Original Article

**SRC3 overexpression promotes glioma proliferation and invasion**

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**Abstract:** Glioma is one of the most common brain tumors. Steroid receptor coactivator3 (SRC3) has been reported to be amplified and overexpressed in breast cancer, prostate cancer, lung cancer, and other types of tumors. However, the role of SRC3 in glioma remains unclear. Objective: Our study aimed to explore the role of SRC3 in glioma proliferation and invasion. Methods: The protein and mRNA expressions of SRC3 in glioma tissues and normal brain tissues were analyzed by western blot and the TCGA database, respectively. Then, the U251 and U87 cell lines were transfected with siRNA-SRC3 and a negative control. After that, an MTS assay and a colony formation assay were performed to investigate the effect of SRC3 in glioma cell proliferation. A Transwell assay was performed to evaluate the role of SRC3 in glioma cell invasion. Moreover, SRC3 mRNA expression in glioma cell lines was determined by RNA extraction and real time-PCR analysis. SRC3 protein expression and the relevant signaling molecules’ protein expressions in glioma cell lines were detected by western blot. Results: We firstly found that SRC3 was overexpressed in glioma tissue samples and glioma cell lines (grade II and IV). Further experiments showed that SRC3 knockdown inhibited cell proliferation, colony formation, and invasion capacity. Moreover, we found that knocking down SRC3 expression reduced PCNA, MMP9, p-Akt, CyclinD1 and CDK2 expressions significantly, while p21 expression was increased dramatically. Conclusion: SRC3 may function as a cancerogenic factor in the malignant progression of glioma and may be a potential target for glioma therapy.

**Keywords:** Glioma, cell proliferation, cell invasion, SRC3

**Introduction**

Gliomas are central nervous system (CNS) neoplasms that affect both the brain and spinal cord. The classification scheme for gliomas has changed dramatically over the past decade, driven by advances in molecular analyses of these tumors. Primary CNS tumors are the most common type of solid neoplasms in children, and are more common in adults (29 per 100,000) than children and adolescents (6 per 100,000). Gliomas make up only 27% of all primary CNS tumors, but importantly, they account for 80% of all malignant primary CNS tumors. 75% of all gliomas are astrocytomas, with the most common being the highest-grade subtype, glioblastoma, and are notable for their dismal overall 5-year survival of 5% [1]. At present, the treatment of glioma consists of three main strategies—surgery, chemotherapy, and radiotherapy. Surgical removal is considered the first choice and is often followed by chemotherapy and concurrent radiotherapy (RT), with continued adjuvant chemotherapy [2-5]. However, high-grade glioma patients cannot completely recover with the current technology because of the procedures’ invasive characteristics. The blood-brain barrier in the brain parenchyma also reduces the sensitivity and efficiency of glioma to chemoradiotherapy [6]. Therefore, high-grade glioma presents an extremely poor prognosis, and the median overall survival is low at approximately 14.6 months [7]. Up to now, researchers have made significant efforts and achievements to develop immunologic therapies or other new and emerging innovations in glioma treatment, but the outcomes need further study.

The steroid receptor coactivator3 (SRC3), which was discovered by Anzick in 1997 [8], is also referred to as AIB1, p/CIP, RAC3, ACTR, NCoA3,
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and TRAM1 and is a member of the p160/steroid receptor coactivator (SRC) family [9]. It has been found amplified and overexpressed in many types of human tumors such as breast, prostate, lung, and gastric cancers as well as esophageal squamous cell carcinomas [10-14]. Previous researchers have shown that SRC3 was involved in promoting cell proliferation, migration and invasion in the cancers [15, 16]. It has been reported that SRC3 overexpression is clinically and functionally relevant to the progression of human ESCC. SRC3 promoted bladder cancer cell proliferation through the IGF/Akt pathway [13]. Others showed that overexpression of SRC3 facilitated human HCC progression by enhancing cell proliferation and invasiveness. So SRC3 may be a regulator of human HCC growth and may also be a useful molecular target for HCC prognosis and treatment [17]. However, studies exploring the relationship between SRC3 and glioma are still limited. Kefalopoulou [18] reported that SRC3 expression was less frequently expressed in grade II astrocytomas compared with grade III and grade IV astrocytomas. He also found that high SRC3 expression was associated with decreased overall survival. Therefore, it is necessary to explore the role of SRC3 in glioma progression, an exploration that might provide new strategies for the clinical treatment of glioma.

Materials and methods

Clinical specimens

Specimens including both the glioma tissues and the adjacent non-tumor tissues were all collected from 14 patients at Affiliated Chenggong Hospital from 2012 to 2014. None of these patients had received chemotherapy or radiotherapy before surgery. Informed consents and approval from patients or family members were all obtained. This study was approved by the Ethical Committee of Affiliated Chenggong Hospital. All tissues were snap frozen and stored in liquid nitrogen before use.

Analysis of SRC3 expression using an online microarray database

The human glioma expression microarray data downloaded from The Cancer Genome Atlas (TCGA) website (http://cancergenome.nih.gov/) were used to analyze the mRNA expression of SRC3 between the glioma tissues and the normal brain tissues.

Cell culture

The CHG5 and SHG44 cell lines were provided by Xiuwu Bian from the Third Military Medical University, China. The U251 cell line was purchased from CCTCC (Chinese Typical Culture Preservation Center). The U87 cell line was purchased from ATCC (American Type Culture Collection). All these cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (GEMINI, Producer, Woodland, California, USA) at 37°C in a humidified atmosphere with 5% CO₂.

RNA interference

The U251 and U87 cell lines were chosen in the knockdown experiment because of their transfection efficiency consideration and background high expression of SRC3. Two siRNAs targeting SRC3 were synthesized and purchased from the RIBBIO company, and the sequences were as follows: siSRC3-1: 5'-TC-GAGACGGAAAAACATTGTA-3'; siSRC3-2: 5'-AA-CACTGCACATGGATTATG-3'. The cells were all seeded into a 6-well plate at 60%-70% confluence and then transfected with oligonucleotides at a final concentration of 20 nM with the Dharma FECT reagent (Thermo Fisher Scientific, Lafayette, CO, USA) according to the manufacturer’s instructions. 6 hours later, the transfection medium was replaced with 3 mL of complete medium for each well. These cells were then harvested for the following assays at the indicated times. All experiments were performed in triplicate.

Cell viability assay

Cell proliferation was detected by an MTS assay which was performed by seeding the cells into a 96-well plate at 4 × 10³ cells per well. 20 μL of MTS was added to each well and then incubated for 1 hour at 37°C. The OD490 nm value of each sample was measured by a microplate reader (Bio-Rad iMark, Hercules, CA, USA). All experiments were performed in triplicate.

Colony formation assay

Glioma cells that were transfected with corresponding miRNAs were seeded into a 6-well plate at a density of 1000 per well and maintained in cell incubators at 37°C for 14 days. The culture medium was replaced with a fresh
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medium every three days. Two weeks later, the cells were washed with PBS twice and stained with Giemsa Stain (Millipore, Billerica, WI, USA). Only colonies with more than 50 cells were counted. Twelve random fields were counted. The formed cell colonies were counted to represent the cell proliferation ability of the glioma cells. All experiments were performed in triplicate.

Cell Invasion assay

The cell invasion assays were performed in the 24-well plate Transwell chambers (Millipore, Billerica, MA, USA). The filters were precoated with Matrigel (BD, Franklin Lakes, NJ, USA) in the upper compartment before seeding the cells. 0.5mL of serum-free suspension of transfected cells (24 h after transfection) at a total count of $2 \times 10^5$ cells was added to the upper chamber, and the lower chamber was filled with 0.7 mL of cell culture medium supplemented with 20% FBS. After incubation in at 37°C for 24 hours, the non-invading cells in the upper surface of the membranes were scraped by a cotton swab, and the invasive cells on the bottom surface of the membranes were stained with Giemsa stain for 40 mins and counted under a microscope. Six random fields from each membrane were counted. All experiments were performed in triplicate.

RNA extraction and real time-PCR analysis

Total RNA was extracted from the cells using Tripure Isolation Reagent (Roche) according to the manufacturer’s instructions. The Transcription First Strand cDNA Synthesis Kit (Roche) was used to synthesize cDNA from 1 μg total RNA. SYBR Green I Master (Roche) and GAPDH were used as an internal control. All experiments were performed in triplicate.

Western blot analysis

Proteins were extracted from transfected cells and tissues using RIPA buffers (radioimmunoprecipitation assay) (Cell Signaling Technology, Danvers, MA), followed by the BCA protein assay kit Protein to quantitate the protein concentrations according to the manufacturer’s instruction (Pierce). Equal amounts of protein lysates were separated to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). After being transferred to a PVDF membrane (Millipore), they were incubated in a blocking buffer for 1 hour on shaking tables at room temperature. After that, the membranes were incubated with anti-SRC3, anti-MMP9, anti-p-Akt, anti-PCNA, anti-p21, anti-p-STAT3, anti-p-ERK1/2, anti-CyclinD1, anti-CDK2, and anti-β-actin at 4°C overnight. The next day, the membranes were incubated with an HRP-conjugated secondary antibody for 2 hours on shaking tables at room temperature. Finally, the membranes were visualized using an enhanced chemiluminescence detection system. Proteins were detected with an HRP-conjugated secondary antibody. An anti-β-actin antibody was employed as a loading control. The ImageJ software (Scion Corporation, Frederick, MD, USA) was used to detect and quantify the immunoreactive bands.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) and SPSS 16. Each experiment was performed at least three times. The data was expressed as the mean ± SD. An unpaired Student’s t-test was used to determine the significant differences of all the results and the differences were considered statistically significant at P < 0.05. The TCGA statistical analysis was performed using a Mann-Whitney test.

Results

SRC3 expression was upregulated in glioma tissues

We analyzed the expression levels of SRC3 in the clinical patient samples using a SRC3-specific antibody, and western blot analysis determined the expression of SRC3 in 14 pairs of glioma and matched tumor-adjacent tissues (Figure 1A). The results showed that the expressions of SRC3 in the glioma tissues were significantly increased compared with the expressions in the matched non-cancerous tissues (Figure 1B, *P < 0.05).

Due to the limited number of fresh samples, we accessed the mRNA expression of SRC3 in the TCGA database (Figure 1C), and it showed that SRC3 mRNA expression was significantly amplified in glioma (***P < 0.01).

SRC3 expression was upregulated in glioma cell lines

To better understand the potential functions of SRC3 in glioma cells, we further analyzed the
expression levels of SRC3 in four human glioma cell lines (CHG5, SHG44, U251, U87) and one normal glial cell line (HEB) (Figure 2A). The CHG5 and SHG44 cell lines were derived from the WHO II glioma tissues, and the U251 and U87 cell lines were from WHO IV glioma tissues. The results showed that SRC3 expressions in the glioma cell lines (CHG5, SHG4, U251 and U87) were significantly upregulated compared with the HEB cells (Figure 2B, **P < 0.01).

RNA interference reduced SRC3 expression in the U251 and U87 cell lines

Since the glioma cell lines in our experiments always showed a relatively higher expression of SRC3, we decided to perform knockdown experiments instead of over expression strategies to explore the potential functions of SRC3.

Then, two different siRNAs against SRC3 were used to interfere with SRC3 expression. Western blot and RT-PCR experiments were used to determine the SRC3 knockdown efficiency. The U251 and U87 cell lines were selected because of a relatively high transfection efficiency (Figure 3A). Compared with the control group, the protein and mRNA expressions of SRC3 in the siSRC3 groups of the U251 and U87 cell lines were significantly decreased (Figure 3B and 3C, **P < 0.01).

SRC3 knockdown inhibited the proliferation and colony formation of glioma cells in vitro

An MTS assay was performed to investigate the effect of SRC3 in glioma cell proliferation. The results showed that the proliferation rate (rele-
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Figure 2. SRC3 was upregulated in four glioma cell lines. A. Western blot was used to detect the expression of SRC3 in four glioma cell lines and one normal glial cell line (HEB). CHG5 and SHG44 are low-grade glioma cell lines, U251 and U87 are high-grade glioma cell lines, β-actin was used as a control; B. A quantification analysis of SRC3 protein expression in four glioma cell lines and one normal glial cell line (HEB), **P < 0.01.

The viability (cell viability) of SRC3 knockdown U251 and U87 cell lines was dramatically reduced to 62% and 61% respectively, compared with the proliferation rate of the scrambled control cells by the third day (Figure 4A, *P < 0.05).

To further confirm the role of SRC3 in proliferation, a colony formation assay was conducted after siRNA transfection. The results showed that SRC3 knockdown dramatically reduced the number of colonies in the U251 and U87 cell lines (Figure 4B and 4C, *P < 0.05, **P < 0.01), indicating that SRC3 promoted anchorage-independent growth and colony formation.

SRC3 knockdown inhibited glioma cell invasion

Invasion capacity is one of the most important features of malignant glioma cells. Therefore, we performed a Transwell assay to investigate the role of SRC3 in glioma cell invasion. SRC3 was first knocked down in both U251 and U87 cell lines after transfection with the specific siRNA against SRC3. The invasive cells were then stained with Giemsa and counted under a microphotographic instrument (Figure 5A). The results demonstrated that knocking down SRC3 exerted a significant decrease in the number of invaded cells in the U251 and U87 cell lines compared to the scramble control (Figure 5B, *P < 0.05). The results indicated that SRC3 might participate in the invasion progression of glioma cells.

SRC3 knockdown reduced the expression of PCNA, CyclinD1, CDK2, MMP9, p-Akt and increased the expression of p21 in glioma cells

To determine the role of SRC3 affecting the tumorigenesis and procession of glioma cells, we then conducted a western blot experiment to detect a series of cell signaling molecules in the signal pathways which were involved in proliferation and invasion (Figure 6A). Compared with the control group, we found that SRC3 knockdown observably decreased the expression of PCNA, CyclinD1, CDK2, MMP9, and p-Akt and increased the expression of p21 in the siSRC3 groups (Figure 6B and 6C, *P < 0.05). These results demonstrated that SRC3 might enhance glioma cell proliferation and invasion ability at least partly through the PCNA, CyclinD1, CDK2, MMP9, and p-Akt signal pathways.

Discussion

SRC3, a vital member of the p160/steroid receptor coactivator (SRC) family, has been shown to be involved in the pathogenesis and development of cancers deriving from a wide range of other organs. Previous studies have demonstrated that SRC3 plays essential roles in regulating cellular functions including proliferation, invasion, cell cycle and DNA damage repair. However, the role of SRC3 in glioma remains vague. In the present study, we found that SRC3 expression in glioma tissues was significantly higher than it was in adjacent non-tumor tissues at the protein level in 14 patients with glioma; and in the TCGA database, it was higher at the mRNA level than in non-tumor tissues as well. By knocking down SRC3 expression with siRNA interfering in U251 and U87 cell lines, we found that SRC3 downregulation could efficiently suppress the cell growth rate, colony formation, and invasion ability. These
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Figure 3. RNA interference reduced SRC3 expression in the U251 and U87 cell lines. A. SRC3 protein expression in the knockdown groups and the control group detected by western blot, with β-actin used as a control; B. Quantification analysis of western blot showed that SRC3 was significantly decreased after siRNA treatment, **P < 0.01; C. RT-PCR showed that SRC3 mRNA expression was significantly downregulated after siRNA treatment, **P < 0.01.

results demonstrated that SRC3 might play essential roles in carcinogenesis and the progression of glioma.

To further explore the regulating role by which the downregulation of SRC3 inhibited glioma cell proliferation, a number of genes were detected in siC and in two siSRC3 cells lines. Cell proliferation plays a pivotal role in all phases of carcinogenesis with multiple genetic changes. PCNA (Proliferating Cell Nuclear Antigen) can be used as an indicator to evaluate cell proliferation status. Previous studies have also shown that PCNA is overexpressed and correlated with tumor grade in glioma patients [19]. As one of the most important members of highly conserved cell cycle family, CyclinD1 has a cyclical change in protein abundance throughout the cell cycle and regulates the cell cycle positively. CyclinD1 can bind cyclin-dependent kinase 4 (CDK4) to form a complex of CyclinD1-CDK4, which can phosphorylate Rb protein and promote cell transformation from G1 phase to S phase. This role can accelerate cell cycle progression and promote abnormal cell proliferation, thus participating in the occurrence and development of tumors. Previous studies have found that CyclinD1 expression in normal tissues is low or not expressed, but it is overexpressed in lung cancer, esophageal cancer, breast cancer, and other malignant tumors. Thus, CyclinD1 might be an important indicator to assess the occurrence and development of tumors.

CDK2 is one of the essential regulators for the transition and progression in the cell-division cycle. Progression through the cell-division cycle is regulated by the coordination of CDKs' activities in complex with their respective cyclin partner [21, 22]. An inhibitor of CDKs p21, also known as p21-waf1/cip1 or p21/CDKN1A [23], is a crucial member of the CIP/Kip family of CDK inhibitors [24]. p21 is a well-known cell cycle inhibitor which can arrest the cell cycle progression in G1/S and G2/M transitions by inhibiting CDK4/CyclinD and CDK2/Cyclin E, respectively. It is believed that the role of p21 regulating cell growth is mediated by the control of E2F activity [25, 26]. Moreover, the mammalian cell cycle progression is regulated by CDKs and regulatory subunits cyclins, and the progression cell cycle is triggered by the partial phosphorylation of Rb by CDK-Cyclin. p21 disrupts this interaction and inhibits cell cycle progression [27]. Our results have shown that SRC3 knockdown significantly decreases the expression of PCNA, CyclinD1, CDK2 and increases the expression...
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Figure 4. SRC3 knockdown suppressed cell proliferation and colony formation ability in vitro. A. An MTS assay indicated that SRC3 knockdown suppressed the cell growth rate in the U251 and U87 cell lines, *P < 0.05; B. A colony formation assay showed that SRC3 knockdown dramatically suppressed the cell colony formation ability in the U251 and U87 cell lines; C. Quantification analysis of colony formation numbers in the control and knockdown groups of the U251 and U87 cell lines, *P < 0.05, **P < 0.01.

of p21 in SRC3 knockdown groups compared with the expression of the control group. These findings indicated that SRC3 promoted cell proliferation at least partially by enhancing these critical cell molecules. However, it is still unclear how SRC3 plays a role by interacting with CyclinD or CDK families. And we are still not sure whether SRC3 was directly bound E2F to regulate p21 expression.

Invasion capacity is one of the most critical features of malignant glioma cells. Metalloproteinases (MMPs) play vital roles in regulating various physiological processes, such as embryonic development and reproduction, disease processes, cancer invasion and metastasis [28]. Matrix metalloproteinase9 (MMP9), a member of the matrix metalloproteinase, is involved in breaking down the basement membrane by degrading type IV collagen and exposing the underlying secretory sites, which, in turn, promote the invasion of cancer cells. The degradation of the extracellular matrix (ECM) in tumor tissue is one of the leading processes of tumor invasion and metastasis [29, 30]. Overexpression of MMP9 has been reported in different cancers and is believed to facilitate tumor cell metastasis. It has been reported that the polymorphism of allele of the MMP promoter is significantly associated with gastric cancer invasion and metastasis [31]. Moreover, studies have shown that the knockdown of MMP9 expression inhibited glioma invasiveness [32-35]. In the present study, we found that SRC3 knockdown significantly reduced the expression of MMP9, indicating that SRC3 promoted glioma cell invasion at least partly through enhancing MMP9 expression.

Downregulation of SRC3 is frequently associated with the inhibition of the Akt signal molecule in several human cancers, including breast cancer, prostate cancer, and HCC as well [36-38]. And it is believed that the Akt signal molecule is involved in a number of tumor cell progressions, including proliferation and invasion. In our study, we detected p-Akt protein expression by western blot analysis. And the results showed that p-Akt was significantly overexpressed compared with the control group, which indicated that SRC3 might regulate glioma cell malignant progression partly through regulating Akt expression.

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor that functions mainly through the phosphoryla-
Oncology research has shown that the malignant transformation of a tumor is closely related to cellular metabolism, mainly through large-scale genetic and protein analyses. Metabolites, downstream of both transcription and translation of tyrosine residues and translocation to the nucleus of dimerization [39]. The STAT3 signal is activated in various malignant human cancers and participates in different cellular processes as well as tumorigenesis [40-42]. Extracellular signal-regulated kinase 1 (44 kDa) and 2 (42 kDa) (ERK1/2 or ERK44/42) are members of the mitogen-activated protein kinase (MAPK) family. They are also important members of the intracellular signaling cascade. The Ras-Raf-MEK-ERK signaling pathway is involved in many aspects of cell physiology, as well as the development of neurons and glial cells [43]. ERK1/2 signaling activity has been shown to be associated with matrix metalloproteinase (MMP) expression and astrocyte activity [44]. However, in the present study, we found that knocking down the expression of SRC3 had no significant effects on the expression of p-STAT3 and ERK1/2, suggesting that SRC3 may not play a role in the proliferation and invasion of glioma by interfering with p-STAT3 and ERK1/2 expression, but it still needs further investigation to confirm.

Figure 5. SRC3 knockdown inhibited the cell invasion of glioma. A Transwell assay showed that the downregulation of SRC3 after siRNA treatment inhibited cell invasion in the U251 and U87 cell lines (Magnification × 100); B. Quantification analysis showed that SRC3 knockdown could suppress cell invasion significantly in the U251 and U87 cell lines, *P < 0.05.
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Researchers have also found that PEGylated silica nanoparticles (PSiNPs) can traverse the blood-brain barrier in vitro and in vivo, which points to the potential application of small sized silica nanoparticles in drug delivery [46]. Since our work proved that SRC3 was upregulated in glioma, we may combine PSiNPs in our future work involving the research and development of drugs targeting SRC3.

In summary, we proved that SRC3 is overexpressed in human glioma tissues and also can demonstrate the molecular mechanism of SRC3 in the malignant progression of glioma.
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regulate cell proliferation and invasion partly by enhancing PCNA, MMP9, p-Akt, CyclinD1, and CDK2 expressions. All these findings indicate that SRC3 could be a potential target for glioma therapy.

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Disclosure of conflict of interest

None.

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