The effect of targeted regulation of LATS2 by LncRNA BCAR4 on proliferation, migration and apoptosis of HCC cells

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Received December 15, 2020; Accepted February 19, 2021; Epub May 15, 2021; Published May 30, 2021

Abstract: Objective: This study explored and analyzed the effects of targeted regulation of LATS2 by LncRNA BCAR4 on the proliferation, migration and apoptosis of hepatocellular carcinoma (HCC). Method: We detected the expression of LncRNA, BCAR4 and LATS2 mRNA in liver hepatocellular carcinoma HepG2 cells and normal hepatocellular cells LO2 by RT-PCR. HepG2 cells were divided into BCAR4-siRNA, NC-siRNA and control groups. We detected the targeted regulation of LncRNA BCAR4 on LATS2 by luciferase gene assay, and measured the proliferation, migration and apoptosis of cells in each group by RT-PCR, MTT, Transwell and flow cytometry, respectively. Results: The relative expression of LncRNA BCAR4 in HepG2 cells was critically higher than that in LO2 cells (P<0.05), while LATS2 mRNA in HepG2 cells was significantly less than that in LO2 cells (P<0.05). Compared with NC siRNA group, the content of luciferase in BCAR4 siRNA group was much higher (P<0.05); The relative expression of LncRNA BCAR4 in BCAR4 siRNA group decreased dramatically than that in NC-siRNA and control group (P<0.05), and the relative expression of LATS2 mRNA increased remarkably than that in NC-siRNA group and control group (P<0.05). The OD value of BCAR4 siRNA group was dramatically higher than that of NC-siRNA group and control group after 48 h and 72 h culture (P<0.05). The quantity of invaded cells in BCAR4 siRNA group was markedly less than that in NC-SIRNA group and control group (P<0.05). Cell apoptosis rate in BCAR4-siRNA group was significantly higher than that of NC-siRNA group and control group (P<0.05). Conclusion: LncRNA BCAR4 can regulate the LATS2 expression, and inhibiting the expression of LncRNA BCAR4 can inhibit proliferation, invasion of HepG2 cells and induce its apoptosis, which finding provides a certain reference for the targeted therapy of liver cancer.

Keywords: LncRNA BCAR4, LATS2, hepatocellular carcinoma, cell proliferation, apoptosis

Introduction

Hepatocellular carcinoma (HCC) is a frequently occurred malignant tumor in clinical practice, and is also the fourth leading cause of cancer-related deaths in China [1]. The treatment of liver cancer has always been a difficult problem that is concerned by medical community, and the malignant degree of liver cancer is largely due to the recurrence of intra- and extra-hepatic metastasis [2]. Therefore, the in-depth study on the mechanism of growth and metastasis of HCC cells is of great significance for the development of new drugs. Long non-coding RNA (LncRNA) is a type of non-coding RNA molecules with a length over 200 bp. LncRNA can interact with DNA, RNA, protein molecules, etc., and is an important regulator of chromatin modification, transcription and post-transcriptional regulation [3]. A number of current studies have shown that a variety of LncRNAs have been proven to be dysregulated in HCC and are associated with the occurrence, progression, prognosis and diagnosis of liver tumor [4, 5]. LncRNA BCAR4 was first discovered to be related to anti-estrogen resistance in breast cancer. There are also scholars who have found that LncRNA BCAR4 is related to multiple tumors such as osteosarcoma and non-small cell lung cancer, and is tightly connected to the occurrence and progression of liver cancer. However, the specific regulatory mechanism of LncRNA BCAR4 in liver cancer has yet to be reported. LATS2, which is located in the centrosome, works for accumulation of γ-tubulin and formation of mitotic spindle, and inhibits tumor gene-
Regulation of LncRNA BCAR4 on hepatocellular carcinoma cells

sis and progression by regulating the cell cycle. Studies have found that LAST2 is closely related to the occurrence, progression and prognosis of a variety of human malignant tumors [6-8]. We adopted the bioinformatics software TargetAcan and miRanda to predict and analyze the possible target genes of LncRNA BCAR4, combined with the known functions of gene, and screened LATS2 as the candidate target gene for further research. This research explored and analyzed the effects of targeted regulation of LATS2 by LncRNA BCAR4 on the proliferation, migration and apoptosis of HCC.

Materials and method

Cells and reagents

The liver cancer cell line HepG2, human liver cell line LO2 and the embryonic kidney cell line HEK 293 used in this experiment were all purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

RPMI 1640 medium (U.S. Thermo Co.), Australian fetal bovine serum (U.S. Thermo Co.), trypsin (U.S. Thermo Co.), Penicillin-Streptomycin solution (U.S. Sigma Co.), Lipofectamine™ 2000 (U.S. Invitrogen Co.), Opti-MEM medium (Invitrogen, USA), Plasmid extraction kit (Invitrogen, USA), Trizol reagent (Invitrogen, USA), One Step SYBR® PrimeScript™ QPCR Kit (Dalianbao Biological Co., Ltd.), PCR primer synthesis (Shanghai Sangon Biotech Co., Ltd.), and Annexin V-FITC/PI apoptosis detection kit (Nanjing KeyGen Biotech. Inc.) were used in this research.

Culture, transfection and grouping of cells

Both HepG2 and LO2 cells were cultured in RPMI 1640 medium with culturing condition at 37°C, 5% CO₂, and digested and passaged after 48 h of cultivation. The cells used in the experiment were in logarithmic growth phase. The HepG2 cells were divided into three groups, the BCAR4-siRNA group, NC-siRNA group, and the control group. BCAR4-siRNA liposome complex was prepared with a concentration of 80 nmol/L, and HepG2 cells were seeded on a 6-well plate at 2×10^5 cells per well. After the cells were grown and fused, the BCAR4-siRNA group and NC-siRNA group were transfected with BCAR4-siRNA sequence and NC-siRNA sequence using Lipofectamine™ 2000 respectively. The control group was treated with PBST to form the blank control group. The forward and reverse primers of BCAR4-siRNA sequence were: 5’-GGGACUUGAGUUAUGUUGUGGCUA-3’ and 5’-UAGCCACCAACAUAACUCAAGUCCC-3’. And those of NC-siRNA sequence were: 5’-UACUGUCUAGUGCAGCAGUA-3 and 5’-GUACGGCGACUAGACAGUA-3’.

The number of cultured cells to be detected were not less than 1×10^6 cells per well. We extracted the total RNA from HepG2 and LO2 cells in each group by Trizol method. Added 1 ml Trizol to each well for cleavage, and transferred the lysis buffer to a clean EP tube. We treated cells with chloroform/isopropanol and collected white RNA precipitates. The precipitate was washed with precooled 75% ethanol, centrifuged at 4°C and 12000 r/min for 10 min, and the supernatant was discarded. After natural drying, dissolved the RNA in DEPC water, and synthesized cDNA by One Step PrimeScript® miRNA cDNA Synthesis Kit in accordance with the instructions. We used One Step SYBR® PrimeScript™ QPCR Kit to perform the reaction on American ABI 7300 QPCR System, with the following specific conditions: deprived at 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s with 40 cycles in total. With GAPDH as the reference gene, the relative expressions of LncRNA BCAR4 and LAST2

Figure 1. Comparison of LncRNA BCAR4 and LATS2 mRNA expression in HepG2 and LO2 cells. Note: compared with LO2, *P<0.05.
Regulation of LncRNA BCAR4 on hepatocellular carcinoma cells

mRNA were calculated by $2^{\Delta\Delta Ct}$ method. The forward and reverse of LncRNA BCAR4 primer sequences were 5'-acagcAGcTTGTTGCTCATCT-3' and 5'-TTGCCTTGGGGA CAGTTCAC-3'. Those of LATS2 mRNA primer sequences were 5'-GCT TCA TCCA CCGAGACATCAA-3' and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

The targeted regulation of LncRNA BCAR4 on LATS2 detected by dual-luciferase reporter assay

The construction of dual luciferase reporter plasmid of LATS2 mRNA 3'-UTR: we cloned the 3'-UTR fragment of LATS2 mRNA containing LncRNA BCAR4 seed sequence from genomic DNA, and inserted this fragment into the downstream of the firefly luciferase reporter gene plasmid to form a wild-type (WT) gene LATS2-3'-UTR-WT. Meanwhile, we adopted StraraGene's QuickChangeTM site-directed mutagenesis kit to perform site-directed mutagenesis of the 3'-UTR in LncRNA BCAR4 seed sequence to form a mutant (MT) gene LATS2-3'-UTR-MT. The HEK 293T cells inoculated in 48-well plates were incubated with BCAR4-siRNA or NC-siRNA for 6 h, and then transfected with 3'-UTR firefly luciferase reporter plasmid and internal control plasmid PRL-CMV (with Renilla Luciferase Reporter Gene). We measured the firefly luciferase signal after 48 h, and evaluated the binding effect of LncRNA BCAR4 to LATS2 mRNA 3'-UTR by the ratio of firefly luciferase and renilla luciferase activity in the reporter gene.

We adjusted the concentration of each group of cells to $1\times10^4$ cells/ml and incubated 100 μL of which in a 96-well plate. After 24 h, 48 h, and 72 h of cultivation, 20 μL of MTT solution was added respectively to each well and incubated at 37°C for 4 h. We aspirated and discarded the culture medium, added 150 μL DMSO in each well, shook the reaction for 15 min after adding to each well, and detected the absorbance (OD) of each well at 490 nm wavelength with a microplate reader.

Cell migration and invasion ability detected by Transwell

We applied 50-100 μL of Matrigel diluent (1:20) evenly to the basement membrane at the bottom of the coated chamber and dried at 4°C; added 100 μL of RPMI 1640 culture medium to each chamber to hydrate the basement membrane, and placed at 37°C for 1 h; collected...
Regulation of LncRNA BCAR4 on hepatocellular carcinoma cells

the transfected cells of each group, washed with serum-free culture medium, digested with 0.25% trypsin and then centrifuged at 1200 r/min for 5 min. After 24-48 h of routine culture, we took out the Transwell chamber, washed with PBS and fixed with 4% paraformaldehyde for 30 minutes. After membrane at the bottom of the chamber was air-dried, the Transwell chamber was placed in crystal violet dye solution for 20 min, and then cells were observed and photographed under an optical microscope. 5 fields were randomly selected in each group.

Cell apoptosis detected by flow cytometry

We seeded HepG2 cells into 6-well plates with a density of $1 \times 10^6$ per well. After 48 h of transfection, the cells were digested with trypsin and collected. After washing with PBS solution according to instructions of Annein V-FITC/PI double staining kit, 5 μL Annein V and 10 μL PI were added in each cell group. After 15 minutes of staining, the cell apoptosis in each group was detected by flow cytometry.

Statistical analysis

Data processing and analysis were conducted by statistical software SPSS 22.0. The measurement data was expressed by mean ± standard deviation ($\bar{x} \pm sd$) and compared with $t$-test. $P<0.05$ indicated that the difference was statistically significant.

Results

Comparison of LncRNA BCAR4 and LATS2 mRNA expression in HepG2 and LO2 cells

The relative expression of LncRNA BCAR4 in HepG2 cells was significantly higher than that in LO2 cells ($P<0.05$), and LATS2 mRNA in HepG2 cells was obviously lower than that in LO2 cells ($P<0.05$) (Figure 1).

Double luciferase experiment

In cells transfected with LATS2-3'-UTR-WT expression vector, the luciferase content in BCAR4-siRNA group increased remarkably than that in NC-siRNA group ($P<0.05$); while in those transfected with LATS2-3'-UTR-MT, the expression degree of luciferase in BCAR4-siRNA group did not change remarkably comparing to the expression in NC-siRNA group ($P>0.05$). These results indicated that LATS2 is the target gene of LncRNA BCAR4, and its expression is negatively regulated by LncRNA BCAR4 (Figure 2).
Regulation of LncRNA BCAR4 on hepatocellular carcinoma cells

Expression of LncRNA BCAR4 and LATS2 mRNA in HepG2 cells of each group

The relative expression of LncRNA BCAR4 in BCAR4 siRNA group decreased dramatically compared to that in NC-siRNA group and the control group \((P<0.05)\), while LATS2 mRNA in BCAR4 siRNA group increased remarkably compared to that in NC-siRNA group and control group \((P<0.05)\) (Figure 3).

Cell proliferation

The OD value of BCAR4 siRNA group was dramatically higher than that of NC-siRNA group and control group after 48 h and 72 h culture \((P<0.05)\) (Figure 4).

Cell migration and invasion

The quantity of invaded cells in BCAR4 siRNA group was markedly less than that in NC-SIRNA group and control group \((P<0.05)\) (Figure 5).

Cell apoptosis

Cell apoptosis rate of BCAR4-siRNA group was significantly higher than that of NC-siRNA group and control group \((P<0.05)\) (Figures 6 and 7).

Discussion

HCC is one of the most common malignant tumors in the world, which is featured by the hidden onset, high malignant degree, easy invasion and metastasis, and poor prognosis in patients [9]. The incidence of liver cancer, due to hepatitis B and hepatitis C virus, is at a high level in Asia-Pacific region. For patients with early primary liver cancer, surgical resection is currently the most effective treatment. However, most patients have missed the optimal period of surgery since their disease states were already in end-stage when clinically diagnosed [10, 11]. Although comprehensive treatment methods such as radiotherapy and chemotherapy have improved the quality of life of patients to a certain extent, tumor recurrence and metastasis cannot be effectively avoided. Survey studies have shown that the 5-year survival of patients with liver cancer is as low as 3 to 5% [12].

LncRNA imposes crucial function in the normal development of human beings and the occurrence and development of diseases. LncRNA expression has tissue specificity, and is usually dysregulated in various types of tumors. Some
LncRNAs have been proved to be associated with tumor recurrence and poor prognosis [13, 14]. LncRNA BCAR4 is a member of the LncRNA family. Studies have shown that the high expression of LncRNA BCAR4 is an independent predictor of progression-free survival in breast cancer patients after tamoxifen treatment [15]. In addition, reports have shown that LncRNA BCAR4 is up-regulated in osteosarcoma tissues, and its up-regulation is correlated with the pathological features of malignancy such as tumor size, stage, lung metastasis, and poor prognosis [16]. It was discovered in this study that the relative expression of LncRNA BCAR4 in HepG2 cells was critically higher than that in LO2 cells. The presence of high expression of LncRNA BCAR4 in liver cancer cells suggested that LncRNA BCAR4 may play a role in the occurrence and progression of HCC, which are consist with those confirmed by other scholars [17].

Hippo signaling pathway plays a significant role in tumorigenesis, and its primary function is to regulate the proliferation and apoptosis of tumor cells. LATS2 is a key factor of Hippo sig-
Regulation of LncRNA BCAR4 on hepatocellular carcinoma cells

In this study, we analyzed the effect of LncRNA BCAR4 on the proliferation, invasion and apoptosis of hepatoma cells by targeting LATS2. However, whether LncRNA BCAR4 can regulate its expression on hepatoma cells through other signaling pathways still needs to be further confirmed. At the same time, this study is only an in vitro cell experiment, and there is no clinical study to verify.

In summary, LncRNA BCAR4 is highly expressed in liver cancer cells HepG2. Inhibiting the expression of LncRNA BCAR4 can inhibit cell proliferation, invasion and promote cell apoptosis, which may likely to be achieved by promoting the expression of tumor-inhibiting factor LATS2.

Disclosure of conflict of interest
None.

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Regulation of LncRNA BCAR4 on hepatocellular carcinoma cells

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