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Effect of downregulated lncRNA NBAT1 on the biological behavior of glioblastoma cells

J. LIU[1], W.-M. WANG[1], X.-L. ZHANG[1], Q.-H. DU[1], H.-G. LI[1], Y. ZHANG[2]

[1]Department of Neurosurgery, The Sixth Affiliated Hospital of Wenzhou Medical University, The People's Hospital of Lishui, Lishui, Zhejiang, P.R. China
[2]Department of Neurosurgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, P.R. China

Abstract. – OBJECTIVE: To investigate the expression of lncRNA neuroblastoma associated transcript 1 (NBAT1) in human glioma cell lines and its underlying mechanism. Effect of NBAT1 on biological behaviors of T98 and U87 cells are also explored.

PATIENTS AND METHODS: The mRNA expressions of NBAT1 in 48 cases of glioblastoma tissues and 30 cases of normal brain tissues were accessed by Real-time fluorescence quantitative PCR (RT-PCR). The relationship between mRNA expression of NBAT1 and tumor size, malignancy, and prognosis were analyzed. Effects of NBAT1 on the proliferation of glioblastoma T98 and U87 cells were determined by CCK-8 assay and colony formation assay, respectively.

RESULTS: NBAT1 expressions in glioblastoma tissues were lower than those in normal brain tissues, which was negatively correlated with malignancy degree ($p<0.01$). Protein levels of Akt were decreased in T98 and U87 cells transfected with si-NBAT1. Meanwhile, proliferation abilities of T98 and U87 cells transfected with si-NBAT1 were significantly decreased as well ($p<0.01$), which were reversed by transfection of si-Akt.

CONCLUSIONS: Upregulated NBAT1 inhibits proliferation of T98 and U87 cells via regulating Akt, indicating that NBAT1 may be related to the malignancy and prognosis of gliomas.

Key Words
Glioma, LncRNA, Neuroblastoma associated transcript 1.

Introduction

Glioma is the most common primary malignancy in the adult central nervous system, accounting for more than 40% of all primary intracranial tumors. Although the traditional treatment of gliomas in recent years have made marked progress, including surgical resection, radiotherapy, and chemotherapy, the overall prognosis of patients with glioma is still unsatisfactory. It has been recognized that the occurrence and development of glioma are regulated by multi-molecular processes. A large number of molecules have been confirmed to be involved in the process of glioma development. The exact molecular mechanism, however, remains unclear. Therefore, it is of great importance to explore the molecular mechanism of the glioma development. Searching for biomarkers that can predict the prognosis of gliomas and new therapeutic strategies are urgently needed.

Long non-coding RNA (lncRNA) is a type of RNA with over 200 nucleotides in length, which doesn't encode proteins. LncRNA was originally thought to be transcriptional noise; recent studies, however, have shown that lncRNA exerted a vital regulatory role in human diseases, including cancer[1]. As a transcriptional regulator, lncRNA is involved in the regulation of gene expression through transcriptional interference and chromatin remodeling[2,3]. Under the transcriptional level, promoter region of lncRNA is expressed by direct binding of transcription factors, such as nuclear factor κB, c-Myc, and Smad family[4]. In addition, lncRNA alters gene transcription by base-pairing and splicing factors. It also regulates gene expression by forming a double-stranded chain with mRNA through a series of regulatory processes, such as processing and modification[5]. Additionally, lncRNA induces epigenetic modifications at chromatin-specific sites by recruiting chromatin complexes. For example, HOTAIR recruits polycomb repressive complex 2 (PRC2) to interact with HOXD site binding, thereby inducing HOXD epigenetic silencing, and inhibiting transcription. LncRNA can regulate downstream gene expressions at multiple levels, including pre-transcriptional, post-transcriptional and epigenetic levels. Recent studies indicated that lncRNA exerted a vi-

Corresponding Author: Yu Zhang, MD; e-mail: drzhyu@126.com

tal regulatory role in embryonic pluripotency, tissue homeostasis, biological processes, and cancer progression[6]. Therefore, we speculate that lncRNA may become a new target of tumor gene therapy. So far, studies have confirmed that lncRNA was involved in the development of neuroblastoma, which significantly affects the malignancy degree and pathogenesis of neuroblastoma.

NBAT1 is down-regulated in neuroblastoma and correlates with the disease prognosis. It inhibits the proliferation, invasion, and differentiation of neuroblastoma[7]. Subsequent studies found that NBAT1 is also down-regulated in breast cancer and renal cell carcinoma, which is correlated with clinicopathological parameters and disease prognosis in cancer patients[8,9]. However, the effect of NBAT1 on predicting and treating glioma remains unclear. In this study, we detected NBAT1 expressions in glioma tissues by RT-PCR and analyzed its relationship with the clinicopathological features of patients. Our study provides a theoretical reference for the pathogenesis and diagnosis of glioma.

Patients and Methods

Clinical Samples

48 glioma tissue samples with different grades were surgically resected in the People's Hospital of Lishui from 2014 to 2016. These patients did not receive any preoperative radiotherapies and chemotherapies (shown in Table I). Part of samples were put into formalin for immunohistochemistry and the remaining were immediately put into liquid nitrogen tank for long-term preservation. The samples were pathologically confirmed as gliomas. Two pathologists from our hospital independently classified the tumor pathology and grading according to the WHO Central Nervous System Tumor Grading Standard (CNS) in 2007. Low-grade gliomas (LGG) was defined as WHO I-II, and high-grade glioma (HGG) was WHO III-IV. Normal brain tissue samples were obtained from 30 patients with open craniocerebral trauma caused by car accidents and without any congenital disease. This study was approved by the Ethics Committee of People's Hospital of Lishui. Signed written informed consents were obtained from the patients and/or guardians.

Cell Lines, Cell Culture and Transfection

Glioma cell lines, including SVGP12, U251, U87, U373, T98, and LZ229, were purchased from the Shanghai Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) complete medium containing 10% fetal bovine serum (FBS) and streptomycin (Gibco, Rockville, MD, USA) and the cell status was observed daily. The cell culture medium was changed every 2-3 days according to the cell

Table I. Correlation between NBAT1 expression and clinicopathological features in patients with glioblastoma (n=48).

Clinicopathologic features	Number of cases	lncRNA NBAT1 expression		*p*-value
		Low (n=24)	High (n=24)	
Age (years)				0.1457
<50	27	11	16	
≥50	21	13	8	
Gender				0.5623
Male	22	10	12	
Female	26	14	12	
Histological type				0.5623
Neuroastrocytoma	26	12	14	
Oligodendroglioma	22	12	10	
Tumor size				0.0088*
<5CM	21	6	15	
≥ 5CM	27	18	9	
WHO stage				0.0204*
I-II	26	9	17	
III-IV	22	15	7	
Lymph node metastasis				0.3827
Absent	27	15	12	
Present	21	9	12	

*$p<0.05$

growth state. The culture plate was placed in a 5% CO_2 incubator at 37°C. Glioma cells in logarithmic growth phase were inoculated into 6-well plates. Cell transfection was performed according to the instructions of Lipofectamine 2000 reagent when cell confluence was up to 80%.

Real-Time Fluorescence Quantitative PCR Reaction

The synthesized cDNA was used as template to amplify NBAT1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes. Primer sequences were as follows: NBAT1, F: 5'-GGAAAGCCTGTGCTCTTGGA-3', R: 5'-TCACAGTGCTGCTCAATCGT-3'; Akt, F: 5'-GGAAAGCCTGTGCTCTTGGA-3', R; 5'-TCACAGTGCTGCTCAATCGT-3; GAPDH, F: 5'-ACCACAGTCCATGCCATCAC-3', R: 5'-TCCACCCTGTTGCTGTA-3'. Reverse transcriptase-polymerase chain reaction (RT-PCR) reaction system was 25 μL, and the reaction conditions were: predenaturation at 95°C for 30 s, followed by denaturation at 95°C for 3 s and renaturation at 60°C for 30 s, for a total of 40 cycles. Three independent PCR amplifications were performed for each experiment, and the transcriptional level of NBAT1 was normalized using the internal standard GAPDH. Results were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Detection of Cell Viability

The proliferative activities of T98 and U87 cells were determined by cell counting kit-8 (CCK-8) after transfection for 6 h, 24 h, 48 h, 72 h, and 96 h, respectively. T98 and U87 glioma cells were digested with 0.25% trypsin for cell counting. 5×10^3 cells were seeded into a 96-well plate, with 6 reduplicated wells in each group. Cells were cultured in a 5% CO_2 incubator at 37°C and transfected with pcDNA-NC or pcDNA-NBAT1 after 12-16 h, respectively. 48 h after transfection, 90 μL of fresh medium containing 10% fetal bovine serum and 10 μL of CCK-8 were simultaneously added to each well for incubating for 2-4 h. The absorbance value (OD value) of each well at 450 nm was determined by immunopotentiator. The experiments were repeated 3 times independently.

Colony formation assay was used to determine long-term cell proliferation. T98 and U87 cells transfected with pcDNA-NC or pcDNA-NBAT1 were inoculated into 6-well plates at a dose of 100 cells per well. Dishes were taken out after cell clones in each well were over 50. Numbers of cell clones were counted after staining with crystal violet.

Western Blot

Glioma tissue or normal brain tissue was lysed with 0.5 mL of Lysis Buffer on ice (Beyotime, Co., Ltd. Shanghai, China) to prepare the tissue homogenate. The homogenate was then transferred to a 1.5 mL centrifuge tube and centrifuged at 12,000 × g at 4°C for 10 min. The supernatant was transferred to a precooled centrifuge tube. Bicinchoninic acid (BCA) method was utilized for the determination of protein concentration. Protein samples were equilibrated, heated and denatured for subsequent experiments. For cell protein sample preparation, T98 and U87 cells transfected with pcDNA-NC, pcDNA-NBAT1 or pcDNA-Akt were digested and washed twice with phosphate buffered saline (PBS). Cells were then centrifuged at 13,000 × rpm at 4°C for 15 min. The supernatant was transferred to a new prechilled centrifuge tube. The protein sample (80 μg) and the standard protein marker were sequentially added for electrophoresis. Protein bands were transferred to a nitrocellulose membrane (NC membrane). After blocked with Tris-buffered saline and Tween (TBS-T) formulated in 5% skimmed milk, primary antibody (Akt, CST Company, Danvers, MA, USA) was utilized for incubation overnight at 4°C. After washed with TBS-T, the secondary antibody was used for incubation for 2 h at room temperature, followed by the image exposure.

Statistical Analysis

We used statistical product and service solutions (SPSS18.0, SPSS Inc., Chicago, IL, USA) statistical software for statistical analysis. GraphPad Prism 5 (La Jolla, CA, USA) and Image-Pro 6.0 (Silver Springs, MD, USA) were introduced for image editing. Statistical data were expressed as mean ± standard deviation. Pearson correlation coefficient method was used to access the correlation between NBAT1 and Akt expression in normal brain tissue and glioma tissue. NBAT1 expression in different glioma cell lines was determined by single factor variance analysis. The Student's *t*-test was used to compare data between the two groups. $p<0.05$ was considered as statistically significant.

Results

NBAT1 is Downregulated in Glioblastoma

Lower mRNA levels of NBAT1 were found in 48 glioblastoma tissues than those of 30 normal brain tissues. Moreover, NBAT1 expression in high-grade gliomas (WHO III-IV) was sig-

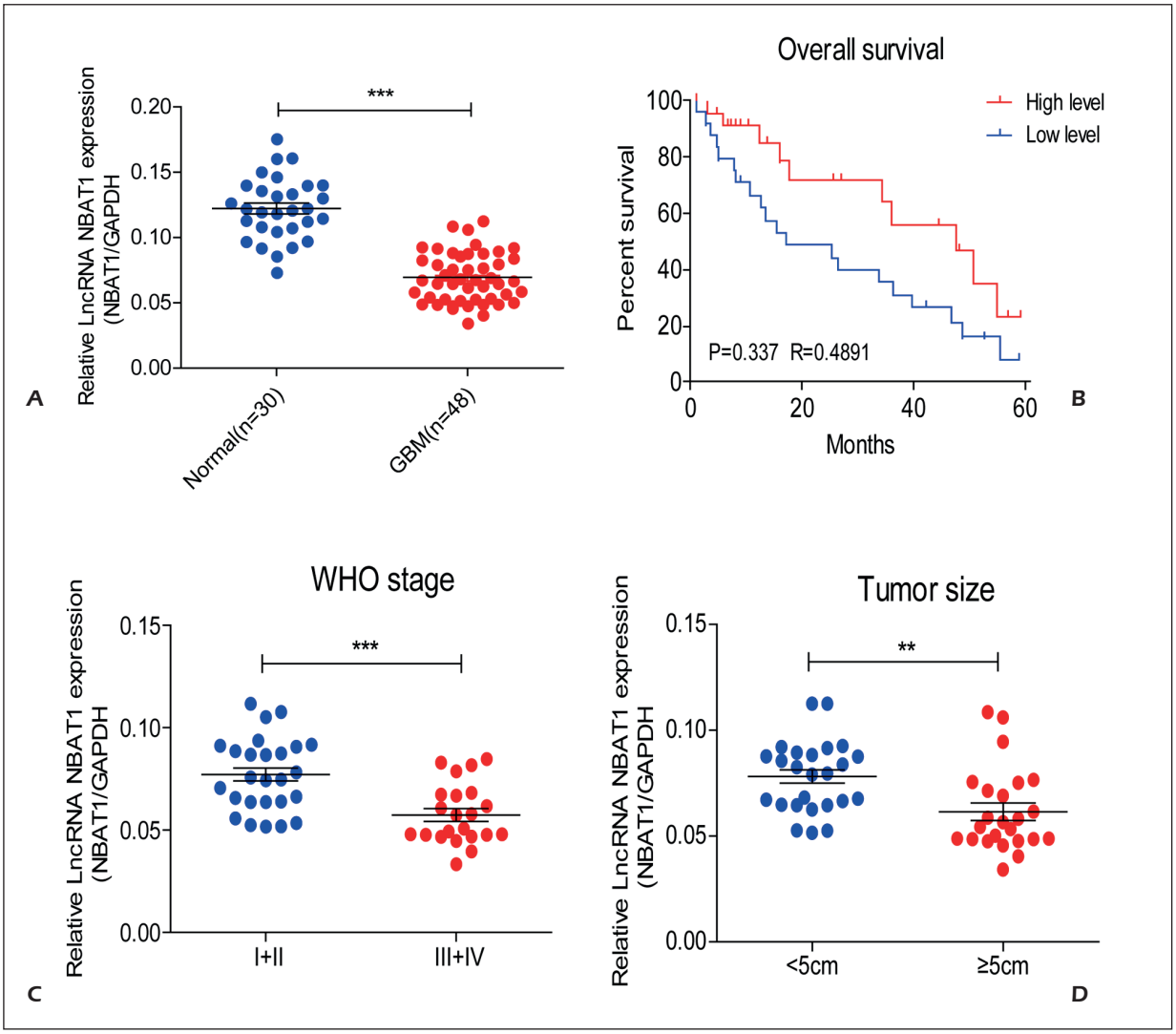

Figure 1. NBAT1 is down-regulated in gliomas. **A**, NBAT1 expressions in 48 glioblastoma tissues were lower than those of 30 normal brain tissues. **B**, The overall survival rate of glioblastoma patients with higher NBAT1 expression was significantly higher than those with lower expression. **C**, The expression of NBAT1 in stage III-IV glioblastoma was lower than that of stage I-II glioblastoma. **D**, NBAT1 expression in glioblastoma tissues larger than 5 cm was higher than those less than 5 cm.

nificantly lower than that of low-grade gliomas (WHO I-II). In addition, NBAT1 expression in glioblastoma tissues larger than 5 cm was significantly lower than that of glioma tissues less than 5 cm. Further analysis found that the overall survival rate in glioblastoma patients with higher NBAT1 expression was apparently higher than those with lower expression (Figure 1).

Overexpression of NBAT1 Inhibits the Proliferative Activity in Glioblastoma Cells

The mRNA expressions of NBAT1 in glioma cell lines were lower than that in normal glial cell line, among which, T98 and U87 cells had the lowest levels (Figure 2A). Therefore, we selected T98 and U87 cells for the following experiments. Transfection of pcDNA-NBAT1 plasmid in T98 and U87 cells significantly increased NBAT1 expressions (Figure 2B and 2C). Overexpressed NBAT1 in T98 and U87 cells attenuated the viability (Figure 2D and 2E), as well as the colony formation ability of glioma cells (Figure 2F).

Akt is Involved in the Glioma Cell Proliferation Regulated by NBAT1

Akt was overexpressed in glioma tissue compared to that of normal brain tissue (Figure 3A). Pearson correlation analysis illustrated that NBAT1 was negatively correlated with Akt in

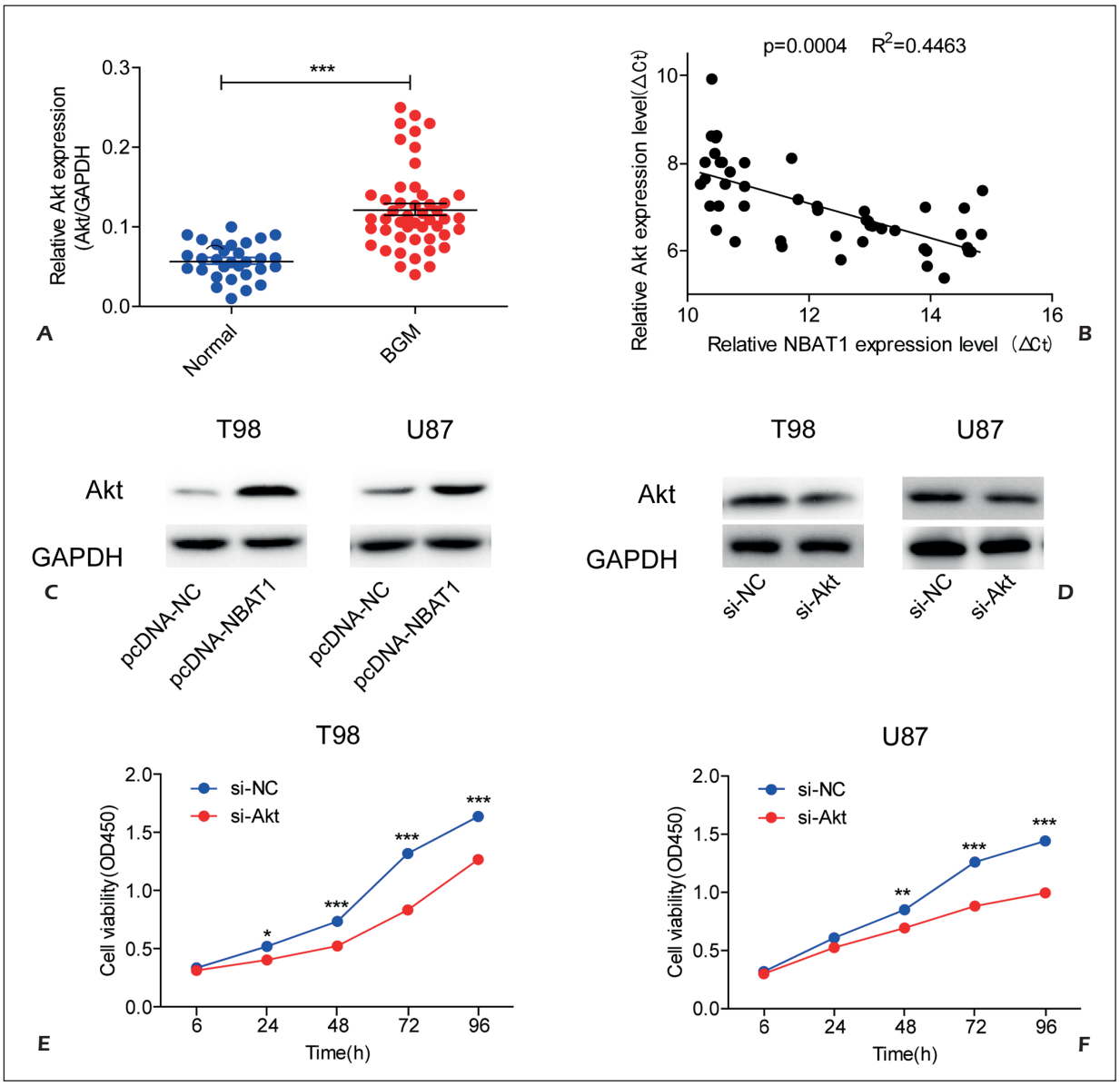

Figure 2. NBAT1 overexpression inhibits glioblastoma cell proliferation. **A**, Expression of NBAT1 in brain normal and cancer cell lines (SVGP12, U251, U87, U373, T98, LZ229). **B**, Transfection of pcDNA-NBAT1 in T98 cell line. **C**, Transfection of pcDNA-NBAT1 in U87 cell line. **D**, CCK-8 results showed that cell viability decreased after NBAT1 overexpression in T98 cell line. **E**, CCK-8 results showed that cell viability decreased after NBAT1 overexpression in the U87 cell line. **F**, Colony formation assay showed that after knockdown of NBAT1, glioblastoma cell proliferation was inhibited.

clinical samples (Figure 3B). Akt expression was decreased after upregulating NBAT1 in T98 and U87 cells (Figure 3C). Therefore, we detected the effect of Akt on cell proliferation. We first constructed si-RNA Akt (si-Akt), and the transfection efficacy of si-Akt in T98 and U87 cells were shown in Figure 3D. Furthermore, inhibition of cell proliferation by overexpression of NBAT1 in glioma cells can be reversed by Akt knockdown (Figure 3E, 3F).

Discussion

Intracranial malignancy is one of the most lethal adult tumors in the world. Glioma accounts for about 32% of all primary CNS tumors and 81% of CNS malignancies, which is the most common primary intracranial tumor. Malignant glioma is characterized as fast growth, poor prognosis and high recurrence rate. Despite the great advances made in clinical surgery, postoperative

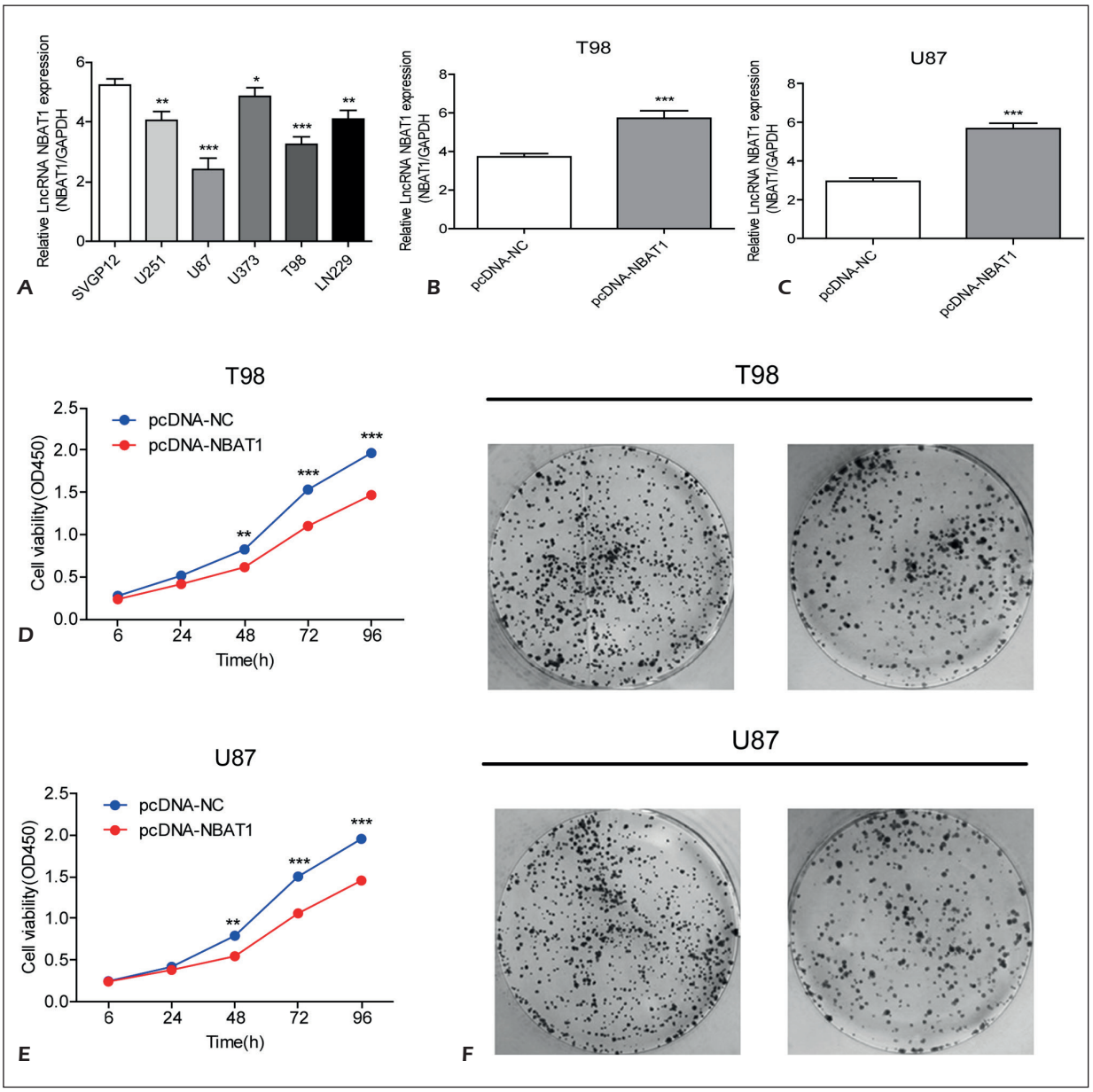

Figure 3. Akt is upregulated in glioblastoma tissues and is regulated by NBAT1. **A**, Akt expression in glioblastoma tissue was higher than that of normal brain tissue. **B**, Pearson correlation analysis showed that NBAT1 and Akt were negatively correlated in clinical samples. **C**, In T98 and U87 cell lines, Akt protein level decreased after NBAT1 was overexpressed. **D**, In T98 and U87 cell lines, Akt protein levels decreased after knockdown of Akt. **E**, In T98 cell line, cell viability decreased after knockdown of Akt. **F**, In the U87 cell line, cell viability was reduced after knockdown of Akt.

radiotherapy and chemotherapy in recent years, the median survival of glioblastoma patients who are newly diagnosed is only 12-18 months[10]. Chemotherapy with temozolomide may prolong patients' survival. However, most patients will relapse within two years[11]. Therefore, it is urgent to explore the molecular mechanism of glioma, so as to improve the diagnosis and treatment efficacy. Studies have showed that 80% of human genome transcriptions were associated with lncRNAs[2]. However, only a small percentage of lncRNAs have been shown to exert biological function[13]. Most of these biologically functional lncRNAs are associated with malignancies[14,15]. LncRNAs were initially considered as by-products or dark matters of gene transcription. Current studies have now pointed out that lncRNAs participate in the tumorigenesis and metastasis of tumor cells

through various pathways. LncRNA NBAT1 is downregulated in neuroblastoma, which is capable of inhibiting the proliferation, invasion and differentiation of neuroblastoma[16]. It has been found that down-regulated NBAT1 may be explained for the pathogenesis of neuroblastoma and breast cancer[8,13]. Moreover, NBAT1 knockdown can activate the neuronal-restrictive silencer factor (NRSF)/repressor element silencing transcription factor (REST) and thus affecting the neuronal differentiation[17]. NBAT1 also inhibits its breast cancer metastasis by regulating DKK1 (Dickkopf-1) expression via Wnt pathway[8]. However, the role of NBAT1 in the pathogenesis and progression of glioma is still unclear.

We found that the expression level of NBAT1 in glioma samples was lower compared with that of normal brain tissue, which is negatively correlated with glioma grade. The results indicated that there was a correlation between NBAT1 and the malignant potential of gliomas. NBAT1 knockdown may promote the progression of glioma. Further analysis of NBAT1 and glioma prognosis found that glioblastoma patients with higher expression of NBAT1 presented a significantly higher overall survival rate than those with lower expression. In the present study, NBAT1 was overexpressed in T98 and U87 cells using plasmid transfection technique. The results illustrated that NBAT1 might inhibit the development of glioma cells. Pearson correlation analysis confirmed that NBAT1 is negatively correlated with Akt in clinical samples. Akt expression was decreased after overexpression of NBAT1 in T98 and U87 cells, suggesting that Akt may be involved in the proliferation of glioma cells regulated by NBAT1. Overexpression of Akt in T98 and U87 cells reversed the inhibitory effect of NBAT1 on cell viability, suggesting that NBAT1 regulates glioma cell proliferation *via* Akt. However, whether there are other pathways that participate in the biological regulation of gliomas remains to be further studied.

Our results demonstrated that NBAT1 may function as tumor suppressor in glioma. NBAT1 expression was closely related with tumor size, degree of malignancy and prognosis of glioma. Hence, we considered that NBAT1 could be served as a molecular marker to evaluate malignant degree and prognosis of glioma. In addition, overexpression of NBAT1 in glioma cells significantly inhibited cell viability, suggesting that lncRNA may be the target agents in treating gliomas.

Conclusions

We showed that NBAT1 was related to the malignancy and prognosis of gliomas. Upregulation of NBAT1 inhibited proliferative activities of T98 and U87 cells via regulating Akt

Conflict of Interest:

The authors declared no conflict of interest.

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