SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 14: 3131-3135, 2017

β-transducin repeat-containing E3 ubiquitin protein ligase inhibits migration, invasion and proliferation of glioma cells

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Received November 28, 2015; Accepted May 11, 2017

DOI: 10.3892/ol.2017.6533

Abstract. β-transducin repeat-containing E3 ubiquitin protein ligase (β-TrCP) serves as the substrate recognition subunit for the Skp1-Cullin1-F-box protein E3 ubiquitin ligase, which recognizes the double phosphorylated DSG $(X)_{2+n}S$ destruction motif in various substrates that are essential for numerous aspects of tumorigenesis and regulates several important signaling pathways. However, the biological significance of β-TrCP in glioma progression remains largely unknown. A previous study by the authors demonstrated that the levels of β-TrCP protein expression in brain glioma tissues were significantly lower compared with non-tumorous tissues and that higher grades of gliomas exhibited lower levels of β-TrCP expression in comparison with lower glioma grades. In addition, low β-TrCP expression was associated with poor prognosis in patients with glioma. Subsequently, the present study aimed to investigate the effect of β-TrCP on migratory, invasive and proliferative abilities of glioma cells. β-TrCP plasmids were transfected into cultured U251 and U87 glioma cells, and changes in migration, invasion and proliferation were analyzed using wound healing, Transwell and EdU assays. It was identified that the overexpression of β-TrCP inhibited migration, invasion and proliferation in glioma cells. In summary, these results indicate that β-TrCP may serve a protective role against the progression of glioma by suppressing cell migration, invasion and proliferation. The potential mechanism of β-TrCP I glioma cells requires additional investigation.

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Key words: β-transducin repeat-containing E3 ubiquitin protein ligase, migration, invasion, proliferation, glioma cells

Introduction

Gliomas are the most frequent and aggressive malignant tumors, with an average survival time of 12 months (1-3). A major cause of the failure of conventional treatments is the highly invasive and diffusively infiltrative nature of these tumors (4,5). Despite advances in surgery and adjuvant therapy, the survival time of patients with malignant glioma has changed little over the past decades (6,7). With the development of molecular biology, gene therapy is becoming the focus of tumor therapy. Therefore, identifying molecular mechanisms and novel tumor therapeutic targets is critical and necessary for this incurable cancer.

β-transducin repeat-containing protein (β-TrCP), as the substrate recognition subunit for the E3 ubiquitin ligases, utilizes seven WD40 repeats to interact with substrates phosphorylated within the DSG $(X)_{2+n}S$ destruction motifs and is involved in the degradation of numerous proteins in cell signaling and cell cycle regulation (8-10). β-TrCP is involved in major regulatory mechanisms, including cell cycle progression, metabolism, development and immunity (11-14). Notably, two β-TrCP proteins are expressed in humans. β-TrCP1 is encoded by BTRC, and β-TrCP2 encoded by FBXW11, also known as HOS or β-TRCP2 (15,16). The ubiquitin proteasome pathway serves a pivotal role in controlling the degradation of the majority of regulatory proteins in mammalian cells (17,18) and regulates a number of cellular processes by facilitating the timely destruction of key regulatory proteins by the 26S proteasome complex (19). In this pathway, protein ubiquitination involves the concerted action of the E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin-protein ligase, of which delivers multiple ubiquitin molecules to the target protein (20-22).

Diverse β-TrCP substrates involved in different normal and malignant pathways have been identified, includingBMI-1 (23), IκB (24), β-catenin (25,26), vascular endothelial growth factor receptor 2 (VEGFR2) (27), metastasis suppressor protein 1 (MTSS1) (28), Emi1 (29), SNAI1 (30), and M-phase inducer phosphatase 1 (Cdc25A) (9). Zhong *et al* (28) reported that β-TrCP targetsMTSS1 for ubiquitination-mediated destruction, to promote breast and prostate cancer cell proliferation and migration. However, Shaik *et al* (27) demonstrated that β-TrCP suppresses angiogenesis and thyroid cancer cell migration by

promoting ubiquitination and destruction of VEGFR2. In addition, β-TrCP may inhibit growth and invasiveness of lung cancer cells (31). Therefore, the roles of β-TrCP are different in different types of tumors.

Previous studies by the authors have demonstrated that β-TrCP protein expression levels were significantly lower in glioma compared with non-tumorous human brain tissues and that low β-TrCP expression indicated poor prognosis in patients with glioma (32). In the present study, it was additionally observed that β-TrCP affected migration, invasion and proliferation of human glioma cells.

Materials and methods

Antibodies and reagents. The rabbit polyclonal anti-β-TrCP antibody was purchased from Abcam (ab71753; 1:500; Cambridge, UK). The rabbit polyclonal anti-Flag antibody was purchased from EarthOx Life Sciences (EO22230; 1:2,000; Millbrae, CA, USA). The rabbit monoclonal anti-β-actin antibody was purchased from EMD Millipore (04-1116; 1:2,000; Billerica, MA, USA).

Cell culture and plasmid transfection. Human glioma U251 and U87 cell lines were purchased from Shanghai Cell Bank of the Type Culture Collection Committee of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM/F-12 media (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Evergreen Biological Engineering Co., Hangzhou, China) in a humidified incubator with 5% CO_2 at 37˚C. For transfection, U251 or U87 cells were transfected with β-TrCP plasmid (1 μg; plasmid 10865; Addgene, Inc., Cambridge, MA, USA) using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's specifications. U251 or U87 cells transfected with an empty plamid were used as controls.

Western blot analysis. Following 24 h transfection with the β-TrCP plasmid, total protein from the transfected U251/U87 cells was extracted using lysis buffer (RIPA, 1 ml; Aprotinin, 1 μl, 2 μg/ml; Leupeptin, (1-10) μl, 10-100 μM; Pepstatin A, 1 μl, 1 μM; PMSF, 5 μl 0.5 mM; Benzamidine, 1 μl, 4 mM; DTT, 1 μl, 1 mM) consisting of protease inhibitors. The protein lysates (80 μg) were subjected to 10% SDS-PAGE, then transferred to polyvinylidene fluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany), and probed with primary antibodies (β-TrCP, 1:500; β-actin, 1:2,000; anti-Flag, 1:2,000) for target bands at 4˚C overnight for blocking and secondary antibodies (7074; 1:4,000; Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 2 h. Bound antibodies were detected by the Pierce ECL Plus Western Blotting substrate (Thermo Fisher Scientific, Inc.) and exposed to X-ray films. The PVDF membranes were washed (3 times for 15 min each) using washing buffer (TBST) following incubation with antibodies. Band densities were quantified using Image J software (1.42q, National Institutes of Health, Bethesda, MD, USA). The relative amount of proteins was determined by normalizing the densitometry value of interest to that of the internal loading control. Western blotting was performed for three times.

Wound healing assay. A total of 24 h following transfection with β-TrCP plasmid ($1.5x10^5$ cells per hole), a rectangular lesion was created using a plastic pipette tip and the monolayer was rinsed twice for 1 min with PBS and incubated in serum-free media [Dulbecco's modified Eagle's medium (DMEM); Gibco; Thermo Fisher Scientific, Inc.] at 37˚C for 24 h. Subsequently, 5 randomly selected fields at the lesion border were acquired under an inverted microscope (magnification, x100; Olympus Corporation, Tokyo, Japan). U251 or U87 cells transfected with empty plasmids were used as controls.

Transwell invasion assay. Cell invasion assays were performed using a Transwell system that incorporated a polycarbonate filter membrane with a diameter of 6.5 mm and pore size of 8 μm (Corning Incorporated, Corning, NY, USA), according to the manufacturer's protocol. To assess invasion, filters were precoated with 10 μg Matrigel (BD Biosciences, Franklin Lakes, NJ). A pretreated cell suspension ($1x10^5$) in serum-free culture media (Gibco; Thermo Fisher Scientific, Inc.) was added into the inserts, and each insert was placed in the lower chamber filled with culture media containing 10% FBS as a chemoattractant. Following 24 h incubation at 37˚C, the non-invasive cells were removed from the upper chamber. The filters were fixed with methanol for 15 min and stained with a 0.1% crystal violet solution at 37˚C for 10 min. A total of 5 fields of adherent cells in each well were randomly photographed under an inverted microscope (magnification, x100; IX71; Olympus, Japan) and counted. The same experimental design was used for the migration experiments, except that filters were not precoated with Matrigel. U251 or U87 cells transfected with empty plamids were used as controls.

5-ethynyl-2'-deoxyuridine (EdU) assay. The effects on proliferative ability of U251 and U87 cells was measured by EdU incorporation assay using the EdU assay kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China) subsequent to β-TrCP plasmids transfection according to the manufacture's protocol. Briefly, U251 or U87cells at $4x10^3$ cells/well were cultured in triplicate in 96-well plates and transfected with β-TrCP plasmids for 24 h. The cells were subsequently exposed to 50 μM EdU for an additional 2 h at 37˚C. The cells were fixed with 4% formaldehyde for 30 min at room temperature and treated with 2 mg/ml glycine to neutralize the formaldehyde, then treated with 0.5% Triton X-100 for 15 min at room temperature for permeabilization. Following 3 washes with PBS for 15 min, 100 μl 1x Apollo reaction cocktail (Guangzhou RiboBio Co., Ltd.) was added into each well for 30 min. Subsequently, the DNA contents of the cells in each well were stained with 100 μl 1X Hoechst 33342 (Beyotime Institute of Biotechnology, Haimen, China) for 30 min and visualized under a fluorescence microscope (magnification, x100; IX71; Olympus, Japan). U251 or U87 cells transfected with empty plamids were used as controls.

Statistical analysis. The SPSS package (version 16.0; SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. To distinguish the difference between the treatment and control groups, the statistical significance was determined using Student's t-test. Data was presented as the

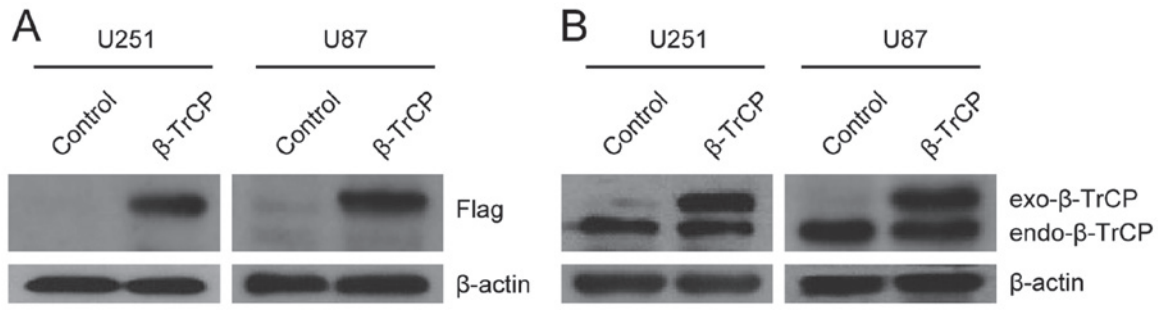

Figure 1. Western blot analysis of exogenous β-TrCP expression in U251 and U87 cells. After 24 h transfection with β-TrCP plasmids, the cells were lysed and protein extraction was performed. Western blot analysis was carried out using (A) Flag or (B) β-TrCP antibody. β-actin was used as the loading control. β-TrCP, β-transducin repeat-containing protein; exo, exogenous, endo, endogenous.

mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

Validation of β-TrCP plasmid transfection. The effect of β-TrCP plasmid transfection was validated in U251 and U87 cells (Fig. 1A and B). The exogenous β-TrCP and endogenous β-TrCP were well-expressed when immunoblotted with anti-Flag or with anti-β-TrCP antibodies.

Effect of β-TrCP on glioma cell migration. Whether overexpression of β-TrCP affects the migration of glioma cells was examined using wound healing assay. As shown in Fig. 2A, 48 h after being scratched, the wound of the control group had clearly healed, and exhibited a tendency to fuse, but any evidence of healing was not observed in the β-TrCP-overexpressing group. Compared with the control group, the migratory cell numbers of the β-TrCP overexpressing group in U251 and U87 cells were decreased to 48.26±3.64 and 58.70±2.31%, respectively (Fig. 2B).

Effect of β-TrCP on glioma cell invasion. Migration and invasion are widely considered to be two closely interrelated processes. The role of β-TrCP in invasion of glioma cells was investigated using Matrigel precoated Transwell chambers. As demonstrated in Fig. 2C, the overexpression of β-TrCP produced a significant reduction in the number of invasive cells. Compared with the control group, the number of invasive cells was reduced to 50.08±3.51 and 42.15±2.43% in U251 and U87 cells, respectively, following β-TrCP overexpression (Fig. 2D). These results demonstrate that β-TrCP is directly involved in suppressing cell migration and invasion.

Effect of β-TrCP on glioma proliferation. Cell proliferation is an important factor in the progression of tumors, so possible changes in cell proliferation following β-TrCP overexpression were next detected by EdU assay. Following 24 h of transfection with β-TrCP plasmid, the U251 and U87 glioma cells were treated with the EdU reagent (Fig. 3). The numbers of positive EdU cells were counted, and the differences between different groups were analyzed. Compared with the control group, the number of proliferative cells was reduced to 44.08±6.12 and 57.6±9.07% in U251 and U87 cells following β-TrCP overexpression, respectively (Fig. 3B and D). The results of the present study demonstrate that overexpressing β-TrCP may suppress glioma cell proliferation.

Discussion

β-TrCP is a well-characterized E3 ubiquitin ligase that is involved in the degradation of a number of proteins involved in cell signaling and cell cycle regulation (8-10). Owing to the diversity in its substrates, β-TrCP was suggested to be responsible for oncogenesis or inhibiting tumorigenesis. In colon cancer cells, β-TrCP has been demonstrated to promote ubiquitination and degradation of PHLPP1, which negatively regulate Akt signaling and promote colon cell growth (33). β-TrCP inhibition reduced prostate cancer cell growth via upregulation of the aryl hydrocarbon receptor (34). However, in lung cancer cell lines, it has been shown that the loss of β-TrCP resulted in the promotion of cell growth and invasion, possibly through the regulation of the levels of CDC25A and the matrix metalloproteinase 11 (31). β-TrCP inhibited the activity of transforming growth factor-β in pancreatic cancer cells by decreasing Smad4 stability (35). β-TrCP suppressed angiogenesis and thyroid cancer cell migration by promoting ubiquitination and destruction of VEGFR2 (27).

However, little is known about the role of β-TrCP in the aggressive behavior of glioma. In a previous study by the authors (32), the protein expression level of β-TrCP in brain glioma tissue was detected by western blot analysis and immunohistochemistry. It was identified that the expression level of β-TrCP protein in human brain glioma samples was significantly lower compared with non-tumor tissues, and the expression level of β-TrCP in high-grade gliomas (grade III and IV) was significantly lower compared with low-grade gliomas (grade I and II). These results demonstrate that β-TrCP probably serves a role in inhibiting the growth of glioma.

Migration, invasion and proliferation are basic features of malignancies. These characteristics reflect the degree of malignancy of tumor cells. Therefore the present study investigated how β-TrCP affects migratory, invasive and proliferative abilities of glioma cells. As the previous study demonstrated that β-TrCP was expressed at low levels in glioma tissues (32). In the present study, β-TrCP was overexpressed by β-TrCP plasmid transfection prior to subsequent experiments.

The effect of β-TrCP plasmid transfection was validated in glioma cells. Exogenous β-TrCP and endogenous β-TrCP

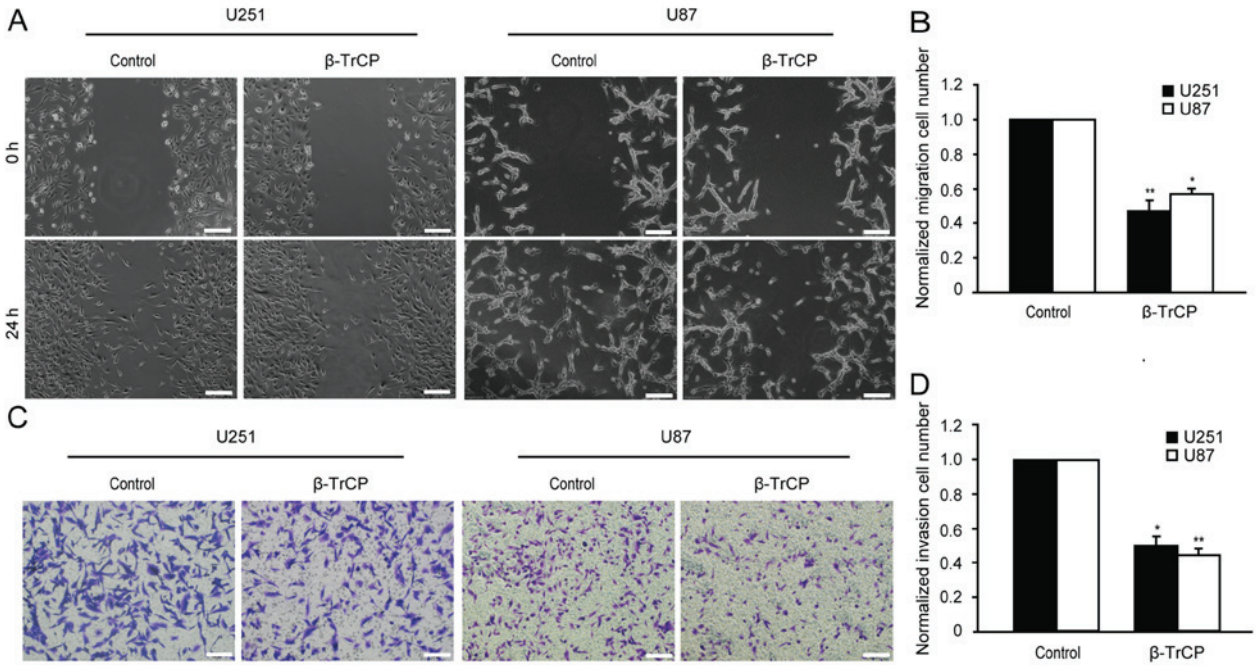

Figure 2. Overexpression of β-TrCP significantly inhibits the migratory and invasive ability of glioma cells. (A) Wound healing assay of U251 and U87 cells at 0 and 24 h following transient transfection with β-TrCP plasmids vs. the control vector group. Scale bar, 100 μm. (B) Cell migration was quantified at 24 h. (C) Representative micrographs of U251 and U87 invasion at 24 h after transient transfection with β-TrCP plasmids. Scale bar, 100 μm. (D) Cell invasion was calculated from Transwell assays with Matrigel. Cell count was normalized to the vacant vector group. *P<0.05, **P<0.01 vs. control. β-TrCP, β-transducin repeat-containing protein. Scale bar, 100 μm.

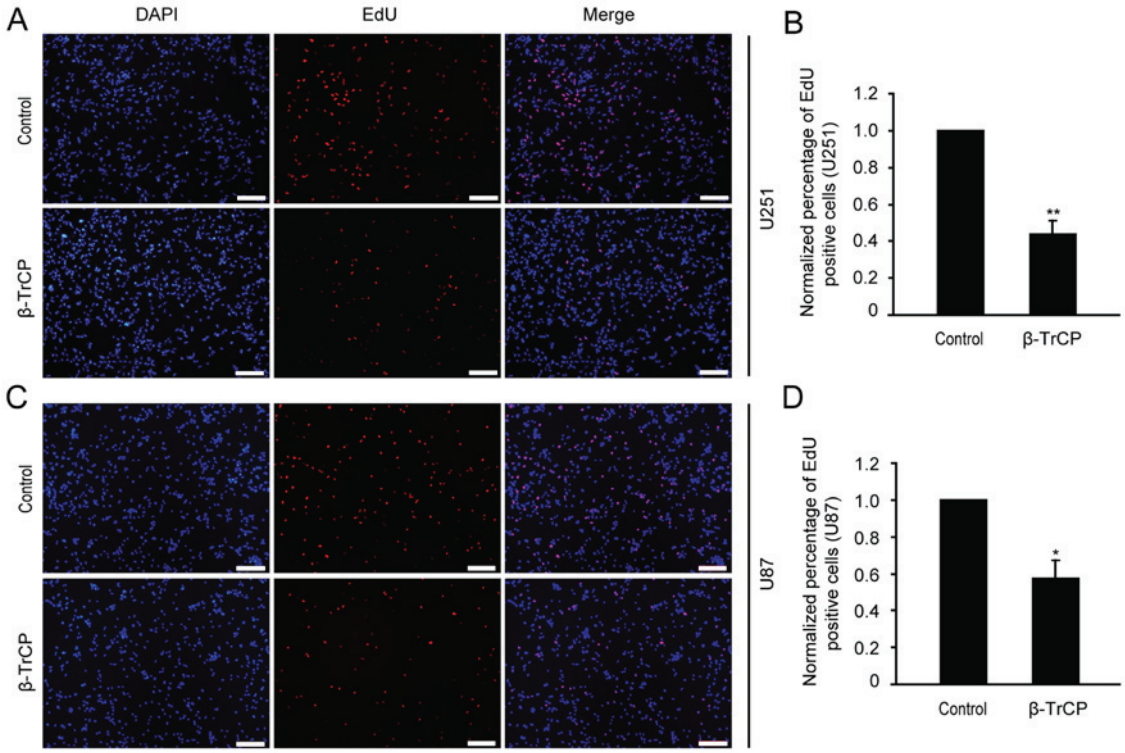

Figure 3. Overexpression of β-TrCP significantly inhibits the proliferative ability of glioma cells. Representative images of EdU positive cells: (A) U251 and (C) U87 cells. Graph presenting the changes between cells overexpressing β-TrCP and the control: (B) U251 and (D) U87 cells. *P<0.05, **P<0.01 vs. control. β-TrCP, β-transducin repeat-containing protein. Scale bar, 100 μm.

were well-expressed, and this formed the basis of subsequent experiments. Subsequently, wound healing assay was used to investigate the effect of β-TrCP on glioma cell migration. It was observed that the ability of wound healing in the β-TrCP

transfection cells group was weaker compared with the control cells. This suggests that the overexpression of β-TrCP inhibits the migration of glioma cells. Invasion is also the main manner of tumor cell movement (36). In the present study, Transwell assay demonstrated that the invasive ability of glioma cells in the β-TrCP transfection group was decreased compared with the control group. Proliferation is the most important feature of tumor cells. Therefore in the present study, the effect of β-TrCP on glioma cell proliferation was also observed using EdU assay. The result demonstrated that the proliferative ability of glioma cells was also significantly decreased following β-TrCP overexpression.

From the above results, it may be hypothesized that β-TrCP protein probably serves as a tumor suppressor to inhibit the growth of glioma. The mechanism may be that β-TrCP acts on different substrates in glioma cells, but this will require additional investigation.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81072072) and a grant from Xuzhou Medical College (grant no. 09KJZ18).

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