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LncRNA RUNX1-IT1 inhibits proliferation and promotes apoptosis of hepatocellular carcinoma by regulating MAPK pathways

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Abstract. – **OBJECTIVE:** The aim of this study was to investigate the exact role of long non-coding RNA (lncRNA) RUNX1-IT1 in regulating the proliferation and apoptosis of hepatocellular carcinoma (HCC) cells, and to explore the possible underlying mechanism.

PATIENTS AND METHODS: LncRNA RUNX1-IT1 expressions in paired HCC tissues (cancer and paired non-cancer tissues) (n=80) and HCC cell lines were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The effects of lncRNA RUNX1-IT1 on the proliferation and apoptosis of HCC cells were estimated by cell counting kit-8 (CCK-8) and tunnel assay, respectively. Furthermore, the association between lncRNA RUNX1-IT1 expression and the overall survival of patients was analyzed by the survival curve.

RESULTS: The expression level of lncRNA RUNX1-IT1 significantly decreased in HCC tissues. The overexpression of lncRNA RUNX1-IT1 remarkably inhibited the proliferation and induced the apoptosis of HCC cells. On the contrary, the knockdown of lncRNA RUNX1-IT1 remarkably enhanced the ability of proliferation and repressed the apoptosis of the HCC cells. In addition, lower expression of lncRNA RUNX1-IT1 indicated the worse prognosis of HCC patients.

CONCLUSIONS: We found that lncRNA RUNX1-IT1 played an important role in the development of HCC by participating in cell proliferation and apoptosis. Thus, lncRNA RUNX1-IT1 might be a powerful candidate as a prognostic biomarker and therapeutic target for HCC.

Key Words:
LncRNA RUNX1-IT1, Liver cancer, Hepatocellular carcinoma (HCC), MAPK pathway

Introduction

Primary liver cancer is one of the most common malignant tumors worldwide. The mortality of male patients with liver cancer ranks second among all tumor-related deaths[1]. However, the molecular mechanism of liver cancer development still remains unclear. Scholars[2-4] have shown that the occurrence of liver cancer is associated with abnormal apoptotic molecules and corresponding signaling pathways. Therefore, the mechanism of the hepatocellular carcinoma (HCC) cell apoptosis needs to be studied at the molecular level. Meanwhile, specific molecules can be used as novel targets in clinical treatment.

The development of liver cancer is a multi-factor and a multi-stage complex process. Increasing evidence has shown that long non-coding RNAs (lncRNAs) are involved in the development of various cancers. LncRNAs are a class of transcripts longer than 200 nt, with no encoding protein or low encoding protein capacity. Meanwhile, they participate in various regulatory processes in tumor cells[5,6]. LncRNAs also play an important role in the regulation of multiple biological processes, including tumorigenesis, proliferation, apoptosis, and metastasis[7-9]. Therefore, lncRNA exerts important function and value in malignancies. For example, the high expression of lncRNA MALAT1 promotes the growth of lung cancer, which is expected to be one of the predictive targets[10]. LncRNA HOTAIR plays a key role in the development of various tumors, including breast

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cancer, thyroid cancer, and bladder cancer[11-13]. LncRNA HULC is the first lncRNA identified in HCC, whose high expression indicates poor prognosis. Subsequent studies have indicated that lncRNA H19 affects the growth of liver tumor by affecting the secretion of CD90+cells[14]. Meanwhile, the high expression of lncRNA MALAT1 significantly predicts an increased recurrence rate of liver cancer[15]. Currently, increasing lncRNAs have been shown to play important roles in the development of liver cancer. However, the underlying regulatory mechanisms have not been fully elucidated yet. In the present study, the expression level of lncRNA RUNX1-IT1 in both HCC tissues and cell lines was detected. Its clinical significance was analyzed with clinical information. Meanwhile, the effects of the over-expression and inhibition of lncRNA RUNX1-IT1 on the proliferation and apoptosis of hepatoma cells were investigated.

Patients and Methods

Tissue Samples

Eighty patients with liver cancer admitted to our hospital for hepatobiliary surgery were recruited in this study. Both cancer tissues and para-cancerous tissues were collected. All patients were examined by the Computed Tomography (CT) scan, and the liver occupying lesions was confirmed. The pathological group was examined and diagnosed as HCC. This study was approved by the Ethics Committee of Wuwei Liangzhou Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Three human hepatoma cell lines (Huh7, HCCLM3, and Hep3B) and one normal liver cell line (LO2) were purchased from the Shanghai Library of Chinese Academy of Sciences (Shanghai, China). All cells were stored in liquid nitrogen according to 50% culture medium, 40% serum, and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured in Hyclone Dulbecco's Modified Eagle's Medium (DMEM; Rockville, MD, USA) containing 10% Gibco fetal bovine serum (FBS; South Logan, UT, USA) and 100 µg/mL streptomycin at 37°C in a 5% CO_2 incubator.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The constructed overexpression plasmid and empty plasmid were transfected into Huh7 and HCCLM3 cells, respectively. After 48 h of transfection, total RNA in cells was extracted with TRIzol[16] (Invitrogen, Carlsbad, CA, USA). The RNA concentration and purity were determined by Nano-Drop 2000C UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA), according to the instructions of the Toyobo kit (QPK-201, Osaka, Japan). QRT-PCR was performed in strict accordance with the TaKaRa qRT-PCR kit (Otsu, Shiga, Japan). Three replicates were set for each sample. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. The relative expression of genes was detected by the 7500 Fast System SDS software (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used in this study were as follows: RUNX1-IT1, F: 5'-GCCAGGAACCGGCCTTACTC-3', R: 5'-GCTAGTGTGCCGAGGAAGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Transfection

The full-length sequence of lncRNA RUNX1-IT1 was constructed on the pcDNA3.1 (+) vector with empty vector pcDNA3.1 (+) as the negative control. Meanwhile, lncRNA RUNX1-IT1 was down-regulated by transfecting shRNA-lncRNA RUNX1-IT1. The number of cells was first adjusted to 1×10^5/mL. Then, the cells were inoculated in 24-well plates with 0.5 mL per well. When the density of cells reached 70%, the cell transfection was performed. An appropriate amount of DNA was added, and the medium was changed for culture after 6 h. QRT-PCR was used to assess the gene expression levels.

Western Blot

The total protein of the cells was extracted and centrifuged at 12,000 r/min for 5 min at 4°C. The supernatant was dispensed into a 1.5 mL centrifuge tube and stored at -20°C for use. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared, and the loading volume was calculated according to protein concentration. After mixing with loading buffer at 1:1, the proteins were boiled in boiling water bath for 30 min. Subsequently, the protein samples were separated by SDS-PAGE and transferred onto membranes. After incubation

with primary antibodies (P-P38, P-ERK, P-JNK, P38, ERK, and JNK) overnight, the membranes were incubated with the corresponding secondary antibody (biotinylated goat anti-rabbit IgG 1:10,000; Abcam, Cambridge, MA, USA). Major proteins in mitogen-activated protein kinase (MAPK) signal pathway, including ERK, P38, JNK, and the phosphorylated proteins, were detected.

Cell Proliferation

The viability of cells at 24, 48, and 72 h after transfection was detected by cell counting kit-8 (CCK-8) method. A specific procedure was conducted according to the instructions of the CCK-8 cell proliferation assay kit (Dojindo Molecular Technoligies, Kumamoto, Japan). Optical density (OD) at 450 nm was detected by a microplate reader.

Cell Apoptosis

The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Then, the cells were incubated with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction (Roche, Basel, Switzerland). After that, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI), and visualized (Sigma-Aldrich, St. Louis, MO, USA). Finally, the number of TUNEL-positive nuclei was expressed as the percentage of total nuclei.

Statistical Analysis

The graphical analysis was statistically analyzed using GraphPad Prism 5 (La Jolla, CA, USA). The relationship between the clinic-pathological features and lncRNA RUNX1-IT1 expression was analyzed using the Chi-square Fisher's test with Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). The survival analysis was performed using the Kaplan-Meier method. $p<0.05$ was considered statistically significant.

Results

LncRNA RUNX1-IT1 Expression Was Significantly Reduced in HCC Tissues and Cell Lines

We first used qRT-PCR to detect the expression level of lncRNA RUNX1-IT1 in HCC tissues and cell lines. The results showed that the expression level of lncRNA RUNX1-IT1 in HCC cell lines (Huh7, Hep3B, and HCCLM3) was significantly lower than that of normal liver cell line L02 (Figure 1A). Huh7 and HCCLM3 cells were selected for subsequent functional experiments. Meanwhile, the expression level of lncRNA RUNX1-IT1 in HCC tissues and adjacent normal tissues was detected as well. We found that the expression level of lncRNA RUNX1-IT1 in HCC tissues was significantly lower than that of adjacent normal tissues (Figure 1B). According to the median expression of lncRNA RUNX1-IT1, HCC patients were divided into two groups. The survival curve indicated that the survival time of patients in lncRNA RUNX1-IT1 high expression group was remarkably higher than those in the low expression group ($p=0.01$, Figure 1C).

The above results suggested that lncRNA RUNX1-IT1 might function as a tumor suppressor in the development of liver cancer. This prompted us to explore the potential role of lncRNA RUNX1-IT1 *in vitro*.

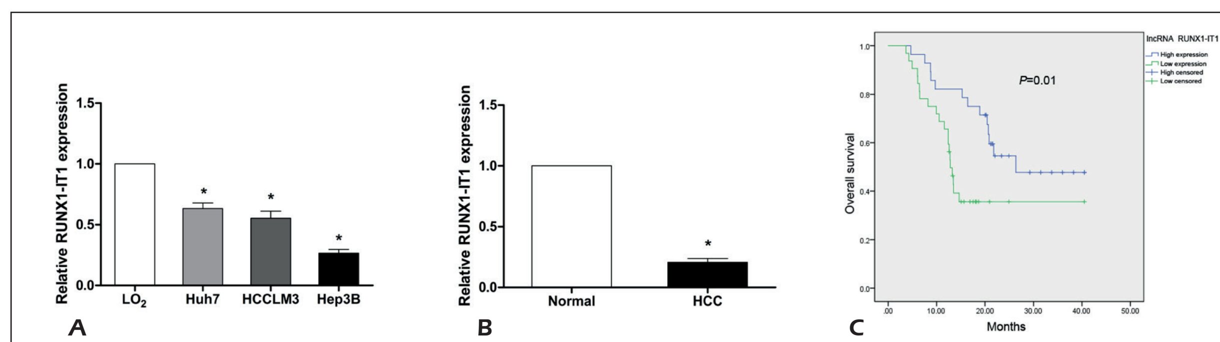

Figure 1. LncRNA RUNX1-IT1 expression was significantly reduced in HCC cell lines and tissues. **A**, We used qRT-PCR to detect the expression level of lncRNA RUNX1-IT1 in HCC cell lines (Huh7, Hep3B, and HCCLM3) and normal cell line (L02). **B**, The expression level of lncRNA RUNX1-IT1 in HCC tissues and adjacent normal tissues from 80 HCC patients was detected as well. **C**, The survival curve showed that the survival time of patients in the lncRNA RUNX1-IT1 high expression group was significantly higher than that of patients in low expression group. $p<0.05$.

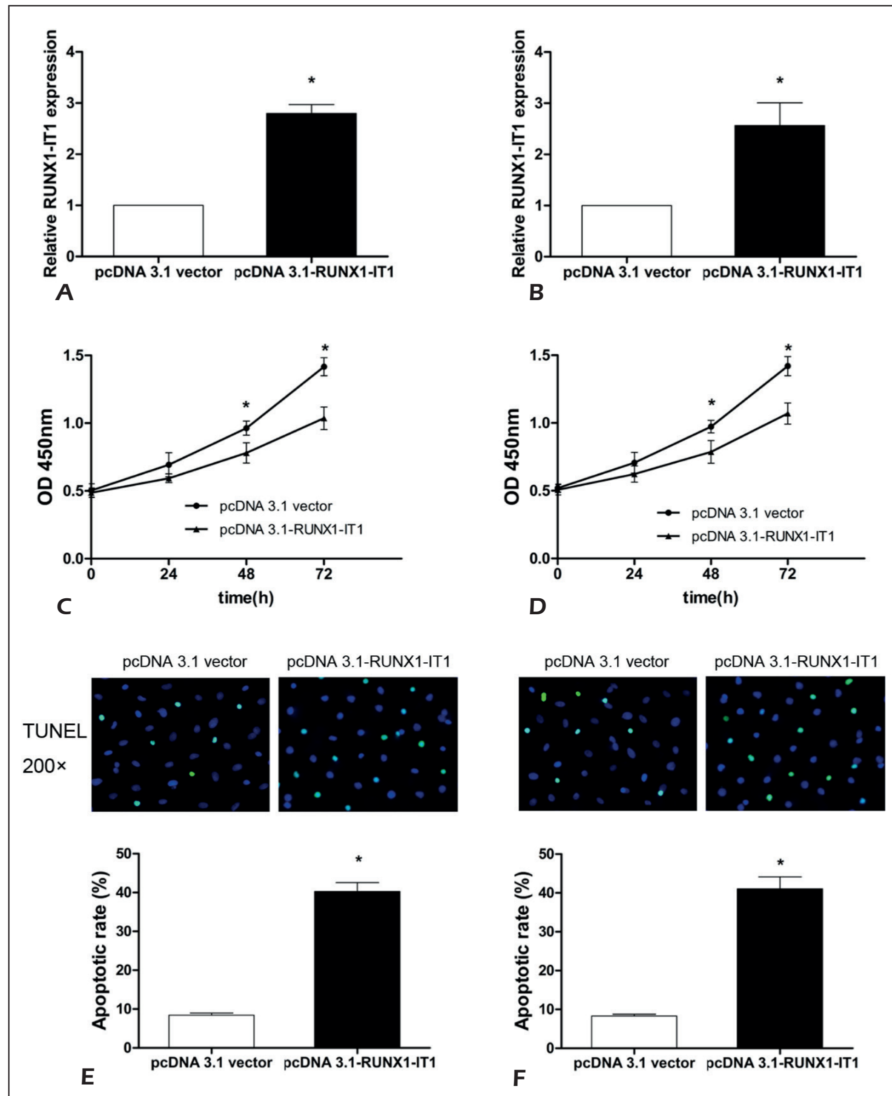

Figure 2. LncRNA RUNX1-IT1 overexpression inhibited cell proliferation and promoted cell apoptosis. **A**, and **B**, We constructed lncRNA RUNX1-IT1 overexpression vector and transfected it into Huh7 and HCCLM3 cell lines. QRT-PCR assay was used to detect the transfection efficiency. **C**, and **D**, The effect of the overexpression of lncRNA RUNX1-IT1 on cell viability was examined by CCK-8 assay. **E**, and **F**, The cell apoptosis assay showed that the number of apoptotic cells significantly increased after the overexpression of lncRNA RUNX1-IT1. $p<0.05$ (magnification: 40×).

LncRNA RUNX1-IT1 Overexpression Inhibited Cell Proliferation and Promoted Cell Apoptosis

LncRNA RUNX1-IT1 overexpression vector was constructed and transfected into Huh7 and HCCLM3 cell lines. QRT-PCR assay was used to verify the transfection efficiency (Figures 2A, 2B). After the overexpression of lncRNA RUNX1-IT1, the viability of the cells was examined by CCK-8 assay. The results demonstrated that the overexpression of lncRNA RUNX1-IT1 significantly reduced the proliferative capacity of Huh7 and HCCLM3 cells (Figures 2C, 2D). The cell apoptosis assay showed that the number of apoptotic cells significantly increased after the overexpression of lncRNA RUNX1-IT1 (Figures 2E, 2F). The results further proved that lncRNA RUNX1-IT1 overexpression could promote the apoptosis of HCC cells.

Overall, these findings indicated that lncRNA RUNX1-IT1 overexpression inhibited cell proliferation and promoted cell apoptosis.

LncRNA RUNX1-IT1 Inhibition Promoted Cell Proliferation But Inhibited Cell Apoptosis

ShRNA was constructed as well, and its inhibitory efficiency was examined in HCC cells of Huh7 and HCCLM3 (Figures 3A, 3B). CCK-8 results showed that the inhibition of lncRNA RUNX1-IT1 significantly promoted the proliferation of HCC cells (Figures 3C, 3D). This indicated that the inhibition of lncRNA RUNX1-IT1 could promote the proliferation of HCC cells.

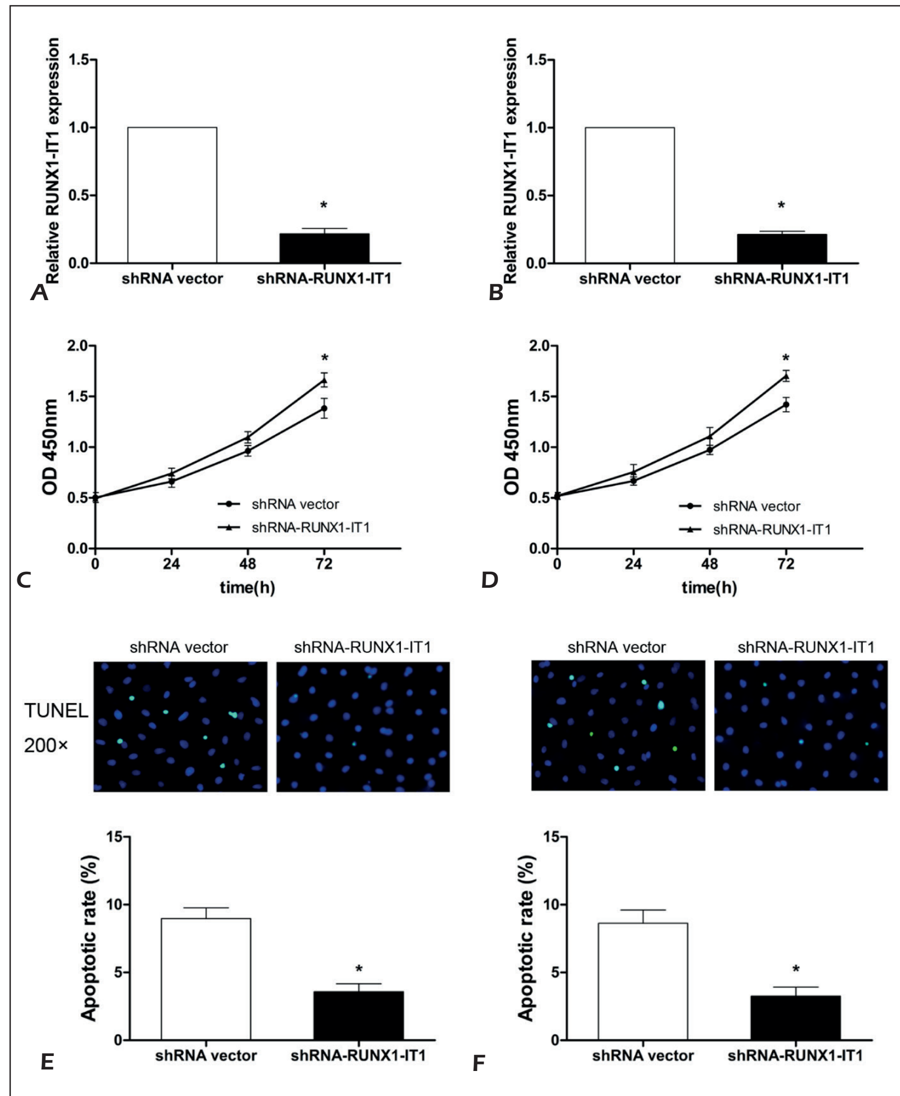

Figure 3. LncRNA RUNX1-IT1 inhibition promoted cell proliferation and inhibited cell apoptosis. **A**, and **B**, shRNA was constructed and its inhibitory efficiency in HCC cells (Huh7 and HCCLM3) was examined. **C**, and **D**, CCK-8 results showed that the inhibition of lncRNA RUNX1-IT1 promoted the proliferation of HCC cells. **E**, and **F**, Cell apoptosis assay showed that the number of apoptotic cells significantly decreased after the low-expression of lncRNA RUNX1-IT1. $p<0.05$ (magnification: 40×).

The cell apoptosis assay found that the number of apoptotic cells significantly decreased after the low downregulation of lncRNA RUNX1-IT1 (Figures 3E, 3F). This further proved that lncRNA RUNX1-IT1 low expression could inhibit the apoptosis of HCC cells.

Collectively, our data revealed that the inhibition of lncRNA RUNX1-IT1 expression promoted cell proliferation and inhibited cell apoptosis.

Relationship Between LncRNA RUNX1-IT1 and MAPK Signaling Pathway

To investigate the relationship between lncRNA RUNX1-IT1 and MAPK signaling pathway, we performed the Western blot assay. The protein changes of P-P38, P-ERK, and P-JNK in cells overexpressing lncRNA RUNX1-IT1 were significantly lower than those of the vector group ($p<0.05$). However, no significant changes were found in the protein expressions of P38, ERK, and JNK (Figures 4A, 4B). In addition, the protein changes of P-P38, P-ERK, and P-JNK in the cells with down-expression of lncRNA RUNX1-IT1 were remarkably higher than those of the vector group ($p<0.05$). Similarly, no significant changes were found in the protein expressions of P38, ERK, and JNK (Figures 4C, 4D). All these data indicated that lncRNA RUNX1-IT1 might be involved in the inhibition of the activation of the MAPK signaling pathway.

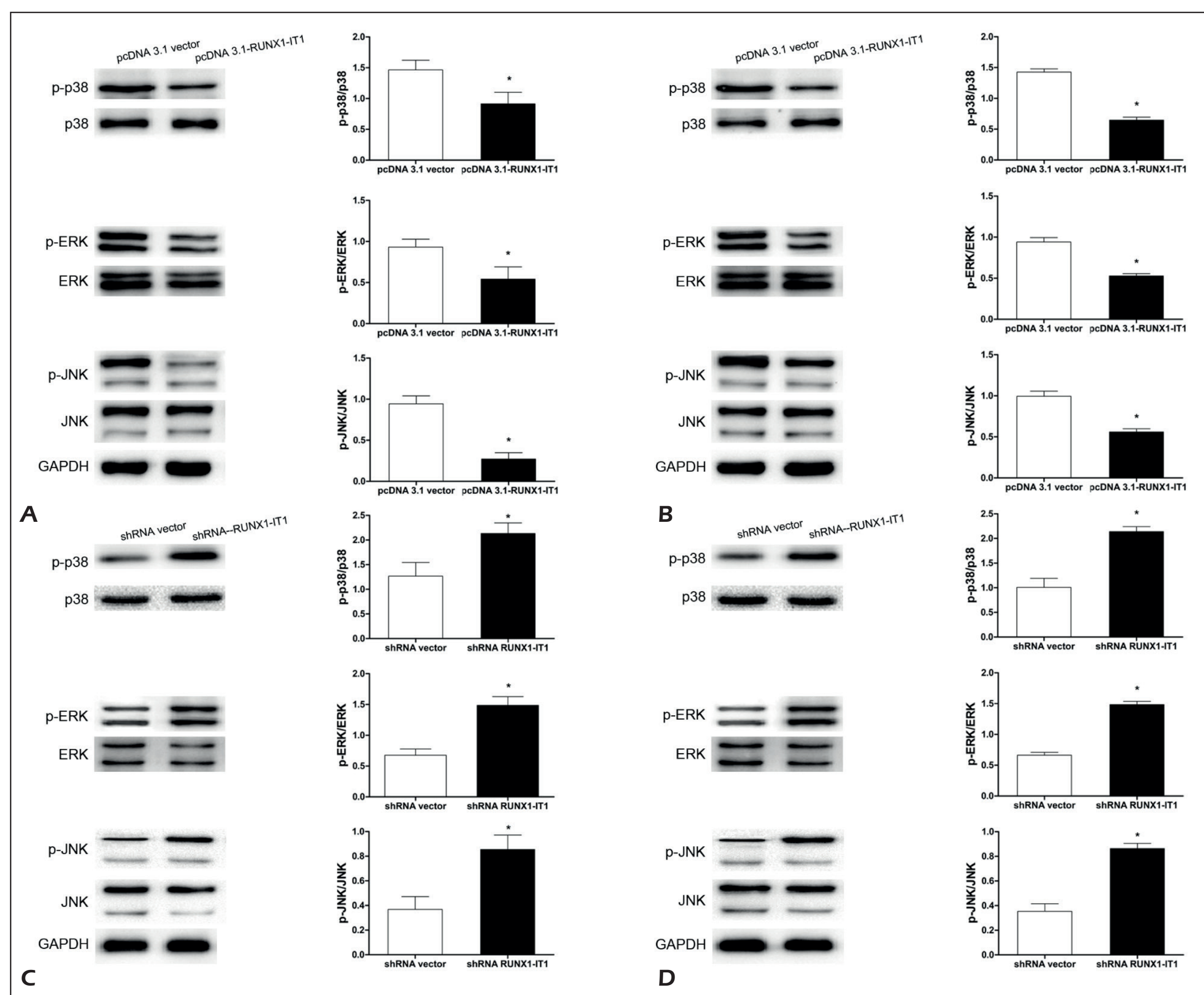

Figure 4. Relationship between lncRNA RUNX1-IT1 and MAPK signaling pathway. **A**, and **B**, The protein changes of P-P38, P-ERK, and P-JNK in Huh7 and HCCLM3 cells overexpressing lncRNA RUNX1-IT1 were detected by Western blot assay. **C**, and **D**, The protein changes of P-P38, P-ERK, and P-JNK in Huh7 and HCCLM3 cells down-expressing lncRNA RUNX1-IT1 were also detected by Western blot assay. $p<0.05$.

Discussion

Our study identified that lncRNA RUNX1-IT1 expression was significantly reduced in both HCC tissues and cell lines. Gain or loss function experiments indicated that the up-regulation of lncRNA RUNX1-IT1 inhibited cell proliferation and promoted cell apoptosis. However, the reverse effects were observed after the downregulation of lncRNA RUNX1-IT1. In addition, lncRNA RUNX1-IT1 might be involved in the inhibition of MAPK signaling pathway activation. Our findings provided a new insight for understanding the molecular (especially lncRNAs) mechanism of HCC progression.

Primary liver cancer is one of the major problems affecting human health worldwide[17]. In recent years, a large number of studies have shown that lncRNA is abnormally expressed in many human tumors, including liver cancer[18,19]. Therefore, the identification of key lncRNAs related to liver cancer is crucial for understanding their roles in the development of liver cancer. Meanwhile, they may also become new therapeutic targets[20].

LncRNA RUNX1-IT1 was first observed significantly down-regulated in HCC cells and tissues. CCK-8 assay indicated that the overexpression of lncRNA RUNX1-IT1 significantly inhibited the proliferation of HCC cells, while the inhibition of lncRNA RUNX1-IT1 promoted cell

proliferation. Therefore, it was speculated that lncRNA RUNX1-IT1 might play an important role in the proliferation of liver cancer cells. At the same time, the survival curve results showed that the survival time of patients in lncRNA RUNX1-IT1 high expression group was significantly higher than that of patients in low expression group. These results suggested that low expression of lncRNA RUNX1-IT1 indicated a poor prognosis.

Apoptosis is a process of active programmed death controlled by related genes. It participates in the regulation of physical development and the maintenance of internal environment[21]. Some researchers believe that the unrestricted growth of tumors is caused by the loss of control of tumor cell apoptosis[22]. Wang et al[23] have indicated that apoptosis-inducing therapy is a safe and effective anti-cancer method, which may become one of the standard therapies for cancer. Therefore, the identification of specific genes that lead to malignant tumor cell apoptosis may help to find novel therapies for cancer patients. In this study, we investigated the relationship between lncRNA RUNX1-IT1 expression and the apoptosis of HCC cells for the first time. After the upregulation of the expression of lncRNA RUNX1-IT1, Tunnel, and Western blot experiments, we showed that lncRNA RUNX1-IT1 could induce the apoptosis of HCC cells and increase the expressions of the apoptosis-related proteins (P-P38, P-ERK, and P-JNK). Meanwhile, it could also inhibit MAPK signaling pathway activation. Jia et al[24] have found that the abnormalities in the MAPK signaling pathway are closely related to the progression of liver cancer and poor prognosis. All these findings indicate that lncRNA and MAPK signaling pathways play an important role in the pathogenesis of liver cancer.

Conclusions

These results showed that the up-regulation of lncRNA RUNX1-IT1 expression was closely related to the development and prognosis of liver cancer for the first time. LncRNA RUNX1-IT1 played an important role in the proliferation and apoptosis of HCC cells as well. Our findings suggested that lncRNA RUNX1-IT1 might be a potential biomarker for the diagnosis and prognosis of liver cancer.

Conflict of Interests

The Authors declared that they have no conflict of interests.

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