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Original Article

Circular RNA circ_0006168 accelerates the development of hepatocellular carcinoma through sponging microRNA-125b

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Abstract

This study explored the role of circular RNA circ_0006168 in the progression of hepatocellular carcinoma (HCC) and its interaction with microRNA-125b. The expression of circ_0006168 was examined in 42 pairs of HCC tumor and adjacent tissue specimens using quantitative polymerase chain reaction (qPCR). Elevated circ_0006168 expression in HCC tissues was significantly associated with advanced pathological staging and lower overall survival rates. Lentivirus-mediated circ_0006168 knockdown in HCC cell lines (Hep3B and Huh7) demonstrated a notable reduction in cell proliferation and an increase in apoptosis. MicroRNA-125b expression exhibited a marked reduction in HCC tissues, negatively correlating with circ_0006168 levels. Luciferase reporting assays indicated that circ_0006168 was a direct target of microRNA-125b. Additionally, cell recovery experiments suggested a reciprocal regulation between circ_0006168 and microRNA-125b, contributing to the accelerated malignant progression of HCC. The study underscored the significantly increased expression of circ_0006168 in both HCC tissues and cell lines, highlighting its association with advanced pathological stages and poor prognosis in HCC patients. Furthermore, circ_0006168 appeared to play a pivotal role in elevating the proliferation rate of HCC cells through its modulation of microRNA-125b. These findings contribute to a deeper understanding of the molecular mechanisms underlying HCC development and may offer potential therapeutic targets for intervention.

Keywords: Circ_0006168, MicroRNA-125b, Hepatocellular carcinoma, Proliferation

1. Introduction

Hepatocellular carcinoma (HCC) represents a highly malignant primary tumor with significant global impact, ranking as the 5th most incident and 2nd most lethal malignancy in men worldwide, according to the latest epidemiological surveys on malignant tumors [1-3]. Notably, in China, liver cancer remains the 3rd most prevalent cancer in men and the 6th in women, underscoring its substantial public health burden [4]. Despite advancements in treatment modalities such as liver cancer resection, transplantation, local ablation, transcatheter arterial intervention, radiation therapy, and systemic therapy, the overall prognosis for HCC remains suboptimal, primarily due to the persistently high rates of postoperative recurrence and metastasis [5,8,9].

Hepatectomy stands as the primary choice for HCC treatment, yet challenges persist, necessitating a deeper understanding of the molecular mechanisms underpinning HCC development [6,7]. This has led to a growing interest in circular RNAs (circRNAs), once considered mere byproducts of RNA splicing with no functional relevance [12,13]. Recent high-throughput sequencing efforts have unveiled a wealth of circRNAs in eukaryotic cells, challenging the notion of their insignificance and highlighting their regulatory roles in diverse biological functions, including those relevant to tumorigenesis [13,14].

More than 10% of genes have been identified as potential producers of circRNAs, and their dysregulation has been implicated in various malignancies, functioning either as tumor suppressors or oncogenes [14-18]. However, the specific involvement of circRNAs in the early stages of liver cancer remains relatively unexplored, necessitating comprehensive investigations into their expression profiles and roles during this critical period [18,19].

In this study, we conducted high-throughput sequencing of circRNAs in HCC tumor tissues and adjacent counterparts. Among the identified circRNAs, circ_0006168 was selected for detailed exploration, aiming to unravel its role and underlying mechanisms in the progression of liver cancer. These findings are anticipated to contribute valuable insights for the clinical application of circ_0006168 as a potential molecular marker and therapeutic target in HCC.

2. Materials and Methods

2.1. Patients and HCC Samples

A total of 42 pairs of HCC tumor tissue samples and paracancerous tissue samples were selected from surgically treated HCC cases and stored at -80°C. This study was

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approved by the Ethics Committee of Second Affiliated Hospital of Soochow University, and patients and their families were fully informed that their specimens would be used for scientific research, and all participating patients had signed informed consent.

2.2. Cell Lines and Reagents

Six human HCC cells (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and one human normal liver cell line (LO2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). The HCC cell lines were cultured with high-glucose DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator. Cells were passaged with 1% trypsin + EDTA for digestion when grown to 80%-90% confluence.

2.3. Transfection

The control (sh-NC) and the lentivirus containing the circ_0006168 knockdown sequences (sh-circ_0006168) were purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%, and then lentiviral transfection was performed according to the manufacturer's instructions. After 48 h, cells were collected for real-time quantitative polymerase chain reaction (qPCR) analysis and cell function experiments.

2.4. Cell Proliferation Assay

The transfected cells after 48 h were collected and plated into 96-well plates at 2000 cells per well. The cells were cultured for 24 h, 48 h, 72 h and 96 h, respectively, and then added with cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan). After incubation for 2 hours, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

2.5. Colony Formation Assay

After 48 h of transfection, cells were collected, and 200 cells were seeded in each well of a 6-well plate and cultured with complete medium for 2 weeks. The medium was changed after one week and then twice a week. The medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, the cells were cloned and then fixed in 2 mL of methanol for 20 minutes. After the methanol was aspirated, the cells were stained with 0.1% crystal violet staining solution for 20 minutes, washed 3 times with phosphate-buffered saline (PBS), photographed and counted under a light-selective environment.

2.6. Flow Cytometry Analysis of the Cell Apoptosis

HCC cells in logarithmic growth phase were seeded into 6-well plates. After 24 h of drug treatment, the cells were collected, washed twice with PBS, resuspended in the binding solution, and incubated at room temperature for 15 min in the dark. 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 5 μL of Propidium Iodide (PI) were added, mixed gently, and apoptosis rate was measured by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

2.7. Quantitative Real-Time PCR (qPCR)

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions, real-time PCR was performed according to the SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) kit instructions, and the PCR was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qPCR: circ_0006168: forward: 5'-ACCAGCAGATAGTAGGAAACA-3', reverse: 5'-TGGCATCCCTATTAGTCTTTC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-GGGAAGCTCACTGGCATGGCCTTCC-3', reverse: 5'-CATGTGGGCCATGAGGTCCACCAC-3'; microRNA-125b: forward: 5'-GAATTTAATTTTCAATATTAT-3', reverse: 5'-AGCTCTGCTGTGCTCCCATGC-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'. Three replicate wells were repeated for each sample and the assay was repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). The GAPDH and U6 genes were used as internal parameters.

2.8. Dual-Luciferase Reporter Assay

A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter plasmid into Hep3B and Huh7 cells. A specific luciferase substrate was added, and luciferase reacted with the substrate to generate fluorescence.

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Differences between two groups were analyzed by the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Independent experiments were repeated at least three times for each experiment and data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

3. Results

3.1. Circ_0006168 Was Highly Expressed in HCC Tissues and Cell Lines

The expression difference of circ_0006168 in 42 pairs of tumor tissue samples and paracancerous ones collected from HCC patients was determined through qPCR detection. As a result, it was found that circ_0006168 level was markedly elevated in the former compared to the latter (Figure 1A and 1B), suggesting that circ_0006168 may act as an oncogene in HCC. In addition, circ_0006168 also showed a higher expression in HCC cell lines than in the human normal liver cell line (LO2), and the difference was statistically significant (Figure 1C). In sum, these results revealed that circ_0006168 is highly expressed in HCC

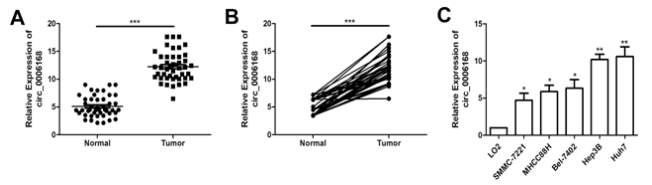

Fig. 1. circ_0006168 is highly expressed in hepatocellular carcinoma tissues and cell lines. (A, B) qRT-PCR was used to detect the difference in expression of circ_0006168 in tumor tissues and adjacent tissues of patients with hepatocellular carcinoma; (C) qRT-PCR was used to detect the expression level of circ_0006168 in hepatocellular carcinoma cell lines. Data are mean ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001.

tissues and cells.

3.2. Circ_0006168 Expression Was Correlated With Pathological Stage and Overall Survival in HCC Patients

According to the mRNA expression of circ_0006168, the above-mentioned tissue samples were divided into two groups, namely, high and low expression groups; meanwhile, the interplay between circ_0006168 expression and some indicators of HCC patients was analyzed, including the age, sex, pathological stage and the lymph node or distant metastasis. As shown in Table 1, high expression of circ_0006168 was positively correlated with the pathological stage, but not with the other related indicators.

3.3. Knockdown of Circ_0006168 Inhibited HCC Proliferation and Promoted HCC Apoptosis

To determine the functional changes of circ_0006168 in HCC cells, we constructed a knockdown circ_0006168 lentiviral vector and transfected it into Hep3B and Huh7 cell lines, respectively, and verified the interference efficiency by qPCR (Figure 2A). Subsequently, CCK-8, plate cloning and flow cytometry experiments were performed to evaluate the proliferation ability and apoptosis rate of HCC cells. The results indicated that compared with that in sh-NC group, the proliferation of HCC cells in sh-circ_0006168 group was remarkably attenuated (Figure 2B and 2C), while the apoptosis rate was oppositely elevated (Figure 2D). These results demonstrated that the knockdown of circ_0006168 inhibits HCC proliferation and promotes HCC apoptosis.

3.4. Circ_0006168 Was Bound to MicroRNA-125b

We predicted miRNAs that may bind to circ_0006168 through databases and found several possible miRNAs with binding relationships, including microRNA-125b. To further validate the binding of microRNA-125b to circ_0006168, pmirGLO-circ_0006168-WT, pmirGLO-circ_0006168-MUT or pmirGLO and microRNA-125b were co-transfected into HCC cells for luciferase reporter gene assay. It was found that overexpression of microRNA-125b remarkably attenuated the luciferase activity of the wild-type circ_0006168 vector (P<0.05) without attenuating that of the mutant vector (P>0.05) or the empty one (P>0.05), further demonstrating that circ_0006168 can be combined with the corresponding site of microRNA-125b (Figure 3A and 3B). In addition, qPCR results revealed an increased expression of microRNA-125b in HCC cells after knocking down circ_0006168 (Figure 3C). These results indicated that microRNA-125b is regulated by circ_0006168 in hepatocarcinoma and that circ_0006168 is bound to microRNA-125b.

3.5. MicroRNA-125b Was Lowly Expressed in HCC Tissues and Cell Lines

Subsequently, we examined microRNA-125b level in the above tissue specimens and found a significantly lowly-expressed microRNA-125b in HCC tumor tissues compared with that in the paracancerous ones, and the difference was statistically significant (Figure 3D). At the same time, the associations of microRNA-125b expression with the clinical parameters and prognosis of HCC patients were

Table 1. Clinicopathologic characteristics of the included patients with hepatocellular carcinoma.

Parameters	Number of cases	circ_0006168 expression		*P*-value	miR-125b expression		*P*-value
		Low (n=21)	High (n=21)		Low (n=16)	High (n=26)	
Age (years)				0.204			0.474
<60	16	10	6		5	11	
≥60	26	11	15		11	15	
Gender				0.355			0.204
Male	21	12	9		6	15	
Female	21	9	12		10	11	
T stage				0.011			0.011
T1-T2	26	17	9		6	20	
T3-T4	16	4	12		10	6	
Lymph node metastasis				0.095			0.159
No	29	17	12		9	20	
Yes	13	4	9		7	6	
Distance metastasis				0.057			0.213
No	26	16	10		8	18	
Yes	16	5	11		8	8	

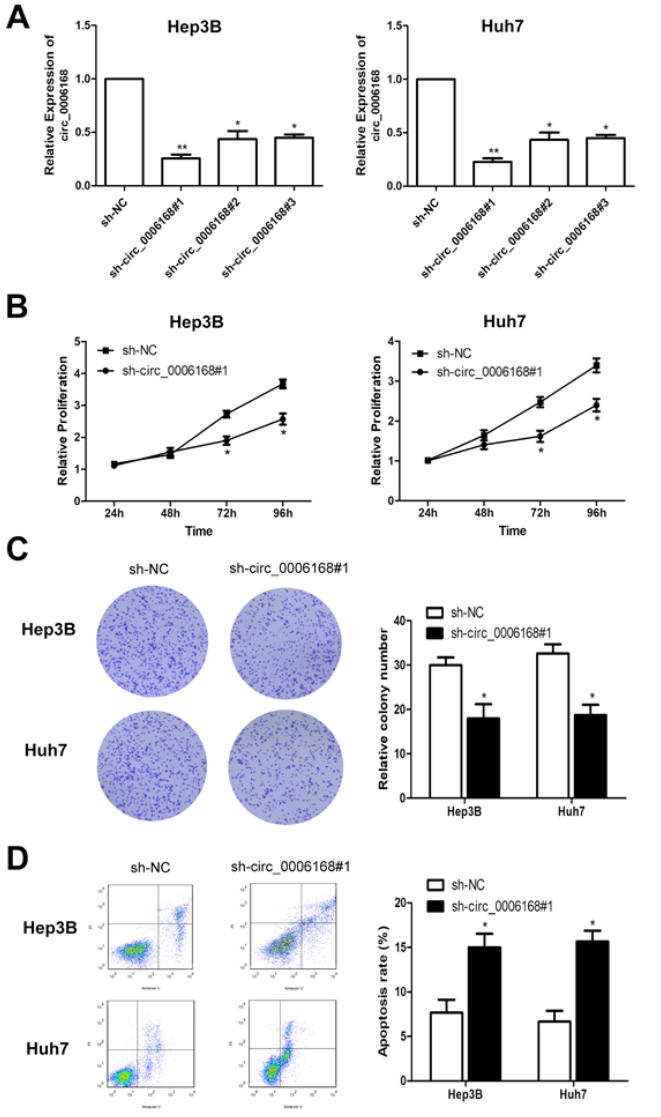

Fig. 2. Silencing circ_0006168 inhibits proliferation of hepatocellular carcinoma cells and promotes apoptosis. (A) qRT-PCR was performed to verify the interference efficiency of circ_0006168 after transfection of circ_0006168 knockdown vector in hepatocellular carcinoma Hep3B and Huh7 cell lines; (B) CCK-8 assay was performed to detect the effect of silencing circ_0006168 on proliferation of hepatocellular carcinoma cells in Hep3B and Huh7 cell lines; (C) Plate cloning experiments were performed to detect the number of CY_1024168-positive hepatocellular carcinoma proliferation-positive cells in Hep3B and Huh7 cell lines (magnification: 10×); (D) Flow cytometry assay was performed to detect the effect of silencing circ_0006168 on apoptosis of hepatocellular carcinoma cells in Hep3B and Huh7 cell lines. Data are mean ± SD, *P<0.05, **P<0.01.

also analyzed. As shown in Table I, low expression of microRNA-125b was found positively correlated with HCC pathological stage, but not with age, gender, or the incidence of lymph node or distant metastasis. In addition, qPCR detection revealed a negative correlation between the expression levels of circ_0006168 and microRNA-125b in HCC tumor tissues (Figure 3E).

3.6. Circ_0006168 Modulated MicroRNA-125b in HCC

Finally, bioinformatics analysis implied that there may be some interaction between circ_0006168 and microRNA-125b. Consequently, to further explore whether circ_0006168 promotes the malignant progression of HCC through microRNA-125b, we simultaneously downregulated these two molecules in HCC cells and confirmed the transfection efficiency by qPCR (Figure 4A). Through CCK-8 and plate cloning experiments as well as the flow cytometry assay, it was found that co-transfection of microRNA-125b and circ_000616 weakened the enhanced cell proliferation ability while elevating the decreased cell apoptosis rate induced by knockdown of circ_0006168 (Figure 4B, 4C, 4D). These results suggested that Circ_0006168 modulates microRNA-125b in HCC.

4. Discussion

Hepatocellular carcinoma (HCC) is a kind of malignant tumor with strong invasive ability, high proliferative capacity and extremely poor prognosis [1-3]. High-risk factors closely related to liver cancer include chronic hepatitis b,

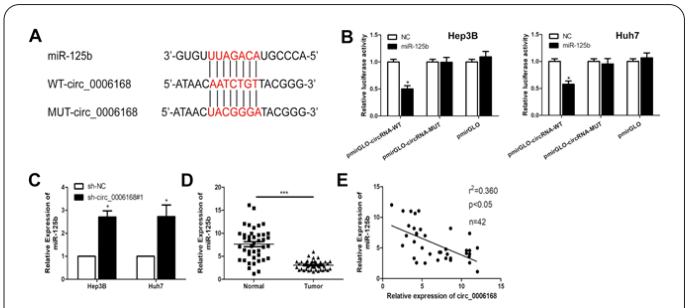

Fig. 3. Circ_0006168 direct targeting of miR-125b. (A) Schematic representation of the targeted binding site of circ_0006168 to miR-125b; (B) Dual-Luciferase reporter gene assay verified the direct targeting of circ_0006168 and miR-125b; (C) qRT-PCR detection revealed that silencing circ_0006168 significantly increased miR-125b expression levels; (D) qRT-PCR was used to detect the differential expression of miR-125b in tumor tissues and paracancerous tissues of patients with hepatocellular carcinoma; (E) There was a significant negative correlation between the expression levels of circ_0006168 and miR-125b in hepatocellular carcinoma tissues. Data are mean ± SD, *P<0.05, ***P<0.001.

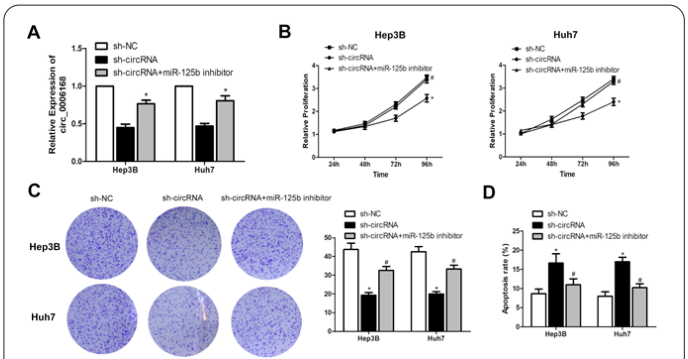

Fig. 4. Circ_0006168 regulates the role of miR-125b in hepatocellular carcinoma. (A) The expression level of circ_0006168 was detected by qRT-PCR after co-transfection of circ_0006168 and miR-125b in Hep3B and Huh7 cell lines; (B) CCK-8 assay was used to detect the proliferation of hepatocyte cancer cells after co-transfection of circ_0006168 and miR-125b in Hep3B and Huh7 cell lines; (C) Plate cloning assay was performed to detect the number of positive cells after co-transfection of circ_0006168 and miR-125b in Hep3B and Huh7 cell lines (magnification: 10×); (D) Flow cytometry was used to detect the apoptosis of hepatocellular carcinoma cells after co-transfection of circ_0006168 and miR-125b in Hep3B and Huh7 cell lines. Data are mean ± SD, *#P<0.05.

chronic hepatitis c, food containing aflatoxin, obesity, type 2 diabetes, alcoholism, non-alcoholic fatty liver disease, smoking, etc. [20]. Currently, the diagnosis and treatment of HCC are faced with many challenges, and the clinical prognosis is often poor [21,22]. Therefore, finding new biological indicators for early diagnosis and prognosis of HCC is of great significance for the treatment and improvement of prognosis of HCC patients [10,11].

In recent years, a large number of non-coding RNAs have received increasing attention for their potential application in the regulation of biological functions and the clinical diagnosis of liver cancer [23]. Among them, the discovery and further cognition of circRNAs have enriched the level of post-transcriptional translation [12,13]. Numerous studies [14-16] have shown that circRNAs play a key role in the progression of HCC through post-transcriptional translation. These findings suggest that these highly expressed circRNAs are not waste products produced during shearing, but functional[16,17]. In this study, circ_0006168 was picked out as a candidate for HCC malignant progression-related circular RNA. Circ_0006168 expression was found to be remarkably higher in HCC tumor tissues than in adjacent tissues, and positively correlated with pathological stage and poor prognosis of HCC patients, suggesting that circ_0006168 may act as a pro-cancer gene in this cancer. To further study the molecular mechanism of circ_0006168 in the occurrence and development of liver cancer, CCK-8, plate cloning and flow cytometry apoptosis experiments were carried out, and the results showed that knockdown of circ_0006168 could inhibit the proliferation ability of HCC and promote its apoptosis.

CircRNA is a competing endogenous RNA (ceRNA) containing miRNA response elements (MERs), it could inhibit the regulation of miRNAs on downstream genes by competitively binding miRNA sites [24]. Many studies have shown that circRNAs can adsorb miRNAs or bind to proteins, and alteration of circRNAs can cause changes in the expression level of tumor-related genes, thereby affecting the occurrence and development of tumors [25]. Previous studies predicted that microRNA-125b might bind to circ_0006168 through bioinformatics analysis. The results of this study showed that microRNA-125b was downregulated in tumor tissues of patients with HCC in comparison with that in adjacent tissues and that microRNA-125b could inhibit the proliferation of HCC cells and promote their apoptosis. Besides, the direct binding of circ_0006168 to downstream microRNA-125b was verified by dual luciferase reporter gene assay. To further investigate the regulation of circ_0006168 on microRNA-125b expression in HCC cell lines, the expression levels of circ_0006168 and microRNA-125b were detected and were shown to be just negatively correlated in HCC tissues. In addition, silencing microRNA-125b was found to counteract the effects of silencing circ_0006168 on proliferation and apoptosis of HCC cells. In summary, this study suggested that circ_0006168 can inhibit the expression of microRNA-125b, thereby promoting the malignant progression of HCC.

5. Conclusions

Circ_0006168 is remarkably increased in HCC tissues and cell lines and is remarkably correlated with pathological stage and poor prognosis of patients with HCC. In addition, circ_0006168 may promote the proliferation ability of HCC by regulating microRNA-125b.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the ethics committee of Second Affiliated Hospital of Soochow University.

Informed Consent

Signed written informed consent were obtained from the patients and/or guardians.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

LZ and WC designed the study and performed the experiments, CH and JG collected the data, ZZ and CL analyzed the data, and LZ and WC prepared the manuscript. All authors read and approved the final manuscript.

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