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Long noncoding RNA CCHE1 indicates a poor prognosis of hepatocellular carcinoma and promotes carcinogenesis via activation of the ERK/MAPK pathway

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ABSTRACT

Survival of patients with hepatocellular carcinoma (HCC) remains poor, which is largely attributed to active carcinogenesis. Accumulating evidence implies that long noncoding RNAs (lncRNAs) could play a pivotal role in cancer biology. However, the clinical valueand biological significance of CCHE1 in HCC carcinogenesis remains to be discovered. Expression of CCHE1was analyzed in 112 HCC tissues and cell lines by qRT–PCR. The higher expression of CCHE1 was significantly correlated with tumor number, tumor size and TNM stage. Multivariate analyses revealed that CCHE1 expression served as an independent predictor for overall survival. Moreover, the effect of CCHE1 on proliferation was evaluated by MTT assays, and cell apoptosis was detected by flow-cytometric analysis. Further experiments demonstrated that CCHE1 knockdown significantly promoted growth arrest and cell apoptosis. Importantly, we further confirmed that ERK/MAPK pathway was found to be inactivated in the HCC cells after CCHE1 knockdown. To our knowledge, this is the first report showed that the role and the mechanism of CCHE1 in the progression of HCC. Together, these results suggest that lncRNA CCHE1 may serve as a candidate prognostic biomarker and target for new therapies in human HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent tumor types with the highest mortality rate and the steadily increasing incidence [1]. The most effective treatment for patients with early-stage HCC remains to be complete surgical resection, providing that the disease is medically operable and adequately staged [2]. However, the effectiveness of surgical resection is limited by high rates of distant recurrence caused by the presence of metastatic disease that is not apparent at the time of surgery [3]. Thus, there is an urgent need to identify valuable diagnostic and prognostic biomarkers to improve the clinical outcome and to develop the effective individual therapeutic strategies for patients with HCC.

Long noncoding RNAs (lncRNAs) are evolutionarily conserved noncoding RNAs that are greater than 200 nucleotides in length with no protein-coding capacity [4,5]. Recent studies have demonstrated that lncRNAs play critical roles in diverse biological processes, such as cell proliferation and apoptosis, as well as in cancer progression [5,6]. Specifically, the lncRNA LUNAR1 has been shown to play a role in diffuse large B-cell lymphoma [7], SPRY4-IT1 has been related to gastric cancer [8], and HULC is reported to contribute to pancreatic cancer [9]. However, the overall patho-physiological contributions of lncRNAs to HCC remain largely unknown.

CCHE1 (cervical carcinoma expressed PCNA regulatory lncRNA, Gene ID: 105682749) encodes a long noncoding RNA and maps to chromosome 10 [10]. Meng et al. revealed CCHE1 plays a pivotal role in cervical cancer cell proliferation and serves as a potential prognostic biomarker and therapeutic target in human cervical cancer [11]. However, the function role and molecular mechanism of CCHE1 in HCC remains unclear.

In the current study, we characterized the function of CCHE1 in HCC development and progression. CCHE1 was found to be overexpressed in HCC tissues and cell lines to a greater extent than in normal tissues and human hepatocyte cell line. High CCHE1 expression was associated with tumor number, tumor size and TNM stage suggesting that CCHE1 might be involved in the tumorigenesis and progression of HCC. In addition, Kaplan–Meier

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and Cox regression analysis showed that high CCHE1 expression was correlated with poor OS and it could be served as an independent prognostic factor for overall survival in HCC. Furthermore, CCHE1 was found to promote HCC carcinogenesis by regulating ERK/MAPK pathway. Together, these results indicate that CCHE1 plays a critical role in HCC and may serve as a candidate target for new therapies in human HCC.

2. Materials and methods

2.1. Tissue samples

This study was approved by the medical ethics committee of Jiangsu Cancer Hospital. Written informed consent was obtained from all patients prior to participation in the study. A total of 112 HCC samples were obtained from patients undergoing surgery in Jiangsu Cancer Hospital or First People Hospital of Yunnan Province between 2007 and 2011. All specimens were immediately frozen in liquid nitrogen and stored at 80 °C until RNA extraction. No patient received chemotherapy or radiotherapy prior to surgery. Follow-up periods ranged from 1 month to 5 years.

2.2. Cell culture

The human HCC cell lines MHCC97H, HepG2, Hep3B, Huh-7 and HCCLM3, and a normal hepatocyte cell line L02 were purchased from the American Type Culture Collection (Manassas, VA, USA). All of these cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Ther) supplemented with 15% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/mL penicillin, and 100 mg/ml streptomycin and incubated in a 37 °C, 5% CO_2 humidified atmosphere.

2.3. Small interfering RNA transfection

Small interfering RNAs (siRNAs) specifically targeting cche1 (si-CCHE1) and a scrambled negative control (si-NC) were provided by Shanghai GenePharma Co. (GenePharma, Shanghai, China). The sequences of si-CCHE1 were as follows: si-CCHE1-1: 5′-GCTTCTGACCAGCGACGCTAGGAGTAGCTG-3′; si-CCHE1-2: 5′-CGAGGGCGAGCATGTTTGTTGTTTA-3′. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells were harvested 48 h after transfection [7,11].

2.4. MTT assay

MTT assays were performed to evaluate the speed of cell proliferation as described previously [12]. Briefly, cells were trypsinized into a single cell suspension and seeded into 96-well plates at a density of 1×10^3 cells/well. After 24, 48, 72 or 96 h of cell cultivation, 20 mL of 3-(4,5)-dimethylthiahiazol (-zy1)-3,5-di-phenytetrazo-libromide (MTT) was added into each well and incubated for 4 h at 37 °C. The supernatant was then aspirated, and 150 mL dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) was added to dissolve the crystals with agitation for 10 min at room temperature. The absorbance values were measured using an ELISA reader (Bio-Rad Laboratories, Richmond, CA) at a wave length of 490 nm. Each experiment was repeated three times.

2.5. Flow-Cytometric analysis of apoptosis

The siRNA-CCHE1 or empty vector-transfected MHCC97H cells were harvested 48 h after transfection for apoptosis analysis [13]. Floating and adherent cells were collected using 0.1% trypsin, washed twice with cold PBS, and suspended in 1000 mL binding buffer (10 mmol/L HEPES buffer, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$). The cells were then treated with fluorescein isothio-cyanate (FITC)-Annexin V and propidium iodide (PI) in the dark at room temperature, according to the manufacturer's recommendations. The cells were then examined by flow cytometry (FACScan; BD Biosciences) on in instrument equipped with CellQuest software (BD Biosciences), and they were discriminated into viable cells, dead cells, early apoptotic cells, and late apoptotic cells. The percentage of early and late apoptotic cells was compared with control groups from each experiment. This assay was repeated three times.

2.6. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cell lines and frozen tumor specimens using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The mRNA expression in HCC cells, HCC samples, and normal liver tissues was measured by qRT-PCR using an ABI7300HT instrument (Applied Biosystems, Foster City, CA). qRT-PCR was done using a SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China) according to the manufacturer's instructions. GAPDH was used as an internal control. The primers used were as follow: Relative mRNA levels were calculated based on the Ct values and normalized using GAPDH expression, according to the equation: 2^{-DCt} [$\Delta Ct = Ct$ (CCHE1) − Ct (GAPDH)]. All experiments were done in triplicate [14].

2.7. Protein preparation and Western blot analysis

Harvested cells (2×10^6) were put into 1.5-mL Eppendorf tubes and homogenized with 400 mL lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mmol/L NaCl, 0.1% SDS, 1% Nonidet P–40, 0.5% sodium deoxycholate 0.02% sodium azide, 100 mg/ml PMSF, 1 mg/ml aprotinin). Cell lysates were centrifuged at 4 °C for 5 min at 10,000 rpm, and the protein–containing supernatant was placed in fresh tubes and quantified using the Bradford protein assay. For western blot analysis, total proteins (50 mg) were electrophoresed on 10% SDS polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% fat-free milk in TBS (20 mmol/L Tris, 0.15 mol/L NaCl (pH 7.0), 0.1% Tween 20), the membranes were incubated with a primary antibody: anti-ERK (ab32537), anti-p–ERK (ab176660), anti-p38 MAPK (ab197348), anti-p-p38 MAPK (ab176664), and anti-Tubulin (ab11304) (Abcam, Massachusetts, US) diluted 1:1000. After repeated washing, the membranes were incubated with horseradish–peroxidase–conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) diluted 1:2000. The bands were visualized using the enhanced chemiluminescence system (Amersham Pharmacia Biotech) and visualized with enhanced chemiluminescence (Millipore, Bedford, MA). Western blot for Tubulin was performed as an internal sample loading control [15].

2.8. Statistical analysis

The statistical analysis was performed with SPSS software version 19.0 (IBM, Armonk, NY, USA). The data are presented as the means ± standard deviation (S.D.). Continuous variables were compared by Student's t test or the ANOVA test. Survival analysis was performed using the Kaplan–Meier method, and the log-rank test was used to compare the differences between patient groups. P-values less than 0.05 were considered statistically significant [16].