Long non-coding RNA NBAT1 inhibits the progression of glioma through the miR-21/SOX7 axis

NING GUAN, RUI WANG, XU FENG, CHENGUANG LI and WENSHI GUO

Department of Neurosurgery, The First Affiliated Hospital of Jinzhou Medical University, Jinzhou, Liaoning 121001, P.R. China

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Abstract. Glioma is one of the most prevalent types of malignancy in the central nervous system worldwide, and the prognosis of patients with late-stage glioma remains poor. Thus, the development of promising therapeutic strategies against glioma is essential. Long non-coding RNAs (lncRNAs) are functional RNA molecules involved in the initiation and progression of tumors, including glioma. Investigation on the regulatory roles of lncRNAs may facilitate the development of effective treatments. lncRNA NBAT1 is associated with the growth and metastasis of cancer; however, its underlying molecular mechanisms remain unknown. Thus, the present study aimed to investigate the effects of NBAT1 in glioma. The expression levels of NBAT1, microRNA (miRNA/miR)-21 and SOX7 in patients with glioma, and healthy donors using reverse transcription-quantitative PCR analysis. Human glioma cells (A172 and AM138) and normal astrocytes were used to establish the NBAT1-knockdown and overexpression models. Cell Counting Kit-8 and Transwell assays were performed to determine whether NBAT1 exerted effects on cell proliferation, migration and invasion. The results demonstrated that NBAT1 expression decreased in glioma tissues compared to normal samples. Additionally, downregulation of NBAT1 was detected in human glioma cells compared with normal astrocytes. Overexpression of NBAT1 inhibited glioma cell proliferation, migration and invasion. In addition, miR-21 was identified as a potential target of NBAT1, and the effects of miR-21-induced cell proliferation and metastasis were reversed following overexpression of NBAT1. Furthermore, SOX7 was predicted as the potential target of miR-21, and its expression was upregulated in glioma cells by overexpression of NBAT1 compared with the vehicle only control. Taken together, the results of the present study provide novel insight into the functions of NBAT1 in glioma, suggesting that the NBAT1/miR-21/SOX7 axis may act as a potential therapeutic target for the treatment of patients with glioma.

Introduction

Glioma is one of the most common and aggressive primary malignancies of the brain, and the global incidence was 5% in 2013; it was reported to account for ~80% of malignant neoplasia and ~30-40% of primary tumors in the central nervous system, worldwide (1). Despite recent advances in adjuvant radiotherapy, hormone therapy, chemotherapy and targeted biologic therapy, the overall survival outcomes of most patients with glioma remain poor (2,3). Furthermore, the aforementioned treatments exhibit side effects, such as anemia and constipation (3); thus, the development of novel therapeutic strategies against glioma remains essential.

Long non-coding RNAs (lncRNAs) are a novel class of non-protein coding RNAs, >200 nt long (4-6). lncRNAs are involved in a number of biological processes, including cell differentiation and gene expression (7). Increasing evidence suggests that lncRNAs are associated with the initiation and progression of different types of tumor, including glioma (8-15). Furthermore, aberrant expression levels of lncRNAs have been reported in tumor cells, indicating the potential role of lncRNAs in tumorigenesis (16-18). Thus, investigation of the regulatory functions of lncRNAs in cancer may help facilitate the development of effective therapies.

lncRNA NBAT1 is involved in carcinogenesis, and previous studies have reported the dysregulation of NBAT1 in different types of cancer (19,20). NBAT1 promotes the initiation and development of tumors via several pathways, including PRC2 signaling (21,22), and thus is considered a promising biomarker and prognostic indicator of glioma (20,23). However, the potential molecular targets of NBAT1 in glioma have not yet been fully investigated.

MicroRNAs (miRNAs/miRs) are endogenously expressed non-coding RNAs that are ~22 nt in length and are considered potential targets of lncRNAs (24,25). Increasing evidence suggests that the levels of miRNAs are dysregulated in cancer, which may contribute to tumor development (26,27). In addition, miRNAs such as miR-140 and miR-152 are able to interact with lncRNAs in glioma (28,29). However, the specific functions of miRNAs in glioma remain unclear.

The present study aimed to investigate the functions of the NBAT1-mediated miR-21/SOX7 signaling pathway in glioma
cell proliferation and metastasis, which may provide novel insight for the effective treatment of patients with glioma.

**Materials and methods**

**Patient samples.** A total of 40 pairs of primary tumor and paracarcinoma tissue samples (>5 cm from tumor margin) were collected from patients with glioma (16 men and 24 women; age range, 45-65 years; mean age, 51 years) undergoing surgical resection at The First Affiliated Hospital of Jinzhou Medical University (Jinzhou, China) between June 2015 and August 2017. Prior to surgery, none of the patients have received chemotherapy or radiotherapy. Metastasis was observed in 14 and absent in 26 patients, according to the World Health Organization grading system (https://www.cancer.net/cancer-types/brain-tumor/grades-and-prognostic-factors). The biopsies were examined by two independent pathologists from the Department of Histopathology, The First Affiliated Hospital of Jinzhou Medical University (Jinzhou, China). All samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until further experimentation. The present study was approved by the Institutional Review Board of The First Affiliated Hospital of Jinzhou Medical University (approval no. JYD159302; Jinzhou, China). Written informed consent was provided by all patients prior to the study start.

**Cell culture.** The human glioma cell lines (AM38, Gli-6, GSC11 and A172) and normal human astrocyte cell line (A735) were purchased from the American Type Culture Collection. Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all purchased from HyClone; Cytiva) at 37°C in a humidified incubator containing 5% CO2.

**Cell transfection.** To establish the NBAT1 knockdown model, short hairpin (sh)RNA sequences targeting NBAT1 (sh-NBAT1), as well as the negative control (sh-NC) were integrated into the lentiviral pU6-Luc-Puro vector (Shanghai GenePharm Co., Ltd.). The shRNAs were annealed, the shRNAs were integrated into the lentiviral vector using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 8 h at 37°C. The culture medium was replaced with fresh DMEM supplemented with 10% FBS at 8 h post-transfection. Subsequent experiments were performed 20 h post-transfection.

**RT-qPCR.** miRNA was extracted from tissues or cell lines using the miRNeasy Mini kit (Qiagen China Co., Ltd.), and miR-21 expression was determined using the TaqMan MicroRNA Assay kit (cat. no. 4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using an Applied Biosystem 7500 PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. U6 small nuclear RNA was used as the internal control.

Total RNA was extracted from clinical samples or cell lines using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) and subsequently reverse-transcribed into cDNA using the PrimeScript™ RT kit (Takara Biotechnology Co., Ltd.). The temperature protocol was as follows: 42°C for 45 min, 99°C for 5 min and 5°C for 5 min. qPCR was performed using the SYBR® Green PCR Master Mix (Takara Biotechnology Co., Ltd.), according to the manufacturer’s protocol. The following primer sequences were used for qPCR: NBAT1 forward, 5'-AGT CCT GGT TCC TCC TC-3' and reverse, 5'-GAA GAG GCC CAAGA GAAG-3' and overexpression of NBAT1 were confirmed by reverse transcription-quantitative (RT-q)PCR analysis.

**Western blotting.** Total protein was extracted from tissues or cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Protein concentration was determined using a bichinchoninic acid assay kit (Beyotime Institute of Biotechnology), and 40 µg protein/lane was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto nitrocellulose membranes (EMD Millipore) and blocked with tris-buffered saline (TBS) containing 5% skimmed milk for 2 h at room temperature. The membranes were incubated with primary antibodies against SOX7 (1:100; cat. no. MAB2766; Novus Biologicals, Ltd.) and GAPDH (1:100; cat. no. sc-47724; Santa Cruz Biotechnology Inc.) overnight at 4°C. Membranes were washed three with TBS + Tween-20 (0.05%) and subsequently incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary
antibody (1:5,000; cat. no. sc-2371; Santa Cruz Biotechnology Inc.) for 1 h at 37°C. Protein bands were visualized using an enhanced chemiluminescence protein detection kit (Pierce; Thermo Fisher Scientific, Inc.) and quantified via densitometric analysis using ImageJ software (version 1.48; National Institutes of Health).

**Cell counting kit-8 (CCK-8) assay.** Transfected A172/AM38 cells were harvested 24 h post-transfection and seeded into 96-well plates at a density of 1x10⁴ cells/well. Briefly, 10 µl of CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to the wells on days 1, 2, 3 and 4 post-inoculation, according to the manufacturer's protocol. Following incubation at 37°C for an additional 2 h, cell proliferation was subsequently analyzed at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

**Transwell assay.** For the migration assay, a total of 1x10⁴ A172/AM38 cells were plated in the upper chamber of Transwell plates with a pore size of 8 µm (BD Biosciences) in FBS-free DMEM medium. For the invasion assay, Transwell membranes were precoated with Matrigel® (Sigma-Aldrich; Merck KGaA) at room temperature for 48 h. DMEM medium (500 µl) supplemented with 10% FBS was added to the lower chambers. Following overnight incubation at 37°C, non-migratory/invasive cells were removed using a cotton swab, whereas the migratory/invasive cells in the lower chambers were fixed with 4% paraformaldehyde for 15 min and stained with 0.5% crystal violet for 20 min at room temperature. Stained cells were counted in five randomly selected fields using an inverted light microscope (magnification, x100; Olympus Corporation).

**Northern blot analysis.** Total RNA was extracted from tissues or cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). An equal amount of RNA (25 µg) was loaded onto 15% TBE-urea gels and separated using a 15% urea-PAGE gel. The separated proteins were subsequently transferred onto positively charged nylon membranes (Cytiva) and cross-linked by UV irradiation. The blots were hybridized by DIG-labelled probe for miR-21 (cat. no. 339546; Qiagen AB) overnight at 42°C. The membranes were washed three times with a low-stringency buffer (2xSSC containing 0.1% SDS; Thermo Fisher Scientific, Inc.), and the RNA levels were determined using the DIG Luminescent Detection kit [Roche Diagnostics (Shanghai) Co., Ltd.] and semi-quantified using Multi Gauge software (version 3.0; Fujifilm Wako Pure Chemical Corporation). U6 (cat. no. 339508; Qiagen) RNA was used as the loading control.

**Bioinformatics prediction and dual-luciferase reporter assay.** TargetScan (www.targetscan.org), miRanda (www.microrna.org/microrna) and LncBase Predicted v2 (http://carolina.imis.athenainnovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted) databases were used to predict the potential binding sites of NBAT1 and SOX7 transcripts on miR-21.

WT fragments of the 3' untranslated region (3'UTR) of NBAT1 and SOX7 containing the potential binding sites of miR-21 were purchased from Shanghai GenePharma Co., Ltd. and cloned into the pmirGLO Dual-Luciferase miRNA Target Expression vector (Promega Corporation) according to the manufacturer's protocol. The QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc.) was used to generate the NBAT1- and SOX7-3'UTR-MUT reporter containing mutant miR-21 binding sites. The constructed luciferase vectors were co-transfected with miR-21 mimics or miR-NC into DH5α competent cells (Thermo Fisher Scientific, Inc.). Following incubation at 37°C for 48 h, firefly and Renilla luciferase activities were detected using a Dual Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

**Statistical analysis.** Statistical analysis was performed using SPSS software 17.0 (SPSS, Inc.), and data are presented as the mean ± standard deviation. Student's t-test (paired and unpaired) was used to compare differences between two groups. One-way analysis of variance followed by Student-Newman-Keuls or Tukey's post-hoc test were performed to compare differences among multiple groups. Pearson's correlation analysis was performed to determine the correlations between mRNA expression levels. All experiments were performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**NBAT1 is downregulated in glioma tissues and cells and associated with poor prognosis.** The expression levels of NBAT1 in 40 paired glioma and paracarcinoma samples were determined using RT-qPCR analysis. The results demonstrated that NBAT1 expression was significantly downregulated in glioma tissues compared with that in the paracarcinoma tissues (P<0.05; Fig. 1A). The effects of NBAT1 on metastasis of patients with glioma were also investigated. The results revealed that NBAT1 expression was significantly decreased in patients with metastatic glioma compared with the controls (patients with non-metastatic glioma; P<0.05; Fig. 1B). In addition, NBAT1 expression was significantly decreased in aggressive (grade III or IV) compared with low-grade (I or II) glioma, suggesting that downregulation of NBAT1 was associated with the development of glioma (P<0.05; Fig. 1C). NBAT1 expression was significantly downregulated in glioma cells compared with A735 cells (P<0.05; Fig. 1D). Taken together, these results suggested that NBAT1 expression was downregulated in glioma, which may also be associated with metastasis.

**Overexpression of NBAT1 suppresses glioma cell proliferation, migration and invasion.** To determine the effects of NBAT1 on glioma cell proliferation, migration and invasion, NBAT1 was overexpressed in A172 and AM38 cells, and transfection efficiency was determined by RT-qPCR (Fig. 2A). The CCK-8 assay demonstrated that the proliferative ability of o/e-NBAT1-transfected A172 and AM38 cells significantly decreased compared with that of the control (P<0.05; Fig. 2B and C). In addition, the Transwell assay results revealed that the migratory and invasive abilities of o/e-NBAT1-transfected A172 and AM38 cells were significantly inhibited compared with those of the control groups (P<0.05; Fig. 2D-G). Collectively, these results suggested that
overexpression of NBAT1 may suppress the proliferation, migration and invasion of glioma cells.

miR-21 is the potential target of NBAT1 in glioma. To determine whether NBAT1 functions by suppressing its target miRNAs in glioma, the potential binding sites of miR-21 in NBAT1 transcripts were predicted using LncBase Predicted v2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=Lncbase2%2Findex-predicted) (Fig. 3A). Luciferase reporter vectors containing WT-NBAT1 and MUT-NBAT1 sequences of predicted miR-21 binding sites were constructed. The results demonstrated that miR-21 mimics significantly attenuated the activity of the luciferase plasmid containing the WT binding sites compared with that of the MUT control (P<0.05; Fig. 3B). RT-qPCR and northern blot analyses indicated that miR-21 expression was upregulated in glioma tissues compared with that in paracarcinoma tissues (P<0.05; Fig. 3C and D). In addition, upregulated miR-21 expression was observed in A172 and AM38 cells compared with that in A735 cells (P<0.05; Fig. 3E and F).

To further investigate the effects of NBAT1 on miR-21 expression, glioma cells were transfected with o/e-NBAT1 and miR-21 mimics, or with sh-NBAT1 and miR-21 inhibitors, and transfection efficiency was determined by RT-qPCR (Fig. 4A-C). Northern blot analysis demonstrated that upregulation of miR-21 expression by transfection with miR-21 mimics was reversed following the addition of o/e-NBAT1 (Fig. 4D). Similarly, downregulation of miR-21 expression by transfection with miR-21 inhibitors was counteracted following co-transfection with sh-NBAT1 (Fig. 4E). Pearson’s correlation analysis demonstrated that the levels of miR-21 and NBAT1 were negatively correlated in glioma tissues (Fig. 4F).

miR-21 serves important roles in the proliferation and metastasis of glioma cells. The effects of miR-21 on the progression of glioma were further assessed. The CCK-8 assay demonstrated that the proliferative ability of glioma cells significantly increased following transfection with the miR-21 mimics compared with the vehicle only control (P<0.05; Fig. 5A and B). In addition, the migratory and invasive abilities of glioma cells were enhanced following transfection with the miR-21 mimics compared with the vehicle only control (P<0.05; Fig. 5C-F). Taken together, these results suggest that miR-21 promoted glioma cell proliferation, migration and invasion.

SOX7 is a downstream target of miR-21. To identify the putative targets of miR-21, the complementary sequence of miR-21 in SOX7 transcripts was predicted (Fig. 6A). The interaction between SOX7 and miR-21 was confirmed using a dual-luciferase assay. Luciferase reporter plasmids containing SOX7-WT) and SOX7-MUT fragments of the predicted miR-21 binding sites were constructed. The results demonstrated that the miR-21 mimics significantly decreased the activity of the luciferase vector containing the SOX7-WT sequence, but not the mutant control (Fig. 6B). RT-qPCR analysis indicated that SOX7 expression was downregulated in glioma tissues compared with that in the paracarcinoma.
tissues (Fig. 6C). In addition, downregulated SOX7 expression was observed in A172 and AM38 cells compared with that in A735 cells (P<0.05; Fig. 6D and E). Pearson’s correlation analysis demonstrated that the levels of SOX7 and miR-21 were inversely correlated in glioma samples (Fig. 6F).

To further investigate the effects of SOX7 on the progression of glioma, the expression levels of SOX7 were assessed in glioma cells transfected with control, o/e-NBAT1 or the miR-21 mimics, or co-transfected with o/e-NBAT1 and the miR-21 mimics. SOX7 expression significantly decreased following transfection with the miR-21 mimics compared with that in the control group, which was reversed following overexpression of NBAT1 (Fig. 6G). Collectively, these results suggested that SOX7 may be a novel target of miR-21, and that
the NBAT1/miR-21/SOX7 signaling pathway may be involved in the progression of glioma.

**Discussion**

lncRNAs are a novel class of non-protein coding RNAs that are >200 nt in length (4). Previous studies on lncRNAs have reported their importance on the pathogenesis of cancer, and increasing evidence indicates that lncRNAs act as key factors in the regulation of cancer cells (25-29). lncRNAs function as oncogenes or tumor suppressors in cancer, and aberrant expression levels of lncRNAs are associated with tumor development (31). For example, lncRNA PVT1 modulates the cell proliferation, invasion and tumor growth in orthotopic xenografts of breast cancer (32). In addition, upregulated LINC01116 expression is associated with unfavorable overall survival and metastasis of patients with breast cancer (33). The expression levels of lncRNA PlncRNA-1 are downregulated in cancer tissues; however, restored PlncRNA-1 expression inhibits proliferation and promotes apoptosis of breast cancer cells via the TGF-β1/phosphoglycerate dehydrogenase axis (34). Furthermore, downregulation of lncRNA SNHG5 suppresses proliferation and migration of gastric cancer cells via the miR-32/Kruppel-like factor 4 axis (35). These findings suggest that lncRNAs are key regulators during the initiation and progression of cancer. Consistent with previous findings, the results of the present study demonstrated the key roles of NBAT1 on the proliferation and metastasis of glioma cells.

A number of lncRNAs can regulate gene expression by interacting with their target miRNAs directly. For example, lncRNA BC032469 binds to miR-1207-5p and human telomerase reverse transcriptase, and induces cancer cell proliferation (36). Furthermore, lncRNA H19 regulates the proliferation, migration and invasion of gastric cancer cells through its target miRNAs, such as miR-675 (37,38). However, the functions and underlying molecular mechanisms of lncRNAs remain largely unknown and require further investigation. Similarly, the present study demonstrated the regulatory function of miR-21 during the proliferation, migration and invasion of glioma cells, and its underlying molecular mechanisms were elucidated.
Increasing evidence suggests that aberrant NBAT1 expression is associated with various types of cancer, such as ovarian cancer, breast cancer and osteosarcoma (19, 21, 22). NBAT1 inhibits the proliferation and promotes apoptosis of ovarian cancer cells (19). NBAT1 regulates the migration and invasion of breast cancer cells by targeting Dickkopf Wnt signaling pathway inhibitor 1/polycomb repressive complex 2 signaling (21). A previous study has reported that miR-21 is a potential target of NBAT1 in osteosarcoma cells (22). NBAT1 has also been demonstrated to be associated with the proliferation and metastasis of glioma cells (20). Consistent with previous findings, the results of the present study demonstrated that NBAT1 was downregulated in glioma tissues and cells. Furthermore, overexpression of NBAT1 suppressed glioma cell proliferation, migration and invasion. However, the effects of sh-NBAT1 on the biological behavior of glioma cells were not investigated in the present study.

miRNAs may function as oncogenes or tumor suppressors by modulating gene expression, and are considered to be targets of lncRNAs (24, 25). Previous studies have reported that the expression levels of miRNAs are impaired in several types of cancer, including glioma, and miR-140 and miR-152 interact with lncRNAs in glioma cells (26-29). The present study aimed to identify novel targets of NBAT1 in glioma. The results of the present study suggested that NBAT1 was able to bind with miR-21, the expression levels of which were notably upregulated in glioma tissues and cells. Additionally, the expression levels were inversely correlated in glioma tissues. Inhibition of miR-21 suppressed the proliferation, migration and invasion of glioma cells. The results of the present study were consistent with a previous study, which suggested that NBAT1 inhibited the progression of osteosarcoma via miR-21 signaling (22).

SOX7 is a member of the SOX transcription factors (39) and is considered to be an essential regulator in cardiovascular development (40). SOX7 functions as a tumor suppressor...
in endometrial cancer and as an oncogene in gastric cancer (41-44). The positive association between SOX7 expression and distant metastasis-free survival has been reported in prostate cancer and breast cancer (45,46). In the present study, SOX7 was identified as a downstream target of miR-21, and the results demonstrated that SOX7 expression levels were decreased in glioma tissues and cells compared with those in paracarcinoma tissues and normal astrocytes, respectively. Of note, downregulated SOX7 expression induced by transfection with miR-21 mimics was reversed following overexpression of NBAT1. Collectively, these results highlighted the key functions of NBAT1/miR-21/SOX7 signaling on the progression of glioma. A major limitation of the present study is that in vivo analysis was not performed, thus prospective studies will focus on performing in vivo experiments to validate these findings. Furthermore, the expression levels of proliferation- and invasion-associated molecules should be investigated to confirm the existing findings.

In conclusion, the results of the present study suggested that NBAT1 may act as a potential tumor suppressor by upregulating SOX7 via suppressing miR-21, thus inhibiting glioma cell proliferation, migration and invasion. These results indicate the essential roles of NBAT1 and
its underlying molecular mechanisms in the proliferation, migration and invasion of glioma cells, providing evidence on the potential functions of NBAT1 in tumorigenesis, and suggest that the NBAT1/miR-21/SOX7 axis may be a promising therapeutic target for the treatment of patients with glioma.

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Availability of data and materials

The datasets generated or analyzed during the present study are included in this published article.

Authors’ contributions

WG conceived and designed the present study. NG, RW, XF and CL performed the experiments and interpreted the data. All authors read and approved the final manuscript.
The role of β-β: A long non-coding RNA: lncRNA PFAR promotes lung fibroblast:  

References

The authors declare that they have no competing interests.

Competing interests

Not applicable.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of The First Affiliated Hospital of Jizhoun Medical University (approval no. JYD159302; Jinzhou, China). Written informed consent was provided by all patients for the use of clinical tissue prior to the study.

Patient consent for publication

Not applicable.

References


