LINC00963 promotes hepatocellular carcinoma progression by activating PI3K/AKT pathway


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Abstract. — OBJECTIVE: To explore the role of LINC00963 in the pathogenesis of hepatocellular carcinoma and its underlying mechanisms.

PATIENTS AND METHODS: The expression level of LINC00963 in 48 cases of hepatocellular carcinoma (HCC) tissues and paracancerous tissues were detected by quantitative Real-time (qRT-PCR). Survival analysis was carried out based on the expression level of LINC00963. The association between the expression level of LINC00963 and clinical characteristics of these subjects was analyzed by x2-test. The proliferation and cell cycle of HCC cells after transfection of LINC00963 overexpression plasmids were evaluated by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively.

RESULTS: The expression level of LINC00963 in HCC tissues was remarkably higher than that in paracancerous tissues, indicating a potential diagnostic significance of LINC00963. The progression-free survival of HCC patients suggested that the expression level of LINC00963 was remarkably associated with the tumor size and TNM stage, but not with age, gender, histological type and lymph node metastasis. Overexpression of LINC00963 significantly enhanced the proliferation ability of HepG2 and HCC cells and prolonged their G0/G1 phase. Furthermore, the PI3K/AKT expression was increased after overexpression of LINC00963, while AKT siRNA effectively reversed the prolonged G0/G1 phase caused by LINC00963 overexpression.

CONCLUSIONS: Our data revealed that LINC00963 was upregulated in HCC, which significantly extended the G0/G1 phase of HCC cells by activating PI3K/AKT pathway and promoting the proliferative ability of HCC cells. LINC00963 may be involved in the HCC development.

Key Words: Liver cancer, LINC00963, PI3K/AKT, Cell proliferation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors at present. Different treatment methods of HCC have been applied, such as surgical resection, liver transplantation, radiotherapy and chemotherapy, radiofrequency ablation, and molecular targeted therapy. However, the 5-year survival of HCC is still lower than 12%. There are about 748,300 new cases and 695,900 death cases of HCC reported worldwide each year, and half of them are reported in China. Researches on prevention and treatment of HCC have been widely carried out in the world due to its high incidence and mortality. Therefore, in-depth investigation of the epidemiology and etiology of HCC is of great significance for formulating a more effective therapy. Long non-coding RNAs (lncRNAs) are a class of highly abundant RNAs in human tissues with little or no function of encoding proteins. Recent researches have revealed that the abnormally expressed lncRNAs are involved in complex physiological and pathological processes. LncRNAs, including HOTTIP, MVIH, MALAT1, UC.338, ZEB1-AS1 and IncRNA-ATB, were demonstrated significantly upregulated in HCC tissues. Furthermore, these lncRNAs were confirmed to participate in the malignant transformation, invasion and metastasis of HCC by regulating apoptosis, angiogenesis, drug resistance and epithelial-mesenchymal transition of tumor cells. LINC00963 is located on chromosome 9 with 25027 bp in length, which is identified to be differentially expressed in a variety of tumors and normal tissues. It was reported that LINC00963 is significantly down-regulated in renal cell carcinoma and exhibits an effective inhibitory effect on the proliferation of renal carcinoma cells. On the contrary, upregulated LINC00963 is observed in non-small cell lung cancer, which is involved in tumorigenesis through activation of PI3K/mTOR signaling pathway. Highly expressed LINC00963 is associated with a poor prognosis of non-small cell lung cancer. In addition, LINC00963 is also highly expressed in prostate cancer, which pro-
promotes the proliferation of tumor cells\textsuperscript{10}. However, little is known about the function of LINC00963 in the HCC progression. The primary of this study was to investigate the role of LINC00963 in the pathogenesis of hepatocellular carcinoma and its underlying mechanisms.

**Patients and Methods**

**Basic Characteristics of Patients**

A total of 48 tissues from HCC patients who underwent hepatic carcinectomy in our hospital were collected. All enrolled HCC patients did not receive preoperative radiotherapy, chemotherapy, radiofrequency ablation or other adjuvant therapy. The collected tissues were pathologically confirmed as HCC after operation. Adjacent normal tissues without any tumor cells confirmed by the postoperative pathology were taken as the controls (at least 3 cm away from the surgical margin). The tumor differentiation level was evaluated according to the Edmondson-Steiner grading criteria, and the tumor stage was assessed following the criteria of the seventh edition of the Staging System released by the American Joint Committee on Cancer (AJCC). Samples were immediately frozen in liquid nitrogen, and then stored in a -80°C refrigerator for the following experiments. This investigation was approved by the Ethics Committee of Peking University Cancer Hospital. Signed written informed consents were obtained from all participants before the study.

**Cell Culture and Transfection**

Normal liver cell line (L-02) and hepatocellular carcinoma cell lines (HepG2, HB611 and HHCC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s modified eagle Medium (DMEM, Gibco, Rockville, MD, USA) high glucose complete medium supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc. Waltham, MA, USA), and maintained in a 5% CO\textsubscript{2} incubator at 37°C. Cells in the logarithmic growth phase were selected and transfected with the LINC00963 overexpression plasmids (Jima, Shanghai, China) according to the instruction.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)**

The total RNA was extracted by TRizol method (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and the RNA concentration was determined by a microtube plate reader. Reverse transcription was performed according to the instructions of SYBR Green method (TaKaRa, Dalian, China). Polymerase chain reaction (PCR) amplification conditions were as follows: 5 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 55°C and 90 s at 72°C. The primer sequences were as follows: LINC00963 (Forward: 5’-GGTAAATCGAGGCCCAGAGAT-3’; Reverse: 5’-ACGTGGATGACAGCGTGTGA-3’).

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<th>LINC00963 expression</th>
<th>( p )-value</th>
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**Table I.** Correlation between the expression level of LINC00963 in and basic characteristics of HCC patients (n = 48).
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Cell Counting Kit-8 (CCK-8) Assay
Cells were digested and collected after 24 h post-transfection. Cells were then seeded into 96-well plates with six replicate wells in each group. After the static adherence, 10 µL of CCK-8 (Dojindo, Kumamoto, Japan) solution were added and cells were incubated at 37°C for 2 h. The optical value of each well at 450 nm was measured by a microplate reader.

Cell Cycle Detection
Cell suspension was prepared after treated cells were digested and washed with phosphate buffered saline (PBS) twice. The supernatant was discarded after centrifugation. Precooled 75% ethanol was added for fixing cells. Before cell cycle detection, the cells were treated with 0.5 µL of propidium iodide (PI) for 20-30 min at 4°C in the dark. The DNA content of cells in each cycle was determined at the wavelength of 488 nm by flow cytometry. Each experiment was repeated for 3 times.

Western Blot
Protein was extracted from cells by radioimmunoprecipitation assay (RIPA, Roche, Basel, Switzerland) method and then quantified using bicinchoninic acid (BCA) based on the instructions (Pierce, Rockford, IL, USA). Proteins were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody overnight. Membranes were incubated with the secondary antibody at room temperature for 1 h. Immuno-reactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis
Statistical product and service solutions (SPSS19.0, Armonk, NY, USA) statistical software was utilized for analyzing data. All data were expressed as mean ± standard deviation. Comparison of measurement data was conducted using t-test. Survival analysis was performed using Graphpad. The correlation between the basic characteristics of patients and the expression level of LINC00963 was analyzed by χ²-test. p<0.05 was considered statistically significant.

Results
LINC00963 Was Upregulated in HCC Tissues
To elucidate the expression level of LINC00963 in HCC, 48 cases of hepatocellular carcinoma tissues and paracancerous tissues were collected. Higher expression level of LINC00963 in HCC tissues was observed compared with that in paracancerous tissues (p<0.001, Figure 1A). The correlation between clinical characteristics of HCC patients and the expression level of LINC00963 was showed in Table I. No significant differences were exerted in age, gender and histological type between the control group and HCC group. Next, patients were assigned into the high expression group and low expression group based on the expression level of LINC00963 in HCC. Shorter progression-free survival was observed in high expression group in comparison with that of the low expression group (p=0.023, Figure 1B). Additionally, the progression-free survival was markedly associated with the expression level of LINC00963, tumor size and TNM stage, but not with the age, gender, histological type and lymph node metastasis (Table II). Besides, higher expression level of LINC00963 in stage III and
IV of HCC was found compared with that in stage I and II (p<0.001, Figure 1C). The area under ROC curve (AUC) was 0.763, indicating a potential diagnostic significance of LINC00963 in HCC (Figure 1D). These results suggested that LINC00963 might be involved in the HCC development.

**LINC00963 Promoted Proliferative Ability of HCC Cells**

To explore the effect of LINC00963 on HCC cells, we first detected the expression of LINC00963 in HCC cells (HepG2, HB611 and HHCC) and normal liver cells (L-02) by qRT-PCR. The expression level of LINC00963 in HCC cells was remarkably higher than that in normal liver cells (Figure 2A). Given the greater abundance of LINC00963, HepG2 and HHCC cells were selected for the following experiments. As shown in Figure 2B, the expression level of LINC00963 in HCC cells was significantly elevated after transfection of LINC00963 overexpression plasmids. Proliferative ability of transfected cells was detected by CCK-8 assay at 6, 24, 48, 72, 96 h, respectively. The results indicated that overexpression of LINC00963 plasmids significantly enhanced the viability of tumor
cells (Figure 2C). Besides, cell cycle results revealed that the G0/G1 phase of HepG2 and HHCC cells was remarkably prolonged after overexpressing LINC00963 (Figure 2D). All above results indicated that LINC00963 promotes the proliferative ability of HCC cells.

**LINC00963 Enhanced the Proliferation of HCC Cells Through the PI3K/AKT Signaling Pathway**

We next explored how LINC00963 regulates the cell cycle thus participating in the regulation of tumor growth. Since AKT pathway was demonstrated to participate in regulating cell cycle, expression levels of AKT and PI3K in HepG2 and HHCC cells were detected after LINC00963 overexpression. Our data suggested that overexpression of LINC00963 significantly up-regulated the expression levels of AKT and PI3K (Figure 3A). To further clarify the effect of LINC00963/AKT/PI3K on cell cycle, cells were co-transfected with the LINC00963 overexpression plasmids and AKT siRNA. It was demonstrated that AKT siRNA partially reverses the prolonged G0/G1 phase

![Figure 2.](image)

**Figure 2.** LINC00963 promoted the proliferation of HCC cells. **A,** The expression of LINC00963 in normal liver cells (L-02) and HCC cells (HepG2, HB611 and HHCC). **B,** Transfection efficiency of LINC00963 overexpression plasmids in HepG2 and HHCC cells. **C,** Viability changes in HepG2 and HHCC cells were detected by CCK-8 assay. **D,** Cell cycle changes in HepG2 and HHCC after overexpression of LINC00963 were detected by flow cytometry.
caused by the overexpressed LINC00963, suggesting that LINC00963 promotes the proliferation of HCC cells through PI3K/AKT signaling pathway (Figure 3B).

**Discussion**

HCC is one of the most common malignant tumors. There is a high incidence of HCC, especially in East Asia, South Asia, Africa and South Europe. More than 700,000 new cases and 600,000 death cases are reported worldwide each year\(^1\)\(^1\). Although a large number of studies have confirmed that the occurrence of HCC is closely related to hepatitis virus infection, aflatoxin intake, smoking, alcoholic cirrhosis, fatty liver and diabetes. The explorations of the molecular mechanisms underlying the invasion and metastasis of HCC cells are still limited\(^13\)-\(^16\). Molecular studies\(^17\)-\(^18\) have also found that genetic mutations, DNA methylation, histone acetylation or methylation and non-coding RNAs exert important roles in the development of HCC.

In 2016, high-throughput sequencing and bioinformatics analysis revealed that there are a large number of non-coding RNAs (ncRNAs) in diverse organisms and diseases\(^19\). According to the molecular weight, these ncRNAs can be divided into long non-coding RNAs (longer than 200 nt) and small regulatory RNAs (shorter than 200 nt) including microRNAs, short silence RNAs, small nuclear RNAs, etc\(^20\). Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs without open reading frames (ORFs), which were initially considered as “junk genes”\(^21\). However, growing evidence indicated that IncRNAs might be capable of regulating gene expression. Abnormally expressed IncRNAs were reported in many diseases, such as HOTAIR in breast cancer, BACE1-AS in Alzheimer’s disease, MEG3 in liver cancer, etc\(^22\)-\(^24\). Therefore, investigating the underlying mechanisms in the HCC pathogenesis is of great significance. LINC00963 was found differentially expressed in a variety of tumors and normal tissues. Our study suggested that up-regulated LINC00963 is significantly related to the poor prognosis of HCC.
The PI3K/AKT pathway is a well-established pathway widely involved in the cell activities, including growth, proliferation, migration and survival. In addition, AKT, as the evolutionarily conserved serine/threonine kinase, is one of the major protein kinases highly activated in human cancers. Studies have already confirmed that AKT signaling pathway participates in the regulation of cell cycle. Inhibition of PI3K/AKT pathway, however, might induce cell apoptosis in human pancreatic cancer cells. In this work, we showed that LINC00963 promoted hepatocellular carcinoma progression in vitro and in vivo. As expected, LINC00963 could both increase cell proliferation and decrease cell apoptosis in HepG2 cells. For example, decreased phosphorylation level of AKT by knockdown of the target gene Nox4 in human pancreatic cancer cells would lead to the increased cell apoptosis, indicating that down-regulation of phosphorylated AKT might induce cell apoptosis. Moreover, LY294002, the specific PI3K inhibitor, could notably arrest the cell cycle in G0/G1 phase, thus inhibiting the proliferation of malignant tumors. In this regard, LINC00963 might increase the proliferation of HCC cells by activating PI3K/AKT signaling pathway. However, there are still some limitations. For instance, phosphorylated levels of PI3K and AKT are needed to explore in the future experiments.

Conclusions

We showed that LINC00963 is upregulated in HCC tissues and negatively correlated with the prognosis of HCC. LINC00963 could promote the proliferative ability of HCC cells through activating PI3K/AKT signaling pathway and extending the G0/G1 phase in HCC cells.

Acknowledgements

This study was supported by International Science & Technology Cooperation Program of China (approval #: 2013DFG32720).

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


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