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Original Research Article

Studies on the expression and biological functions of ZIC5 in hepatocellular carcinoma

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Abstract

Purpose: *To study the expression of zinc finger protein of the cerebellum 5 (ZIC5) and its biological functions in hepatocellular carcinoma (HCC).*
Methods: *Sixty-five patients undergoing HCC surgery were selected. Expression of ZIC5 in HCC and para-carcinoma tissue was examined by quantitative real time-polymerase chain reaction (qRT-PCR) and western blotting. The relationship between ZIC5 expression and clinicopathological features, postoperative survival rate, and prognosis of liver cancer patients was analyzed by t-test, Kaplan-Meier method, and Cox regression analysis, respectively. The effects of ZIC5 silencing on Huh-7 cell proliferation, migration, invasion, and apoptosis were assessed using Cell Counting Kit-8 (CCK-8), wound healing assay, Transwell assay, and flow cytometry, respectively.*
Results: *ZIC5 expression in liver cancer tissue was significantly higher than in the para-carcinoma tissue and was significantly correlated with TNM stage and differentiation degree ($p < 0.001$). The overall survival rate of patients with high ZIC5 expression level was significantly lower than that of patients with low ZIC5 expression ($p < 0.01$). ZIC5 expression, TNM stage, and differentiation degree were independent prognostic factors. ZIC5 silencing significantly inhibited the proliferative, migratory, invasive, and anti-apoptotic capacity of Huh-7 cells ($p < 0.01$).*
Conclusion: *ZIC5 is highly expressed in HCC, and this can promote liver cancer cell proliferation, migration, and invasion.*

Keywords: *Hepatocellular carcinoma, Cerebellar zinc finger structure 5, Prognosis, Migration, Proliferation*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of death in the world, accounting for 90 % of primary liver cancers. There are about 1 million new cases of HCC worldwide each year, and 750,000 people died from HCC [1]. The pathogenesis and clinical treatment plan of HCC have always been research hotspots in the medical field. In the past 20 years, with continuous developments in medical technology, great progress has been made in the diagnosis and treatment of HCC. However, the prognosis of HCC is not good, and the 5-year recurrence rate can still reach 70 % [2]. Therefore, it is urgent to elucidate the mechanism of HCC

occurrence and development. Genetic studies believe that the occurrence and development of HCC is a complex biological process involving multiple genes. Therefore, identification of critical genes can be useful not only to determine tumor recurrence, but also to serve as potential candidate therapeutic targets for HCC [3].

Zinc finger protein 5 of the cerebellum 5 (ZIC5) is an important member of the ZIC family, and it has a critical regulatory role in biological development [4]. Studies have shown that ZIC5 is closely related to the occurrence and development of prostate cancer, colon cancer, and gliomas [4,5], but its role in HCC has been less studied. Therefore, this study aims to investigate the expression of ZIC5 in HCC tissue and its relationship to the clinicopathological features of HCC, and to study the effect of *ZIC5* silencing on the biological behavior of HCC cells, so as to provide experimental evidence for use of ZIC5 as a potential target for HCC diagnosis and treatment.

EXPERIMENTAL

Tissue samples

Liver cancer tissue samples and corresponding para-carcinoma tissue samples were taken from 65 patients who underwent surgical treatment in the hepatobiliary surgery department at Renmin Hospital of Wuhan University. Postoperative pathological diagnoses of those patients were all liver cancer, and no radiotherapy or chemotherapy were applied before surgery. Participants were 35 males and 30 females, with an average age of 60.3 ± 5.2 years. The TNM stage of liver cancer was determined according to the standards established by the Union for International Cancer Control in 2010 [6]. There were 39 cases of stage I and II, and 26 cases of stage III and IV. This study has been approved by the Ethics Committee of Renmin Hospital of Wuhan University (approval no. WDRY2019-K005), and all patients and their families gave informed consent to participate in this study [7].

Cells and main reagents

The liver cancer cell line Huh-7 was purchased from Shanghai Cell Bank of Chinese Academy of Sciences. siRNA targeting human *ZIC5* (si-ZIC5) and negative control siRNA (si-NC) were synthesized by Shanghai Jima Pharmaceutical Technology Co. Ltd. Liposome transfection reagent Lipofectamine 2000, TRIzol extraction kit, and CCK-8 cell proliferation detection kit were purchased from Invitrogen (USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Gibco (USA). 4',6-diamidino-2-phenylindole (DAPI), rabbit anti-human ZIC5 polyclonal antibody, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (secondary antibody) were purchased from Thermo Fisher Scientific (USA). SYBR® Premix Ex Taq™ II kit was purchased from TaKaRa (Japan).

Cell culture

Huh-7 liver cancer cells were suspended in DMEM containing 10 % fetal bovine serum, and cultured in an atmosphere of 5 % CO_2 at 37 °C. After the cells had grown to 80 % confluence, passage or subsequent experiments were performed.

ZIC5-siRNA transfection

ZIC5-specific siRNAs were designed. Huh-7 cells at logarithmic growth phase were plated in 6-well plates. At 80 % confluence cells were transfected with si-NC and si-ZIC using Lipofectamine according to the manufacturer's instructions. After 24-hour incubation, ZIC5 mRNA and protein levels were determined.

Real time (quantitative) polymerase chain reaction (qPCR)

Total RNA of transfected cells, liver cancer, and para-carcinoma tissue were extracted using the TRIzol method, and reverse transcribed to generate cDNA. The resulting cDNA was used as the template in PCR to detect the expression of *ZIC5* (Table 1).

ZIC5 forward primer	5'- GCG CUG AGA UUA GCG GAU UTT -3'
ZIC5 reverse primer	5'- AAU CCG CUA AUC UCA GCG CTT -3'
β-actin forward primer	5'- TGG CAC CCA GCA CAA TGA A-3'
β-actin reverse primer	5'- CTA AGT CAT AGT CCG CCT AGA AGC A -3'

Western analysis

Cells were lysed in 2× protein loading buffer by heating at 100 °C for 5 min, and then centrifuged at 12,000 × g for 5 min. Proteins in the cell lysates were separated through a 10 % SDS-PAGE gel and then transferred to PVDF membrane. The PVDF membrane was then incubated in blocking buffer (Tris-buffered saline with Tween 20 [TBST] containing 5 % skim milk powder) at room temperature for 1 h. Primary antibody (1:2000 dilution) incubation was performed at 4 °C overnight, and then the membrane was washed three times with TBST.

Incubation with HRP-conjugated IgG secondary antibody was performed at room temperature for 1 h. After washing the membrane with TBST three times, proteins on the membrane were detected by the enhanced chemiluminescence (ECL) method and photographed on the gel imaging system. The densities of the protein bands corresponding to ZIC5 and internal reference β-actin were then analyzed.

Evaluation of cell proliferation

Twenty-four hours after transfection, the cells were digested with 0.25 % trypsin and seeded in 96-well plates at a density of 1×10^3 cells/well. After 24 h of incubation, 10 μL CCK-8 reagent was added. After 2 h of further incubation, the OD_{450} value of each well was determined by ELISA analysis instrument. Each group had six replicate wells, and the experiment was repeated three times.

Cell migration and invasion assay

Wound healing assay: Twenty-four hours after transfection, the cells were seeded in a 6-well plate at a density of 1×10^5 cells/well. After 24 h of incubation, a wound in the form of a linear scratch was generated using a 200-μL pipette tip. Observation and imaging of the wounds was performed at 0 h and 24 h. Each group had three replicate wells, and the experiment was repeated three times. Transwell assay: Twenty-four hours after transfection, the cells were resuspended in DMEM at 1×10^5 cells/mL. Cell suspension (200 μL) was added to the upper chamber of the Transwell chamber, and 600 μL DMEM containing 10% fetal bovine serum was added to the lower chamber. After 24 h of incubation, five fields of view of each group were randomly selected under an inverted microscope for imaging and counting the number of cells passing through the upper chamber. The experiment was repeated three times.

Flow cytometry

Twenty-four hours after transfection, cells were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS containing 5 μL annexin V-FITC and 5 μL propidium iodide, mixed well, and incubated at room temperature (20–25 ºC) in the dark for 15 min. Apoptosis was examined by flow cytometry within 1 h, and the experiment was repeated three times.

Bioinformatics analysis

The mRNA sequencing data of the liver cancer patients were downloaded from The Cancer Genome Atlas (TCGA) database. *ZIC5* expression in liver cancer tissue and normal liver tissue, and its relationship with the patient's prognosis were analyzed.

Statistical analysis

The experimental data were statistically analyzed using SPSS 20.0 software. Measurement data are expressed as mean ± standard deviation (SD). A comparison between two groups was done with independent sample *t*-test. The Kaplan-Meier method was employed to analyze the relationship between ZIC5 expression and the patient's overall survival. Independent factors affecting the prognosis of patients were analyzed using the Cox proportional risk model.

RESULTS

ZIC5 expression in liver cancer

Analysis of ZIC5 expression in liver cancer tissue (369 cases) and normal liver tissue (160 cases) from the TCGA database showed that the expression level of ZIC5 in liver cancer tissue was significantly higher than that in para-carcinoma tissue ($p < 0.05$, Figure 1 A). *ZIC5* mRNA level of 65 cases of liver cancer tissue and corresponding para-carcinoma tissue was examined by qPCR. As shown in Figure 1 B, the expression level of *ZIC5* mRNA in liver cancer tissue was significantly higher than that in the para-carcinoma tissue ($p < 0.05$).

The ZIC5 protein expression level in liver cancer tissue and corresponding para-carcinoma tissue from five patients was examined by western analysis, and the results showed that the ZIC5 protein level in liver cancer tissue was significantly higher than that in para-carcinoma tissue ($p < 0.05$, Figure 1 C and D).

Correlation between ZIC5 expression and clinicopathological features of liver cancer

The relationship between *ZIC5* mRNA the expression in 65 cases of liver cancer tissue and the clinicopathology of liver cancer is shown in Table 1. No statistical difference in ZIC5 expression was observed dependent on patient age, gender, tumor size, presence/absence of cirrhosis, presence/absence of viral infection, or alpha-fetoprotein content ($p > 0.05$). *ZIC5* mRNA expression level increased with the progression of TNM stage and differentiation degree, and the differences were statistically significant ($p < 0.05$).

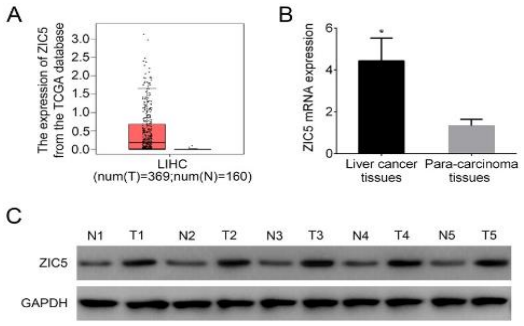

Figure 1: ZIC5 expression in liver cancer and corresponding para-carcinoma tissue**.** (A) The expression levels of ZIC5 in liver cancer and normal liver tissue from the TCGA database were compared. (B) The *ZIC5* mRNA levels in liver cancer tissue and corresponding para-carcinoma tissue were examined by qPCR (n = 65). (C) The ZIC5 protein expression level in liver cancer tissue and corresponding para-carcinoma tissue was examined by western analysis (n = 5); **p* < 0.05, as compared with the corresponding para-carcinoma tissue

Relationship between ZIC5 expression and the prognosis of liver cancer patients

The Kaplan-Meier plotter was used to analyze the relationship between ZIC5 expression and overall survival rate of 175 liver cancer patients from the TCGA database. The results showed that patients with high ZIC5 expression had lower overall survival rates than those with low ZIC5 expression ($p < 0.001$, Figure 2A). At the same time, the relationship between the expression of *ZIC5* mRNA and the overall survival rate of 65 liver cancer patients was also analyzed. As shown in Figure 2B, liver cancer patients with high *ZIC5* mRNA expression levels had lower overall survival rate than those with low *ZIC5* mRNA expression ($p < 0.001$). Cox proportional risk model analysis showed that ZIC5 expression level, TNM stage, and differentiation degree were independent prognostic factors in HCC patients (Table 2).

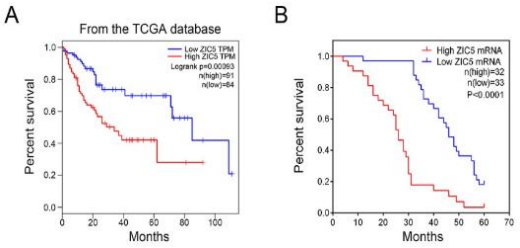

Figure 2: Survival rate of HCC patients with different ZIC5 expression levels. (A) Overall survival rate of HCC patients with different ZIC5 expression levels (n = 175, TCGA database). (B) Overall survival rate of HCC patients with different *ZIC5* mRNA expression levels (n = 65). HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas

Table 1: Correlation between *ZIC5* mRNA expression in liver cancer tissue and clinicopathological features of liver cancer

Clinical pathology	Number of cases	*ZIC5* mRNA expression level	*t*/*F*	*P*-value
Age (years)				
>60	32	4.28 ± 1.01	1.173	0.245
≤60	33	4.59 ± 1.16		
Gender				
Male	33	4.32 ± 1.20	0.844	0.402
Female	32	4.55 ± 0.96		
Tumor size (cm)				
>5	35	4.29 ± 1.09	1.138	0.259
≤5	30	4.60 ± 1.08		
TNM stage				
I-II	39	3.99 ± 0.97	4.559	0.000
III-IV	26	5.09 ± 0.93		
Differentiation				
Medial and High	35	4.17 ± 1.20	2.138	0.036
Low	30	4.74 ± 0.86		
Cirrhosis				
No	33	4.46 ± 1.11	0.220	0.827
Yes	32	4.40 ± 1.09		
Viral infection				
No	28	4.50 ± 1.09	0.095	0.909
Hepatitis B virus	27	4.37 ± 1.11		
Hepatitis C virus	10	4.41 ± 1.15		
Alpha-fetoprotein (ng/ml)				
≤80	58	4.44 ± 1.09	0.180	0.857
>80	7	4.36 ± 1.21		

Table 2: Cox regression analysis of the prognosis of HCC patients

Influencing factors	B	SE	Wald	df	Sig.	Exp (B)	95.0 % CI for Exp (B)	
							Lower	Upper
ZIC5 expression level	0.891	0.168	28.22	1	0.000	2.437	1.754	3.385
TNM stage	0.852	0.330	6.68	1	0.010	2.345	1.228	4.477
Differentiation degree	0.703	0.303	5.38	1	0.020	2.020	1.115	3.659

ZIC5 gene silencing

After transfection of Huh-7 cells with si-ZIC5 or si-NC for 24 h, gene silencing was measured by qPCR and western blot analysis. Compared with the si-NC group, ZIC5 mRNA and protein levels in the si-ZIC5 group were significantly reduced ($p < 0.05$, Figure 3).

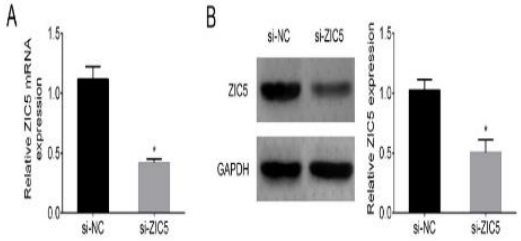

Figure 3: *ZIC5* gene silencing effect**.** (A) *ZIC5* mRNA expression levels examined by qPCR. (B) ZIC5 protein expression levels were determined by western analysis; *$p < 0.05$ compared with the si-NC group

Effect of *ZIC5* silencing on cell proliferation and apoptosis of Huh-7 cells

CCK-8 analysis showed that the cell proliferation rate of the si-ZIC5 group was reduced compared with the si-NC group, and the difference was statistically significant ($p < 0.05$, Figure 4 A). Flow cytometric analysis showed that the rate of apoptosis in the si-ZIC5 group was statistically significantly increased compared with the si-NC group ($p < 0.05$, Figure 4 B).

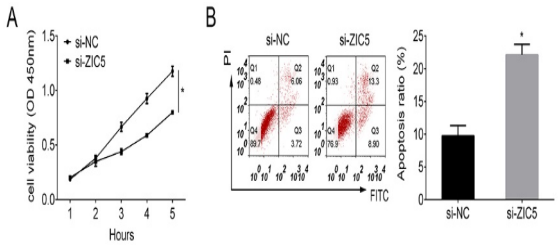

Figure 4: Effects of *ZIC5* silencing on Huh-7 cell proliferation and apoptosis. (A) Effect of *ZIC5* silencing on proliferation of Huh-7 cells determined by CCK-8 assay. (B) Effect of *ZIC5* silencing on apoptosis of Huh-7 cells determined by flow cytometry; *$p < 0.05$, as compared with the si-NC group

Effect of *ZIC5* silencing on Huh-7 cell migration and invasion

Cell migratory capacity was determined by the wound healing assay. As shown in Figure 5 A, the wound healing rate of cells in si-ZIC5 group was significantly reduced at 24 h compared to the si-NC group ($p < 0.05$). Cell invasive capacity was determined with the Transwell assay. The number of cells migrating to the lower chamber through the filter in the si-ZIC5 group was decreased significantly compared to the si-NC group ($p < 0.05$, Figure 5 B).

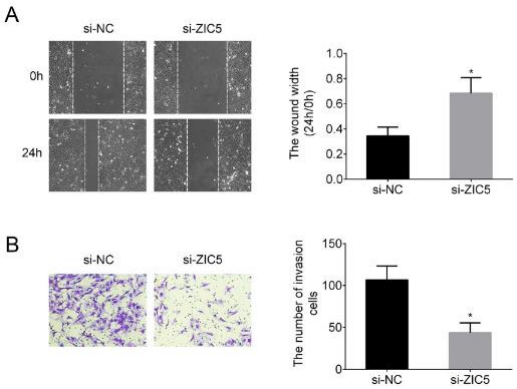

Figure 5: Effects of *ZIC5* silencing on Huh-7 cell migration and invasion. (A) Effect of *ZIC5* silencing on migration of Huh-7 cells examined by wound healing assay. (B) Effect of *ZIC5* silencing on the invasive capacity of Huh-7 cells determined by Transwell assay; *$p < 0.05$, as compared with the si-NC group

DISCUSSION

Hepatocellular carcinoma is a common malignant tumor type worldwide, with the characteristics of high malignancy, frequent recurrence, easy metastasis, and poor prognosis [8] . China belongs to a high HCC-incidence region, and the high morbidity and mortality rate of HCC has seriously affected the public health in China [9]. Currently, the main treatment plan for HCC is surgical resection; however, most HCC patients do not receive the benefit of surgical treatment because diagnosis takes place at an advanced stage [10]. Although radiochemotherapy and targeted therapy help HCC patients by improving their quality of life, recurrence and metastasis

limit the therapeutic effect [11]. Therefore, finding new HCC treatment methods is urgently needed. In the past several years, with rapid developments in biotechnology and tumor diagnostic technology, specific drugs targeting tumor biomarkers have drawn scientists' attention for developing new strategies for the treatment of HCC [12]. Therefore, studying the pathogenesis of HCC and looking for new specific molecular biomarkers are very important for the diagnosis and treatment of HCC.

The *ZIC5* gene was first discovered in African toads by Nakata *et al* [13] and was thought to be involved in the early stages of development of the nervous system. At present, studies have confirmed that *ZIC5* is a potential new oncogene that is associated with various tumors. Increased expression levels of ZIC5 in non-small–cell lung cancer (NSCLC) have been reported by Sun *et al* [14] and positively correlated with the TNM stage of this cancer type. After silencing of *ZIC5*, the proliferation of NSCLC cells was effectively inhibited and apoptosis was promoted. It was also reported that ZIC5 was highly expressed in human melanoma and induced epithelial-mesenchymal transition of melanoma cells and promoted cell migration and drug resistance. Li *et al* [4] found that *ZIC5* also acted as an oncogene in gliomas, and ZIC5 overexpression promoted cell proliferation and migration. Moreover, the association between ZIC5 and Has_circ_0007534 and miR-761 was identified by luciferase experiment, and it was found that *ZIC5* is the target gene of the Has_circ_0007534/miR-761 axis.

In this study, ZIC5 mRNA and protein expression levels in liver cancer tissue were shown to be higher than in the corresponding para-carcinoma tissue, which is consistent with the findings of Liu *et al* [15]. Considering the small sample size in this study, ZIC5 expression in liver cancer and normal liver tissue from the TCGA database was also analyzed, and the result showed that ZIC5 expression level in liver cancer tissue was significantly higher than in normal liver tissue.

By comparing the differences in ZIC5 expression in the liver cancer tissue of different patients, it was found that ZIC5 expression was not statistically different in patients of different age, gender, tumor size, with or without liver cirrhosis, with or without viral infection, or with different alpha-fetoprotein content, but there were statistical differences in patients with different degrees of differentiation and TNM stage, suggesting that ZIC5 may affect the occurrence and development of HCC. To further elucidate the relationship between ZIC5 and HCC patient prognosis, the Kaplan-Meier method was employed. The overall survival rate of 175 liver cancer patients from the TCGA database and 65 collected liver cancer tissue samples was analyzed in the study, and the result showed that patients with high ZIC5 expression had lower overall survival rate and poor prognosis.

The relationship between ZIC5 expression, TNM stage, liver cancer differentiation, and patients' prognosis was analyzed with the Cox proportional risk model, and it was found that ZIC5 expression, TNM stage, and differentiation degree were all independent factors for the prognosis of HCC patients. These results indicate that *ZIC5* is an oncogene in HCC. Thus, it can be used as a biomarker for the prognosis of HCC patients. As reported by Satow *et al*, ZIC5 is highly expressed in prostate cancer tissue [16].

After *ZIC5* silencing, the proliferation, migration, and invasive capacity of PC3 and LNCaP prostate cancer cells were inhibited. In this study, a *ZIC5*-gene–silenced cell line was established, and the CCK-8 method, wound healing assay, Transwell assay, and flow cytometry were employed to determine the effect of *ZIC5* silencing on HCC cell proliferation, migration and invasion, and apoptosis, respectively. The results showed that *ZIC5* silencing inhibits the proliferation, migration and invasion of Huh-7 cells and promotes apoptosis, which indicates that targeted interference with *ZIC5* expression can regulate the biological characteristics of HCC cells, suggesting its potential as a new target for HCC therapy. At present, there are few studies exploring the mechanism by which ZIC5 regulates tumor cell proliferation and migration.
Focal adhesion kinase (FAK) is a non-receptor tyrosine protein kinase that participates in proliferation, migration, and apoptosis through multiple pathways, such as the MAPK/STAT3 pathway. E-cadherin is a type of calcium-dependent transmembrane glycoprotein and is involved in regulating tumor cell growth and metastasis [17]. Satow *et al* [18] found that silencing of *ZIC5* downregulates FAK, STAT3, and E-cadherin expression in melanoma cells, and FAK and STAT3 inhibitors inhibited ZIC5 expression, indicating that ZIC5 expression is positively regulated by FAK and STAT3, which form a positive feedback loop.

Therefore, it is possible that ZIC5 may regulate cell proliferation and migration through FAK and E-cadherin. Some studies have found that overexpression of ZIC5 promotes the proliferation and migration of liver cancer cells and activates the Wnt/β-catenin signaling

pathway. Silencing of β-catenin inhibits the promotional effect of ZIC5 on cancer cells, indicating that ZIC5 may promote the proliferation and migration of liver cancer cells via the Wnt/β-catenin pathway [15]. Proliferation and migration of tumor cells are complex processes regulated by various genes and signaling pathways. The detailed mechanism by which ZIC5 regulates the proliferation and migration of liver cancer cells needs to be further studied.

This study has demonstrated the high expression levels of ZIC5 mRNA and protein in HCC patients. High expression of ZIC5 indicates that the patient would have a poor prognosis of HCC, and ZIC5 is an independent prognostic factor. It was also found that the silencing of *ZIC5* inhibited the proliferation, migration and invasion, and anti-apoptotic ability of liver cancer cells, indicating that ZIC5 might be a new target for clinical treatment of HCC; however, to elucidate the detailed mechanism further investigation is needed.

DECLARATIONS

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Competing interests

There is no conflict of interest to disclose.

Contribution of authors

Pingpo Ming and Weixing Wang designed the study, supervised the data collection, and analyzed the data. Chunyan Li interpreted the data and prepared the manuscript for publication. Yongfa Zheng and Wei Ge supervised the data collection, analyzed the data, and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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