Downregulation of Src enhances the cytotoxic effect of temozolomide through AKT in glioma

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Abstract. Src is an attractive target since it is overexpressed in a number of malignancies, including glioma. However, the mechanism of Src signaling as well as its silencing effect on temozolomide in glioma is not well known. We hypothesized that downregulation of Src may enhance the cytotoxic effect of temozolomide on glioma. As expected, Src was overexpressed in glioblastoma multiforme (GBM) compared with normal brain tissues. Src silencing suppressed tumor proliferation and induced apoptosis in glioma. In addition, Src silencing combined with temozolomide treatment resulted in significant inhibition of tumor growth. These effects may be mediated by AKT which is a downstream effector of Src, since downregu-lation of AKT exhibited a similar effect as Src siRNA when combined with temozolomide. Finally, we demonstrated that overexpression of AKT suppressed the enhanced cytotoxic effect of temozolomide mediated by Src silencing. Thus, the present study demonstrated that Src plays a biologically signif-icant role in tumor proliferation and apoptosis and enhances the cytotoxic effect of temozolomide through AKT supression in glioma.

Introduction

Glioblastoma, the most aggressive type of central nervous system tumor, is considered to be one of the deadliest of human cancers. Despite advances in surgery, radiation therapy and chemotherapy, the 1-year overall survival rate is less than 30%, and the median survival time of patients with high-grade glioma is approximately 15 months (1,2). To develop more optimized and effective treatment strategies for glioblastomas, it is critical to gain a deeper understanding of the molecular mechanisms underlying gliomagenesis and to identify targets for therapeutic intervention (3,4).

Src family kinases (SFKs) comprise a subclass of membrane-associated non-receptor tyrosine kinases that are involved in a variety of cellular processes, such as cell division, motility, adhesion, angiogenesis and survival (5,6). Several of the SFKs have been shown to be upregulated in cancer and topromote progression and metastasis. Among the SFKs, Src has arguably been the best characterized and has been the most commonly implicated in cancer, including glioblastoma (7). Src mediates many critical proteins that when dysregulated highly contribute to prolif-eration, invasion and vasculogenesis through interconnected signaling cascades. These proteins include focal adhesion kinase (FAK), which colocalizes with p130CAS, integrin avh3, and paxillin to form focal adhesions, as well as AKT, which is a downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway (8-12). In addition, Src appears to activate the mitogen-activated protein kinase (p44/42 MAPK) in cooperation with Grb2/PI3K regulation via activation by platelet-derived growth factor receptor, ultimately inducing tumorigenic transcription and gene expression (13,14).

In the present study, we demonstrated the inhibitory effect of Src siRNA on cell proliferation and apoptosis in multiple glioma cell lines via the blockage of Src. A mechanistic assay demonstrated that downregulation of Src suppressed AKT and FAK autophosphorylation. Furthermore, we showed that downregulation of Src and temozolomide had a synergistic effect on the inhibition of cell proliferation partly through AKT suppression.

Materials and methods

Glioblastoma cell lines. The human glioblastoma (GBM) cell lines U87 and LN229 were purchased from the Chinese Academy of Sciences Cell Bank. The cells were maintained at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Reagents. Temozolomide (TMZ) was purchased from Sigma (USA). TMZ was dissolved in 10% dimethyl sulfoxide (DMSO; Sigma) to make a stock solution (TMZ, 100 mg/ml). DMSO concentration was maintained below 0.1% in all the

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cell cultures and did not exert any detectable effect on cell growth or cell death.

Target sequences for Src (5'-GGGCGAACCACCUGAA CAA-3') and the control (5'-UUCUCCGAACGUGUCACGU-3') were purchased from Sigma Genosys. Rabbit anti-human polyclonal antibodies against AKT, p-AKT, FAK, P-FAK and GAPDH; and the horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assay. For the cell viability assay, cells were plated on 96-well plates in triplicate and incubated for 24 h at 37°C in 5% CO_2. After incubation, cells were washed, serum and antibiotic-free medium were added and treated with the control or Src siRNA. After 6 h, cells were washed and incubated in serum-containing medium overnight. Afterwards, cells were washed and either regular or docetaxel-containing medium was added. After 72 h, the cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Apoptosis assays. U87 and LN229 cells were plated in 6-well plates and transfected with oligonucleotide. The apoptosis ratio was analyzed 48 h post-transfection using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Annexin V-FITC and propidium iodide (PI) double staining was used to evaluate the percentage of apoptotic cells. Annexin V⁻/PI⁻ cells were used as the controls. Annexin V⁺/PI⁻ cells were evaluated as apoptotic and Annexin V⁺/PI⁺ cells were evaluated as necrotic. Tests were repeated in triplicate.

Western blotting. For western blotting, protein was abstracted from the treated cells. After the protein concentration was determined, equivalent amounts of proteins were boiled in sample buffers with DL-dithiothreitol (DTT) and then centrifuged at 12,000 rpm for 15 min at 4°C. The protein was separated by SDS-PAGE (PAGE, 15% gel for MAP1LC3; 10% gel for Beclin 1). Separated proteins on the gel were transferred onto PVDF membranes by an electroblot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Filters were blocked for 2 h in 5% low-fat milk and incubated with the rabbit anti-human antibody for Src, AKT, p-AKT, FAK, p-FAK and GAPDH at 4°C for 12 h. Membranes were then washed with PBS-T and incubated with HRP-conjugated anti-goat antibody for 1 h. Specific signals were detected from the quantitative gel and western blot imaging system (Becton-Dickinson, Franklin Lakes, NJ, USA).

Subcutaneous tumor assay. Female nude mice, 5-weeks old, were subcutaneously injected with LN229 and U87 glioma cells. When the tumor volumes reached 50 mm³, the mice were randomly divided into groups: the control groups, Src siRNA group, TMZ group, Src siRNA plus TMZ group, AKT siRNA group, AKT siRNA plus TMZ group and Src siRNA, TMZ plus AKT group and received the relevant treatment with a local injection in the xenograft tumor in multiple sites. The treatment was performed once every 2 days for 20 days, and the tumor volume was measured with a caliper every 2 days,
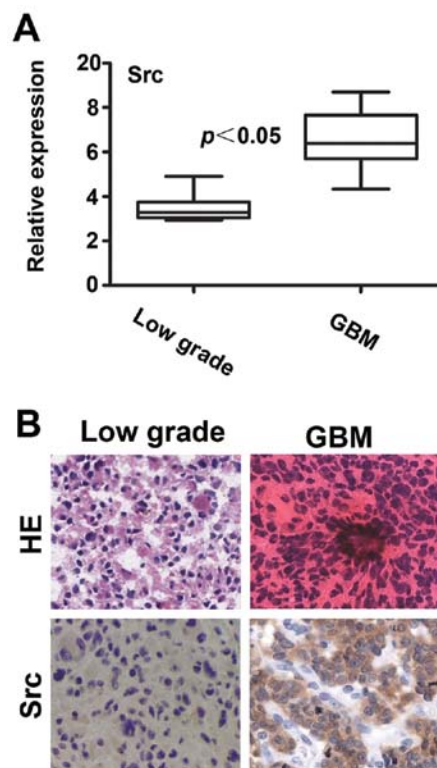

Figure 1. Src is overexpressed in GBM clinical samples. (A) The expression of Src was detected in glioma tissues by RT-PCR. The levels of mRNA Src increased markedly in GBM in comparison to low-grade gliomas. (B) The expression of Src was detected in glioma tissues by IHC. The levels of Src increased markedly in GBM in comparison to low-grade gliomas.

using the formula: Volume = length x width²/2. At the end of the 20-day observation period, the mice bearing xenograft tumors were sacrificed.

Statistical analysis. Statistical analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). Multiple groups were compared using analysis of variance (ANOVA) followed by post hoc Fisher's least significant difference (LSD) testing when appropriate. Data were considered statistically significant at $P < 0.05$. All data are expressed as the means ± standard deviation (SD).

Results

Src is overexpressed in GBM clinical samples. To determine the expression of Src in glioblastoma and low-grade glioma tissue, the mRNA and protein expression of 10 GBM and 10 low-grade gliomas was determined using RT-PCR and immunohistochemistry (IHC). Both mRNA and protein expression of Src was overexpressed in glioblastoma relative to that in the low-grade gliomas (Fig. 1). Thus, Src is overexpressed in GBM, and may contribute to its metastatic progression.

In vitro Src silencing. As demonstrated, Src was overexpressed in the GBM samples. Therefore, we subsequently evaluated the *in vivo* biological effect of Src gene silencing. U87 and LN229 cells were harvested 48 h after transfection with Src siRNA, and the expression of total Src was determined by RT-PCR
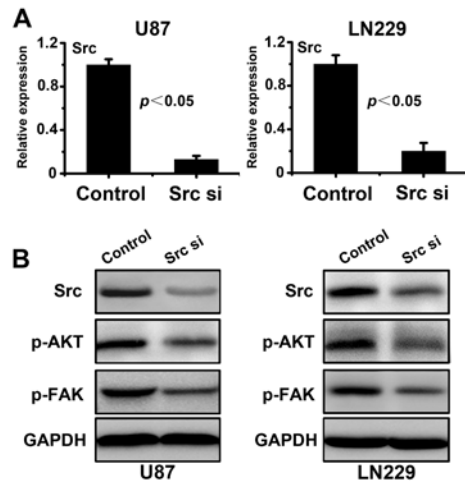
Figure 2. Effect of Src silencing on expression of Src signaling. (A) U87 and LN229 cells were transfected with the Src siRNA or with a control siRNA. Expression of Src was evaluated 48 h later by RT-PCR. As shown, the levels of Src were significantly decreased in the presence of Src siRNA, but not in the presence of control siRNA. (B) Western blot analysis of total cell lysates of U87 and LN229 cells transfected with the Src siRNA. As shown, transfection with Src siRNA induced a decrease in Src, p-FAK and p-AKT.
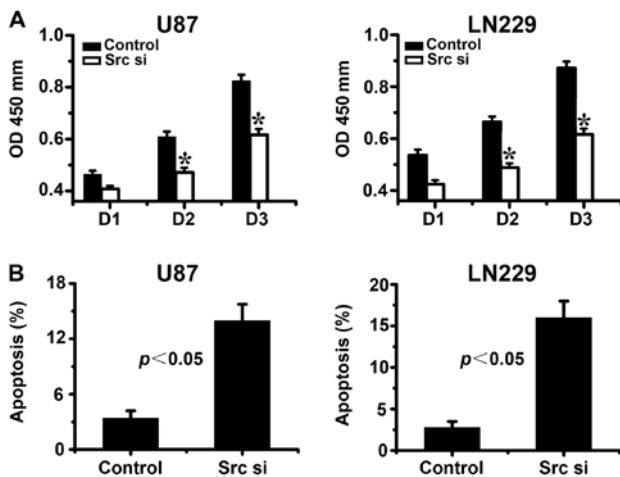

Figure 3. Src silencing impacts the proliferation and apoptosis of glioma cells. (A) Representative histograms showing cell proliferation regulated by Src siRNA. (B) Representative histograms showing apoptosis regulated by Src siRNA. *P<0.05.
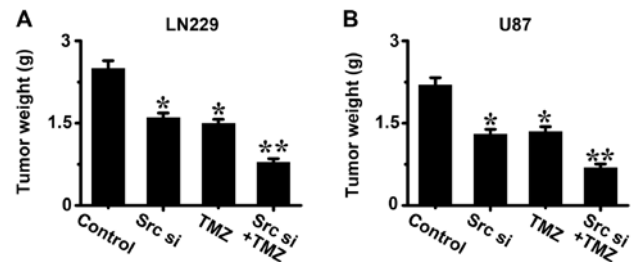

Figure 4. Therapeutic efficacy of Src silencing and TMZ *in vivo*. (A) Tumor weight analysis showed that mice injected with U87 cells and treated with Src siRNA and TMZ presented with tumors with significantly lower weights than the other groups. (B) A similar effect was detected in the mice injected with LN229 cells treated with Src siRNA and TMZ. *P<0.05; **P<0.01.
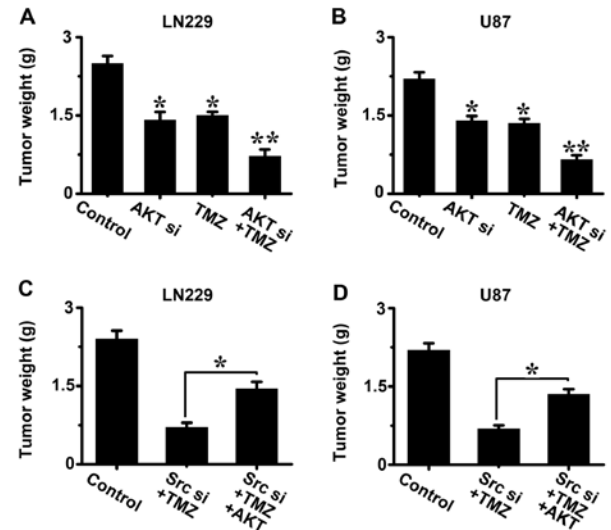

Figure 5. Therapeutic efficacy of AKT silencing or overexpression with TMZ *in vivo*. (A and B) Tumor weight analysis showed that mice injected with U87 and LN229 cells and treated with AKT siRNA and TMZ presented with tumors with significantly lower weights than the other groups. (C and D) Overexpression of AKT could recover the survival of glioma cells after Src silencing with TMZ in the U87 and LN229 *in vivo* model. *P<0.05; **P<0.01.

and western blot assay. Both mRNA and protein expression of Src was significantly reduced both in the U87 and LN229 cells (Fig. 2). As expected, Src silencing resulted in decreased phosphorylation of downstream proteins such as FAK and AKT (Fig. 2B). Next, we examined the effects of Src gene silencing on cell viability. In both cell lines, Src silencing resulted in lower cell viability compared with the untreated and control siRNA groups (P<0.05; Fig. 3A).

Low expression of Src induces apoptosis in glioma cells. To measure the effect of Src expression on tumor cell apoptosis, we transfected U87 and LN229 cells with Src siRNA or a scrambled control. After 48 h of transfection, apoptosis was measured by flow cytometry. Statistically significant increases in Annexin V+ apoptotic cells were observed in the Src siRNA-treated group compared to the untreated or scramble controls (Fig. 3B).

Therapeutic efficacy of Src silencing with TMZ in vivo. Based on prior *in vitro* data, we next assessed the *in vivo* therapeutic efficacy of Src silencing. Seven days after i.p. injection of tumor cells, mice were randomly allocated to 1 of 4 treatment groups. Mice were sacrificed when animals in any group became moribund. As expected, tumor growth was significantly reduced in the control siRNA TMZ group (Fig. 4A). Src siRNA also resulted in significant growth inhibition compared with the control siRNA group (Fig. 4). Combination of Src siRNA plus TMZ resulted in the greatest tumor reduction compared with the control siRNA grop (Fig. 4).

To determine the potential mechanisms responsible for the therapeutic effects of Src silencing and TMZ, we examined the *in vivo* therapeutic efficacy of AKT suppression. Tumor growth was significantly reduced in the AKT siRNA group. Combination of AKT siRNA and TMZ resulted in the greatest tumor reduction compared with the control siRNA (Fig. 5A and B). In addtion, we found that enhanced expression of

AKT could recover the survival of glioma cells after Src silencing and TMZ (Fig. 5C and D). Thus, we concluded that Src enhances the cytotoxic effect of temozolomide by AKT regulation in glioma.

Discussion

A growing body of evidence indicates that Src is an attractive target because it is overexpressed in a number of malignancies, including glioma. However, the mechanism of Src silencing and temozolomide treatment in glioma is not well known. The key findings of our study are that Src silencing enhanced the cytotoxic effect of temozolomide *in vivo*. These effects were achieved, in part, through decreased tumor cell proliferation and increased tumor cell apoptosis that was mediated by decreased FAK and AKT activity.

Accumulating data demonstrate that Src kinases are promising targets for anticancer therapy. The viral homolog of the *Src* gene, *v-Src*, was discovered as an extension of studies to identify a transmissible non-cellular agent that promoted formation of chicken sarcomas (15). The Rous sarcoma virus, which encoded *v-Src* was responsible for malignant transformation (16). Subsequent research found a gene in normal avian DNA closely related to *v-Src* that was named cellular *Src*, which was the first proto-oncogene to be identified (17). In additional, investigators have found elevated Src activity in GBM compared with normal brain samples using both western blotting for phosphorylated Src and Src kinase activity assays (18). The increased SFK activity in GBM samples is largely (>90%) due to elevated Lyn activity (18). In our study, we showed that Src was elevated in GBM samples when compared to that in low-grade glioma samples. Thus, Src activity was confirmed to be increased in GBMs, likely due to increased activation of cell surface growth factor receptors.

Previous experimental evidence indicates that constitutive activation of the P13K/AKT signaling pathway is associated with tumor cell resistance to conventional chemotherapy (19,20). The prominent role of Src in activating downstream signaling through the Ras/MAPK and PI3K pathways in promoting tumor proliferation and invasion makes it an attractive target for chemotherapy in glioblastoma. To our knowledge, our study is the first to investigate whether the blockade of Src increased the cytotoxic effect of temozolomide in glioma. We found that Src silencing using siRNA in combination with temozolomide resulted in significant inhibition of glioma growth. These effects may be mediated by AKT.

Thus, the present study demonstrated that Src plays a biologically significant role in tumor proliferation and apoptosis. Moreover, downregulation of Src combined with temozolomide synergistically inhibited cell proliferation in glioma.

Acknowledgements

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