Inhibition of SHP-2 promotes radiosensitivity in glioma

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Abstract. As a phosphatase, SHP-2 has been identified to be involved in regulating several cell functions, including growth, division, adhesion and motility. Therefore, SHP-2 may affect the response of glioma to radiotherapy, such as via enhancing angiogenesis. The present study aimed to investigate the function of SHP-2, a protein tyrosine phosphatase, in the radiosensitivity of glioma. U251, U87 and SHG44 glioma cell lines were transfected with small interfering (si)RNA against SHP-2 and cell proliferation was assessed using a cell counting kit 8 assay, cell apoptosis was assessed by fluorescence-activated cell sorting and immunoblotting, cell invasion was determined by an invasion assay, and the vasculogenic mimicry capacity was assessed by a tube formation assay. SHP-2 siRNA transfection reduced the proliferation and increased apoptosis in the glioma cell lines. Downregulation of SHP-2 suppressed glioma cell invasion and vasculogenic mimicry. These results demonstrated that no significant difference was observed between glioma tissues and normal brain tissues, however, silencing of SHP-2 inhibited cell proliferation, invasion and vasculogenic mimicry in the glioma cell lines. SHP-2 may be a novel therapeutic target for glioma.

Introduction

Glioma is the most common and aggressive type of brain tumor. Although patients undergo comprehensive treatment, including maximal micro-neurosurgical resection, radiotherapy and chemotherapy with temozolomide, tumor recurrence is almost inevitable and the 5-year survival rate is <10% (1,2). Several types of tumor, which have been considered by histology to be equivalent have been identified through molecular and genetic investigations to be teleologically and ontologically diverse, suggesting that their treatment requires an increased understanding of their biology and the use of a targeted therapeutic approach (3). Poor prognosis is associated with diffused infiltrative growth in the surrounding brain tissue. SHP-2 is a ubiquitously expressed cytoplasmic protein tyrosine phosphatase (PTP), encoded by the *PTPN11* gene (4). Mutations in *PTPN11* have been identified in several human afflictions, including Noonan syndrome (NS), Leopard syndrome and childhood hematologic malignancies (5). Activating mutations in *PTPN11* have also been identified in solid tumors, including lung cancer, liver carcinoma, colon cancer, neuroblastoma and melanoma (6,7). The present study aimed to investigate whether the inhibition of SHP-2 increases the radiosensitivity of glioma cell lines. The results of the present study may improve the understanding of the functions of SHP-2 in the pathogenesis of glioma and assist in the development of future therapeutic strategies for the treatment of glioma.

Materials and methods

Tissue collection. Glioma cases (n=21; 10 male; 11 female) were used in the present study. Patient age ranged between 25 and 65 years, with an average age of 42 years. Each patient underwent primary surgical resection of glioma between 2008 and 2012. Normal brain tissue was obtained from nine patients (five male and four female; age range, 18-55 years) who endured decompressive surgical procedures for severe head injury within the same time period. This study was approved by the Harbin Medical University (HMU) Ethics Committee (Heilongjiang, China) and informed consent was obtained from the patients. Pathological grading was performed, according to the 2007 WHO classification (8). The tumor samples were consisted of three grade I, seven grade II, five grade III and six grade IV. The pathological review was diagnosed at The Fourth Affiliated Hospital of Harbin Medical University, (Heilongjiang, China) by three pathologists and two neurosurgeons as a routine study.

Immunoblot analysis. Glioma cells ($5x10^6$) were directly lysed with radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot analysis was performed by transfer of the proteins onto polyvinylidene fluoride membranes (Schleicher & Schuell Microscience, Riviera Beach , FL, USA) using a mini

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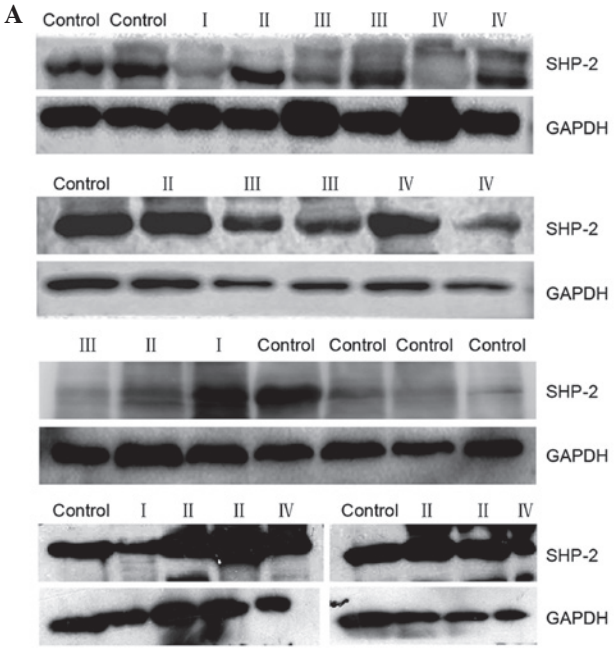
Trans-Blot apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking for 2 h, the membranes were incubated overnight at 4˚C with the following specific primary human antibodies: Anti-SHP-2 (1:1,000; cat. no. 3752; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Caspase 3 (1:1,000; cat. no. ab2302) Abcam, Cambridge, UK), anti-Bax (1:1,000; cat. no. ab7977; Abcam), anti-Bcl2 (1:1,000; cat. no. ab33862; Abcam) and anti-GAPDH (1:1,000; cat. no. ab9485; Abcam). Following washing, the membrane was incubated with the appropriate fluorescein-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:200; cat. no. ZF-0311; ZSGB-BIO, Beijing, China) for 1 h at room temperature. Following extensive washing, the signals were visualized by enhanced chemilluminescence substrate (Pierce Chemical, Rockford, IL, USA).

Cell culture, transient transfection and radiation exposure. The U251, U87 and SHG44 human glioma cell lines were obtained from the Shanghai Cell Collection (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all Gibco Life Technologies, Carlsbad, CA, USA) at 37˚C in a humidified incubator containing 5% CO_2. The specific SHP-2 small interfering (si)RNA (SHP-2.si) was designed and synthesized by Invitrogen Life Technologies (Shanghai, China). Once the cells reached 70-80% confluence, the glioma cells were transfected with SHP-2.si using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Following 24 h transfection, the glioma cells were exposed to different doses of radiation at a dose rate of 200 Gy/min at room temperature. An X linear accelerator (Varian Medical Systems, Inc., Palo Alto, CA, USA) was used for radiation treatment. Following irradiation (IR), the glioma cells were returned immediately to a 37˚C incubator.

Cell proliferation assay. The glioma cells were seeded into 96-well plates (6,000 cells/well) and were transfected with SHP-2.si as described above. Following incubation for 24 h, the 96-well plates were irradiated with 0, 2, 4, 6 or 8 Gy and the glioma cells were subsequently incubated for 24 or 48 h at 37˚C, and cell proliferation was analyzed using a cell counting kit (CCK)8 assay. CCK8 solution (10 ml) was added to each well of the 96-well plates and the plates were incubated at 37˚C for 4 h prior to measuring the absorbance at 450 nm using a plate reader (iMark microplate absorbance reader; Bio-Rad Laboratories, Inc.).

Cell apoptosis analysis. To assess the induction of apoptosis, Annexin V and propidium iodide (PI) double staining was performed using the Annexin-V-FLUOS Staining kit (Roche Diagnostics, Shanghai, China). Following transfection of SHP-2.si, the glioma cells were incubated at 37˚C for 24 h. The cells were subsequently irradiated at doses of 0, 2 or 6 Gy. Following incubation for 24 h, the glioma cells were stained with Annexin V and PI, and analyzed using a FACS Calibur (BD Bioscience, Franklin Lakes, NJ, USA).

Cell invasion assay. Matrigel (BD Biosciences) was added to the upper chamber of the Transwell apparatus with 8-μm pore size membrane (Corning Costar, Corning, NY, USA). When the Matrigel had solidified at 37˚C, serum-free DMEM, containing $1x10^3$ glioma cells in 100 μl was added into the upper chamber. The lower chamber was loaded with 500 μl DMEM, containing 10% FBS. Following incubation at 37˚C for 24 h, the membranes coated with Matrigel were wiped with a cotton swab and fixed using 100% methanol for 10 min. The membranes with cells were soaked in 0.1% crystal violet (Beyotime Institute of Biotechnology) for 10 min and subsequently washed with distilled water. The number of cells attached to the lower surface of the polycarbonate filter was counted at a magnification of x400 under a light microscope (Eclipse E200; Nikon Instruments, Inc., Melville, NY, USA). The results are expressed as the mean of triplicate experiments.



B

	WTO Grades				
	I	II	III	IV	Control
Cases	3	7	5	6	9

Figure 1. No significant difference in the expression of SHP-2 was observed between glioma tissues and normal brain tissues. (A) The expression of SHP-2 in human glioma tissues and normal brain tissues. (B) The number of samples of human glioma tissues of different grades and of normal brain tissues. WTO, 2007 World Health Organization central nervous system tumor classification.

Vasculogenic mimicry (VM) assay. Immediately prior to use, 24-well plates were coated with high-concentration Matrigel (BD Biosciences; 200 μl/well) and incubated at 37˚C for 40 min until the Matrigel was solid. The glioma cells were transfected and pre-incubated in DMEM without serum overnight. The cells were lifted using 0.05% trypsin (Beyotime Institute of Beyotechnology), which was neutralized with DMEM, containing 10% FBS. The cells were centrifuged at 800 x g for 5 min, resuspended and seeded onto the Matrigel-coated wells at a density of $3x10^4$ cells/well. Photomicrographs were captured (OLS4100; Olympus Corporation, Tokyo, Japan)

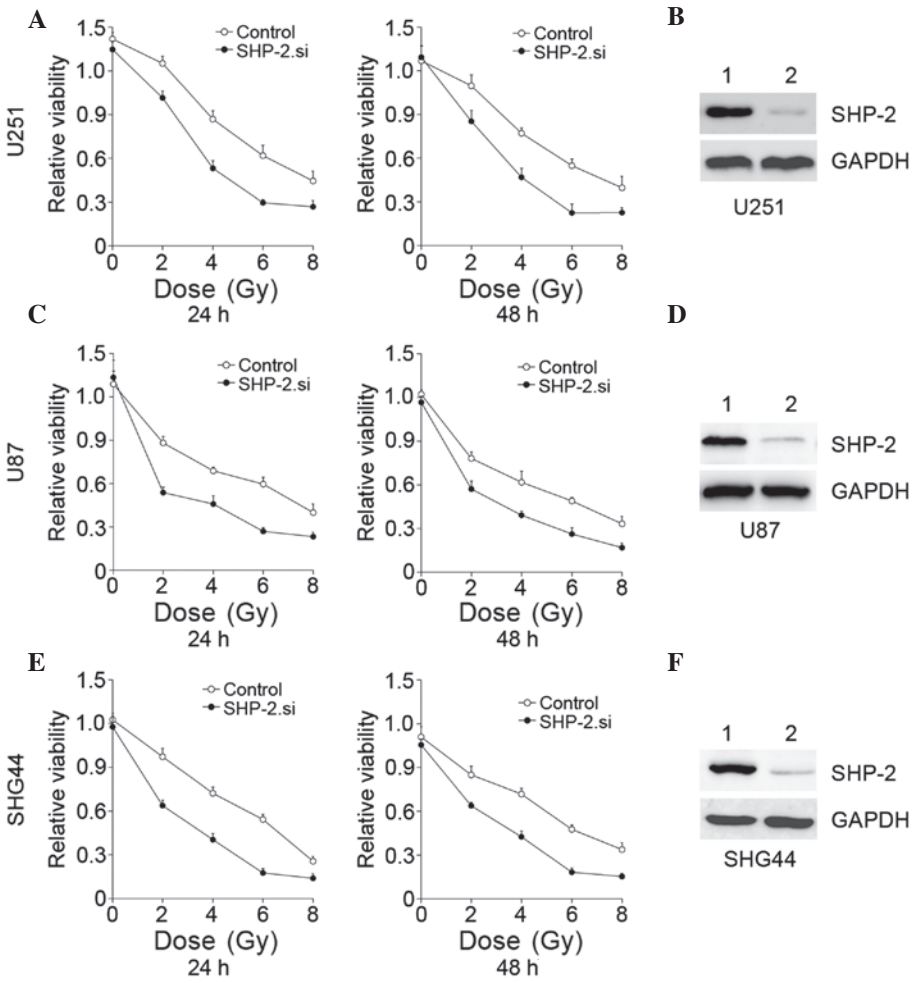


Figure 2. Downregulation of SHP-2 inhibits the proliferation of glioma cell lines combined with radiotherapy. Following transfection for 24 h with SHP-2 siRNA, the glioma cells were administered different doses of IR. Cell counting kit-8 analysis was used to determine the cell viability of the different groups. (A) U251 cells were assessed 24 and 48 h following IR and (B) the expression levels of SHP-2 in different glioma cell lines following transfection of siRNA. (C) U87 cells were assessed 24 and 48 h following IR and (D) the expression levels of SHP-2 in different glioma cell lines following transfection of siRNA. (E) SHG44 cells were assessed 24 and 48 h following IR and (F) the expression levels of SHP-2 in different glioma cell lines following transfection of siRNA. 1, Control; 2, SHP-2.si. IR, ionising radiation; si, small interfering.

following 16 h of incubation from each well and the number of tubes (complete circular structures) was counted. The mean of three readings of each well was used as the final reading from that well.

Statistical analysis. All data were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of SHP-2 in human glioma tissues. The expression of SHP-2 in the 21 cases of human glioma and nine normal brain tissue samples was assessed by immunoblotting. The results demonstrated that there was no significant difference between the glioma tissue and the normal brain tissue (Fig. 1).

Suppression of SHP-2 in combination with IR improves the antiglioma effect in vitro. Glioma cell lines were transfected with SHP-2.si. Following incubation for 24 h, the cells were exposed to IR at different doses (0, 2, 4, 6 or 8 Gy), and the cell proliferation was analyzed by a CCK8 assay at different time points (24 and 48 h). The results demonstrated that cell proliferation was significantly suppressed by the downregulation of SHP-2 in combination with IR (Fig. 2). IR inhibited glioma cell proliferation and in the SHP-2.si groups, the inhibitory effects were markedly increased compared with treatment with IR alone. A significant difference was observed between SHP-2.si and the control groups of each glioma cell line, particularly 24 h following IR. The results indicated that the suppression of SHP-2 improved the antiglioma effect of IR.

Inhibition of SHP-2 in glioma cell lines upregulates IR-induced cell apoptosis. Glioma cell lines were transfected with SHP-2.si and exposed to IR (0, 2 or 6 Gy). Following incubation for 24 h, cell apoptosis was assessed by FACS. The apoptotic rate increased in the SHP-2.si groups, which was significantly difference compared with the control group ($P<0.05$). Additional proteins associated with cell apoptosis, including Bcl-2, Bax and Caspase-3, were assessed. The pro-apoptotic proteins, Bax and Caspase-3, were upregulated

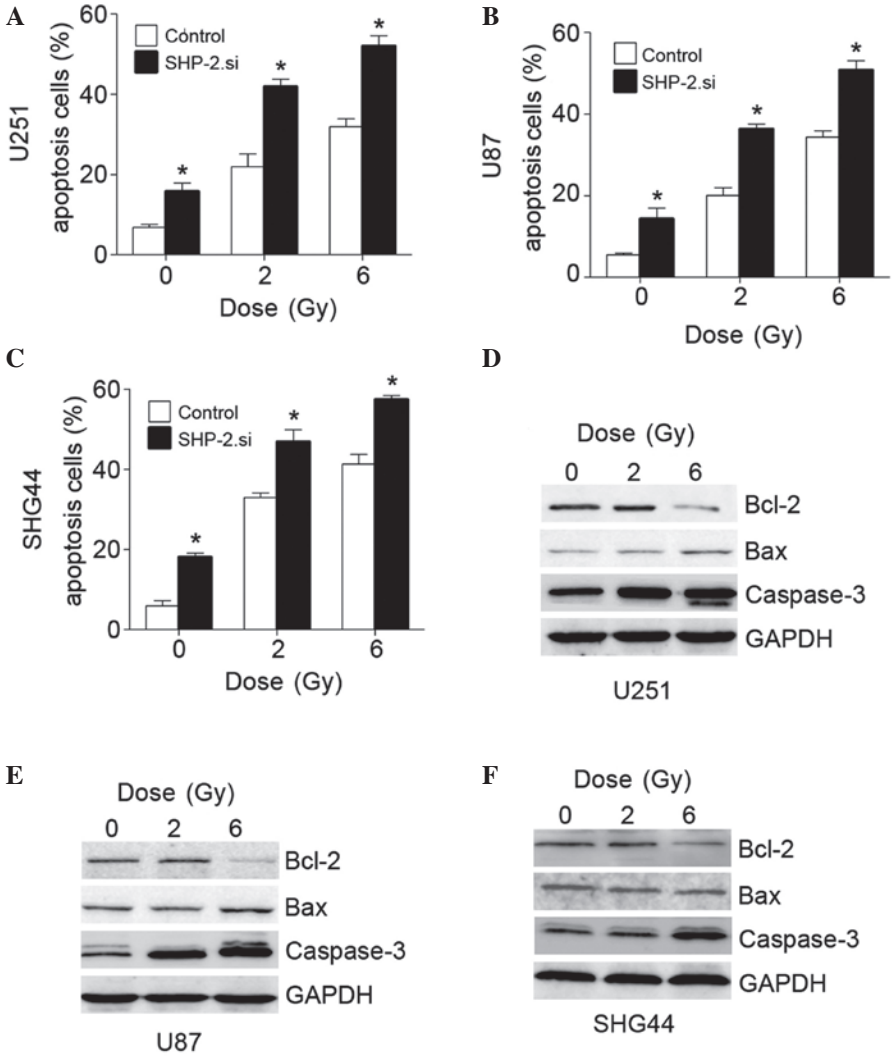


Figure 3. Suppression of SHP-2 increases the apoptosis induced by IR. Cell apoptosis assay of (A) U251, (B) U87 and (C) SHG44 cells. *P<0.05 between the control group and SHP-2 siRNA group. Immunoblot analysis was performed to demonstrate the expression levels of Bcl-2, Bax and Caspase-3 in the SHP-2 siRNA (D) U251, (E) U87, (F) SHG44 glioma cells cells treated with different doses of IR. IR, ionising radiation; si, small interfering.

and the anti-apoptotic protein, Bcl-2, was downregulated in the SHP-2.si groups (Fig. 3). These results suggested that SHP-2.si transfection induced cell apoptosis.

siRNA-mediated silencing of SHP-2 suppresses the invasive ability of glioma cells in vitro. Following transfection with SHP-2.si, an invasion assay was performed in the glioma cell lines. The results of Matrigel Transwell analysis demonstrated that the invasive capacity of glioma cells was significantly reduced by transfection with SHP-2.si (Fig. 4). The number of cells in the SHP-2.si group passing through the Matrigel was significantly lower compared with the control group. The results demonstrated that siRNA-mediated SHP-2 silencing suppressed the metastasis and inhibited the invasion of glioma cells.

Downregulation of SHP-2 inhibits VM in glioma cell lines. A VM assay was conducted in the SHP-2.si and the control groups of each glioma cell line. The VM assay revealed that the tube formation capacity of glioma cells was inhibited following transfection with SHP-2.si and the microtube density was significantly lower compared with the control group (Fig. 5).

Discussion

In glioma, radiotherapy is the most common treatment following microsurgery. For several solid tumors, IR is a treatment tool, which offers a clear survival benefit (9). However, glioblastoma multiforme and pancreatic carcinoma represent types of tumor, which are resistant to conventional radiochemotherapeutical regimes (10). A previous study indicated that glioma demonstrates low sensitivity to radiation therapy and that glioma cells have the capacity of repairing IR-induced apoptosis (11). Glioma progression and resistance to radiotherapy has been associated with angiogenesis (12-14). Neovascularization has long been implicated as a salient feature of glioma progression. Therefore anti-angiogenic therapy may be useful to increase the radiosensitivity of glioma (15). Despite intensive research and development of novel targeted therapies, the prognosis for patients with these types of tumor remains poor (16,17). Novel therapeutic approaches are therefore required.

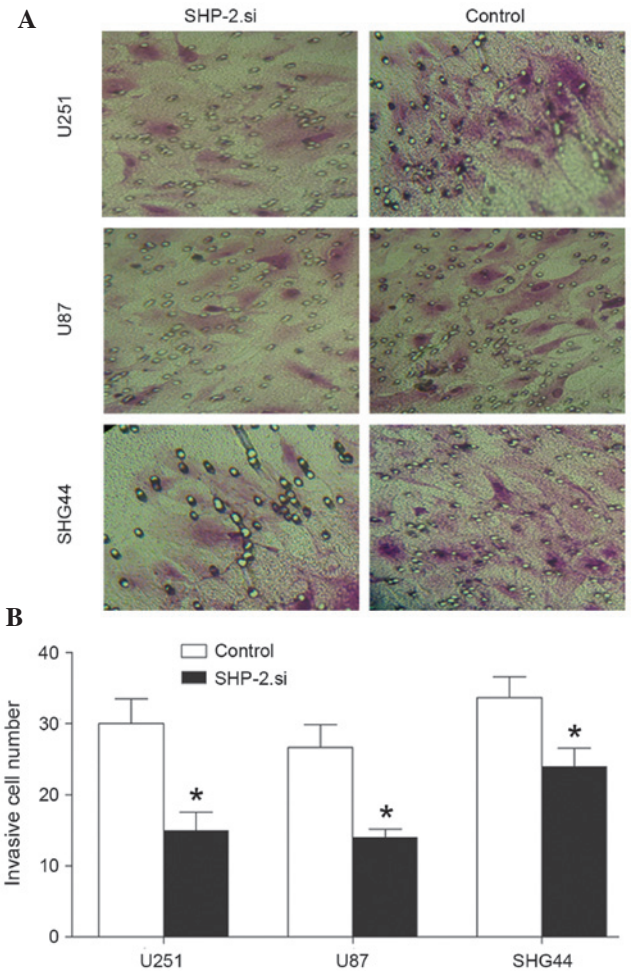


Figure 4. (A) Matrigel Transwell assays for analysis of invasion. The membranes were incubated with crystal violet for 10 min, and the microscopy images represent the glioma cells migrating through the Matrigel (magnification, x400). (B) The graph demonstrates the invading cell numbers in the SHP-2 siRNA and the control cell from each group. *P<0.05 between the control group and the SHP-2 siRNA group. si, small interfering.

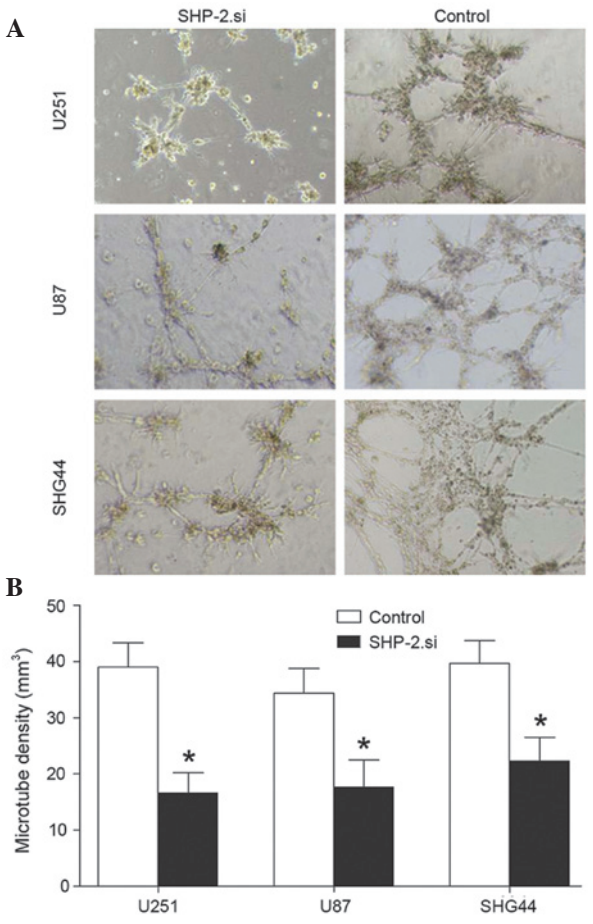


Figure 5. Silencing of SHP-2 inhibits VM in glioma cells *in vitro*. (A) The results of VM in U251, U87 and SHG44. Microscopy images were captured, and the number of tubes (complete circular structures) were counted (magnification, x400). (B) Statistical analysis was performed on the microtube density of each group. *P<0.05 between the control group and SHP-2 siRNA group. si, small interfering; VM, vasculogenic mimicry.

Neovascularization is the most important feature of glioma. High-grade gliomas are among the most vascular of all solid types of tumor, and vascular proliferation is a pathological hallmark of glioma (18). Glioma resistance to radiotherapy and chemotherapy leads to poor clinical outcomes and this resistance was demonstrated to be associated with the tumor microenvironment (19,20). Decades of research improved the understanding of the tumorigenesis of gliomas at the molecular level, through the identification of VEGF and its associated pathways, novel therapeutic targets may be developed (21). Therefore, anti-angiogenic therapies are used in patients with glioma in combination with chemotherapy and radiotherapy (22,23). The most well established anti-angiogenic therapy is bevacizumab (24,25). Anti-angiogenic agents are hypothesized to be significant in the treatment of glioma in the future.

SHP-2 is a protein phosphatase identified in the early 1990s, which is expressed in all tissue types (26). It contains two tandem SH2 domains, a PTP domain and a COOH-terminal hydrophobic tail with two tyrosine phosphorylation sites. SHP-2 is a positive signaling component downstream of growth factor, cytokine and extracellular matrix receptors, and is important in regulating cell growth, transformation, differentiation and migration. Protein phosphorylation and dephosphorylation are fundamental cellular events mediated by kinases and phosphatases, respectively, which govern a host of cell functions, including growth, division, adhesion and motility (27). Mutations in PTPs and/or altered expression of PTPs may contribute to cancer, autoimmune disorders and inflammation. Previous studies demonstrated that SHP-2 is involved in the RAS-MAPK, PI3K-AKT, JNK, JAK-STAT, NF-κB, RHO and NFAT signaling pathways (27,28). It has been revealed that SHP-2 is important in tumor proliferation and invasion in several types of cancer, including glioma. Zhan *et al* (7) revealed that SHP-2 is required for EGFRvIII oncogenic transformation in human glioblastoma cells. SHP-2/PTPN11 mediates glioma genesis driven by PDGFRA and INK4A/ARF aberrations in mice and humans (29). The present study demonstrated that inhibition of SHP-2 increases the antiglioma effect of IR by suppressing the proliferation of, and upregulating the apoptosis of glioma cells. Previous studies have demonstrated that SHP-2 increases cell migration and angiogenesis in NS and leukemia. Since the invasion and abundant vasculature are the biological features of glioma, the present study investigated the invasion and angiogenesis ability of glioma cells when SHP-2 is downregulated. The results

demonstrated that silencing of SHP-2 inhibited the migratory ability and VM of glioma cells. However, the expression of SHP-2 was not upregulated in human glioma tissues, which indicated that SHP-2 may control the biological functions of glioma by other means, for instance, the downstream cell signaling pathways of SHP-2, including the Ras-MAPK, PI3K-AKT, JAK-STAT, NF-κB and NFAT signaling pathways.

These findings suggested that SHP-2 is important in glioma radiosensitivity by regulating cell proliferation, apoptosis, migration and neoangiogenesis. Thus, SHP-2 may be a potential therapeutic target for the treatment of human glioma in the future.

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