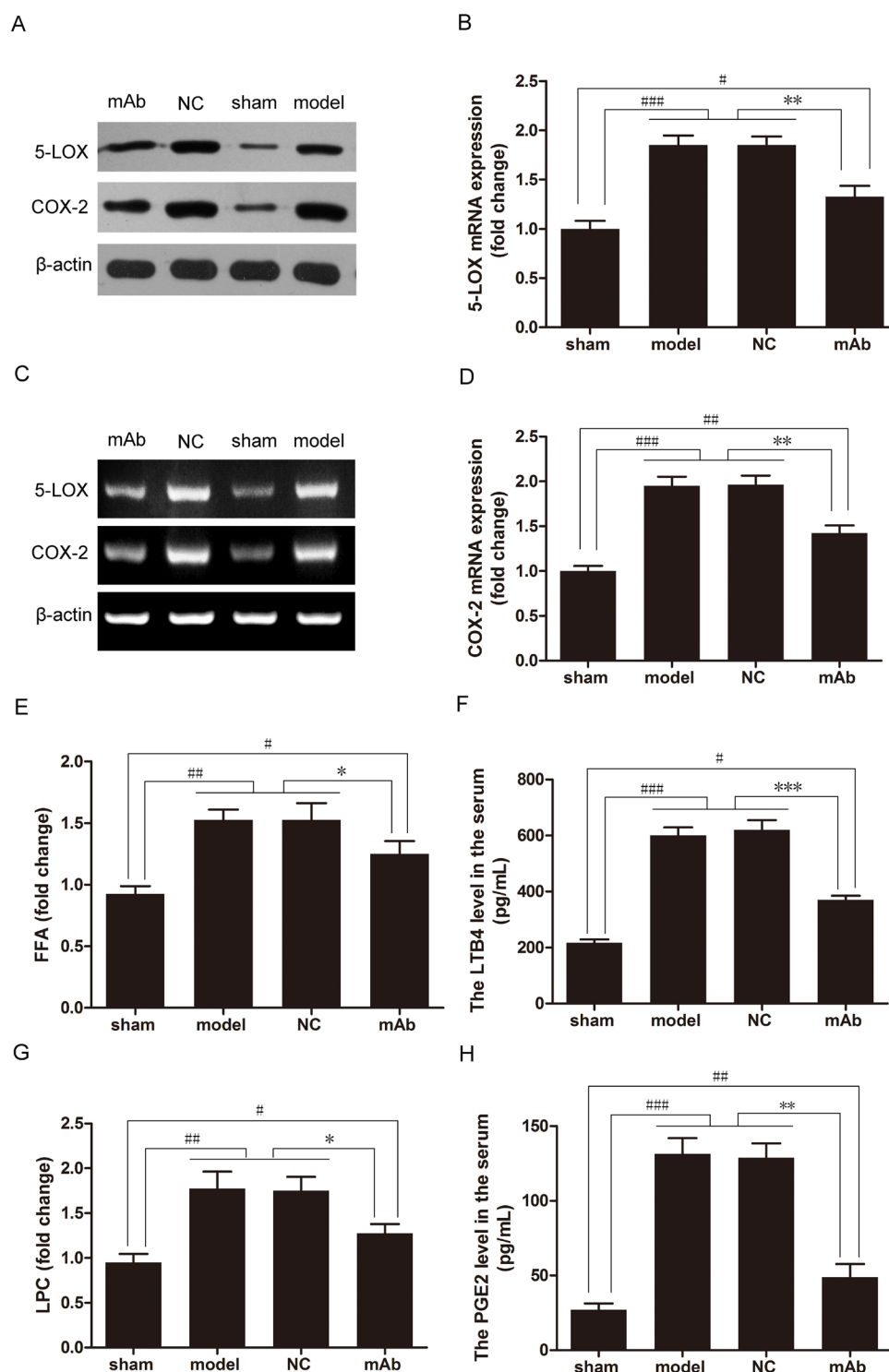


Figure 7. The effect of anti-cPLA2α mAb on the expression of 5-LOX, COX-2, FFA, LPC, PGE2, and LTB4. (Panel A) A representative western blot of 5-LOX and COX-2 protein levels in whole brain lysates of mice 24 h after reperfusion following a stroke. **(Panels B, C, and D)** Levels of 5-LOX and COX-2 mRNA expression in brain tissue from mice 24 h after surgery. Anti-cPLA2α mAb treatment significantly reduced the levels of 5-LOX and COX-2 in brain tissue. **(Panel C)** **(Panels E and G)** Levels of FFA and LPC in brain tissue from mice 24 h after surgery. Anti-cPLA2α mAb treatment decreased FFA and LPC compared to levels in the NC group and the model group. **(Panels F and H)** Levels of LTB4 and PGE2 in serum from mice 24 h after surgery. Animals in the mAb group had significantly lower levels of LTB4 and PGE2 in serum than did the NC group or the model group 24 h after surgery. Anti-cPLA2α mAb treatment decreased the levels of FFA, LPC, PGE2, and LTB4. Data are presented as the mean $\pm$ SD of three independent experiments. Statistically significant differences are indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mAb group vs. the NC group or model group; # $p < 0.05$, ### $p < 0.001$, sham-operated group vs. the model group, NC group, or mAb group.

4. Discussion

Cerebral ischemia injury is a common clinical condition that poses a substantial burden to a patient's family and society. The goal of clinical treatment of cerebral ischemia is to restore blood flow and oxygen supply, suppress inflammation of the ischemic area, and maintain the integrity of the neuronal structure and function. A growing body of evidence has demonstrated that cPLA2 α contributes to the biosynthesis of eicosanoids by releasing AA and that it is associated with various inflammatory diseases (2,5-7,27,28). The current study provides evidence that cPLA2 α is essential for speedy recovery from cerebral ischemia and that it is closely related to inflammation and neurological deficits.

cPLA2 are a group of hydrolytic enzymes that act on membrane phospholipids to release free unsaturated fatty acids. The activation of cPLA2 contributes to an increase in AA, and followed by the generation of FFA and lysophospholipids upon injury. AA itself can cause BBB dysfunction and subsequent brain edema (10,11) and can be converted into lipid mediators *via* COX and LOX pathways (8,9). The final products are prostaglandins, thromboxanes, and leukotrienes, which are members of a group of biologically active oxygenated fatty acids. These oxygenated fatty acids are important modulators of pain in inflammation and immunomodulatory agents. cPLA2 plays an important role in several physiological and pathophysiological processes, including immunity, reproduction, cancer, and inflammation (2,12,26,29-34). Inhibition of the expression of cPLA2 has been proven to improve functional recovery in stroke, Alzheimer's disease, and multiple sclerosis (35,36). The current study and other studies have found that cPLA2 signaling is predominant in cerebral ischemia, suggesting that upregulation of this enzyme after trauma may be responsible for secondary injury and thus inflammation, nociception, and functional deficits (6).

Patients with ischemic brain damage are prone to partial or complete loss of motor function, clinically known as paralysis. Seventy-five percent of patients with cerebral ischemia have varying degrees of neurological deficits (37), and motor function deficits are an important manifestation of neurological deficits (38). Neurological deficits are a characteristic of cerebral ischemia. Motor function recovery is an index to determine the therapeutic efficacy of a drug. cPLA2 α plays a significant role in causing neurological injury following ischemic brain injury. In the current study, a significant neurological deficit was observed 2 h after cerebral IR injury surgery, validating the cerebral ischemia model. The mAb group had a lower NDS than the NC group and the model group, corroborating the contention that cPLA2 α contributes to functional injury (Figure 3).

A previously suggested mechanism is one whereby BBB disruption in mice after cerebral IR injury surgery is associated with increased cPLA2 α activity 24 h after reperfusion (39). The current study evaluated whether the use of anti-cPLA2 α mAb would reduce stroke injury in this mouse model. The expression of cPLA2 α peaked 24 h after reperfusion (Figure 2C), and cPLA2 α activity decreased markedly as a result of anti-cPLA2 α mAb at the same time point. Treatment with anti-cPLA2 α mAb significantly reduced the expression of cPLA2 α for up to 72 h after reperfusion. cPLA2 plays an important role in physiological and pathophysiological processes. The current results indicate that anti-cPLA2 α mAb may ameliorate pathological changes in animals after ischemic stroke by inhibiting apoptosis after surgery (Figures 5 and 6), and anti-cPLA2 α mAb treatment effectively decreased cerebral injury caused by cerebral IR injury surgery with a significant reduction in infarct volume in the ischemic hemisphere (Figure 4).

Inhibition of cerebral ischemia by cPLA2 α treatment confers anti-inflammatory and neuroprotective effects, leading to increased functional recovery. Many mediators of inflammation are mediated by COX and LOX cascades. cPLA2 α plays an important role in the release of AA from membrane phospholipids linked to eicosanoid production in various pathological states. FFA are the product at the start of the COX and LOX cascades, and FFA are subsequently metabolized to PGE by COX-2 and to LTB4 by 5-LOX. PGE are potent vasodilators that contribute to the increased blood flow in inflamed areas. LTB4 is a chemoattractant for leukocytes and may initiate monocyte recruitment. LPC is a signaling molecule involved in chronic inflammation and tissue damage (40). The current study found that inhibition of cPLA2 decreased the expression and production of both COX-2 and 5-LOX and that anti-cPLA2 α mAb treatment effectively reduced the expression of those proinflammatory lipid mediators (Figure 7), indicating that both originated from cPLA2. PLA2-derived proinflammatory lipid mediators such as PGE2, LTB4, LPC, and FFA are implicated in ischemic brain damage, and anti-cPLA2 α mAb treatment may reduce COX-2 and 5-LOX-derived inflammatory mediators in ischemic brain damage.

Together, the current findings suggest that cPLA2 α plays a significant role in effectively and efficiently protecting cells from adverse effects of a stroke and that cPLA2 α inhibition may ameliorate pathological changes by reducing inflammation and inhibiting apoptosis after cerebral IR injury. The beneficial effects of cPLA2 α treatment may be primarily due to the inhibition of cPLA2 and the reduction of proinflammatory lipid mediators originating from the activation of cPLA2. The neuroprotection conferred by anti-cPLA2 α mAb treatment has noteworthy implications that since mAb against cPLA2 α may one day be used as a treatment for stroke in clinical settings.

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Poly(U) and CpG ameliorate the unbalanced T cell immunity and pneumonia of mice with RSV vaccine-enhanced disease

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Summary

Respiratory Syncycial Virus (RSV) is the most important pathogen responsible for children's severe lower respiratory tract infection. So far no RSV vaccine has yet been authorized for clinical use. The main impediment that blocked development of RSV vaccine is that inactivated RSV vaccine could cause RSV vaccine-enhanced disease (RVED). The mechanism of RVED remains unclear. Recently some researchers found that insufficient activation of innate immunity, including Toll-like receptors (TLRs), might be associated with the onset of RVED. Based on the above findings, this research was conducted to further study the mechanism of RVED. We first vaccinated mice with formalin-inactivated RSV vaccine (FIRSV) and then exposed them to RSV to establish a RVED mouse model. Consequently, we found that mice previously inoculated with FIRSV showed obvious weight loss and extensive pneumonia, as well as T helper 2 cells (Th2)-biased immunity and suppressed CD8⁺T cell immunity after viral exposure, suggesting that we have successfully established a RVED mouse model. Then based on this model, we further added Poly(U) (TLR7/8 agonist) and CpG (TLR9 agonist) in FIRSV to see if RVED could be ameliorated. As a result, mice inoculated with FIRSV supplemented with Poly(U) and CpG had a much relieved weight loss and pneumonia, as well as suppressed Th2-biased immunity and strengthened CD8⁺T cell function. Thus, the insufficient stimulation of TLR7/8 and (or) TLR9 might play a role in the development of RVED, which could provide evidence for using TLR agonists as vaccine adjuvants to confer a protective immune response against RSV.

Keywords: Respiratory syncytial virus (RSV), RSV vaccine-enhanced disease, Th2-biased immune response, Toll-like receptor

1. Introduction

Respiratory Syncycial Virus (RSV) is the leading cause of severe lower respiratory tract infection in infants, the elderly and immune-compromised individuals (1). As has been reported, 200,000 children under 5 years old have died of RSV infection every year (2). Nonetheless, the

deleterious impact of RSV infection on pediatric health is still difficult to avoid because no RSV vaccine has yet been authorized for clinical use (3). The development of RSV vaccine has been hampered by concerns about its safety and effectiveness, given that the first RSV vaccine clinical test in the 1960s showed that children previously inoculated with formalin-inactivated RSV vaccine (FIRSV) consequently suffered with enhanced respiratory disease after subsequent RSV exposure (4,5). Since it was only seen in vaccine recipients, the above-mentioned disease was called RSV vaccine-enhanced disease (RVED).

Current findings have revealed that RVED is characterized by T helper 2 cells (Th2)-biased immunity, non-neutralizing antibodies and lung eosinophilia

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(5,6). Some researchers demonstrated that the RSV-specific antibodies elicited by inactivated RSV vaccine, including FIRSV and ultraviolet-inactivated RSV vaccine (UVRSV), had relatively low avidity and was considered nonprotective (7,8). It is without a doubt that formalin impaired the immunogenicity of virus antigens to some extent. However, the impaired immunogens were usually sufficient to confer protection in other formalin-inactivated virus vaccines, for example, influenza virus vaccine. Furthermore, it's worth mentioning that vaccine-enhanced disease only exists in RSV, measles virus and metapneumovirus, which all belong to the paramyxoviridae family (9,10). We have no idea whether some inherent properties of the paramyxoviridae family make them more likely to cause vaccine-enhanced disease, and the mechanism of RVED has not been identified yet.

Recently some researchers found that the ineffectiveness of FIRSV was associated with its insufficient stimulation of host's innate immunity (11-13). Toll-like receptors (TLRs), which are considered to be doorkeepers in recognition of exogenous pathogens, play a critical role in induction of host's innate immunity (14). In accordance with their functions, TLRs are mainly expressed inside (e.g. TLR7/8, TLR9) or on the surface (e.g. TLR2, TLR4) of antigen presenting cells (APCs). Among the ten TLRs found in humans, TLR4 is known to be the first TLR to recognize RSV F protein, while TLR7/8 and TLR9 are considered to recognize single strand RNA (ssRNA) and alien DNA of invading pathogens (10,15). MyD88 is a downstream adaptor of most TLRs, including TLR4 and TLR7/8. Recently MyD88 has been found to be important in the maturation of protective antibodies and viral control against RSV (7,16). Moreover, with the help of TLR agonists (e.g. LPS) and TLR4 knockout mice (e.g. C3H/HeJ mice), the critical role of TLR4 in the pathogenesis of RSV-associated diseases, including RVED, has been confirmed (7,12,14,17,18). Therefore, as a supplement to the above findings, we conducted this research to further study the possible function of TLR7/8 and TLR9 in the onset of RVED.

In this study, we established a classic mouse model of RVED at first and then examined whether RVED could be ameliorated by strengthened stimulation of TLR7/8 and TLR9. Here we used two widely-accepted exogenous TLR ligands, namely Poly(U) and CpG. Poly(U) is a synthetic ssRNA which can substitute for viral RNAs to stimulate TLR7/8 (19), while CpG is a synthetic oligonucleotide which can be recognized by TLR9 (20).

2. Materials and Methods

2.1. Viruses and vaccines

RSV A2 strain was kindly offered by Prof. Shibo

Jiang (Key Lab of Medical Molecular Virology of MOE/MOH in Shanghai Medical College of Fudan University) and was propagated in HEp-2 cells. RSV stocks were collected when the cytopathic effect (CPE) appeared in over 90% of the HEp-2 cells. FIRSV was made following the general procedure used to produce the vaccine used in earlier clinical trials (3). Briefly, RSV stocks of 1×10^6 plaque-forming units (PFU)/mL were incubated with formalin (1:4,000) at 37°C and condensed (1/25 of original volume) after centrifugation at 50,000×g. Then the vaccine antigens were adsorbed onto aluminum hydroxide adjuvant at a final concentration of 4mg/mL. The mixture was then centrifuged at 3,000×g and resuspended in DMED (1/4 of original volume) and stored at 4°C. For further study, we added Poly(U) (2 µg/mL) or CpG (100 µg/mL) to FIRSV in the second part of the study. FI-HEp-2 was prepared in the same way as FIRSV but substitute RSV stocks for supernatants of lysed HEp-2 cells were used.

2.2. Immunization and viral challenge

Female 6-8 weeks old BALB/c mice were chosen to conduct the research. The animal study was approved by the institutional review board at Children's Hospital of Fudan University.

In the establishment of the RVED mouse model, mice were divided into 3 groups, namely, group FV, group VV and group BV. On day 1 and day 14, mice of group FV were intramuscularly inoculated with 50 µL FIRSV, while mice of group VV were intranasally infected with 50 µL live RSV (1×10^6 PFU/mL) and mice of group BV were intramuscularly innoculated with 50 µL FI-HEp-2. For the TLR-associated experiments in our second part, group Poly(U) and group CpG were added for a total of five groups. On day 1 and day 14, mice of group Poly(U) and group CpG were intramuscularly inoculated with FIRSV supplemented with Poly(U) and CpG respectively. On day 28, mice of all groups were challenged with live RSV (1×10^6 PFU/mL) intranasally and the daily body weights of all mice were recorded from that time. All mice were sacrificed on day 32 (4th day after challenge).

2.3. Pulmonary histopathology

The lung lobes were removed on the 4th day after challenge, fixed in 4% formalin and then embedded in paraffin. The fixed lung tissues were sliced into 4 µm slides and stained with hematoxylin and eosin (H&E). Pulmonary pathology was observed with light microscopy.

2.4. T cell immunity in splenocytes

Spleens were isolated aseptically and ground tenderly through a 75 µm nylon mesh, then erythrocytes were

lysed to get mononuclear cell suspensions. The cells were stimulated by PMA and Ionomycin at 37°C for 1 hour. Golgi-stop was added for the next 2 hours in order to withhold the secreted cytokines. Afterwards the cells were stained with fluorescent antibodies and assayed using a BD LSR Fortessa™ cell analyzer.

2.5. Quantification of RSV RNA in lungs

On the 4th day after viral challenge, lungs were isolated aseptically and ground thoroughly to get lung homogenates. Total lung RNA was extracted by TRIzol according to the manufacturer's instructions. RT-PCR was conducted with random primers, and quantitative real-time PCR (qPCR) was performed to detect levels of RSV F protein using the primers RSV-F1 (3'-CAARTCAAYATTGAGATAGAATCTA GAA-5'), RSV-F2 (3'-GCTATACAYARTATTATCATCCCACA-5') and the probe (3'-FAM-CTCCAGAATAYAGGCAT GATTCTCC-BHQ-5')

2.6. Statistical analysis

Data were analyzed with statistical software (STATA). Comparisons were made using ANOVA and *t*-test. *p* values < 0.05 were considered statistically significant.

3. Results

3.1. FIRSV recipients developed enhanced disease after RSV challenge

After RSV challenge, mice of group FV showed greater weight loss and slower rehabilitation than mice of group VV and group BV (Figure 1). Pneumonia of different severity existed in mice of all groups, but only mice of group FV suffered with extensive perivascular and peribronchiolar lymphocytic pneumonia as well as lung eosinophilia. The eosinophils dissipated in the inflammatory area are identified by arrows (Figure 2). In contrast, mice of group VV and group BV didn't show eosinophilic infiltration in lungs. Real-time PCR showed that lung RSV load of mice in group FV was much lower than that of group VV and group BV, and mice of group VV showed lower RSV load than that of group BV (Figure 3).

3.2. Mice of group FV showed aberrant T cell immune response

Flow cytometry analysis of splenocytes showed that the proportion of CD4⁺T cells in mice of group FV was highest among the three groups (Figure 4A), as were the levels of IL-4, IL-13 and IL-4/IFN-γ ratio in CD4⁺T cells of group FV (Figure 4B and Figure 4C). No statistical difference was found in the secretion of IFN-γ in CD4⁺T cells. However, the levels of TNF-α

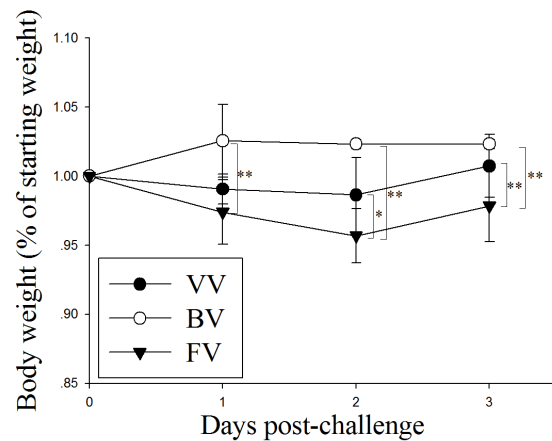


Figure 1. Body weight changes of mice in group FV, group VV and group BV after RSV challenge. On day 1 and day 14, mice of group FV and group BV were intramuscularly inoculated with 50 μL FIRSV and 50 μL FI-HEp-2 respectively, while mice of group VV were intranasally infected with 50 μL live RSV (1×10⁶ PFU/mL). On day 28, mice of all groups were challenged with live RSV (1×10⁶ PFU/mL) intranasally and the daily body weights of all mice were recorded from that time. (**p* < 0.05, ***p* < 0.001)

and IFN-γ in CD8⁺T cells in mice of group FV were much lower than that of group VV. Granzyme B in CD8⁺T cells was too low to be detected with significant differences (Figure 4D).

3.3. The performances of RVED were ameliorated by the admixture of FIRSV and TLR agonists

Similar to the results we mentioned previously, mice of group FV still suffered with the largest weight loss and the most severe pneumonia after RSV exposure. However, mice of group Poly(U) and group CpG showed milder weight loss and pneumonia than that of group FV (Figure 5 and Figure 6). Lung RSV loads in mice of group FV, group Poly(U) and group CpG, although no significant differences were found among the three groups, were much lower than that of group VV and group BV (Figure 7).

3.4. The supplementation of Poly(U) and CpG in FIRSV relieved the aberrant T cell immunity of RVED

As we expected, mice of group FV had polarized Th2 immune response and dampened CD8⁺T cell function, as well as elevated IL-17 secretion. Compared with group FV, the proportions of CD4⁺T cells in mice of group Poly(U) and group CpG were much higher (Figure 8A), while the levels of IL-4 and IL-13 in CD4⁺T cells were much lower (Figure 8B). In group CpG, the secretion of IL-17 in CD4⁺T cells was inhibited compared with that of group FV (Figure 8B). IL-4/IFN-γ ratio in CD4⁺T cells in mice of group Poly(U) and group CpG were also lowered as a concomitant of decreased IL-4 (Figure 8C). The levels

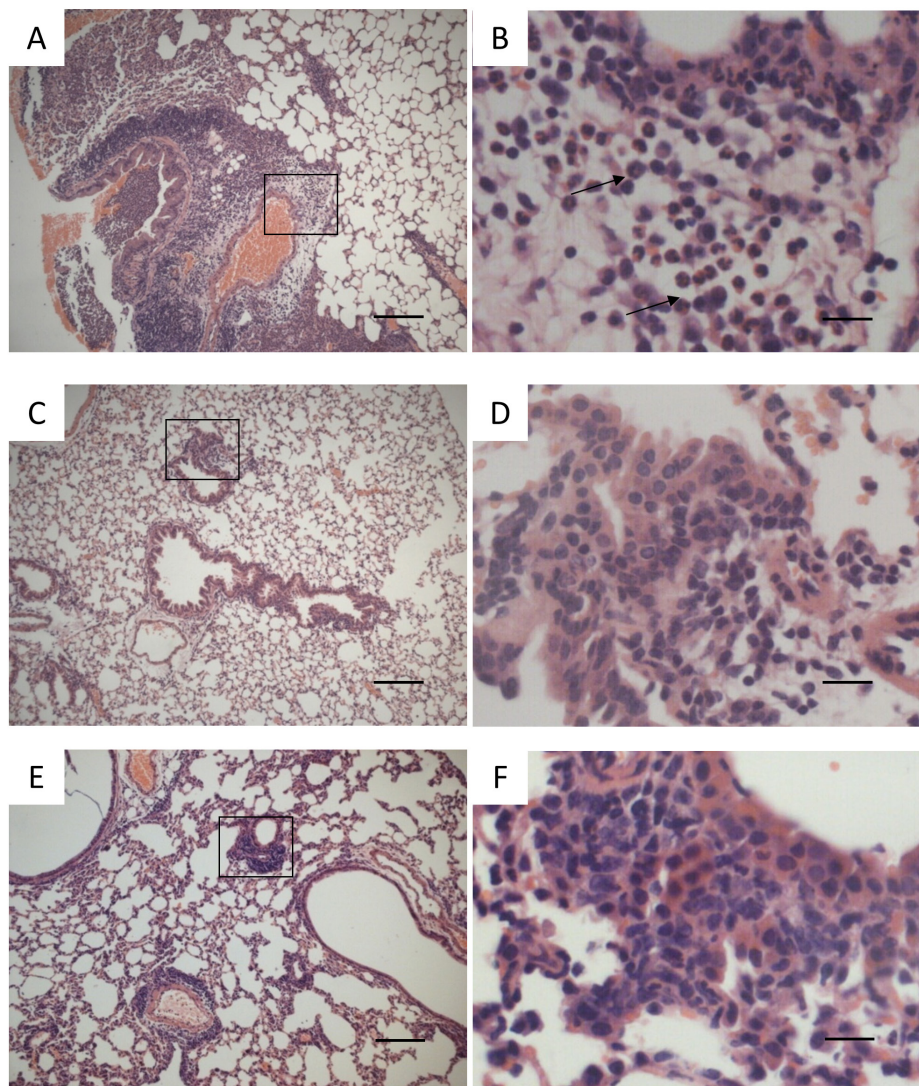


Figure 2. Pulmonary pathology of mice on the 4th day after RSV challenge. On the 4th day after challenge, lungs of mice were isolated and stained with hematoxylin and eosin (H&E). Pulmonary pathology was observed using light microscopy at an original magnification of 100 $\times$ and 400 $\times$. The figure shows the H&E staining of lung tissue in mice of group FV (A), group VV (C) and group BV (E) at an original magnification of 100 $\times$, as well as the 400 $\times$ magnification of the selected area in A (B), C (D) and E (F). Scale bar in A, C and E: 100 μ m. Scale bar in panel B, D and F: 25 μ m.

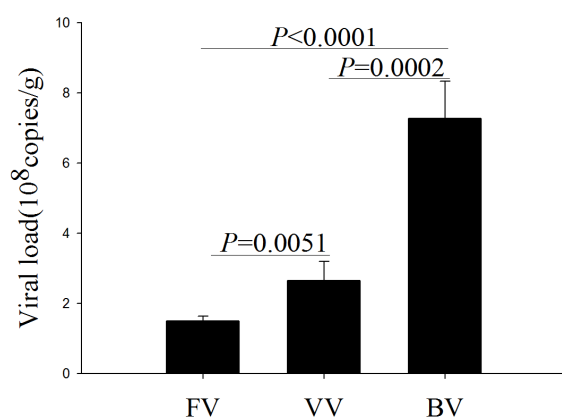


Figure 3. Lung RSV loads in mice of group FV, group VV and group BV on the 4th day after RSV challenge. On the 4th day after viral challenge, lungs of mice were isolated aseptically and ground thoroughly to obtain lung homogenates. Total lung RNA was extracted by TRIzol and then RT-qPCR was conducted to detect the level of RSV in lungs.

of both IFN- γ and TNF- α in CD8⁺T cells in mice of group CpG were upregulated compared to group FV, while for group Poly(U), only TNF- α was obviously elevated (Figure 8D).

4. Discussion

RVED is the main impediment that hampered the development of a RSV vaccine. Therefore the study of the mechanism of RVED is of paramount importance to the prevention and control of RSV infection. In this study, mice of group FV showed largest weight loss, extensive pneumonia and lung eosinophilia after subsequent viral exposure, which highly resembled the performance of children's RVED in the 1960s (21,22). In addition, mice of group FV also demonstrated Th2-biased immunity and CD8⁺T cell dysfunction compared with that of group VV, which were supposed

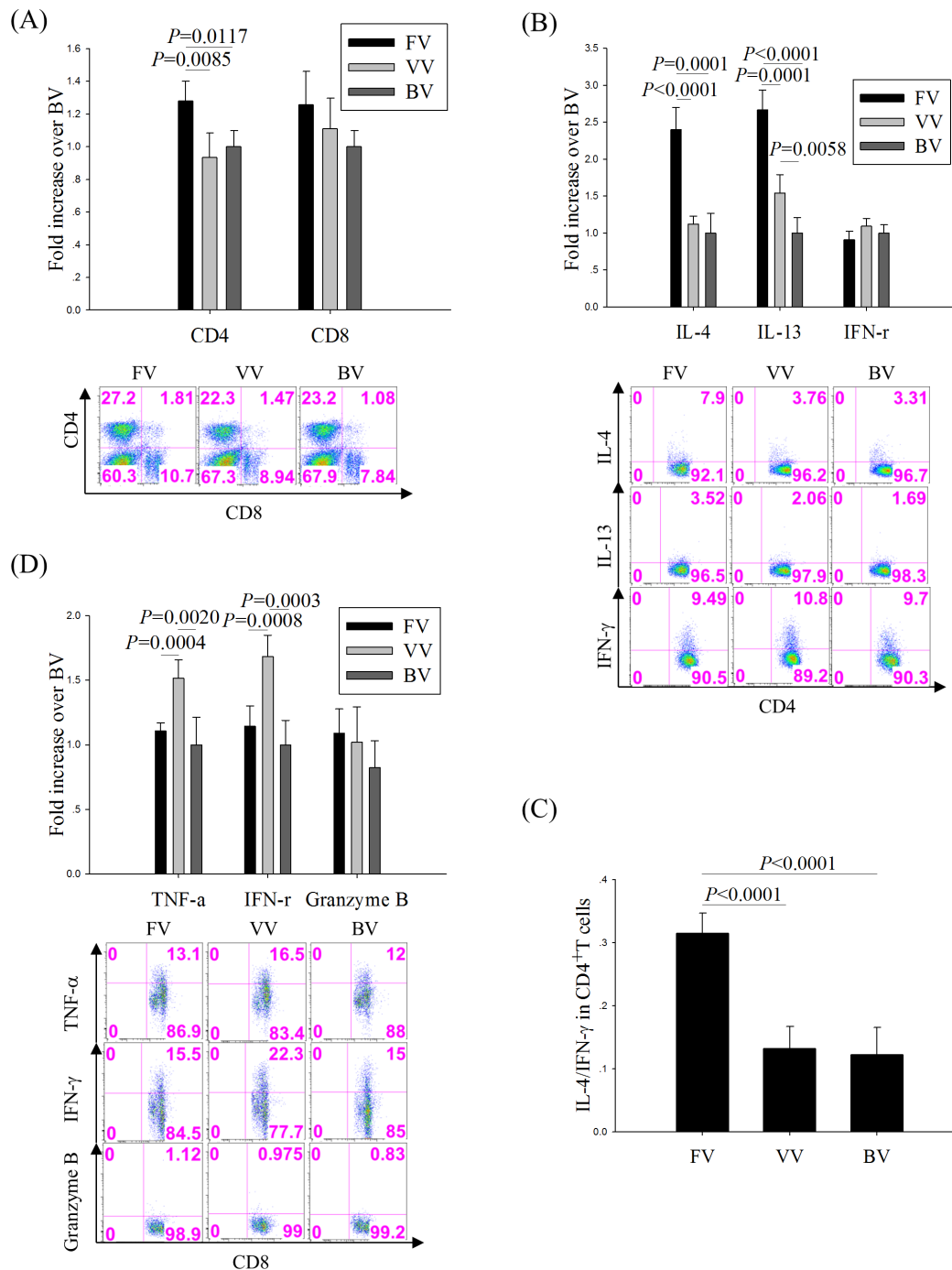


Figure 4. T cell immune responses in mice of three groups. On the 4th day after challenge, spleens of mice were isolated aseptically and ground tenderly to get a cell suspension. The cells were then stained with different fluorescent antibodies for flow cytometry analysis. **(A)** Percentage of CD4⁺T cells and CD8⁺T cells in splenocytes. **(B)** Levels of IL-4, IL-13 and IFN-γ in CD4⁺T lymphocytes. **(C)** Ratio of IL-4/IFN-γ in CD4⁺T lymphocytes. **(D)** Levels of TNF-α, IFN-γ and Granzyme B in CD8⁺T lymphocytes.

to generate relatively effective immunological memory against RSV after their primary infection. Thus we have successfully established a RVED mouse model. Furthermore, we found that FIRSV supplemented with Poly(U) and CpG could efficiently ameliorate the overall condition of RVED mice, suggesting that the insufficient activation of TLR7/8 and TLR9 might play a role in the pathogenesis of RVED.

While other researchers mainly studied the

ineffective humoral immunity of FIRSV recipients (7,23), we highlighted the aberrant cellular immunity. With the help of flow cytometry, we were able to examine the secretions of the representative cytokines in different subtypes of T cells with more sensitivity and accuracy. T helper cells (Th) could promote the proliferation and differentiation of virus-specific B lymphocytes as well as the production of specific antibodies, and thus play a critical role in the

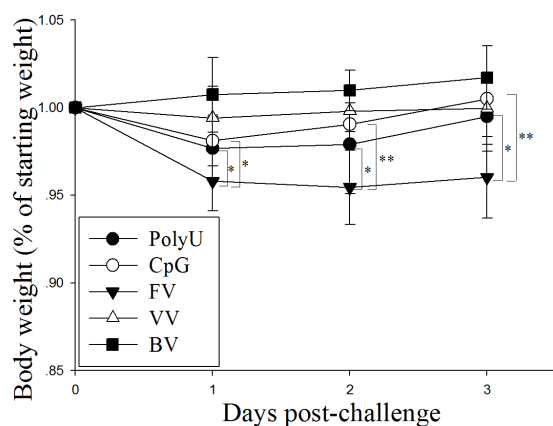


Figure 5. Body weight changes in mice of five groups after RSV challenge. On day 1 and day 14, mice of group Poly(U) and group CpG were intramuscularly inoculated with 50 μ L FRSV supplemented with Poly(U) or CpG respectively. Mice of other groups were treated in the same way as the first part. On day 28, mice of all groups were challenged with live RSV (1×10^6 PFU/mL) intranasally and the daily body weights of all mice were recorded from that time. (* $p < 0.05$, ** $p < 0.001$)

activation of anti-virus immunity (24,25). After antigen stimulation, primary $CD4^+$ T lymphocytes differentiate into Th0 lymphocytes, which then differentiate into Th1, Th2 and Th17 lymphocytes. As one of the most important Th2 cytokines, IL-4 could inhibit Th0 lymphocytes' differentiation into Th1 lymphocytes and promote reconstruction of the respiratory tract and development of pneumonia (26). Besides, IL-17 was reported to increase airway mucus by upregulating expression of the *MUC5B* gene and cause airway hypersensitivity (13,27). As the main source of IL-17, Th17 lymphocytes could also work synergistically with Th2 lymphocytes and inhibit the cytotoxic effect of $CD8^+$ T cells, which was consistent with the increased levels of Th2 and Th17 cytokines as well as the suppressed Th1 and $CD8^+$ T cell cytokines of RVED mice in our data (28). Therefore it's reasonable to think that Th2 cells and Th17 cells might be the main promoters in development of RVED.

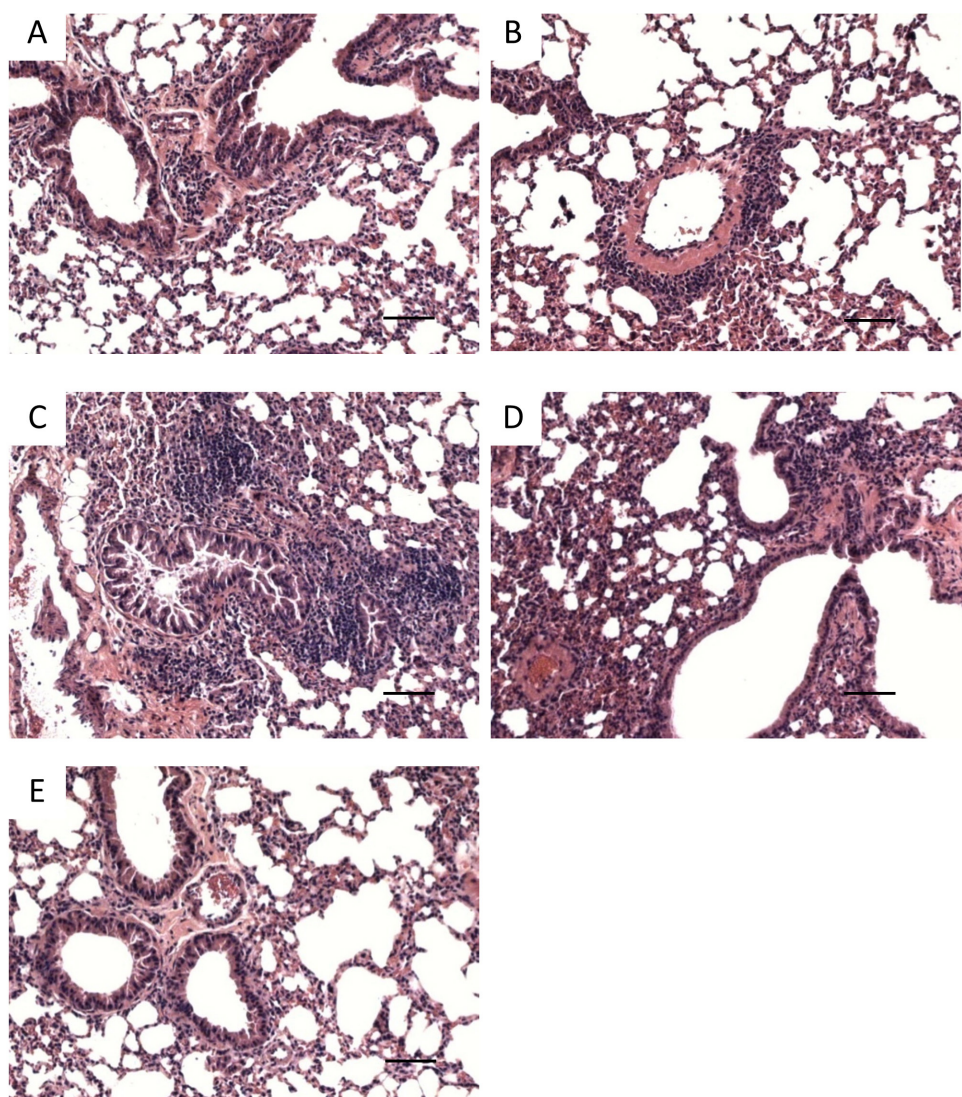


Figure 6. Pulmonary pathology of mice on the 4th day after RSV challenge. On 4th day after challenge, mice of all groups were sacrificed and their lungs were isolated and stained with (H&E). The figure shows the H&E staining photos of group Poly(U) (A), group CpG (B), group FV (C), group VV (D) and group BV (E) at an original magnification of 100 $\times$. Scale bar, 100 μ m.

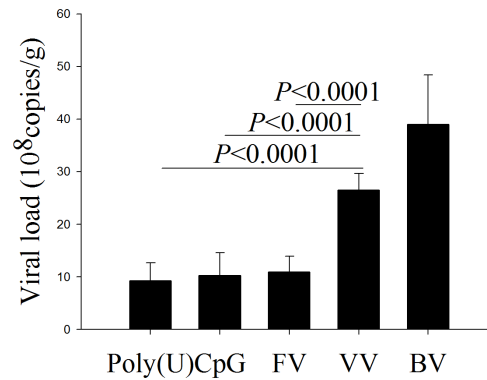


Figure 7. Lung RSV loads in mice of five groups on the 4th day after RSV challenge. On the 4th day after viral challenge, total lung RNA was extracted from lung tissue as described before and RT-qPCR was conducted to detect the level of RSV in mice of each group.

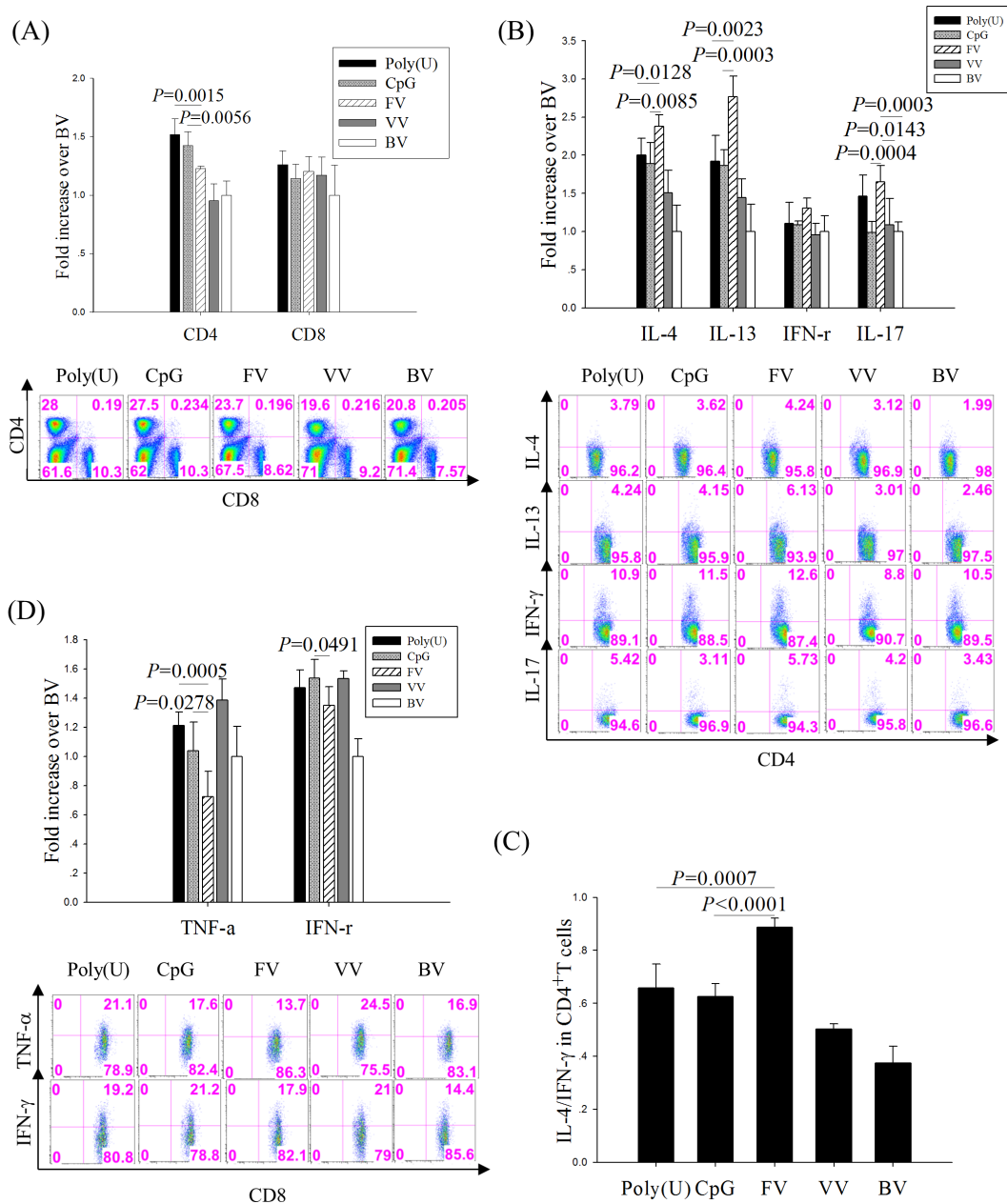


Figure 8. T cell immune responses in mice of five groups on the 4th day after RSV challenge. On the 4th day after challenge, spleens of mice were isolated for flow cytometry analysis. (A) Percentage of CD4⁺T cells and CD8⁺T cells in splenocytes. (B) Levels of IL-4, IL-13, IFN-γ and IL-17 in CD4⁺T cells. (C) Ratio of IL-4/ IFN-γ in CD4⁺T cells. (D) Levels of TNF-α and IFN-γ in CD8⁺T cells.

Lung eosinophilia is one of the main characteristics of RVED (29). Some researchers found that deficient Th2 cytokines, especially IL-13, could significantly reduce the recruitment and activation of eosinophils (30), suggesting that eosinophilic accumulation in airway epithelium might be associated with overproduced Th2 cytokines. As the most important cells responsible for asthma and other allergic diseases, eosinophils could not only impair the airway epithelium by releasing inflammatory mediators and cause airway hyperresponsiveness (31), but also help with the RSV clearance and apoptosis of virus-infected cells by upregulating production of IFN- α/β and nitric oxide (32). In our data, RSV loads in mice of group FV were obviously lower than that of the other groups, which was beyond our expectations. Since RVED mice had been proved to develop nonprotective anti-RSV immunity (7), we tend to attribute the high RSV clearance in RVED mice to the large amount of eosinophils in their lungs. However, further studies on the relationship between eosinophilia and RSV clearance are needed to confirm our assumption.

As a well-known TLR9 agonist, CpG has been proved to promote the activation of Th1 lymphocytes and CD8⁺T lymphocytes (33). Our data demonstrated that the overall conditions of RVED mice including symptoms and aberrant T cell immunity were finely ameliorated by the admixture of FIRSV and CpG. In our study, Poly(U) had a similar impact on RVED mice as CpG except for its stronger effect on TNF- α and weaker effect on IFN- γ and IL-17. However, Johnson *et al.* found that the pneumonia and Th2 cytokines could be inhibited when FIRSV was assisted by both R848 (TLR7/8 agonist) and CpG or by CpG only, while FIRSV assisted by only R848 didn't have a similar effect, which differs from our results (34). We thought that this might be associated with the inherent properties of the two different TLR7/8 ligands. R848 is a synthetic guanine (G) analogue of low molecular weight (35), while Poly(U) is a single-stranded polyuridylic acid. Florian *et al.* compared the uridine (U)-rich ssRNA, G-rich ssRNA and GU-rich ssRNA and concluded that U-rich ssRNA and GU-rich ssRNA had a more prominent effect on TLR7/8 than G-rich ssRNA, thus U-rich ssRNA and GU-rich ssRNA were more suitable for vaccine adjuvants and immune therapy (19). Therefore, the different base sequences endowed R848 and Poly(U) with different immunostimulatory effects on TLR7/8, which could be the explanation for the discrepancy between Johnson's results and ours.

Noticeably, although Poly(U) and CpG added in FIRSV remarkably improved the overall conditions of RVED, the disease still couldn't be completely avoided in this way. As our data showed, weight loss and pneumonia in mice of group Poly(U) and group CpG were still more severe than that of group VV, and Th2 cytokines in mice of Poly(U) and CpG, although much

lower than that of group FV, were still dominant in cellular immunity. Therefore, further studies are needed to figure out whether the combination of Poly(U) and CpG or other TLR agonists as adjuvants of FIRSV could generate a more protective and powerful immune response in RVED mice.

In conclusion, we established a RVED mouse model with Th2-biased immune response and impaired CD8⁺T cell function, and then based on the mouse model, we found that Poly(U) and CpG could effectively ameliorate the unbalanced T cell immunity as well as the pneumonia in RVED mice, which supports the idea of using exogenous ligands of TLRs as vaccine adjuvants to generate a protective immune response against RSV.

Acknowledgements

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Clinical data analysis of genotypes and phenotypes of deafness gene mutations in newborns: A retrospective study

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Summary

We retrospectively analyzed newborns with deafness gene mutations and summarized the relationship between genotype and phenotype to provide a basis for genetic counseling. We studied 582 subjects positive for deafness gene mutations that were treated in the otology outpatient department of Beijing Tongren Hospital, Capital Medical University, between April 2012 and April 2016. The subjects were divided into 3 categories: a diagnosed group (group A), which was further subdivided into subgroups A1 (homozygous and compound heterozygous *GJB2* mutations) and A2 (homozygous and compound heterozygous *SLC26A4* mutations); a drug-induced deafness group (group B, mitochondrial (Mt) gene mutations); and a mutation carrier group (group C), which was further subdivided into the subgroups C1 (*GJB2* heterozygous mutations), C2 (*SLC26A4* heterozygous mutations), C3 (*GJB3* heterozygous mutations), and C4 (double gene mutations). Partial sequences positive for *GJB2* or *SLC26A4* were sequenced and analyzed for mutations. Subjects underwent otoscopic examination and comprehensive audiological evaluation, and temporal bone computerized tomography and/or inner ear magnetic resonance imaging were performed. *GJB2* 235delC was the most common mutation locus. The highest proportion of deafness detected during universal newborn hearing screening was for drug-induced deafness, whereas the lowest was for the diagnosed group. *GJB2* gene mutations mainly resulted in flat-type, profound-to-severe sensorineural hearing loss (SNHL). *SLC26A4* gene mutation was mainly associated with high-frequency drop-type and profound-severe SNHL and was closely related to enlargement of the vestibular aqueduct.

Keywords: Gene, screening, *GJB2*, *SLC26A4*, hearing loss

1. Introduction

Deafness, which refers to varying degrees of hearing loss, is one of the most common sensory disorders. In

2016, the World Health Organization reported that the rate of disabling deafness was as high as 360 million, accounting for approximately 5.14% of the world's population. Among them, more than 32 million are children. Thus, deafness has become a global public health problem.

Although deafness is ascribed to many causes, genetic factors account for approximately 50-60% of cases (1), and the incidence of neonatal congenital deafness is approximately 1-3‰ (2,3). With rapid advances in science and technology, several deafness genes have been identified. By the end of May 2015, 97 non-syndromic deafness genes and 152 non-syndromic deafness genetic loci were identified. These findings

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underscore the importance of detecting deafness genes in children at a minimized cost.

Green *et al.* (4) proposed the use of deafness gene chip screening in the diagnosis of neonatal deafness in 2000. Soon after, Morton *et al.* (2) and Wang *et al.* (3) proposed deafness gene screening as a part of newborn hearing screening, leading to increased awareness. Using large-scale national deafness disease molecular epidemiology survey data (5,6), together with data on mutations for non-syndromic deafness in the Chinese population, allele-specific primer extension polymerase chain reaction (PCR) and universal chip technology were combined to develop gene chip technology for 4 genetic deafness genes (7). In these 4 genes, 9 loci were screened, including *GJB2* c.235delC, c.299delAT, c.176del6, and c.35delG; *GJB3* c.538C>T; *SLC26A4* c.IVS7-2A>G and c.2168A>G; and *Mt 12S rRNA* m.1555A>G and m.1494C>T. The whole experimental process takes approximately 5 h, which is conducive to rapid detection in the clinical setting or for large-scale population screening (7).

Newborn deafness gene screening can enable early detection of deafness in children and guide necessary management as soon as possible. In 2007, China put forth the concept of "newborn deafness gene screening" for the first time (3), and neonatal deafness gene screening and joint hearing screening were gradually actualized. In 2012, Beijing became the first city in China to implement a neonatal deafness gene screening project in the resident population. In the preliminary statistics of our research group, blood samples of 62,560,000 newborns were screened in Beijing by December 2014. The positivity rate for the 9 common deafness gene mutations was 4.59%. The ototoxic drug susceptibility rate was 2.37%, and the rate of diagnosed congenital deafness was 0.24%. Of single heterozygous mutations, 4.34% might be associated with late-onset deafness. Currently, many provinces and cities nationwide are equipped to screen for newborn deafness genes.

Owing to the increasing number of newborns being diagnosed with deafness gene mutations, otologists are facing great challenges. The purpose of this study, therefore, was to investigate the nature, degree, and curves of hearing loss in neonates positive for deafness gene mutations, and to provide the basis for clinical genetic counseling.

2. Materials and Methods

Subjects' parents provided written informed consent for their participation in the study. The protocol was approved by the Declaration of Helsinki principles and Ethics Committee of Beijing Tongren Hospital, Capital Medical University.

2.1. Subject recruitment

Between April 2012 and April 2016, 1258 Chinese newborns who underwent deafness gene screening were recruited from among patients seeking genetic testing and counseling at the Department of Otolaryngology, Head and Neck Surgery, Tongren Hospital (Beijing, China). We screened 9 loci in 4 genes, including *GJB2* c.235delC, c.299delAT, c.176del6, and c.35delG; *GJB3* c.538C>T; *SLC26A4* c.IVS7-2A>G and c.2168A>G; and *Mt 12S rRNA* m.1555A>G and m.1494C>T. In total, 582 cases were finally included in the study.

According to the gene mutations, the subjects were divided into the following 3 groups: diagnosed group (group A), which was further subdivided into A1 (homozygous and compound heterozygous mutations in *GJB2*) and A2 (homozygous and compound heterozygous mutations in *SLC26A4*); drug-induced deafness group (group B): mitochondrial gene mutations; and gene mutation carrier group (group C), which was further subdivided into C1 (*GJB2* heterozygous mutations), C2 (*SLC26A4* heterozygous mutations), C3 (*GJB3* heterozygous mutations), and C4 (double gene non-pathogenic mutations).

2.2. Clinical evaluation

The following demographic information was collected for each patient: sex, birth date, pregnancy, relevant family history, and date of initial otolaryngological consultation, major comorbidities, and the result of newborn hearing screening (8).

2.3. DNA analysis

Genomic DNA was extracted from 2 ml of whole blood from each patient, using the Blood DNA kit (Tiangen Biotech, Beijing, China). All exons and flanking splice sites of the *GJB2* and *SLC26A4* genes were screened for mutations by PCR amplification and bidirectional sequencing.

2.4. Auditory evaluation

Subjects underwent physical examination, including otoscopic examination, with special attention to hearing. Comprehensive audiological evaluation included auditory brainstem response (ABR), 40-Hz auditory event-related potential, distortion product otoacoustic emission, auditory steady-state response (ASSR), acoustic immittance, and pediatric behavioral audiometry. According to Liden/Jerger, the classification of acoustic immittance (226 Hz) was as follows: A (including As and Ad), B, and C. A was considered normal. The classification of acoustic immittance (1,000 Hz) was unimodal, bimodal, or flat type (9,10).

We evaluated the audiology according to Mazzoli *et al.*, who described the term non-syndromic hearing

Table 1. Comparison of basic parameters among the diagnosed, drug-induced deafness, and mutation carrier groups (*n* = 582 cases)

Group	Sub-group	Number (case)	Sex (case)		Age (months)		Age at first visit (months)		Family history (case,%)
			Male	Female	Section	Mean	Section	Mean	
A	A1	85	54	31	8-58	35.4 ± 13.02	1-44	8.19 ± 9.14	8 (9.41)
	A2	32	14	18	9-58	36.8 ± 15.69	1-46	13.2 ± 13.74	3 (9.38)
B	B	69	35	34	14-56	42.1 ± 10.29	2-17	3.83 ± 2.73	19 (27.54)
C	C1	188	114	74	11-56	7.15 ± 5.02	1-25	7.15 ± 5.02	15 (7.98)
	C2	133	72	61	11-57	37.4 ± 10.83	2-43	7.28 ± 6.49	7 (5.26)
	C3	48	18	30	16-54	40.58 ± 9.60	2-16	5.46 ± 3.71	1 (2.08)
	C4	27	17	10	16-56	39.5 ± 12.18	1-33	6.26 ± 6.64	5 (18.52)
Total		582	324	258	/	/	/	/	58 (9.97)

loss in 2003 (11). The nature of hearing loss was divided into SNHL, conductive hearing loss (CHL), and mixed hearing loss (MHL). The hearing threshold was calculated as the average hearing level at 0.5, 1.0, 2.0, and 4.0 kHz according to the World Health Organization standard (1997). The severity of hearing impairment was defined as mild (26-40 dB), moderate (41-60 dB), severe (61-80 dB), or profound (>80 dB). Owing to the subjects' young age, the ABR threshold and/or ASSR were recorded, and mean thresholds at frequencies in the 0.5-4 kHz range were averaged to obtain an approximation for directional conditioned reflex. For children lacking behavioral thresholds and ASSR results, the ABR threshold is considered the high-frequency auditory threshold (12,13). We excluded patients with discriminated hearing loss curves. Hearing loss curve types are divided into ascending-type, U-type, drop-type, and flat-type curves. When the maximum sound output evoked no response, the default could not be determined (12).

2.5. Image evaluation and statistical analysis

Computerized tomography of the temporal bone or magnetic resonance imaging of the inner ear was performed. SPSS21.0 statistical software was used for data analysis using the chi-squared (χ^2) test.

3. Results

3.1. Demographic data

Of the 582 cases, male and female subjects accounted for 55.67% and 44.33%, respectively. The age ranged from 8 to 58 months (mean, 39.20 ± 11.26 months). The age at first visit ranged from 1 to 46 months (mean, 7.10 ± 6.89 months). In total, 58 (9.97% of the total) subjects had a family history of deafness.

Table 1 presents the clinical characteristics of the 3 groups. With regard to age at first visit, patients in group B visited the clinic the earliest, followed by group A, then C. The rate of family history of mitochondrial gene mutation (group B) was the highest, accounting for 27.54% of the observed mutations.

3.2. Genetic testing

Table 2 shows the mutation detection results of the 3 groups. The decreasing order of the proportion of gene detection was as follows: C > A > B, accounting for 68.04% (396/582), 20.10% (117/582), and 11.86% (69/582), respectively. c.235delC was the most common mutation locus, accounting for 22.76% (265/1164) of the mutations. Then, c.IVS7-2A>G accounted for 22.76% (265/1164). The c.35delG mutation was not detected.

3.3. Comparison of the results of gene detection and universal newborn hearing screening (UNHS)

Table 3 shows the comparison of the UNHS results of the 3 groups. For UNHS, 378 subjects had the pass outcome, whereas 204 were referred for UNHS, including 40 with single reference.

The UNHS rates of groups A, B, and C were 21.37%, 98.55%, and 77.02%, respectively ($\chi^2 = 143.47$, $P < 0.01$). Pair-wise comparisons between groups showed significant differences: group A, B ($\chi^2 = 126.75$, $P < 0.01$); group A, C ($\chi^2 = 62.75$, $P < 0.01$); and group B, C ($\chi^2 = 22.917$, $P < 0.01$).

3.4. Genetic testing results and the nature of hearing loss

Genetic testing results and the nature of hearing loss are shown in Table 4. The normal hearing rates of groups A, B, and C were 8.97%, 97.10%, and 79.17%, respectively ($\chi^2 = 219.269$, $P < 0.01$). Group-wise comparison for any 2 groups showed significant differences ($\alpha = 0.05/3 = 0.01667$; $P1 = P2 = P3 < 0.01$).

A few patients in group B had hearing loss; therefore, this group was not compared with the other groups. Both groups A and C demonstrated mostly SNHL (81.20% and 18.56%, respectively; $\chi^2 = 76.88$, $P < 0.01$). The rates of SNHL in their subgroups A1, A2, C1, and C2 were 85.29%, 70.31%, 78.72%, and 71.05%, respectively. The difference between A1 and A2 was significant ($\chi^2 = 6.45$, $P < 0.01$). However, the comparisons of C1 and C2, A1 and C1, and A2 and C2 showed no significant difference ($P1 = P2 = 0.19$, $P3 = 0.88$).

Table 2. Results of gene mutation analyses of the diagnosed, drug-induced deafness, and mutation carrier groups ($n = 582$ cases)

Group	sub-group	Gene mutation	Number (case,%)	Total (case,%)			
A	A1	<i>GJB2</i> 109G>A / 235delC CHM	8	85 (14.60)			
		<i>GJB2</i> 109G>A / 299delAT CHM	4				
		<i>GJB2</i> 176del16 / 235delC CHM	5				
		<i>GJB2</i> 176del16 / 299delAT CHM	1				
		<i>GJB2</i> 235delC / 299delAT CHM	29				
		<i>GJB2</i> 235delC / 512G>A CHM	1				
		<i>GJB2</i> 235delC Hom M	34				
		<i>GJB2</i> 299delAT Hom M	1				
		<i>GJB2</i> 9G>A/235delC CHM	2				
	A2	<i>SLC26A4</i> 1641T>G / 2168AT CHM	1	32 (5.50)			
		<i>SLC26A4</i> IVS7-2A>G / 1226G>A CHM	3				
		<i>SLC26A4</i> IVS7-2A>G / 2168A>G CHM	5				
		<i>SLC26A4</i> IVS7-2A>G Hom M	18				
		<i>SLC26A4</i> IVS7-2A>G / 916dupG CHM	1				
		<i>SLC26A4</i> IVS7-2A>G / 2000T>C CHM	1				
		<i>SLC26A4</i> IVS7-2A>G / 2106delG CHM	1				
		<i>SLC26A4</i> IVS7-2A>G / 1522A>G CHM	1				
		B	B		<i>Mt 12s rRNA</i> 1494C>T Hom M	4	69 (11.86)
					<i>Mt 12s rRNA</i> 1555A>G Hom M	49	
					<i>Mt 12s rRNA</i> 1555A>G Het M	16	
C	C1		<i>GJB2</i> 176del16 Het M	8	188 (32.30)		
			<i>GJB2</i> 235delC Het M	133			
			<i>GJB2</i> 299delAT Het M	47			
	C2		<i>SLC26A4</i> 2168A>G Het M	29	133 (22.85)		
			<i>SLC26A4</i> IVS7-2A>G Het M	104			
	C3		<i>GJB3</i> 538C>T Het M	48	48 (8.25)		
	C4	<i>GJB2</i> 176del16 Het M / <i>SLC26A4</i> IVS7-2A>G Het M	1	27 (4.64)			
		<i>GJB2</i> 176del16 Het M / <i>Mt 12s rRNA</i> 1555A>G Hom M	1				
		<i>GJB2</i> 235delC Het M / <i>Mt 12s rRNA</i> 1555A>G Hom M	1				
		<i>GJB2</i> 235delC Het M / <i>GJB3</i> 538C>T Het M	2				
		<i>GJB2</i> 235delC Het M / <i>SLC26A4</i> 2168A>G Het M	3				
<i>GJB2</i> 235delC Het M / <i>SLC26A4</i> IVS7-2A>G Het M		13					
<i>GJB2</i> 299delAT Het M / <i>Mt 12s rRNA</i> 1555A>G Het M		1					
<i>GJB2</i> 299delAT Het M / <i>SLC26A4</i> IVS7-2A>G Het M		1					
<i>GJB3</i> 538C>T Het M / <i>SLC26A4</i> 2168A>G Het M		1					
<i>GJB3</i> 538C>T Het M / <i>SLC26A4</i> IVS7-2A>G Het M		2					
<i>SLC26A4</i> IVS7-2A>G Het M / <i>Mt 12s rRNA</i> 1555A>G Hom M		1					
Total	/	582	582 (100.00)				

Hom M: homozygous mutation; CHM: compound heterozygous mutations; Het M: heterozygous mutation.

Table 3. Comparison of UNHS results of the diagnosed, drug-induced deafness, and mutation carrier groups ($n = 1,164$ ears)

Group	Sub-group	UNHS (ear, %)		Total (case,%)
		Pass	Refer	
A	A1	24 (14.12)	146 (85.88)	170
	A2	26 (40.63)	38 (59.38)	64
	Total (ear,%)	50 (21.37)	184 (78.63)	234
B	B	136 (98.55)	2 (1.45)	138
C	C1	265 (70.48)	111 (29.52)	376
	C2	203 (76.32)	63 (23.68)	266
	C3	94 (97.92)	2 (2.08)	96
	C4	48 (88.89)	6 (11.11)	54
	Total (ear,%)	610 (77.02)	182 (22.98)	792

3.5. Genetic testing results and degree of hearing loss

In total, 382 ears had hearing loss (Table 5). Profound, severe, moderate, and mild hearing loss occurred

in 169, 113, 62, and 38 ears, respectively. Severe-profound hearing loss was frequent in all groups, and profound hearing loss had the highest prevalence. The rates of profound hearing loss in groups A, B, and C were 44.60%, 50.00%, and 43.64%, respectively (Table 5). Two patients in group B had mild hearing loss, and 2 had profound hearing loss; therefore, group B was not analyzed with the other groups. χ^2 analysis between profound hearing loss in group A and that in group C ($P = 0.13$) revealed no significant difference. Severe-profound hearing loss in group A (80.28%) and group C (66.06%) ($P < 0.01$) differed significantly.

Profound hearing loss was most frequent in A1, C1, and C2, and severe hearing loss was most frequent in A2. There was no significant difference between A1 and A2 or between A2 and C2 ($P_1 = 0.06$, $P_2 = 0.10$). The difference between C1 and C2 and that between A1 and C1 were statistically significant ($P_3 = 0.04$, $P_4 < 0.01$).

Table 4. Nature of hearing loss across the diagnosed, drug-induced deafness, and mutation carrier groups ($n = 1,164$ ears)

Group	Sub-group	The nature of Hearing Loss (ear,%)				Total (case,%)
		Normal	SNHL	CHL	MHL	
A	A1	17 (10.00)	145 (85.29)	2 (1.18)	6 (3.53)	170
	A2	4 (6.25)	45 (70.31)	6 (9.38)	9 (14.06)	64
	Total (ear,%)	21 (8.97)	190 (81.20)	8 (3.42)	15 (6.41)	234
B	B	134 (97.10)	3 (2.17)	0 (0.00)	1 (0.72)	138
C	C1	296 (78.72)	74 (19.68)	6 (1.60)	0 (0.00)	376
	C2	189 (71.05)	69 (25.94)	1 (0.38)	7 (2.63)	266
	C3	92 (95.83)	0 (0.00)	3 (3.13)	1 (1.04)	96
	C4	50 (92.59)	4 (7.41)	0 (0.00)	0 (0.00)	54
	Total (ear,%)	627 (79.17)	147 (18.56)	10 (1.26)	8 (1.01)	792

Table 5. Levels of hearing loss across the diagnosed, drug-induced deafness, and mutation carrier groups ($n = 382$ ears)

Group	Sub-group	The curve type of Hearing Loss (ear,%)				Total (case,%)
		Mild	Moderate	Severe	Profound	
A	A1	5 (3.27)	27 (17.65)	49 (32.03)	72 (47.06)	153
	A2	5 (8.33)	5 (8.33)	27 (45.00)	23 (38.33)	60
	Total (ear,%)	10 (4.69)	32 (15.02)	76 (35.68)	95 (44.60)	213
B	B	2 (50.00)	0 (0.00)	0 (0.00)	2 (50.00)	4
C	C1	14 (17.50)	20 (25.00)	17 (21.25)	29 (36.25)	80
	C2	9 (11.69)	8 (10.39)	19 (24.68)	41 (53.25)	77
	C3	3 (75.00)	1 (25.00)	0 (0.00)	0 (0.00)	4
	C4	0 (0.00)	1 (25.00)	1 (25.00)	2 (50.00)	4
	Total (ear,%)	26 (15.76)	30 (18.18)	37 (22.42)	72 (43.64)	165

Table 6. Curve type of hearing loss across three groups ($n = 382$ ears)

Group	Sub-group	The curve type of Hearing Loss (ear,%)					Total (case,%)
		Flat	Drop	U	Ascending	Discriminated	
A	A1	84 (54.90)	19 (12.42)	3 (1.96)	11 (7.19)	36 (23.53)	153
	A2	24 (40.00)	30 (50.00)	0 (0.00)	2 (3.33)	4 (6.67)	60
	Total (ear,%)	108 (50.70)	49 (12.42)	3 (1.96)	13 (7.19)	40 (23.53)	213
B	B	2 (50.00)	2 (50.00)	0 (0.00)	0 (0.00)	0 (0.00)	4
C	C1	44 (55.00)	21 (26.25)	1 (1.25)	5 (6.25)	9 (11.25)	80
	C2	26 (33.77)	37 (48.05)	2 (2.60)	5 (6.49)	7 (9.09)	77
	C3	4 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	4
	C4	3 (75.00)	0 (0.00)	0 (0.00)	1 (25.00)	0 (0.00)	4
	Total (ear,%)	77 (46.67)	58 (35.15)	3 (1.82)	11 (6.67)	16 (9.70)	165

3.6. Genetic testing results and hearing loss curves

Genetic testing results were compared with hearing loss curves (Table 6). Of 382 ears with hearing loss, 187 ears, 109 ears, 24 ears, and 6 ears, respectively, had flat-type, drop-type, ascending-type, and U-type hearing loss curves, whereas the curves could not be identified in 56 ears.

Few subjects in group B had hearing loss curves; therefore, this group was excluded from the comparative analysis with the other groups. Groups A and C mostly demonstrated the flat-type curve (50.70% and 46.67%, respectively; $\chi^2 = 0.32$, $P = 0.57$). The rates of flat-type hearing loss curves were highest in groups A1 and C1 (54.90% and 55.00%, respectively). The rates of drop-type hearing loss curves were highest in groups A2 and C2 (50.00% and 48.05%, respectively). The differences

in these rates were significantly different between A1 and A2, A1 and C1, and C1 and C2 ($P_1 < 0.01$, $P_2 = 0.04$, $P_3 = 0.048$), but not between A2 and C2 ($P_4 = 0.38$).

3.7. Imaging results for SLC26A4 gene mutation

SLC26A4 mutations were detected in 165 cases – 32 cases in A2 and 133 in C2. Among them, 61 cases had imaging results (A2, 23 cases; C2, 38 cases). In total, 50 cases were abnormal, and 11 cases had no obvious abnormalities. The abnormality was an enlarged vestibular aqueduct (EVA) in 47 (94 ears) cases; thus, the overall abnormality rate was 77.05%. EVA was present in 95.65% (22/23) of patients in group A2 and 65.79% of patients in group C2 (25/38). EVA with Mondini deformity (MD) occurred in 2 (3.28%) cases.

4. Discussion

The number of positive results in newborn deafness gene screening is large, and genetic counseling work is increasingly important. Deafness gene detection can: *i*) clarify the cause of congenital hereditary deafness and improve adherence to deafness intervention; *ii*) detect delayed deafness early to enable intervention and early prevention of hearing loss; *iii*) identify individuals susceptible to drug-induced deafness and prevent the occurrence of deafness in these individuals; and *iv*) allow genetic counseling that reduces the birth of deaf children, thereby reducing the burden on families and society. Deafness gene screening in newborns is important for early detection of deafness in children to provide early guidance for hearing care.

4.1. Demographic data

In 2006, Huang *et al.* studied 265 children (0-6 years old) with hearing loss, and found that the mean age at first visit was 28.01 ± 13.41 months (14). In 2014, Yang (15) studied 122 children diagnosed with an EVA. Among them, 84 had undergone UNHS, and the mean age at first visit was 17.24 ± 17.08 months; however, the mean age at first visit for 37 children who had not undergone UNHS was 30.92 ± 18.21 months.

The age at first visit in our current study was earlier than that of the previous study. These findings indicate that screening of newborn deafness genes in concert with UNHS can advance the timing of the first visit for investigation of hearing loss. Drug-related deafness had the lowest mean age at first visit, and the highest mean age at first visit was observed for the *GJB3* gene heterozygous mutation. This may be associated with the highest proportion of patients with a family history of drug-induced deafness (27.54%), indicating that a similar situation at home can make parents pay more attention to hearing loss.

4.2. Genetic testing

GJB2 is the most frequently mutated gene in cases of hereditary hearing loss, but the mutation spectrum varies among ethnic groups. For example, among Caucasians, the most common *GJB2* gene mutation is c.35delG, with a carrier frequency of 2-4% (16). However, c.235delC is most frequently observed among Asians, with an allele frequency ranging from 5% to 22% (17-19), which is consistent with our present results.

SLC26A4 is the most important pathogenic gene underlying deafness in large vestibular aqueduct syndrome (LVAS), and there are obvious regional and racial differences in hot spot mutations. Wang *et al.* conducted a screening of the *SLC26A4* gene in 107 patients with deafness with LVAS, and found that the C.IVS7-2A>G mutation is the most common mutation

in the Chinese population, accounting for 57.63% of the total mutations, followed by c.2168A>G (9.04%). Up to 97.9% of patients had at least one *SLC26A4* mutation, and 88.4% of patients had bi-allelic mutations (20). In the Japanese and Korean populations, the main mutation of the *SLC26A4* gene was c.2168A>G, and the c.IVS7-2A>G mutation in the Korean population was also common (21,22). In Caucasian populations in Europe and the United States, the most common three mutations were observed to be p. L236P (16%), p. T416P (15%), and IVS8 + 1G>A (14%). The sum of the prevalence of these three mutations is approximately half of that of the total mutation, but these three mutations are very infrequent in the East Asian population (23).

In this study, the rate of *GJB2* gene mutation reached 46.91%; followed by that of *SLC26A4* gene mutation, accounting for 28.35%. The mutation rate of c.235delC was 22.75%, followed by the c.IVS7-2A>G mutation (14.59%). The c.35delG mutation was not detected.

4.3. Comparison of the results of gene detection and UNHS

The most fundamental purpose of UNHS is to realize "early detection, early diagnosis, and early intervention" in children with congenital hearing loss. Therefore, UNHS benefits children, families, and society as a whole. In 2010, China made UNHS a routine inspection. Before the introduction of UNHS, children with hearing loss were diagnosed at an average age of 23 months (8 months later in rural areas than in urban areas) (14). Congenital hearing loss, especially moderate or heavy hearing loss, prevents babies from hearing voices and hence leads to speech and cognitive developmental disorders. Newborn deafness gene screening in combination with UNHS effectively identifies children with hearing loss because both approaches complement each other.

The UNHS rates of groups A, B, and C were 21.37%, 98.55%, 77.02%, respectively, differing significantly from one another. The UNHS pass rate in group B was the highest, suggesting that warning against ototoxic drugs was effective. The UNHS pass rate for group A was lower than that of group C, corresponding to the nature of the corresponding gene mutations. However, the homozygous or compound heterozygous mutations of group A are pathogenic. Children in group A might have had hearing loss in theory, but the UNHS pass rate was 21.37%. This could be attributed to late-onset hearing loss or technical and personnel factors when performing UNHS, leading to false pass rates. Thus, positivity for newborn deafness genes should warn parents to pay close attention to the child's hearing even if they pass the UNHS.

4.4. Genetic testing results and the nature of hearing loss

The normal hearing rate of group B was the highest,

suggesting that caution was being practiced against the use of deafness-causing drugs. Few patients in group B had hearing loss, and these were not included in the comparison with other groups. Both group A and group C demonstrated mostly SNHL. On comparing SNHL of A1 and A2, C1 and C2, A1 and C1, and A2 and C2, the difference was statistically significant only between A1 and A2. This result may be related to the pathogenic mechanism of the different genes, which is explained below.

GJB2 encodes the connexin-26 gap junction protein (Cx26). Cx26 protein forms a membrane channel with 6 subunits, thus constituting the cell gap junction, and is distributed in the cochlear stria, basal cells, spiral limbus convex, nerve conduction fiber, and cochlear sensory epithelium. It is an important channel for electrolytes, second messengers, and metabolites, playing an important role in the exchange of information and materials (24). Therefore, *GJB2* mutations mainly lead to SNHL. However, other factors such as otitis media and ear deformities might result in a different nature of hearing loss.

SLC26A4 encodes pendrin, a protein with complex structure and function. It is expressed mainly in the inner ear lymph sac and lymphatic vessels, mediating the transport of Cl^- , HCO_3^- , and I^- to maintain the ionic balance of the inner ear lymph and playing an important role in inner ear lymph reuptake (25). *SLC26A4* mutations can cause syndromic or non-syndromic SNHL (26), but the pathogenesis is not clear.

The pathogenesis in group C might be explained as follows. First, some patients might not have undergone gene sequencing, or they might have harbored other deafness mutations. Second, interaction with other genes might have occurred. In an earlier study, a single *GJB2* mutation was detected in 10-42% of patients with deafness (27). Other genes might also be involved in the phenotypic expression of deafness. *GJB6* and *GJB2* are both expressed in the cochlea, and their mutations can affect gap junction formation (27). Furthermore, the pathogenesis of *SLC26A4* mutations might be related to mutations in *FOXI1* and *KCNJ10* (28,29). In addition, other unknown reasons may underlie the pathogenesis, warranting further studies on patients with *SLC26A4* mutations.

4.5. Genetic testing results and the degree of hearing loss

Severe-profound hearing loss was the most common across the groups, and profound hearing loss had the highest rate. The rates of profound hearing loss did not differ significantly between group A and group C, whereas severe-profound hearing loss rates were significantly different between group A and group C. Furthermore, the degree of hearing loss but not that of severe-profound hearing loss differed significantly

between group A1 and A2. Severe-profound hearing loss significantly differed between C1 and C2, between A1 and C1, and between A2 and C2.

A national molecular epidemiological investigation of deafness showed *GJB2*, *SLC26A4*, and mitochondrial DNA to be the genes most commonly associated with severe-profound non-syndromic hearing loss in China. The hearing loss resulting from *GJB2* mutations is generally congenital, bilateral, non-progressive, and severe or profound (30). The hearing loss related to *SLC26A4* mutations is bilateral or non-bilateral, progressive, and with differing degrees (31). In the present study, *GJB2* and *SLC26A4* mutations caused severe-profound hearing loss, consistent with the literature. It is noteworthy that mild and moderate deafness was associated with *GJB2* and *SLC26A4* mutations. In addition, to some extent, the degree of hearing loss with *SLC26A4* mutations was milder than that with *GJB2*. Nevertheless, the relationship between genotype and hearing loss varies across patients (32,33).

4.6. Genetic testing results and the hearing loss curve

In a previous study in 77 deaf individuals, the hearing loss curves of cases positive for *GJB2* mutations were mainly of the drop type and flat type, with little U shape and no ascending curve (34). The ascending type was found in 14.93% of 297 cases with *GJB2* mutations, but the *GJB2* hearing loss curve still generally indicated the drop type (26.27%) and flat type (25.16%) (35). In this study, the flat type of *GJB2* gene mutations was the most common, followed by drop type, ascending type, and finally the U type was less, which is basically the same pattern as reported in the literature.

SLC26A4 mutations often lead to LVA, with the hearing loss curve most often being of the drop type, followed by the flat type (36). This report showed that the hearing loss curves of *SLC26A4* mutations were predominantly of the drop type, followed by flat type, ascending type, and U type.

4.7. Analysis of imaging results of the *SLC26A4* mutations

SLC26A4 mutation was found to be associated with LVA, which is characterized by vestibular aqueduct and SNHL. Huang *et al.* studied MD (28 cases), MD combined with EVA (50 cases), EVA alone (50 cases), and patients with other internal ear deformity (16 cases) by performing *SLC26A4* gene sequence analysis. The results showed that mutations in the *SLC26A4* gene are common in children with or without EVA, but there was no evidence of MD being associated with mutations in the *SLC26A4* gene (37). Zhu *et al.* sequenced the *SLC26A4* gene in 14 patients with EVA, 6 patients with MD (with EVA), and 7 patients with other internal ear deformity (without EVA). In total, there were

14 cases of EVA children, 12 cases of double allelic *SLC26A4* mutation, 2 cases of single heterozygous mutation, 6 cases of MD (with EVA) with double mutation of *SLC26A4*, and 7 patients with other internal ear deformity (without EVA) in which the *SLC26A4* mutation was not detected. They concluded that the incidence of MD with EVA or EVA is closely related to the *SLC26A4* mutation (38). In this study, of 165 cases with the *SLC26A4* gene mutation, a total of 61 cases had imaging results showing EVA (77.05%), and EVA with MD accounted for 3.28%.

5. Conclusions

Among positive results in genetic testing of deafness, *GJB2* gene mutations are prevalent, and the most common mutation is c.235delC. The drug-induced deafness group, gene mutation carrier group, and diagnosed group had high rates of patients who passed the universal newborn hearing screening. The hearing loss features of the *GJB2* gene mutation suggest a flat type, severe-profound sensorineural effect. The hearing loss features of the *SLC26A4* gene mutation include drop type and severe-profound sensorineural effects, as well as an enlarged vestibular aqueduct.

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Coagulopathy associated with poor prognosis in intrahepatic cholangiocarcinoma patients after curative resection

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Summary

As a rare type of liver cancer, intrahepatic cholangiocarcinoma (ICC) has become an increasingly important malignancy and continues to present significant therapeutic challenges. Since coagulopathy is associated with poor prognosis in hepatocellular carcinoma (HCC), and prognostic factors of ICC after curative resection were still not clear, we aim to analyze the characteristics of ICC patients with coagulopathy and its correlation to prognosis. From January 2000 to June 2011, 541 ICC patients, after curative resection, were enrolled in our study. Survival curves were depicted by the Kaplan-Meier method and analyzed by the log-rank test. The Cox proportional hazard regression was adopted for multivariate survival analysis. Student's *t* test was performed to analyze the difference between the coagulopathy group and the normal group. The correlation between coagulation parameters and prognosis was also evaluated. The incidence rate of at least one coagulation parameter abnormality was 22.6% (122/541) while PT was the most common factor (8.87%, 48/541). The one-year survival rate of patients with coagulopathy was significantly lower than that of patients with normal coagulation ($p < 0.01$). In a univariate analysis, patients with prolonged PT was associated with shortened DFS ($p < 0.05$). Meanwhile, PT was negatively correlated with pre-albumin level. TNM stage, CA19-9, GGT, and pre-albumin level were independent prognostic factors of DFS in the multivariate analysis. In conclusion, the incidence rate of coagulopathy of ICC patients is lower than HCC patients. Prolonged PT, advanced TNM stage, low pre-albumin level, and high CA19-9 and GGT level were correlated with high recurrence rate and poor prognosis.

Keywords: Intrahepatic cholangiocarcinoma, coagulopathy, prognosis

1. Introduction

Intrahepatic cholangiocarcinoma (ICC), a rare type of liver cancer, is different from hepatocellular carcinoma (HCC) and extrahepatic bile duct carcinoma. The

incidence rate of ICC accounts for 10% of primary liver cancer, which is far below than that of HCC (1). Although the rate of curative resection increased significantly due to the improvement of diagnosis and treatment (2,3), the postoperative recurrence rate is still high and the long-term survival rate is unsatisfactory (4-6).

Hypercoagulability is a well-known condition in patients with cancer, but it is exceedingly rare in HCC because liver is the main organ that synthesizes proteins (7,8) which include fibrinogen, prothrombin, and factors V, VII, IX, X, XI, and XII, and the reduction of the clotting factors can also reflect impaired liver function (9). Previous research revealed that

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coagulopathy is associated with poor prognosis in HCC (10,11). However, prognostic factors of ICC after curative resection were still not clear. On one hand, ICC patients have little impaired liver function and better reversed liver function due to the rare combination with cirrhosis. On the other hand, ICC is a malignant tumor that may lead to hypercoagulability for many reasons.

As a result, the correlation between coagulation abnormality and prognosis in ICC patients is still unclear. This triggers our interest to follow the changing pattern of some coagulation factors in patients with ICC and to test whether there is a correlation between some hemostatic variables and the prognosis of patients.

In this study, we retrospectively analyzed 541 ICC patients in Zhongshan Hospital of Fudan University from January 2000 to June 2011, following up for 4 to 5 years. The coagulopathy and its prognosis were further investigated.

2. Materials and Methods

2.1. Patients

From January 2000 to December 2011, 541 ICC patients at the Liver Cancer Institute of Fudan University were enrolled in our study and retrospectively analyzed. The median age of patients was 58 years (27 to 89 years). There were 331 men (61%, 331/541) and 210 women (39%, 210/541). The median course of disease was 1 month. Median hospital stay time was 15 (5 to 94) days. All patients underwent curative surgical treatment and were diagnosed as ICC by postoperative pathology. This study was approved by the institutional review board of Zhongshan Hospital and complied with the standards of the Declaration of Helsinki and current ethical guidelines.

2.2. Coagulation parameters and clinicopathological factors

Coagulation parameters and clinicopathological factors that were potentially related to survival were selected in this study. Coagulation parameters include prothrombin time (PT), activated partial thromboplastin time (APTT), prothrombin time ratio (PTR), international normalized ratio (INR), and blood biochemistry parameters including gamma-glutamyl transferase (GGT), total bilirubin (TB), carbohydrate antigen 19-9 (CA19-9), albumin (ALB), and pre-albumin (PA) which were all tested according to regular methods. Tumor related characteristics were also recorded including TNM stage, tumor volume, regional lymph node metastasis, vascular invasion, and distant metastasis. Vascular invasion refers to trunk or branch vascular invasion of portal vein and/or hepatic vein. All pathological specimens were reviewed by two pathologists to confirm the histological type, differentiation, lymph node

metastasis, and neural invasion. The staging of tumors was determined according to the 7th edition of the TNM classification system (12). Coagulation and demographic characteristics of the groups are shown in Tables 1 and 2.

2.3. Follow-up

All patients were followed up every 2 months till December 31, 2015 at the Outpatient Department and prospectively monitored for recurrence by a standard protocol. Overall survival (OS) refers to the period between initial diagnosis and last follow-up or death. Disease free survival (DFS) refers to the length of time after primary treatment that the patient survives without tumor recurrence.

2.4. Statistical analysis

OS time and DFS time were calculated by the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazard regression was performed for a multivariate survival analysis. For continuous variables, the Student's *t*-test was used to compare the differences in indexes between the normal group and the coagulopathy group. The SPSS 18.0 software was used to perform statistical analysis. Two-tailed $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Clinical characteristics of patients with coagulopathy

Patients were divided into 2 groups according to their coagulation parameters: the normal group and the coagulopathy group (patients with at least 1 abnormal coagulation parameters). At least 1 abnormal coagulation parameters (PT, APTT, PTR, INR) counted for 22.6% (122/541), 8.87% of patients (48/541) with PT > 13 s, 4.8% of patients (26/541) with APTT < 23.5 s, 5.7% of patients (31/541) with APTT > 37.5 s, 0.18% of patients (1/541) with PTR < 0.8, 2.22% of patients (12/541) with PTR > 1.2, and 3.0% of patients (16/541) with INR > 1.2 (Table 1). There were no statistically

Table 1. Coagulation characteristics of 541 ICC patients

Coagulation parameters	Cases	Percent (%)
At least 1 abnormal	122	22.60
PT > 13 s	48	8.87
APTT		
< 23.5 s	26	4.80
> 37.5 s	31	5.73
PTR		
< 0.8	13	2.40
> 1.2	13	2.40
INR > 1.2	16	3.00

PT, prothrombin time; APTT, activated partial thromboplastin time; PTR, prothrombin time ratio; INR, international normalized ratio.

Table 2. Clinical characteristics of 541 ICC patients

Clinical Characteristics	Normal group (n = 419)	Coagulopathy group (n = 122)	Chi-square value/t value	p value
Age yr, (range)	57 (27,89)	59 (28,80)	- 0.326	0.745
Gender			0.846	0.358
Male	252	79		
Female	167	43		
TNM Stage			6.658	0.247
Stage I	197	65		
Stage II	61	21		
Stage III	9	2		
Stage IVa	70	11		
Stage IVb	61	14		
Unknown	21	9		
Tumor Differentiation			4.62	0.202
I-II	2	48		
III	182	47		
IV	144	1		
HbsAg (positive)	174	53	0.135	0.714
GGT > 40 U/L	309	85	0.941	0.332
AFP > 20 ng/mL	37	17	2.639	0.104
CA199 > 37 U/mL	240	73	0.027	0.87
ALB < 40 g/L	274	84	0.461	0.497
PA < 0.25 g/L	209	74	0.139	0.709
TBIL > 17 µmol/L	101	35	1.027	0.311
Overall Survival	10 (0,112)	10 (0,106)	1.589	0.113
Disease Free Survival	8 (0,111)	8 (0,106)	1.167	0.244
Survival Rate				
1-year	48.02	34.43	8.267	0.004**
3-year	19.81	17.21	0.41	0.522
5-year	6.92	5.74	0.213	0.644

HbsAg, Hepatitis B surface antigen; GGT, gamma-glutamyl transferase; AFP, alpha fetoprotein; CA19-9, carbohydrate antigen 19-9; ALB, albumin; PA, pre-albumin; TBIL, total bilirubin; ** $p < 0.01$.

significant differences in age, gender, TNM stage, tumor differentiation, GGT, TB, and CA19-9 between the two groups (Table 2).

3.2. Following-up postoperative survival status and survival analysis

By December 31, 2015, there were 352 deaths. There was significant difference in 1- year survival rate between the normal group (48.02%) and coagulopathy group (34.43%) ($p < 0.01$). However, there were no statistically significant differences between 3- and 5-year survival rates ($p > 0.05$) (Table 2). The average OS time of the normal group was 20.72 months comparing to 17.2 months of the coagulopathy group, and the average DFS time of the normal group was 15.78 months comparing to 13.41 months of the coagulopathy group. However, both differences were not statistically significant (Table 2). Univariate analysis was used to analyze the effect of the single coagulation factor (PT, APTT, PTR, and INR) on prognosis (OS, DFS). We found that PT had an effect on DFS ($p < 0.05$) (Table 3). The multivariate analysis was used to further verify the relation between coagulation factors and DFS. The results showed that PT was an independent factor affected DFS, while APTT, PTR and INR were not (Table 4). Kaplan-Meier survival analysis showed that

there was significant difference in the DFS rate between patients with prolonged PT (17.2%) and patients without it (27.7%) ($p < 0.05$) (Figure 1). However, the effect of PT on OS time had not been observed. APTT, PTR, and INR played no effect neither on OS nor DFS in univariate analysis ($p > 0.05$) (Table3).

3.3. Correlation analysis between other prognostic factors and DFS

We used univariate analysis in order to further analyze the correlation between DFS and other prognostic factors including the TNM stage, ALB, PA, GGT, TB, and CA19-9. We found that the TNM stage, PA, GGT, TB, and CA19-9 significantly influenced DFS ($p < 0.05$) (Table 5). The Cox proportional hazards model was used to detect potential predictors of DFS based on variables selected by univariate analysis. The results showed that TNM stage, PA, GGT, and CA19-9 were independent factors affected DFS, while ALB and TB were not (Table 4). Correlation analysis indicated that there was a negative correlation between PT and PA level (correlation coefficient: -0.088, $p < 0.05$) (Table 6). However, there was no statistically significant difference between PT and other liver function indicators (including GGT, TB) and tumor marker (CA19-9) ($p > 0.05$).

Table 3. Relation between coagulopathy and prognosis

Variable	Cases	Overall survival	<i>p</i> value	Disease free survival	<i>p</i> value
PT					
≥ 13 s	48	8 (0,91)	0.976	6.5 (0,51)	0.041*
< 13 s	493	11 (0,112)		8 (0,111)	
APTT					
23.5-37.5	484	11 (0,112)	0.215	8 (0,111)	0.762
Abnormal	57	8 (0,106)		7 (0,103)	
PTR					
0.8-1.2	515	11 (0,112)	0.611	8 (0,111)	0.322
Abnormal	13	9 (3,53)		9 (2,93)	
INR					
≥ 1	219	10 (0,112)	0.446	7 (0,112)	0.259
< 1	322	11.5 (0,112)		8.5 (0,112)	

PT, prothrombin time; APTT, activated partial thromboplastin time; PTR, prothrombin time ratio; INR, international normalized ratio; **p* < 0.05.

Table 4. Multivariate analysis

Variable	Regression coefficients	Standard error	<i>p</i> value	Relative risk	95% CI
PT	0.436	0.192	0.023*	5.159	1.062 - 2.254
APTT	- 0.179	0.190	0.345	0.892	0.577 - 1.212
PTR	- 0.355	0.373	0.342	0.902	0.337 - 1.458
INR	- 0.060	0.114	0.601	0.273	0.753 - 1.178
TNM stage	0.193	0.036	< 0.001***	28.841	1.131 - 1.302
CA19-9	0	0	< 0.001***	19.881	1
TB	- 0.001	0.001	0.245	1.351	0.997 - 1.001
ALB	- 0.003	0.003	0.417	0.657	0.99 - 1.004
GGT	0	0	0.011*	6.395	1.0 - 1.001
PA	0.001	0.001	0.022*	5.281	1.0 - 1.002

PT, prothrombin time; APTT, activated partial thromboplastin time; PTR, prothrombin time ratio; INR, international normalized ratio; CA19-9, carbohydrate antigen 19-9; TB, total bilirubin; ALB, albumin; GGT, gamma-glutamyl transferase; PA, pre-albumin. **p* < 0.05; ****p* < 0.001.

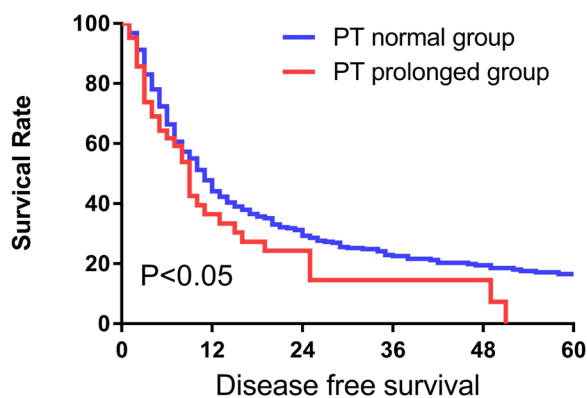


Figure 1. Disease free survival comparison between PT normal group PT prolonged group.

4. Discussion

PT, APTT, PTR, and INR are common parameters for the coagulation function evaluation. PT mainly represents the content and function of factor VII while APTT relates to factors VIII, IX, XI, and XII which is the most common screening test for the intrinsic pathway (13). In HCC patients, the global effect of liver disease with regard to hemostasis is complex because

Table 5. Other factors' affection to disease free survival

Variables	Cases	Disease free survival	<i>p</i> value
TNM Stage			
Stage I	262	9 (0,111)	0.001**
Stage II	82	7 (0,103)	
Stage III	11	7 (2,21)	
Stage IVa	81	7 (0,81)	
Stage IVb	75	6 (0,108)	
PA			
< 0.25 g/L	283	7 (0,111)	0.012*
≥ 0.25 g/L	127	11 (0,109)	
ALB			
< 40 g/L	358	8 (0,111)	0.795
≥ 40 g/L	182	7 (0,81)	
GGT			
< 40 U/L	145	10 (0,111)	< 0.001***
≥ 40 U/L	394	7 (0,108)	
TB			
< 17 μmol/L	404	8 (0,111)	0.001**
≥ 17 μmol/L	136	7 (0,79)	
CA19-9			
< 37 U/mL	207	9 (0,106)	0.004**
≥ 37 U/mL	313	7 (0,111)	

PA, pre-albumin; ALB, albumin; GGT, gamma-glutamyl transferase; TB, total bilirubin; CA19-9, carbohydrate antigen 19-9; **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

of impaired liver function, which may result in reduced plasma levels of procoagulant and anticoagulant clotting factors and reduced capacity to clear activated

Table 6. Correlation analysis

Variable	PT/CC [#]	<i>p</i> value	APTT/CC [#]	<i>p</i> value	PTR/CC [#]	<i>p</i> value	INR/CC [#]	<i>p</i> value
TNM Stage	- 0.042	0.347	- 0.022	0.626	- 0.063	0.158	- 0.051	0.249
TB	- 0.017	0.693	- 0.031	0.468	- 0.023	0.590	- 0.026	0.553
ALB	0.039	0.362	- 0.200	0.001**	- 0.019	0.658	- 0.024	0.573
GGT	- 0.014	0.738	0.002	0.969	- 0.043	0.321	- 0.083	0.055
PA	- 0.088	0.044*	0.051	0.243	- 0.092	0.034*	- 0.060	0.168
CA19-9	- 0.016	0.718	0.019	0.668	0.041	0.350	0.020	0.646

CC[#]: correlation coefficient; TB, total bilirubin; ALB, albumin; GGT, gamma-glutamyl transferase; PA, pre-albumin; CA19-9, carbohydrate antigen 19-9; **p* < 0.05; ***p* < 0.01.

hemostatic proteins and protein inhibitor complexes from the circulation, so that patients can experience severe bleeding or even thrombotic complications (14,15). However, few researchers analyzed coagulopathy in ICC patients.

ICC is a rare primary liver cancer which has become a malignancy of increasing importance and continues to present significant biological and therapeutic challenges (16-18). The clinical features of ICC are diverse and often advanced at the time of diagnosis, often precluding surgical treatment. Hepatic resection is regarded as the treatment of choice, but tumor recurrence is common after curative resection (19-22). Different from HCC, ICC patients have little impaired liver function and with better reversed liver function because of rare combination with cirrhosis. According to our study, the incidence of at least 1 abnormal coagulopathy was 22.6% (122/541 cases). Prolonged PT counted for most (*n* = 48, 8.8%) and prolonged APTT counted for second (*n* = 31, 5.7%), which are far lower than those of HCC (15,23).

Long-term survival is rare in ICC due to high malignancy and poor prognosis. The first optional treatment for ICC is surgical resection. Few patients can survive over 3 years without operation (24). So far, the prognostic factors of post resection outcomes in ICC studies are still inconsistent and even conflicting, probably due to the relatively small number of patients studied. In a validation study with patients from Chinese and Japanese centers, TNM stage, GGT and CA19-9 were related with prognosis. Lymph node metastasis, invasion of peripheral nerves, and tumor size over 5cm are related to low DFS rate (25). In our study, we found that the 1-year survival rate of patients with coagulopathy was lower than that of patients with normal coagulation (*p* < 0.01). Univariate analysis showed that patients with prolonged PT had shorter DFS (*p* < 0.05). Meanwhile, prolonged PT was negatively correlated with pre-albumin level. Because PT and pre-albumin are sensitive factors of reversed liver function, we speculate that more tumor infiltration was in patients with prolonged PT, whatever more tumor burden, massive type, multiple lesions, intrahepatic micrometastases or tumor thrombus that can't be detected by current methods, that leads to a

decrease in liver reverse function. It seems to be the same reason for the lower 1-year survival rate in patient with abnormal coagulopathy (*p* < 0.01). However, no significant differences were found in 3- and 5-year survival rate between patients with abnormal coagulopathy and patients with normal coagulopathy. One possible reason is that the long-term survival rate of ICC was very low, which was lower than 20% in both groups. More cases need to be analyzed due to the small difference between the two groups.

Interestingly, as a test for intrinsic pathway, APTT was not an independent factor that affected DFS. The reason may be coagulation factor VIII is synthesized mainly by the hepatic but also nonhepatic sinusoidal endothelial cells (26), thus the plasma concentration of VIII did not decrease with liver disease and may have even increased, as many chronic liver diseases including ICC are associated with chronic inflammation (27). In this study, we also found that that TNM stage, CA19-9, GGT, and pre-albumin level acted as independent factors of DFS, which indicated that higher TNM stage, lower pre-albumin level, and increased CA19-9 and GGT levels were correlated with earlier tumor recurrence and poor prognosis. In presence of prolonged PT, patients were in high-risk of recurrence and we provided them with strict postoperative supervision which is beneficial for the treatment once tumor recurred.

In conclusion, this study found that PT is an independent factor related with ICC recurrence. Higher TNM stage, lower pre-albumin level, and increased CA19-9 and GGT levels suggest earlier tumor recurrence and poor prognosis.

Acknowledgements

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A comparison of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and enzyme-multiplied immunoassay technique (EMIT) for the determination of the cyclosporin A concentration in whole blood from Chinese patients

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Summary

Cyclosporin A (CyA) is an immunosuppressive agent widely used in clinical therapy. In the therapeutic process, the blood concentration of CyA should be monitored to avoid or prevent rejection and toxicity. The objectives of this study were to compare the correlation of liquid chromatography-tandem mass spectrometry (LC-MS/MS) and enzyme-multiplied immunoassay technique (EMIT) for the determination of the CyA concentration in human blood and to provide evidence for the rational usage of EMIT in clinical practice. Blood samples collected from 132 patients undergoing a liver or kidney transplant or patients with aplastic anemia at Qilu Hospital of Shandong University were tested using the two methods. The calibration curve was linear from 25-500 ng·mL⁻¹ for LC-MS/MS and from 50-450 ng·mL⁻¹ for EMIT. The inter- and intra-day RSDs were less than 15%. The CyA blood concentration according to EMIT was 3.5 ng·mL⁻¹ more than that according to LC-MS/MS. The 95% confidence interval was -10.0~16.9 ng·mL⁻¹. The CyA blood concentration according to the two methods did not differ significantly ($p > 0.05$). LC-MS/MS and EMIT were suitable methods for determining the CyA blood concentration. The two methods were closely correlated ($r^2 = 0.969$), but the CyA blood concentration according to EMIT was slightly higher than that according to LC-MS/MS. The clinical significance of this finding needs to be further evaluated.

Keywords: Cyclosporin A, LC-MS/MS, enzyme-multiplied immunoassay technique, comparison, therapeutic drug monitoring

1. Introduction

Cyclosporin A (CyA) is a highly lipophilic cyclic peptide consisting of 11 amino acids with selective and potent immunosuppressive activity, but it also causes a number of untoward adverse reactions. Therapeutic applications of CyA include kidney, liver, heart, lung, pancreas, and

bone marrow transplants, treatment of autoimmune and rheumatoid diseases, and uses in dermatology and pulmonology (1). Immunosuppressive therapy with CyA follows a narrow path between the risk of rejection as a result of too little immunosuppression and toxic organ damage as a result of too much immunosuppression. Therapeutic drug monitoring (TDM) is recommended for monitoring blood/plasma CyA levels to avoid or prevent rejection and toxicity (2,3). Dose adjustment based on TDM leads to a better clinical outcome, especially in transplant recipients. During treatment with CyA, TDM and subsequent dosage adjustment for individual patients are required after liver transplantation to prevent rejection and over-immunosuppression. This leads to severe infection and adverse reactions including nephro-, hepato- and neurotoxicity (4-

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6). Many commercially available assays are used to monitor CyA levels; these assays involve techniques such as an enzyme-multiplied immunoassay (EMIT), an enzyme-linked immunosorbent assay (ELISA), and a fluorescence polarization immunoassay (FPIA) (7), that utilize the same monoclonal antibody against CyA. However, these methods are not specific enough as they cannot distinguish CyA from its metabolites, which may increase in concentration during CyA treatment or during liver dysfunction. Thus, more specific assays with better sensitivity are required to verify the CyA concentration.

High-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used extensively in clinical laboratories over the last 10-15 years. This technique offers analytical specificity superior to that of immunoassays or conventional high-performance/pressure liquid chromatography (HPLC) for low molecular weight analytes. LC-MS/MS has superior analytical specificity and lower reagent cost, especially if one considers the ability to multiplex several different immunosuppressive drugs (8-10). LC-MS/MS is a sensitive and selective technique that could prove useful in TDM for CyA.

The current study created and validated a LC-MS/MS assay for CyA and the results of that assay were compared to those obtained with an EMIT assay. This was done in order to determine whether the EMIT assays that are currently available are suitable for determining the concentration of CyA in blood as part of TDM and to provide an experimental basis for individualized CyA treatment.

2. Materials and Instruments

2.1. Reagents and equipment

CyA (Lot No. 30517, 95%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Clarithromycin (Internal standard, IS, Lot No.130558-200501, 97.5%) of analytical grade was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Analytical ammonium formate (Lot No. F990215) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Zinc sulfate heptahydrate (Lot No. 20090210) of analytical grade was obtained from Tianjin Beifang Tianyi Chemical Reagent Factory (Tianjin, China). Acetonitrile (Lot No. 0000059829) and methanol (Lot No.0000118131) of chromatographic grade were obtained from J.T. Baker Company (Phillipsburg, New Jersey, USA), and pure water was obtained from the Hangzhou Wahaha Group Co., Ltd. (Hangzhou, Zhejiang Province, China). CyA-specific calibrators (Lot No. 6R119UL-H2), control CyA (Lot No. 6019), and a CyA sample preparation reagent (Lot No. J2) were obtained from Siemens Healthcare Diagnostic Ltd (Newark, New

Jersey, USA).

The Agilent 1200 series HPLC system (Agilent Technology, Santa Clara, CA, USA), equipped with a G1367C auto-sampler connected to an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source, was used in this study. The Siemens Viva-E automatic biochemical analyzer (Siemens Healthcare Diagnostics, Inc.) was also used. The PROIND centrifuge (Heraeus, Connaught, Germany), the XW-80A vortex mixer (Shanghai Jingke Industrial Co., Ltd., Shanghai, China), the PK514BP ultrasonic cleaner (BANDEL, Henstedt-Ulzburg, Germany), and the BHW-IV thermostatic water tank (Beijing Medical Equipment Factory, Beijing, China) were also used.

2.2. Samples

Blood samples used in this study were routinely collected from patients undergoing a liver or kidney transplant or patients with aplastic anemia receiving CyA treatment at Qilu Hospital of Shandong University. The study was conducted in accordance with the Declaration of Helsinki and patient consent was obtained. One hundred and thirty-two samples were collected from patients (males: 76, females: 56). Patient age ranged from 1 to 75 years, with a mean of 40.8 ± 18.6 years. All samples were collected between June and July 2016. Frozen blood samples were supplied in routine blood collection tubes and stored in the laboratory at -20°C prior to analysis.

2.3. EMIT assay

2.3.1. Sample preparation

A 300- μL extraction solution was added to 100 μL of whole blood. The mixture was then vortexed for 1 min and centrifuged at 10,800 rpm for 5 min. The supernatant was injected for analysis.

2.3.2. Assay performance

The CyA-EMIT kit consists of a CyA-specific assay, standard calibrators, controls (three levels), and a wash buffer. Samples were processed according to the kit's instructions.

2.4. LC-MS/MS assay

2.4.1. Preparation of stock and working solutions

A primary solution of CyA and the internal standard (IS) were separately prepared in acetonitrile at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$. Stock solutions of CyA were diluted from the primary solution with acetonitrile and then further diluted to working solutions of 250-

5,000 ng·mL⁻¹. A quality control (QC) working solution of CyA was diluted from the stock solution (1 mg·mL⁻¹) with acetonitrile to reach a concentration of 500, 2,000, or 4,000 ng·mL⁻¹. A working solution of IS (100 ng·mL⁻¹) was prepared by dilution of the stock solution (20 µg·mL⁻¹) with acetonitrile. All standard solutions were stored at 4°C. The stability of the standard solutions was verified throughout the study. A solution of zinc sulphate was prepared in water at a concentration of 100 mM to serve as a cell lysis buffer.

2.4.2. Chromatography and mass spectrometry conditions

CyA and clarithromycin (IS) in whole blood were separated on a Diamonsil C18 column (150 × 4.6 mm, 5 µm, Waters, Milford, MA, USA) and eluted with a mobile phase of 15 mM ammonium formate and 0.5% formic acid:acetonitrile (12:88, v/v) at a flow rate of 0.8 mL/min. The temperature of the auto-sampler was set at room temperature and the temperature of the column oven was set at 70°C.

The source parameters were as follows: sprayed gas (nitrogen) at a temperature of 350°C, a spray flow of 9 L/min, a nebulizer pressure of 40 psi, and a capillary voltage of 4,000 v. A full scan mass spectrum was obtained over a range of m/z 200 to 1,300. The fragmentor voltage was 100 V for CyA and 90 V for the IS, and the collision energy was 50 eV for CyA and 20 eV for the IS. The delta EMV was 300V. Multiple reaction monitoring (MRM) mode was used to detect CyA and the IS.

2.4.3. Preparation and disposition of samples

Calibration and QC samples were prepared during the validation of the two methods and testing of whole blood samples. The concentration of the calibration samples was 25, 50, 75, 100, 200, 300, 400, and 500 ng·mL⁻¹. The concentration of the QC samples was 50, 200, and 400 ng·mL⁻¹.

Protein precipitation was used in sample preparation. A zinc sulfate heptahydrate solution (50 µL, 100 mM) was added to blood samples (150 µL) and mixed. The IS working solution (10 µL) was then added, and the mixture was vortexed for 1 min. Afterwards, 700 µL of methanol was also added and the mixture was vortexed again for 2 min and then centrifuged at 10,800 rpm for 5 min. The supernatant was injected into the LC-MS/MS system for analysis.

2.4.4. Method validation

The LC-MS/MS method was validated in terms of specificity, the matrix effect and extract recovery, linearity and lowest limit of quantitation (LLOQ), intra- and inter-day precision, and storage stability. Validation

strictly conformed to the Chinese Food and Drug Administration guidance on the validation of methods of biomedical analysis.

Specificity. The specificity of method was evaluated by comparing chromatograms of blank blood, a standard solution of CyA and the IS, blank blood spiked with CyA and the IS, and blood from patient 88 after oral administration of CyA.

Matrix effect and extraction recovery. The matrix effect and extraction recovery of CyA and the IS were evaluated with five replicates of the QC samples at three concentrations (50, 200, and 400 ng·mL⁻¹) and five replicates of the IS (100 ng·mL⁻¹). Extraction recovery was evaluated by comparing peak areas of CyA or the IS in mixed blank blood samples to which CyA or IS had been added after extraction.

Calibration curve and LLOQ. A calibration curve was generated with concentrations of 25, 50, 75, 100, 200, 300, 400, and 500 ng·mL⁻¹ in the blood. Samples of each concentration were analyzed in five replicates. A calibration curve was derived by plotting the peak area ratios of CyA to the IS as a function of concentration of CyA. The calibration curve was described by the equation $y = ax + b$, where y corresponds to the peak-area ratio and x to the ratio of the concentration of CyA to IS. The linearity of the calibration curve was assessed using linear regression with the reciprocal of the concentration squared ($1/x^2$) serving as a weighting factor. The LLOQ was evaluated by analyzing five replicates of mixed whole blood samples at the concentration of 25 ng·mL⁻¹.

Accuracy and precision. Accuracy and precision were determined using the same data set. Intra-day precision was determined based on 3 determinations: the LQC (25 ng·mL⁻¹), the MQC (200 ng·mL⁻¹), and the HQC (400 ng·mL⁻¹). The inter-day precision of each assay was analyzed based on the LQC, MQC, and HQC on three different days over a period of one week. Precision was expressed as the coefficient of variation (RSD).

Stability. The stability of CyA in the biological matrix was evaluated as follows and the results were expressed as the percentage recovery. The stability of CyA in blood samples was examined when samples were stored at -20°C for 60 days. Freeze-thaw stability was determined after two cycles of freezing (-20°C) and thawing (25°C) QC samples. The stability of samples on the bench after extraction and in the LC-MS/MS auto-sampler was also determined for 6 and 12 h at room temperature.

2.5. Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was performed using regression analysis. A p -value of < 0.05 was considered statistically significant. The statistical software SPSS was used to

evaluate the correlation between the two assays and Med-Cal software was used to draw a Bland-Altman plot. The Bland-Altman plot is useful in revealing the relationship between the differences and the magnitude of measurements, indicating any systematic bias, and in identifying possible outliers.

3. Results

3.1. EMIT assay

Calibration curve. A calibration curve was automatically obtained from the Viva-E automatic biochemical analyzer. A four-point logarithmic curve was used to obtain the formula for the calibration curve, which was A

$= Ro + K * (1 / [1 + \exp(-a + b * (\ln C))])$. The parameters were $Ro = 2.61010 \times 10^2$, $K = 8.19348 \times 10^1$, $a = -7.22146$, and $b = 1.40956$.

Precision and accuracy. Intra-day precision was determined based on LQC, MQC, and HQC on 3 different days, and the inter-day precision of each assay was analyzed based on the LQC, MQC, and HQC. The precision of QC for CyA was expressed as the coefficient of variation (RSD). The inter- and intra-day RSDs were less than 15% (Table 1).

3.2. LC-MS/MS assay

Specificity. The full scan and product ion mass spectrum of CyA and the IS are shown in Figure 1, while typical

Table 1. The intra-day and inter-day precision of the CyA concentration according to EMIT ($n = 5$)

Concentration (ng mL ⁻¹)	Intra-day			Inter-day		
	Mean $\pm$ SD	Accuracy (%)	RSD (%)	Mean $\pm$ SD	Accuracy (%)	RSD (%)
76.6	67.7 $\pm$ 5.0	88.4	7.4	75.6 $\pm$ 8.0	98.8	10.6
190.4	201.6 $\pm$ 26.7	105.9	13.2	192.9 $\pm$ 17.7	101.3	9.2
350.2	307.9 $\pm$ 41.1	87.9	13.4	325.1 $\pm$ 31.3	92.8	9.6

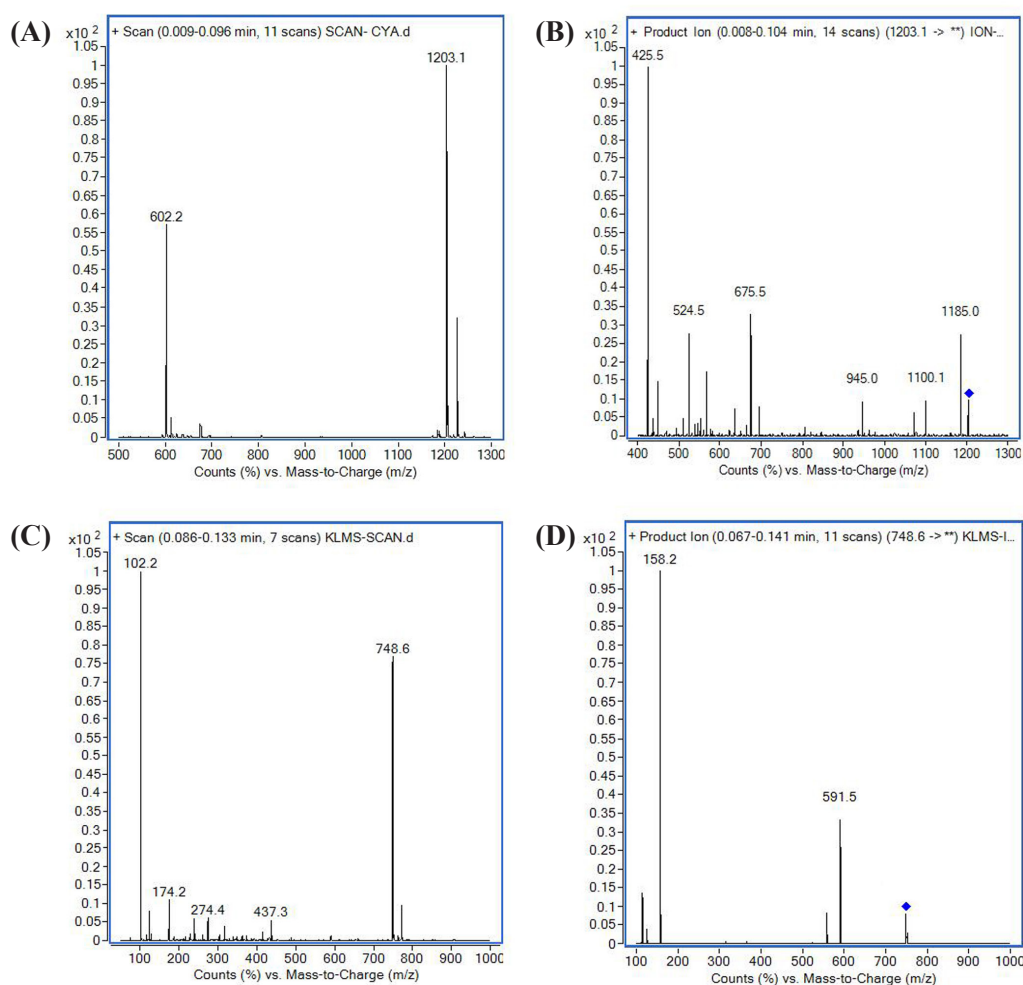


Figure 1. Full scan and product ion mass spectra for CyA (A, B) and an IS (C, D)

MRM chromatograms are shown in Figure 2. MRM mode was used to detect CyA and the IS, with an ion transition of m/z 1203.1 $\rightarrow$ 425.5 (CyA) and 748.6 $\rightarrow$ 158.2 (IS), respectively. The retention time was about 1.8 min for the IS and 5.5 min for CyA. There was no significant interference with endogenous blank human blood at the retention time for the analyte and the IS.

Calibration curve and LLOQ. The calibration curve for CyA was linear over the concentrations governed by the regression equation (weight = $1/x^2$), which was $y = 0.016x + 0.0408$. The correlation coefficient was 0.9950. The LLOQ of CyA was 25 $\text{ng}\cdot\text{mL}^{-1}$ ($n = 5$) with a precision of 3.1% and an accuracy of 106%.

Recovery and matrix effect. A high percentage of

CyA and the IS were recovered and results of both methods were highly reproducible. No significant matrix effect was observed when the CyA concentration was determined at three different QC levels and when the IS was tested at a concentration of 100 $\text{ng}\cdot\text{mL}^{-1}$. The mean recovery was 75.73% and the matrix effect was 93.75% for CyA, and the mean recovery was 94.33% and the matrix effect was 97.19% for the IS. The RSD was less than 15%, indicating that the analytical methods were free of endogenous substances in human blood.

Precision and accuracy. The intra- and inter-day precision and accuracy of the CyA concentration were acceptable for analysis. Results are shown in Table 2.

Stability. The stability of CyA in whole human

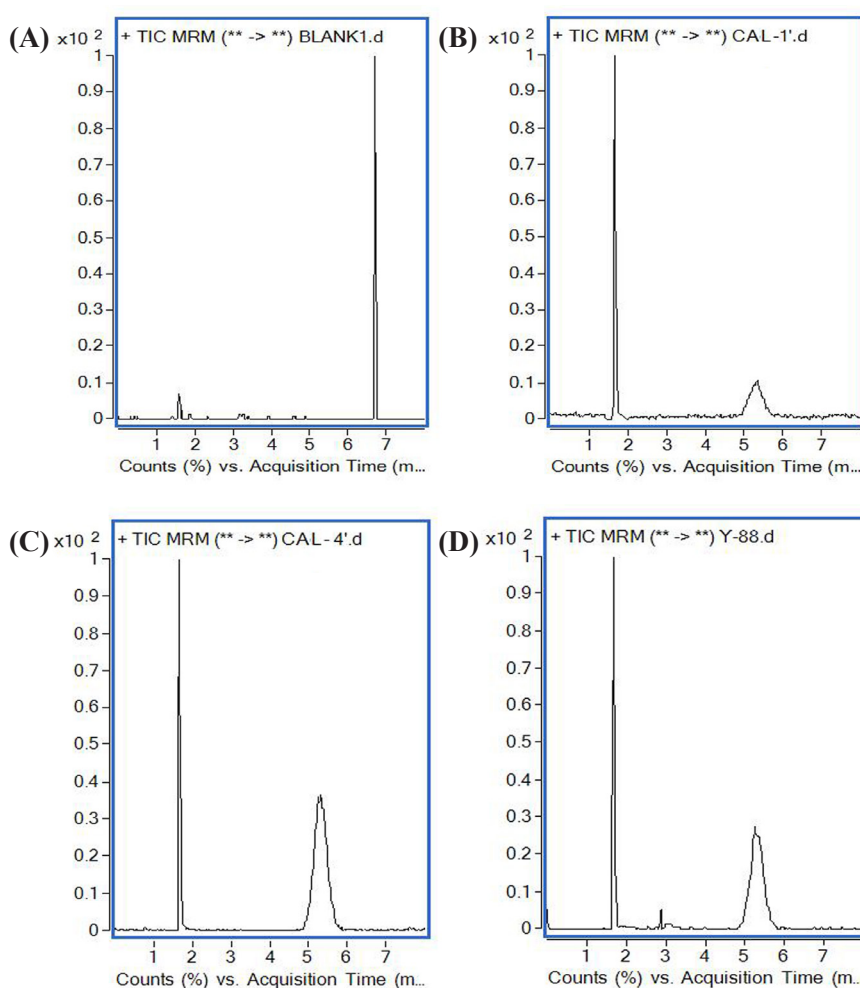


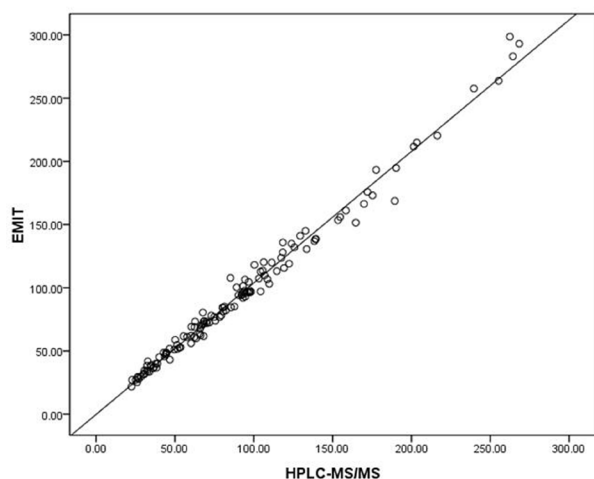
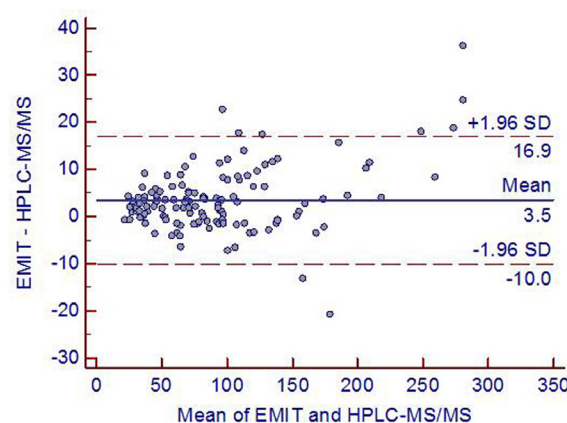
Figure 2. Typical MRM chromatograms for CyA and an IS. (A) blank blood; (B) LLOQ-blank blood spiked with CyA (25 ng/mL) and the IS (100 ng/mL); (C) blank blood spiked with CyA (200 ng/mL) and the IS (100 ng/mL); (D) blood spiked with the IS from patient 88 after oral administration of CyA.

Table 2. The intra-day and inter-day precision of the CyA concentration according to LC-MS/MS ($n = 5$)

Concentration (ng mL^{-1})	Intra-day			Inter-day		
	Mean $\pm$ SD	Accuracy (%)	RSD (%)	Mean $\pm$ SD	Accuracy (%)	RSD (%)
50	49.22 $\pm$ 2.08	98.4	4.24	49.45 $\pm$ 3.41	98.9	6.89
200	192.17 $\pm$ 1.61	96.1	0.84	202.65 $\pm$ 9.06	101.3	4.47
400	410.85 $\pm$ 19.15	102.7	4.66	390.48 $\pm$ 28.35	97.6	7.26

Table 3. Stability of CyA in human blood under different conditions ($n = 5$)

Conditions	50 ng·mL ⁻¹		200 ng·mL ⁻¹		400 ng·mL ⁻¹	
	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
-20°C/60 d	48.77 ± 2.18	4.47	189.43 ± 7.75	4.09	384.89 ± 8.70	2.26
-20°C/1 freeze-thaw cycle	50.63 ± 3.50	6.91	199.38 ± 8.21	4.11	394.90 ± 46.90	11.88
-20°C/2 freeze-thaw cycles	49.28 ± 2.00	4.06	201.27 ± 13.42	6.67	368.40 ± 6.64	1.80
25°C/6 h (extracted samples)	50.43 ± 1.25	2.48	191.86 ± 17.27	9.00	383.69 ± 25.43	6.63
25°C/12 h (extracted samples)	51.18 ± 2.75	5.37	184.68 ± 4.50	2.44	358.48 ± 8.69	2.42

**Figure 3. Correlation of the regression curve for LC-MS/MS and EMIT ($n = 132$).****Figure 4. The Bland-Altman deviation graph of the CyA concentration according to LC-MS/MS and EMIT ($n = 132$).**

blood was investigated under different storage conditions and processing. CyA was stable after 6 h and 12 h on the bench or in the auto sampler, it was stable after two freeze-thaw cycles, and it was stable after freezing at -20°C for 60 days. These results are shown in Table 3.

3.3. Comparison of EMIT and LC-MS/MS

One hundred and thirty-two patient samples were tested with EMIT and LC-MS/MS. Both methods can be used to determine the CyA concentration in blood. The LLOQ was 25 ng·mL⁻¹ for LC-MS/MS and 50 ng·mL⁻¹ for EMIT. EMIT had less reproducibility than LC-MS/MS did, but the two assays were closely correlated over a range of concentrations from 26.24–293.06 ng·mL⁻¹ ($R^2 = 0.969$, Figure 3).

In addition, a plot of the differences between the two methods in terms of the mean concentrations they yielded indicated a close relationship between the two methods, albeit with slight differences. The Bland-Altman deviation graph of CyA is shown in Figure 4. As shown in the plot, the 95% confidence interval was -10.0 – 16.9 ng·mL⁻¹, and most of the data were within the 95% confidence interval. Thus, there was no systematic bias in terms of differences in CyA concentrations and the magnitude of those

measurements.

The CyA blood concentration according to the EMIT assay was slightly higher than that according to the LC-MS/MS assay. One potential explanation for this would be non-specific binding to antibodies in the EMIT assay. The LC-MS/MS assay had excellent reproducibility, suggesting that sample handling was an unlikely source of error.

4. Discussion

The LC-MS/MS technique devised here is a reliable and sensitive assay of the CyA concentration in human blood with an LLOQ of 25 ng·mL⁻¹, which is 2-fold lower than that of EMIT (50 ng·mL⁻¹) assays that are currently available.

Simple protein precipitation with methanol was a robust way to prepare samples for LC-MS/MS and provided clean samples in this study. Previous studies have used liquid-liquid extraction with ethyl ester (11) or tert-butyl-methyl-ether (12) as extraction agents. Solid-phase extraction has been used to extract CyA from biological samples in other studies (13,14). Procedures for liquid-liquid extraction and solid-phase extraction are quite complicated, time-consuming, and potentially dangerous both to the environment and the experimenter. In contrast, protein precipitation is rapid,

environmentally friendly, and convenient for continuous batch analysis, making this technique suitable for analysis of the CyA concentration in human blood.

LC-MS/MS has proven to be useful in determining the CyA concentration as part of TDM since it is more reliable, sensitive, and rational than EMIT. In the current study, the CyA blood concentration according to the EMIT assay was slightly higher than that according to the LC-MS/MS assay. LC-MS/MS had excellent reproducibility, suggesting that sample handling is an unlikely source of error. The most likely explanation for these results would be non-specific binding to antibodies in the EMIT assay. EMIT depends on the reaction between an analyte and a biological antibody, so it may involve more inherent imprecision than other methods of pharmaceutical analysis (*e.g.* chromatography). The specificity of immunoassays depends mainly on the antibody targeting the analyte, but some immunoassays are not highly selective and they may respond to a group of compounds (*e.g.* metabolites) rather than individual compounds. Due to the cross-reaction between a drug and metabolites, EMIT may overestimate the CyA concentration, so monitoring the concentration of CyA with EMIT might lead to too low a dosage of CyA. This could affect the effective administration of CyA, and especially in patients with a low concentration of CyA in the blood since CyA is already a narrowly defined therapeutic index.

Techniques such as a chemiluminescent enzyme immunoassay (CLIA) and an electro-chemiluminescence immunoassay (ECLIA) have recently been used in TDM and have become key methods of measuring the CyA concentration in blood. CLIA systematically yielded a higher CyA concentration than UPLC-TMS did (15). Compared to EMIT, CLIA and ECLIA have a higher sensitivity and a higher precision when measuring the tacrolimus concentration in blood (16). However, no study has compared EMIT and CLIA or ECLIA when measuring the CyA concentration as part of TDM, so this topic should be studied further.

The current study found a close correlation between the LC-MS/MS assay and previous immunoassays. This finding is reassuring since clinical estimates of the boundaries of CyA therapy, which were identified using immunoassays, remain intact. In addition, the increased sensitivity of the LC-MS/MS assay will allow an accurate determination of the CyA concentration in other patients treated with lower doses.

5. Conclusion

In conclusion, a comparison of LC-MS/MS and EMIT to determine the concentration of CyA in human blood indicated that EMIT slightly overestimated the CyA concentration. This finding is consistent with cross-reactivity of the EMIT antibodies with one or more CyA metabolites. However, the two methods were closely

correlated. Therefore, the EMIT assay is suitable for TDM of CyA.

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Organ-preserving surgery for locally advanced duodenal gastrointestinal stromal tumor after neoadjuvant treatment

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Summary

This report aims to investigate the feasibility and outcomes of neoadjuvant imatinib mesylate (IM) administration followed by organ-preserving surgery (OPS) for patients with locally advanced duodenal gastrointestinal stromal tumor (GIST). Between 2012 and 2015, 10 consecutive patients with locally advanced duodenal GISTs were treated in Peking University Cancer Hospital. Multidisciplinary assessment was implemented, and pancreaticoduodenectomy (PD) was initially indicated as the most probable surgical procedure for all 10 patients. To attempt to create opportunities of less-invasive OPS for patients, neoadjuvant IM was administered followed by radical resection. All data were prospectively collected, and the short- and long-term outcomes of the treatment strategy were analyzed. The median treatment duration of neoadjuvant IM administration was 5 mo (range 2-18 mo). Significant tumor shrinkage (from 9.2 to 5.9 cm on average) was observed in all patients, and partial response was achieved in eight patients (80.0%) according to the Response Evaluation Criteria in Solid Tumors 1.1. No tumor perforation occurred, and nine patients (90.0%) underwent successful OPS with four different operation types. Postoperative morbidity rate of OPS was 55.6% (5/9), and no mortality occurred. After a median follow-up of 36 mo, one patient developed multiple distant metastases, but no local recurrence was observed. For long-term follow-up, patients who underwent OPS did not show any degradation in quality of life, whereas the patient who underwent PD suffered weight loss of ~10 kg. In conclusion, in patients with locally advanced duodenal GISTs, neoadjuvant IM administration followed by OPS is a feasible treatment strategy which leads to favorable short- and long-term outcomes.

Keywords: Organ-preserving surgery, duodenal gastrointestinal stromal tumor, imatinib mesylate, pancreaticoduodenectomy, neoadjuvant therapy

1. Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor arising in the gastrointestinal tract. GISTs occur most frequently in the stomach (50-60%), followed by the small intestine

(~30%) (1). Duodenal GISTs are relatively uncommon and comprise ~5% of all GISTs, but represent ~20% of primary small intestine cases (2).

More than 80% of GISTs harbor *c-KIT* or platelet-derived growth factor receptor (*PDGFR*)A gene mutations (3,4). Imatinib mesylate (IM), an inhibitor of *c-KIT* and *PDGFR*A, can effectively reduce tumor size and improve prognosis (5). However, IM treatment cannot completely replace surgery, and complete surgical resection with clear margins remains the only curative approach for resectable GISTs.

Pancreaticoduodenectomy (PD) is the standard surgical procedure for malignant tumors in the periampullary area. Despite advances in surgical

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techniques and perioperative management, the rates of postoperative complications (30-50%) and mortality (1-7.8%) for PD remain high (6). For these reasons, and because of its significant effect on long-term quality of life, less invasive organ-preserving surgery (OPS) might be more beneficial for patients with low-grade malignancies such as duodenal GISTs. GISTs frequently show expansive growth with a clear border, and they rarely exhibit lymph node metastasis, for which complete excision with negative margins is indicated; therefore, extensive resection is usually not required.

However, for advanced duodenal GISTs that exhibit large tumor size, the separation of the pancreas and the major papilla from the tumor is complicated. These features hamper OPS, and PD remains the most common procedure in patients with locally advanced duodenal GISTs. The treatment strategy of neoadjuvant IM followed by OPS was designed specifically for these patients. It is expected that IM-induced tumor shrinkage to produce clearer borders would facilitate the successful application of OPS in patients who were initially indicated for PD.

The objective of the present study was to evaluate the feasibility and short- and long-term outcomes of this treatment strategy in patients with locally advanced duodenal GISTs.

2. Materials and Methods

2.1. Study population

We reviewed the prospectively collected data of 10 consecutive patients with locally advanced duodenal GISTs who were treated with preoperative IM followed by radical resection from August 2012 to September 2015 at the Department of Hepato-Pancreato-Biliary Surgery, Peking University Cancer Hospital & Institute, Beijing, China. All patients were definitively diagnosed with GIST by preoperative endoscopic or abdominal ultrasound-guided fine needle aspiration. The incidence of mutations (*c-KIT* 9, 11, 13 and 17, and *PDGFRA* 12 and 18) was evaluated by sequencing of polymerase chain reaction products, as described previously (7).

Data concerning clinical information, surgical procedures, pathological findings, complications after surgery, and long-term outcomes were extracted from patient records. Written informed consent, as required by the Institutional Review Board of Peking University Cancer Hospital & Institute, was obtained from all patients.

2.2. Preoperative treatment

Following a discussion among the surgeons, oncologists, radiologists and pathologists, all cases were classified as advanced stage and PD was initially

indicated as the most probable surgical procedure for all 10 patients. After careful multidisciplinary assessment, IM (400 mg/d) was administered preoperatively in all patients to reduce tumor burden. Thereafter, tumor response was evaluated every 2 mo according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 (8), and surgical resection was performed when tumor shrinkage reached a plateau.

2.3. Surgical procedures

After exploratory laparotomy and confirming the possibility of R0 resection, the right part of the gastrocolic ligament was divided and the Kocher procedure was performed to expose the second portion of the duodenum and the pancreatic head. When necessary, the Cattell-Braasch maneuver was performed to expose the third portion of the duodenum.

The position of the major papilla and its relationship to the tumor were subsequently determined by careful palpation and insertion of a tube in the cystic duct after cholecystectomy. If tumor invasion of the major papilla was observed, PD was performed. However, if conservation of the major papilla was possible, one of the four OPS procedures was performed (Figure 1). The decision for resection type was made at the discretion of the operating surgeon. Type 1, for tumors located at the second portion, the area of the duodenal wall containing the base of the tumor was completely resected and primary closure was performed. Type 2, for tumors located at the medial wall of the second portion, when separation of the pancreas from the tumor was complicated. *En-bloc* radical resection including partial duodenal wall and partial pancreatic parenchyma resection were performed, and to avoid intractable postoperative pancreatic fistula formation, as well as for primary closure of the duodenal wall, side-to-side anastomosis of the pancreatic wound surface with the jejunum was performed by running suturing via Roux-en-Y reconstruction. Type 3, for large tumors located at the third portion, segmental resection of the third and fourth portions, and end-to-end anastomosis between the residual duodenum and the proximal jejunum were performed. Type 4, for large tumors located at the border of the second and third portions that invaded the head of the pancreas, when the Wirsung duct had to be transected but the major papilla and intrapancreatic common bile duct could be preserved, *en-bloc* radical resection, including the third and fourth portions, and partial pancreatic parenchyma resection were performed. The pancreatic wound surface was anastomosed to the jejunum by a Wirsung duct-to-mucosa anastomosis, and the duodenojejunal end-to-side anastomosis in the same jejunal loop was also performed.

Systematic nodal dissection around the pancreatic head was not performed routinely. All procedures were performed by the same senior hepato-pancreato-biliary surgeon (CY Hao). The resected specimens

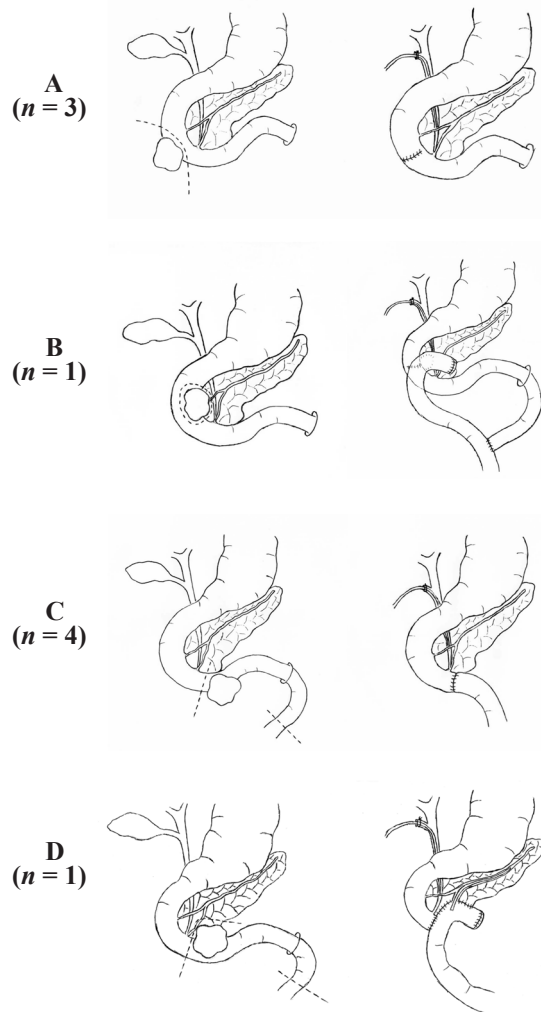


Figure 1. The four OPS procedures. (A) Type 1: for tumors located at the second portion, the area of the duodenal wall containing the base of the tumor was completely resected (left), and primary closure was performed (right). (B) Type 2: for tumors located at the medial wall of the second portion, *en-bloc* radical resection including partial duodenal wall and partial pancreatic parenchyma resection were performed (left). Primary closure of the duodenal wall and side-to-side anastomosis of the pancreatic wound surface with the jejunum was performed by runningsuturing *via* Roux-en-Y reconstruction (right). (C) Type 3: for large tumors located at the third portion, segmental resection of the third and fourth portions (left), and end-to-end anastomosis between the residual duodenum and proximal jejunum was performed (right). (D) Type 4: for large tumors located at the border of the second and third portions that invaded the head of the pancreas, when the Wirsung duct had to be transected but major papilla and intrapancreatic common bile duct could be preserved, *en-bloc* radical resection, including the third and fourth portions, and partial pancreatic parenchyma resection were performed (left). The pancreatic wound surface was anastomosed to the jejunum by a Wirsung duct-to-mucosa anastomosis, and the duodenojejunal end-to-side anastomosis in the same jejunal loop was also performed (right).

were examined pathologically. Risk stratification was determined according to the revised National Institutes of Health (NIH) classification (9).

2.4. Postoperative complications and follow-up

Complications were recorded and classified according

to the criteria proposed by Clavien *et al* (10); grade ≥ 2 complications were recorded. Postoperative pancreatic fistula (POPF) was defined according to the International Study Group on Pancreatic Fistula recommendations (11); grade $\geq B$ POPF was recorded. Delayed gastric emptying (DGE) was defined as prolonged aspiration of 500 mL/d from the nasogastric tube for ≥ 10 postoperative days, the need for reinsertion of a nasogastric tube, or the failure to maintain oral intake by postoperative day 14 (12).

Postoperative adjuvant therapy with IM at 400 mg/d was administered in all cases. Systemic follow-up included postoperative abdominal ultrasound or computed tomography scanning at 3-mo intervals. Long-term quality of life data regarding general health, weight loss, gastrointestinal symptoms, and dietary restrictions were obtained.

3. Results and Discussion

Ten patients with locally advanced duodenal GISTs (6 men and 4 women; median age, 50 years; range, 43-65 years) were enrolled in the analysis. Tumors were diagnosed based on chief complaints (Table 1). The primary tumor was located at the second portion of the duodenum in five cases, at the third portion in three cases, and at the border of the second and third portions in two cases. The most common c-KIT mutation was the exon 11 deletion mutation (8/10 patients), whereas two patients harbored the exon 9 duplication (Table 1).

Preoperative IM was administered in all 10 patients for 2-18 mo (median, 5 mo). Significant tumor shrinkage was observed in all patients, with a maximum mean diameter decrease from 9.2 cm (range, 4.7-16.0 cm) to 5.9 cm (range, 3.0-12.8 cm) (Table 1, Figure 2). No tumor perforation occurred. According to RECIST 1.1 criteria, eight cases (80.0%) achieved a partial response (PR), and two cases had stable disease (SD) with $< 30\%$ tumor shrinkage. Surgical resection was performed at a mean of 22 d (range, 14-36 d) after IM discontinuation.

Nine patients (90.0%) underwent successful OPS (Table 2). In one patient, PD was performed because the major papilla was invaded by the tumor, which failed to be separated. R0 resection was achieved in all cases. For OPS, the median estimated blood loss was 450 mL (range, 100-800 mL), and the median operating time was 385 min (range, 273-540 min). None of the patients received intraoperative blood transfusion. For the patient undergoing PD, the blood loss was 400 mL, and operating time was 380 min. Overall, 4 units of red blood cells were transfused because of preoperative anemia.

The mean maximal tumor diameter of all surgical specimens was 6.8 cm (range, 3.5-14.0 cm). The mitotic counts and Ki67 proliferation indices of all specimens are shown in Table 2. All 10 cases were classified as high-risk at the time of surgical resection. Surgical

Table 1. Clinicopathological characteristics and preoperative treatment of patients with advanced duodenal GIST

No.	Sex/age (yr)	Chief complaint	Gene mutation	Tumor size (cm) pre-/post-IM	IM treatment duration (mo)	Response (RECIST 1.1)	Revised NIH classification
1	F/44	Abdominal pain	Exon 11 Del 569-576	13.5/7	18	PR	High
2	M/50	Asymptomatic	Exon 11 Del 559-574	4.7/3	4	PR	High
3	M/43	Black stool	Exon 11 Del 553-574	6.7/4.6	4	PR	High
4	F/50	Abdominal pain	Exon 11 Del 557-571	5.0/3.5	2	PR	High
5	M/60	Abdominal discomfort	Exon 11 Del 563-571	6.5/4.1	6	PR	High
6	M/44	Abdominal discomfort	Exon 11 PM V559D	16/12.7	6	SD	High
7	F/50	Black stool	Exon 9 Dup 502-503	8/4.7	8	PR	High
8	M/44	Black stool	Exon 11 Del 566-575	11/6.5	4	PR	High
9	M/65	Black stool	Exon 11 Del 557-571	8/3.7	17	PR	High
10	F/64	Abdominal pain	Exon 9 Dup 502-503	12/9	4	SD	High

F, female; M, male; Del, deletion; PM, point mutation; Dup, duplication.

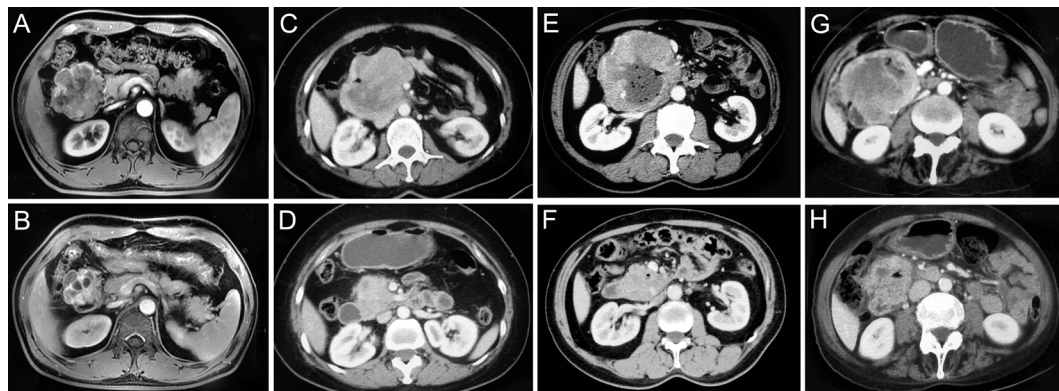


Figure 2. Comparison of radiological appearance before and after preoperative IM treatment for advanced duodenal GIST. (A, B): A 6.7-cm lesion at the second portion (A) reduced to 4.6 cm after 4 mo preoperative treatment (B). A type 2 surgical procedure was performed. **(C,D):** An 8.0-cm lesion at the third portion (C) reduced to 4.7 cm after 8 mo preoperative treatment (D). A type 3 surgical procedure was performed. **(E,F):** An 11.0-cm lesion at the border of the second and third portions (E) reduced to 6.5 cm after 4 mo preoperative treatment (F). A type 4 surgical procedure was performed. **(G,H):** A 12.0-cm lesion at the third portion (G) reduced to 9.0 cm after 4 mo preoperative treatment (H). Because the major papilla was invaded by the tumor, PD was performed.

Table 2. Surgical characteristics, pathological findings, and short and long-term outcomes of patients with advanced duodenal GISTs

No.	Location	Surgical procedure	OT (min)	EBL (mL)	Mitotic counts (/50 HPF)	Ki67 (%)	POPF/GI leakage/DGE	Recurrence or metastasis	Status/follow-up (mo)
1	D3	Type 3	390	500	5-10	5	N/N/Y	None	Alive/36
2	D2	Type 1	274	200	> 10	10	N/N/N	None	Alive/50
3	D2	Type 2	356	700	< 5	5	N/N/N	None	Alive/44
4	D2/3	Type 3	273	100	5-10	5	N/N/N	None	Alive/47
5	D2	Type 1	353	800	> 10	60	N/Y/N	Liver (6 mo)	Dead/17
6	D2	Type 1	405	600	> 10	10	Y/N/N	None	Alive/36
7	D3	Type 3	420	300	> 10	10	Y/N/Y	None	Alive/17
8	D2/3	Type 4	540	200	< 5	3	Y/Y/N	None	Alive/16
9	D2	Type 3	458	550	5-10	10	N/N/N	None	Alive/16
10	D3	PD	380	400	< 5	15	Y/N/N	None	Alive/41

D2, second portion of the duodenum; D3, third portion of the duodenum; OT, operating time; EBL, estimated blood loss; HPF, high-power field; GI, gastrointestinal; N, no; Y, yes.

margins were negative in all patients.

Postoperative morbidity rate of OPS was 55.6% (5/9). POPF, gastrointestinal leakage, and DGE were the most common complications (Table 2). Two patients were successfully treated with ultrasound-guided percutaneous drainage, and others were resolved after conservative therapy consisting of complete drainage and antibiotic therapy. In the patient who underwent PD, POPF was observed and was successfully treated with ultrasound-guided percutaneous drainage. Subsequent surgery was not required in any patient, and there was no perioperative mortality. The median duration of hospital stay was 19 d (range, 13-36 d).

The median follow-up duration was 36 mo (range, 16-50 mo). At 6 postoperative months, multiple liver and peritoneal metastases were detected in one patient who underwent OPS, and second-line treatment with sunitinib was administered. The patient was dead at 17 postoperative months. The remaining nine patients were alive with no evidence of local recurrence or metastasis at the end of the follow-up period (Table 2).

OPS was not associated with late comorbidity such as reflux cholangitis, pancreatitis, weight loss, or diarrhea. In addition, none of the patients who underwent OPS reported any degradation in quality of life. The patient who underwent PD reported a weight loss of ~10 kg. There were no IM-related grade 3 or 4 adverse events.

The outcomes of limited resection or pancreas-sparing duodenectomy of duodenal GISTs (13,14), and the results of neoadjuvant IM therapy in advanced GISTs (15,16) have been reported. However, to the best of our knowledge, the present study is the first to describe preoperative IM as neoadjuvant therapy followed by OPS for patients with locally advanced duodenal GISTs who would otherwise require PD.

Duodenal GISTs frequently involve the second portion of the duodenum and less often the third, fourth, and first portions (17). With less invasiveness and a low incidence of nodal metastasis (18), the goal of surgery for GISTs is complete tumor resection with negative surgical margins. Therefore, extensive resection including nodal dissection is usually not required, and OPS presents an attractive alternative to PD for duodenal GISTs.

IM is the first globally approved effective nonsurgical treatment for inoperable or metastatic GIST with a response rate of > 80% (5,19). IM has been approved for adjuvant therapy in patients with GIST who have a high risk of postoperative recurrence (20,21). Subsequently, the development of an IM-based neoadjuvant treatment strategy has been proposed (22). In selected cases of locally advanced GIST, IM facilitates resection and decreases surgical morbidity by reducing the need for extensive resection and the perioperative risk of tumor rupture (15,23).

Candidates for preoperative IM include patients who

may benefit from preoperative tumor downstaging, and such selection processes require careful multidisciplinary assessment. This strategy is especially attractive in difficult anatomical locations (duodenum, distal rectum, or gastroesophageal junction) where resection of the primary tumor may cause significant morbidity or functional deficits. Therefore, patients with locally advanced duodenal GISTs, in which PD is considered as the standard surgical approach, represent ideal candidates for preoperative IM therapy. Tumor shrinkage facilitates visualization of the relationship of the tumor with the major papilla and pancreatic parenchyma, thereby enhancing the likelihood of successful OPS. In the present study, preoperative IM induced tumor shrinkage in all cases, with most patients demonstrating a PR and undergoing successful OPS. Although, previous studies have shown that 3-5% of patients with advanced GISTs who were treated with IM had gastrointestinal and tumor hemorrhage that required earlier surgical intervention (5), in the present study IM was well tolerated with no cases of tumor perforation, hemorrhage, or grade 3/4 adverse events.

Because of the acquisition of additional activating *c-KIT* or *PDGFRA* mutations in tumor clones, which usually accounts for secondary resistance, a refractory response to IM occurs at a median of 2 years after treatment initiation (24). Therefore, surgery cannot be completely replaced, and surgical intervention is required for resectable cases following tumor shrinkage. The optimal duration for preoperative IM therapy is usually 4-12 mo (15). At this time, a plateau in tumor shrinkage is usually seen and the risk of developing secondary resistance to IM is still low. In our series, the mean duration of IM was within these suggested boundaries.

Johnston *et al* (25) supposed that recurrence of duodenal GISTs was mostly dependent on the tumor biology rather than the surgical approach. A retrospective review of 114 patients from the French Sarcoma Group (26) revealed that limited resection results in similar survival and lower morbidity rates compared with PD. In the present study, local recurrence was not observed in the median 36-mo follow-up. At 6 postoperative months, patient 5 developed liver and peritoneal multiple metastases. It is worth noting that the Ki67 index reached up to 60%. This might explain the poor prognosis and confirm that the recurrence in that case was dependent on tumor biology rather than surgical approach. POPF, gastrointestinal leakage and DGE occurred in three, two and two cases respectively, but all cases were successfully treated with ultrasound-guided percutaneous drainage or conservative therapy, indicating the acceptable safety of OPS. Furthermore, the patients who underwent OPS have demonstrated good quality of life thus far, while the patient who underwent PD suffered obvious weight loss, suggesting that OPS might be more advantageous than PD in terms of long-term quality of life.

Indications for OPS depend not only on tumor size, but also on tumor location and proximity to nearby structures (pancreatic duct, distal common bile duct, and major papilla). Because of proximity to these structures, OPS is a technically demanding procedure. In the present study, the major indication for OPS was successful preservation of the major papilla and intrapancreatic common bile duct. Some studies (13) have suggested that, to achieve adequate tumor clearance, conventional PD should be performed in cases of partial pancreatic parenchyma invasion. However, in our experience, this may not be a contraindication to OPS. Resection of adequate partial pancreas parenchyma followed by anastomosis of the pancreatic wound surface to the jejunum (type 2 procedure), and even transection of the Wirsung duct and duct-to-mucosa anastomosis (type 4 procedure) resulted in favorable progression-free survival. It is worth noting that in patient 10, although obvious tumor shrinkage was seen, OPS was not performed because of tumor invasion to the major papilla.

Various OPS techniques for duodenal GISTs have been described (13,14,27,28), however, to the best of our knowledge, the type 4 procedure is a creative surgical procedure that has not been reported before. Besides the four techniques described in the present study, for large tumors involving the second portion of the duodenum, where the resulting defect is too large to close, both proximal segmental duodenectomy and Roux-en-Y anastomosis of the duodenal defect with the jejunum have been proposed. Pancreatic head resection with segmental duodenectomy has been reported as OPS for benign or low-grade malignant tumors of the pancreatic head (29). However, OPS with reimplantation of the major papilla is technically demanding, and carries a higher morbidity risk. Therefore, in our institution, we perform PD when tumor invasion prevents major papilla preservation.

The study had some limitations such as small sample size and short follow-up period. However, our results indicate that preoperative IM effectively downsizes the tumor in patients with locally advanced duodenal GISTs, facilitating complete tumor resection via less invasive OPS.

In conclusion, in patients with locally advanced duodenal GISTs, neoadjuvant IM administration followed by OPS is a feasible treatment strategy that provides favorable short- and long-term outcomes.

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Latest advances in the efficacy, tolerability, and monotherapy of integrase inhibitors

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Summary

More than 30 drugs for antiretroviral therapy (ART), including integrase inhibitors (INIs), have been approved by the U.S. Food and Drug Administration (FDA) as of 2017. Integrase is the third essential enzyme in the cycle of human immunodeficiency virus (HIV) replication. INIs can effectively inhibit the replication of HIV and HIV is less prone to develop resistance to INIs clinically. Previous studies based on 7 phase III clinic trials indicate that INIs have satisfactory efficacy and tolerability in patients infected with HIV. The latest advances in INIs indicate that: *i*) dolutegravir (DTG)-based regimens are more efficacious, tolerable, and safer forms of first-, second-, and third-Line ART; *ii*) current studies have indicated that DTG monotherapy fails both virologically and clinically; and *iii*) whether the most cost-effective treatment for DTG is to replace efavirenz (EFV) as a first-line ART, to replace protease inhibitors (PIs) in second-line ART, or to replace both as a monotherapy is unclear. Given these circumstances, further study of INIs in terms of drug interactions, dose reduction, drug convenience, and drug costs is warranted.

Keywords: Dolutegravir; drug resistance; monotherapy; antiretroviral therapy

1. Introduction

Due to the complexity and lethality of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), a total of 36.7 million people are living with HIV and 1.8 million people worldwide were initially infected with HIV in 2016; there were a total of 708,158 people with HIV/AIDS and 219,050 AIDS-related deaths in China as of May 31, 2017 (1-2).

Antiretroviral therapy (ART) is a chronic suppressive treatment that effectively provides lifelong treatment for patients with HIV, improving their quality of life (3). In 2015, the World Health Organization (WHO) recommended that ART should be initiated in all HIV-infected adults, regardless of their CD4⁺ cell count (4).

Because of the expanded scale of ART worldwide, AIDS-related deaths declined by 48% from a peak of 1.9 million in 2005 to 1.0 million in 2016 (5).

Since zidovudine (AZT) was used as the first drug for treatment of AIDS in 1987, more than 30 drugs have been approved by the U.S. Food and Drug Administration (FDA) for ART as of 2017. These include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INs), fusion inhibitors (FIs), entry inhibitors (EIs), HIV integrase strand transfer inhibitors (INSTIs), and multi-class combination products (Table 1) (6).

Integrase is the third essential enzyme in the cycle of HIV replication. INIs can effectively inhibit the replication of HIV. The drugs mainly used in initial ART at present are NRTIs, NNRTIs, and PIs, but HIV mutates and develops resistance to these drugs. In contrast, HIV is less prone to develop resistance to INIs clinically (7-8). Functional analogues of integrase have yet to be identified in the human body thus far, so integrase has become an ideal new target for anti-HIV therapy, ushering in an era of ART using INIs (9).

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Table 1. Anti-HIV drugs approved by the U.S. FDA over the past 30 years

Approved In	Brand Name	Generic Name	Manufacturer
Nucleoside reverse transcriptase inhibitors (NRTIs)			
1987	Retrovir	Zidovudine (AZT) + Zidovudine (ZDV)	GlaxoSmithKline
1991	Videx	Didanosine (ddI)	Bristol-Myers Squibb
1992	Hivid	Zalcitabine (ddC)	Roche Pharmaceuticals
1994	Zerit	Stavudine (d4T)	Bristol-Myers Squibb
1995	Epivir	Lamivudine (3TC)	GlaxoSmithKline
1997	Combivir	3TC + AZT	GlaxoSmithKline
1998	Ziagen	Abacavir (ABC)	GlaxoSmithKline
2000	Trizivir	ABC + 3TC + AZT	GlaxoSmithKline
2000	Videx EC	ddI	Bristol-Myers Squibb
2001	Viread	Tenofovir Disoproxil Fumarate (TDF)	Gilead Sciences
2003	Emtriva	Emtricitabine (FTC)	Gilead Sciences
2004	Epzicom	ABC + 3TC	GlaxoSmithKline
2004	Truvada	FTC + TDF	Gilead Sciences
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)			
1996	Viramune	Nevirapine (NVP)	Boehringer Ingelheim
1997	Rescriptor	Delavirdine (DLV)	Pfizer
1998	Sustiva	Efavirenz (EFV)	Bristol-Myers Squibb
2008	Intelence	Etravirine (ETR)	Tibotec
2011	Viramune XR	NVP	Boehringer Ingelheim
2011	Edurant	Rilpivirine (RPV)	Tibotec
Protease inhibitors (PIs)			
1995	Invirase	Saquinavir (SQV)	Roche Pharmaceuticals
1996	Norvir	Ritonavir (RTV)	Abbott Laboratories
1996	Crixivan	Indinavir (IDV)	Merck
1997	Viracept	Nelfinavir (NFV)	Pfizer
1997	Fortovase	SQV Mesylate	Roche Pharmaceuticals
1999	Agenerase	Amprenavir (APV)	GlaxoSmithKline
2000	Kaletra	Lopinavir and Ritonavir (LPV/r)	Abbott Laboratories
2003	Reyataz	Atazanavir (ATV)	Bristol-Myers Squibb
2003	Lexiva	Fosamprenavir (FPV)	GlaxoSmithKline
2005	Aptivus	Tipranavir (TPV)	Boehringer Ingelheim
2006	Prezista; Prezcobix	Darunavir (DRV)	Tibotec
Integrase inhibitors (INIs)			
2007	Isentress	Raltegravir (RAL)	Merck
2012	Stribild	Elvitegravir (EVG)	Gilead Sciences
2013	Tivicay	Dolutegravir (DTG)	GlaxoSmithKline & Shionogi Pharma
Fusion inhibitors (FIs)			
2003	Fuzeon	enfuvirtide, T-20 (ENF)	Roche Pharmaceuticals & Trimeris
Entry inhibitor (EI) - CCR5 co-receptor antagonists			
2007	Selzentry; Celsentri	Maraviroc (MVC)	Pfizer
HIV integrase strand transfer inhibitors (INSTIs)			
2007	Isentress	RAL	Merck & Co.
2013	Tivicay	DTG	ViiV Healthcare
2014	Vitekta	EVG	Gilead Sciences
Multi-class combination products			
2006	Atripla	EFV+FTC+TDF	Gilead Sciences
2011	Complera	FTC+RPV+TDF	Gilead Sciences
2012	Stribild	EVG+Cobicistat+FTC+TDF	Gilead Sciences
2015	Evotaz	ATV Sulfate+Cobicistat	Bristol-Myers Squibb
2015	Prezcobix	Cobicistat + DRV Ethanolate	Janssen Pharmaceuticals

2. Clinical trial on INIs, and dolutegravir (DTG) in particular

At the current point in time, 7 clinical trials have demonstrated that INIs, and DTG in particular, has satisfactory efficacy and tolerability in newly diagnosed patients, treated patients, and patients receiving multiple regimens (Table 2).

Walmsley *et al.* conducted a randomized controlled trial (RCT) that divided 833 untreated patients with HIV

into a DTG group and an efavirenz (EFV) group. Results indicated that DTG plus abacavir (ABC)-lamivudine (3TC) was significantly superior to EFV-tenofovir (TDF)-emtricitabine (FTC) at 48, 96, and 144 weeks, with better tolerability and fewer dropouts (10-12).

Raffi *et al.* conducted an RCT that divided 822 treatment-naïve patients with HIV into a DTG group and an Raltegravir (RAL) group. Results indicated that once-daily DTG was comparable to twice-daily RAL in untreated patients in week 48 and week 96 (13-15).

Table 2. Efficacy and tolerability of INIs in 7 clinical trials

Authors	Study Design	Sample Size	Group	Drug Dose	Treatment Stage	Primary End Point	Outcomes	Tolerability*	Ref.
Walmsley SL, <i>et al.</i>	Randomized, double-blind, phase 3 study.	414 419	DTG-ABC-3TC EFV-TDF-FTC	Once daily Once daily	Initial treatment	HIV-1 RNA level of less than 50 copies per milliliter in week 48.	88% 88%	4% 10%	(17-19)
Raffi F, <i>et al.</i>	Phase 3, randomized, double-blind, active-controlled, non-inferiority study.	411 411	DTG RAL	50 mg once daily 400 mg twice daily	Initial treatment	Proportion of participants with HIV-1 RNA less than 50 copies per mL at 48 weeks with a 10% non-inferiority margin.	88% 85%	2% 2%	(20-22)
Feinberg J, <i>et al.</i>	Open-label, phase 3b, non-inferiority study.	242 242	DTG + 2 NRTIs DRV plus RTV + 2 NRTIs	50 mg once daily 800 mg plus 100 mg once daily,	Initial treatment	Proportion of patients with HIV-1 RNA concentration lower than 50 copies per mL in week 48 with a 12% non-inferiority margin.	90% 83%	2% 4%	(23-24)
Cahn P, <i>et al.</i>	Phase 3, randomized, double-blind, active-controlled, non-inferiority study.	354 361	DTG RAL	50 mg once-daily 400 mg twice-daily	Second- or third-line treatment	Proportion of patients with plasma HIV-1 RNA less than 50 copies per mL in week 48.	71% 64%	– –	(25)
Nichols G, <i>et al.</i>	Single-arm, open-label phase III study.	183	DTG while continuing a failed regimen (without RAL or EVG) through day 7	50 mg	Multiple treatments	i) Mean change from baseline in plasma HIV-1 RNA on day 8. ii) Proportion of subjects with HIV-1 RNA <50 c/mL in week 24.	-1.43 log10 c/mL 69%	–	(26)
Trottier B, <i>et al.</i>	Randomized, open-label, Phase IIIb study.	275 278	Early switch group Late switch group	ABC/DTG/3TC once daily for 48 weeks Continue ART for 24 weeks and then switch to ABC/DTG/3TC	Second- or third-line treatment	Proportion of subjects with HIV-1 RNA <50 copies/mL in week 24.	85% 88%	– –	(27)
Orrell C, <i>et al.</i>	Randomised, open-label, multicenter, active-controlled, parallel-group, non-inferiority phase 3b study.	250 249	DTG+ABC+3TC ATV+TDF+ FTC	Once a day once a day	Second- or third-line treatment	Proportion of participants with HIV-1 RNA viral loads of less than 50 copies per mL in week 48.	82% 71%	11% 13%	(28)

*Treatment discontinuation due to adverse events.

Once-daily dosing with no need for a pharmacokinetic booster makes DTG-based therapy an attractive treatment option for treatment-naïve patients infected with HIV-1.

Feinberg *et al.* conducted an RCT that divided 484 therapy-naïve adults into a DTG group and a darunavir plus ritonavir (DRV/r) group (16-17). Results indicated that once-daily DTG was superior to once-daily DRV/r. Once-daily DTG in combination with fixed-dose NRTIs represents an effective new treatment option for treatment-naïve patients infected with HIV-1.

Cahn *et al.* conducted an RCT that divided 719 INI-naïve adults with resistance to at least two classes of drugs into a DTG group and an RAL group (18). Results indicated that once-daily DTG was well-tolerated and had a greater virological effect than twice-daily RAL in patients who had previously been treated.

Nichols *et al.* conducted a RCT in which 183 adults with resistance to RAL and/or EVG received DTG 50 mg twice daily while continuing their failing regimen through day 7, after which the regimen was optimized with ≥ 1 fully active drug and continued administration of DTG (19). Results indicated that the mean change in HIV-1 RNA on day 8 decreased from the baseline and that HIV-1 RNA was < 50 c/mL in 69% of subjects in week 24. DTG 50 mg twice daily therapy was effective in this highly treatment-experienced population with INI-resistant virus.

A study by Trottier *et al.* randomly assigned subjects to switch to ABC/DTG/3TC once daily for 48 weeks (early-switch group) or to continue current ART for 24 weeks and then switch to ABC/DTG/3TC (late-switch group) (20). Data revealed that switching to ABC/DTG/3TC was a comparable alternative to continuing ART support ABC/DTG/3TC when considering whether to switch regimens in HIV-1-infected adults with stable viral suppression.

Subjects in the study by Orrell *et al.* were randomly assigned to a DTG group or an atazanavir (ATV) group (21). The regimen that included DTG had comparable efficacy and a similar safety profile to the ATV regimen, substantiating the use of DTG to treat HIV-1 infection in treatment-naïve women.

Based on international guidelines and the large clinical trials mentioned earlier (22-24): *i*) most international guidelines recommend DTG as an integral part of initial treatment; *ii*) data from a number of clinical trials support the use of DTG, which has a high level of antiviral efficacy; *iii*) DTG is administered once daily in a small dose with no need for synergistic action of other drugs and can be administered at any time; *iv*) DTG interacts little with commonly used drugs; and *v*) HIV is unlikely to develop resistance to DTG.

Although the above studies have indicated the advantages of INIs (and especially DTG), there are still many problems with the tolerability and independent use of INIs. These issues were touched upon by national

experts and researchers at the 9th International AIDS Society Conference on HIV Science (IAS 2017).

3. Latest advances in INIs according to IAS 2017

Efficacy as initial treatment. Taiwo *et al.* conducted a clinic trial of once-daily DTG (50 mg) + 3TC (300 mg) in treatment-naïve participants who were infected with HIV-1 and who had a viral load (VL) $\geq 1,000$ and $< 500,000$ copies/mL (25). Results indicated that the median change in the CD4 count from entry to week 24 was +167 (86,275) cells/mm³, which means once-daily DTG plus 3TC was efficacious and welltolerated. However, an RCT of this regimen versus standard treatment is warranted.

Figueroa *et al.* conducted a pilot study to evaluate the antiviral efficacy of a dual therapy regimen with DTG plus 3TC as initial ART in 20 treatment-naïve adults infected with HIV-1 (26). Results indicated that there were no new virologic failures, no new AIDS-defining illnesses, and no treatment discontinuations through the extension phase. Dual therapy with DTG plus 3TC was efficacious and tolerated through 96 weeks of treatment, so this approach is being examined in a large randomized, doubleblinded trial.

Efficacy as second- or third-line treatment. Aboud *et al.* published interim data from the DAWNING study, which is a non-inferiority study of DTG plus 2 NRTIs compared to lopinavir/ritonavir (LPV/r) plus 2 NRTIs as second-line treatment (27). Results indicated that HIV-1 RNA was < 50 c/mL in 78% of subjects on DTG versus 69% of those on LPV/r in week 24. The Independent Data Monitoring Committee (IDMC) recommended discontinuation of the LPV/r arm due to the superior efficacy of DTG+2NRTIs and the potential of LPV/r to harm subjects based on available data. These findings provide important information to help guide second-line treatment decisions in resource-limited settings.

Moh *et al.* conducted the ANRS 12269 THILAO study to evaluate the efficacy of a third-line treatment based on TDF plus RAL regimen at 48 weeks in HIV-infected adults in sub-Saharan Africa who failed to respond to a second-line protease inhibitor-based regimen (28). Of 44 patients who received the TDF 3TC/FTC-NNRTI regimen, 27 had a viral load < 50 copies/mL. The third-line regimen of TDF plus RAL is highly efficient as salvage therapy.

Tolerability. Anstett *et al.* hypothesized that the R263K substitution interferes with some actions of integrase by dysregulating the acetylation of nearby residues (29). That study is the first to describe how post-translational modifications affect the drug resistance of HIV. The study's results indicated that HIV-1 resistance to DTG is modulated by epigenetic signals, suggesting that some drugresistant viruses may respond differently to histone deacetylase inhibitors.

Pham *et al.* evaluated mutations associated with INI resistance by sequencing the virus both prior to and at the time of virologic failure (30). That study found that virological failure involving DTG monotherapy can occur due to replication of a virus containing a novel S230R substitution that confers a modest level of resistance to DTG and other INSTIs.

Monotherapy and virus inhibition. Heredia *et al.* conducted two studies that respectively evaluated the efficacy of 20-week monotherapy with DTG or RAL and dual therapy with DTG plus lamivudine (3TC) in humanized mice (HSC-NSG) infected with HIV_{BaL} (31). Results indicated that DTG monotherapy does not maintain HIV suppression, suggesting that streamlining of ART may require dual therapy.

Liang *et al.* studied the status of viral infection during suppression achieved by ART in HIV-positive individuals receiving DTG-based ART (32). That study found that patients treated with DTG had more robust levels of antibody-dependent cellular cytotoxicity (ADCC) responses and earlier recovery of neutralization than those treated with EVG. This means that HIV would be less likely to evolve following exposure to DTG.

4. Prospects for the future

As a result of continued efforts by national experts for close to 10 years, INIs have gradually become a new class of ART drugs to manage patients infected with HIV. IAS 2017 highlighted the latest advances in the efficacy, tolerability, and monotherapy of INIs (32-39) based on many groundbreaking studies by national experts and researchers: *i)* dolutegravir (DTG)-based regimens are more efficacious, tolerable, and safer forms of first-, second-, and third-Line ART; *ii)* current studies have indicated that DTG monotherapy fails both virologically and clinically; and *iii)* whether the most cost-effective treatment for DTG is to replace efavirenz (EFV) as a first-line ART, to replace protease inhibitors (PIs) in second-line ART, or to replace both as a monotherapy is unclear.

Based on recent studies, further study of INIs in terms of drug interactions, dose reduction, drug convenience, and drug costs is warranted.

Although numerous studies are underway overseas, full clinical trials of INIs and related studies of clinical efficacy involving Chinese samples have yet to be conducted. Clinical trials on INIs in Chinese samples in the "real world" are anticipated.

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