

Interference of Notch 2 inhibits the progression of gliomas and induces cell apoptosis by induction of the cell cycle at the G0/G1 phase

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Abstract. Glioblastoma is the most common type of malignant brain tumor with a poor prognosis. The Notch signaling pathway is often aberrantly activated in glioma cells. In order to determine the expression of Notch 2 and to evaluate its possible prognostic value in malignant glioblastoma, specimens from 32 patients and 20 controls were analyzed using immunohistochemical staining and reverse transcription quantitative polymerase chain reaction. The expression of Notch 2 in the glioma tissues was significantly higher compared with that in the normal brain tissues ($P < 0.01$). Subsequently, endogenous Notch 2 interference was effectively performed by specific small hairpin (sh)RNA in the glioma cancer cell line U251. The results from an MTT assay and from Annexin V-fluorescein isothiocyanate/propidium iodide staining indicated that interference of Notch 2 significantly inhibited the proliferation and induced the apoptosis of U251 cells. In addition, the cell cycle was analyzed using flow cytometry and the results revealed that Notch 2 shRNA induced cell cycle arrest at the G0/G1 phase in U251 cells. Additionally, proteins associated with the cell cycle and cell proliferation were detected using western blot analysis. The data demonstrated that the expression of P21, cyclin D and phosphorylated retinoblastoma was significantly inhibited in the Notch 2 shRNA-transfected U251 cells. The results of the present study provide further insights into the effects of Notch 2 and a molecular reference for brain tumor therapy.

Introduction

A glioma is a type of malignant brain tumor that mainly arises from glial cells. The most common site of gliomas is in the brain (1). Gliomas represent ~30% of tumors of the brain and central nervous system and 80% of malignant tumors in the brain (2). The main types of glioma are ependymomas, astrocytic gliomas, oligodendrogliomas, brainstem gliomas, optic nerve gliomas and mixed gliomas (3,4). At present, due to the characteristics of glioma, including a rapid growth rate, unclear boundaries and frequent relapse following surgery, it remains one of the most intractable diseases in the field of neurosurgery (5). Therefore, it is particularly important to identify and develop new and effective treatment methods for glioma. Investigation of the targets of key molecules in the tumor signal transduction system has provided new hope for patients with gliomas.

The Notch signaling pathway is well known and is highly conserved in the majority of multicellular organisms (6). It has been confirmed that the occurrence of a variety of diseases, including cardiovascular disease and cancer, is closely associated with the abnormal activation of the Notch signaling pathway (7,8). The abnormal activation of Notch is found in several types of cancer, including lung cancer (9), colon cancer (10), cervical cancer (11) and pancreatic cancer (12). Notch 2 is expressed in normal neuroblasts and controls neuronal differentiation (13). It is also one of the receptors with a single-pass transmembrane receptor protein (13). Notch signaling enhances the proliferative effects during neurogenesis in mammals (14,15).

The present study aimed to clarify the effect of the Notch 2 protein on the proliferation of glioma cells. Initially, specimens from glioma tissues and normal brain tissues were obtained and the expression of Notch 2 was compared between them. Subsequently, RNA interference technology was used to knock down the expression of the Notch 2 protein in the human glioma cell line U251. Cell proliferation and cell cycle arrest were detected using an MTT assay and fluorescence-activated cell sorting (FACS). Additionally, proteins associated with the cell cycle and cell apoptosis

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were detected and compared using western blot analysis. Therefore, the present study aimed to provide new information to assist in the therapy of human gliomas.

Materials and methods

Specimens. A total of 32 glioma tumor samples and 20 normal tissue samples were obtained by surgical resection of traumatized brain tissue following traumatic brain injury. All the specimens were obtained from the Institute of Neurosurgery, Nanfang Hospital Affiliated to Southern Medical University (Guangzhou, China). All the experiments were performed on patients in compliance with the Helsinki Declaration and study approval was obtained from the Ethics Committee of Nanfang Hospital Affiliated to Southern Medical University. The patients and their families were well informed of the details and written informed consent was obtained prior to the study.

Immunohistochemical staining. The specimens, obtained from the glioma tumor and normal brain tissues, were fixed in neutral buffered paraformaldehyde and processed for hematoxylin and eosin staining. The process of immunohistochemical staining was performed, as described previously (16,17). The primary antibody used was rabbit polyclonal anti-Notch 2 (cat no. NB600-879; Novus Biologicals, Littleton, CO, USA), which was diluted (1:200) for use in the immunohistochemical analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to detect the Notch 2 mRNA expression levels in the glioma and normal brain tissues. Firstly, RNA was reverse transcribed into cDNA and the resulting cDNA was used as templates for PCR amplification. The primer sequences were as follows: Upstream, 5'-ATGACTGCCCTAACACAGG-3' and downstream, 5'-TGCAGTCATCTCCACTCCAG-3' for Notch 2; upstream, 5'-AGAGCTACGAGCTGCCTGAC-3' and downstream, 5'-AGCACTGTGTTGGCGTACAG-3' for β -actin. Here, β -actin was used as the internal control. A PCR kit (Invitrogen Life Technologies) was used and the cycle conditions were an initial step (hot start) of 95°C for 10 min prior to amplification cycles, followed by the PCR conditions of denaturation (95°C for 15 sec), annealing (59°C for 20 sec) and elongation (72°C for 15 sec) for a total of 25 cycles.

Cell line and short hairpin (sh)RNA. The human glioma cell line U251 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, UT, USA), penicillin and streptomycin (100 U/ml; Gibco-BRL, Carlsbad, CA, USA) at 37°C in 5% CO₂. Notch 2 shRNA (human) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA; cat no. sc-40135-V) and the control shRNA of Lentiviral Particles-A was obtained from Santa Cruz Biotechnology, Inc.; cat no. sc-108080).

Western blot analysis. The U251 cells were seeded into 48-well plates. After 3 days, the whole-cell extracts were prepared and the proteins were separated by PAGE, as previously described (18-20).

MTT assay. An MTT assay was performed, as described previously (21-23). Human glioma U251 cells (1×10^5) were seeded into a 48-well plate. Following culture for different time periods (1-7 days), the plates were read on a microplate reader (Corning, Inc., Acton, MA, USA) with a test wavelength of 490 nm.

Flow cytometric analysis. The apoptosis of human glioma U251 cells was determined using annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) dual staining according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc.) and cell cycle analysis was performed using PI staining, as described previously (24,25). Cells were then subjected to FACS analysis and >10,000 events were recorded in every example. The experiment was performed at least three independent times.

Statistical analysis. Basic statistical analyses were performed using the statistical software, SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). All results are expressed as the mean \pm standard deviation. $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Positive Notch 2 staining is observed in the majority of gliomas. In order to confirm the expression of Notch 2 in gliomas and in normal brain tissues, immunohistochemical staining analysis was performed. As shown in Fig. 1A-D, positive staining for Notch 2 appeared as brown granules, which were predominantly located in the cell membrane, nucleus and cytoplasm. However, only a small quantity of visible staining, indicating expression of Notch 2, was observed in normal brain tissues.

Subsequently, RT-qPCR was performed to further confirm the expression of Notch 2 in the glioma and normal brain tissues. As shown in Fig. 1E, the expression of Notch 2 in the glioma tissues was increased significantly compared with the normal brain tissues ($P < 0.01$).

Notch 2 shRNA decreases the expression of Notch 2 in U251 cells. Notch 2 shRNA was used for interference of endogenous Notch 2 gene expression. The silencing effect was then determined using RT-qPCR. As shown in Fig. 2, the Notch 2 shRNA effectively interfered with the expression of Notch 2. The control shRNA was used as a negative control.

Knocking down the expression of Notch 2 inhibits the growth of the glioma cell line U251. In order to determine whether Notch 2 knockdown inhibited the growth of U251 cells, an MTT assay was performed. As shown in Fig. 3, 4 days after transfection with shRNA, the optical density (OD)₄₉₀ values in the U251 cells transfected with Notch 2 shRNA were lower compared with the control cells ($^*P < 0.05$ and $^{**}P < 0.01$). Additionally, inhibition of U251 cell proliferation occurred in a time-dependent manner.

Transfection of Notch 2 shRNA induces the apoptosis of U251 cells. The present study then aimed to assess whether treating cancer cells with Notch 2 shRNA further induced

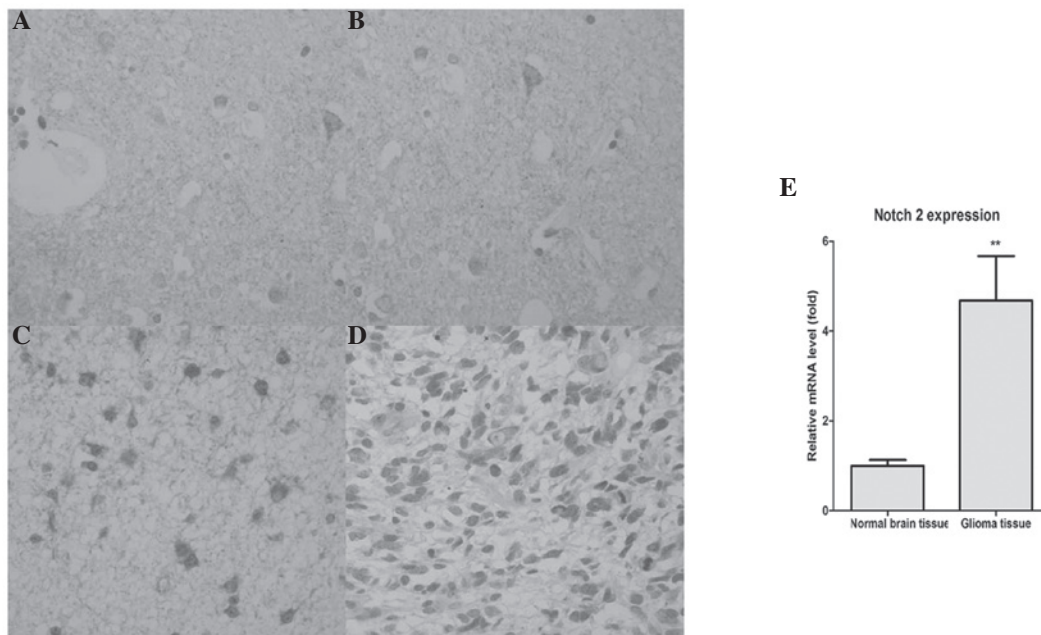


Figure 1. Paraffin-embedded samples were analyzed using histochemical staining for Notch 2. (A and B) Normal expression of Notch 2 was detected in the normal brain tissues and images were captured (magnification, x400). (D) Expression of Notch 2 was detected in the glioma tissues and images were captured (magnification, x400). (E) Total RNA was extracted and the expression of Notch 2 was assessed using reverse transcription quantitative polymerase chain reaction in the normal brain tissues and glioma tissues. **P<0.01, compared with normal brain tissues.

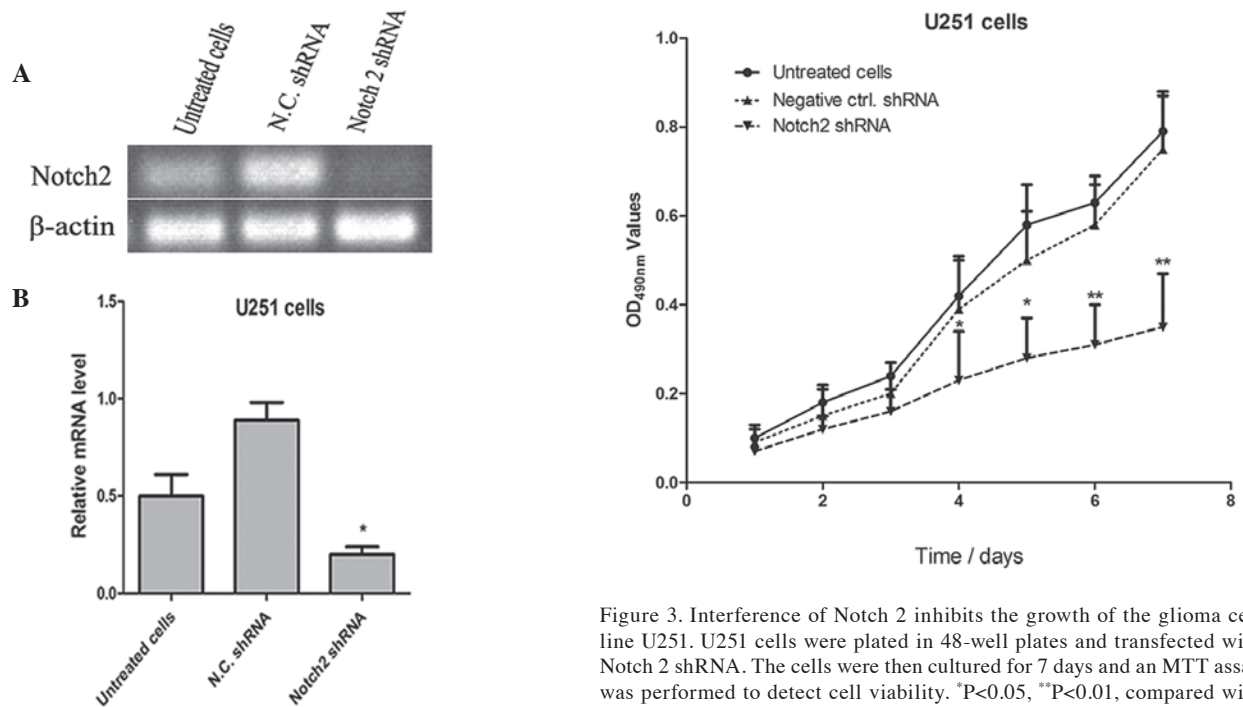


Figure 2. Expression of Notch 2 is effectively suppressed. The U251 cells were plated in 6-well plates. After 8 h, Notch 2 shRNA or NC shRNA was transfected into the U251 cells. After 48 h, total RNA was extracted and reverse transcribed into cDNA. Reverse transcription quantitative polymerase chain reaction was used to detect the expression of Notch 2. β -actin was used an internal reference. shRNA, short hairpin RNA; NC, negative control. *P<0.05, compared with untreated cells.

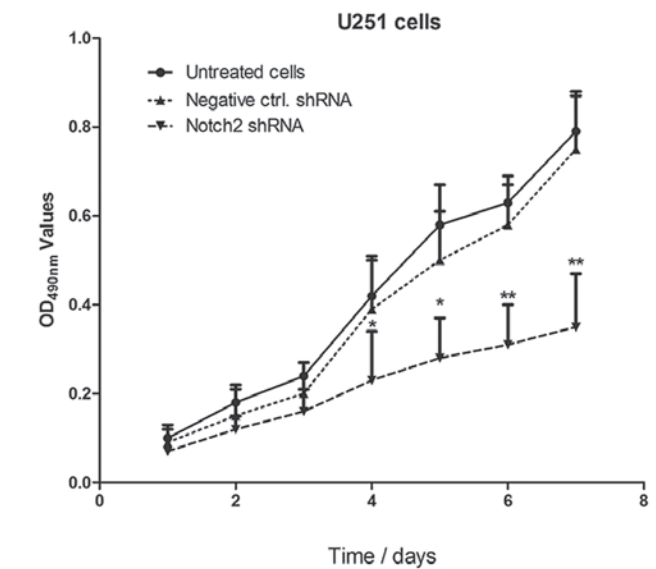


Figure 3. Interference of Notch 2 inhibits the growth of the glioma cell line U251. U251 cells were plated in 48-well plates and transfected with Notch 2 shRNA. The cells were then cultured for 7 days and an MTT assay was performed to detect cell viability. *P<0.05, **P<0.01, compared with the untreated cells. shRNA, short hairpin RNA; ctrl, control; OD, optical density.

rate of the cells transfected with Notch 2 shRNA was significantly higher in the Notch 2 shRNA group compared with the negative control shRNA group (36.7±4.5% and 6.8±2.8%, respectively; n=5; P=0.0027).

apoptosis. As shown in Fig. 4, Annexin V-FITC/PI dual staining was used to detect the apoptotic rate of the U251 cells. After 5 days, the results demonstrated that the apoptotic

Notch 2 shRNA1 induces cell cycle arrest at the G0/G1 phase in U251 cells. Subsequently, cell cycle distribution was assessed in the different groups of cells. As shown in Fig. 5,

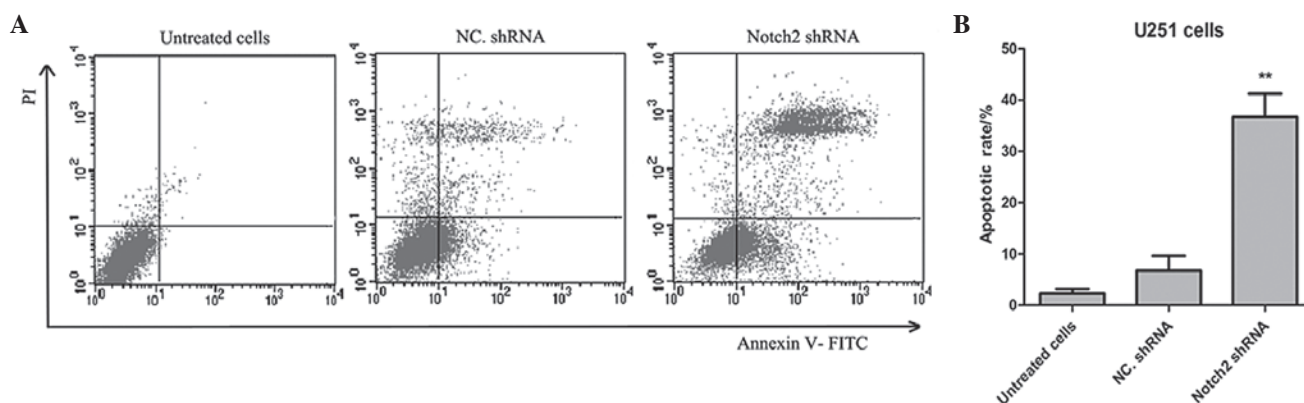


Figure 4. Transfection of Notch 2 shRNA induces the apoptosis of U251 cells (A) U251 cells were plated into a 6-well plate and transfected with Notch 2 shRNA. After 3 days, cell apoptosis was detected using Annexin V-FITC/PI staining. (B) Histogram of the cell apoptotic rate. Untreated cells or cells transfected with NC shRNA were used as negative controls. ** $P < 0.01$, compared with the untreated cells. FITC, fluorescein isothiocyanate; PI, propidium iodide; shRNA, small hairpin RNA; NC, negative control.

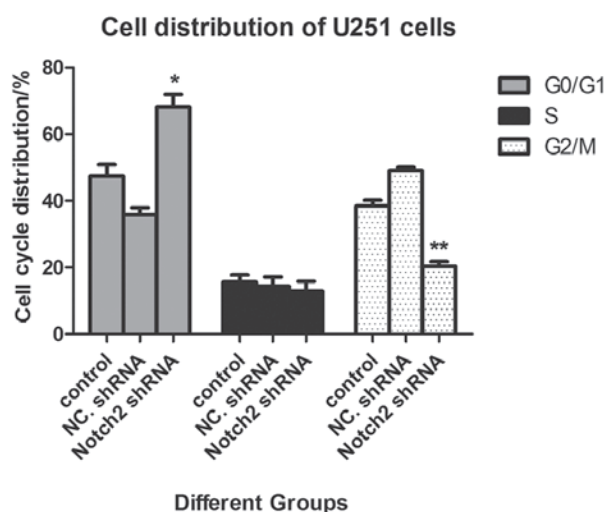


Figure 5. Notch 2 shRNA induces cell cycle arrest at the G0/G1 phase in U251 cells. Cells were stained with propidium iodide and analyzed for cell cycle distribution using flow cytometry. The histogram demonstrates cell cycle distribution. * $P < 0.05$ and ** $P < 0.01$, compared with the untreated cells.

the cell cycle was arrested at the G0/G1 phase following transfection with the Notch 2 shRNA compared with the negative control shRNA. The percentage of cells in the G0/G1 phase was increased from 47.40 to 68.20%, however, the percentage of cells in the G2/M phase was decreased from 38.40 to 20.40% ($P < 0.01$).

Detection of proteins associated with cell proliferation, the cell cycle and apoptosis. The expression levels of proteins associated with cell proliferation, the cell cycle and apoptosis were determined using western blot analysis. As shown in Fig. 6, the expression of Notch 2 was effectively suppressed in the Notch 2 shRNA-transfected U251 cells. In addition, the expression of P21 was also markedly increased compared with the untreated and negative control shRNA-transfected U251 cells, however, cyclin D and phosphorylated retinoblastoma (p-Rb) were significantly inhibited in the Notch 2 shRNA-transfected U251 cells.

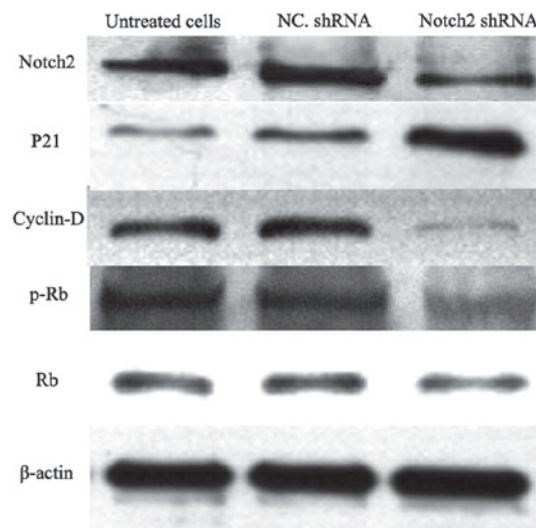


Figure 6. Notch 2 shRNA affects the expression of proteins associated with the cell cycle. Cell lysates were prepared and the expression of P21, cyclin D1 and p-Rb were detected using western blot analysis. β-actin was used as an internal reference. The untreated cells and the cells transfected with negative control shRNA were used as negative controls. Rb, retinoblastoma; p-Rb, phosphorylated retinoblastoma; shRNA, short hairpin RNA; NC, negative control.

Discussion

Gliomas are the most common and malignant type of tumor in the central nervous system (26). Glioma is particularly difficult to treat, with high rates of recurrence and low median survival rates. However, the treatment of gliomas is constantly improving and combination therapy, surgical and concurrent radiotherapy and chemotherapy are used. Despite this, the rate of recurrence is almost 100% (27,28), with little improvement in efficacy and a poor prognosis. The present study demonstrated that the Notch 2 protein exhibited abnormal and high expression of the Notch 2 protein in human glioma tissues. Following interference of Notch 2, the proliferation of the glioma cell line U251 was significantly inhibited, the cell cycle was arrested at the G0/G1 phase and apoptotic rates were markedly increased. These results suggested that the Notch 2

protein may be an effective target molecule in the therapy of gliomas. Additionally, it is key in several important pathways in tumor development and progression.

Purow *et al* (8) successfully reduced the expression of Notch 1 protein in the human glioma cell line U251 using RNA interference technology. The cells were then implanted into nude mice, which significantly prolonged the survival rate of the mice compared with the control group. Several studies have also demonstrated that activation of the Notch signaling pathway can stimulate cell proliferation in acute T lymphoblastic leukemia, breast cancer, renal epithelial tumor with transitional cells and pancreatic cancer (12,29-33). In the present study, the effects of depletion of Notch 2 by RNA interference on glioma cell cycle progression was investigated. The results revealed that loss of Notch 2 led to cell cycle arrest at the G0/G1 phase in U251 cells, with a significant decrease in the proportion of U251 cells in the G2/M phase. In addition, cell cycle arrest at the G0/G1 phase was accompanied by an accumulation of p21 and a decrease in cyclin D and p-Rb. All these results were consistent with each other.

In conclusion, the results of the present study demonstrated that interference of Notch 2 may provide a useful therapeutic approach in the treatment of human brain tumors and, to a certain extent, provides theoretical support for the use of gene therapy in the treatment of human gliomas.

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