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ORIGINAL ARTICLE

NBR2 promotes the proliferation of glioma cells *via* inhibiting p15 expression

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Summary

Purpose: *The purpose of this study was to investigate whether NBR2 can affect the proliferation of glioma cells by inhibiting the expression of p15, so as to promote the occurrence and development of glioma.*

Methods: *The expression of NBR2 in 44 glioma tissue specimens was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The effects of NBR2 on cell viability, cell colony formation as well as cell migration and invasion abilities were examined by cell counting kit-8 (CCK-8) assay, plate cloning assay and Transwell assay. p15 protein was detected using Western blot. After simultaneous knockdown of NBR2 and p15, qRT-PCR, CCK-8, and plate cloning experiments were adopted to analyze p15 gene level, cell viability and proliferation ability, respectively.*

Results: *NBR2 was highly expressed in glioma tissues, and the level in stage III/IV glioma tissues was conspicuously higher than that in stage I/II. The overall survival rate of glioma patients with high NBR2 level was conspicuously lower than those with low NBR2 expression. Clinical data analysis revealed that NBR2 expression was correlated with the WHO stage of clinical patients. After knockdown of NBR2, it was found that NBR2 level, cell viability, cell proliferation ability as well as migration and invasion abilities were all conspicuously reduced. In addition, the protein level of p15 was significantly increased after NBR2 was inhibited. Meanwhile, knockout of p15 could reverse the inhibitory effect of NBR2 on glioma cell proliferation.*

Conclusions: *The highly-expressed NBR2 inhibits the expression of p15, thus promoting the proliferation of glioma cells.*

Key words: *glioma, NBR2, p15*

Introduction

Glioma originates from glial cells in the brain, and its morbidity and mortality rates rank first among malignant tumors of the central nervous system [1]. Glioma has numerous histologic subtypes, which can generally be divided into hairy cell astrocytoma, diffuse star cell tumor, less variant between pilocytic astrocytoma, oligodendrocytes tumor and glioblastoma, and can be divided into grade I-IV according to WHO on the basis of its malignant degree [2]. At present, the commonly used glioma tumor markers include classic serum squamous cell carcinoma antigen (SCCA), carbohydrate antigen (CA) 19-9, carcinoembryonic antigen (CEA), but lacking sensitivity and specificity make these markers unable to be used in early diagnosis of glioma [3]. Therefore, it is urgent to explore new markers for early detection of glioma.

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As a major transcriptional regulator, long non-coding RNA (lncRNA) can regulate gene level through transcriptional interference and chromatin remodeling [4]. LncRNA is often dysregulated in many tumors, and its abnormal expression can be used as a potential diagnostic or prognostic biomarker for multiple human malignant tumors such as breast cancer, lung cancer and liver cancer [5-7]. LncRNA BRCA 1 gene 2 (NBR2) is an important lncRNA newly discovered in recent years because it is adjacent to the tumor suppressor gene BRCAl on the chromosome [8]. Studies have shown that lncRNA NBR2 level is negatively correlated with the development of various cancers, especially breast cancer [9]. However, no related studies between NBR2 and glioma have been reported.

P15 is located on the human chromosome 9p21 region, and the encoded protein contains 137 amino acids with a molecular weight of 14.7 kDa. In gliomas, the gene loss rate is high [10]. P15 is a cell cycle-dependent protein kinase inhibitor that binds to CDK4 or CDK6 and inhibits cells to enter from G1 to S phase, arresting the cell cycle in G1 phase. Deletion of p15 protein can cause a large number of cells to enter S phase, and the abnormally active proliferation can lead to tumor occurrence [11]. Moulton et al [12] reported that p15 deletion is associated with malignant transformation of glioma. Therefore, this study investigated whether NBR2 can regulate p15 protein to affect the development of glioma, and thus provide new clinical ideas for finding potential targets for glioma diagnosis and treatment.

Methods

Pathological tissue collection

The tumor tissues diagnosed as glioma by preoperative pathological examination in our hospital were collected, and the adjacent normal tissues 2 cm away from the cancer lesion were taken at the same time. Tissue samples were rapidly stored in liquid nitrogen after sampling. All patients were not treated with neoadjuvant chemotherapy or radiotherapy before surgery, no surgical contraindications were observed, and their survival time and status were followed up. This study was approved by the Ethics Committee of Tianjin Medical University General Hospital, and informed consent was signed by patients and their families before surgery.

Cell culture

Glioma cell lines (U87, LN229, U251 and T98) and normal glial cells (HA) were cultured at 37°C in 5% CO_2 in air in medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin along with 1% streptomycin. The medium was changed once every 3 days until the cells covered with the bottom of the culture bottle. All of the following experiments were performed on cells in the logarithmic growth phase.

Cell transfection

The cells were washed with phosphate buffered saline (PBS) for 3 times, trypsinized for 2 min, transferred to a 15 mL sterile centrifuge tube, centrifuged and counted, and seeded in 6-well plates at 4×10^5 cells per well. When the fusion rate reached about 80%, transfection was performed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). U87 cells were transfected with sh-NBR2 and si-NBR2, while LN229 cells were transfected with Lv-NBR2 and si-NBR2+si-p15. After transfection for 24 h, fresh medium was added in. (The sequences of these siRNAs are as follows: siRNA-NBR2 5'-GGUUGGCGAACACAUCCAUTT-3' (sense) and 5'-AUGGAUGUGUUCGCCAACCTT-3' (antisense); si-p15: CGCCUCUUCGAAUUUAAAUUU.)

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

The total RNA of the cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the levels of NBR2 and p15 were detected using a PCR detection kit. Primer sequences are as follows: NBR2: F: 5'-CTTCCAGTTGCGGCTTAT-3', R: 5'-ATCTTCCTCATC CAGGTATT-3'; p15 F: 5'-GGTGAACCCACAACTTAGGC-3'R: 5'-TTAGCATCTGTCGTCGCTTG-3'; Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) F: 5'-GAAGGTGAAGGTCGGAGTC-3'R: 5'-GAAGATGGTGATGGGATTTC-3'.

Cell counting kit-8 (CCK-8) test

The cells were plated into 96-well plates with four replicate wells each group, and CCK8 assay (Dojindo, Kumamoto, Japan) was performed after 6, 24, 48, 72, or 96 h of culture. Subsequently, 20 μL of CCK-8 reagent was added, and after 3 h, the optical density (OD) at 450 nm of each well was measured by an ultraviolet spectrophotometer.

Plate cloning experiment

After digested and counted, the cells were seeded in a 96-well plate at a number of 5.000 cells per well with three replicate wells in each group, and cultured in a 37°C incubator. After 24 h, the state of the cells was observed, and the medium was aspirated. The cells were added to lentivirus-transfected cells protected from light, incubated at 37°C for 24 h, and observed with a microscope (low magnification).

Transwell assay

Cell migration ability test: After transfected for 48 h, the cells were trypsinized and centrifuged, and the cell pellet was taken. The cell suspension was prepared with serum-free medium at a density of 1×10^4 cells/mL. 100 μL of cell suspension was added to the upper Transwell chamber, while 600 μL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) was added to the lower chamber. After cultured at 37°C in 5% CO_2 for 24 h, they were fixed with formaldehyde and subsequently

stained with 0.1% crystal violet staining. After the scattered cells were removed, cells were photographed and counted under high magnification.

Cell invasion ability test: Martrigel was diluted 1:6, coated with Transwell chamber, air-dried at 4°C, and incubated at 37°C for 60 min to coagulate. The cells were cultured for 48 h after transfection, trypsinized, and the remaining steps were same to above cell migration ability test.

Western blot

Protein lysate was added in cell culture plate on ice to extract cell total protein, which was then quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The equal amounts of protein samples in each group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After membrane transferring and blocking, rabbit anti-human BCL2 antibody (1:2000) was added to incubate the proteins on the membrane overnight at 4°C, with mouse anti-human GAPDH (1:6000) as an internal reference antibody. The membrane was washed the next day, then horseradish peroxidase-labeled goat anti-rabbit or mouse IgG (diluted as 1:5000) was added, incubated for 1 h at room temperature, and imaging was performed using a gel imaging system.

Statistics

Data analysis was performed using SPSS 22.0 statistical software package (IBM, Armonk, NY, USA). The difference between the two groups was compared using t-test. P<0.05 was considered to be statistically significant.

Results

NBR2 has a high expression in glioma tissues with a poor prognosis

NBR2 was found to be highly expressed in glioma tissues by qRT-PCR, and its level in stage III/IV glioma tissues was conspicuously higher than that in stage I/II ones (Figure 1A). Further analysis of the interplay between NBR2 level and glioma prognosis revealed that the overall survival time of patients with a high level of NBR2 was significantly lower than that of patients with a low one (Figure 1B HR=2.129, p=0.0324). The results of clinical data analysis showed that NBR2 level was correlated with WHO stage, but not with age and gender of patients (Table 1). The above results indicated that NBR2 was relevant to the develop-

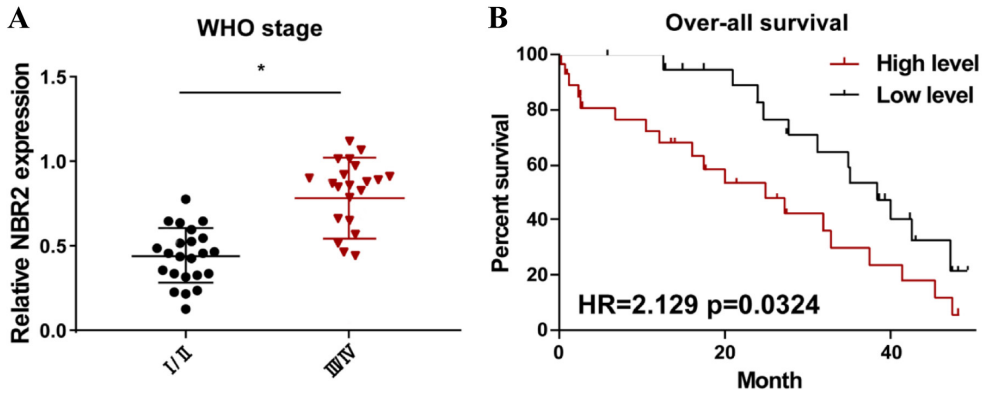

Figure 1. NBR2 is highly expressed in glioma tissues, leading to a poor prognosis. **A:** qRT-PCR detection of NBR2 expression in stage III/IV glioma tissues was significantly higher than that in stage I/II (*p<0.05). **B:** The overall survival of patients with high expression of NBR2 glioma was significantly lower than that of patients with low expression of NBR2.

Table 1. Correlation between NBR2 expression and clinicopathological features in glioma patients

Clinicopathologic features	*Number of cases*	*LncRNA NBR2 expression*		*p value*
		Low (n=22)	*High (n=22)*	
Age (years)				0.7726
≤50	23	11	12	
>50	21	11	10	
Gender				0.7679
Male	17	9	8	
Female	27	13	14	
WHO stage				0.0092
I/II	23	16	7	
III/IV	21	6	15	

ment of glioma, and the high level of NBR2 was not conducive to the prognosis of glioma.

NBR2 expression in glioma cells and the knockdown effect

QRT-PCR revealed that NBR2 expression in glioma cell lines (U87, LN229, U251 and T98) was found conspicuously higher than that in normal glial cells. Among the four cell lines, LN229 cells showed the highest expression, followed by U87 cells (Figure 2A), so the two cell lines were selected for subsequent experiment. After knocking down NBR2 in U87 and LN229 cells, the level of NBR2 was detected conspicuously decreased, confirming the transfection efficiency was good (Figure 2B).

Inhibition of glioma cell proliferation after NBR2 knockdown

In order to explore the relationship between NBR2 and glioma, CCK-8 test was performed and showed that U87 cell viability decreased after transfected with sh-NBR2 (Figure 3A), so did the LN229 cell viability (Figure 3B), indicating that NBR2 could enhance cell viability. In addition, results of plate cloning experiment demonstrated that proliferation of the U87 and LN229 cells transfected with sh-NBR2 decreased (Figure 3C), indicating that NBR2 promoted glioma cell proliferation.

NBR2 knockdown inhibits migration and invasion of glioma cells

After transfection of si-NBR2 in U87 and LN229, Transwell assay was performed and the result revealed a decrease in migration and invasion of U87 (Figure 4A) as well as LN229 cells (Figure 4B). The above results verified that NBR2 could enhance cell migration and invasion abilities.

Overexpression of NBR2 promotes the proliferation, migration and invasion of LN229 cells

To further verify the interplay between NBR2 and glioma cells, we overexpressed NBR2 in LN229, and qRT-PCR detected that NBR2 level was conspicuously increased in LN229 (Figure 5A), in-

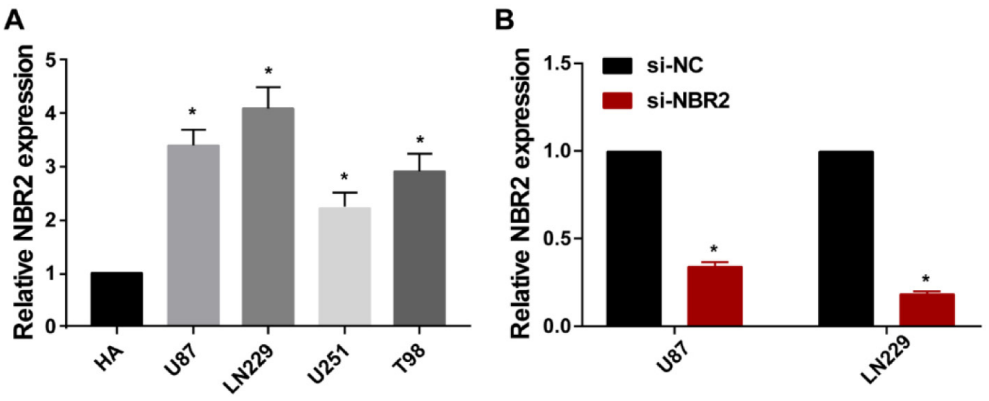

Figure 2. Expression of NBR2 in glioma cells and knockdown effect. **A:** qRT-PCR detection of NBR2 expression in glioma cell lines (U87, LN229, U251 and T98) was significantly higher than that in normal glial cells. **B:** After knocking down NBR2 in U87 and LN229 cells, the expression of NBR2 was significantly decreased (*p<0.05).

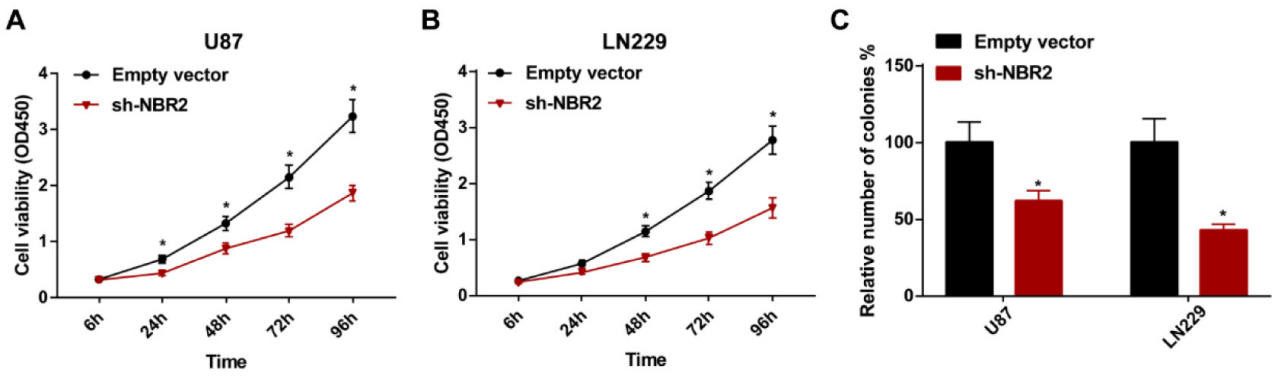

Figure 3. Glioma cell proliferation is inhibited after NBR2 knockdown. After transfection of sh-NBR2 in U87 and LN229, CCK-8 detected the decrease of cell viability of U87 cells **(A)** and reduced cell viability of LN229 cells **(B)**; **C:** Plate cloning assay showed that the proliferation of U87 and LN229 cell clones decreased (*p<0.05).

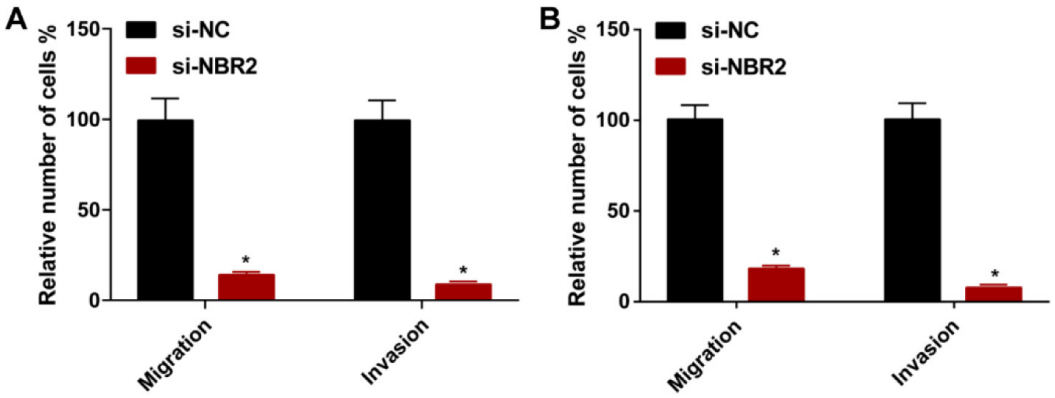

Figure 4. Migration and invasion of glioma cells are inhibited after NBR2 knockdown. After transfection of si-NBR2 in U87 and LN229, Transwell assay showed that the migration and invasion ability of U87 cells **(A)** and LN229 cells decreased **(B)** (*p<0.05).

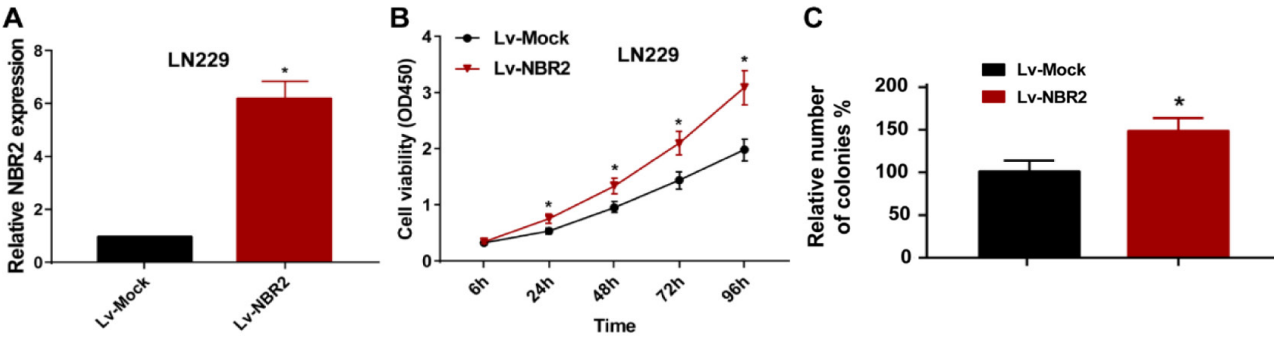

Figure 5. Overexpression of NBR2 promotes the proliferation, migration and invasion of LN229 cells. After overexpression of NBR2 in LN229, qRT-PCR detected that NBR2 expression was significantly increased in LN229 **(A)**, while CCK8 detected LN229 cell viability was significantly enhanced **(B)**; **C:** Plate cloning assay detected increased LN229 cell proliferation; **D:** Transwell experiment detected enhanced migration and invasion ability of LN229 cells (*p<0.05).

dicating that the transfection was successful. And the cell viability and capacities of clone formation (Figure 5B), migration and invasion (Figure 5C) were all found conspicuously enhanced. The above results suggested that NBR2 was able to enhance the cell functions of glioma cells mentioned above.

Knockdown of NBR2 can elevate p15 protein level

Western blot revealed that p15 protein expression was strikingly increased after knockdown of NBR2 in U87 and LN229 cells (Figure 6), which suggested that NBR2 might have a regulatory effect on p15, and knockdown of NBR2 may play a role of promoting p15 expression.

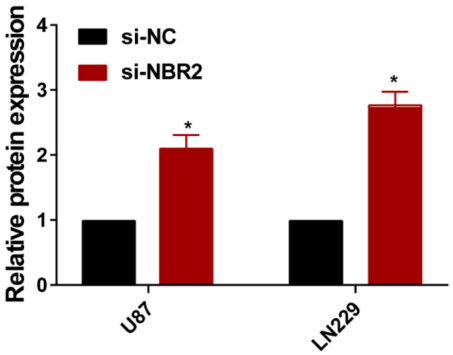

Figure 6. Protein expression of p15 was enhanced after knockdown of NBR2. After knockdown of NBR2 in U87 and LN229 cells, the expression of p15 was significantly increased confirmed by Western blot (*p<0.05).

Knockdown of p15 can reverse the inhibition effect of NBR2 knockdown on glioma cell proliferation

In order to clarify the regulatory association between NBR2 and p15 in the development of glioma, we divided LN229 cells into three groups, namely, control group, NBR2 knockdown group and simultaneous knockdown of NBR2 and p15 group. qRT-PCR detection revealed that knockdown of p15 reversed the increased p15 expression, and decreased cell viability and proliferation ability of LN229 cells induced by NBR2 knockdown (Figures 7A, 7B, 7C). The above results demonstrated that NBR2 can promote the occurrence and development of glioma by reducing p15 level.

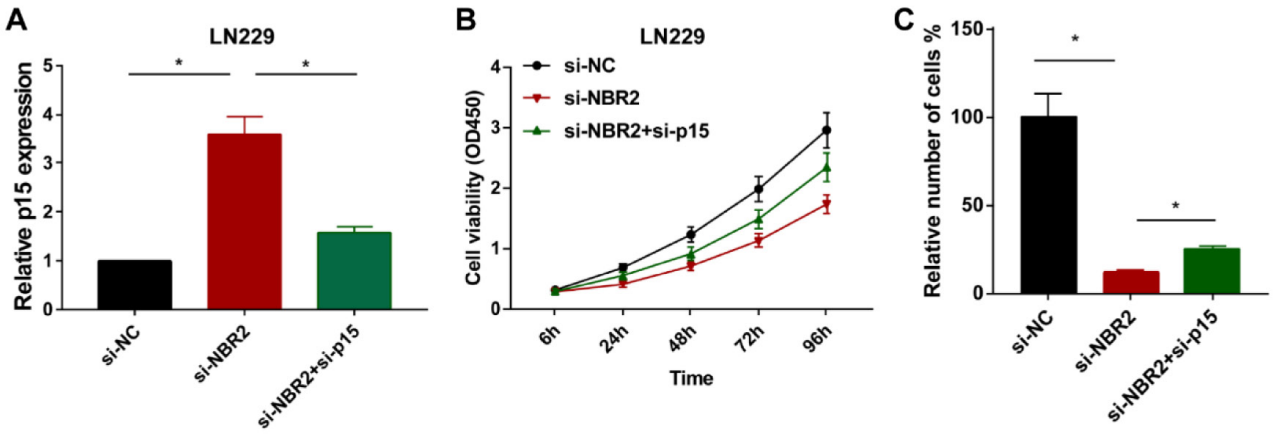

Figure 7. Knockdown of p15 reversed the inhibition effect of knockdown of NBR2 on glioma cell proliferation. LN229 cells were divided into three groups, which were control group, NBR2 knockdown group and NBR2&p15 simultaneous knockdown group. **A:** qRT-PCR detection of p15 gene expression; **B:** CCK8 was used to detect the decreased activity of LN229 cells after simultaneous knockdown of p15 and NBR2; **C:** Plate cloning assay was used to detect the decreased proliferation of LN229 cells after simultaneous knockdown of NBR2 and p15 (*p<0.05).

Discussion

Glioma is an intracranial neoplastic disease induced by malignant proliferation of glial cells caused by changes in genes and environment. It is a common neurosurgical disease that can cause headache, vomiting, epilepsy, speech disorder, hemiplegia, paresthesia and other clinical symptoms, and even death due to cerebral hernia in severe cases [13]. Therefore, the research on the pathogenesis of glioma has broad research prospects and important clinical significance.

LncRNA can not only serve as a diagnostic marker for a variety of tumors, but also participate in and regulate tumorigenesis [14]. Studies have shown that lncRNA AB073614 is more highly expressed in glioma cells than in normal tissues and is associated with tumor malignancy. Kaplan Meier survival analysis suggested that patients with a high level of AB073614 had a worse prognosis [15]. LncRNA HULC level was positively correlated with glioma grade, while inhibition of its expression can reduce glioma cell proliferation and invasion ability as well as suppress angiogenesis. In human glioma cells like U87MG and U251, HULC knockdown induces cell apoptosis and arrests the cell cycle in the G1/S phase *via* P13K/Akt/mTOR signaling pathway, but this process can be reversed by ESM-1 overexpression, meaning that ESM-1 is involved in the HULC process [16]. Similar to these conclusions, in this study, we found that NBR2 was highly expressed in glioma tissues, and led to a poor prognosis of glioma patients through promoting the proliferation, migration and invasion of glioma cells.

P15 gene, also known as cycle-dependent kinase suppressor gene, is a tumor suppressor related to a variety of tumors [17]. It is a direct transcription target of RB/E2F pathway in cell cycle process and DNA synthesis, which can over-stimulate cell division and proliferation to induce normal cells finally develop into cancer ones [18]. Studies have found that cells in some tumors such as liver cancer [18] and lung cancer [19] overexpress P15, making the prognosis of such patients poor. In this experiment, we found that NBR2 could inhibit the expression of p15 and thus promote the occurrence of glioma.

In summary, this study revealed that highly-expressed NBR2 can inhibit p15 expression and promote the proliferation of glioma cells, thus promoting the occurrence of glioma. This result may provide a theoretical basis for the pathogenesis and treatment of glioma.

Conclusions

The high expression of long non-coding RNA NBR2 is able to promote the proliferation of glioma cells by inhibiting the expression of p15, which provides many guidelines for the diagnosis and treatment of glioma.

Conflict of interests

The authors declare no conflict of interests.

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