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LncRNA NEAT1 regulates proliferation, apoptosis and invasion of liver cancer

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Abstract. – OBJECTIVE: The occurrence and progression of hepatocellular carcinoma (HCC) is a multi-step complex process and the exact molecular mechanisms remain to be elucidated. LncRNA NEAT1 is involved in tumorigenesis and progression. However, the role of LncRNA NEAT1 in HCC remains unclear.

PATIENTS AND METHODS: The tumor tissues and adjacent tissues of HCC patients were collected and LncRNA NEAT1 expression was detected by Real time PCR. The hepatoma cell line HepG2 was cultured and transfected with lnc RNA NEAT1 siRNA or lnc RNA NEAT1 plasmid followed by analysis of LncRNA NEAT1 expression, cell proliferation by MTT assay, as well as Caspase 3 activity. In addition, cell apoptosis and cell cycle were assessed by flow cytometry and cell invasion was measured by transwell chambers. The expression of EGFR, Bax and Bcl-2 was detected by Western blot.

RESULTS: LncRNA NEAT1 expression was significantly increased in HCC tissues compared with adjacent tissues ($p < 0.05$). Compared with the siRNA group, transfection of lncRNA NEAT1 siRNA into HepG2 cells significantly inhibited cell proliferation, increased Caspase 3 activity and apoptosis, reduced cell invasion, as well as arrested cell cycle ($p < 0.05$). Meanwhile, lncRNA NEAT1 siRNA also significantly decreased Bcl-2 and EGFR expression and increased Bax expression ($p < 0.05$). Transfection of lncRNA NEAT1 plasmid in hepatoma cells HepG2 reversed the above changes, compared with vector group, the differences were statistically significant ($p < 0.05$).

CONCLUSIONS: LncRNA NEAT1 expression is increased in liver cancer tissues. Down-regulation of LncRNA NEAT1 can inhibit EGFR expression and promote hepatoma cell apoptosis, inhibit cell cycle, thus inhibiting tumor proliferation and invasion.

Key Words:
LncRNA NEAT1, Liver cancer, EGFR, Apoptosis, Proliferation, Invasion.

Introduction

Liver cancer is the second most common malignant tumor in the world. Recently, nearly 700,000 people worldwide have died of liver cancer each year[1,2]. As one of the most common malignant gastrointestinal tumors, the incidence rate in China is high throughout the year, and hepatocellular carcinoma (HCC) is the most important type of liver cancer[3]. With the development of medical diagnosis and treatment technology, the treatment of liver cancer is diverse, including surgery combined with radiotherapy, chemotherapy, immunotherapy and other approaches, but currently liver cancer patients, especially patients with advanced liver cancer, have poor therapeutic effects due to late appearance of liver cancer symptoms and not significant early symptoms, so the diagnosis is often late, resulting in treatment difficulty[4,5]. Liver cancer is prone to metastasis, with a high recurrence rate, poor prognosis, low survival rate and poor quality of life, which brings a heavy burden to the global economy. Therefore, research on the occurrence, development, diagnosis and treatment of liver cancer has become a medical problem to be solved[6,7]. Important risk factors currently known include chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol abuse, autoimmune hepatitis, diabetes, obesity and several metabolic diseases. The increased incidence of HCC in developed countries is attributed to hepatitis C virus (HCV) infection, obesity and diabetes mellitus[8,9]. The accumulation of reactive oxygen species, inflammatory cytokines and fibrosis may be important factors contributing to genetic and epigenetic changes in HCC cells[10,11]. The occurrence and progression of HCC is a multi-step complex process, so the mechanism of liver cancer discovery is conducive to the early detection, early diagnosis and timely treatment of liver cancer[12].

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Long-chain non-coding RNAs (LncRNAs) are transcripts with a length of more than 200 nucleotides that can participate in biological progression through transcriptional and post-transcriptional regulation, but do not have the function of encoding proteins[13]. They play key roles in the regulation of various diseases and biological processes, including cell differentiation, proliferation, apoptosis, gene regulation, and tumor development[14]. LncRNA NEAT1 is abnormally expressed in various tissues and organs, and can participate in the occurrence and development of various diseases such as tumor and cardiovascular disease[15,16]. However, the expression and regulatory mechanism of LncRNA NEAT1 in liver cancer has not been elucidated.

Patients and Methods

Research Object

From January 20 to 2018, we collected 62 patients with primary liver cancer who underwent surgical resection of the primary liver cancer and underwent total or subtotal resection. There were 42 males and 20 females, aged 40-75 years. The average age (51.5 ± 18.5) years old. The intraoperative criteria for pathological diagnosis and TNM staging were confirmed by pathological diagnosis[6]; exclusion criteria included metastasis liver cancer receiving surgery or other treatment; combined with other tumors or metabolic diseases on admission[6]. The tumor tissues were removed and the adjacent tissues more than 5 cm away from the tumor were collected and frozen at -80°C. The investigation was approved by the Medical Ethics Committee of Beijing Chaoyang Hospital, Capital Medical University, Beijing, China and all selected subjects signed informed consent.

Main Materials and Instruments

The liver cancer cell line HepG2 was supplied by our laboratory and stored in liquid nitrogen. Dulbecco's Modified Eagle's Medium (DMEM) medium, fetal bovine serum (FBS), and cyan chain double antibody were purchased from HyClone (South Logan, UT, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder was purchased from Gibco (Grand Island, NY, USA); trypsin-Ethylenediaminetetraacetic acid (EDTA) digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Life Sciences (Port Washington, NY, USA), EDTA was purchased from HyClone, Western blot related chemical reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), rabbit anti-human epidermal growth factor receptor (EGFR) monoclonal antibody, rabbit anti-human Bax Monoclonal antibody, rabbit anti-human Bcl-2 monoclonal antibody, mouse anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody was purchased from Cell signaling Technology (Danvers, MA, USA). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (Union City, CA, USA). LncRNA NEAT1 siRNA negative control and LncRNA NEAT1 siRNA sequence, vector and LncRNA NEAT1 plasmid were synthesized by Shanghai Jima Company (Shanghai, China). The lipo2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). The transwell chamber was purchased from Corning (Corning, NY, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Corporation (Hercules, CA, USA). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (Waltham, MA, USA). The ultra-clean workbench was purchased from Suzhou Sutai Purification Equipment Engineering Co., Ltd (Suzhou, China). The DNA amplification instrument was purchased from the US PE Gene Amp PCR System 2400 (Foster City, CA, USA). Flow cytometry was purchased from BD Bioscences (San Jose, CA, USA).

Hepatoma Cell Line HepG2 Cell Culture and Grouping

The HepG2 cell line was preserved in liquid nitrogen and resuscitated and cultured. The cultured 2-8 generation logarithmic growth phase HepG2 cells were used for the experiment, randomly divided into 4 groups, siRNA group, in which siRNA negative control was transfected to HepG2 cell; si-NEAT1 group, in which LZTR1 NEAT1 siRNA was transfected into HepG2 cells; vector group, which was transfected with NEAT1 empty plasmid in HepG2 cells; NEAT1 group, in which NEAT1 plasmid was transfected into HepG2 cells.

Liposome Transfection of NEAT1 siRNA and NEAT1 Plasmid In HepG2 Cells

The cell density was fused to 70-80% in a 6-well plate; according to the instructions,

empty plasmid, NEAT1 plasmid NEAT1 siRNA negative control and LZTR1 siRNA liposome were separately added to 200 μl of serum-free medium, thoroughly mixed, and incubated for 15 min at room temperature. The mixed lipo2000 was separately mixed and incubated for 30 min at room temperature. The serum of the cells was removed, PBS was gently rinsed, 1.6 ml of serum-free medium was added, and each system was added to each system, and cultured in a 5% CO_2 incubator at 37°C for 6 hours. The serum culture solution was replaced and cultured for 48 hours for experimental research.

Real-Time PCR Detection of NEAT1 Expression

The liver cancer and adjacent tissues and each group of HepG2 tumor cells were used to extract RNA with TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. The primers were designed according to each gene sequence by PrimerPremier 6.0 and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). Real-time PCR reaction conditions: 56°C 1 min, 92°C 30 s, 58°C 45 s, 72°C 35 s, a total of 35 cycles. Data were collected using the PCR reactor software and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and then the semi-quantitative analysis was carried out by the $2^{-\Delta Ct}$ method.

MTT Assay to Detect the Proliferation of Cells In Each Group

The logarithmic growth phase HepG2 cells were inoculated into the 96-well culture plate with 10% fetal bovine serum, DMEM culture medium at a dose of 5×10^3 cells. After 24 hours of culture, the supernatant was discarded and randomly divided into 3 groups and grouped according to the above treatment. After 48 h of each group of cells, 20 ml of sterile MTT was added to the wells to be tested, and 3 replicate wells were set in each treatment group. After 4 hours of continuous culture, the supernatant was completely removed, 150 μl/well of DMSO was added, and the shaker was shaken for 10 min. After the purple crystals were sufficiently dissolved, the absorbance (A) value was measured at a wavelength of 570 nm by a microplate reader, and the proliferation rate of each group was calculated. The experiment was repeated three more times.

Caspase 3 Activity Assay

The changes in Caspase 3 activity in each group of cells were examined according to the kit instructions. Trypsin digested cells were centrifuged at 600 g at 4°C for 5 min, and cell lysate was lysed on ice for 15 min followed by centrifugation at 20000 g at 4°C for 5 min. After addition of 2 mM Ac-DEVD-pNA, OD value at 405 nm was measured to calculate Caspase 3 activity changes.

Transwell Chamber Test to Detect the Invasion of Cells In Each Group

After 48 h of transfection, the cells in each group were cultured in DMEM medium supplemented with 10% fetal bovine serum, and then cultured for 24 h in serum-free medium. The bottom of the transwell chamber and the upper chamber of the membrane were coated with 50 mg/L Matrigel l:5 dilution and air dried at 4°C. The residual liquid in the plate was aspirated, and 50 ml of serum-free medium containing 10 g/L of bovine serum albumin (BSA) was added to each well at 37°C for 30 min. Then, 500 ml of 10% fetal bovine serum was added to DMEM medium in a small chamber and 100 μl of tumor cell suspension was added to the chamber and cells were cultured in serum-free medium. 3 samples per group were repeated.

Western Blot Detection of EGFR, Bax, Bcl-2 Expression Changes

Each group of HEPG2 cell proteins was extracted: radio immunoprecipitation assay (RIPA) lysate containing protease inhibitor was added, and the cells were lysed on ice for 15-30 min, 5 s × 4 times sonicated, and centrifuged at 4°C, 10 000 × g for 15 min. After clearing the

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGATCTCCAGTGGCTGG	TAGGGACATGATATGCTGT
NEAT1	TCATCATCTCCAGTGCTG	TGAGCAGAGGTGATACGTT

new Eppendorf (EP) tube, the protein was quantified and stored at -20°C for Western blot. The isolated protein was electrophoresed on a 10% sodium dodecyl sulfate and polyacrylamide gel (SDS-PAGE), transferred to a PVDF membrane by semi-dry transfer, blocked with 5% skim milk to remove the non-specific background, and incubated with 1:1000, 1:1000, 1:1000 dilution of primary anti-EGFR, Bax or Bcl-2 monoclonal antibody at 4°C, overnight. After Phosphate Buffered Saline with Tween™ 20 (PBST) washing, the membrane was incubated with 1:2000 goat anti-rabbit secondary antibody under dark for 30 min followed by washing with PBST, addition of chemiluminescence for 1 min, and X-ray exposure imaging. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4) and statistical analysis.

Flow Cytometry Analysis of Cell Cycle

Cells were collected through digestion with 0.25% trypsin to digest the cells and the cell suspension was transferred to a centrifuge tube, centrifuged at 1500 rpm for 5 min. The cells were washed with PBS, fixed overnight followed by addition of 100 µl of 100 µg/ml PI staining solution (in PBS) for 20 min incubation under dark and subsequent analysis of cell cycle by flow cytometry.

Flow Cytometry Analysis of Apoptosis

The culture medium was collected in a flow tube, washed once with PBS, and 1 ml of 0.25% trypsin-digested cells were added, followed by being centrifuged at 1500 rpm for 5 min. Cells were resuspended in 3 ml of 4°C pre-cooled PBS, centrifuged at 1500 rpm for 5 min, and the pellet was resuspended in 300 µL of Binding Buffer followed by addition of 5 µL of Annexin V-FITC and 5 µL of Propidium Iodide under dark for 5-15 min incubation at room temperature. After that, cell apoptosis was analyzed by flow cytometry.

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). The mean values of the two groups were compared by *t*-test test, analyzed by SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA) and the differences between groups were analyzed by ANOVA test with Bonferroni post-hoc analysis. The count data were analyzed by χ^2-test. Correlation analysis was performed using Pearson correlation analysis. $p < 0.05$ indicated a statistically significant difference.

Results

Expression of LncRNA NEAT1 In Liver Cancer

The expression of LncRNA NEAT1 in liver cancer was analyzed by Real time PCR. The results showed that LncRNA NEAT1 was significantly increased in HCC tissues compared with that in adjacent tissues ($p < 0.05$; Figure 1).

Regulation of NEAT1 Expression by LncRNA NEAT1 In Hepatoma Cells

Real time PCR analysis showed that LncRNA NEAT1 siRNA transfection significantly inhibited the expression of LncRNA NEAT1 in HCC cells ($p < 0.05$). The transfection of LncRNA NEAT1 plasmid significantly promoted the expression of LncRNA NEAT1 in HCC cells compared with the vector group ($p < 0.05$; Figure 2).

Effect of LncRNA NEAT1 on Proliferation of Hepatoma Cells

The effect of LncRNA NEAT1 on the proliferation of hepatoma HepG2 cells was detected by MTT assay. LncRNA NEAT1 siRNA significantly inhibited the expression of LncRNA NEAT1 in hepatocarcinoma cells and inhibited the proliferation of tumor cells. Compared with siRNA group, the difference was statistically significant ($p < 0.05$). Transfection of LncRNA NEAT1 plasmid

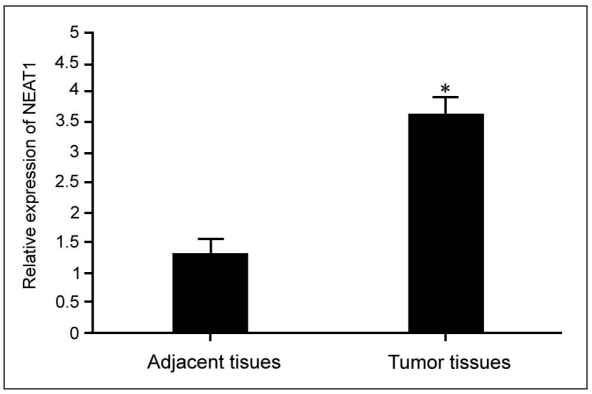

Figure 1. Expression of LncRNA NEAT1 in liver cancer. Compared with the paracancerous group, *$p < 0.05$.

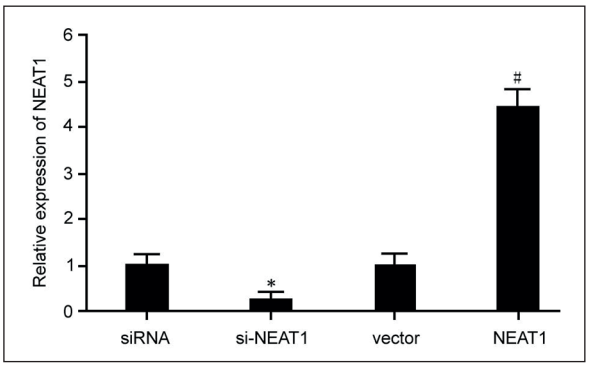

Figure 2. Effect of regulating LncRNA NEAT1 on NEAT1 expression in hepatoma cells. Compared with siRNA group, $^{*}p < 0.05$; compared with vector group, $^{\#}p < 0.05$.

into hepatoma cells significantly promoted the expression of LncRNA NEAT1 in hepatoma cells and further promoted the proliferation of hepatoma cells. Compared with the vector group, the difference was statistically significant ($p < 0.05$; Figure 3).

Effect of LncRNA NEAT1 on Caspase 3 Activity In Hepatoma Cells

LncRNA NEAT1 siRNA significantly inhibited the expression of LncRNA NEAT1 in hepatocellular carcinoma cells and promoted the increase of Caspase 3 activity. Compared with siRNA group, the difference was statistically significant ($p < 0.05$). Transfection of LncRNA NEAT1 plasmid into hepatoma cells significantly promoted the expression of LncRNA NEAT1 in hepatoma cells and further inhibited Caspase 3 activity ($p < 0.05$; Figure 4).

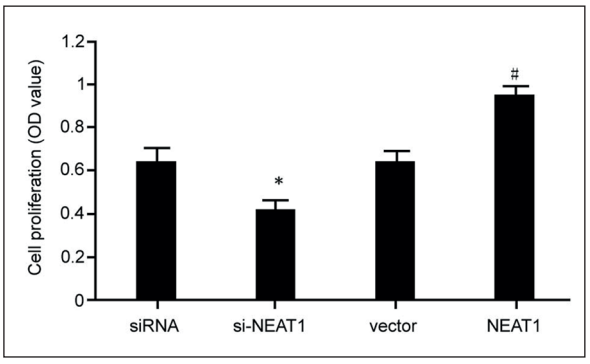

Figure 3. Effect of regulating LncRNA NEAT1 on proliferation of hepatoma cells. Compared with siRNA group, $^{*}p < 0.05$; compared with vector group, $^{\#}p < 0.05$.

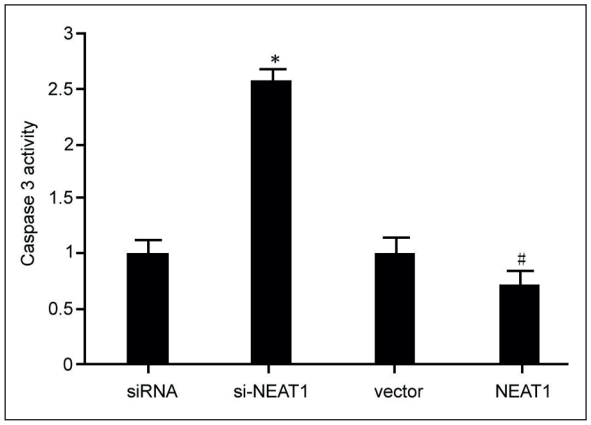

Figure 4. Effect of LncRNA NEAT1 on Caspase 3 activity in hepatoma cells. Compared with siRNA group, $^{*}p < 0.05$; compared with vector group, $^{\#}p < 0.05$.

Effect of LncRNA NEAT1 on Apoptosis of Hepatoma Cells

LncRNA NEAT1 siRNA significantly inhibited the expression of LncRNA NEAT1 in hepatocellular carcinoma cells, promoted apoptosis, and increased apoptosis rate. Compared with siRNA group, the difference was statistically significant ($p < 0.05$). Transfection of LncRNA NEAT1 plasmid into hepatoma cells significantly promoted the expression of LncRNA NEAT1 in hepatoma cells and further inhibited apoptosis. Compared with the vector group, the difference was statistically significant ($p < 0.05$; Figure 5).

Effect of LncRNA NEAT1 on Cell Cycle of Hepatoma Cells

LncRNA NEAT1 siRNA significantly inhibited the expression of LncRNA NEAT1 in hepatocellular carcinoma cells, inhibited cell cycle, and cell cycle was arrested in S phase. Compared with siRNA group, the difference was statistically significant ($p < 0.05$). Transfection of LncRNA NEAT1 plasmid into hepatoma cells significantly promoted the expression of LncRNA NEAT1 in hepatoma cells, and further increased cell cycle and S phase cells. Compared with vector group, the difference was statistically significant ($p < 0.05$; Figure 6).

Effect of LncRNA NEAT1 on Invasion of Hepatoma Cells

LncRNA NEAT1 siRNA significantly inhibited the expression of LncRNA NEAT1 in hepatocellular carcinoma cells and inhibited

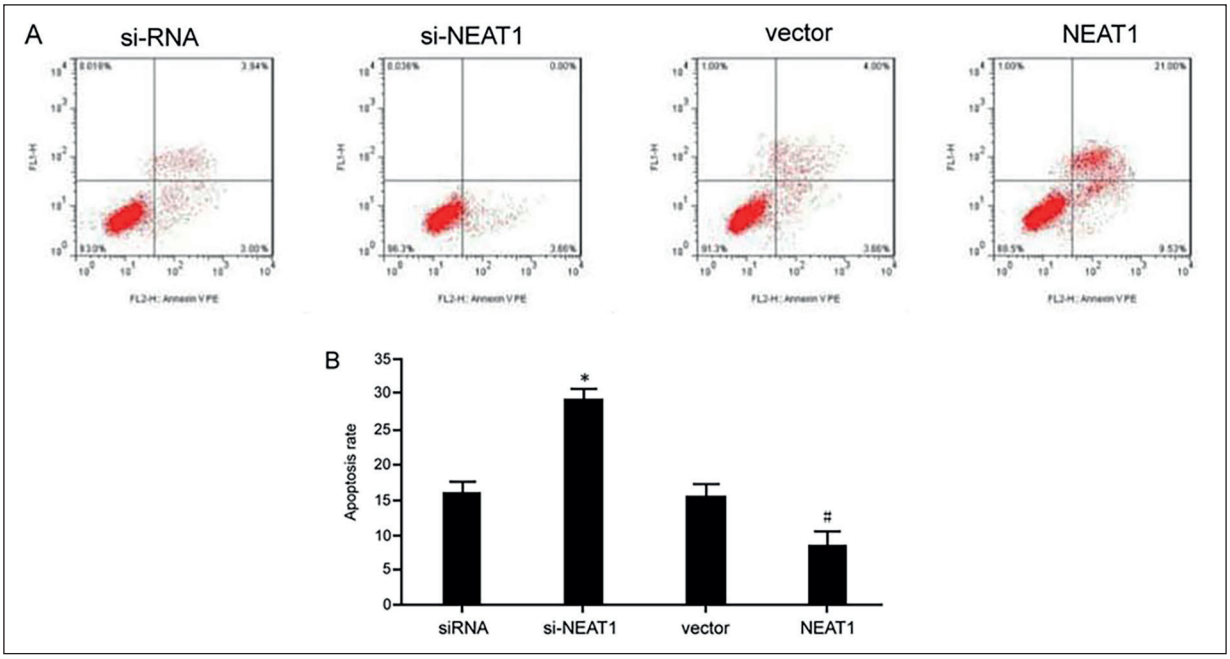

Figure 5. Effect of LncRNA NEAT1 on apoptosis of hepatoma cells. **A**, Flow cytometry analysis of apoptosis; statistical analysis of **B** cell apoptosis rate, compared with siRNA group, $*p < 0.05$; compared with vector group, $^{\#}p < 0.05$.

cell invasion. Compared with siRNA group, the difference was statistically significant ($p < 0.05$). Transfection of LncRNA NEAT1 plasmid into hepatoma cells significantly promoted the expression of LncRNA NEAT1 in hepatoma cells and further promoted cell invasion. Compared with the vector group, the difference was statistically significant ($p < 0.05$; Figure 7).

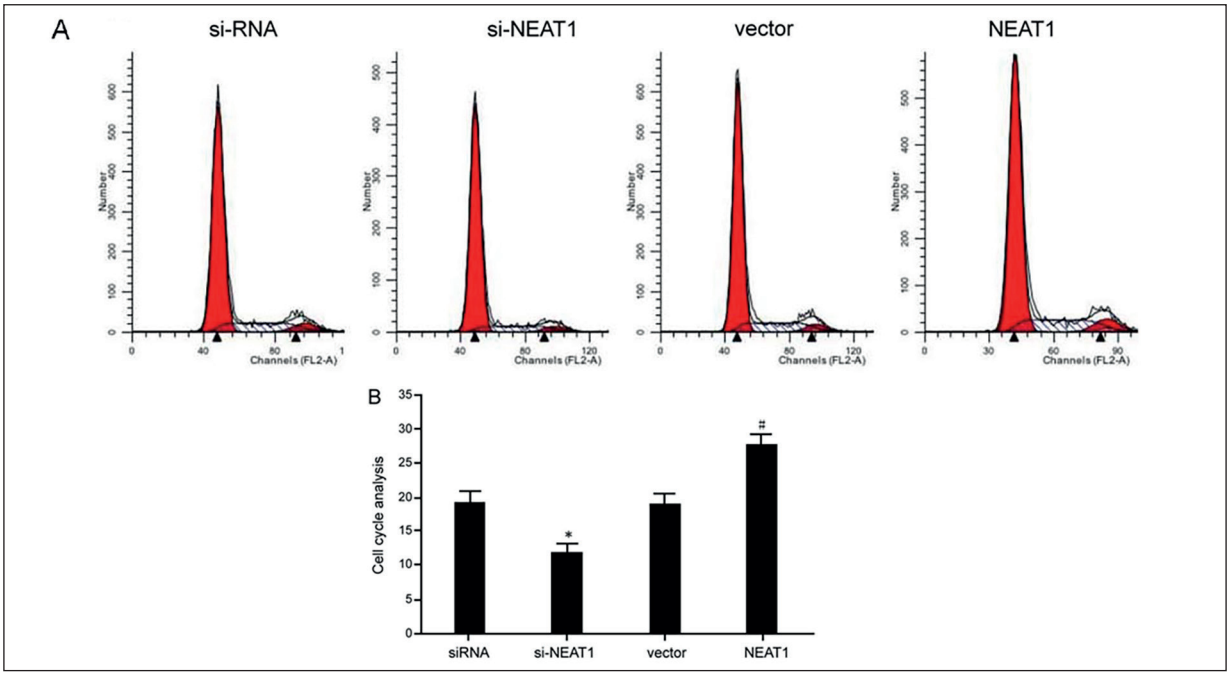

Figure 6. Effect of LncRNA NEAT1 on cell cycle of hepatoma cells. **A**, Flow cytometry analysis of cell cycle; **B**, cell cycle statistical analysis, compared with siRNA group, $*p < 0.05$; compared with vector group, $^{\#}p < 0.05$.

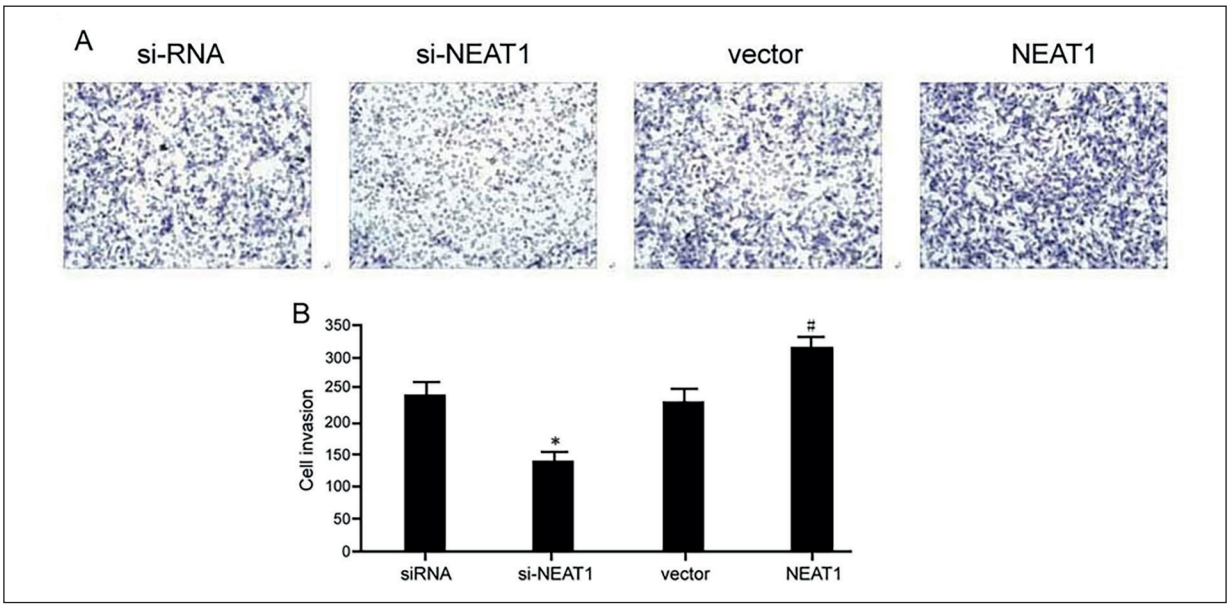

Figure 7. Effect of regulating LncRNA NEAT1 on invasion of hepatoma cells. **A**, Transwell chamber analysis regulates the effect of LncRNA NEAT1 on hepatocarcinoma cell invasion (magnification 200×); statistical analysis of invasion ability of **B** hepatoma cells compared with siRNA group, $^{*}p < 0.05$; compared with vector group, $^{\#}p < 0.05$.

Effects of LncRNA NEAT1 on the Expression of EGFR, Bax and Bcl-2 in Hepatocellular carcinoma cells

LncRNA NEAT1 siRNA significantly inhibited the expression of LncRNA NEAT1 in hepatocellular carcinoma cells, decreased Bcl-2 expression, increased Bax expression, and decreased EGFR expression. The difference was statistically significant ($p < 0.05$). Transfection of LncRNA NEAT1 plasmid into hepatoma cells significantly promoted the expression of LncRNA NEAT1 in hepatoma cells, increased Bcl-2 expression, decreased Bax expression, and increased EGFR expression ($p < 0.05$; Figure 8).

Discussion

The pathogenesis of liver cancer is complex and related genes are numerous. HCC is a multi-factor, multi-gene, multi-step regulation process. It is of great clinical significance to determine the molecular targets of HCC for the early diagnosis and prevention of liver cancer[17,18]. LncRNA NEAT1 plays an important role in tumors and other diseases, and can participate in tumorigenesis, invasion, metastasis, etc., while LncRNA NEAT1 can participate in the development of tissue and organogenesis and non-tumor diseases[15,16]. This study confirmed that LncRNA NEAT1 expression was increased in HCC tissues, suggesting that LncRNA NEAT1 might play an important role in the development and progression of HCC.

By manipulating the expression of LncRNA NEAT1 in hepatoma cells, its effect on hepatoma cells was analyzed. The results indicated that transfection of lncRNA NEAT1 siRNA into hepatoma cell HepG2 down-regulated the expression of lncRNA NEAT1, inhibited cell proliferation, promoted the increase of Caspase 3 activity, increased apoptosis rate, decreased cell invasion, arrested cell cycle, and decreased Bcl-2 expression, increased Bax expression. Transfection of lncRNA NEAT1 plasmid into hepatoma cell HepG2, up-regulation of lncRNA NEAT1 expression, can promote cell proliferation, inhibit Caspase 3 activity, decrease apoptosis rate, increase cell invasion, and increase Bcl-2 expression and reduce Bax expression. Bcl-2 is a key regulatory gene of anti-apoptotic protein family, and its apoptosis-inhibiting gene is closely related to its anti-apoptosis. Bax, as an apoptotic gene, not only antagonizes the inhibitory effect of Bcl-2 on apoptosis, but also promotes cell apoptosis. Bax plays a key role in apoptosis induced by mitochondrial stress and in the activation of the enzymatic cascade of the Caspase protease family, in particular increasing Caspase 3 activity, and inducing cell

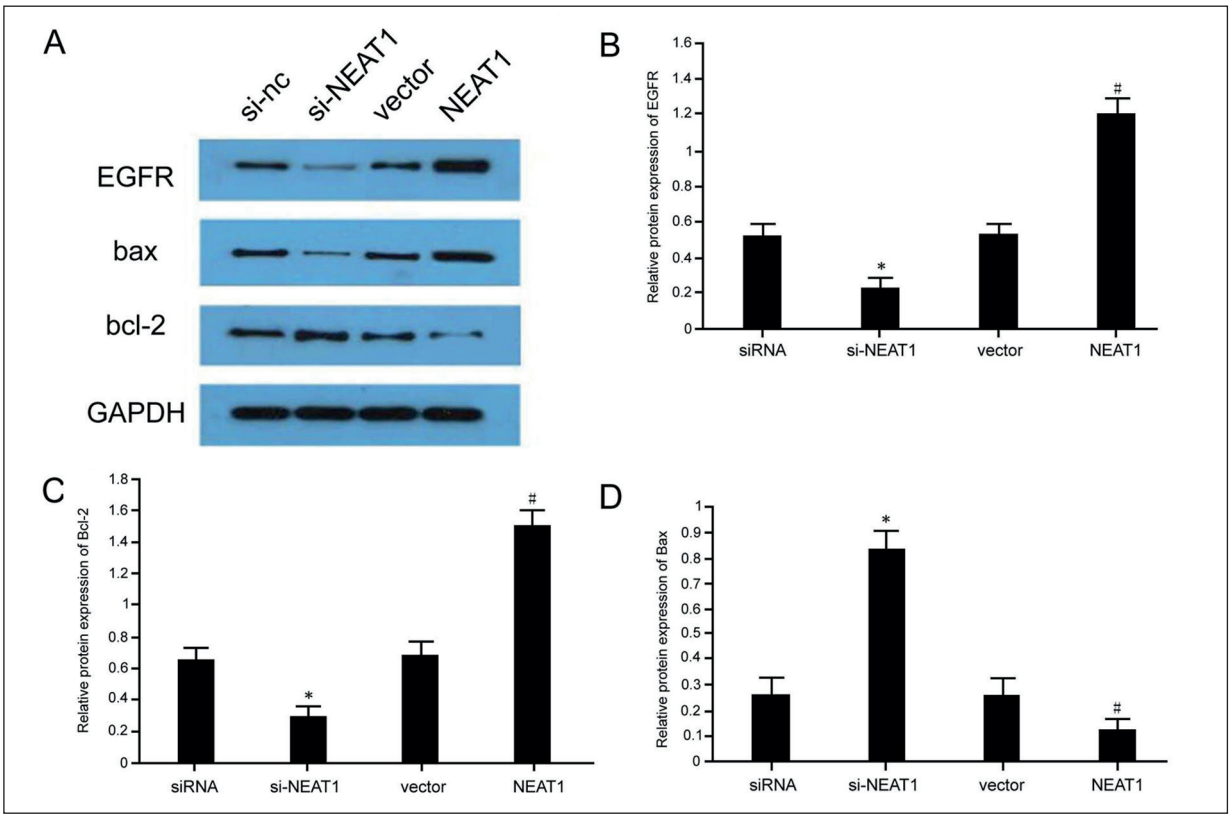

Figure 8. Effects of LncRNA-mediated NEAT1 on the expression of EGFR, Bax and Bcl-2 in hepatocellular carcinoma cells. (**A**) Western blot analysis of the regulation of LncRNA NEAT1 on the expression of EGFR and Bax and Bcl-2 in hepatoma cells; statistical analysis of EGFR expression (**B**); Bax expression (**C**) and Bcl-2 expression (**D**); compared with siRNA group, $^{*}p < 0.05$; compared with the vector group, $^{\#}p < 0.05$.

apoptosis[19-21]. EGFR is a member of the epidermal growth factor receptor (HER) family and belongs to the tyrosine kinase receptor. Overexpression of EGFR plays an important role in the progression of malignant tumors. Glial cells and kidneys EGFR are overexpressed in tissues such as cancer, lung cancer, prostate cancer, pancreatic cancer, and breast cancer. After overexpression, the pathway that controls cell proliferation, differentiation, and survival is activated[22,23]. This study confirmed that transfection of lncRNA NEAT1 siRNA to hepatoma cell HepG2 inhibited EGFR expression, thereby inhibiting tumor cell proliferation and invasion. Up-regulation of lncRNA NEAT1 expression promotes EGFR expression and promotes tumor cell proliferation and invasion.

Conclusions

Above all, lncRNA NEAT1 is overexpressed in liver cancer tissues. Down-regulation of LncRNA NEAT1 expression can inhibit EGFR expression and promote hepatoma cell apoptosis, inhibit cell cycle, and thus inhibiting tumor cell proliferation and invasion.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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