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Linc-POU3F3 is overexpressed in hepatocellular carcinoma and regulates cell proliferation, migration and invasion

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ABSTRACT

Linc-POU3F3 showed an up-regulated tendency and functioned as tumor promoter in glioma, esophageal cancer and colorectal cancer. There was no report about the expression pattern and clinical value of linc-POU3F3 in hepatocellular carcinoma. Thus, the purpose of our study is to explore the clinical significance and biological role of linc-POU3F3 in hepatocellular carcinoma. Our results suggested that levels of linc-POU3F3 were dramatically increased in hepatocellular carcinoma tissues and cell lines compared with paired normal hepatic tissues and normal hepatic cell line, respectively. Levels of linc-POU3F3 were positively correlated with clinical stage, tumor size, vascular invasion and metastasis. Moreover, high-expression of linc-POU3F3 was an independent prognostic factor for hepatocellular carcinoma patients. The gain- and loss-of-function experiments showed that linc-POU3F3 expression significantly promoted tumor cell proliferation, migration and invasion. In addition, linc-POU3F3 expression was negatively correlated with POU3F3 mRNA and protein expressions in hepatocellular carcinoma tissues, and negatively regulated POU3F3 mRNA and protein expressions in hepatocellular carcinoma cells. In conclusion, our study supports the first evidence that linc-POU3F3 plays an oncogenic role in hepatocellular carcinoma, and represents a potential therapeutic strategy for hepatocellular carcinoma patients.

1. Introduction

Liver cancer is the sixth most common malignancy and the second cause of cancer death following lung cancer worldwide [1]. In China, liver cancer is the fifth most common cancer and the second leading cause of cancer-related death [2]. According to the 2012 Global Cancer Statistics, about 782,500 newly diagnosed liver cancer cases and 745,500 liver cancer deaths were appeared worldwide, with China alone accounting for about 50% of the total number of cases and deaths [1]. Hepatocellular carcinoma (HCC) accounts for 70–90% of all liver cancer cases [3]. Although a significantly decreasing incidence and mortality trend for hepatocellular carcinoma was observed in China, a huge population base and rapid population growth still led to a large number of new hepatocellular carcinoma case [4]. Recently, the diagnosis and therapeutic strategy for hepatocellular carcinoma has a rapid development such as novel chemotherapeutic interventions, molecular targeted therapy (sorafenib) and liver transplantation, but the overall survival of hepatocellular carcinoma remains disappointing due to high rate of recurrence and metastasis [5–7]. Therefore, it is necessary to explore the molecular mechanisms of hepatocellular carcinoma progression and identify credible biomarkers for cancer treatment.

Long intervening noncoding RNAs (lincRNAs), which are a subtype of long noncoding RNAs (lncRNA), are transcript units located within genomic intervals between two protein coding genes [8,9]. Nowadays, more and more evidence shows that lincRNAs are not transcriptional "noise", and has been identified to be involved in multiple biological processes such as development, differentiation and carcinogenesis [10–12].

Linc-POU3F3 is located in the human chromosome 2q12.1 (Chr2q12.1) with 747bp transcript on the reverse strand, which is only about 4-kb upstream from the POU class 3 homeobox 3 (POU3F3) gene, a member of the class III POU family of transcription factors [13]. Linc-POU3F3 has been found overexpressed in glioma [14,15], esophageal cancer [16–18] and colorectal cancer [19]. Early, we screened ten kinds of lncRNA expression in hepatocellular carcinoma tissues and cell lines, and found linc-POU3F3 was conservatively overexpression in

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hepatocellular carcinoma tissues and cell lines. Thus, the aim of our study is to explore the clinical significance of linc-POU3F3 in hepatocellular carcinoma patients, and identify the biological role of linc-POU3F3 in regulating hepatocellular carcinoma cell proliferation, migration and invasion.

2. Materials and methods

2.1. Clinical sample collection

Fresh clinical samples of twenty paired normal hepatic tissues and one hundred and four hepatocellular carcinoma tissues were obtained from Jining No.1 People's Hospital, Jining Traditional Chinese Medicine Hospital and Third Affiliated Hospital of Shandong Academy of Medical Sciences. All specimens had confirmed diagnosis and were staged according to the AJCC Cancer Staging Manual (7th Edition). No hepatocellular carcinoma patient underwent anti-tumor therapy before diagnose. Prior to use of these clinical materials for research purposes, consent was obtained from the patients and approved by the Ethics Committee of Jining No.1 People's Hospital, Jining Traditional Chinese Medicine Hospital and Third Affiliated Hospital of Shandong Academy of Medical Sciences.

2.2. Cell culture

The human hepatocellular carcinoma cell lines (Li-7, HepG2, SNU-449 and Bel-7402) and the human normal hepatic cell line (LO-2) were purchased from the Chinese Academy of Sciences, and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% fetal calf serum (FCS, Gibco, USA). All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO2.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR) assay

RNA was extracted from tissues and cells using Trizol (Takara, Japan) per the manufacturer's protocol. The cDNA was synthesized from 1 μg of total RNA with PrimeScript® RT reagent Kit (Takara, Japan). Real-time PCR was performed with the SYBR® Premix Ex TaqTM II (Takara, Japan) detection method on an ABI-7500 RT-PCR system. Specific sense primers for linc-POU3F3 and GAPDH are shown as follows: linc-POU3F3 forward, 5'-AATCACTGCAATTGAAGGAAAAA-3', reverse, 5'-CCTTGTTTTCCAACCCTTAGACT-3'; GAPDH forward, 5'-GGGAGCCAAAAGGGTCAT-3', reverse, 5'-GAGTCCTTCCACGATAC CAA-3'. GAPDH gene was used as gene internal control.

2.4. Plasmid construction and cell transfection

The target sequence of linc-POU3F3 gene was 5'-GATCCGTGCTGG AGAGTTGAGAATTTCAAGAGAATTCTCAACTCTCCAGCACTTTTTTAC GCGTA-3', and the resultant vector was designated as pSilencer-linc-POU3F3. The negative control siRNA plasmid (pSilencer-NC) encodes a siRNA, which has no significant sequence similarity to human gene sequences. The coding sequence region of human linc-POU3F3 was amplified from cDNA and cloned into pcDNA3.1 express vector (pcDNA-linc-POU3F3). Transfections were carried out using lipofectamine 3000 (Invitrogen, USA) based on the manufacturer's protocol. Following the transfection, the cells were incubated for 48 h to 72 h and then were used to investigate the transfection efficiency, and were harvested for the subsequent experiments.

2.5. Cell proliferation analysis

MTT assay was used to evaluate cell proliferation *in vitro*. A density of 1000 cells/well was seeded in 96-well plates. Transfected cells were incubated for 4 days. 20 μl of MTT (5 mg/ml, Sigma, USA) was added to each well and incubated for 4 h. At the end of incubation, supernatants were removed, and 150 μl of DMSO (dimethyl sulfoxide, Sigma, USA) was added to each well. The absorbance value (OD) of each well was measured at 490 nm. Experiments were performed thrice.

2.6. Cell migration and invasion assays

For cell migration assays, 1×10^5 transfected cells suspended 100 μl RPMI 1640 medium without FCS were plated into a fibronectincoated polycarbonate membrane insert in a transwell apparatus (Costar, USA), and media supplemented with 10% FCS was placed into the lower chamber. After the cells were incubated for 12 h at 37 °C in a 5% CO2 atmosphere, lower surfaces of the insert were washed with PBS, were fixed with methanol, stained with Giemsa solution and counted under a microscope in five predetermined fields. For the cell invasion assay, the procedure was similar to the cell migration assay, except that the membranes of transwell apparatus were pre-coated with 24 μg/μl Matrigel. All assays were independently repeated three times.

2.7. Immunohistochemical staining

Forty paraffin-embedded hepatocellular carcinoma sections were used for immunohistochemistry to detect protein expression levels of POU3F3 through POU3F3 antibody (1:200, Abcam, USA). The indirect streptavidin-peroxidase method was utilized based on the manufacturer's instructions. Stained tissue sections were reviewed and scored separately by two pathologists. The staining intensity was scored as previously described [20]. The final score was calculated by multiplication of intensity and extent score to assess and grade expression of POU3F3 into two groups: low expression (≤ 6 scores) and high expression (> 6 scores).

2.8. Western blot

The culture medium of cells was discarded 48 h after transfection. Transfected cells were lysed, and total proteins were extracted using RIPA protein extraction reagent (Beyotime, China) and were quantified using the BCA protein assay kit (Beyotime, China). Equal amounts (50 μg) of protein extracts were separated by 10%SDS-PAGE and transferred on PVDF membrane. The PVDF membranes was incubated for 12 h in blocking solution, and then probed with the following primary antibodies: POU3F3 and β-actin antibodies (1:1000, Abcam, USA) at 4 °C overnight. An HRP-conjugated IgG antibody was used as the secondary antibody (Abcam, USA). Signals were detected using enhanced chemiluminescence reagents (Pierce, USA).

2.9. Statistical analysis

Statistical analyses were performed using SPSS 17.0 software. The Wilcoxon Signed Rank test was performed to compare the expression of linc-POU3F3 between hepatocellular carcinoma tissues and paired normal hepatic tissues. Associations between linc-POU3F3 and clinicopathological characteristics of hepatocellular carcinoma patients were analyzed using Chi-square test. Two-tailed Student's *t*-test was used for comparisons of two independent groups. The correlation between linc-POU3F3 expression and POU3F3 mRNA expression was analyzed by using Spearman's correlation coefficient analysis. Survival analysis was performed by Kaplan-Meier method and log-rank test. Univariate and multivariate Cox proportional hazards method was used for analyzing the relationship between variables and patient survival time. A P value of less than 0.05 was considered statistically significant.