Tropical Journal of Pharmaceutical Research July 2019; 18 (7): 1519-1526
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i7.23

Original Research Article

Mechanism of *Klebsiella pneumoniae* resistance to carbapenem antibiotics

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Sent for review: 23 April 2019 *Revised accepted: 29 June 2019*

Abstract

Purpose: *To investigate the mechanism of action of Klebsiella pneumoniae (Kpn) resistance to carbapenem.*
Methods: *The susceptibility of six Klebsiella pneumoniae strains to antibiotics was determined using K-B assay. Six isolated strains which were resistant to carbapenem were identified and collected using modified Hodge test. The phenotypes of metal enzyme were evaluated by ethylene diamine tetraacetic acid (EDTA) disk diffusion method. The genes for beta-lactamases, including KPC gene, were examined.*
Results: *The six carbapenem-resistant strains of Klebsiella pneumoniae were resistant to imipenem, meropenem and aztreonam, but were sensitive to amikacin, fosfomycin, minocycline, and polymyxin. The six pathogens did not produce metal enzyme, but they produced carbapenemases. Moreover, the six strains partially carried blaTEM or blaSHV gene, but all had blaKPC-2 gene.*
Conclusion: *These results suggest that the pathogens that contain blaKPC-2 gene may be involved in the mechanism of Klebsiella pneumoniae (Kpn) resistance to carbapenem.*

Keywords: *Klebsiella pneumoniae, β-Lactamase, Carbapenemase, Resistance mechanism*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

The resistance of some *Enterobacteriaceae* strains to extended-spectrum cephalosporins has become a thorny issue in clinics all over the world [1]. *Klebsiella pneumoniae* and *Escherichia coli* produce extended spectrum β-lactamases (ESBLs) which have led to the use of more carbapenems in the treatment of serious infections in hospitalized patients [2]. Later on, carbapenemases were discovered, including class B metallo-β-lactamases VIM, IMP, SPM and GIM [3]. These enzymes contain zinc in their active sites. They are mainly found in *Acinetobacter* and *Pseudomonas* species, but are rarely detected in *Escherichia* coli and *Klebsiella pneumoniae*. In the late 1990s, *Klebsiella* strains that were less susceptible to

carbapenem antibiotics were reported in New York City, North Carolina and Maryland [4]. Many hospitals in New York City had reported strains that were less susceptible to carbapenem antibiotics. Initially, some researchers tried to assay metal beta-lactamases in these strains, but the MICs of carbapenem on these strains were not inhibited by EDTA as expected, which confirmed that the bacteria produced KPC carbapenem-hydrolyzing enzymes [5].

The clinical isolation rate of imipenem-resistant *Enterobacteriaceae* (imipenem-resistant *Enterobacter*, IRE) has continued to rise [6]. The emergence of carbapenem-resistant *Enterobacteriaceae* was of great importance to clinical anti-infective therapy [7]. One of the main mechanisms of carbapenem antibiotic resistance was bacterial production of *Klebsiella pneumoniae* carbapenemases (KPCs) [8]. The first KPC was reported in the United States in 2001 [9]. *Enterobacteriaceae* bacteria that subsequently produce KPC enzymes are continuously being identified in the United States [10]. Twenty-four states in the United States have reported production of KPC enzyme strains that have caused epidemics in the states of New York and New Jersey in the eastern United States. Moreover, clinical cases of infection with KPC-producing strains have been reported in France, Colombia, Greece, United Kingdom, Argentina, Brazil, Norway, Sweden and China [11]. Currently, majority of *Klebsiella pneumoniae* producing KPC-2 or KPC-3 are mainly cloned [12]. The KPC-producing strains are not easily detected in the laboratory, because many strains may be with similar phenotypic characteristics of KPC-producing strains, and sensitive to imipenem or meropenem [13]. Ertapenem is a better indicator of KPC enzyme than other carbapenem antibiotics [14]. The KPC genes are often located on larger plasmids, which in turn often carry other drug-resistant genes, resulting in multiple drug resistance and increasing the difficulty of treatment [15].

The resistance to extended-spectrum cephalosporins in *Enterobacteriaceae* strains has become a serious medical issue in recent years. Therefore, the current study was carried out to investigate the mechanism involved in *Klebsiella pneumoniae* (Kpn) resistance to carbapenem.

EXPERIMENTAL

Klebsiella pneumoniae strains

The *Klebsiella pneumoniae* strains were collected in Guangdong Second Provincial General Hospital, Southern Medical University, from January 2017 to September 2017. The *Klebsiella pneumoniae* strains were derived from sputum or airway secretions of six different patients with lung infection. All the six specimens of *Klebsiella pneumoniae* strain were resistant to carbapenem (imipenem or meropenem). The ages of the six patients ranged from 70 to 90 years, and they comprised 4 males and 2 females. They were admitted with infectious diseases, and there were large areas of shadow in their lungs. The patients had dyspnea, in addition to different degrees of increases in white blood cell count and C-reactive protein (CRP). There were invasive procedures and history of high-dose broad-spectrum antibiotic use before admission. Specimens were identified by the API Method Bacteria Identification System from Merial, France. The control strain used was *Escherichia coli* ATCC25922. This research was approved by the Ethical Committee of Center of Faculty Development and Educational Technology, Information Science School and School of Statistics and Mathematics, Guangdong University of Finance and Economics, Guangzhou, China (approval no. 201815165), and followed the guidelines of the Declaration of Helsinki promulgated in 1964 as amended in 1996 [16].

Drug-sensitive test strips and main reagents

Drug-sensitive paper was purchased from Beijing Tiantan Biological Products Co., Ltd. The main antibacterial agents were cefotaxime CTX, ceftazidime, cefuroxime, amoxicillin/clavulanic acid (AMC), ampicillin (AM), ampicillin/sulbactam (GEN), cotrimoxazole (SXT), cefoperazone/sulbactam (SCF), piperacillin/azobactam (TZP), ATM, cefepime (FEP), levofloxacin (LEV), meropenem (MEM), imipenem (IPM), polymyxin B (PO), fosfomycin (P), amikacin (AN) and minocycline. Extraction Kit for DNA Plasmid and DNA Gel Recovery Kit were purchased from Wiegers Biotech (Beijing) Co., Ltd. Taq enzyme was purchased from Shanghai Dingguo Biotechnology Co., Ltd, while T4 DNA ligase and IPTG were purchased from Promega. Restriction endonuclease and DNA Marker were purchased from TaKaRa (Dalian). The other reagents used were X-α-gal (Clontech, Japan); DNA ligase, pGEM-T, IPTG and X-β-gal (Promega, USA); EcoR I (Japan TaKaRa), and PCR reaction system.

Phenotypic screening and phenotype confirmation

Specimen collection, isolation and culture were carried out in strict compliance with *National Clinical Laboratory Procedures* (3rd edition). The

resistance of CRKP was analyzed using WHONET 5.6 software. Clinical screening of the 6 CRKP phenotypes was performed according to the procedure described in *Clinical and Laboratory Standards Institute (CLSI*, 2009), and the *NDM-l Pan-resistant Enterobacteriaceae Bacterial Infection Diagnosis and Treatment Guidelines'* from China's Ministry of Health and Chinese Medicine Administration [17]. The modified Hodge test confirmed carbapenemases, while the metalloenzyme phenotype was confirmed with EDTA inhibition test.

Extraction of bacterial DNA plasmid

Colonies cultured overnight on plates were inoculated into 5 ml of LB broth and cultured at 37 °C for 12-16 h with shaking at 12 000 rpm. The cells were pelleted by centrifugation for 2 min, and then the supernatant was completely discarded. All procedures were strictly according to the instructions for plasmid extraction.

PCR amplification and gene sequence analysis

The primers used for the amplification of the resistant carbapenemase-resistant and ESBLs-resistant genes, and the size of the expected products were selected based on data in GenBank (http://www.ncbi.nlm.nih.gov). Various types of bla gene sequence design primers are shown in Table 1. These primers were synthesized by Invitrogen, USA. The PCR reaction system (total volume = 25 μL) contained 2 μL of DNA template, 12.5 μL of Premix Taq DNA Polymerase, and a mixture of DNase, buffer, dNTP at 2-fold concentrations for all PCRs (Dalian Bao Bioengineering Co., Ltd.), and 1 μL of the corresponding primer. The PCR reaction conditions were: 95 ^{o}C for 5 min, 35 cycles; 94 ^{o}C for 1 min, 56 °C for 30 sec, 72 °C for 1 min, and finally 72 °C for 10 min. The PCR amplification products were electrophoresed on a 1 % agarose gel and stained with Gold View dye. The results were observed with a gel imaging system and photographed. The imaging strip was cut out of the DNA strip under long-wave UV lamp for DNA recovery.

Connection and transformation

The DNA recovery products were connected with T vector using T4 ligase-dubbed system. After a water bath overnight at 16 ℃, the ligation products were transformed with *E. coli* competent cells, with IPTG as an inducer, and X-gal as a staining reagent. The plates were incubated overnight at 37 °C. Blue and white screening were conducted. The white colonies were picked and shaped, and then the bacteria plasmid was obtained. All PCR products were purified and sequenced using Beijing Jinweizhi Technology Co. Ltd. Blast sequence alignment software confirmed the sequencing results.

Statistical analysis

All experiments were repeated at least 5 times. Data are presented as mean ± standard deviation (SD). GraphPad Prism program (GraphPad Software, USA) was used for statistical analyses.

Table 1: Primer sequences of drug resistance genes, and the sizes of the amplified fragments

Drug resistance gene	Primer (5′→3′)	Size of amplified fragment (bp)
bla_{KPC-2}	GCTACACCTAGCTCCACCTTC ACAGTGGTTGGTAATCCATGC	989
bla_{SHV}	GGGTTATTCTTATTTGTCGC TTAGCGTTGCCAGTGCTC	927
bla_{TEM}	ATGAGTATTCAACATTTCCGTG TTACCAATGCTTAATCAGTGAG	861
bla_{VEB}	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	642
bla_{PER}	TGACGATCTGGAACCTTT AACTGCATAACCTACTCC	850
$bla_{CTX-M-1}$	ATGGTTAAAAAATCACTGCGC TCCCGACGGCTTTCCGCCTT	944
$bla_{CTX-M-2}$	ATGATGACTCAGAGCATTCG TCCCGACGGCTTTCCGCCTT	900
$bla_{CTX-M-9}$	CGGCCTGTATTTCGCTGTTG TCCCGACGGCTTTCCGCCTT	877
bla_{OXA-1}	CTGTTGTTTGGGTTTCGCAAG CTTGGCTTTTATGCTTGATG	720
bla_{OXA-10}	GTCTTTCAAGTACGGCATTA GATTTTCTTAGCGGCAACTTA	720

Treatment groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Drug susceptibility

The results of drug susceptibility test revealed that 6 *Klebsiella pneumoniae* tested *in vitro* were resistant to cefotaxime, ceftazidime, cefuroxime, ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, gentamicin, cotrimoxazole, cefepime (FEP), piperacillin/tazobactam, levofloxacin (LEV) and aztreonam. Moreover, the inhibition zones of meropenem, imipenem and cefoperazone/sulbactam were small, while the inhibition zones of polymyxin, minocycline, amikacin, fosfomycin were larger. At the same time, the resistant strains were determined with MIC method (Table 2).

Carbapenemase production by strains

The modified Hodge test showed positive results for all strains, which confirmed carbapenemase production from the strains. In the test, ATCCBAA-1705 was employed as positive control, while ATCCBAA-1706 was used as negative control. The positive result of strain 089 is shown in Figure 1. Similar results were obtained for the remaining five strains of bacteria.

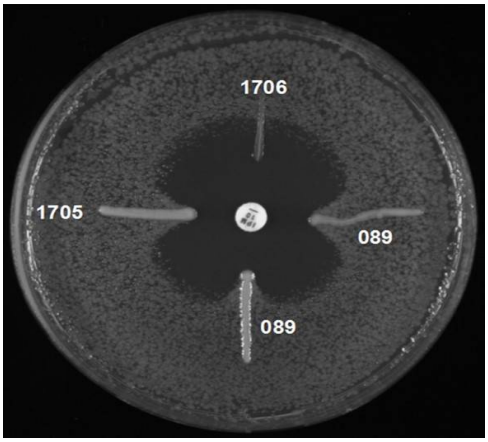

Figure 1: Improved Hodge test results. Strain 089 was positive. ATCCBAA-1705 was employed as the positive control, while ATCCBAA-1706 was used as the negative control

EDTA inhibition

Double paper synergy tests were performed in the direction of EDTA paper, but imipenem inhibition zone did not expand. Thus, the result of EDTA inhibition test for strain 102 was negative. The other five strains all achieved the same result (Figure 2).

Table 2: Diameters of inhibition zone and breakpoints

Antibacterial	Diameter of inhibition zone (mm)	Breakpoints (K-B) (mm)	MIC (mm)	Breakpoints (MIC) (mm)
Cefotaxime	6	22-26	—	—
Ceftazidime	6	17-21	≥ 64	4-16
Cefuroxime	6	14-23	≥ 64	8-32
Ampicillin	6	13-17	≥ 32	8-32
Amoxicillin / Clavulanic acid	6	13-18	—	—
Ampicillin / Sulbactam	6	11-15	≥ 32	8/4-32/16
Gentamicin	6	12-15	≥ 16	4-16
Cotrimoxazole	6	10-16	≥ 320	2/38-4/76
Piperacillin / Tazobactam,	6	17-21	≥ 128	16/4-128/4
Aztreonam	6	17-21	≥ 64	4-16
Cefepime (FEP)	6	14-18	≥ 64	8-32
Levofloxacin (LEV)	6	13-17	≥ 8	2-8
Meropenem	8	13-16	≥ 16	4-16
Imipenem	9	13-16	≥ 16	4-16
Cefoperazone / Sulbactam	16	15-21	—	—
Polymyxin	18	—	—	—
Minocycline	20	12-16	—	—
Amikacin	24	14-17	≤ 2	1664

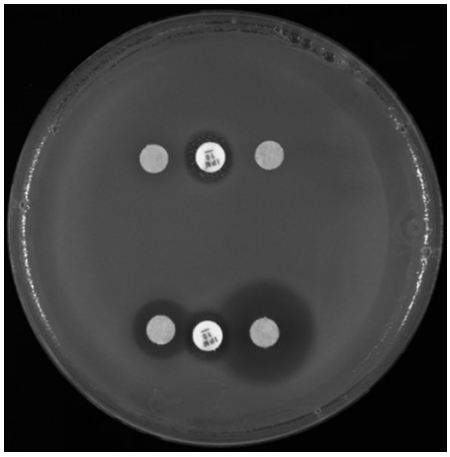

Figure 2: EDTA inhibition results. Double paper synergy tests were performed in the direction of EDTA paper. EDTA inhibition test showed negative result

MIC test results

The results of MIC on strain 102 are shown in Figure 3. The other five strains all achieved the same result.

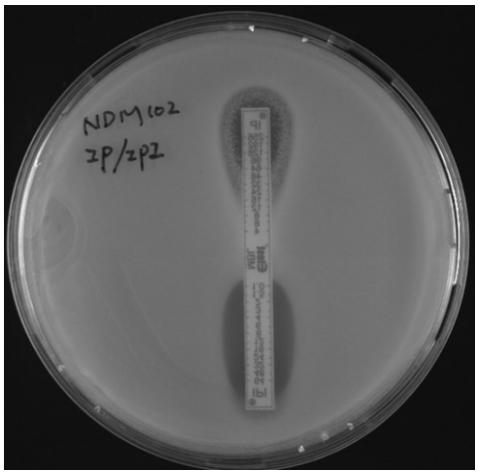

Figure 3: MIC test results for strain 102. The other five strains yielded similar results

FGE maps

The result of pulsed field gel electrophoresis (PFGE) is shown in Figure 4. The PFGE maps showed that the chromosome bands of the six *Klebsiella pneumoniae* strains were basically same as those of other strains, and may have originated from the same clone.

Confirmation of drug resistance gene

The results showed that the six *Klebsiella pneumoniae* strains all carried drug resistance genes i.e. carbapenemase genes, of which six strains all carried KPC-2 gene, five strains carried SHV gene, and three strains carried TEM gene. Moreover, two strains also carried three kinds of resistance genes.

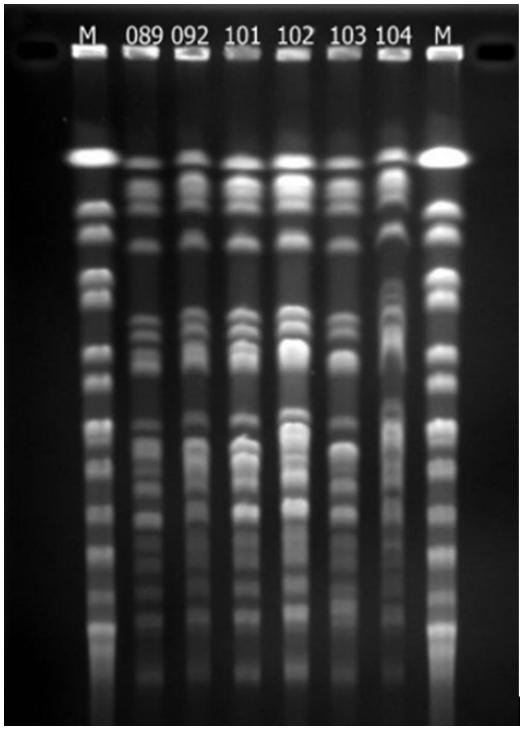

Figure 4: PFGE maps. The chromosome bands of the 6 strains of *Klebsiella pneumoniae* were basically same as those of other strains. (M: DNA marker; 089, 091, 101, 102, 103 and 104 are the six *Klebsiella pneumoniae* strains)

DISCUSSION

Carbapenem antibiotics play an important role in the treatment of clinically serious infections, especially *Enterobacteriaceae* [18]. However, with the increasing use of clinical carbapenem antibiotics, resistant strains have emerged, mainly against *Pseudomonas aeruginosa* and *Acinetobacter*. In recent years, reports of imipenem-resistant *Enterobacteriaceae* (imipenem-resistant *Enterobacter*, IRE) have been on the increase [19]. The mechanisms of bacterial antibiotic resistance to carbapenem involve the following (1) high-yielding AmpC enzyme with loss of porin or decreased expression levels, resulting in decreased permeability of antibiotics through the cell membrane; (2) efflux pump-mediated decrease in intracellular antibiotic concentration; (3) alteration of target of carbapenem antibiotics, and (4) production of carbapenemase [20]. In addition, the L1 enzyme of *Stenotrophomonas maltophilia* has three types of carbapenem antibiotic β-lactamase (Ambler classification of A, B, D) [21].

Klebsiella pneumoniae carbapenemases (KPC) is a newly discovered carbapenemase belonging

to the Ambler class A, which can hydrolyze almost all β-lactam antibiotics [1,7]. A total of nine subtypes (KPC 1 - 9) have been identified. However, KPC-1 sequencing was confirmed to be incorrect (http://www.1ahey.org/Studies/) on the grounds that it is actually the same genotype as KPC-2 [7].

Klebsiella pneumoniae carbapenemase-producing strain was first discovered in the United States, after which KPC enzyme producing strains were found in France, Colombia, Israel, China, Norway, Brazil, Britain, Greece and other regions. These strains were *Enterobacteriaceae* bacteria, including pneumonia grams *Salmonella, Salmonella, Escherichia coli, Enterobacter cloacae, Citrobacter freundii, Serratia marcescens* and *Proteus mirabilis*, and also *Pseudomonas aeruginosa* and *Pseudomonas putida* [8]. The KPC-producing strains are resistant to carbapenem-based antibiotics and also resistant to many other antibacterial agents based on transmission of plasmids. The carbapenem-resistant *Enterobacteriaceae* bacteria have become a major clinical challenge [10].

Klebsiella pneumoniae which produces KPC enzymes in hospitals, is found mainly in contaminated medical devices, hands of medical staff, or in the patient's gastrointestinal tract [10]. *Klebsiella pneumoniae* strains that produce KPC enzymes have been isolated in venous cannulas, bed railings, and sphygmomanometers in patients' rooms [22]. The spread of KPC-producing strains has been reported in the same hospital, between different hospitals and in different regions, and even in different countries. These findings indicate that environmental factors may be involved in infection or bacterial colonization.

In the present study, PFGE results showed that the six isolated strains of *Klebsiella pneumoniae* belonged to the same clone. The six patients were hospitalized from January 2017 to September 2017, and the cultures of patients who had lived in the same ward yielded the same clone. This indicates that carbapenemase-producing *Klebsiella pneumoniae* had a brief clonal spread in the ward during this period. The clonal spread may be due to a variety of medical interventions. Surveillance of nosocomial infections, detection of drug-resistant strains and the timely introduction of precautionary measures are prerequisites for preventing a wider spread.

Detection of KPC enzyme is not only an important part of the control of its spread, but also a basis for clinical drug selection. However, the identification of KPC enzymes is a difficult process because many strains producing KPC enzymes are sensitive to meropenem and imipenem. Moreover, the characteristics of KPC enzymes and ESBLs are similar, and easily misjudged as ESBLs. In addition, the phenotypic identification of local resistant strains is constrained by presence of other carbapenemases (e.g. metal β-lactamase), culture media, strains and drugs. The susceptibility results showed that the six strains of *Klebsiella pneumoniae* were resistant to aztreonam, piperacillin/tazobactam, cefotaxime, cefoperazone/sulbactam, and imipene. The sensitivity to meropenem and ertapenein was decreased. The results of PCR showed that the six strains of *Klebsiella pneumoniae* contained genes of SHVβ-lactamase and TEM gene in addition to KPC-2 gene.

Patient-susceptibility factors to isolates of KPC strains include age, intensive care unit hospitalization, and ventilator use. The six patients used in this study were all aged above 65 years and lived in the same ward, similar to those reported in the literature. In addition, all the six patients had underlying disease. Regarding the use of antibacterial agents in relation to the production of KPC enzymes, it has been reported that only 20 % of 60 patients infected with KPC pathogens used carbapenem antibiotics before the pathogens were isolated, and 60 % of the patients used beta-lactam antibiotics or antibiotics with beta-lactamase inhibitors, while 60 % used quinolone antibiotics [22].

These data suggest that prior treatment with carbapenem antibiotics does not appear to be necessary for the infection or colonization by KPC pathogens. Most of the patients in this study had a history of using antibiotics and antifungal drugs. As confirmed by PFGEE, the six strains of *Klebsiella pneumoniae* were the same clone. Thus, infection-related pathogens such as hospital-acquired infections may be more important than prior use of antibacterial agents.

The results of drug susceptibility test indicated that polymyxin, timentin, and SMZ-sensitive, combined with polymyxin B and tylinin in the treatment of KPC bacterial infections have been the conventional treatment in some institutions. This treatment is difficult to implement due to difficulty in accessing medicines. Moreover, the treatment is not too effective, resulting in death of patients. Thus, sensitive antibiotics should be sought to strengthen the treatment of underlying diseases and to strengthen the supportive care so as to improve treatment outcomes. Timely release of indwelling needles, intubation and

other infection-related factors such as comprehensive treatment measures seem to be particularly important. More critical is the rational use of antibiotics to strengthen the monitoring of drug-resistant strains.

CONCLUSION

The results suggest that the pathogens that contain bla_{KPC-2} gene may be involved in the mechanism of *Klebsiella pneumoniae* resistance to carbapenemFurther studies may help to solve the challenge of resistance to extended-spectrum cephalosporins in *Enterobacteriaceae strains* in globule. In addition, further studies should lead to the development of important guidance for clinical medication.

DECLARATIONS

Acknowledgement

This work was supported by the National Natural Science Foundation of China (nos. 30600524 and 81341067), Guangdong Provincial Science and Technology Program (no. 2017A040405051), Education Project of Industry-University Cooperation (no. 201801186008), and Higher Education Teaching Reform Project of Guangdong in 2017.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. HanHui Lin conceived and designed the study, Danxia Chen, Yan Zhu, Liangshan Hu, Xiaoyun Wu, Mingyou Li, HanHui Lin collected and analysed the data, while Danxia Chen wrote the manuscript.

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