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Linc00601 upregulation promotes hepatocellular carcinoma development by activating MAPK signaling pathway

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Abstract. – OBJECTIVE: To study the expression of linc00601 in hepatocellular carcinoma (HCC) tissues and cells, and to study the biological function and downstream mechanism of linc00601 in HCC using *in vitro* experiments.

PATIENTS AND METHODS: The expression of linc00601 in HCC was predicted *via* bioinformatics, and the expression of linc00601 in HCC tissues and cells was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). After interference with the expression of linc00601, the interference efficiency was determined using qRT-PCR, and the changes in HCC cell proliferation, cycle distribution, and apoptosis were determined through Cell Counting Kit-8 (CCK-8) assay and flow cytometry, respectively. Finally, the expressions of molecular markers in downstream signaling pathway were determined through Western blotting.

RESULTS: It was found *via* bioinformatics that the expression of linc00601 was upregulated in HCC. The results of qRT-PCR revealed that the expression of linc00601 was upregulated in 36 cases of HCC tissues compared with that in para-carcinoma tissues, and it was also upregulated in HCC cells. According to the results of CCK-8 assay, HCC cell proliferation was inhibited after interference with the expression of linc00601. In the si-linc00601 group, the apoptosis rate rose, and the cell cycle was arrested at the G1/G0 phase compared with those in the si-NC group. The results of Western blotting revealed that after the knockdown of linc00601 in HCC cells, the expressions of molecular markers (p-P38, p-ERK) in the downstream mitogen-activated protein kinase (MAPK) signaling pathway were downregulated.

CONCLUSIONS: Linc00601 is upregulated in HCC, which promotes the development of HCC *via* activating the MAPK signaling pathway.

Key Words:

Hepatocellular carcinoma, Linc00601, Biological function, MAPK signaling pathway.

Introduction

Hepatocellular carcinoma (HCC) is one of the malignant tumors seriously threatening human health. About 383,000 people die of HCC every year in China, accounting for 51% of the total deaths in the world. Due to its high mortality and morbidity rates, it is urgent to search for effective prevention and treatment means for HCC[1,2]. Studying the molecular mechanism of the occurrence and development of HCC will contribute to the exploration of new targets for early diagnosis and targeted therapy of HCC.

With the rapid development of second-generation sequencing technique, thousands of long non-coding ribonucleic acids (lncRNAs) have been quickly identified in mammals[3]. LncRNAs possess a wide range of functions and participate in gene expression and protein translation and stabilization[4]. Moreover, lncRNAs are involved in pathological changes by regulating chromatin remodeling, DNA methylation and RNA metabolism, so they are closely related to the occurrence and development of many tumors, such as lung cancer, breast cancer, and gastric cancer[5-8].

According to Schmitz et al[9], lncRNAs can act as important regulators in the occurrence and development of HCC. Yuan et al[8] found that lncRNA-ATB competitively binds to miR-200, thereby activating the expressions of ZEB1 and ZEB2. Then, ZEB1 and ZEB2 interact with interleukin-11 mRNA to enhance the STAT3 signal, promote HCC metastasis, and facilitate organ involvement. Chen et al[10] reported that lncRNA NR027113 is downregulated in HCC, which suppresses cell proliferation and metastasis *via* the PTEN/PI3K/AKT pathway. However, there have been no reports about the correlation between linc00601 and HCC yet. In this study, it was

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first found *via* bioinformatics that the expression of linc00601 was upregulated in HCC, which was verified using *in vitro* experiments. Furthermore, the functional experiments showed that si-linc00601 could inhibit proliferation and promote apoptosis of HCC cells for the first time.

Patients and Methods

Sample Collection and Objects of Study

A total of 42 cases of HCC samples were collected *via* surgical resection in the Yantai Yuhuangding Hospital between January 2015 and December 2016. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). All tissues were diagnosed and confirmed as HCC tissues by two pathologists. Neither of patients underwent chemotherapy, radiotherapy, and targeted therapy before surgery. All tissue samples were harvested during surgery and immediately stored in liquid nitrogen at -80°C. This study was approved by the Medical Ethics Committee of Yantai Yuhuangding Hospital, and informed consent was signed by patients before sample detection.

Cell Culture

Four kinds of HCC cell lines (Hep3B, MHCC97-H, HCCLM3, and Huh7) and normal hepatocytes were purchased from ZQXZ Biotechnology (Shanghai, China). All cells were cultured with high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and double antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in an incubator with 5% CO_2 at 37°C. The original medium was replaced with fresh one every 1-2 days, and the cells were subcultured when 80-90% of them were fused.

Cell Transfection

Well-growing HCC cells were taken and inoculated into an antibiotic-free culture dish (60 mm) at 1 d before transfection when the cell density was 30-40%. The interference sequences (20 μmol/L) were diluted and mixed evenly with 500 μL of serum-free medium, while 10 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was diluted in 500 μL of serum-free medium and incubated at room temperature for 5 min. Then, the diluted Lipofectamine 2000 was mixed evenly with the interference sequences and incubated at room temperature for 20 min. 1 mL of the mixture was added into the culture dish, and added with serum-free medium until the total volume of 5 mL. After 6 h, the original medium was discarded and replaced with the medium containing 10% FBS. After 48 h, the cells were collected for biological experiments. Linc00601 siRNA: 5'-CACCGCCCAGATTTAATCAAGCTGGGCCTTTTTTG-3', si-NC: (5'-CACCGTTCTCCGAACGTGTCACGTC-3'.

RNA Extraction and Reverse Transcription

The total RNA was extracted with TRIzol in strict accordance with the instructions of RNA extraction kit (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA, 1 μL of 10 μM Oligo (dT), and diethyl pyrocarbonate (DEPC)-treated water were added into an Eppendorf (EP; Hamburg, Germany) tube and mixed evenly, followed by heating in the PCR instrument at 65°C for 5 min and ice bath for 3 min. After the reaction reagent was added into the EP tube, the mixture was incubated at 42°C for 60 min first and then at 70°C for 5 min. The resulting reaction solution was complementary deoxyribose nucleic acid (cDNA), and stored in a refrigerator at -80°C for later use.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The expression level was measured using the 7900HT fluorescence quantitative PCR system under 20 μL of reaction system (2 μL of cDNA diluted at 1:1, 10 μL of 2 × SYBR premix, 6 μL of distilled water, 1 μL of forward primers, and 1 μL of reverse primers). The reaction conditions are as follows: pre-denaturation at 95°C for 10 min, 95°C for 10 s, 60°C for 20 s, 72°C for 10 s, a total of 40 cycles. The relative expression of linc00601 was calculated using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated for 3 times. Linc00601 F: 5'-GAGCTGCACTG-ACCAGTAGG-3' R: 5'GTGCTGGCAGATGGATCACT-3', GAPDH F: 5'-CATCACCATCTTCCAGGAGCG-3' R: 5'-TGACCTTGCCCACAGCCTTG-3'.

Cell Counting Kit-8 (CCK8) Assay

The cells in the experimental group and control group were digested into single cell suspension and inoculated into a 96-well (2×10^3 cells/well). At 1-5 d after inoculation, 10 μL of CCK-8 buffer (Dojindo Molecular Technologies, Kuma-

moto, Japan) was added into each well every day for incubation in the incubator with 5% CO_2 at 37°C for 1 h. Then, the absorbance (A) value of each well was measured at 450 nm using a microplate reader. 5 repeated wells and blank control wells were set in each group. The experiment was repeated for 3 times, the average was taken, and the cell proliferation curve was plotted.

Detection of Apoptosis Via Flow Cytometry

After transfection, the cells were collected and resuspended, and the cell concentration was adjusted to 10^6 cells/mL, followed by centrifugation. After the supernatant was discarded, the cells were resuspended again, mixed evenly with 5 μL of Annexin V-fluorescein isothiocyanate (FITC), and 5 μL of Propidium Iodide (PI) dye, and incubated for 15 min in the dark. Finally, the apoptosis rate was determined for 3 times.

Detection of Cell Cycle Via Flow Cytometry

After transfection, the cells were collected, washed and digested, followed by centrifugation. After the supernatant was discarded, the cells were fixed with ethanol at 4°C overnight, centrifuged again, and resuspended. Then, 300 μL of PI dye was added for staining for 0.5 h, and the cells were incubated at 4°C for 30 min in the dark. Finally, the cell cycle was determined for 3 times.

Western Blotting

After transfection for 48 h, HCC cells in each group were collected, from which the total protein was extracted on ice. The protein concentration was determined using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). A total of 40 μg of protein samples were loaded and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the protein samples were transferred onto a membrane, sealed with 5% skim milk powder at room temperature for 1 h, and incubated with the primary antibody (1:5000; CST, Danvers, MA, USA) at 4°C overnight. After the membrane was washed with Tris-Buffered Saline and Tween (TBST) for 3 times (10 min/time), the protein samples were incubated again with the secondary antibody (1:5000) at room temperature for 2 h, and the membrane was washed again with TBST for 3 times (10 min/time). Finally, the enhanced chemiluminescence (ECL) solution was added for image development.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The measurement data were expressed as mean ± standard deviation (SD). The differences between two groups were analyzed by using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). The test level was set as $p<0.05$.

Results

Linc00601 was Upregulated in HCC

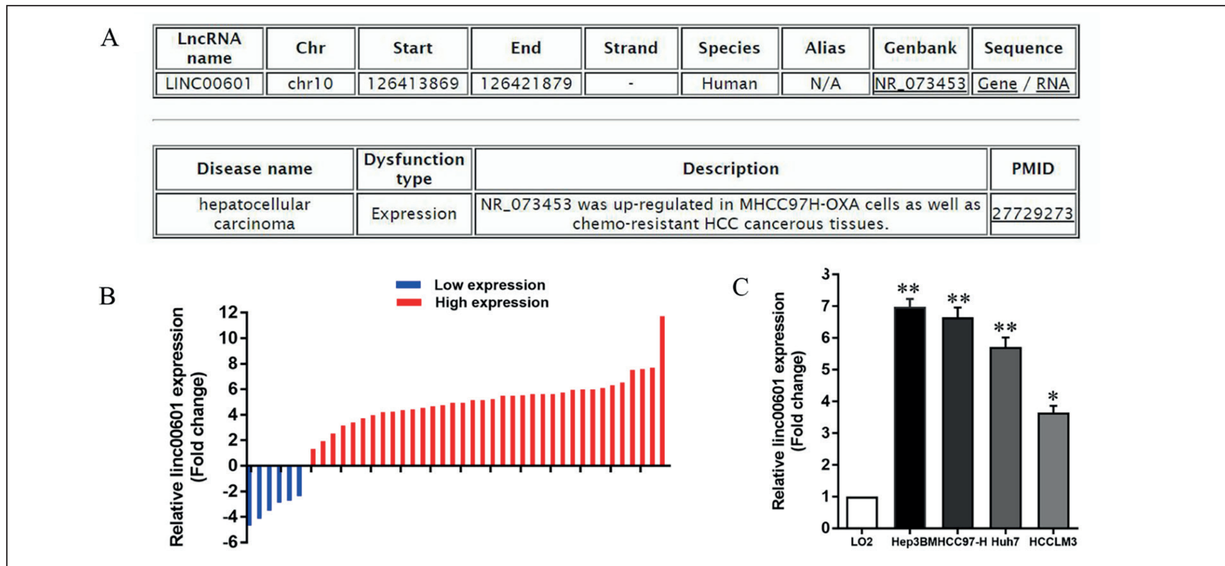
It was found *via* bioinformatics (http://www.cuilab.cn/lncrnadisease) that the expression of linc00601 was upregulated in HCC (Figure 1A). To verify the above finding, RNA was extracted from 42 cases of HCC tissue samples, and the relative expression of linc00601 in HCC tissues was detected *via* qRT-PCR. The results showed that linc00601 was upregulated in 36 cases of HCC tissues compared with that in para-carcinoma tissues (Figure 1B). Then, the relative expression of linc00601 in HCC cells was also detected, and the upregulated expression was also observed (Figure 1C).

Effects of Linc00601 on Proliferation and Cycle of HCC Cells

As indicated above, linc00601 was upregulated in HCC tissues and cells. To study its biological function in HCC, the linc00601-specific interference sequences were designed and synthesized, and transiently transfected into HCC cells. The interference efficiency was determined using qRT-PCR (Figure 2A). According to the results of CCK-8 assay, HCC cell proliferation was inhibited in si-linc00601 group compared with that in the si-NC group (Figure 2B and 2C). The results of flow cytometry manifested that the HCC cell cycle was arrested at the G1/G0 phase after interference with linc00601 expression (Figure 2D and 2E).

Effects of Linc00601 on HCC Cell Apoptosis and Downstream Signaling Pathway

The HCC cell apoptosis was determined through flow cytometry, and it was observed that the apoptosis rate rose after interference with linc00601 expression (Figure 3A and 3B). The



LncRNA name	Chr	Start	End	Strand	Species	Alias	Genbank	Sequence
LINC00601	chr10	126413869	126421879	-	Human	N/A	NR_073453	Gene / RNA

Disease name	Dysfunction type	Description	PMID
hepatocellular carcinoma	Expression	NR_073453 was up-regulated in MHCC97H-OXA cells as well as chemo-resistant HCC cancerous tissues.	27729273

Figure 1. Linc00601 is upregulated in HCC. **A,** It is found *via* bioinformatics that the expression of linc00601 is upregulated in HCC. **B,** The expression of linc00601 in 42 cases of HCC tissues and corresponding para-carcinoma tissues is detected *via* qRT-PCR. The results show that linc00601 is upregulated in 36 cases of HCC tissues. **C,** The upregulated expression of linc00601 is also observed in HCC cells according to qRT-PCR.

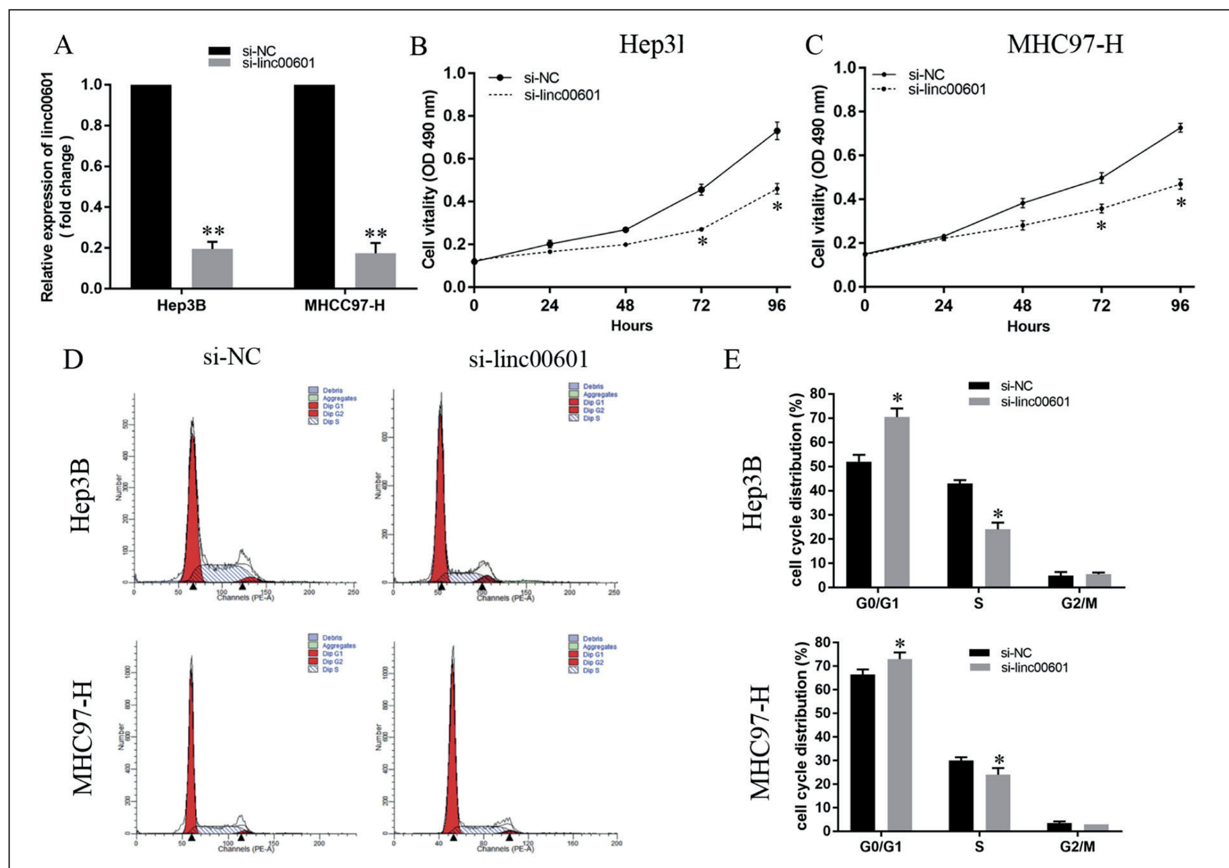


Figure 2. Effects of linc00601 on proliferation and cycle of HCC cells. **A,** Si-linc00601 and si-NC are transiently transfected into HCC cells, and the interference efficiency is detected after 48 h. **B,** and **C,** According to the results of CCK8 assay, HCC cell proliferation is inhibited after interference with linc00601 expression. **D,** and **E,** The results of flow cytometry manifest that the cell cycle is arrested at the G1/G0 phase in si-linc00601 group compared with that in si-NC group.

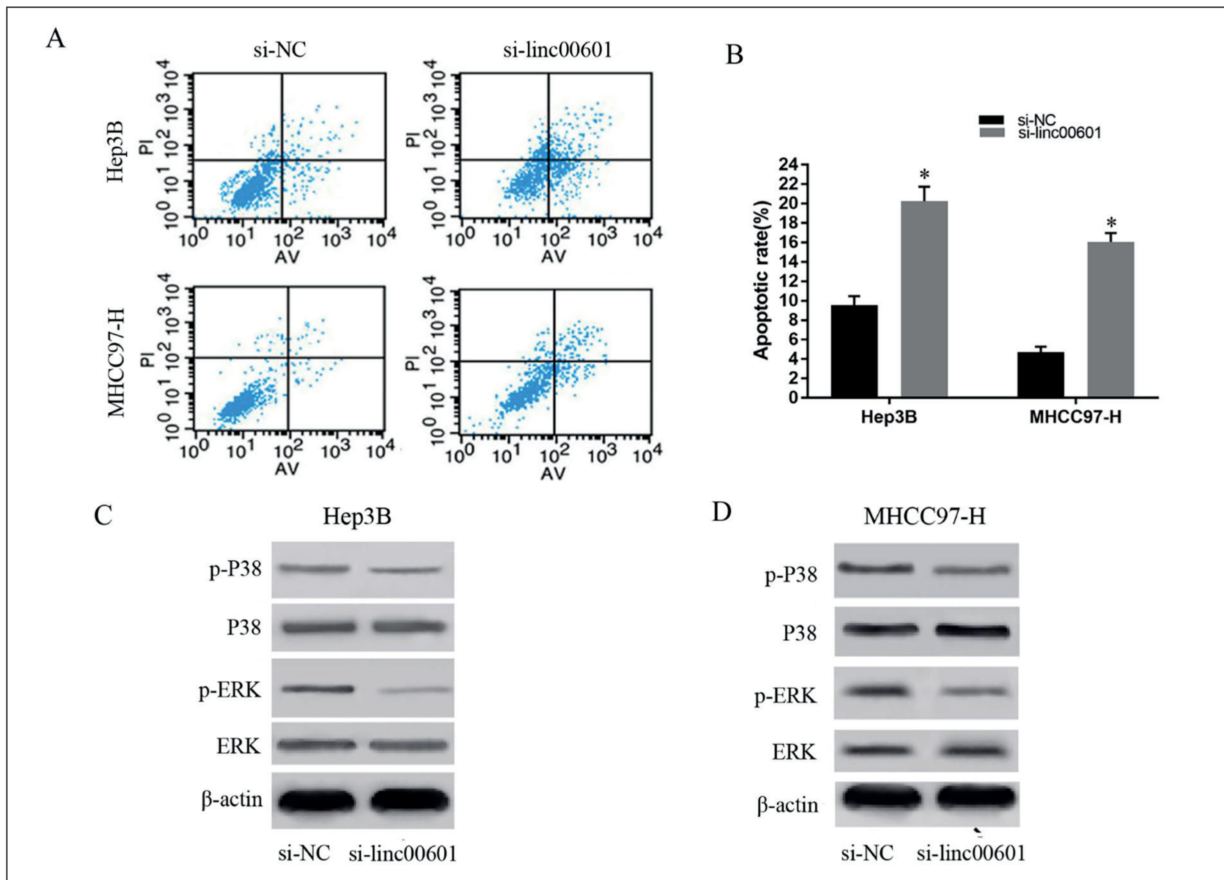


Figure 3. Effects of linc00601 on HCC cell apoptosis and downstream signaling pathway. **A,** and **B,** The results of flow cytometry confirm that the apoptosis rate of HCC cells rises after interference with linc00601 expression. **C,** and **D,** The results of Western blotting reveal that the expressions of molecular markers in downstream MAPK signaling pathway are changed after interference with linc00601 expression (si-linc00601).

above results demonstrate that the expression of linc00601 is upregulated in HCC and plays a similar role to "oncogene". Then, its possible molecular mechanism was further explored. The results of Western blotting revealed that after interference with linc00601 expression (si-linc00601), the expressions of molecular markers phosphorylated-extracellular signal-regulated kinase (p-ERK) and p-p38 in the mitogen-activated protein kinase (MAPK) signaling pathway were inhibited (Figure 3C and 3D).

Discussion

Primary liver cancer is one of the most common malignant tumors of the digestive system. According to the annual report of cancer in 2017, the morbidity and mortality rates of primary liver cancer in China rank 3rd and 2nd, respectively, in malignant tumors, and HCC is dominated in primary liver cancer[11,12]. Therefore, understanding the related mechanisms of HCC occurrence and development and searching for new molecular targets are of important significance for the prevention and treatment of HCC. The occurrence of HCC is a long-term process involving a large number of genes and epigenetic mutations[13,14].

LncRNAs are RNAs with more than 200 nucleotides in length, which cannot be translated into protein due to the lack of promoters. There are increasing studies[15-17] proving that lncRNAs regulate the proliferation, apoptosis, differentiation, invasion, and migration of tumor cells *via* participating in the regulation of chromatin modification, transcriptional activation and intranuclear interference. LncRNAs also play an important regulatory role in the occurrence and development of HCC. In this study, it was found for the first time using *in vitro* experiments that

linc00601 promoted HCC cell proliferation and cell cycle distribution at the G1/G0 phase, and inhibited apoptosis.

MAPK is a serine/threonine kinase in cells, whose main members include ERK, p38 MAPK, and JNK[18]. Abnormally activated MAPK signaling pathway is closely related to the occurrence, development, invasion, and metastasis of HCC[19,20]. In this study, in order to explore the role of linc00601 in the MAPK signaling pathway in HCC cells, the protein and phosphorylation levels of key molecules (ERK and p38) of the MAPK signaling pathway were determined in HCC cells with linc00601 downregulation. The results manifested that the protein expression levels of p-ERK and p-p38 were downregulated, while those of ERK and p38 had no evident changes. The above findings suggest that the downregulation of linc00601 (si-linc00601) can suppress the activation of MAPK signaling pathway.

Conclusions

Briefly, linc00601 may affect the HCC cell proliferation, cycle and apoptosis *via* activating the MAPK signaling pathway. Our findings provide a novel biomarker for the diagnosis and treatment of HCC patients and treatment strategies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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