MicroRNA-766 promotes cancer progression by targeting NR3C2 in hepatocellular carcinoma

Chao Yang,* Xiang Ma,† Ge Guan,‡ Huan Liu,§ Yuling Yang,* Qinghui Niu,* Zehua Wu,‡ Yueping Jiang,* Cheng Bian,* Yunjin Zang,‡,§ and Likun Zhuang‡,1

*Department of Infectious Diseases, †Department of Hepatobiliary and Pancreatic Surgery, ‡Organ Transplantation Center, and §Institute of Transplantation Science, Affiliated Hospital of Qingdao University, Qingdao, China

ABSTRACT: MicroRNAs (miRNAs) have been reported to play important roles in tumor progression of various cancers. However, the clinical significance and biologic function of miR-766 in hepatocellular carcinoma (HCC) remain unknown. In this study, we investigated the roles of miR-766 in HCC progression using HCC cell lines and a xenograft mouse model. miR-766 expression in tumor tissues and adjacent nontumorous liver tissues of patients with HCC was evaluated by quantitative RT-PCR. Our results showed that miR-766 promoted proliferation and metastasis of HCC cells in vitro and in vivo and that NR3C2 was a direct target of miR-766 and involved in miR-766–mediated proliferation and metastasis of HCC cells. We also found that miR-766 affected the β-catenin signaling pathway by targeting NR3C2. Furthermore, miR-766 was significantly up-regulated in HCC tissues and was correlated with the prognosis of patients with liver cancer. Taken together, our results show that miR-766 affects HCC progression by modulating NR3C2 expression and is a possible new therapeutic target for patients with HCC.

†Ge Guan,‡ Huan Liu,§ Yuling Yang,* Qinghui Niu,* Zehua Wu,‡ Yueping Jiang,* Cheng Bian,* Yunjin Zang,‡,§ and Likun Zhuang‡,1

*Department of Infectious Diseases, †Department of Hepatobiliary and Pancreatic Surgery, ‡Organ Transplantation Center, and §Institute of Transplantation Science, Affiliated Hospital of Qingdao University, Qingdao, China

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Noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs, have been reported to affect progression of tumors by regulating the expression of downstream genes (1–3). miRNAs, with sizes of 18–25 nt, suppress the expression of target genes by directly binding to the 3′-untranslated region (3′-UTR) of target miRNAs. Studies have suggested that the abnormal expression of miRNAs could play crucial roles in the pathogenesis of many cancers (4–6). In addition, miRNAs have been indicated to be promising diagnostic biomarkers and therapeutic targets for cancers (7). In the past few years, many studies have revealed the relationships between miRNAs and disease progression of hepatocellular carcinomas (HCCs) (8, 9). However, only a limited number of miRNAs were investigated in these studies, and the roles of more miRNAs in HCC occurrence and progression should be explored.

HCC is one of the leading causes of cancer-related death worldwide. During the past few years, the clinical diagnosis and treatment for patients with HCC have made great progress. However, a large proportion of patients with HCC are diagnosed at a late stage of disease, and recurrence or metastasis of HCC remains common after liver resection (10). Therefore, studies focusing on the abnormal expression and biologic roles of miRNAs in HCC may lead to new diagnostic biomarkers and therapeutic targets.

miR-766 was reported to act as an oncogene or a tumor suppressor gene in different tumors, and it was related to cell proliferation, metastasis, and apoptosis by targeting different downstream genes and signaling pathways (11, 12). However, the roles and molecular mechanisms of miR-766 in HCC progression remain unknown. Moreover, we have shown, by small RNA sequencing, that miR-766, miR-92b, and miR-23b are expressed differently in tumor tissues and adjacent nontumorous liver tissues (ANLTs) of patients with HCC (13, 14). In this study, we investigated the expression, roles, and mechanisms of miR-766 in the tumorigenesis of HCC in vitro and in vivo and revealed potential values of miR-766 in the clinical diagnosis and treatment of patients with HCC.

ABBREVIATIONS: **3′-UTR, 3′-untranslated region; ANLT, adjacent nontumorous liver tissue; HCC, hepatocellular carcinoma; miRNA, microRNA

1 Correspondence: Institute of Transplantation Science, the Affiliated Hospital of Qingdao University, 16 Jiangsu Rd., Qingdao 266003, China. E-mail: zlk0823@163.com
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MATERIALS AND METHODS

Cell culture

SMMC-7721 and HCCLM3 cell lines were obtained from the Cell Resource Center (Shanghai Institutes for Biologic Sciences,
Chinese Academy of Sciences, Shanghai, China). MHCC97H and MHCC97L cell lines were obtained from Cobioer Biosciences Company (Nanjing, China). They were cultured in DMEM containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere with 5% CO₂ at 37°C.

**RNA extraction and quantitative RT-PCR**

Total RNAs were extracted using Trizol (Thermo Fisher Scientific) according to the manufacturer’s instructions. After reverse transcription, cDNA was amplified by using SYBR-Green Premix (Takara, Otsu, Japan). The expression of miR-766 and other genes was normalized to the expression of U6 and β-actin, respectively. The relative expression levels were calculated using the 2−ΔΔCt method. Primers for miR-766 and U6 were obtained from the Hairpin-it Quantitation PCR Kit (GenePharma, Shanghai, China). The sequences of the other primers used in this study are listed in Supplemental Table 1.

**Transfection and luciferase reporter assay**

Oligonucleotides were purchased from GenePharma. NR3C2 expression plasmids were purchased from Biogot Technology (Nanjing, China). NR3C2 3’-UTR sequences, including 2 putative miR-766 complementary sites, were fused into a modified pcDNA3.1 vector containing a luciferase cDNA. Reporter plasmids with mutant sites were constructed using the Mutagenesis Kit (Stratagene, La Jolla, CA, USA). TOP/FLASH reporter gene including β-catenin binding sites was obtained from MilliporeSigma (Burlington, MA, USA). Oligonucleotides were transfected into cells using Hiperfect transfection reagent (Qiagen, Valencia, CA, USA), and the transfection of plasmids were conducted using Lipofectamine 3000 (Thermo Fisher Scientific). Luciferase reporter analysis was conducted using the Dual Luciferase Assay System (Promega, Madison, WI, USA), and pRL-TK plasmid (Promega) was used to normalize the transfection efficiency.

**Cell proliferation assay**

Cell proliferation was evaluated by CCK-8 (Dojindo, Kumamoto, Japan) assay. Cells were plated into 96-well plates and cultured for 12 h. Cells were transfected with the corresponding oligonucleotides or plasmids. At the indicated time points, CCK-8 solution was added into the wells of the plates, and cells were incubated for 1 h. The absorbance at 450 nm was measured.

**Colony formation assay**

For the colony formation assay, HCC cells after transfection were seeded in 6-well plates (1000 cells/well). The cells were cultured for 10 d, fixed with methanol for 15 min, and stained with 0.1% crystal violet for 20 min. Visible cell colonies were counted.

**Wound-healing assay**

HCC cells were cultured in 12-well plates. After 24 h, cell layers were wounded by a 10-μl tip to create a wounded gap. Cells were gently washed to remove the cellular debris and incubated in serum-free DMEM. At the indicated time points, the wounded gaps were photographed using light microscopy, and the results were analyzed with Imagel software (National Institutes of Health, Bethesda, MD, USA).

**Transwell invasion assay**

Twenty-four-well transwell chambers with 8.0-μm pore polycarbonate membrane (Corning, Corning, NY, USA) were used in the cell invasion assays. Cells suspended by DMEM were added into the upper chamber, which was coated with matrigel (BD, Franklin Lakes, NJ, USA). DMEM with 10% fetal bovine serum was added to the lower well. After the indicated time points, noninvaded cells were removed from the upper chamber membrane with cotton-tipped swabs. The invaded cells on the lower surface of the chamber membrane were fixed in methanol for 10 min and stained in 0.1% crystal violet for 20 min. Cells were counted under a light microscope.

**Western blot assay**

Total protein was extracted by RIPA lysis, separated by SDS-PAGE, and transferred to a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% milk and incubated with the following primary antibodies: antibodies for NR3C2 (ab64457; Abcam, Cambridge, MA, USA), vimentin (3390; Cell Signaling Technology, Danvers, MA, USA), β-actin (3700; Cell Signaling Technology), Cyr61 (14479; Cell Signaling Technology), C-myc (ab17356; Abcam), and β-catenin (8480; Cell Signaling Technology). Then the membrane was incubated with the corresponding secondary antibodies. Protein bands were visualized using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

**Xenograft mouse model**

BALB/c nude mice at 6–8 wk of age were obtained from the Shanghai Institute of Materia Medica (Shanghai, China). The mice were subcutaneously injected with HCCLM3 cells infected with miR-766 knockdown lentivirus. Ten days later, the mice were euthanized. Tumor volumes were measured and calculated using the formula: tumor volume = (length × width²)/2. For the second step, the subcutaneous tumor tissues were cut into small pieces of the same size (2 mm diameter), and the pieces were implanted into the livers of new nude mice. Four weeks later, the mice were euthanized, and the lungs of mice were paraffin embedded, sectioned, and stained with hematoxylin and eosin. All animal studies were conducted in the Animal Institute of Affiliated Hospital of Qingdao University according to the protocols approved by the Medical Experimental Animal Care Commission of Qingdao University.

**Immunohistochemistry**

Paraformaldehyde-fixed liver tissues from nude mice were paraffin embedded and sectioned. NR3C2 antibody (ab64457; Abcam) and Ki-67 antibody (bs-23103R; Bioss, Beijing, China) were used in immunohistochemistry with the streptavidin peroxidase-conjugated method.

**Patients and tissue samples**

Specimens of tumor tissues and ANLTs were randomly collected from patients with HCC who underwent surgical resection at the
Department of Hepatobiliary and Pancreatic Surgery, the Affiliated Hospital of Qingdao University during January 2015 to March 2017. The clinicopathological data of patients with HCC are listed in Supplemental Table 2. This study was approved by the Ethics Committee of Affiliated Hospital of Qingdao University, and written informed consent was obtained from each participant.

Statistical analysis

Statistical analysis was performed using the Prism 7 software (GraphPad Software, La Jolla, CA, USA). Experimental data are presented as mean ± SD. Comparisons were performed using a Student’s t test or 1-way ANOVA. Kaplan-Meier analysis of overall survival and disease-free survival were conducted based

**Figure 1.** Effects of miR-766 on proliferation and colony formation of HCC cells in vitro. A) Relative miR-766 expressions in different HCC cell lines were determined by quantitative RT-PCR. The data were normalized to the expression level of miR-766 in SMMC-7721 cells. B, C) miR-766 was overexpressed in SMMC-7721 cells and knocked down in HCCLM3 cells. D, E) CCK-8 assays at different time points were performed. F, G) Colony formation assays were conducted. Cell colonies were counted and compared in the diagrams. NC, negative control. Results are represented as means ± SD (n ≥ 3). *P < 0.05, **P < 0.01.
on data from Kaplan-Meier Plotter (www.kmplot.com/analysis). A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

**miR-766 promotes proliferation and metastasis of HCC cells in vitro**

To explore the biologic function of miR-766 in HCC cells, we measured the expression levels of miR-766 in HCC cell lines, including HCCLM3, MHCC97H, MHCC97L, and SMMC-7721. The expression of miR-766 in HCCLM3 cells, which possessed a higher potential of proliferation and metastasis, was significantly higher than that in SMMC-7721 cells, which possessed a lower potential of proliferation and metastasis (Fig. 1A). Then miR-766 was overexpressed in SMMC-7721 cells using miR-766 mimics and knocked down in HCCLM3 cells using miR-766 inhibitor to carry out the further experiments (Fig. 1B, C).

CCK-8 assays were performed to assess the roles of miR-766 in proliferation of HCC cells. The results revealed that ectopic expression of miR-766 significantly promoted proliferation of SMMC-7721 cells. Consequently, proliferation of HCCLM3 cells transfected with miR-766 inhibitor was significantly suppressed (Fig. 1D, E). Colony formation assays also showed that up-regulation of miR-766 promoted the colony formation capacity of SMMC-7721 cells and down-regulation of miR-766 inhibited the colony formation capacity of HCCLM3 cells (Fig. 1F, G). Collectively, these results showed that miR-766 enhanced the proliferation and colony formation of HCC cells in vitro.

We performed wound healing and transwell assays to explore the effects of miR-766 on migration and invasion in HCC cells. Up-regulation of miR-766 promoted the migration and invasion of SMMC-7721 cells, and down-regulation of miR-766 suppressed the migration and invasion of HCCLM3 cells (Fig. 2). Together, these results demonstrate that overexpression of miR-766 promotes the proliferation, colony formation, and metastasis of HCC cells in vitro.

**miR-766 promotes tumor growth and metastasis of HCC in vivo**

To further investigate the effects of miR-766 on tumor growth and metastasis of HCC in vivo, HCCLM3 cells with

![Figure 2.](image-url) Effects of miR-766 on metastasis of HCC cells *in vitro*. miR-766 was overexpressed in SMMC-7721 cells and knocked down in HCCLM3 cells. **A, B** Wound-healing assays were performed. The percent of wound closure was calculated and compared in the right diagrams. **C, D** Transwell invasion assays were conducted. The number of cells passing through the membrane was counted and compared in the diagrams. NC, negative control. Results are represented as means ± sd (n = 3). *P < 0.05, **P < 0.01.
miR-766 knockdown were subcutaneously injected into the nude mice. The expression of miR-766 in xenografted tumor tissues is shown in Fig. 3A. As expected, miR-766 knockdown substantially attenuated the growth of tumors in nude mice (Fig. 3B, C). Immunohistochemistry assay showed that the proliferation marker Ki67 was also down-regulated in the miR-766 knockdown group (Fig. 3D).

The subcutaneous tumors were implanted into the livers of new nude mice. Four weeks later, we measured lung metastasis rates of mice. Our results showed that knockdown of miR-766 significantly reduced the incidence of lung metastasis in mice (Fig. 3E, F). Taken together, these data suggest that miR-766 promotes tumor growth and metastasis of HCC in vivo.

**miR-766 directly targets NR3C2**

To further explore the mechanisms underlying miR-766–mediated promotion of cell proliferation and metastasis in HCC, we searched for potential downstream genes of miR-766 using 3 different miRNA target prediction

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**Figure 3.** Effects of miR-766 on tumor growth and metastasis of HCC in vivo. The mouse model was constructed using HCCLM3 cells infected with the corresponding lentiviruses. A) miR-766 expression in xenograft tumor tissues was measured by quantitative RT-PCR. B, C) The sizes of subcutaneous tumors were calculated and compared. Results are represented as mean ± SD (n = 4). D) Expression of Ki67 in subcutaneous tumors was analyzed by immunohistochemistry. E) Typical pictures for lung metastasis of HCC mouse model. F) The percentage of mice with or without lung metastasis was calculated (n = 8). NC, negative control. *P < 0.05, **P < 0.01.
algorithms (Targetscan, microRNA.org, and miRDB) and focused on NR3C3 as a potential target gene for miR-766. It has been reported that NR3C2 was involved in the proliferation and metastasis of cancer cells and can act as a tumor suppressor gene in cancer progression (15). To investigate whether NR3C2 was a direct target for miR-766, we constructed the reporter plasmids coupled with 3'-UTR of NR3C2 mRNA containing wild-type or mutant potential miR-766 binding sites (Fig. 4A). The results of luciferase assays showed that miR-766 repressed the expression of reporter gene containing wild-type 3'-UTR but not that containing mutant 3'-UTR of NR3C2 (Fig. 4B). Our results showed that the expression of NR3C2 in SMMC-7721 cells was higher than that in HCCLM3 cells (Fig. 4C and Supplemental Fig. 1A), having an inverse relationship with the miR-766 expression in these 2 cell lines (Fig. 1A). Furthermore, transfection with miR-766 mimics significantly down-regulated NR3C2 expression in SMMC-7721 cells, whereas knockdown of miR-766 increased the NR3C2 expression in HCCLM3 cells (Fig. 4D, E and Supplemental Fig. 1B, C). We also found that NR3C2 expression was increased in the miR-766 knockdown group of the HCC mouse model (Fig. 4F). These results show that NR3C2 was a direct target for miR-766.

NR3C2 is essential for miR-766–mediated HCC progression

To identify whether miR-766 promoted proliferation and metastasis of HCC cells by targeting NR3C2, we carried...
out gain- and loss-of-function experiments, miR-766 mimics and NR3C2 expression plasmids were cotransfected into SMMC-7721 cells, and miR-766 inhibitor and small interfering RNAs targeting NR3C2 were cotransfected into HCCLM3 cells. Our results showed that overexpression of NR3C2 markedly attenuated miR-766–mediated promotion of proliferation and colony formation in SMMC-7721 cells (Fig. 5A, C), whereas knockdown of NR3C2 abolished miR-766 inhibitor–mediated inhibition of proliferation and colony formation in HCCLM3 cells (Fig. 5B, D). Meanwhile, NR3C2 overexpression inhibited miR-766–mediated HCC cell migration and invasion (Fig. 6A, C), and NR3C2 knockdown attenuated the inhibition of HCC cell migration and invasion mediated by miR-766 inhibitor (Fig. 6B, D). These data suggest that NR3C2 was involved in miR-766–mediated proliferation and metastasis of HCC cells.

Figure 5. NR3C2 attenuates the effects of miR-766 on proliferation and colony formation of HCC cells in vitro. SMMC-7721 cells were cotransfected with miR-766 mimics and NR3C2 expression plasmids, and HCCLM3 cells were cotransfected with miR-766 inhibitor and NR3C2 siRNA. A, B) CCK-8 assays at different time points were performed. C, D) Colony formation assays were conducted. The cell colonies were counted and compared in the diagrams. NC, negative control. Results are represented as means ± sd (n = 3). *P < 0.05, **P < 0.01.

miR-766 activates the β-catenin pathway by targeting NR3C2 in HCC cells

The β-catenin pathway was reported to play important roles in proliferation and metastasis of cancer cells (16, 17). To investigate whether miR-766 and NR3C2 regulate the β-catenin pathway in HCC cells, we measured the protein expression of β-catenin in SMMC-7721 cells transfected with miR-766 mimics and NR3C2 expression plasmids. Our results showed that miR-766 increased β-catenin expression and NR3C2 attenuated miR-766–mediated up-regulation of β-catenin expression (Fig. 7A and Supplemental Fig. 1D). Luciferase analysis also showed that miR-766 significantly increased the transcriptional activity of β-catenin in HCC cells and that NR3C2 inhibited miR-766–mediated β-catenin activation (Fig. 7B). The expression of a series of downstream genes, including Cyr61, C-myc, and

Figure 7A. Luciferase reporter assay. A) Luciferase activities in SMMC-7721 cells transfected with indicated plasmids. The Firefly luciferase activity was normalized to Renilla luciferase activity. B) Luciferase activities in HCCLM3 cells transfected with indicated plasmids. The Firefly luciferase activity was normalized to Renilla luciferase activity.
vimentin, in β-catenin pathway was evaluated. Our results showed that mRNA and protein expressions of Cyr61, C-myc, and vimentin were up-regulated by miR-766 and that NR3C3 attenuated the effects of miR-766 on the promotion of these downstream genes in HCC cells (Fig. 7C–F and Supplemental Fig. 1E, F). These results suggest that miR-766 activates the β-catenin pathway by targeting NR3C2 in HCC cells.

Figure 6. NR3C2attenuates the effects of miR-766 on metastasis of HCC cells in vitro. SMMC-7721 cells were cotransfected with miR-766 mimics and NR3C2 expression plasmids, and HCCLM3 cells were cotransfected with miR-766 inhibitor and NR3C2 small interfering RNA. A, B) wound-healing assays were performed. The percent of wound closure was counted and compared in the right diagrams. C, D) Transwell invasion assays were conducted. The cells passing through the membrane were counted and compared in the diagrams. NC, negative control. Results are represented as means ± sd (n = 3). *P < 0.05, **P < 0.01.
miR-766 expression is up-regulated in tumor tissues of HCC

To analyze the expression of miR-766 and NR3C2 in patients with HCC, we measured and compared miR-766 and NR3C2 expression in 30 pairs of tumor tissues and ANLTs of patients with HCC. Our results showed that the expression of miR-766 was significantly higher in tumor tissues than in ANLTs of patients with HCC, and HCC tissues showed a reduced expression of NR3C2 (Fig. 8A, B). The prognostic value of miR-766 for liver cancer, evaluated using an online database for prognostic analysis (Kaplan-Meier Plotter, www.kmplot.com), indicated that high miR-766 expression in patients with liver cancer was positively correlated with poor overall survival and disease-free survival.

Figure 7. Effects of miR-766 on the β-catenin signaling pathway in HCC cells. SMMC-7721 cells were cotransfected with miR-766 mimics and NR3C2 expression plasmids, and HCCLM3 cells were cotransfected with miR-766 inhibitor and NR3C2 small interfering RNA. A) Western blot analysis of β-catenin was performed. B) Reporter gene analysis for β-catenin transactivity was conducted. C) Quantitative RT-PCR analysis of Cyr61, C-myc, and vimentin expression was performed. D) Western blot analysis of Cyr61, C-myc, and vimentin expression was performed. NC, negative control. *P < 0.05, **P < 0.01.
HCC accounts for about 90% of primary liver cancer. These results indicate that increased expression of miR-766 might serve as a prognostic factor for poor outcome in patients with HCC.

**DISCUSSION**

Many miRNAs have been reported to be abnormally expressed in tumor tissues and to play important roles in the tumorigenesis of cancers (18). miRNAs could also be detected in the tissues and blood of patients and have great potential in the clinical diagnosis of cancers. Our previous study showed that miR-92b was higher in tumor tissues and plasma of patients with HCC, which suggested that miR-92b might be a potential biomarker in the diagnosis of HCC (13). Because adeno-associated virus and exosomes have been reported to act as the delivery carriers for miRNAs, miRNAs also have immense potential in the gene therapy of cancers (19, 20). Increasing the knowledge of the expression and roles of various miRNAs in the progression of cancers could improve the development of tools for diagnosis and therapy of cancers. In this study, our results showed that miR-766 could affect the proliferation and metastasis of HCC cells. miR-766 was also significantly up-regulated in tumor tissues of patients with HCC. These data suggest that miR-766 might act as one of the potential diagnostic biomarkers and therapeutic targets for HCC.

One miRNA usually targets multiple genes, including oncogenes or tumor suppressor genes in cancer cells. Consequently, the roles of a specific miRNA in a specific cancer type mainly depend on the gene expression patterns specific to the cancer cells. miRNAs might act as oncogenes or tumor suppressor genes in different cancer types. Many studies have
shown that miR-766 exerted different functions in different cancers. For instance, miR-766 was reported to promote cell proliferation in colorectal cancer by targeting SOX6 expression (12). In renal cell carcinoma, miR-766 overexpression attenuated cell-cycle progression by down-regulating SF2 expression (21). In this study, our results showed that miR-766 was up-regulated in HCC tissues and that up-regulation of miR-766 promotes the proliferation and metastasis of HCC cells by directly decreasing the NR3C2 expression. Therefore, we identified the oncogenic role of miR-766 in HCC progression.

NR3C2, also known as mineralocorticoid receptor, is a cytoplasmic steroid-responsive receptor that controls electrolyte and water balance (22). NR3C2 is a cytoplasmic steroid-responsive receptor that plays a role in the specific mechanism underlying the regulatory effects of miR-766 and NR3C2 on β-catenin expression should be further explored. In summary, our results showed that miR-766 promotes proliferation, migration, and invasion of HCC cells in vitro and in vivo. NR3C2 was one of the direct targets of miR-766 and was involved in miR-766–mediated tumorigenesis of HCC by regulating the β-catenin signaling pathway. Furthermore, miR-766 was up-regulated in tumor tissue of patients with HCC. This study revealed the importance of miR-766 in HCC progression and implicated the potential role of miR-766 in the clinical diagnosis and treatment of patients with HCC.

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AUTHOR CONTRIBUTIONS

Y. Zang and L. Zhuang conceived and designed the study; C. Yang, X. Ma, G. Guan, H. Liu, Y. Yang, and Z. Wu performed the experiments; C. Yang, Q. Niu, and L. Zhuang analyzed the data and drafted the manuscript; Y. Jiang, C. Bian, and Y. Zang revised the manuscript; and all the authors read and approved the final manuscript.

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