Original Article
NPTX1 promotes tumorigenesis of glioma through augmentation of IRS-1/PI3K/AKT signaling

Leiming Huo1, Wentao Kong2, Maohua Zheng1, Yonghong Zhang1, Jiguang Xu1, Gang Yang1, Quanlin Guan1

1Department of Neurosurgery, The First Hospital of Lanzhou University, Lanzhou, Gansu, China; 2Department of Neurosurgery, The First People’s Hospital of Tongwei County, Gansu, China

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Abstract: A mount of previous research have focused on glioma, but very few combined its clinical characteristics with fundamental research. Thus, it is not clear about the exact mechanisms of glioma. In order to explore the potential mechanisms of glioma, 57 pairs of tumor tissues and the adjacent non-tumor tissues were collected, and through qRT-PCR and western blot (WB) analysis, the NPTX1 expression level was significantly elevated in tumor tissues in comparison with that in the normal tissues. According to long-term follow up visits, the survival rate of patients with high expression levels of NPTX1 was significantly decreased compared with the patients with low expression level of NPTX1 (P=0.0361). Afterwards, several glioma cell lines-U87, U373, and SHG44 were cultured, and qRT-PCR and WB analysis were performed, it was confirmed that the expression of NPTX1 was obviously elevated in these cells compared with that in the normal human glial cell-HEB. Additionally, after NPTX1 expression levels in U373 and SHG44 cells were increased respectively with lentivirus transfection, it showed that the proliferation rate of the cells was significantly promoted and the expression of IRS-1/PI3K/AKT signaling was also elevated. Furthermore, after NPTX1 expression was inhibited with NPTX1 siRNA, it was found the proliferation rate of the cells was notably decreased. In addition, the expression level of IRS-1/PI3K/AKT signaling was also significantly down-regulated. The IRS-1/PI3K/AKT signaling proteins were detected with WB in the tumor tissues; it showed that the signaling proteins were all statistically elevated. The results demonstrated that NPTX1 may promote tumorigenesis of glioma through augmentation of IRS-1/PI3K/AKT signaling.

Keywords: Glioma, NPTX1, tumorigenesis, IRS-1, PI3K, AKT

Introduction

Gliomas are the most prevalent malignant tumors taking up 46-70% of all the central nervous system (CNS) tumors [1]. Glioma is characterized by its invasive growth and a high rate of recurrence after intracranial surgery, leading to a poor prognosis [2, 3]. The World Health Organization (WHO) has classified CNS gliomas into four grades. Grade I and Grade II belong to low grades tumors while Grade III and Grade IV belong to high grades tumors [4]. Despite significant advances in surgical techniques and chemotherapeutic treatment, the median survival rate of glioma patients is extremely poor [5-7]. It is reported that a 5-year survival rates of patients with Grades I and II range from 30% to 70% while survival of 9 to 12 months for patients with Grades III and IV [8]. Hence, it is crucial to explore the molecular mechanisms underlying the development of glioma and to identify novel biomarkers for predicting progression of glioma and seeking for targeted therapy. Neuronal pentraxin-1 (NPTX1), locating on chromosome 17q25.3, is identified as a binding protein and regulates a series of cellular process such as cell immortalization, neural induction [9, 10]. It has been reported that NPTX1 is potentially involved in pancreatic cancer development and progression through methylation of its 5’ CpG island [10]. The alterations at NPTX1 epigenetic level may serve as potential diagnostic and prognostic biomarkers in lung cancer [11]. In previous research of cervical cancer and colorectal cancer, it has been found that NPTX1 plays a crucial role in the progression and metastasis of the tumors as a methylation marker [12, 13]. Though NPTX1 was involved in a variety of cancers and regulated growth, development and metastasis of
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tumor cells, the relationship between NPTX1 and glioma has not been reported yet. Thus, this study was the first to explore the role of NPTX1 in glioma carcinogenesis.

The insulin receptor is a protein with intrinsic tyrosine kinase activity that, following activation by insulin, undergoes rapid autophosphorylation. Subsequently, it phosphorylates intra-cellular protein substrates, including insulin receptor substrate (IRS) proteins 1 and 2. IRS-1 and IRS-2 activate several proteins, including phosphatidylinositol-3 kinase (PI3K). This, in turn, activates, among some downstream targets, the Akt/PKB pathway, which plays a pivotal role in regulation of various biological processes, including apoptosis, proliferation, differentiation, and intermediary metabolism. Juliet C. Russell, reported that expression of a dominant-negative inhibitor of Akt (Akt-kd) blocked phosphorylation of GSK-3α/β and subsequently enhanced NPTX1 induction [14]. So we wanted to study the effect of IRS-1/PI3K/AKT signaling pathway on NPTX1.

In this study, it has been found the expression level of NPTX1 was elevated in glioma patients' tumor tissues and the survival rate of patients with high expression levels of NPTX1 was obviously decreased. Through a series of in vitro and in vivo experiments, it was confirmed that IRS-1/PI3K/AKT signaling pathway was activated in glioma tissues and tumor cells. Therefore, NPTX1 promotes glioma growth and development through activating IRS-1/PI3K/AKT signaling pathway. It could be a therapeutic target in the treatment of glioma.

Materials and methods

The glioma patients and their tissue samples

During 2009 to 2016, a total of 57 pairs of glioma tumors and the adjacent normal tissues were collected from the Department of Neurosurgery of The First Hospital of Lanzhou University. Inclusion Criteria were as follows: (1) all patients were suspected with glioma on pre-operative CT and MRI; (2) the selected gliomas were those which had less clear border and supratentorial gliomas, especially those that had edema; (3) the diameter of gliomas was <5.5 cm; (4) all of the patients voluntarily joined this study and were older than 18 years. Exclusion criteria were as follows: (1) the patients who refused to participate in this study; (2) the patients who were allergic to contrast agent composition; (3) the patients that were not suitable for participation in the study because of severe heart or lung disease; (4) the quality of conventional ultrasound image was not satisfactory; (5) the patients who had received other treatment before surgery, such as radiotherapy and chemotherapy.

Tumor and the corresponding normal tissues were rapidly frozen in liquid nitrogen and preserved at -70°C till use. Influencing factors including blood transfusion, chemotherapy or radiotherapy had never been existed before surgery. All the samples used for experiments were with the patients' written informed consent and the approval of the ethic committee of First Hospital of Lanzhou University (Ethic Number: FM20090C03). In addition, the follow-up visit for the patients was as long as five years.

Cells culture and transfection

Human glioma cell line U87, U373, SHG44 and normal human glial cells-HEB purchased from the Chinese Academy of Sciences (Shanghai Cell Bank, China) were cultured in MEM/EBSS (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone), 50 U/mL penicillin, and 50 mg/mL streptomycin (Hyclone) at 37°C in a humidified 5% CO2 atmosphere. Replacing the medium of the cells plated in a 6-well plate (5*10^5 cells/well) every 3 days and till the cell fusion reaching to around 70%. Glioma cells were infected by adding NPTX1 Lentiviral Activation Particles (Santa Cruz, USA) in complete medium with polybrene (4 μg/mL) and incubated overnight. Stable activated clones were selected via 5 μg/mL puromycinhydrochloride (Sigma-Aldrich, USA). NPTX1 siRNA regent (100 nM, Sangon, Shanghai, China) and its negative control were transfected into the cells, respectively. The medium was exchanged for fresh complete medium after 6 h and 36 h later, total protein and RNA were extracted, respectively. The expression of NPTX1 mRNA and protein version was detected by reverse transcription polymerase chain reaction (RT-PCR) and western blot, respectively.

Quantitative real-time PCR analysis

Total RNA was extracted from glioma tissues or transfected cells using Trizol reagent
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(Invitrogen, Carlsbad, CA, USA), and then total RNA (lug) was reverse transcribed using a reverse transcription kit (Takara, Dalian, China) according to the manufacturer’s protocol. Sequences of all the primers were as follows: NPTX1 forward, 5’-TTG ACA GTT GCA TCA CAA CG-3’, NPTX1 reverse, 5’-CCA GCT ATG GCC TGC GAC CG-3’; β-actin forward, GCT GTC CCT GTA TGC CTC T-3’, β-actin reverse, TGT CAC GCA CGA TTT CC-3’. Subsequently, 1 µl of NPTX1 primers (Sangon Biotech, Shanghai, China), 10 µl of SYBR (Takara, Dalian, China), 6 µl of DEPC water (Sangon, Shanghai, China) and 2 µl of cDNA production, which was in a total volume of 20 µl reactions and then performed by the Real-Time PCR System (Roche, Basel, Switzerland). The following thermal cycling conditions: 5 sec at 95°C; 40 cycles of 1 sec at 95°C and 20 sec at 65°C reaction conditions. The comparative 2-ΔΔCt cycle threshold method was used for relative quantification of gene expression [14]. Each sample was analyzed in three times. The ratio of NPTX1 expression in tumor tissues and in the adjacent tissues over 3 was considered to be relatively high expression.

Western blot analysis

Total protein was extracted from collected tissues and cells using RIPA Lysis Buffer (Beyotime, Shanghai, China) supplemented with 100× proteinase inhibitor and phosphatase inhibitor, and then the proteins were separated with 10% SDS-PAGE. Afterwards, the gel was transferred to a PVDF membrane (Merck Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk for 1 h at room temperature. The primary antibodies: anti-NPTX1 (#3725, 1:1000 dilution), anti-IRS-1 (#2382, 1:500 dilution), anti-p-IRS-1 (Tyr895) (#3070, 1:500), anti-p-AKT (ser473) (#12694, 1:1000 dilution), anti-AKT (#2938, 1:1000 dilution), anti-β-actin (#4970, 1:1000 dilution) were all rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA). The secondary antibody (Beyotime, Shanghai, China) was at diluted at 1:5000. Signal was visualized with the ECL kit (Invitrogen, Carlsbad, CA, USA). The gray bands were analyzed with Image J software (NIH, Bethesda, MD, USA) to compare the expression between targeted proteins and internal controls.

U373 and SHG44 cells proliferation assay

To detect the proliferation activity of transfected U373 and SHG44 cells, the 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using a Cell Proliferation Kit I (Sigma-Aldrich, St.Louis, MO, USA) in a 96-well plate at a density of 4×10^4 per well according to the manufacturer’s instructions. After 24, 48, and 72 hours of incubation, MTT-formazan production was estimated by VersaMax (Molecular Devices, CA) at 570 nm to evaluate the proliferation rate. All experiments were performed in triplicates.

Statistical analysis

Data are presented as mean ± SEM. All statistical analyses were performed using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA). The survival rate of patients was stratified using the Kaplan-Meier method and statistically analyzed using the log rank statistic. The characteristics of the patients were analyzed using a two-sided chi-square test or a one-way analysis of variance. The differences between two groups were compared using Student’s t-tests, and those among three or more groups using one-way ANOVA analysis. Statistical significance was accepted at P<0.05.

Results

The expression of NPTX1 was elevated in glioma tissues or cells and was intimately associated with survival rates

From 2009 to 2016, a total of 57 glioma patients underwent brain tumor resection in the Department of Neurosurgery of the First Hospital of Lanzhou University. The tumor and the paratumor tissues were collected. qRT-PCR showed that NPTX1 was statistically promoted in tumor tissues in comparison with paratumor tissues at any mRNA or protein level (Figure 1A, 1B). Actually, it was elevated 1.7 folds at transcriptional level and 1.3 folds in translational level, respectively. In order to further clarify the relationship, several of human glioma cell lines were cultured and, it was found that at any mRNA or protein level, the expression of NPTX1 was significantly elevated in glioma cells compared with in normal brain glial cell (Figure 1C, 1D). Thus, the patients were classified into two groups according to the differential expression
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It was found that there were no statistical differences in age, gender between the two groups; but in the Grade and TNM stage, the differences were statistical, and P value was 0.039 and 0.012, respectively. A 5-year follow-up visit was conducted for the two groups, and it was demonstrated that the survival rate was significantly decreased in the group with NPTX1 high expression levels in comparison with the group with NPTX1 low expression levels (Figure 1E).

Proliferation rates of the U373 and SHG44 cells were elevated after NPTX1 was overexpressed

Two human glioma cell lines—U373 and SHG44 cells were cultured and transfected with lentivirus particles with NPTX1 gene. NPTX1 was successfully inserted into the cells; the total RNA and protein was extracted; through qRT-PCR and WB analysis, it was showed that in mRNA level, the expression level of NPTX1 was elevated 2.3 folds in U373 cells (Figure 2A) and 3.2 folds in SHG44 cells (Figure 2B). In protein level, the expression of NPTX1 was elevated 1.4 folds in U373 cells (Figure 2C) and 1.5 folds in SHG44 cells (Figure 2D). Thus, the expression of NPTX1 was significantly promoted in any transcriptional or translational level in the two human glioma cells. The proliferation rate of the cells was detected using the MTT assay; it was found the proliferation rate of the cells was significantly up-regulated after NPTX1 was overexpressed in U373 cells (Figure 2E) and in SHG44 cells (Figure 2F).

The IRS-1/PI3K/AKT signaling pathway was intimately associated with NPTX1

In order to further explore the potential mechanisms involved in glioma. The expression of NPTX1 was elevated after the glioma cells was transfected with lentivirus, while the NPTX1 expression was decreased after NPTX1 siRNA was transfected into the cells. After NPTX1

Figure 1. The expression of NPTX1 was promoted in glioma tissues and cells and associated with survival rates. A. The expression of NPTX1 mRNA was significantly up-regulated in glioma tissues in comparison with its adjacent non-tumor tissues. B. At protein level, the expression of NPTX1 was obviously promoted in the tumor tissue compared with its normal tissue. C. In cultured human glioma cells, the expression of NPTX1 mRNA was also higher than in normal brain glial cell. D. At protein level, the expression of NPTX1 was notably elevated in human glioma cells compared with the normal brain glial cell. E. The survival rate of the patients with high expression of NPTX1 was decreased in comparison with the patients with low expression of NPTX1. Number of independent samples n=3, **P<0.01, ***P<0.001.
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siRNA was transfected into the U373 cells, the mRNA expression level of NPTX1 was significantly decreased 48% (Figure 3A). Furthermore, the proliferation rate of the U373 cells was decreased 55% (Figure 3B). According to WB analysis, the expression of NPTX1/IRS-1/PI3K/AKT signaling pathway was obviously inhibited.

(Figure 3C). In addition, it was clear that the expression level of NPTX1 was down-regulated by almost 20%, and phosphorylated IRS-1 (p-IRS-1) was decreased by 32%. The expression level of PI3K and p-AKT was down-regulated by 30% and 38%, respectively. However, after NPTX1 was overexpressed, the expression level of IRS-1/PI3K/AKT signaling pathway was statistically elevated (Figure 3D). Actually, the expression level of p-IRS-1 was up-regulated by 2.1 folds, and the expression of PI3K and p-AKT was elevated by 1.7 and 1.5 folds, respectively.

The expression level of p-IRS-1, PI3K and p-AKT were increased in the tissues of glioma patients

It was found that the IRS-1/PI3K/AKT signaling pathway was significantly promoted in human glioma cells after NPTX1 was overexpressed. And then IRS-1/PI3K/AKT signaling was also activated in the tissues of glioma patients. Therefore, by WB analysis, it was demonstrated that in tumor tissues, the expression of p-IRS-1 was up-regulated by 4.6 folds, and the PI3K expression was elevated by 6.2 folds. In addition, the expression of p-AKT was up-regulated by 2.1 folds (Figure 4A and 4B) while the expression levels of IRS-1 and AKT were not significantly different. The results again demonstrated that in the development of glioma, the IRS-1/PI3K/AKT signaling pathway was indeed activated.

Discussion

It has been reported that in the United States of America (USA), approximately 6 persons per...
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Figure 3. IRS-1/PI3K/AKT signaling pathway was involved in the growth and development of glioma. A. The expression of NPTX1 mRNA was statistically reduced as NPTX1 was inhibited by NPTX1 siRNA transfection with U373 cells. B. The proliferation rate of the U373 cells was significantly decreased after NPTX1 siRNA was transfected into the cells. C. The expression of p-IRS-1, PI3K, p-AKT was notably decreased after the expression of NPTX1 was obviously suppressed. D. After NPTX1 was overexpressed, the expression of p-IRS-1, PI3K, p-AKT was notably elevated. Number of independent samples n=3, *P<0.05, ***P<0.001.

Figure 4. The expression of p-IRS-1, PI3K and p-AKT was up-regulated in glioma tissues. A. Though WB analysis, the expression of p-IRS-1, PI3K and p-AKT was significantly promoted in glioma tumor tissues in comparison with the adjacent normal tissues. B. Through Image J software analysis, the expression of p-IRS-1, PI3K and p-AKT was elevated 4.6 folds, 6.2 folds, 2.1 folds respectively in tumor tissues compared with the normal tissues. Number of independent samples n=3, *P<0.05, ***P<0.001.

NPTX1 is served as a binding protein which is involved in the increase of synaptic strength when neuronal activity is suppressed [19]. It has been demonstrated that synergistic action of metformin and aspirin regulates the transcriptional profile of pancreatic cancer cells, and NPTX1 is up-regulated by more than 10 folds [20]. NPTX1 has been found to be located within the 17q25.3 breakpoint region and which is significantly associated with bipolar disorder [21]. Specific gene expression signatures have been identified in individuals and Nptx1 expression is decreased in strains, which is susceptible to pulmonary adenomas [22]. NPTX1 was found to be intimately associated with colorectal cancer, high-grade cervical intraepithelial neoplasia and cervical cancer as biomarkers involved in methylation [13, 23]. These previous studies demonstrate that NPTX1 is closely associated with a variety of tumors, but the relationship between NPTX1 and glioma has not been studied. In this study, 57 pairs of glioma patients’ tumor tissues and the adjacent normal tissues were collected and by qRT-PCR, it was found that the expression of NPTX1 was significantly elevated in tumor tissues compared with in normal tissues. What’s more, the expression of NPTX1 was also higher in cultured human glioma cells than in the normal brain glial cells. In addition, the survival rate of patients with high expression level of NPTX1 was significantly decreased compared with the patients with low NPTX1 expression levels. These results clearly illustrate that NPTX1 was closely associated with glioma and it could be a biomarker and therapeutic target for the treatment of glioma. Furthermore, after the expression of NPTX1 was overexpressed or suppressed through lentivirus or siRNA trans-
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Infection, it was found that the proliferation rate of the cells was obviously elevated or decreased. The results show that in growth and development of glioma cells, NPTX1 played an essential role.

Klotho regulates IGF-1R phosphorylation, subsequent activation of IRS-1/PI3K/Akt/mTOR signaling, tumor cell proliferation, apoptosis, and autophagy in gastric cancer [24]. It has been showed that leptin stimulates the migration of human prostate cancer cells; one of the mechanisms was transcriptional up-regulation of αvβ3 integrin expression through the OBR1/IRS-1/PI3K/Akt/NF-kB signal transduction pathway [25]. In previous studies, leptin promotes the migration of chondrosarcoma cells by increasing alphavbeta3 integrin expression through the OBR1/IRS-1/PI3K/Akt/NF-kB signal transduction pathway [26]. In this study, after the glioma cells was transfected with lentiviral particles with NPTX1 gene, the expression of NPTX1 was significantly elevated. It shows that the expression of p-IRS-1, PI3K and p-AKT was obviously promoted while the results turned to be opposite as NPTX1 was notably inhibited via NPTX1 siRNA transfection. What’s more, as we detected the expression of p-IRS-1, PI3K and p-AKT in the tumor tissues by WB, the results clearly clarified that IRS-1/PI3K/AKT was significantly activated. All the results show that the IRS-1/PI3K/AKT signaling pathway was up-regulated in the glioma and it was positively associated with the growth, proliferation and metastasis of the glioma.

This research provided a new biomarker-NPTX1 for the diagnosis of glioma and it could be a brand new therapeutic target in the treatment of glioma. In the regulation of glioma’s growth, proliferation and migration, NPTX1 plays a vital role through activating the expression of IRS-1/PI3K/AKT signaling pathway. More data is still needed to further illustrate this relationship.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Quanlin Guan, Department of Neurosurgery, The First Hospital of Lanzhou University, Gansu 730000, China. Tel: 86-0931-8356740; Fax: 86-0931-8619797; E-mail: guanuanl@163.com

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