High Expression of PTPN3 Predicts Progression and Unfavorable Prognosis of Glioblastoma

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Background: PTPN3 was demonstrated to be involved in the progression of several types of cancers, such as gastric adenocarcinoma, lung cancer, and intrahepatic cholangiocarcinoma. However, its clinical significance in glioblastoma (GBM) has not been elucidated.

Material/Methods: We investigated the expression of PTPN3 in 95 cases of GBM with immunohistochemistry and in 8 pairs of fresh GBMs and their adjacent tissues with qualitative polymerase chain reaction. Moreover, the correlation between PTPN3 and clinicopathological factors was evaluated by chi-square test. The prognostic value of PTPN3 was investigated with univariate analysis and multivariate analysis. With MTT assay and Transwell assay, the oncogenic functions of PTPN3 in GBM proliferation and invasion were further investigated.

Results: Expression of PTPN3 in GBM tissues was significantly higher than in their corresponding adjacent tissues. High expression of PTPN3 was significantly associated with unfavorable prognosis of GBM. Moreover, in GBM cell lines, PTPN3 promoted cell proliferation and invasion, and the PTP common inhibitor pervanadate suppressed GBM proliferation and invasion.

Conclusions: Our experiments show that PTPN3 is an independent prognostic factor in GBM and indicated that postoperative detection of PTPN3 can be used to identify high-risk patients and guide individual treatment.

MeSH Keywords: Cell Proliferation • Glioblastoma • Neoplasm Invasiveness • Prognosis • Protein Tyrosine Phosphatase, Non-Receptor Type 3

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Background

Malignant glioma is an aggressive and highly invasive primary brain tumor [1]. Glioblastoma (GBM) is the most common and lethal subtype (grade IV), with the highest morbidity. GBM accounts for more than 50% of all detected malignant primary brain cancers [2]. The standard treatment of GBM is usually surgery, followed by radiation therapy or/and chemotherapy [3]. Unfortunately, the overall survival rate of GBM is still very poor, although the surgical equipment has been improved and new chemical drugs such as temozolomide have been applied. The median survival time of GBM is 2.1–14.6 months, and the 2-year survival rate is approximately 26% [4]. Moreover, there is no available targeted drug to GBM in this era of precise treatment. Based on the unsatisfactory prognosis of GBM, new biomarkers and drugs are in urgent need to improve survival time of patients.

Tyrosine protein phosphorylation is essential in many cellular processes, including cell growth, differentiation, cell cycle regulation, apoptosis, and invasion. The reversible tyrosine phosphorylation is coordinated and controlled by protein tyrosine kinases (PTKs) and phosphatases (PTPs). The roles of tyrosine phosphorylation in human cancer are controversial. Some PTPs have potential tumor suppressor functions in human cancers, while some PTPs are considered to be oncoproteins in cancers [5]. Interestingly, even the same PTP has opposite functions of progression in different tumor types. For example, PTPRA has an oncogenic function in colon cancer [6], but it is regarded as a tumor suppressor in breast cancer [7]. In humans, there are 107 types of PTPs, and PTPN3 (also called PTPH1) is a non-receptor PTP [8]. PTPN3 regulates the immune response by inhibiting T cell activation and promoting tumorigenesis in several solid tumors [9], and it was demonstrated to be involved in the progression of several types of cancers, such as gastric adenocarcinoma, lung cancer, and intrahepatic cholangiocarcinoma [10–12]. In glioma, PTPN3 was proved to promote tumor growth and metastasis with in vitro experiments [12]. However, the clinical significance of PTPN3 in GBM is unclear.

In the present study, we investigated the expression of PTPN3 in 95 cases of GBM and in 8 pairs of fresh GBMs and their adjacent tissues. Moreover, we evaluated the clinical significance of PTPN3 by analyzing its correlation with clinicopathological factors and its prognostic value. Using in vitro experiments, we further investigated the oncogenic function of PTPN3 in GBM proliferation and invasion.

Material and Methods

Patients and follow-up

A total of 212 patients who were diagnosed as having GBM and who underwent surgical resection in Yidu Central Hospital and Harbin Medical University Cancer Hospital were enrolled into the primary cohort. The validation cohort was selected from the primary cohort if patients had available follow-up and specimen for immunohistochemistry (IHC) detection. The validation cohort consisted of 95 patients, including 52 male patients and 43 female patients. The average follow-up time of the validation cohort was 8.1 months. In the 95 patients, 8 pairs of GBM tissues and the adjacent tumor tissues were obtained during the operations and preserved in liquid nitrogen immediately for qualitative polymerase chain reaction (qPCR) detection. The study was approved by the Ethics Committee of Yidu Central Hospital and Harbin Medical University Cancer Hospital. All paraffin-embedded specimens were obtained from the Department of Pathology with consent of patients. All the patients underwent evaluation using the Karnofsky Performance Scale (KPS), and all tissues were confirmed according to the World Health Organization classification, 2007 version. The overall survival time was calculated from the operation to the date of death or censored.

Immunohistochemistry and evaluation

The expression of PTPN3 was detected with IHC. The paraffin-embedded slides were first deparaffinized with xylene and then incubated in citrate buffer heated in a microwave oven for 30 min for optimal antigen retrieval. We used 3% H2O2 to immerse the specimens for 20 min for blockage of endogenous peroxidase enzyme. The primary antibody of PTPN3 (sc-515181, Santa Cruz Biotechnology, CA, USA) with dilution at 1: 100 was used to incubate the specimens at 4°C overnight. After being rinsed with phosphate-buffered saline (PBS) 3 times, slides were incubated in biotinylated secondary antibodies (Sangon, Shanghai, China) at room temperature for 1 h. We used 3,3’-diaminobenzidine solution for visualization.

The IHC results were semi-quantified by 2 senior pathologists who were unaware of the clinical data. The total IHC score system, consisting of 2 parts – the staining intensity and the positive cells percentage – was used to evaluate the staining results. The score for staining intensity from 0 to 4 referred to negative staining, weak staining, medium staining, and strong staining, respectively. The score for positive cells was defined as: 1, <25% positive cells; 2, 25–50% positive cells; 3, 50–75% positive cells; and 4, 75–100% positive cells. The final IHC score was defined as the product of the score for positive cells multiplied by the score for staining intensity. The verification cohort was divided into groups with high/low PTPN3 expression.
according to the cut-off defined by the ROC curve [13]. In our study, the cut-off was defined as 3.5, meaning that a score ≥4 was regarded as high expression of PTPN3.

**Cell culture and transfection**

Human GBM cell lines U118 MG and A172 and glioma cell line U251 MG were all purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with streptomycin (100 μg/ml) and penicillin (100 U/ml). The siRNA of PTPN3 and the scrambled siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection was performed using Lipofectamine 2000 according to the manual.

**RNA extraction and qPCR**

The mRNA was extracted from GBM tissues and adjacent tissues with the TRizol agent RNeasy Protect MiniKit (Qiagen, Hilden, Germany). Synthesis of cDNA was achieved using the qPCR-RT-Kit of ToyoBio Company (Osaka, Japan) and quantitative PCR was performed using the StepOnePlus Real-Time PCR system (Applied Biosystems) in SYBR Greenway according to the manual. Glycereraldehyde 3-phosphate dehydrogenase (GAPDH) was applied as an internal control. The following primers were used: PTPH1, Forward: 5’-AGACCTCCCCCCGTTACGTCGTTG-3’ and Reverse: 5’-GCTGGCCTCCTCGGTACGGCACCTTTG-3’; GAPDH Forward: 5’-GCTGGGACATCCGGCAAAGC-3’ and Reverse: 5’-AAAGGGTGAACGCACTA-3’.

**Western blot analysis**

The whole-cell lysates of GBM cells were generated with RIPA lysis (Beyotime Biotechnology, Shanghai). After electrophoresis, proteins were transferred to a nitrocellulose membrane (PALL Cooperation, Port Washington, NY, USA). After blocking in 5% bovine serum albumin for 1 h, the membrane was incubated in the primary antibody of PTPN3 at a dilution of 1: 1000 at 4°C overnight. Followed by washing with TTBS buffer, the secondary antibodies of actin or PTPN3 (Beyotime Biotechnology, Shanghai) were applied at room temperature for 1 h. Enhanced chemiluminescent (ECL) agent (Merck Millipore, Darmstadt, Germany) was used for visualization of proteins. The whole-cell lysates of GBM cells were generated with RIPA lysis (Beyotime Biotechnology, Shanghai). After electrophoresis, proteins were transferred to a nitrocellulose membrane (PALL Cooperation, Port Washington, NY, USA). After blocking in 5% bovine serum albumin for 1 h, the membrane was incubated in the primary antibody of PTPN3 at a dilution of 1: 1000 at 4°C overnight. Followed by washing with TTBS buffer, the secondary antibodies of actin or PTPN3 (Beyotime Biotechnology, Shanghai) were applied at room temperature for 1 h. Enhanced chemiluminescent (ECL) agent (Merck Millipore, Darmstadt, Germany) was used for visualization of proteins.

**Proliferation assay**

Cell proliferation was evaluated with MTT assay according to previous study [14]. After transfection of PTPN3 siRNA or scrambled siRNA, U118 cells were planted into the 96-well plates at the density of 4000 cells per well and incubated for 4 h for cell adhesion. We used 100 μM pervanadate to incubate the cells for 24 h, and then cells were cultured for another 48 h. MTT at a concentration of 10 mg/ml was added to each well and the crystals at bottom were resolved by 100 μL DMSO after 6 h. The optical density (OD) at 570 nm was read. OD570 of the control group (without transfection and pervanadate stimulation) was set as the baseline and the proliferation indexes of other groups were calculated as ratio to the baseline.

**Invasion assay**

Cell invasion was evaluated with Matrigel invasion assay. Cells were transfected 24 h before transfer into the upper chamber of an 8-μm Transwell plate (BD, Franklin Lakes, NJ, USA), and 100 μM pervanadate was used to incubate the cells for 12 h. Cells were totally cultured in the Transwell plates for 24 h, and the upper cells were removed after that. Cells at the bottom were fixed with methanol and stained with hematoxylin. Cells of different groups were counted in 8 random visual fields under a microscope. The number of invaded cells in the control group was set as the baseline and the invasion indexes of other groups were calculated as ratios to the baseline.

**Statistical analysis**

All data were analyzed with SPSS 22.0 software (IBM, Chicago, IL, USA) without special illustration. The chi-square test was used to evaluate the correlations between PTPN3 and the clinicopathological factors. The Kaplan-Meier method was applied to display the survival curves, while the log-rank test was used to evaluate the statistical differences of survival curves. The Cox proportional hazards regression model was used to identify the independent prognostic factors. The statistical significance of differences among groups in proliferation or Transwell assay was evaluated with ANOVA test or t test. P-value <0.05 was considered to be statistically significant.

**Results**

**Expression of PTPN3 in GBM and adjacent tumor tissues**

The expression of PTPN3 in 8 pairs of GBM tissues and the adjacent tumor tissues was investigated by qPCR. The PTPN3 mRNA in GBM tissues was significantly higher than that in adjacent tumor tissues (Figure 1A). In the cohort with 95 cases of GBM, PTPN3 expression was detected with IHC. According to the cut-off of IHC score, the patients with GBM were divided into groups with low expression of PTPN3 and high expression of PTPN3 (Figure 1B, 1C). In GBM, expression of PTPN3 was mainly observed in the cytoplasm, which was consistent with previous studies.
Correlation between PTPN3 expression and clinicopathological factors

The correlation between PTPN3 expression and the clinicopathological factors was analyzed with the chi-square test (Table 1). The enrolled clinicopathological factors included patient sex, age, KPS score, and surgical resection status. Among these factors, there was no factor significantly associated with the expression of PTPN3.

High expression of PTPN3 was correlated with low survival rates

In a previous study, PTPN3 was demonstrated to promote the proliferation and invasion of glioma, and GBM was the most common type of glioma, so we further evaluated the prognostic value of PTPN3 in GBM. Univariate analysis was performed to investigate the correlation between survival rates and clinicopathological factors (Table 2). We found that PTPN3 was a prognostic factor of GBM. Patients with high expression of PTPN3 had lower survival rates compared with patients with low expression of PTPN3 (P<0.001) (Figure 2A). Additionally, incomplete

Table 1. Correlation between PTPN3 expression and clinicopathological factors.

| Parameters       | PTPN3 | P*   |
|------------------|-------|------|
|                  | Low   | High |
| Age              |       |      |
| ≤50              | 33    | 19   | 0.946 |
| >50              | 27    | 16   |      |
| Sex              |       |      |
| Male             | 30    | 22   | 0.287 |
| Female           | 30    | 13   |      |
| KPS              |       |      |
| <80              | 19    | 16   | 0.192 |
| ≥80              | 41    | 19   |      |
| Extent of resection |      |      |
| Total resection (95%) | 33    | 19   | 0.946 |
| Subtotal resection | 27    | 16   |      |

* Means calculated by chi-square test.
resection \( (P=0.003) \) and low KPS \( (P<0.016) \) were also associated with poor prognosis of patients with GBM (Figure 2B, 2C).

The independent prognostic factors were identified with multivariate analysis. The prognostic factors, including KPS, surgical resection status, and PTPN3, were enrolled into the Cox regression model. PTPN3 was an independent prognostic biomarker indicating unfavorable prognosis by itself \( (P<0.001) \). Besides PTPN3, incomplete surgical margin was also identified as an independent prognostic factor \( (P=0.002) \).

### Table 2. Prognostic value of PTPN3 was analyzed with univariate and multivariate analysis.

| Parameters                | Univariate analysis | Multivariate analysis |
|---------------------------|---------------------|-----------------------|
|                           | 1-year OS (%)       | HR                    |
|                           | \( P^* \)           | \( 95\% CI \)         |
|                           |                     | \( P^\# \)           |
| Age                       |                     |                      |
| \( \leq 50 \)              | 38.4                | 1                     |
| \( >50 \)                  | 22.8                | 0.287                 |
| Sex                       |                     |                      |
| Male                      | 41.3                | 1                     |
| Female                    | 21.6                | 0.637                 |
| KPS                       |                     |                      |
| \( \geq 80 \)              | 48.8                | 1                     |
| \( <80 \)                  | 21.4                | 0.016                 |
| Extent of resection       | 40.8                | 1                     |
| Total resection           | 19.9                | 0.003                 |
| Subtotal resection        | 2.37                | 1.37–3.87             |
| PTPN3                     |                     |                      |
| Low                       | 50.4                | 1                     |
| High                      | 4.1                 | \( <0.001 \)          |
|                           | 3.82                | 2.18–6.82             |

* Means calculated by Log-rank test, \( ^* \) means calculated by Cox proportional hazards regression.

#### Figure 2. Overall survival curves of different PTPN3 expression, surgical margin, and KPS. (A–C) Patients with high expression of PTPN3 (A), incomplete surgical resection (B), and low KPS (C) had poorer prognosis compared with those with low expression of PTPN3 \( (P<0.001) \), complete surgical resection \( (P=0.003) \), and high KPS \( (P=0.016) \).

PTPN3 can promote GBM cell proliferation and invasion

The intrinsic expression of PTPN3 in different GBM cell lines were detected with Western blotting (Figure 3A). U118 MG cells had the highest PTPN3 expression and were selected for PTPN3 silencing test. PTPN3 siRNA and scrambled siRNA were transfected into U118 and successful knockdown was verified with Western blotting (Figure 3B). To evaluate the role of PTPN3 in GBM progression, we investigated the function of PTPN3 in cell proliferation with MTT assay and in invasion

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with Transwell assay. After silencing PTPN3, cells had a significantly lower proliferative rate (Figure 3C). Pervanadate, the PTP common inhibitor, was also applied to decrease the catalytic activity of PTPN3. Interestingly, pervanadate attenuated proliferation more than PTPN3 knockdown, suggesting other PTPs may also be involved in GBM proliferation. Similarly, the invasion of GBM cells transfected with PTPN3 siRNA was impaired compared to cells with scrambled siRNA transfection (Figure 3D). In cell invasion, pervanadate had no significant difference in PTPN3 knockdown.

Discussion

Mutations of PTPN3 are commonly observed in human cancers, especially in colon cancer [12]. The genetic mutation of PTPN3 is considered to be correlated with oncogenesis of colon cancer, but the function of PTPN3 in cancer progression and prognosis is still unknown. Some previous studies indicated the role of PTPN3 as an oncogenic factor or tumor suppressor in different cancers. In cholangiocarcinoma, PTPN3 was proved to be an oncogenic protein and could promote proliferation, invasion, and poor prognosis [15]. On the contrary, PTPN3 inhibited lung cancer cell proliferation and migration by promoting EGFR endocytic degradation [11]. The only evidence supporting the oncogenic role of PTPN3 in glioma was limited to in vitro experiments and lacked clinical data. Our study is the first to identify PTPN3 as an independent prognostic factor in GBM, suggesting that postoperative PTPN3 detection can identify patients who are more predisposed to poor prognosis.

Besides the C-terminal tyrosine phosphorylation catalytic domain, PTPN3 consists of a PDZ domain and a N-terminal FERM domain. Among the 107 human PTPs, PTPN3 has a PDZ domain. A previous study demonstrated that the PDZ domain could mediate the interaction between PTPH1 and p38. PTPH1 could dephosphorylate p38 to increase Ras oncogenesis through PDZ-mediated interaction [16]. Interestingly, the aberrant activation of Ras-MAPK and PI3K-AKT signaling is a well-recognized characteristic of GBM [17]. Whether PTPN3 functions as an oncogenic protein via facilitating Ras-MAPK and PI3K-AKT signaling pathway requires further experiments. In our study, we proved that high expression of PTPN3 could promote progression and predict poor prognosis of GBM. However, we did not explore the underlying molecular mechanisms. As a PTP, PTPN3 functions via dephosphorylating its substrates. However, the substrates and functions of PTPN3 are little known. Our findings may initiate more interest in PTPN3 and improve the identification of PTPN3 substrates.

Figure 3. PTPN3 could promote GBM cells proliferation and invasion. (A) Expression of PTPN3 in glioma cell line U251 and GBM cell lines A172 and U118. (B) Successful knockdown of PTPN3 in U118 was verified by Western blotting. (C) PTPN3 knockdown by siRNA or inhibition by pervanadate attenuated proliferation of U118 cells. (D) PTPN3 knockdown by siRNA or inhibition by pervanadate attenuated invasion of U118 cells.
Several specific small-molecular inhibitors have been developed in recent years, like PTPN1 and PTPN9 [18,19]. However, no specific inhibitor of PTPN3 is available now, which increases the difficulty of studying PTP3 substrates and mechanisms. As a common inhibitor of PTPs, pervanadate was applied in our study and was demonstrated to inhibit proliferation and invasion of GBM cells. Interestingly, the cells treated with pervanadate had lower proliferation than PTPN3 knockdown, but this tendency disappeared in cell invasion. This result suggests that there are more PTPs besides PTPN3 are involved in GBM proliferation than in invasion.

Conclusions

By investigating the expression of PTPN3 in GBM tissues and the adjacent tissues, we demonstrated that PTPN3 is highly expressed in GBM tissues. By evaluating its prognostic value with univariate analysis and multivariate analysis, we demonstrated that PTPN3 is an independent prognostic factor of GBM. Postoperative detection of PTPN3 can identify patients who are more predisposed to poor prognosis, and anti-PTPN3 therapy may be a promising treatment for GBM in the future.

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