

Expression of β -transducin repeat-containing E3 ubiquitin protein ligase in human glioma and its correlation with prognosis

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Abstract. β -transducin repeat-containing E3 ubiquitin protein ligase (β -TrCP) targets a number of substrates essential for specific aspects of tumorigenesis. In addition, β -TrCP regulates various important signaling pathways. As β -TrCP is involved in regulating the ubiquitination and degradation of multiple oncogenes and tumor suppressors, the function of β -TrCP varies between cancer types. At present, the association between β -TrCP expression and clinicopathological factors in glioma is unknown. Therefore, the current study used western blotting and immunohistochemistry to investigate the expression of β -TrCP protein in glioma tissue specimens. It was identified that β -TrCP protein expression levels were significantly lower in glioma compared with non-tumorous human brain tissues. Furthermore, the higher the grade of glioma, the lower the level of β -TrCP expression. Kaplan-Meier analysis demonstrated that patients with low β -TrCP expression experienced significantly worse overall survival compared with patients with high β -TrCP expression. The results indicate that downregulation of β -TrCP may be associated with poor survival in patients with glioma. Together, the current data indicates that β -TrCP may be applied as a useful indicator of glioma prognosis and may serve as an anticancer therapeutic target for glioma, however further investigation is required.

Introduction

The highly invasive and diffusively infiltrative nature of glioma, an aggressive malignant cancer of the central nervous system, is the major cause of conventional treatment failure and results in a mean survival time of 12 months (1-5). Although advances

have been made in surgery and adjuvant therapy, patients with malignant glioma have experienced little change in survival time over recent decades (6,7). However, with the development of molecular biology, gene therapy is becoming a major focus of tumor therapy. Therefore, the identification of molecular mechanisms and novel therapeutic targets to combat tumor invasion are critical for the treatment of this currently incurable type of cancer.

The ubiquitin proteasome pathway is pivotal for controlling the degradation of the majority of regulatory proteins in mammalian cells (8,9). Furthermore, the pathway regulates various cellular processes by facilitating the prompt destruction of key regulatory proteins by the 26S proteasome complex (10). Protein ubiquitination in the proteasome pathway involves the concerted action of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-protein ligase, the latter of which delivers multiple ubiquitin molecules to the target protein (11-13). β -transducin repeat-containing E3 ubiquitin protein ligase (β -TrCP) is characterized by a ~40-amino acid motif. β -TrCP utilizes its seven WD40 repeats to interact with substrates phosphorylated within the DSG(X)_{2+n}S destruction motif, and is involved in the degradation of numerous cell signaling and cell cycle regulation proteins (14-16).

In recent years, a number of β -TrCP substrates have been identified, including Bmi1 (17), inhibitor of κ B (18), β -catenin (19,20), Emi1 (21), nuclear factor- κ B p105 subunit (22) and cell cycle division 25A (15). It was previously reported that β -TrCP is able to promote breast and prostate cancer cell proliferation and migration (23). However, β -TrCP may also suppress angiogenesis and thyroid cancer cell migration (24), as well as pancreatic cancer cell growth (25). In addition, β -TrCP appears to inhibit lung cancer cell growth and invasiveness (26). Although there have been substantial advances in the understanding of the basic biology and pathogenesis of β -TrCP, little is known with regard to the possible role and clinical significance of β -TrCP in glioma. Therefore, the aim of the present study was to explore the protein expression levels of β -TrCP in glioma tissues by performing western blot analysis and immunohistochemical staining. In addition, the study evaluated the association between the expression of β -TrCP and survival time during the four-year follow-up period. The aim of the

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Table I. Clinical features and overall survival of 66 glioma patients with different β -TrCP expression levels.

Characteristic	Patients, n	β -TrCP expression		P-value
		High (n=29)	Low (n=37)	
Gender				0.803
Male	42	19	23	
Female	24	10	14	
Age, years				0.599
<60	45	21	24	
\geq 60	21	8	13	
KPS				0.068
<80	22	6	16	
\geq 80	44	23	21	
Post-operative treatment strategy				0.493
Radiotherapy plus chemotherapy	56	26	30	
No radiochemotherapy	10	3	7	
Overall survival				0.006
Mortalities, n	38	11	27	
Censored ^a , n	28	18	10	

^aNumber of surviving patients at the end of the follow-up period. β -TrCP, β -transducin repeat-containing E3 ubiquitin protein ligase; KPS, Karnofsky performance status (27).

present study was to provide important data with regard to the possible role of β -TrCP in human glioma progression and to determine whether β -TrCP could serve as a novel prognostic marker for patients with glioma.

Materials and methods

Tissue specimens

Western blot specimens. To detect the protein expression levels of β -TrCP using western blot analysis, 32 glioma tissue specimens (obtained during surgical resection) and 16 non-tumorous brain tissue specimens (obtained from the same brain region during internal decompression in cases of cerebral trauma) were collected from the Affiliated Hospital of Xuzhou Medical College (Xuzhou, China). Sections of the surgically removed tissue specimens were analyzed to determine a histological diagnosis, and the remaining tissues were immediately frozen and stored in liquid nitrogen for subsequent analysis. Clinical staging was performed according to the 2007 World Health Organization (WHO) classification of tumors of the central nervous system (28), revealing six grade I astrocytomas, nine grade II astrocytomas, eight grade III astrocytomas and nine grade IV glioblastomas. Written informed consent was obtained from each patient and the study was approved by the Research Ethics Committee of the Affiliated Hospital of Xuzhou Medical College.

Immunohistochemical specimens. Paraffin-embedded tissue sections for immunohistochemical analysis were obtained from the pathology files of the Department of Pathology at the Affiliated Hospital of Xuzhou Medical College between March 2007 and January 2010. According to the 2007 WHO classification of tumors, the 66 glioma

samples included 35 grade I and II astrocytomas, 12 grade III anaplastic astrocytomas and 19 grade IV glioblastomas. The available clinical data indicated that none of the tissue specimens were recurrent glioma, and 56 cases had received radiotherapy and chemotherapy. The other 10 cases had not received radiotherapy or chemotherapy due to the patient's poor physical condition following surgery (Table I). Non-tumorous brain specimens were acquired from 25 patients undergoing surgery for internal decompression in cerebral trauma. Written informed consent was obtained from each patient and the study was approved by the Research Ethics Committee.

Antibodies and reagents. Rabbit polyclonal anti- β -TrCP antibody (cat no. ab71753) was purchased from Abcam (Hong Kong, China) and rabbit monoclonal anti- β -actin antibody (cat no. 04-1116) was purchased from EMD Millipore (Billerica, MA, USA). The bound antibodies were detected using a rabbit streptavidin-peroxidase kit and diaminobenzidine (DAB; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China).

Western blot analysis. The 32 glioma and 16 non-tumorous human brain tissues were weighed and ground into small pieces. For protein analysis, the tissues were homogenized (Pro 200 homogenizer; Promega Corporation, Madison, WI, USA) in lysis buffer [0.1% SDS, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Triton X-100 and 2 mM EDTA] containing complete protease inhibitor (0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 1 mM NaF). The protein lysates were then concentrated using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). After the tissues were lysed for 30 min, equal quantities of

protein lysate were subjected to 10% SDS-PAGE, then transferred to polyvinylidene difluoride membranes with a 0.45- μ m pore size (EMD Millipore). The membranes were saturated with 3% bovine serum albumin (BSA) and incubated for 2 h at 37°C. Subsequently, the membranes were incubated with primary rabbit polyclonal anti- β -TrCP antibody and rabbit monoclonal anti- β -actin antibody at 4°C overnight. Subsequent being washed three times with 0.1% PBS (10 min each), the membranes were incubated with anti-rabbit secondary antibody at 37°C for 2 h. After washing three times with 0.1% PBS (10 min each), the bound antibodies were detected using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) and exposed to X-ray film. Band densities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The relative quantity of protein was determined by normalizing the densitometry value of interest to that of the internal loading control (β -actin).

Immunohistochemistry (IHC). Following collection, the paraffin-embedded tissue blocks were cut in a microtome to a thickness of 4 μ m and affixed onto slides. IHC was performed using a rabbit streptavidin-peroxidase kit and DAB, according to the manufacturer's instructions. Briefly, the tissue sections were dewaxed in xylene and rehydrated through graded alcohol concentrations using standard procedures. The sections were subsequently submerged in EDTA (pH 8.0) and autoclaved at 121°C for 5 min to retrieve antigenicity. After washing three times in phosphate-buffered saline (PBS; 0.1 M (pH 7.4)] for 5 min, endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 15 min at room temperature. Next, incubation with β -TrCP antibody (Abcam), at a dilution of 1:150 in PBS containing 0.5% BSA, was conducted overnight at 4°C. After washing the sections in PBS three times (5 min each), the bound antibodies were detected by applying the streptavidin-peroxidase kit for 30 min at room temperature. Subsequently, the sections were washed with PBS and DAB coloration was applied, followed by the application of a DAB solution until the color developed. Staining was monitored under a bright-field microscope (XSP-17C; Shanghai Changfang Optical Instrument Co., Ltd., Shanghai, China) and the reaction was terminated by washing with distilled water. The slides were then counterstained with hematoxylin, dehydrated with ethanol and xylene, and covered with coverslips.

Immunohistochemical staining evaluation. Three independent experienced pathologists, who were blinded to the clinicopathological data, evaluated the immunostained slides. The proportion of stained tumor cells in each selected field was determined by counting individual tumor cells in three randomly-selected high-magnification fields (magnification, x400) using light microscopy (IX71+DP721; Olympus Corporation, Tokyo, Japan). Expression was quantified using a visual grading system based on the extent and intensity of the staining. The percentage of positively-stained tumor cells was scored as follows: 0, no positive tumor cells; 1, \leq 25% positive tumor cells; 2, 26-50% positive tumor cells; 3, 51-75% positive tumor cells; and 4, \geq 76% positive tumor cells. Staining intensity was scored as follows: 0, no staining; 1, very weak staining; 2, weak staining; 3, moderate staining;

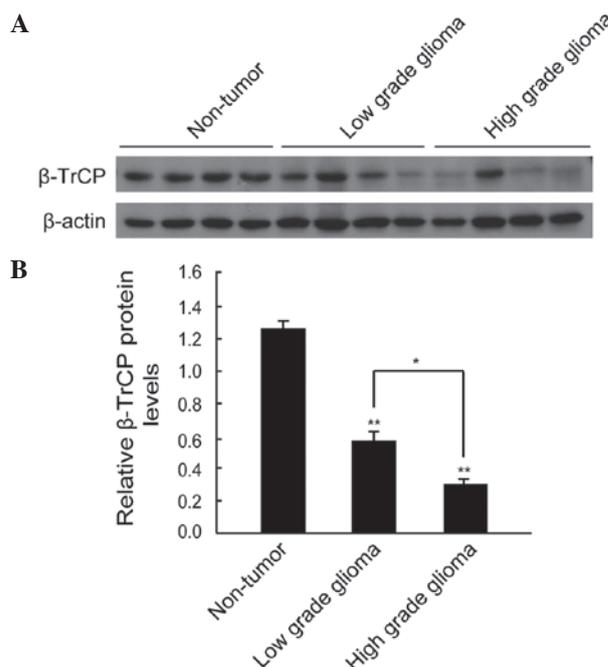


Figure 1. Expression of β -TrCP in glioma tissues and non-tumorous human brain tissues. (A) Representative western blot analysis of the total extracts isolated from human brain glioma tissues (eight samples shown) and non-tumorous tissues (four samples shown) using human β -TrCP antibody. β -actin served as the internal control. (B) Quantitative analysis of the protein levels of β -TrCP in glioma tissues (n=32) and non-tumorous brain tissues (n=16), represented as a bar graph. *P<0.05. **P<0.01 vs. non-tumorous brain tissues. β -TrCP, β -transducin repeat-containing E3 ubiquitin protein ligase.

and 4, strong staining. The final score was calculated by multiplying the positive percentage score by the staining intensity score. A score of 0-3 was considered to indicate low expression and a score of \geq 4 was considered to indicate high expression.

Statistical analysis. SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA) was used to perform all statistical analyses. In all analyses, quantitative data were obtained from a minimum of three independent experiments and are expressed as the mean \pm standard error of the mean. One-way analysis of variance was used to compare β -TrCP protein expression levels between glioma and non-tumorous tissue specimens, and a χ^2 test was used to compare clinical features between the low and high β -TrCP expression groups. In addition, overall survival (OS) was calculated from the day of surgery to the date of last follow-up or mortality. Postoperative survival curves were plotted using the Kaplan-Meier method and differences in survival rates were assessed by performing the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

β -TrCP protein expression levels in glioma and non-tumorous human brain tissue samples, as determined by western blotting. Western blot analysis identified that the expression level of β -TrCP protein was lower in the majority of the glioma samples compared with the non-tumorous specimens (Fig. 1A). By performing quantitative analysis, it was determined that the

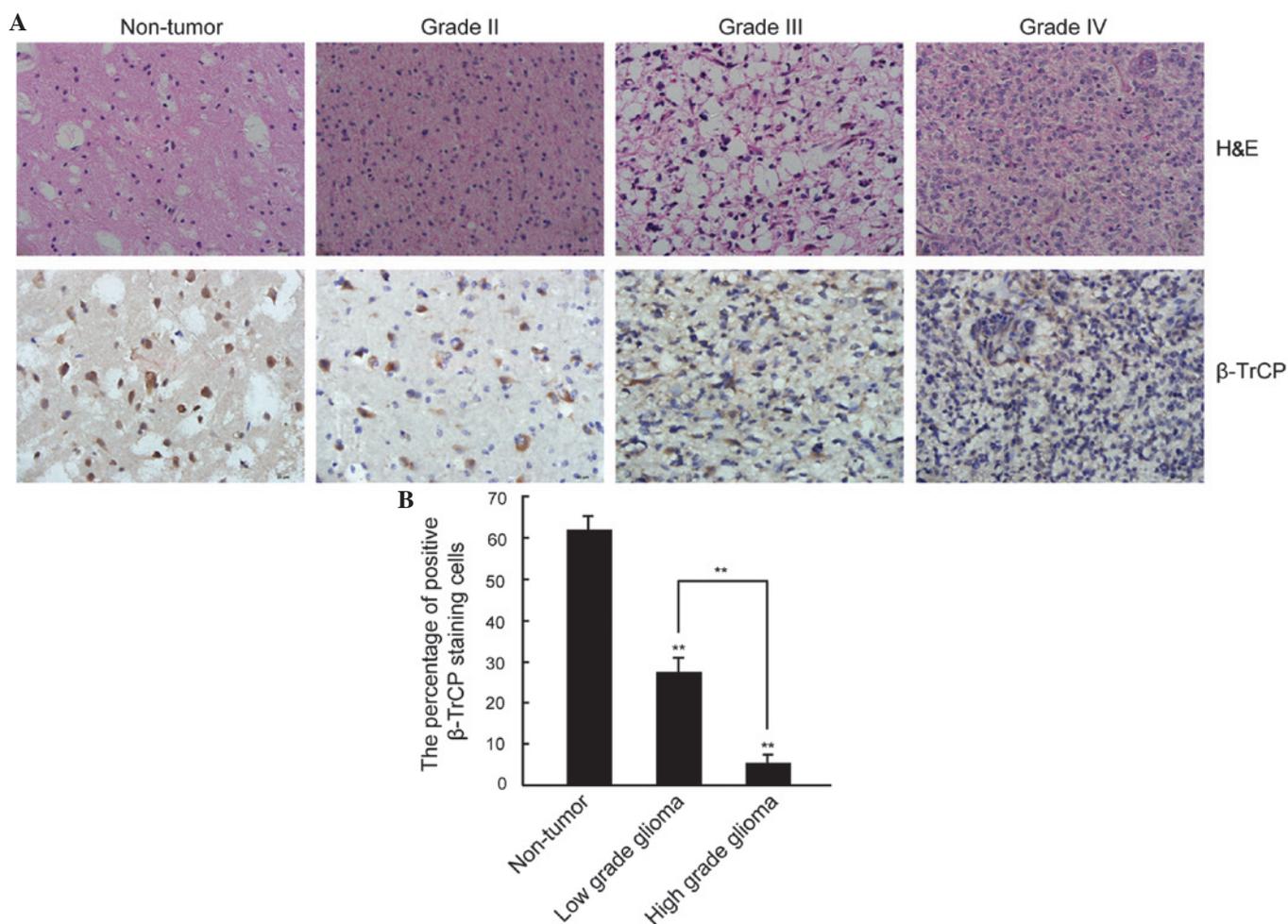


Figure 2. Immunohistochemical analysis of β -TrCP expression in non-tumorous and glioma human brain specimens of varying World Health Organization grades. (A) H&E and β -TrCP immunohistochemically-stained non-tumorous specimens, and grade II, III and IV glioma specimens (magnification, x400). Immunohistochemical staining resulting in similar staining patterns was achieved a minimum of two times in each tumor specimen. (B) Histogram indicating quantitative analysis of the percentage of β -TrCP-positive cells. ** $P < 0.01$ vs. non-tumour tissues. H&E, hematoxylin and eosin; β -TrCP, β -transducin repeat-containing E3 ubiquitin protein ligase.

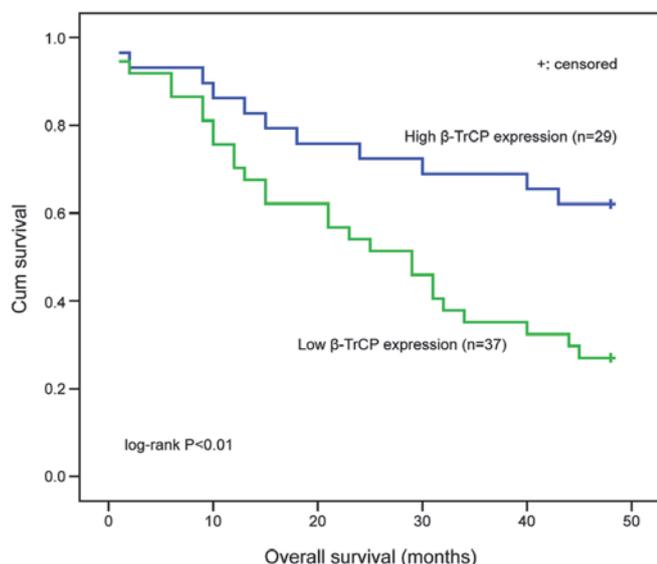


Figure 3. Kaplan-Meier analysis of overall survival in patients with glioma according to β -TrCP protein expression levels in glioma tissues, as determined by immunohistochemical staining. Glioma patients with lower β -TrCP expression exhibited significantly shorter overall survival times compared with those exhibiting higher β -TrCP expression levels ($P < 0.01$; log-rank test). Cum, cumulative; β -TrCP, β -transducin repeat-containing E3 ubiquitin protein ligase.

expression level of β -TrCP protein was significantly lower in the glioma tissues compared with the non-tumorous human brain tissues ($P < 0.01$), and the expression level of β -TrCP protein in high-grade glioma (grades III and IV) was significantly lower than that in low-grade glioma (grades I and II; $P < 0.05$) (Fig. 1B).

Immunohistochemical detection of the expression and location of β -TrCP protein in glioma and non-tumorous human brain tissues samples. To clarify the association between β -TrCP expression and the pathological grade of glioma, the expression level and intracellular location of β -TrCP protein was analyzed in glioma and non-tumorous human brain tissues by immunohistochemical staining. As indicated in Fig. 2A, β -TrCP staining appeared as brown particles and was predominantly located in the cytoplasm. Furthermore, the expression level of β -TrCP was significantly lower in the glioma tissue samples compared with in the non-tumorous human brain tissue samples. In addition, the high grade glioma was associated with a significantly lower level of β -TrCP expression compared with low grade glioma, with weak expression of β -TrCP only identified in a small number of cell nuclei (non-tumorous nuclei, $62.09 \pm 2.36\%$ positive

β -TrCP staining; low grade nuclei, $27.65\pm 2.75\%$ positive β -TrCP staining; high grade nuclei, $5.41\pm 1.94\%$ positive β -TrCP staining; $P < 0.01$; Fig. 2B).

Impact of β -TrCP expression on the OS of patients with glioma. The prognostic value of β -TrCP expression on the OS of patients with glioma was evaluated in the present study. There were no statistical differences in the pre-operative clinical data and postoperative treatment strategies between the low and high β -TrCP expression groups (Table I). Kaplan-Meier and log-rank analysis determined that glioma patients with high β -TrCP expression had significantly improved OS compared with those with low expression ($P < 0.01$; Fig. 3). These results indicate that β -TrCP protein expression levels are downregulated in human glioma tissue and that β -TrCP expression levels are significantly associated with the malignant state of glioma. These data provide early evidence for β -TrCP as an important factor in the maintenance of normal conditions in brain tissue and as a negative factor in the development of human glioma.

Discussion

Previous studies have identified that β -TrCP recruits phosphorylated substrates to the Skp, Cullin, F-box ubiquitin ligase complex (18,29). Considering the diversity in its substrates, β -TrCP may be responsible for oncogenesis or the inhibition of tumorigenesis. For example, previous studies have determined that β -TrCP is involved in the oncogenesis of gastric, prostate and breast cancer cell lines (30-32), with overexpression of β -TrCP mRNA and protein expression levels observed in colorectal cancer (33). β -TrCP has been identified to act as a tumor suppressor gene in various types of solid cancer, such as thyroid and lung cancer (24,26). However, to the best of our knowledge, no studies have thus far investigated β -TrCP expression levels in human glioma tissue.

In the present study, western blot analysis identified that the levels of β -TrCP protein expression in the glioma tissues were significantly lower than those in the non-tumorous tissues, and that an increase in glioma grade was significantly associated with a gradual reduction in the overall expression of β -TrCP protein. IHC supported the observation that the level of β -TrCP was significantly lower in the glioma tissues compared with the non-tumorous brain tissues, and identified that β -TrCP was predominantly located in the cytoplasm. Furthermore, weak β -TrCP expression was observed in a small number of cell nuclei. Warfel *et al* (34) identified higher β -TrCP1 protein expression levels in the cytosol compared with the nucleus in astrocyte and astrocytoma cell lines. However, in contrast to the present study, the expression level of β -TrCP1 protein was lower in the cytosol of glioblastoma cells compared with the nuclei. These findings indicate that β -TrCP may be an important factor for the maintenance of normal conditions in glioma tissue, and that its nuclear deficiency in astrocytes may contribute to glioma formation and progression.

Furthermore, in the present study, Kaplan-Meier survival analysis determined that the OS period of patients exhibiting tumors with low β -TrCP expression was significantly worse than that of patients with high β -TrCP expression. These results indicated that the detection of decreased β -TrCP expression may facilitate the identification of glioma patients

with a poor prognosis. Thus, β -TrCP may be a novel prognostic marker for patients with glioma. However, due to the limited sample size and short duration of patient follow-up in the present study, more studies with larger sample sizes are required to clarify these findings. A previous study identified that β -TrCP regulates Bmi-1 protein turnover via ubiquitination and degradation in a human osteosarcoma cell line (17). Furthermore, Bmi-1 appears to be highly expressed in patients with glioma, and promotes glioma cell invasion, migration and proliferation (35,36). The high expression of Bmi-1 also predicted poor OS in patients with glioma (37-39). Therefore, it is proposed that the function of β -TrCP in glioma may be mediated by Bmi-1. Additional studies are required to clarify the specific molecular mechanism underlying the association between β -TrCP expression and Bmi-1.

In conclusion, the data obtained in the current study indicates that the β -TrCP expression level is low in glioma and is associated with the poor prognosis of glioma patients. Therefore, β -TrCP may serve as a novel prognostic marker for patients with glioma.

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