

# Combination therapy with Gamma Knife radiosurgery and antisense EGFR for malignant glioma *in vitro* and orthotopic xenografts

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**Abstract.** Accumulating evidence has indicated that epidermal growth factor receptor (EGFR) signaling pathway plays a potentially important role in mediating radiation resistance in human gliomas. In this study, we aimed to evaluate the effect of combination therapy with Gamma Knife radiosurgery (GKR) and antisense EGFR for malignant glioma *in vitro* and orthotopic xenografts. GKR and down-regulation of EGFR expression by antisense EGFR resulted in significant suppression of cell proliferation and induction of cell apoptosis *in vitro*. Moreover, the growth of the C6 glioma in orthotopic xenografts was significantly inhibited by this combination treatment. Thus, our results indicate that down-regulation of EGFR expression enhanced the radiosensitivity of glioma and GKR in combination with antisense EGFR is a potential strategy for glioma therapy.

## Introduction

Malignant gliomas, the most common primary brain tumors in adults, are characterized by aggressive and invasive growth. The natural history of untreated glioblastoma multiforme results in a median survival of 3 months (1). Despite advances

in surgery, radiation and chemotherapy, the prognosis for patients with malignant glioma remains poor. Therefore, there is an urgent need to develop new therapeutic strategies for malignant glioma.

Gamma Knife radiosurgery (GKR), developed by Dr Lars Leksell in the late 1960s, has become an indispensable neurosurgical tool that is used for the primary or adjuvant treatment of glioma (2). GKR differs from radiation therapy in that it is not based on variable tissue response to fractionated radiation. Instead, GKR delivers a high dose of radiation in a single session to a stereotactically defined target by converging multiple beams of ionizing radiation. By creating a rapid radiation dose fall-off around the target, radiation can be targeted to the lesion itself, and damage to surrounding structures can be minimized. Previous studies have demonstrated that radioresistance of glioma is associated with overexpression of EGFR or EGFRvIII and radiation-induced release of TGF $\alpha$  activates the EGFR and MAPK pathway, leading to increased proliferation and protection from radiation-induced cell death (3-5). The amplification, overexpression or mutation of EGFR is the early and major molecular event in many human tumors. Our previous and other studies have also indicated that EGFR was overexpressed in malignant glioma (6,7). Targeting EGFR through antisense RNA, RNA interference and specific tyrosine kinase inhibitors could inhibit significantly cell proliferation of malignant glioma *in vitro* and *in vivo* (8-11).

In the current study, we explored the effect of combination therapy with GKR and antisense EGFR on glioma growth. We used rat C6 and human TJ905 glioma cells following combined GKR and antisense EGFR treatment *in vitro*. A tumor intracerebral model of C6 cells for glioma was also employed to investigate the treatment effect *in vivo*. Our data showed that using this approach could inhibit glioma growth *in vitro* and orthotopic xenografts.

## Materials and methods

**Cell line and plasmid.** Rat C6 glioma cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Human TJ905 glioblastoma cells were

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established and characterized in the Neuro-oncology laboratory, Tianjin Neurological Institute (12). C6 cells and TJ905 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO<sub>2</sub>. The EGFR cDNA in plasmid pactQsR3 was kindly provided by Dr Laura Beguinot, Italy (13). The antisense orientation of EGFR cDNA fragment (552 bp), complementary to the 3'-coding region of EGFR mRNA, was double digested by *Xba*I/*Xho*I and subcloned into pcDNA3, which was named as panti-EGFR.

**Antisense-EGFR transduction.** For gene transfection, 2x10<sup>5</sup> cells per well were planted into six-well plates and grown overnight until they were 50-80% confluent. Plasmids panti-EGFR (2 µg each) were transfected into C6 and TJ905 cells with lipofectamine according to the protocol. Stably resistant cells were selected using G418 as previously described (10). The panti-EGFR transfected clones were selected and expanded as cell pools for further studies.

**Western blot analysis.** Total protein lysates (40 µg/sample), were separated by SDS-PAGE. The separate proteins were transferred to PVDF membranes (Millipore, USA). The blot was incubated with primary antibody against EGFR (1:500 dilution, Santa Cruz Biotechnology), followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution). The specific protein was detected using a Super signal protein detection kit (Pierce, USA). After washing with stripping buffer, the PVDF membrane was reprobed with antibody against β-actin (1:500 dilution, Santa Cruz Biotechnology).

**GKR for glioma cells in vitro.** The transfected or control cells (4x10<sup>7</sup>) were planted and grown overnight in a 10-cm Petri dish. Cells in the log phase of growth were harvested, put into Eppendorf tube (4x10<sup>6</sup>/ml), centrifuged (500 x g, 2 min), and then fixed to the Leksell G stereotactic frame for GKR. High-resolution MR images were obtained. Leksell Gamma Plan software (versions 5.31) was used to attain target localization for the radiosurgery (Fig. 1). A margin dose of 15 Gy was delivered to the 75% isodose line using a 4-mm collimator of the 201-source <sup>60</sup>Co gamma unit. Radiosurgery was performed in the Leksell gamma knife unit. After radiosurgery, cells were cultured quickly in DMEM supplemented with 10% fetal calf serum. Cell proliferation and apoptosis were detected 48 h post-radiosurgery by MTT, immunohistochemical and TUNEL assay, respectively.

**MTT assay.** Cells growth inhibition was evaluated using the MTT assay. Briefly, cells (4x10<sup>3</sup> per well) were plated in 96-well plates and grown overnight. Each day of the consecutive 6 days, 20 µl of MTT (5 mg/ml, Sigma) was added to each well, and the cells were incubated at 37°C for an additional 4 h. The reaction was stopped by lysing the cells with 200 µl of DMSO for 5 min. Quantitation was obtained at wavelength of 570 nm and expressed as a percentage of control. The data are presented as the mean ± SD, derived from triplicate samples of at least three independent experiments.

**Antitumor activity of GKR and antisense EGFR in vivo.** Adult male Sprague-Dawley rats weighing between 150 and 200 g each were purchased from the animal center of the Cancer

Institute of Chinese Academy of Medical Sciences. The guidelines for animal welfare were approved by the Ethics Committee on Animal Research of Tianjin Medical University. Sixty-four rats were anesthetized by an intra-peritoneal injection of 10% chloral hydrate (300 mg/kg), and then placed in a stereotactic apparatus. C6 cells (1x10<sup>6</sup>) in 10 µl serum-free DMEM were injected into the right frontal lobe of rat using a guide-screw system implanted within the skull as described previously (14). As the tumor formation demonstrated in MRI on day 6 post-implantation, all rats were divided randomly into four groups: control group, As-EGFR group, GKR group and combined group.

**Control group:** 10 µl PBS was injected into the tumor site by using the same coordinates with stereotactic guidance on day 6 and 8 post-implantation, respectively. **As-EGFR group:** 2 µg of plasmids panti-EGFR mixed with 10 µl lipofectamine were injected into the tumor site by using the same coordinates with stereotactic guidance on day 6 and 8 post-implantation, respectively. **GKR group:** Radiosurgery was performed in the Leksell Gamma Knife unit on day 10 post-implantation. High-resolution MR images were obtained. Leksell Gamma Plan software was used to attain target localization for the radiosurgery (Fig. 1). A margin dose of 15 Gy was delivered to the 50% isodose line using a 4-mm collimator of the 201-source <sup>60</sup>Co gamma unit.

**Combined group:** 2 µg of plasmids panti-EGFR mixed with 10 µl lipofectamine were injected into the tumor site on day 6 and 8 post-implantation, and radiosurgery was performed with a margin dose of 15 Gy on day 10 post-implantation. Two days after GKR, six rats of each group were euthanized, and brains were removed for proliferation (PCNA) and apoptosis (TUNEL) examination. The other ten rats were observed for the survival period and MR images until 8 weeks post-radiosurgery. After 8 weeks, all the rats were sacrificed, and then we performed a survival period analysis.

**Immunohistochemical analysis.** Cell proliferation was evaluated by PCNA immunostaining using ABC-peroxidase method. Briefly, the sections were incubated with primary PCNA antibody (1:100 dilution, Santa Cruz Biotechnology) overnight at 4°C, then incubated with a biotinylated secondary antibody (1:200 dilution) at room temperature for 1 h, followed by the incubation with ABC-peroxidase reagent (1:200 dilution, Vector, USA) for an additional 1 h. After washing with Tris-buffer, the sections were stained with DAB (3,3 diaminobenzidine, 30 mg dissolved in 100 ml Tris-buffer containing 0.03% H<sub>2</sub>O<sub>2</sub>) for 5 min, rinsed in water and counterstained with hematoxylin. The percentage of the positive staining cells in a total number of 500-1000 cells was determined under x400 magnification by a light microscope. Apoptosis was detected by TUNEL method using *in situ* cell death kit (Boehringer Mannheim, Germany) according to the supplier's instructions. Apoptotic Index (AI) was calculated as the number of apoptotic cells per 500-1000 total cells counted under the x400 magnification by a light microscope.

**Statistical analysis.** The data obtained in this study were analyzed by one way ANOVA test and t-test using SPSS 11.0 software. Survival was analyzed by a log-rank test based on the Kaplan-Meier test. P<0.05 was considered as significant.

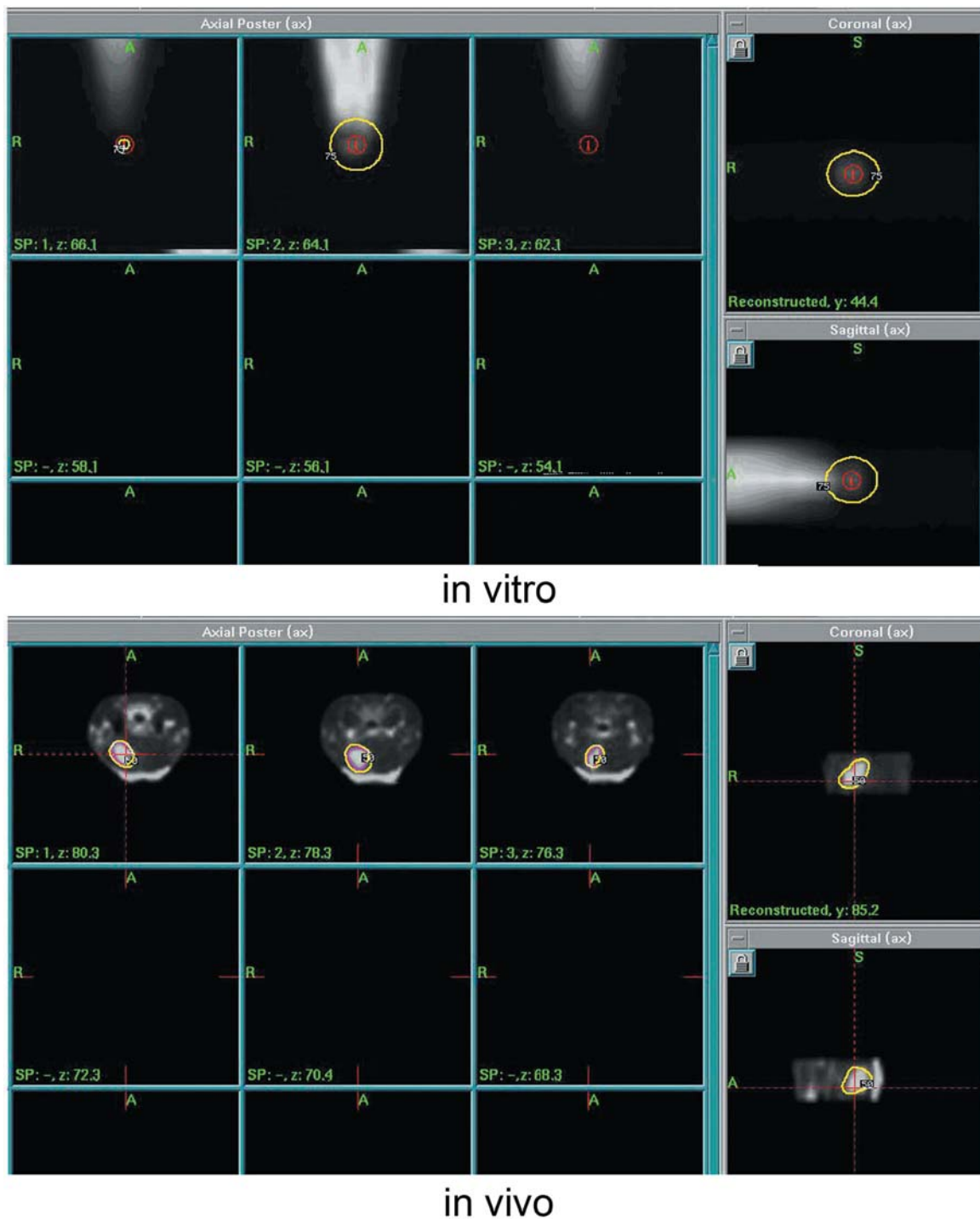


Figure 1. Leksell Gamma Plan (version 5.31) was used to design a conformal treatment plan *in vitro* and *in vivo*.

## Results

**Down-regulation of EGFR expression by antisense EGFR.** In order to efficiently and stably knock down EGFR expression in glioma cells, antisense EGFR expression plasmids (panti-EGFR) were transfected into C6 and TJ905 cells. After selection for 2 weeks, G418 resistant cell pools were obtained and cell lysates were analyzed by Western blot analysis. As shown in Fig. 2, antisense EGFR triggered a marked decrease in the protein levels for the target proteins both in C6 and TJ905 cells, with minimal effect on the expression of  $\beta$ -actin.

These results indicated that antisense EGFR potently and specifically down-regulated EGFR protein expression in glioma cells.

**Effects of GKR and antisense EGFR on glioma cell proliferation and apoptosis in vitro.** To evaluate the impact of GKR and antisense EGFR on glioma cell proliferation *in vitro*, MTT assay was measured. As shown in Fig. 3A, statistically significant cell proliferation inhibition of C6 and TJ905 cells was found in As-EGFR, GKR and combined groups compared with control group for 2 days after GKR. The growth



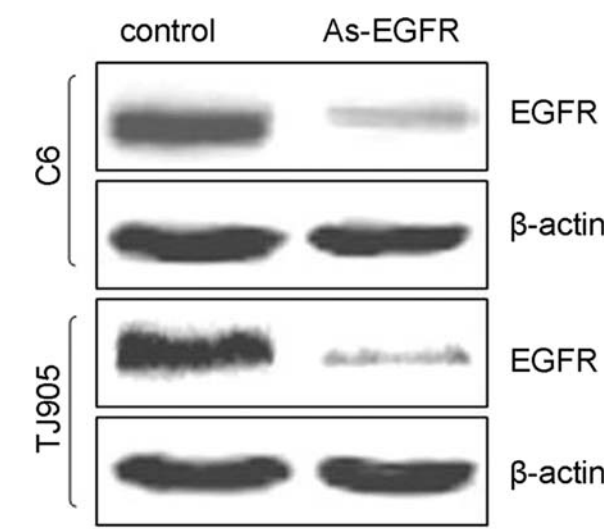


Figure 2. Antisense EGFR down-regulates EGFR expression in glioma cells. EGFR expression in C6 and TJ905 cells transfected with panti-EGFR was determined by Western blot analysis. The quantitative data show relative protein levels of EGFR (normalized to  $\beta$ -actin level).

rate of cells in combined group was the lowest among all four groups. There was no difference between the As-EGFR and GKR group in the experiments. Additionally, using PCNA as a biomarker for cell proliferation, we examined the effect of GKR and antisense EGFR on glioma cell proliferation *in vitro* by observing PCNA immunohistochemical staining. The PCNA positive rate was  $86.78 \pm 4.41$ ,  $59.35 \pm 3.79$ ,  $50.71 \pm 2.61$  and  $29.01 \pm 1.87\%$ , respectively for control, As-EGFR, GKR and combined group ( $P < 0.01$ ). Similar trend of cell proliferation inhibition was also detected in TJ905 cells (Fig. 3B). These results suggest that GKR combined with antisense EGFR may more effectively inhibit glioma cell proliferation than treatment with either of them.

Next, we determined the biological function of GKR and antisense EGFR on cell apoptosis of glioma cells. TUNEL staining showed that average AI of C6 cells was  $0.60 \pm 0.26$ ,  $12.73 \pm 0.48$ ,  $18.39 \pm 1.06$  and  $28.38 \pm 1.20\%$ , respectively for control, As-EGFR, GKR, and combined group ( $P < 0.01$ ). The apoptosis rate of TJ905 cells was also induced significantly following treatment with GKR and antisense EGFR (Fig. 3C).

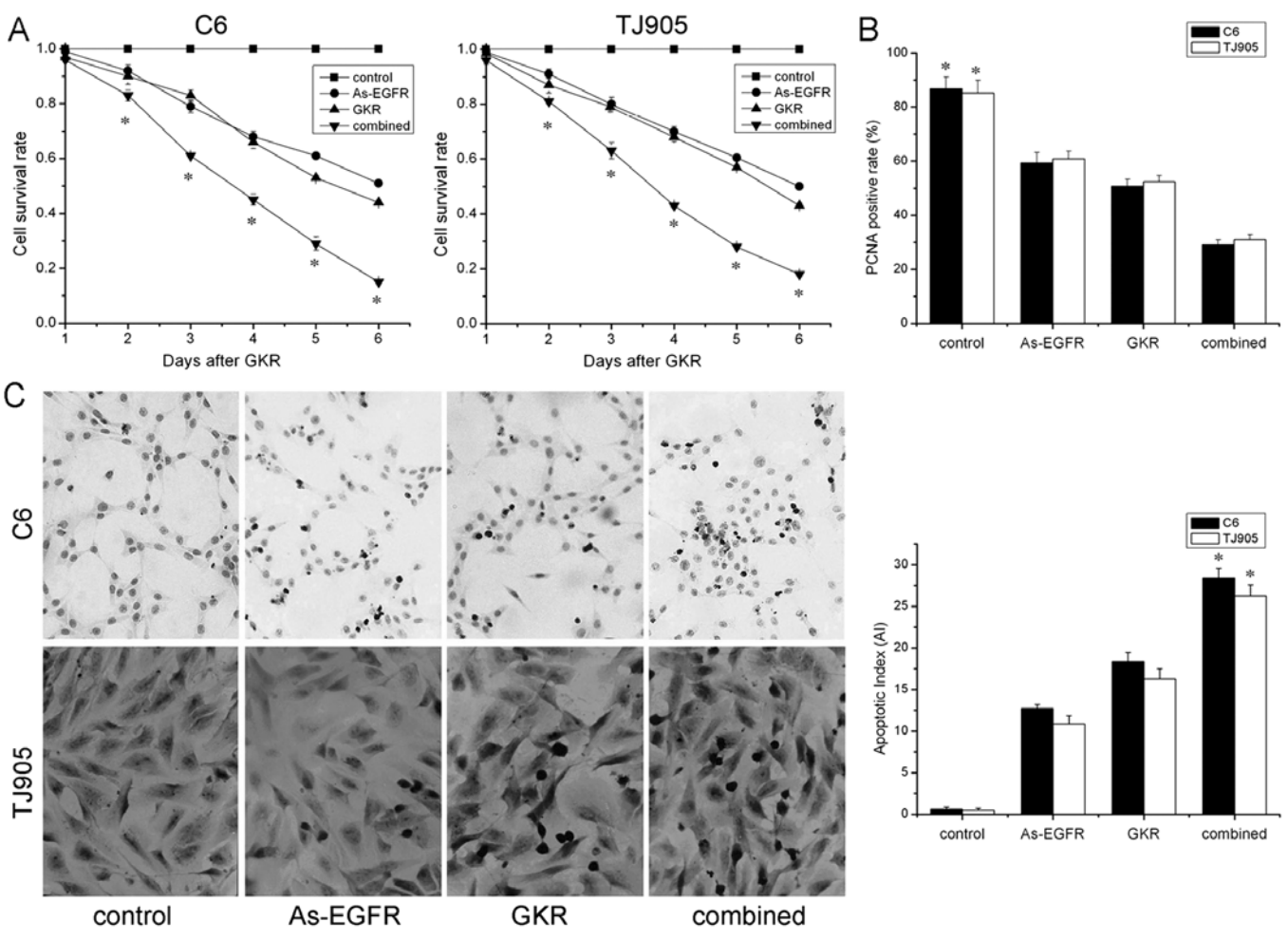


Figure 3. GKR and antisense EGFR inhibit glioma cell proliferation and apoptosis. (A) C6 and TJ905 cells in As-EGFR, GKR and combined groups proliferate at a significantly lower rate than control cells measured by MTT assay. The survival rate of combined group has the most robust cell proliferation inhibition. (B) Similar trend of cell proliferation inhibition is observed by PCNA immunohistochemical staining. The data are calculated and presented as the mean  $\pm$  SD. (C) TUNEL staining shows that combination therapy with GKR and antisense EGFR induce significant apoptosis. Apoptotic Index (AI) is calculated and presented as the mean  $\pm$  SD. \* $P < 0.01$ .

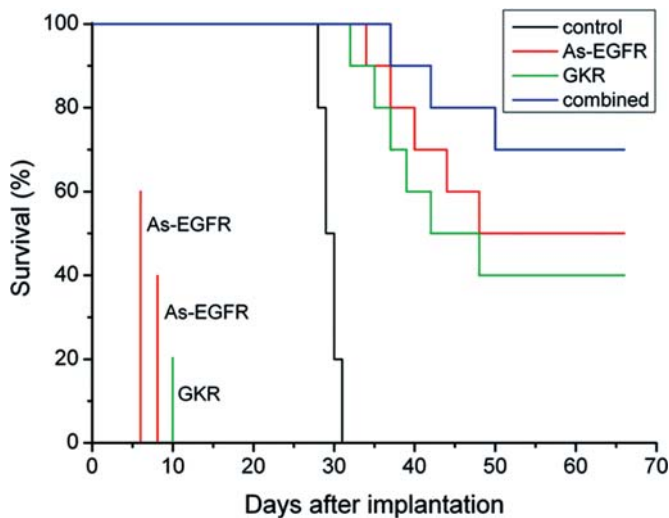


Figure 4. Graph displaying Kaplan-Meier curves for rats implanted with C6 glioma that underwent radiosurgery using 15 Gy to the tumour margin and antisense EGFR therapy. Rats of combined group have a longer survival time compared to the control group, As-EGFR group and GKR group ( $P < 0.01$ ).

The data indicated that combination treatment with GKR and antisense EGFR had the most robust effect on cell apoptosis.

*GKR and antisense EGFR prolong the survival period in vivo.* Our *in vitro* experiments demonstrated that GKR and antisense EGFR can efficiently inhibit cell proliferation and

induce apoptosis of glioma cells. Therefore, we further investigated the antitumor effect of such approaches *in vivo* using a C6 intracerebral tumors model. Intracerebral gliomas were well-established on day 6 post-implantation, plasmids panti-EGFR were injected into the tumor site on day 6 and 8 post-implantation, and GKR was performed on day 10 post-implantation. All surviving rats underwent MRI scan to allow for observation of tumor changes at 1, 2 and 8 weeks after GKR. Figs. 4 and 5 revealed that all the rats in control group died of tumor progression, and the median survival time of these rats was 17.5 days. Five rats in As-EGFR group died on day 22, 25, 28, 32 and 36, respectively, and the other 5 rats survived over 66 days after implantation (the end of observation period). In GKR group, 4 rats had prolonged survival of more than 66 days after implantation. In combined group, 7 rats were alive at the end of observation period, with no visible brain tumors by MRI scan at 8 weeks after GKR. The other 3 animals died on day 25, 30 and 38, respectively. The results suggested that rats of the combined group had a longer survival time compared to the control group, As-EGFR group and GKR group.

Two days after GKR, the tumor samples were taken out and paraffin-embedded section were prepared for proliferation (PCNA) and apoptosis (TUNEL) examination. Representative sections in the control group, As-EGFR group, GKR group and combined group are shown in Fig. 6. The expression of PCNA was greatly reduced in tumor tissues derived from the combined group, compared with that in control, As-EGFR and GKR groups ( $P < 0.01$ ). TUNEL staining showed that there were nearly no apoptotic cells found in control group. However, apoptosis was prominently increased

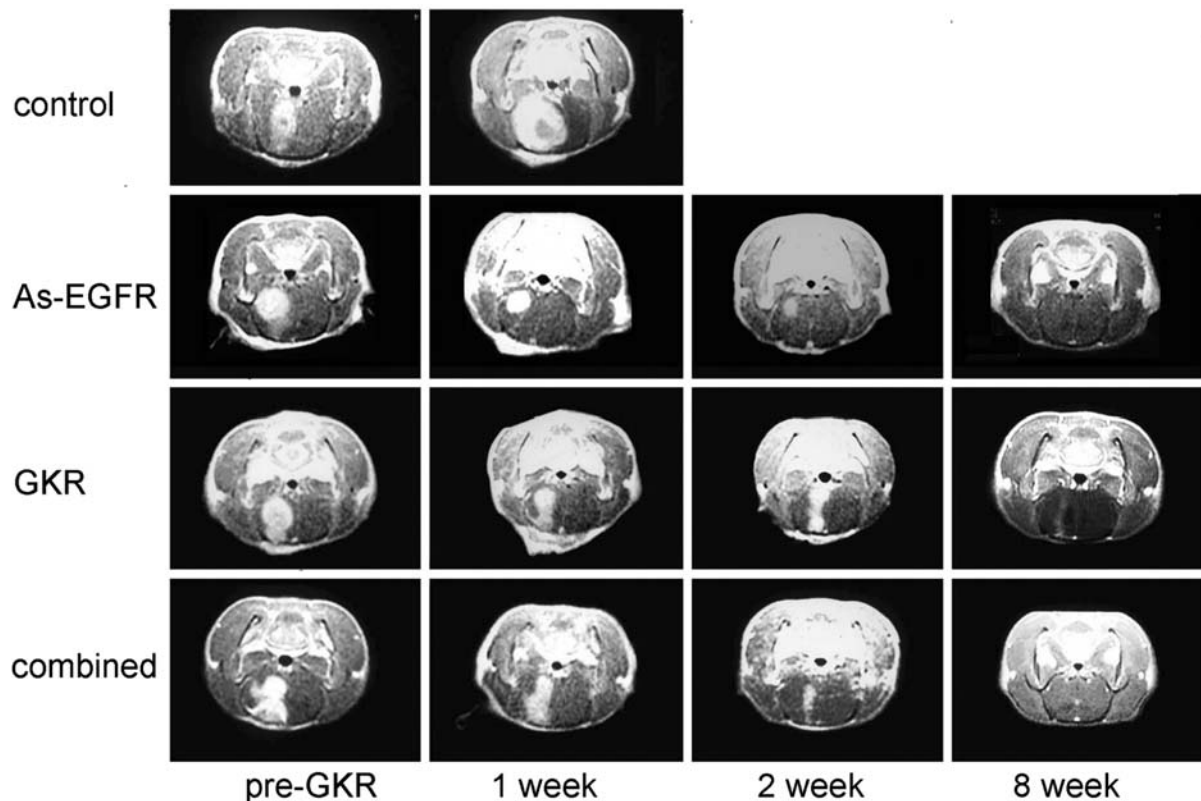


Figure 5. Dynamic MRI of glioma in orthotopic xenografts. The tumor formation in orthotopic xenografts demonstrated in MRI on day 6 post-implantation. After GKR and antisense EGFR treatment, MRI was observed until 8 weeks post-radiosurgery.



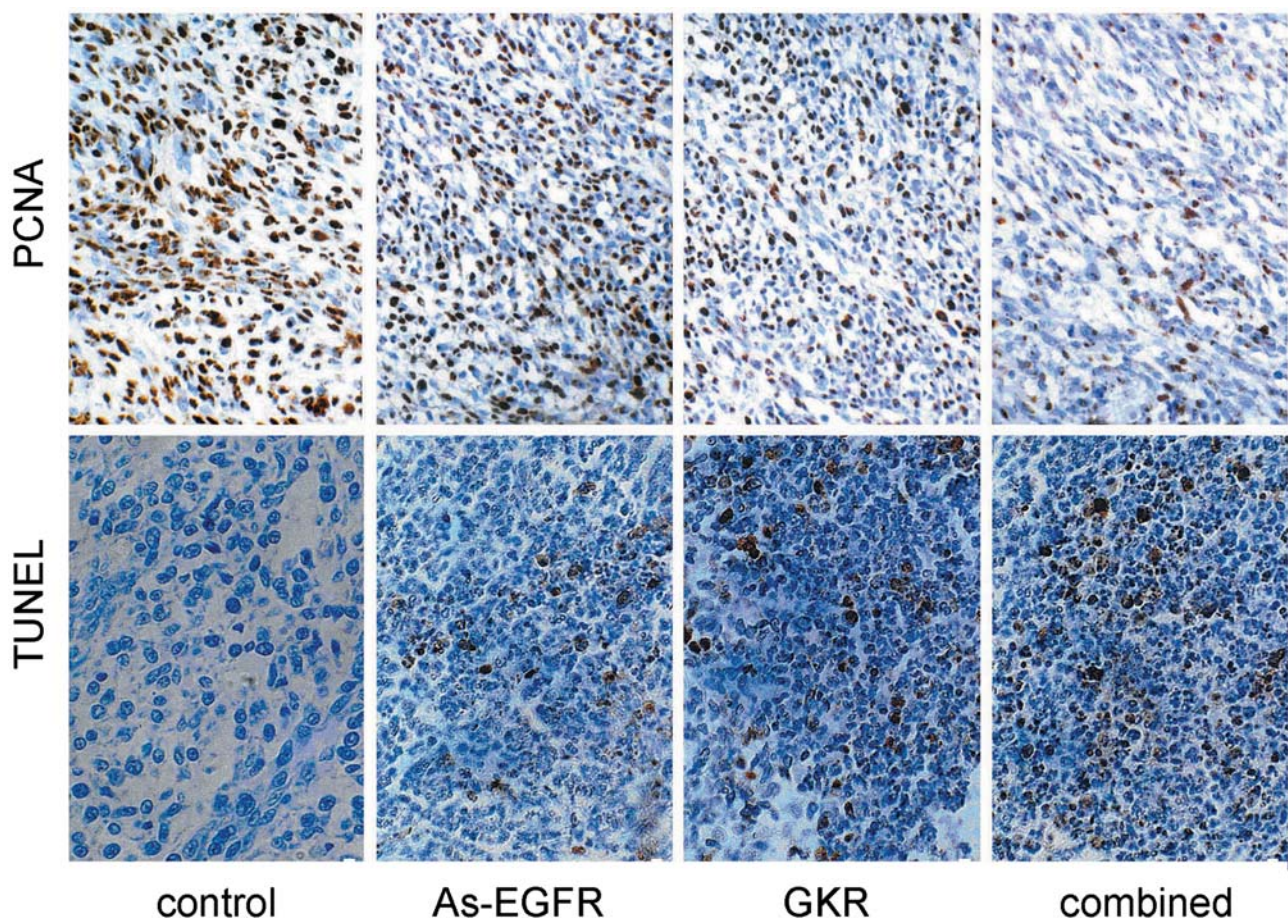


Figure 6. Immunohistochemistry analysis of tumors after treatment. Cell proliferation and apoptosis were examined by PCNA and TUNEL assay in rats bearing C6 glioma, respectively.

in the As-EGFR and GKR groups, but mostly aggregated in combined group ( $P < 0.01$ ).

## Discussion

GKR is an effective method to a selected group of patients with small to medium sized, well defined, histologically proven low grade cerebral gliomas. GKR is a minimally invasive technique designed to produce a destructive radiobiological response within an imaging-defined target volume in a single session. It is capable of delivering a high dose of radiation to the tumor site with a sharp dose fall off to normal surrounding tissue. It has been demonstrated that GKR may prolong the survival period of tumor-harboring animals by inducing apoptosis and inhibiting proliferation and the cell cycle (15). The results of GKR against the 9L glioma *in vivo* revealed that apoptosis was initially induced following radiosurgery at ~6 h, peaked ~48 h, and returned to baseline levels by 72 h.

In our study, TUNEL staining showed that apoptosis was significantly increased 48 h after GKR. Although GKR can effectively kill the malignant glioma cells in the target area and prevent tumor growth, it can not control the growth of the tumor cells outside the target area or prevent the recurrence of the tumor (16). Tumor cells infiltrating into the surrounding normal brain tissue, cannot be targeted by radiosurgery for fear of causing additional neurological

damage. Thus, combination therapy is the hot spot in the radiotherapy research. Our previous study found that GKR in combination with adenoviral p53 can improve antiglioma efficacy (14).

The EGFR pathway is frequently up-regulated in high-grade gliomas via gene amplification and specific mutations. Dysregulation of EGFR-mediated signaling pathways contribute to tumor development and progression. EGFR and its ligands, activate intracellular signaling pathways, including RAS/MAPK, PI3K/AKT, and STAT-3 signal transduction pathways. The stimulation of these downstream signal pathways contribute to promote the malignant phenotype of cells, such as proliferation, anti-apoptosis, invasion, metastasis and angiogenesis (6,17,19). In LN229 and U87 glioma cells, blockade of EGFR inhibits the proliferation through induction of G1 arrest, and this process correlates with inhibition of PI3K/Akt/mTor signaling pathway (19).

Extensive evidence indicates that EGFR signaling plays a critical role in mediating radiation resistance in human gliomas. EGFR overexpression correlates with relative resistance to radiation therapy in malignant glioma (3). Moreover, several studies have demonstrated that ionizing radiation exposure induces EGFR autophosphorylation and increases EGFR expression, and this radiation-inducible activation of EGFR may contribute to radiation resistance of tumors including cell cycle alteration, decreased apoptosis,

and enhanced proliferation and DNA damage repair (5,20). The combination EGFR blockage with radiation therapy has been evaluated by multiple laboratories. Inhibition of EGFR signaling in combination with radiotherapy has been studied with small molecule inhibitors that target the kinase domain (21,22), and through gene therapy using an EGFR deletion mutant (EGFR-CD533) (23). Although each strategy has a different mechanism of action, all have shown promise in preclinical studies. However, studies on combination therapy with GKR and down-regulation of EGFR expression for glioma have not been reported.

In the present study, we demonstrated that C6 and TJ905 cells of GKR in combination with antisense EGFR showed much more decreased proliferation activity than that detected by MTT and PCNA assay *in vitro* of either of them. Simultaneously, the cell apoptosis increased following the combined group as examined by TUNEL method. Furthermore, the survival time of rats in the combined treatment were more prolonged in the orthotopic xenografts glioma model, consistent with dynamic MRI of glioma in orthotopic xenografts. Therefore, disruption of EGFR signaling could increase the sensitivity of glioma cells to death by GKR.

Collectively, the combined GKR and antisense EGFR treatment were more effective than treatment with either of them *in vivo* and in orthotopic xenografts. Our data support the potential for down-regulation of EGFR expression to enhance the radiosensitivity of glioma. However, the further mechanism is not clear and still under investigation. The findings of this study suggest that GKR in combination with EGFR-based therapy may be superior to single modality treatment of malignant glioma and provides an effective approach for glioma therapy.

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