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ORIGINAL ARTICLE

Low expression of lncRNA NBAT-1 promotes gastric cancer development and is associated with poor prognosis

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Summary

Purpose: *This study aimed to investigate the regulatory effect of long non-coding RNA (lncRNA) NBAT-1 (neuroblastoma associated transcript 1) on the development and prognosis of gastric cancer (GC), and its underlying mechanism.*

Methods: *NBAT-1 expression in GC tissues, paracancerous tissues and normal gastric tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR). We also detected NBAT-1 expression in GC cell lines. Correlation between NBAT-1 expression and TNM stage was analyzed. Overexpression plasmid of NBAT-1 was transfected into SGC-7901 and MGC-823 cells, followed by detection of proliferation and cell cycle by CCK-8 (cell counting kit-8) assay and flow cytometry. NBAT-1 localization in GC cells was accessed by chromatin fractionation. Finally, PTEN expression in GC cells overexpressing NBAT-1 was determined by Western blot analysis.*

Results: *NBAT-1 and PTEN were lowly expressed in GC tissues compared with paracancerous tissues and normal gastric tissues. GC patients with stage III-IV presented lower expression of NBAT-1 than those with stage I-II. Besides, lower expression of NBAT-1 was found in GC patients with N2-N3 compared with those with N0-N1. NBAT-1 expression was not correlated with TNM stage and distant metastasis in GC patients. Upregulation of NBAT-1 in GC cells inhibited proliferation and arrested cell cycle. Chromatin fractionation results indicated that NBAT-1 was mainly localized in the cytoplasm of SGC-7901 and MGC-823 cells. NBAT-1 overexpression remarkably upregulated PTEN expression in GC cells.*

Conclusions: *Low expression of NBAT-1 promotes GC development by downregulating PTEN expression*

Key words: *gastric cancer, LncRNA, NBAT-1, PTEN*

Introduction

Gastric cancer (GC) is one of the malignant tumors of the digestive system that endangers human life. GC is highly prevalent in East Asia, West Asia and Latin America [1]. At present, the diagnosis of GC is generally based on clinical symptoms combined with related examinations, including endoscopy, X-ray barium meal, B-ultrasound, CT, MRI and exfoliative cytology. However, these methods have certain flaws that prevent the early diagnosis of GC [2,3]. Specific and sensitive indicators for the early screening of GC are still lacking. As a result, most GC patients are already in middle to late stages at the time of diagnosis. Moreover, the therapeutic efficacy in GC is unsatisfactory, which brings great difficulties to the clinical treatment of GC. Hence, it is of great significance to fully elucidate the pathogenesis of GC, so as to improve the efficacy of early prevention and diagnosis.

Recently, with the rapid development of sequencing technologies, multiple long non-coding RNAS (lncRNAs) and their biological functions have been identified [4]. LncRNAs exert different regulatory effects. It has been shown that lncRNAs could regulate gene transcription and translation,

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cell differentiation and growth, genetic and epigenetic variation [5,6]. At present, lncRNAs have been confirmed to be differentially expressed in various tumors, such as breast cancer, bladder cancer, melanoma, liver cancer and colorectal cancer, which exert a key regulatory role in tumorigenesis [7-11]. GC-related lncRNAs have been well studied. For example, H19/miR-675 promotes the proliferation and inhibits the apoptosis of GC cells by downregulating the RUNX1 expression [12]. The abnormal expression of HOTAIR is associated with tumor stage, peritoneal metastasis, nodal metastasis, vascular invasion, and prognosis of GC patients [13]. The overexpression of HOTAIR promotes the proliferation, migration and invasion of GC cells [14]. ANRIL can inhibit miR-99a/miR-44a by recruiting the PRC2 complex, thereby affecting the proliferation of GC cells through activating the mTOR and CDK6/E2F1 pathways [15].

Neuroblastoma associated transcript 1 (NBAT-1) is a tumor suppressor gene identified in neuroblastoma. NBAT-1 is lowly expressed in neuroblastoma and is associated with the prognosis of neuroblastoma patients. Functionally, NBAT-1 inhibits the proliferation, invasion and differentiation of neuroblasts [16]. Subsequent studies have found that NBAT-1 is also lowly expressed in breast cancer and renal cell carcinoma, which serves as a prognostic indicator [17,18]. At present, the specific role of NBAT-1 in GC has not been reported yet. This study aims to discuss the regulatory effect of NBAT-1 on the pathogenesis of GC.

Methods

Sample collection

45 pairs of GC tissues and paracancerous tissues, as well as 23 cases of normal gastric tissues were collected in The Third Affiliated Hospital of Soochow University from May 2016 to May 2017. The tissues were preserved in liquid nitrogen. All of the enrolled patients were pathologically diagnosed with GC. Informed consent was signed before the study. The Ethics Committee of the Third Affiliated Hospital of Soochow University approved this study.

Cell culture and transfection

Gastric mucosal cell line (GES-1) and GC cell lines (SGC-7901, BGC-823 and MGC-803) were obtained from the ATCC (American Type Culture Collection) (Manassas, VA, USA). The cells were cultured in RPMI-1640 (Roswell Park Memorial Institute 1640) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). The cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed using trypsin until reaching a confluence of 80-90%.

The cells were transfected with corresponding plasmids following the instructions for Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The culture medium was replaced 4 hrs later. The transfection plasmids were constructed by GenePharma, Shanghai, China.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA in treated cells was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions for the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). The RNA concentration was detected using a spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions for SYBR Premix Ex Taq TM (TaKaRa, Tokyo, Japan). The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. The primers used in the study were as follows: NBAT-1 F: 5'-GGAAAGCCTGTGCTCTTGGA-3', R: 5'-TCACAGTGCTGCTCAATCGT-3'; PTEN F: 5'-ACCAGGACCAGAGGAAACCT-3', R: 5'-GCTAGCCTCTGGATTTGACG-3'.

CCK-8 (cell counting kit-8) assay

GC cells were seeded into 96-well plates at a density of 2×10^3/ml. Ten μl of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) were added in each well. Four hrs later, fresh medium was added and the cells were incubated for 1 hr. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell cycle detection

GC cells were diluted to 1×10^5/mL and fixed with 75% ethanol at -4°C overnight. After double PBS (phosphate buffered saline) wash, the cells were incubated with 100 μL of RNaseA at 37°C in a water bath in the dark. Subsequently, the cells were incubated with 400 μL of PI at 4°C in the dark. Thirty min later, cell cycle was detected using flow cytometry at 488 nm wavelength.

Chromatin fractionation

The cells were fully lysed in RLA, incubated on ice for 20 min and centrifuged at 3000 r/min for 15 min. The supernatant contained cytoplasmic proteins. The precipitant was washed with RLA three times and lysed with RIPA (radioimmunoprecipitation assay) (Beyotime, Shanghai, China) for incubation on ice for a total of 20 min. The mixture underwent vortex oscillation every 5 min for 30 s. Finally, the mixture was centrifuged at 12500 rpm/min for 15 min and the supernatant contained nuclear proteins.

Western blot

The cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibodies,

the immunoreactive bands were exposed by enhanced chemiluminescence method.

Statistics

SPSS 19.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. The data were expressed as mean±standard deviation (x±s). The correlation coefficient (r) was calculated by Pearson correlation analysis. Measurement data were compared using the *t*-test. A statistically significant difference was considered when $p<0.05$.

Results

NBAT-1 was lowly expressed in GC

We detected the NBAT-1 expression in 45 pairs of GC tissues and paracancerous tissues by qRT-PCR. The data showed that NBAT-1 WAS lowly expressed in GC tissues compared to paracancerous tissues (Figure 1A). Meanwhile, we detected the NBAT-1 expression in 23 cases of normal gastric tissues. Similarly, the NBAT-1 expression was lower in GC tissues than in normal gastric tissues (Figure 1B). The clinical data of the enrolled GC patients were collected for correlation analyses. We found that GC patients with stage III-IV presented A lower expression of NBAT-1 than those with stage I-II (Figure 1C). Besides, A lower expression of NBAT-1 was found in GC patients with N2-N3 disease compared with those with N0-N1 (Figure 1D). However, the NBAT-1 expression was not correlated with TNM stage and distant metastasis in GC patients (Figure 1E and 1F). It was concluded

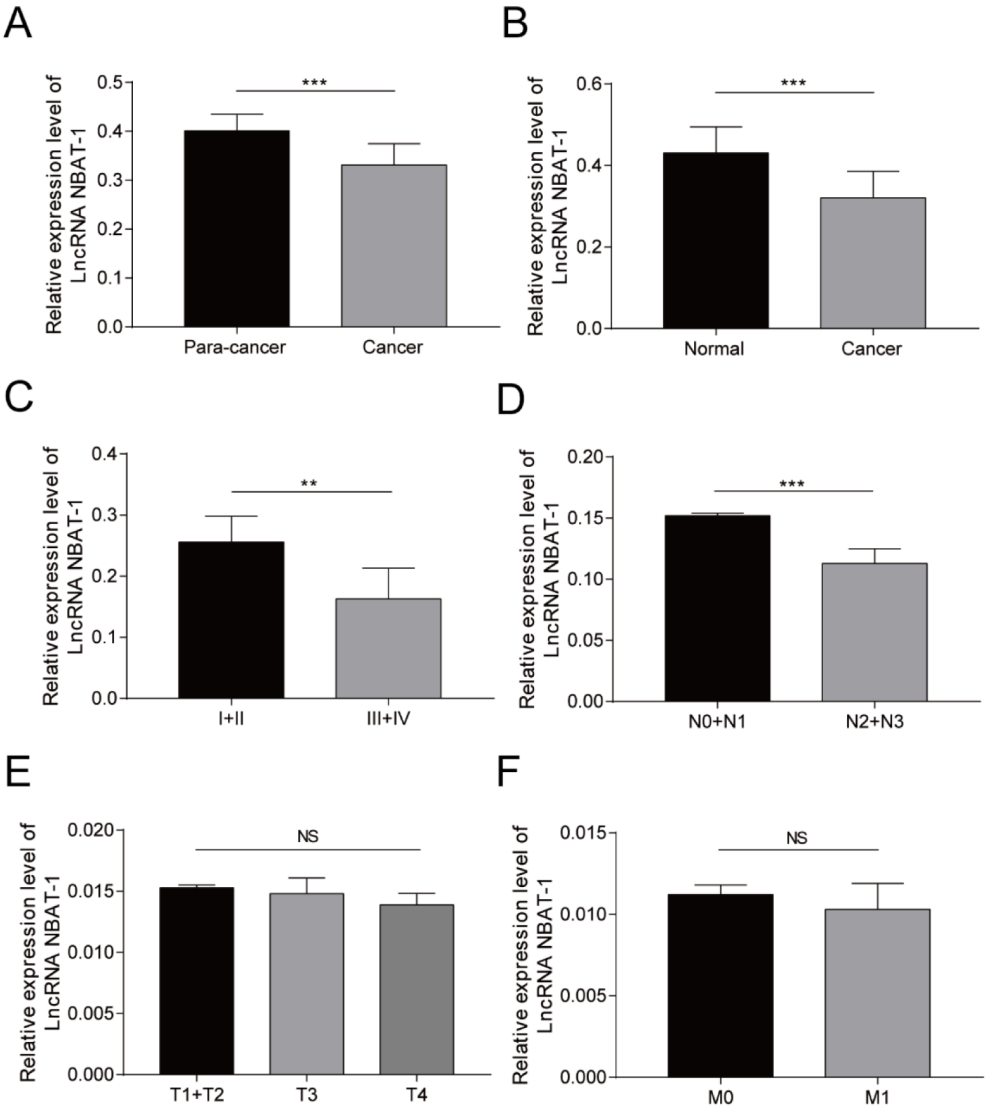

Figure 1. NBAT-1 was lowly expressed in GC. **(A)** NBAT-1 WAS lowly expressed in GC tissues compared to paracancerous tissues. **(B)** NBAT-1 expression was lower in GC tissues than in normal gastric tissues. **(C)** GC patients with stage III-IV presented lower expression of NBAT-1 than those with stage I-II. **(D)** NBAT-1 expression was lower in GC patients with N2-N3 compared with those with N0-N1. **(E,F)** NBAT-1 expression was not correlated with TNM stage and distant metastasis in GC patients. NS: non significant. **p<0.01, ***p<0.001.

that A low expression of NBAT-1 was related to poor prognosis in GC.

NBAT-1 inhibited the proliferation of GC cells

QRT-PCR was performed to detect the NBAT-1 expression in gastric mucosal cell line (GES-1) and GC cell lines (SGC-7901, BGC-823 and MGC-803). NBAT-1 was lowly expressed in GC cells compared to gastric mucosal cells (Figure 2A). SGC-7901 and MGC-823 cells were selected for the following experiments since they had a relatively low expression of NBAT-1. The overexpression plasmid of NBAT-1 was transfected into SGC-7901 and MGC-823 cells, and the NBAT-1 expression was remarkably upregulated (Figure 2B). CCK-8 assay elucidated that the NBAT-1 overexpression inhibited the proliferative capacities of SGC-7901 and MGC-823 cells (Figure 2C). Moreover, the NBAT-1 overexpression arrested the cell cycle of GC cells (Figure 2D).

NBAT-1 downregulated the PTEN expression

Chromatin fractionation results indicated that NBAT-1 was mainly localized in the cytoplasm of SGC-7901 and MGC-823 cells (Figure 3A). Subsequently, qRT-PCR data indicated that the PTEN

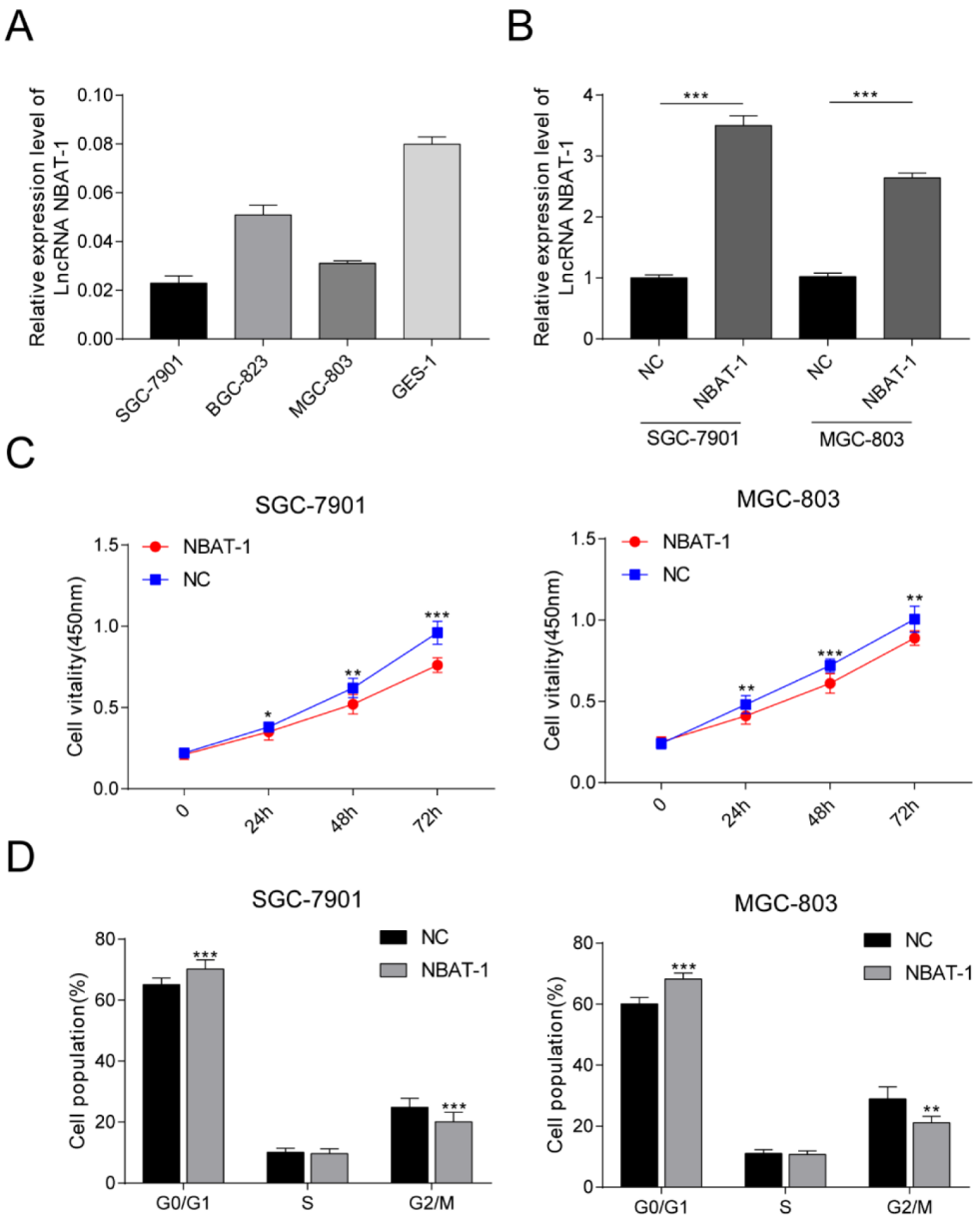

Figure 2. NBAT-1 inhibited the proliferation of GC cells. **(A)** NBAT-1 was lowly expressed in GC cells compared to gastric mucosal cells. **(B)** Transfection of overexpression plasmid of NBAT-1 in SGC-7901 and MGC-823 cells remarkably upregulated the NBAT-1 expression. **(C)** CCK-8 assay elucidated that NBAT-1 overexpression inhibited the proliferative capacities of SGC-7901 and MGC-823 cells. **(D)** NBAT-1 overexpression arrested the cell cycle of GC cells. NC: normal control plasmid of NBAT-1. **p<0.01, ***p<0.001.

expression was lower in GC tissues than in paracancerous tissues (Figure 3B). Correlation analyses pointed out the positive correlation between the NBAT-1 expression and the PTEN expression in GC cells (Figure 3C). Western blot further verified that the NBAT-1 overexpression upregulated the protein expression of PTEN (Figure 3D). It was indicated that the expression of NBAT-1 promoted GC development by upregulating the PTEN expression.

Discussion

The occurrence and development of GC is a multi-factor and multi-stage process. Environmental or genetic risk factors lead to the dysregulation of relative genes in GC pathogenesis [19,20]. Environmental risk factors mainly include infectious factors, dietary factors, occupational exposure, smoking, and drinking. Helicobacter pylori infection is a definite risk factor for GC [21,22]. With advances in research, the diagnostic and prognostic values of lncRNAs have been well recognized. Since some lncRNAs are differentially expressed in GC with tissue-specific characteristics and can be detected in body fluids, they may be potential diagnostic markers for GC. For example, the high expression of HOTAIR is not only an independent prognostic indicator of GC, but also a risk factor for tumor metastasis [23].

PTEN is a homolog of protein tyrosine phosphatase, which is located in the human chromosome 10q23 region and serves as a tumor suppressor gene in many types of tumors [24]. PTEN can inhibit tumor growth by regulating the apoptosis, proliferation, migration and invasion of tumor cells [25,26]. Recent studies have shown that the upregulation of PTEN increases caspase-3 expression, thereby inhibiting the apoptosis of tumor cells [27]. Studies have shown that PTEN is lowly expressed in GC and closely related to tumor size and lymph node metastasis. On the contrary, PI3K, AKT, p-AKT

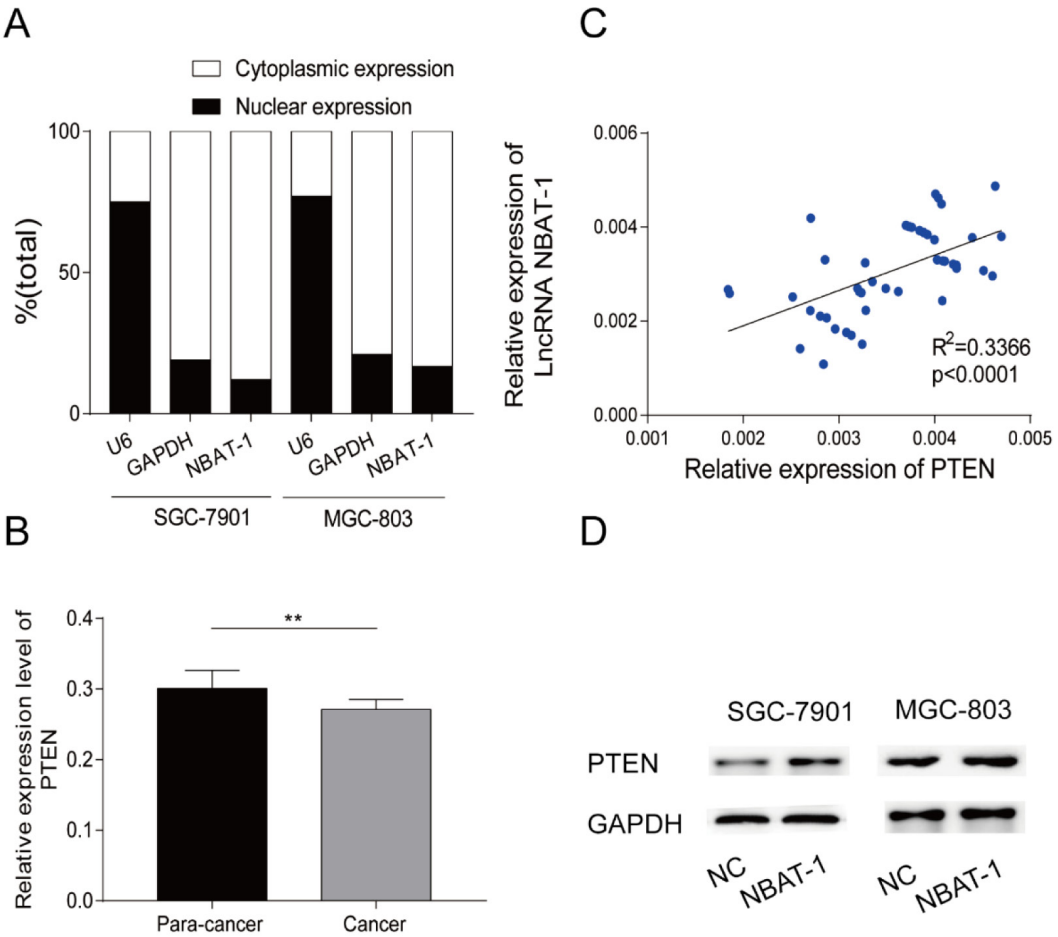

Figure 3. NBAT-1 downregulated PTEN expression. **(A)** Chromatin fractionation results indicated that NBAT-1 was mainly localized in the cytoplasm of SGC-7901 and MGC-823 cells. **(B)** QRT-PCR data indicated that PTEN expression was lower in GC tissues than in paracancerous tissues. **(C)** Correlation analyses pointed out the positive correlation between NBAT-1 expression and PTEN expression. **(D)** Western blot verified that NBAT-1 overexpression upregulated the protein expression of PTEN. **p<0.01.

and p-mTOR are highly expressed in GC, indicating the activated PI3K/AKT/mTOR pathway in GC [28]. Chen et al. detected the PTEN expression in Asian GC patients by immunohistochemistry and they found that the PTEN expression is correlated with tumor size, differentiation, stage, invasion, lymph node metastasis, distant metastasis and vascular invasion. Their results suggested that the immunohistochemical detection of PTEN can predict GC invasion and metastasis [29].

In this study, we found that, compared with paracancerous tissues and normal gastric tissues, the expression level of NBAT-1 significantly decreased in GC tissues. The NBAT-1 expression was negatively correlated with tumor stage and lymph node metastasis in GC patients. NBAT-1 was lowly expressed in GC cells as well. After the ovexpression of NBAT-1 in GC cells, cell proliferation was inhibited and cell cycle was blocked. NBAT-1 was confirmed to be mainly present in the cytoplasm. Further studies found that PTEN was lowly expressed in GC tissues, which was positively correlated with the NBAT-1 expression. The NBAT-1 overexpression remarkably increased the PTEN expression in GC cells.

Conclusions

The low expression of NBAT-1 promotes GC development by downregulating the PTEN expression. NBAT-1 may serve as a potential hallmark in predicting the prognosis of GC patients.

Conflict of interests

The authors declare no conflict of interests.

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