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Original Article

Decreased expression of long noncoding RNA NBAT1 indicates a poor prognosis and promotes cell proliferation and EMT in bladder cancer

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Abstract: Objective: Dysregulation of long non-coding RNAs (lncRNAs) may exert critical roles in tumor progression in human. In the current research, we focused on lncRNA NBAT1 and explored its expression pattern and biological function in bladder cancer (BCa). Methods: NBAT1 expression in 79 BCa tissues was assessed through quantitative Real-time PCR (qRT-PCR) and its correlation with clinicopathological features and prognosis was later analyzed. Moreover, *in vitro* assays, including MTT assay, transwell migration and invasion assays, Annexin V-binding assay and Cleaved Caspase-3 ELISA assay were performed to measure the effect of NBAT1 overexpression in BCa cell lines. Protein levels of epithelial-mesenchymal transition (EMT) markers were determined by western blot. Results: NBAT1 expression was remarkably decreased in BCa tissues and cell lines, and NBAT1 levels were closely associated with tumor grade, tumor stage, and unfavorable overall survival. Besides, the results of *in vitro* experiments demonstrated that up-regulation of NBAT1 significantly inhibited BCa cell proliferation, migration, invasion, and promoted cell apoptosis. Overexpression of NBAT1 was observed to suppress EMT through the regulation of E-cadherin and N-cadherin expression. Conclusion: Our present study suggested that lncRNA NBAT1 might be a novel molecule involved in BCa progression, which provide a potential diagnostics or predictive biomarker and therapeutic approach.

Keywords: Bladder cancer, NBAT1, metastasis, prognosis, EMT

Introduction

Around the world, bladder cancer (BCa) is one of the leading malignancies with about 386,300 new cases and 150,200 deaths every year [1, 2]. As a heterogeneous malignancy, BCa could be mainly categorized into the low-grade papillary tumors and the high-grade invasive tumors [3]. Patients with invasive bladder tumor usually have a much worse prognostic outcome, with a five-year survival rate at only 50%-60% [4]. Despite the current achievements in the early diagnosis and surgical therapy, the overall survival time of BCa patients has not been improved remarkably. Accordingly, it is of vital importance to clarify novel molecular and cellular mechanisms involved in the development of BCa for the establishment of novel diagnostic indicators and therapeutic approaches.

The rapid development of whole-genome and transcriptome sequencing technologies has highlighted the critical role of long non-coding RNAs (lncRNAs) in a wide variety of human diseases, especially in carcinogenesis [5, 6]. LncRNAs with more than 200 nucleotides in length are extensively identified as a diverse class of transcripts with a limited or no protein-coding capacity that modulate several signaling pathways to serve oncogenic or tumor suppressive roles various types of carcinomas [7-9]. Moreover, in clinic, lncRNAs can be used with potential value in cancer diagnosis and prognosis and function as promising therapeutic targets [10, 11]. For example, Chen et al. revealed that a group of patients with up-regulated lncRNA-n336928 (noncode database ID: n336928) expression in BCa might have a relatively unfavorable prognosis [12].

Table 1. Correlation between NBAT1 expression and clinicopathological characteristics of bladder cancer patients

Characteristics	Total number	NBAT1 expression		*P* value
		Low (n=34)	High (n=45)	
Age				0.807
<60	50	21	29	
≥60	29	13	16	
Gender				0.580
Male	46	21	25	
Female	33	13	20	
Tumor size				0.179
<3 cm	44	16	28	
≥3 cm	35	18	17	
Tumor grade				0.043
G1	31	9	22	
G2, G3	48	25	23	
Tumor stage				0.027
T1, T2	27	7	20	
T3, T4	52	27	25	
Lymph nodes metastasis				0.073
No	57	21	36	
Yes	22	13	9	

In accordance to the literature, neuroblastoma associated transcript 1 (NBAT1), located on chromosome 6p22, is a recently identified lncRNA that was originally identified to control neuroblastoma development through modulating cell proliferation and neuronal differentiation [13]. Xue et al. also suggested that NBAT1 is a novel molecular correlated with poor prognosis in patients with clear cell renal cell carcinoma [14]. However, to date, the relationship between NBAT1 expression and BCa is completely elusive. In the present article, we aimed to detect NBAT1 expression pattern in BCa tissues and cell lines and evaluated its association with clinicopathological features of 79 BCa patients. Further experiments illustrated the role of NBAT1 in cell proliferation and apoptosis of BCa cell lines, which might open up a novel approach for treating BCa in the near future.

Materials and methods

Patients and specimens

Paired BCa tissues and corresponding non-neoplastic bladder mucosal tissues, which were collected from more than 5 cm away from the tumors, were obtained from a total of 79 BCa patients who received surgery in the Department of the second people's Hospital of Three Gorges University (Yichang, China). None of the patients had undergone preoperative treatment, including chemotherapy or radiotherapy. After surgical resection, all collected tissue samples were snap-frozen in liquid nitrogen immediately and stored at -80°C until further process. Data were retrieved from their operative and pathological reports, and follow-up data were obtained by our clinical database. Clinical pathology information of all patients was summarized in **Table 1**. The research was approved by the Clinical Experiment Ethical Committee of the second people's Hospital of Three Gorges University, and written informed consent from each participant was also obtained.

Cell lines and cell culture

Three human BCa cell lines (J82, T24 and SW780), and a normal human uroepithelial cell line (CRL-9520) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco) at 37°C with a humidified atmosphere of 5% CO_2 in incubator. Cells at low to passage 10 were applied for the subsequent trials.

Cell transfection

The expression vector pcDNA3.1 (+) vector (Transheep, Shanghai, China) that express NBAT1 (pcDNA3.1-NBAT1) and a scrambled negative control (pcDNA3.1-NC) were constructed by Hanhen Co. Ltd. (Shanghai, China). After culture in antibiotic-free DMEM for 24 h and cell confluency reaching over 80%, the cells were transfected with matched pcDNA using Lipofectamine™ 2000 transfection kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from BCa tissues and cell lines using a standard protocol with TRIzol

reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration was determined by a NANO DROP spectrophotometer (ND-1000, Thermo Scientific, USA), and the integrity of RNA was investigated by agarose gel electrophoresis (2% agarose gel). Reaction mixture (20 μl) containing 2 μg of DNA-free total RNA was reversely transcribed to complementary DNA (cDNA) by using a Reverse Transcription Kit (Takara, Dalian, China). qRT-PCR analyses were performed with Power SYBR Green (Takara) on an Applied Biosystems 7900HT (Applied Biosystems, Grand Island, NY, USA). The primer sequences synthesized by Sangon Biotech, Co., Ltd (Shanghai, China) were listed as follows: NBAT1, Forward 5'-GCTCTACATGACGGGAAAGC-3' and Reverse 5'-AAGCAGCCTCTGATCCATGA-3'; GAPDH, Forward 5'-ACAGTCAGCCGCATCTTCTT-3' and Reverse 5'-GACAAGCTTCCCGTTCTCAG-3'. The endogenous gene glyceraldehyde-phosphate dehydrogenase (GAPDH) as an internal reference was applied to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values are represented using the comparative cycle threshold (CT) ($2^{-\Delta\Delta Ct}$) method [15].

Cell proliferation assay

Cell proliferation was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 10 μL, 5 mg/mL, Sigma, St Louis, MO, USA) following the manufacturer's protocol. Briefly, 5×10^3 cells/well were allowed to grow in a 96-well plate for 24 h, then transfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC and cultured in DMEM. At 0, 24, 48 and 72 h after transfection, 100 μg MTT was added into each well and the cells were cultured for 4 h then measured the absorbance (OD) value of each well at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA), and the cell proliferation curves were drawn with time as x-axis and absorbance as y-axis.

Transwell assay

24 h after transfection with pcDNA3.1-NBAT1 or pcDNA3.1-NC, a total of 5×10^4 cells were first starved in 200 μl serum-free DMEM and then seeded on the upper chamber of a transwell insert (8-μm pore size; Corning Co. NY, USA). The lower chamber was filled with 500 μl of DMEM supplemented with 10% FBS as chemo-attractant. The cells were incubated at 37°C in 5% CO_2 for 48 h, and then the cells that had migrated or invaded to the bottom surface of the filter membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet (Sigma, St Louis, MO) and photographed in five randomly selected fields under an IX71 inverted microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis of cell apoptosis

After transfection with pcDNA3.1-NBAT1 or pcDNA3.1-NC for 48 h, the cells were stained with propidium iodide (PI) together with Annexin V/FITC (BD Bioscience, USA). After incubation in the dark for 15 min at room temperature, cell apoptosis condition was detected and analyzed by using a flow cytometry (FACS Calibur flow, BD Company, USA) with CellQuestPro software (BD Biosciences, Franklin Lakes, NJ, USA).

Cleaved caspase-3 ELISA assay

Cell apoptosis was also investigated through ELISA assay. Briefly, 5×10^5 cells/well were placed in a 6-well plate for 24 h, then transfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC, respectively. At 48 h after transfection, Caspase-3 Colorimetric Assay kit (Abcam, Cambridge, UK) was used to detect cleaved caspase-3 activity in cells following the manufacturer's protocol.

Western blot analysis

Cells were lysed 48 h post-transfection with RIPA lysis buffer (Beyotime, Jiangsu, China) containing protease inhibitor; the proteins were then harvested. Total protein content was quantitated through a BCA assay (Pierce, Rockford, IL, USA). Equal amounts of protein extracts (30 to 40 ng) were separated using 8% SDS-PAGE gels (BioDev-Tech. Co., Ltd, Beijing, China) at 80 V for 2 h and transferred onto PVDF membranes (Millipore, Billerica, MA, USA) for 2 h. Afterwards, blots were blocked with 5% fat-free milk powder for 1 h. The membranes were probed with antibodies against GAPDH, E-cadherin, and N-cadherin (Cell Signaling Technology Inc., Danvers, MA) at 4°C overnight. The blots were subsequently incubated with respective horseradish peroxidase-conjugated secondary antibodies and visualized using the enhanced chemiluminescence system (ECL) (Millipore, Wisconsin, USA). Protein expression was assessed using Alpha Innotech imaging

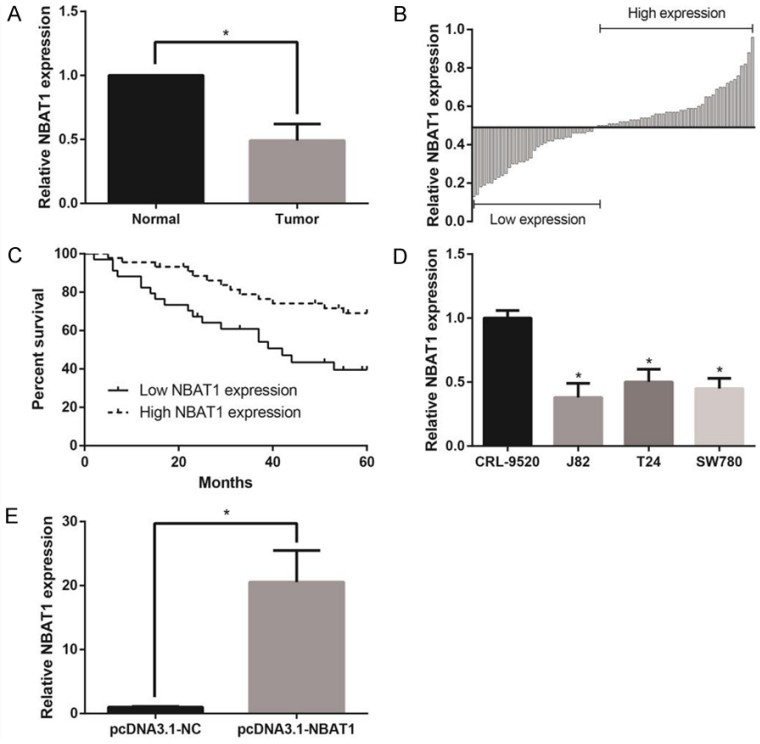

Figure 1. NBAT1 was down-regulated in BCa tissues and cell lines. A. Levels of NBAT1 were determined in 79 surgical specimens of BCa tissues and their matched normal tissues. B. The fold changes of relative NBAT1 level in each matched samples. C. Kaplan-Meier plot of overall survival of BCa patients based on expression of NBAT1. D. Levels of NBAT1 were determined in two BCa cell lines (J82, T24 and SW780) and a normal human uroepithelial cell line (CRL-9520). E. NBAT1 expression in BCa cells transfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC. Values are the mean ± SD; *: $P<0.05$.

software (San Leandro, CA). GAPDH was applied as an endogenous control for normalization.

Statistical analysis

Data were analyzed using independent two-tailed Student's t-test (for continuous data) and two-side chi-square test (for categorical data) in this study. Overall survival curves were plotted following the Kaplan-Meier method, and the log-rank test was used to evaluate for the statistical significance. All statistical analyses were carried out using SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and Graph PAD prism 6 (GraphPad Software, Inc., CA, USA). A two-sided P values<0.05 were considered to indicate statistical significance.

Results

NBAT1 was down-regulated in BCa tissues and cell lines

Firstly, in order to determine the levels of NBAT1 in BCa, NBAT1 expression in 79 paired invasive BCa specimens and pair-corresponding adjacent noncancerous bladder tissues was profiled through using qRT-PCR approach. Our results indicated that the average expression level of NBAT1 in BCa specimens was dramatically reduced compared to that in their normal counterparts ($P<0.05$, **Figure 1A**). Approximately 43% (34 out of 79) patients whose NBAT1 levels were below 0.49, the median ratio of relative NBAT1 expression, were classified as low NBAT1 expression group, and the remaining 45 cases were assigned as high NBAT1 expression group (**Figure 1B**). Correlation between NBAT1 expression and clinicopathological features of 79 BCa patients was illustrated in **Table 1**. Noticeably, down-regulated NBAT1 expression was significantly associated with aggressive tumor features, including higher tumor grade ($P=0.043$) and advanced tumor stage ($P=0.027$); but not correlated with other clinicopathologic features including patient's age ($P=0.807$), gender ($P=0.580$), tumor size ($P=0.179$) and lymph node metastasis ($P=0.073$).

For overall survival, death from any cause was regarded as an event, and the follow-up was investigated from the date of diagnosis to the date of death. Kaplan-meier survival analysis based on NBAT1 expression showed that BCa patients with high NBAT1 expression were inclined to have dramatically longer overall survival than patients with low NBAT1 expression (log-rank test: $P=0.007$, **Figure 1C**), indicating that down-regulation of NBAT1 might be associated with unfavorable prognosis of BCa patients.

The relative expression pattern of NBAT1 was also detected by using qRT-PCR in different cell lines. Using a normal urothelial cell line CRL-9520 as a reference, NBAT1 was remarkably down-regulated in BCa cell lines (J82, T24 and SW780) (all $P<0.05$, **Figure 1D**). BCaT24 cell-lines were cultured and thus NBAT1 expression

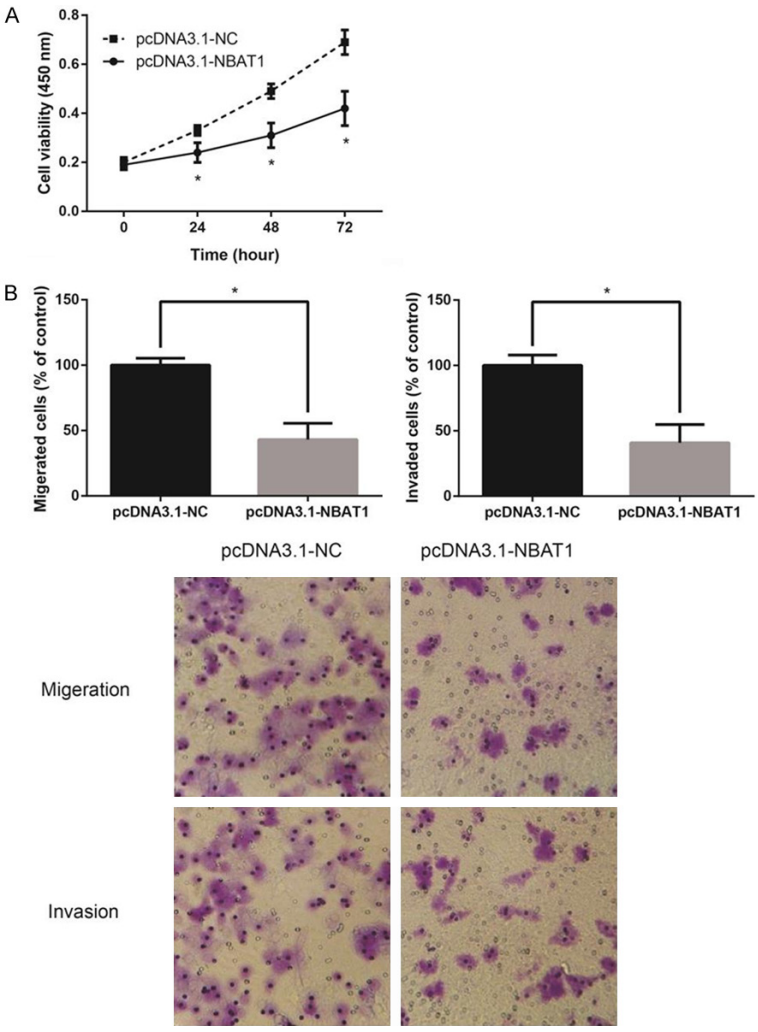

Figure 2. NBAT1 inhibits BCa cell proliferation, migration and invasion *in vitro*. A. MTT assay was conducted in BCa cells transfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC. B. Migration assay and invasion assay were conducted in BCa cells transfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC. Stained cells were counted in five randomly selected fields under a light microscope. Representative photographs and quantification are demonstrated. Values are the mean ± SD; *: $P<0.05$.

was enhanced by transfecting a NBAT1 expression vector (pcDNA3.1-NBAT1). At 48 h after transfection, NBAT1 expression level was determined by qRT-PCR and the results revealed that the relative level of NBAT1 in T24 cells was remarkably up-regulated after the transfection with pcDNA3.1-NBAT1 ($P<0.05$, **Figure 1E**).

NBAT1 inhibits in vitro BCa cell proliferation, migration and invasion

The effect of NBAT1 on BCa cell proliferation was assessed by MTT assay. As demonstrated in **Figure 2A**, in comparison to the NC group, NBAT1 significantly inhibited the proliferation of T24 cells after transfection with pcDNA3.1-NBAT1 ($P<0.05$).

Transwell assays were carried out to evaluate whether NBAT1 has a direct functional role in cell metastasis capacity. The results demonstrated that cell migration and invasion capacity were greatly impaired when T24 cells were transfected with pcDNA3.1-NBAT1 (all $P<0.05$, **Figure 2B**). The results indicated that miR-128 suppressed the *in vitro* migration and invasion of BCa cells.

NBAT1 promotes BCa cell apoptosis in vitro

Next, to explore whether apoptosis was a critical factor to cell growth inhibition, we determined whether NBAT1 could inhibit cell apoptosis in BCa. Cells undergoing early apoptosis bind only to annexin V, and cells binding both are either in the late stages of apoptosis or already dead. As shown in **Figure 3A**, up-regulation of NBAT1 increased proportions of annexin V-positive only T24 cells compared to scramble control group ($P<0.05$). Similarly, a significantly increased activity of cleaved caspase-3 protein in T24 cells transfected with pcDNA3.1-NBAT1 than that of pcDNA3.1-NC was also observed ($P<0.05$, **Figure 3B**). These results demonstrated that NBAT1 inhibits cell apoptosis in BCa.

NBAT1 inhibits EMT of BCa cells

In order to identify the role of NBAT1 involved in BCa metastasis via partly regulating EMT progress, we investigated the expression of EMT-related proteins in the BCa T24 cellstransfected with synthesized pcDNA3.1-NBAT1. As shown in **Figure 4**, western blot analysis

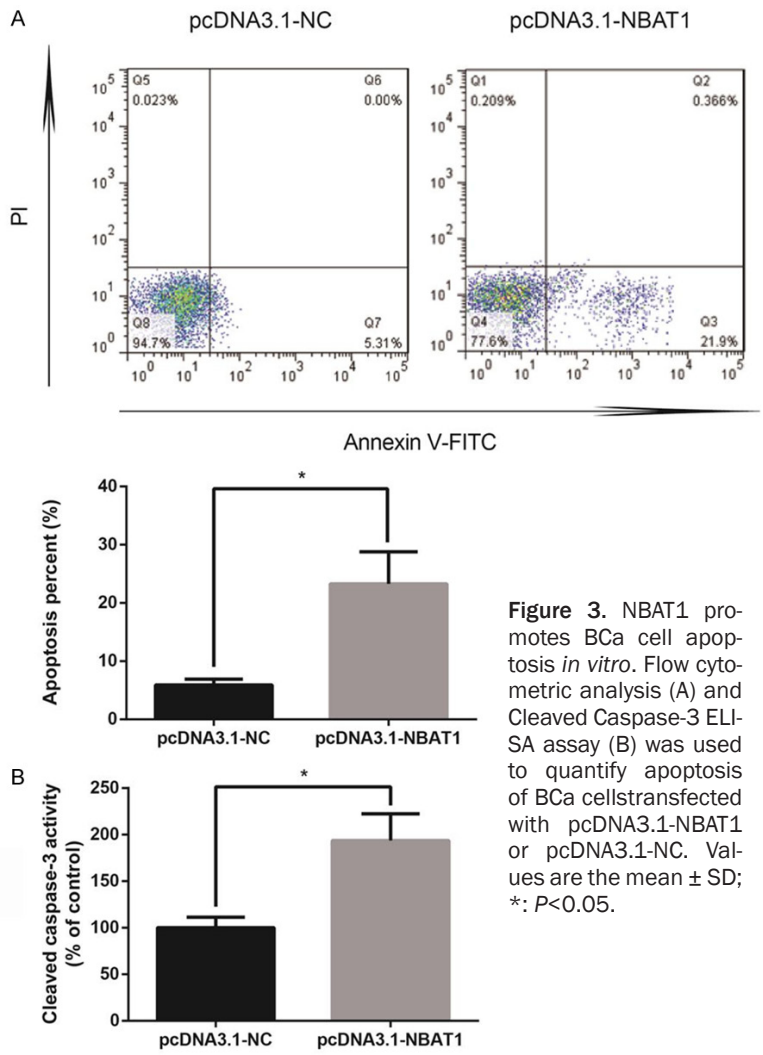

Figure 3. NBAT1 promotes BCa cell apoptosis *in vitro*. Flow cytometric analysis (A) and Cleaved Caspase-3 ELISA assay (B) was used to quantify apoptosis of BCa cellstransfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC. Values are the mean ± SD; *: $P<0.05$.

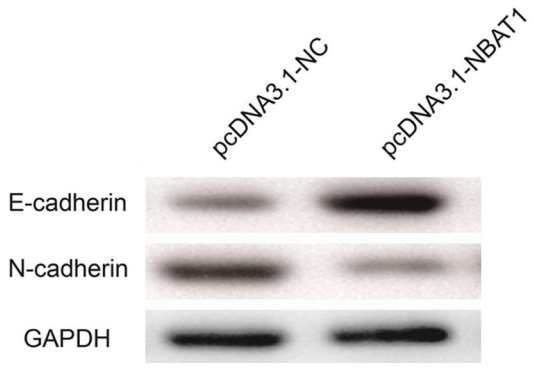

Figure 4. NBAT1 inhibits EMT of BCa cells. Western blot analysis of E-cadherin and N-cadherin expression in BCa cells transfected with pcDNA3.1-NBAT1 or pcDNA3.1-NC. β-actin was usedas a loading control.

revealed that at the protein level, T24 cells transfected with pcDNA3.1-NBAT1 led to a robust increase in the expression of E-cadherin, which is a typical protein expressed by epithelial cells, and led to adecrease in the expression of N-cadherin, which is atypical protein expressed by mesenchymal cells. Collectively, these results indicated that NBAT1 can inhibit EMT event in BCa.

Discussion

During the past several decades, large-scale sequencing efforts and the ENCODE project have suggested that a large fraction of the human noncoding genome is transcribed [16]. Although a number of publications demonstrated aberrant expression of lncRNAs contributes to the pathogenesis of most human malignancies [17, 18], only a small number of BCa-associated lncRNAs have attracted much attention from urological researchers. For example, Chen et al. demonstrated that lncRNA NEAT1 might serve as an oncogene and can be considered as a therapeutic target for treating BCa [19]. Zhan et al. revealed that lncRNA SUMO1P3 was remarkably up-regulated in BCa tissues [20]. Determination of the expression profile and function of aberrant lncRNAs in BCa would be particularly helpful to further understand how lncRNAs serve roles in the development of the disease. Therefore, in the present research, we focused on the function of lncRNA NBAT1 in BCa.

NBAT1, previously reported to be down-regulated in neuroblastoma, epigenetically controls the expression of target genes associated to cell proliferation and cell invasion through interacting with EZH2 [13], an oncogenic regulator which has been functionally linked to tumor malignancy and a poor prognosisin many human cancers, such as neuroblastoma [21] and nonsmall cell lung cancer [22]. Hu et al. also indicated that NBAT1 expression was obser-

ved to be down-regulated in various cancers including breast cancer, lung squamous cell carcinoma and hepatic cell carcinoma, and NBAT1 might exert this tumor-suppressing function in breast cancer through regulating EZH2 [23]. However, to date, we understand limited information about the correlation between NBAT1 and BCa development.

In the current study, we observed that NBAT1 expression is dramatically down-regulated in BCa tissues and cell lines. From clinical perspective, dysregulated NBAT1 expression was significantly associated with tumor grade, tumor stage and overall survival of BCa patients, suggesting NBAT1 might be useful as a novel diagnostic and prognostic biomarker for BCa. However, our relatively small clinical sample size might have limited the power of our clinical analysis. As is commonly understood, uncontrolled cell proliferation and apoptosis are the important mechanisms for neoplastic progression [24]. In comparison to the control group, inhibited proliferation, suppressed migration and increased apoptosis were remarkably demonstrated in NBAT1 overexpressed BCa cells *in vitro*, thus indicating that NBAT1 functioned as a tumor suppressor in BCa cells and involved in BCa progress.

To explore the underlying mechanism through which NBAT1 contributes to the invasion and migration of BCa cells, we subsequently investigated the effects of NBAT1 on EMT. Distant metastasis is the major cause of death in BCa patients. Epithelial-to-mesenchymal transition (EMT), featured by loss of cell apicobasolateral polarity, cell-cell junctions and gain of mesenchymal cell-like characteristics, is one of the key molecular steps permits invasion and metastasis in various cancers [25, 26]. Main hallmarks of EMT include the loss of E-cadherin expression and enhanced expression of N-cadherin [27]. Accumulating evidence suggested that lncRNAs act like master regulators for EMT in different kinds of cancers [28-30]. Our current study uncovered that NBAT1 affected the expression of EMT markers such as E-cadherin and N-cadherin in BCa cells, indicating NBAT1 might exert a central role in the mediation of metastatic processes in BCa.

To the best of our knowledge, this was for the first time to demonstrate the functional significance of NBAT1 expression involved in a cohort of human BCa. Herein, our results revealed that the down-regulation of NBAT1, as a tumor suppressive gene, promoted BCa malignant progression. Accordingly, NBAT1 held great promise as a novel diagnostic and prognostic marker and therapeutic target for BCa in the near future.

Disclosure of conflict of interest

None.

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