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Original Research Article

LncRNA NBAT-1 inhibits the progression of hepatocellular carcinoma by interacting with CYLD

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Abstract

Purpose: *To illustrate the biological influences of long non-coding RNA (lncRNA) NBAT-1 (neuroblastoma associated transcript 1) on HCC progression and the molecular mechanism of action.*
Methods: *NBAT-1 levels in HCC tissues and cell lines were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between NBAT-1 and prognosis in HCC was analyzed. After knockdown of NBAT-1 in HepG2 and Hep3B cells, proliferative and migratory changes were assessed by cell counting kit-8 (CCK-8) and Transwell assay, respectively. The interaction between NBAT-1 and CYLD was confirmed by subcellular fraction determination and RNA binding protein immunoprecipitation (RIP). Rescue experiments were conducted to verify the involvement of CYLD in HCC cell functions regulated by NBAT-1.*
Results: *NBAT-1 was downregulated in HCC tissues. Its level was much lower in metastatic or advanced stage HCC patients ($p < 0.05$), showing a certain prognostic potential. Knockdown of NBAT-1 stimulated proliferative and migratory potentials in HepG2 and Hep3B cells. NBAT-1 was mainly distributed in the cell cytoplasm. The mRNA and protein levels of CYLD were downregulated in HCC cells by NBAT-1 knockdown, displaying a positive interaction. CYLD was involved in the regulatory effect of NBAT-1 on HCC progression.*
Conclusion: *Through a positive interaction with CYLD, NBAT-1 inhibits the malignant progression of HCC. These findings provide a potential approach to the development of targeted therapies for HCC.*

Keywords: *Hepatocellular carcinoma, NBAT-1, CYLD, Cell proliferation, Cell migration*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent subtype of primary liver cancer. It is the sixth most-common, and the second most-fatal cancer in the world [1,2]. Chronic liver diseases, infection of hepatitis B virus (HBV), hepatitis C virus (HCV) or aflatoxin, and overconsumption of alcohol markedly increase the susceptibility to HCC [3]. HCC is featured by high level of malignancy, insidious onset and rapid aggravation. The detection rate of middle stage or advanced HCC is very high, leading to a poor prognosis [4].

In recent years, people have made great efforts on improving diagnostic and therapeutic strategies for HCC. Currently, there are many optional therapeutic strategies for HCC patients, including surgical resection, liver transplantation, percutaneous ethanol injection, radiofrequency ablation and transarterial chemoembolization [5]. However, the high rates of recurrence and metastasis, as well as drug resistance largely restrict clinical outcomes of HCC [6]. Therefore, to understand the potential mechanisms underlying the occurrence and progression of HCC is necessary to improve survival in HCC patients.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nt. They are differentially expressed in tissues and tumors, presenting epigenetic regulations. Previous studies have shown many tumor-associated lncRNAs involved in carcinogenesis [7,8]. LncRNAs are evolutionarily conserved, and they are able to regulate cell growth, differentiation and other biological functions [9,10]. In addition, lncRNA-protein interaction is of significance in affecting subcellular distribution and function of a protein. For example, lncRNA MALAT1 competitively binds PSF with the PSF/PTB complex, thus driving colorectal cancer growth and metastasis [11].

NBAT-1 (neuroblastoma associated transcript 1) is a tumor suppressor identified in neuroblastoma, which is able to inhibit proliferative, invasive and differentiation abilities. It is lowly expressed in neuroblastoma profiling, and closely linked to its prognosis [12]. NBAT-1 was later found to be downregulated in gastric cancer and renal cell carcinoma as a potential prognostic factor [13,14]. The expression pattern of NBAT-1 and its clinical significance in HCC are unclear.

Our findings showed that NBAT-1 was downregulated in HCC samples. Knockdown of NBAT-1 triggered HCC to proliferate and migrate *via* interaction with CYLD. This study demonstrated a novel mechanism underlying HCC metastasis, and provided a potential target for HCC intervention and treatment.

PATIENTS AND METHODS

Sample collection and ethics statement

Fresh cancer tissues and paracancerous ones were collected from 30 HCC patients. Samples were pathologically confirmed after surgery. This study was approved by the Ethics Committee of Tianjin First Center Hospital (no. 15-TJ-EC01-TJ-1206). The study was conducted by following the Declaration of Helsinki. Signed written informed consents were obtained from all participants before the study. Samples were immediately stored at -80 °C. All the patients did not receive any treatment before surgery, neither had a family history, that were pathologically diagnosed with HCC.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HCC samples and cell lines using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured by a spectrophotometer (NanoDrop™ ND-1000). Total RNA (1 μg) was reversely transcribed using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). QRT-PCR was performed using RNA-direct TMSYBR® GreenReal-time PCR Master Mix (Toyobo, Osaka, Japan). The amplified primers were shown in Table 1. Relative level was measured with the ABI 7900HT qRT-PCR system (Applied Biosystems, Foster City, CA, USA) and calculated by $2^{-\Delta\Delta Ct}$ method.

Table 1: Primers used in PCR

Gene		Primer sequence
NBAT-1	Forward	5'-GGAAAGCCTGTGCTCTTGGA-3'
	Reverse	5'-TCACAGTGCTGCTCAATCGT-3'
CYLD	Forward	5'-CACCAAGATGCCCAATACCA-3'
	Reverse	5'-CTTCAGCCAATGAGCCCACT-3'
GAPDH	Forward	5'-CTGCCAACGTGTCAGTGGTG-3'
	Reverse	5'-TCAGTGTAGCCCAGGATGCC-3'

Cell culture

The human immortalized normal hepatocyte cell line (LO2) and HCC cell lines (HepG2, Hep3B, Huh7, SMMC-7721) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in dulbecco's modified eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA), 100 μg/mL streptomycin and 100 U/mL penicillin (Sigma, St-Louis, MO, USA) in a humidified incubator containing 5 % CO_2 at 37 °C.

Cell transfection

Cells were inoculated in 6-well plates and cultured overnight. They were transfected with 50-100 nM plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Six hours later, fresh medium was replaced.

Cell counting kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 5 × 10^3 cells per well, and six replicates were set. At 24, 48 and 72 h, 10 µL of CCK-8 solution (Dojindo, Kumamoto, Japan) was applied per well for 1 h incubation and optical density at 450 nm was measured.

Transwell assay

Cells were collected and suspended in serum-free medium at density of 1-10×10^5 cells/ml. 100 - 200 µL of suspension and 500 µL of complete medium were respectively applied at the top and bottom chambers. After 24 h cell culture, transwell chambers were taken out. Cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Migratory cells were counted in 5 randomly selected fields per sample.

Subcellular fraction determination

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. U6 was the internal reference of nucleus and GAPDH was that of cytoplasm.

RNA binding protein immunoprecipitation (RIP)

The interaction between NBAT-1 and CYLD was tested using the Magna RIP Kit (Millipore, Billerica, MA, USA). Cells were lysed in radioimmunoprecipitation assay (RIPA) and incubated with magnetic beads conjugated with anti-CYLD or anti-IgG at 4°C for 6 h. Subsequently, magnetic beads were washed and incubated with Proteinase K for clearing proteins. Purified RNAs were subjected to qRT-PCR.

Statistical analysis

Statistical analysis was conducted using Statistical Product and Service Solutions (SPSS) 17.0 (IBM, Chicago, IL, USA). Data were expressed as mean ± SEM (Standard Error of Mean). Analysis of variance and independent sample *t*-test were conducted to assess differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

NBAT-1 is downregulated in HCC tissues

Expression of NBAT-1 in 30 pairs of HCC and paracancerous tissues were detected. QRT-PCR data showed lower levels of NBAT-1 in HCC tissues than normal ones (Figure 1 A). To analyze the relationship between NBAT-1 and clinical features in HCC, we detected differential levels of NBAT-1 in HCC patients classified by metastasis state and tumor stage. Lower level of NBAT-1 was detected in metastatic HCC patients than those of non-metastatic ones (Figure 1 B). Meanwhile, stage III-IV HCC patients expressed lower level of NBAT-1 than those of stage I-II patients (Figure 1 C). Furthermore, survival analysis uncovered that lowly expressed NBAT-1 predicted a poor prognosis in HCC (Figure 1 D). The above data suggested that NBAT-1 exerted a certain potential as HCC biomarker.

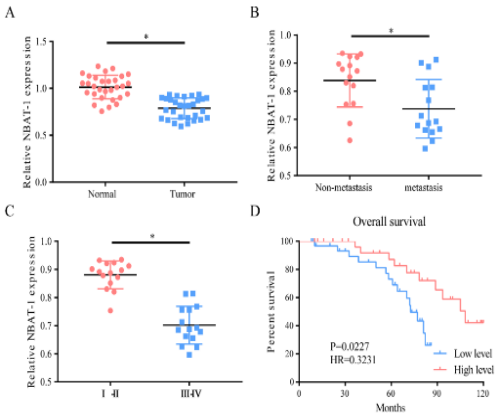

Figure 1: NBAT-1 is downregulated in HCC tissues. (A) NBAT-1 was downregulated in HCC tissues (n = 30) than paracancerous ones (n = 30); (B) NBAT-1 level was lower in metastatic HCC group than non-metastatic group; (C) NBAT-1 level was lower in stage III-IV HCC patients than those stage I-II HCC ones; (D) Kaplan-Meier curves indicated that low level of NBAT-1 predicted poor prognosis in HCC ($p = 0.0227$, HR = 0.3231); *$p < 0.05$

Knockdown of NBAT-1 promotes proliferative and migratory abilities in HCC

NBAT-1 levels in HCC cell lines and the human normal hepatocytes were determined. It is revealed that NBAT-1 was lowly expressed in HCC cell lines than that of LO2 cells (Figure 2 A). In particular, NBAT-1 level was much lower in HepG2 and Hep3B cells compared with other tested HCC cell lines, and they were used for the following experiments. Transfection of sh-NBAT-1 effectively downregulated NBAT-1 in HepG2 and Hep3B cells (Figure 2 B). CCK-8 assay showed that knockdown of NBAT-1 reduced viability in HCC cells (Figure 2 C and D). In addition, migratory cell number was higher in HCC cells with NBAT-1 knockdown (Figure 2 E). It is suggested that NBAT-1 inhibited proliferative and migratory abilities in HCC.

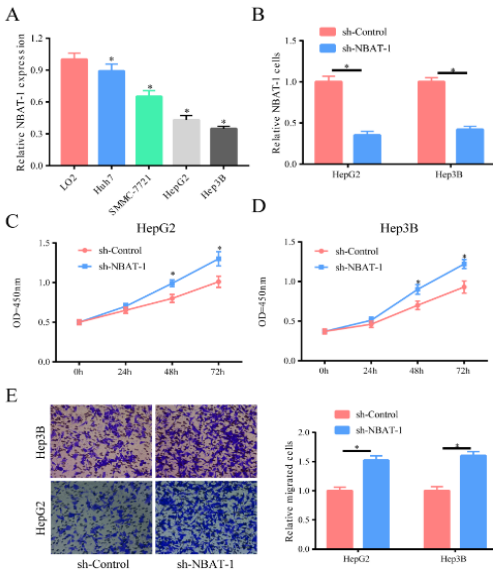

Figure 2: Knockdown of NBAT-1 promoted proliferative and migratory abilities in HCC. (A) NBAT-1 was downregulated in HCC cell lines; (B) NBAT-1 was effectively downregulated in HepG2 and Hep3B cells transfected with sh-NBAT-1; (C, D) Proliferation was enhanced by knockdown of NBAT-1 in HepG2 and Hep3B cells; (E) Migration was enhanced by knockdown of NBAT-1 in HepG2 and Hep3B cells. (magnification: 40×); *$p < 0.05$

NBAT-1 positively interacts with CYLD

To analyze the potential molecular mechanism of NBAT-1 in regulating HCC cell functions, we predicted potential targets of NBAT-1 through bioinformatics analysis and the tumor-suppressor gene CYLD was identified. Transfection of sh-NBAT-1 markedly downregulated mRNA level of CYLD in HepG2 and Hep3B cells (Figure 3 A and B). Similarly, the protein level of CYLD was downregulated by knockdown of NBAT-1 in HCC cells (Figure 3 C). We further detected expression pattern of CYLD in HCC tissues, which was downregulated compared with paracancerous ones (Figure 3 D). Pearson correlation test showed a positive correlation between NBAT-1 and CYLD levels in HCC tissues (Figure 3 E).

NBAT-1 exerts its biological functions through interacting with CYLD

It was found that NBAT-1 was mainly distributed in the cytoplasm of HepG2 and Hep3B cells (Figure 4 A and B). Furthermore, RIP assay confirmed that NBAT-1 could interact with CYLD (Figure 4 C). Results indicated that the role of NBAT-1 in regulating HCC cell functions was required for the interaction with CYLD. Transfection efficacy of pcDNA-CYLD was tested in HCC cells co-transfected with sh-NBAT-1 (Figure 4 D). Notably, overexpression of CYLD partially abolished the increased viability in HepG2 and Hep3B cells with NBAT-1 knockdown (Figure 4 E). Therefore, NBAT-1 regulated HCC cell viability by positively interacting with CYLD.

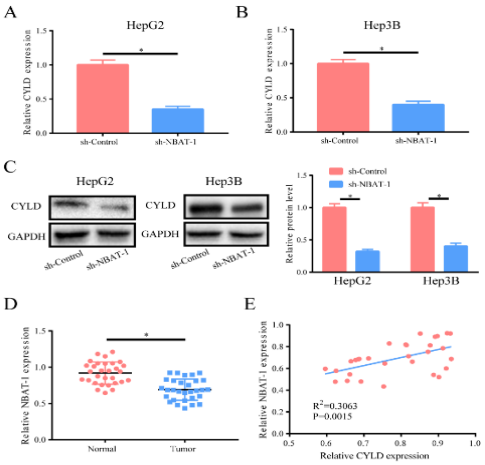

Figure 3: NBAT-1 positively interacts with CYLD. (A, B) The mRNA level of CYLD was downregulated in HepG2 and Hep3B cells transfected with sh-NBAT-1; (C) The protein level of CYLD was downregulated in HepG2 and Hep3B cells transfected with sh-NBAT-1; (D) CYLD was downregulated in HCC tissues (n = 30) than paracancerous ones (n = 30); (E) Pearson correlation test showed a positive correlation between NBAT-1 and CYLD levels in HCC tissues ($R^2 = 0.3063$, $p = 0.0015$); *$p < 0.05$

DISCUSSION

The carcinogenesis and cancer progression of HCC involve diverse factors, pathways and mutations. The pathogenesis of HCC remains unclear. It is considered that hepatocyte injury, inflammatory response, genomic instability and abnormal signaling are all risk factors for HCC. Molecular biology research has improved our understanding of the pathogenesis of HCC and promoted the development of targeted therapy, creating a novel therapeutic strategy for HCC instead of the conventional systematic treatment.

LncRNAs are distributed in the cytoplasm or nucleus. Their subcellular distributions determine different regulatory patterns on genomes. In recent years, a growing number of studies have identified the role of lncRNAs in tumor metastasis through downstream gene transcription, mRNA splicing and post-transcriptional modification [16]. Our study uncovered that NBAT-1 was downregulated in HCC samples. *In vitro*

experiments demonstrated that knockdown of NBAT-1 markedly stimulated proliferative and migratory abilities in HCC cells. It is suggested that NBAT-1 was involved in the malignant progression of HCC as a tumor suppressor.

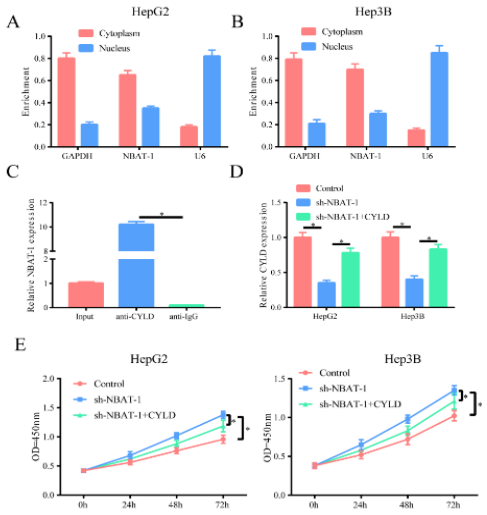

Figure 4: NBAT-1 exerts its biological functions through interacting with CYLD. (A, B) NBAT-1 was mainly distributed in the cytoplasm of HepG2 and Hep3B cells; (C) RIP assay showed the interaction between NBAT-1 and CYLD; (D) Co-transfection of sh-NBAT-1 and pcDNA-CYLD could abolish the downregulated CYLD in HepG2 and Hep3B cells transfected with sh-NBAT-1; (E) Co-transfection of sh-NBAT-1 and pcDNA-CYLD could abolish the increased viability in HepG2 and Hep3B cells transfected with sh-NBAT-1; *$p < 0.05$

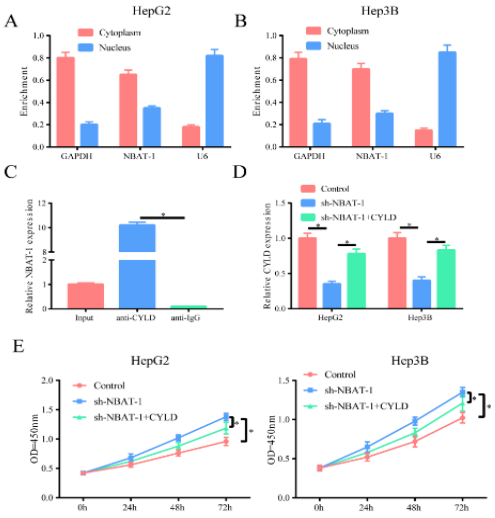

Figure 4: NBAT-1 exerts its biological functions through interacting with CYLD. (A, B) NBAT-1 was mainly distributed in the cytoplasm of HepG2 and Hep3B cells; (C) RIP assay showed the interaction between NBAT-1 and CYLD; (D) Co-transfection of sh-NBAT-1 and pcDNA-CYLD could abolish the downregulated CYLD in HepG2 and Hep3B cells transfected with sh-NBAT-1; (E) Co-transfection of sh-NBAT-1 and pcDNA-CYLD could abolish the increased viability in HepG2 and Hep3B cells transfected with sh-NBAT-1; *$p < 0.05$

Some HCC-associated lncRNAs exert vital functions in carcinogenesis *via* the formation of ribonucleoproteins. NF90 is a double-strand RNA-binding protein, which is functional in mediating mRNA stabilization, transportation and translation. Silence of lncRNA-LET in HCC cells can stabilize NF90, which further induces hypoxia and cancer cell metastasis [17]. LncRNA-PVT1 drives HCC cell growth and cell cycle progression by stabilizing NOP2 [18]. By blocking the interaction between HOTAIR and PRC2 or LSD1, the metastatic potential in breast cancer markedly decreases [19]. LncRNA-protein interaction exerts either oncogenic or tumor-suppressive function, which may be utilized for developing targeted therapy for cancers.

Bioinformatics analysis showed the interaction between NBAT-1 and CYLD (Cylindromatosis). CYLD is a ubiquitinated and microtubule binding protein. It is the vital regulator in immune response, cell proliferation, and inflammatory cascade through catalyzing activities of multiple key intermediates of ubiquitination. A tumor-suppressive gene. CYLD mutation is the major cause for hereditary familial cylindroma dermatosis [20]. In addition, CYLD is downregulated in many other types of tumors [21]. After transfection of sh-NBAT-1 in HCC cells, we detected the mRNA and protein levels of CYLD. The results showed that CYLD was downregulated after knockdown of NBAT-1. Meanwhile, RIP assay confirmed the interaction between CYLD and NBAT-1. In HCC tissues, CYLD was downregulated and positively correlated to NBAT-1 level. Overexpression of CYLD abolished the enhanced viability in HCC cells with NBAT-1 knockdown. To sum up, NBAT-1 inhibited HCC progression by positively interacting with CYLD.

CONCLUSION

NBAT-1 and CYLD are downregulated in HCC tissues. The low level of NBAT-1 is related to the progression of HCC. The interaction between NBAT-1 and CYLD is responsible for inhibiting the malignant proliferative and migratory activities in HCC. These findings not only provide an insight into the mechanisms operating in HCC, but also provide a potential approach to the development of targeted therapies for HCC.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jisan Sun and Guoyao Li contributed equally to this work.

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