

EGFRvIII and DNA Double-Strand Break Repair: A Molecular Mechanism for Radioresistance in Glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is the most lethal of brain tumors and is highly resistant to ionizing radiation (IR) and chemotherapy. Here, we report on a molecular mechanism by which a key glioma-specific mutation, epidermal growth factor receptor variant III (EGFRvIII), confers radiation resistance. Using Ink4a/Arf-deficient primary mouse astrocytes, primary astrocytes immortalized by p53/Rb suppression, as well as human U87 glioma cells, we show that EGFRvIII expression enhances clonogenic survival following IR. This enhanced radioresistance is due to accelerated repair of DNA double-strand breaks (DSB), the most lethal lesion inflicted by IR. The EGFR inhibitor gefitinib (Iressa) and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 attenuate the rate of DSB repair. Importantly, expression of constitutively active, myristylated Akt-1 accelerates repair, implicating the PI3K/Akt-1 pathway in radioresistance. Most notably, EGFRvIII-expressing U87 glioma cells show elevated activation of a key DSB repair enzyme, DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Enhanced radioresistance is abrogated by the DNA-PKcs-specific inhibitor NU7026, and EGFRvIII fails to confer radioresistance in DNA-PKcs-deficient cells. *In vivo*, orthotopic U87-EGFRvIII-derived tumors display faster rates of DSB repair following whole-brain radiotherapy compared with U87-derived tumors. Consequently, EGFRvIII-expressing tumors are radioresistant and continue to grow following whole-brain radiotherapy with little effect on overall survival. These *in vitro* and *in vivo* data support our hypothesis that EGFRvIII expression promotes DNA-PKcs activation and DSB repair, perhaps as a consequence of hyperactivated PI3K/Akt-1 signaling. Taken together, our results raise the possibility that EGFR and/or DNA-PKcs inhibition concurrent with radiation may be an effective therapeutic strategy for radiosensitizing high-grade gliomas. [Cancer Res 2009;69(10):4252–9]

Introduction

Glioblastoma multiforme (GBM) is the most aggressive form of brain tumors with extremely poor prognosis (1). Effective treatment of GBM is complicated by the diffuse infiltrative nature of the disease and extreme radioresistance of these tumors. However, a clear survival advantage of post-resection radiation has been established by randomized trials, showing that the median survival of GBM patients was improved, from ~6 months to 10 to 12 months, following near-maximal brain-tolerated doses of ionizing radiation (IR; 50–60 Gy). The central role that radiation plays in treating GBM is also illustrated by the landmark Stupp study (2) showing that concurrent radiation and temozolomide, an alkylating agent, can further increase median survival from 12.1 to 14.6 months. It is clear that an improved molecular understanding of GBM radioresistance is needed for the development of rational, tumor-selective radiosensitizing drugs.

As a first step toward understanding the genetic basis of GBM radioresistance, we focused on epidermal growth factor receptor variant III (*EGFRvIII*), the most commonly amplified/mutated gene in GBMs (1, 3, 4). Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase (RTK) whose ligands include epidermal growth factor and transforming growth factor α (5, 6). The *EGFR* gene is amplified in ~50% of GBMs, and of these, about half express a truncated version of the receptor, EGFRvIII (1, 3, 4). Although EGFRvIII lacks the ligand-binding domain (7), it is constitutively active (8), stimulating downstream signaling effectors that include phosphatidylinositol 3-kinase (PI3K), Akt-1, Ras, and mitogen-activated protein kinase (MAPK). Several studies have shown that EGFRvIII promotes malignant growth (9) and is associated with poor prognosis (10, 11).

Previous *in vitro* studies using established glioma cell lines have shown that EGFRvIII confers resistance to IR (12–14). Xenograft studies have shown that EGFR-specific inhibitors (small-molecule as well as α -EGFR antibodies) significantly enhance the efficacy of radiotherapy (15, 16). However, the signaling pathways directly involved in EGFRvIII-mediated radiation resistance have not been completely elucidated. We report here that EGFRvIII expression enhances radioresistance in tumor suppressor-deficient primary mouse astrocytes and in U87 human glioma cell lines by promoting the rapid repair of radiation-induced DNA double-strand breaks (DSB). Proficient DSB repair is facilitated by hyperactivation of the DSB repair enzyme, DNA-dependent protein kinase catalytic subunit (DNA-PKcs; refs. 17, 18). We extend these *in vitro* mechanistic studies to an orthotopic model and show that, following whole-brain radiotherapy, intracranial U87-EGFRvIII tumors show proficient DSB repair compared with U87 parental

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tumors. The possible clinical effect of hyperactivating such DSB repair pathways due to EGFRvIII expression was assessed by Kaplan-Meier survival analysis. Nude mice bearing U87-EGFRvIII intracranial tumors receiving whole-brain radiotherapy showed no evidence of improved survival, whereas mice bearing U87 parental tumors showed a remarkable increase in survival following radiation. Taken together, our results suggest that DNA-PKcs inhibitors and/or EGFR inhibitors administered concurrently with radiation may be an effective therapeutic strategy for radiosensitizing these recalcitrant tumors.

Materials and Methods

Isolation of primary astrocytes. Primary astrocytes were isolated from 5-d-old wild-type or *Ink4a/Arf*^{-/-} pups as described previously (19). Primary wild-type astrocytes were immortalized by retroviral expression of the SV40-large T antigen (SV40-LT). Plasmid construction, virus production, and infection protocols have previously been described in detail (20). Transfection of astrocytes with retroviruses expressing mutant, constitutively active EGFR (EGFRvIII), wild-type EGFR (EGFRwt), or kinase-dead EGFR (EGFRkd) was carried out as described (19).

Cell culture. Mouse astrocytes, mouse embryonic fibroblasts (MEF), and human U87 glioma lines were all maintained in α -MEM containing 10% fetal bovine serum (FBS) in a humidified 37°C incubator in the presence of 5% CO₂.

Drug treatments. For drug treatments, DNA-PKcs inhibitor [10 μ mol/L NU7026 (Calbiochem)], EGFR inhibitor [5 μ mol/L gefitinib (Iressa; AstraZeneca Co.)], or PI3K inhibitor [50 μ mol/L LY294002 (Sigma)] was added to cells (*Ink4a/Arf*^{-/-} cohort, SV40-LT cohort, or U87 cohort) 1 h before irradiation. Control cells were treated with DMSO.

Irradiation of cells and animals. For γ -ray irradiation of cells (*Ink4a/Arf*^{-/-} cohort, SV40-LT cohort, U87 cohort, or MEFs), a ¹³⁷Cs source (JL Shepherd and Associates) was used. Midbrains of mice were irradiated with an X-ray device (Pantak, 300 kV, 12 mA, 1.65 mm Al) fitted with a specifically designed collimator providing a 1-cm-diameter field size iso-dose exposure.

Colony formation assays. Three hundred cells (*Ink4a/Arf*^{-/-} cohort, SV40-LT cohort, U87 cohort, or MEFs) were plated in triplicate under reduced serum (0.5% FBS) conditions. Cells were irradiated at different doses (0–8 Gy) after allowing recovery for 4 h. Medium containing 10% serum was added back 24 h later. Colonies were allowed to form for ~8 d. Cells were then fixed and stained with crystal violet. Colonies with >50 cells were scored, and mean values for triplicate counts determined as described (21).

DSB repair assay. DSB repair rates were assessed by quantifying the rates of dissolution of 53BP1 foci as described (22). Approximately 4 \times 10⁴ cells (*Ink4a/Arf*^{-/-} cohort, SV40-LT cohort, or U87 cohort) were seeded overnight in glass chamber slides under reduced serum (0.5% FBS) conditions. The following day, cells were irradiated with a total dose of 1 Gy, fixed at the indicated times, and immunostained with α -53BP1 primary antibody (Cell Signaling) and FITC-conjugated goat anti-rabbit secondary antibody (Molecular Probes) as described (22). The number of 53BP1 foci was determined for each time (average of 100 nuclei), and after subtracting background (number of foci in unirradiated population), the percentage foci remaining was plotted against time to obtain DSB repair kinetics.

Western blotting. To detect EGFR, EGFRvIII, Akt-1, and phosphoAkt-1, whole-cell extracts were prepared from cells (*Ink4a/Arf*^{-/-} cohort, SV40-LT cohort, U87 cohort, or MEFs) grown overnight in medium containing reduced serum (0.5%) as described (19), separated by 8% PAGE, blotted onto nitrocellulose, and probed with α -EGFR (Santa Cruz), α -phosphoAkt-1(Ser473; Cell Signaling), and α -Akt-1 (Cell Signaling). For detection of DNA-PKcs and phospho-DNA-PKcs (in mock-irradiated or γ -irradiated U87 cells), nuclear extracts were prepared as described (23), separated by 8% PAGE (low BIS), blotted onto nitrocellulose, and probed with α -DNA-PKcs (Neomarkers), α -phosphoDNA-PKcs(T2647) (kind gift from Dr. Benjamin Chen, Department of Radiation Oncology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX), and α -actin (Sigma) antibodies.

Stereotactic injection of cell lines. Orthotopic tumors were generated as described (9, 19). U87 parental or U87-EGFRvIII cells were infected with a puromycin-selectable retrovirus expressing luciferase. Equivalent levels of luciferase expression in the two cell lines were verified by Western blotting with an α -luciferase antibody (Sigma). For intracerebral stereotactic inoculation, 5 \times 10⁵ cells were suspended in PBS (5 μ L) and injected into the right corpus striatum of the brains of 4- to 5-wk-old Nu/Nu mice (Charles River). Tumors were allowed to develop and monitored by luciferase bioluminescence imaging. All animal studies were done under protocols approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

Noninvasive intracranial bioluminescence imaging. Serial bioluminescence images of tumor-bearing mice were obtained using the IVIS Lumina System (Xenogen Corp.) coupled to Living Image data acquisition software (Xenogen). During imaging, mice were anesthetized with isoflurane (Baxter International, Inc.) and a solution of D-luciferin (450 mg/kg in PBS; total volume, 250 μ L; Biosynthesis) was administered s.c. in the neck region. Images were acquired between 10 and 20 min after

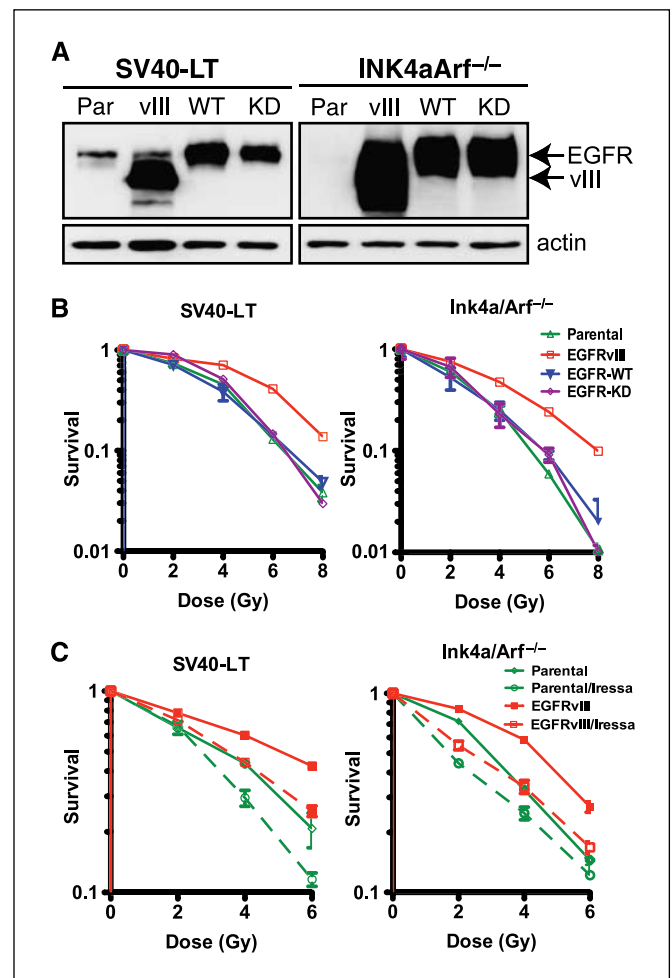


Figure 1. EGFRvIII expression renders astrocytes radioresistant irrespective of tumor-suppressor background. *A*, expression of EGFRvIII (*vIII*), EGFRwt (*WT*), or EGFRkd (*KD*) in SV40-LT-expressing mouse astrocytes or in *Ink4a/Arf*^{-/-} primary mouse astrocytes was assayed by Western blotting with α -EGFR and α -actin (loading control) antibodies. *Par*, parental cells. *B*, radiation survival of astrocyte lines was quantified by colony formation assays. The fraction of surviving colonies (*y* axis) was plotted against corresponding radiation dose (*x* axis). Bars, SE of experiments done three or more times. Please note increased survival after EGFRvIII expression (*red lines*). *C*, radiation survival of astrocytes pretreated with gefitinib (Iressa) was quantified as above. Note reduced survival after Iressa pretreatment (*dashed lines*).

luciferin administration and peak luminescence signals were recorded. The bioluminescence image signals emanating from the tumors were quantified by measuring photon flux within a region of interest using the Living Image software package.

Brain sectioning and immunohistochemistry. For pathologic analyses and immunohistochemistry, brains were fixed in 10% formaldehyde and processed for H&E staining by standard techniques. Entire brains were sectioned in 1- to 2-mm coronal blocks and submitted in one cassette for paraffin embedding and sectioning. Sections (10 μ m) were treated with xylene and washed with ethanol. Antigen retrieval was done by sodium citrate (10 mmol/L, 20 min) treatment. Sections were then permeabilized in Triton X-100 and blocked with 5% goat serum. After incubation with α -53BP1 antibody (Cell Signaling; at 4°C, overnight), cells were treated (at room temperature, 2 h) with FITC-conjugated goat anti-rabbit antibody (Molecular Probes). Sections were washed and mounted in Vectashield containing 4',6-diamidino-2-phenylindole (Vector Labs).

Results

EGFRvIII, in cooperation with glioma-relevant tumor suppressor loss, confers IR resistance to primary murine astrocytes. We examined the contribution of EGFRvIII to radioresistance in primary astrocytes, the presumptive cells of origin of GBMs (19). For analyses of IR resistance, we used either Ink4a/Arf-null primary mouse astrocytes (henceforth referred to as Ink4a/

Arf^{-/-} astrocytes) or astrocytes in which p53/Rb tumor suppressors were inactivated by retroviral expression of SV40-LT (henceforth referred to as SV40-LT astrocytes). Ink4a/Arf (p16/p19) and p53/Rb tumor suppressors are frequently lost in GBM (1, 3, 4). Therefore, these two cell lines, with two distinct, GBM-relevant tumor suppressor backgrounds, are most appropriate for this study. To assess the effect of EGFRvIII on IR resistance, we compared EGFRvIII to equivalently expressed levels of EGFRwt or EGFRkd (Fig. 1A). Resistance to IR was quantified by standard colony formation assays. Expression of EGFRvIII, but not EGFRwt or EGFRkd, resulted in dramatically increased survival of both Ink4a/Arf^{-/-} astrocytes and SV40-LT astrocytes (Fig. 1B). Radioresistance conferred by EGFRvIII was abrogated by the small-molecule inhibitor gefitinib (Iressa), which binds to the ATP-binding pocket of EGFR (ref. 6; Fig. 1C). These data suggest that EGFRvIII signaling is involved in the increased resistance to IR irrespective of the tumor suppressor background.

EGFRvIII-induced IR resistance correlates with proficient DSB repair in primary murine astrocytes. The major mechanism by which IR induces lethality is by introducing DSBs. We examined whether the increased resistance to IR conferred by EGFRvIII expression might be due to enhanced DSB repair. SV40-LT and Ink4a/Arf^{-/-} parental and EGFRvIII-expressing lines were

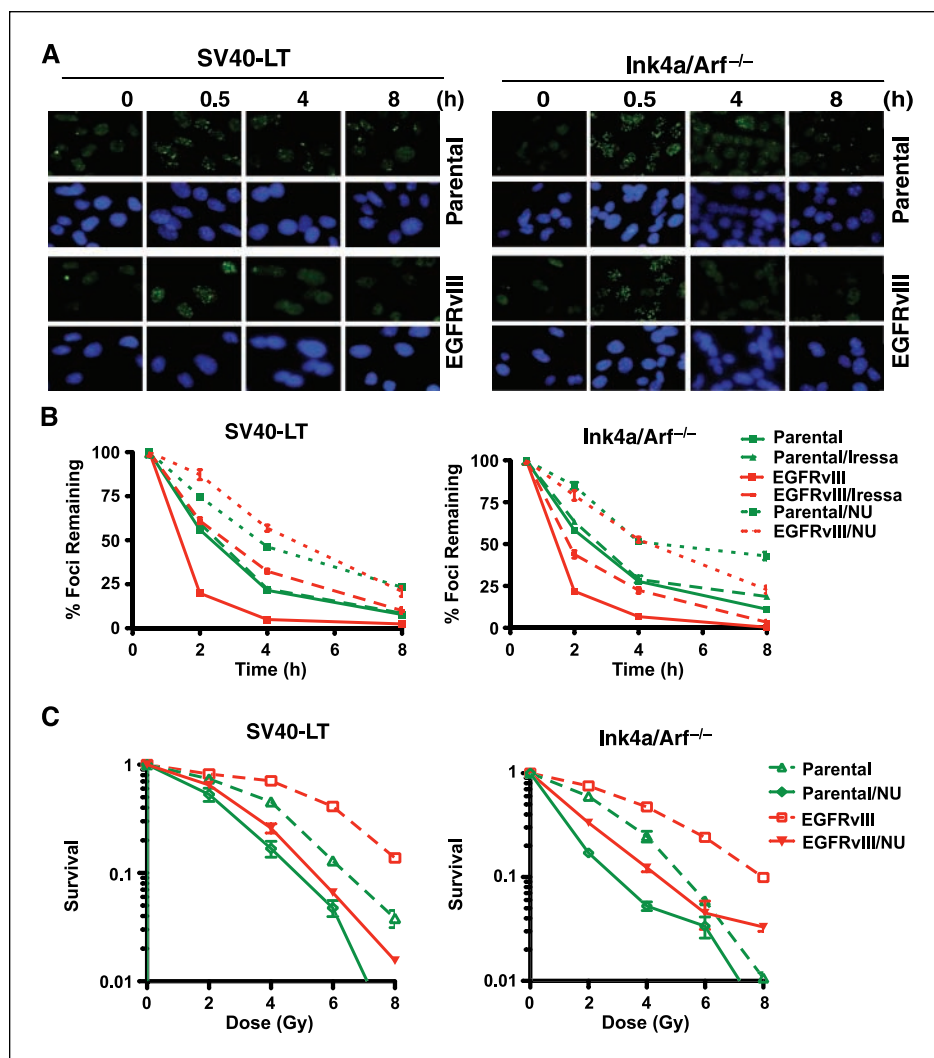


Figure 2. Increased radioresistance of EGFRvIII-expressing astrocytes correlates with proficient DSB repair. **A**, SV40-LT-expressing mouse astrocytes or Ink4a/Arf^{-/-} primary mouse astrocytes were irradiated with 1 Gy of γ -rays and immunostained for 53BP1 foci at various time points ranging from 0.5 to 8 h postirradiation. The pictures depict initial DNA damage (0.5 h) and DNA damage remaining at 4 and 8 h. **B**, 53BP1 foci were scored at 0.5, 2, 4, and 8 h postirradiation. The number of 53BP1 foci was determined for each time point (average of 100 nuclei), and after subtracting background (number of foci in unirradiated nuclei), the percentage foci remaining was plotted against time to obtain DNA repair kinetics. Bars, SE. Note nearly complete repair by 4 h in cells expressing EGFRvIII. Pretreatment of cells with the EGFR inhibitor Iressa (dashed lines) or with the DNA-PKcs inhibitor NU7026 (dotted lines) abrogated DSB repair. **C**, radiation survival of astrocytes pretreated with NU7026 was quantified by colony formation assays. The fraction of surviving colonies (*y* axis) was plotted against corresponding radiation dose (*x* axis). Bars, SE of experiments done three or more times.

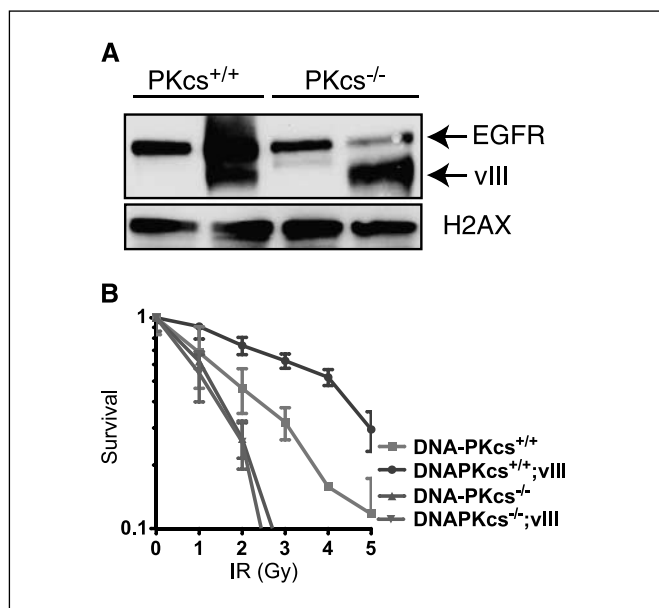


Figure 3. A genetic link between EGFRvIII and DNA-PKcs in the repair of radiation-induced DSBs. *A*, ectopic expression of EGFRvIII in DNA-PKcs^{+/+} and DNA-PKcs^{-/-} MEFs was assayed by Western blotting with α -EGFR and α -H2AX (loading control) antibodies. *B*, radiation survival was quantified by colony formation assays. The fraction of surviving colonies (*y* axis) was plotted against corresponding radiation dose (*x* axis). Bars, SE. Note increased survival of DNA-PKcs^{+/+} cells expressing EGFRvIII, whereas DNA-PKcs^{-/-} cells expressing EGFRvIII show no enhanced survival compared with parental lines.

irradiated with a total dose of 1 Gy, and induction and rate of repair of DSBs were visualized by immunofluorescence staining for 53BP1 foci as described (ref. 22; Fig. 2*A*). Compared with parental lines, the EGFRvIII-expressing lines displayed significantly faster DSB repair kinetics, completing repair by 4 hours (Fig. 2*B*). Pretreatment of these cells with gefitinib (Iressa), which inhibits EGFRvIII signaling (6), slowed down repair to the extent observed in parental lines. These data clearly link improved repair kinetics with signaling from the EGFRvIII receptor.

Small-molecule inhibition of DNA-PKcs counters improved repair and survival exhibited by EGFRvIII-expressing cells. DNA-PKcs is a critical component of the predominant DSB repair pathway in mammalian cells—nonhomologous end joining (17, 18). We tested the hypothesis that EGFR signaling promotes DSB repair via efficient activation of DNA-PKcs (24–26). To investigate a possible link between EGFRvIII and DNA-PKcs activity in glioblastomas, we pretreated EGFRvIII-expressing astrocytes with NU7026, a potent and specific DNA-PKcs inhibitor (27). Pretreatment with NU7026 blocked fast repair kinetics observed in EGFRvIII-expressing astrocytes (Fig. 2*B*). The slower kinetics of DSB repair, in turn, correlated with increased radiation sensitivity in survival assays (Fig. 2*C*), underscoring the potential usefulness of small-molecule inhibitors of DNA-PKcs as radiosensitizers for GBM treatment.

A genetic link between EGFRvIII and DNA-PKcs in DSB repair. Because pharmacologic agents like NU7026 may have nonspecific effects beyond simply blocking their target protein, a genetic strategy was used to establish a mechanistic link between EGFRvIII expression and DNA-PKcs activation. We expressed EGFRvIII in MEFs from DNA-PKcs knockout mice (ref. 28; Fig. 3*A*) and assayed for radiation sensitivity by colony formation

assays. Expression of EGFRvIII in DNA-PKcs^{+/+} MEFs led to a considerable increase in radiation resistance (Fig. 3*B*). Significantly, expression of EGFRvIII in DNA-PKcs^{-/-} cells failed to confer any increase in radioresistance. These results strongly suggest that DNA-PKcs is required for the radiation resistance conferred by EGFRvIII.

Activation of Akt-1 in mouse astrocytes mimics the effects of EGFRvIII expression on DSB repair. EGFRvIII preferentially signals through the PI3K/Akt-1 pathway (29, 30). Pharmacologic inhibition of this pathway can block DSB repair and radiosensitize mammalian cells (31, 32). Conversely, activation of this pathway, due to loss of PTEN, results in proficient DSB repair and radioresistance (33). Moreover, Akt-1 is reported to translocate into the nucleus on irradiation and interact and colocalize with DNA-PKcs at DSBs (34–36). Therefore, we investigated whether EGFRvIII expression directly affected DSB repair via the PI3K/Akt-1 pathway. Toward this end, we ectopically expressed constitutively active, myristylated Akt-1 in SV40-LT astrocytes (Fig. 4*A*). Expression of myristylated Akt-1 resulted in efficient DSB repair similar to that noted on EGFRvIII expression (Fig. 4*B*). Conversely, treatment of EGFRvIII-expressing SV40-LT astrocytes with LY294002, a specific inhibitor of PI3K (37), resulted in slower DSB repair, similar to that seen in parental cells (Fig. 4*B*). These results raise the possibility that EGFRvIII expression might influence DSB repair via the PI3K/Akt-1 pathway.

EGFRvIII expression results in hyperactivation of DNA-PKcs in response to IR. Having established a genetic link between EGFRvIII and DNA-PKcs, we investigated whether EGFRvIII overexpression resulted in hyperactivation of DNA-PKcs in

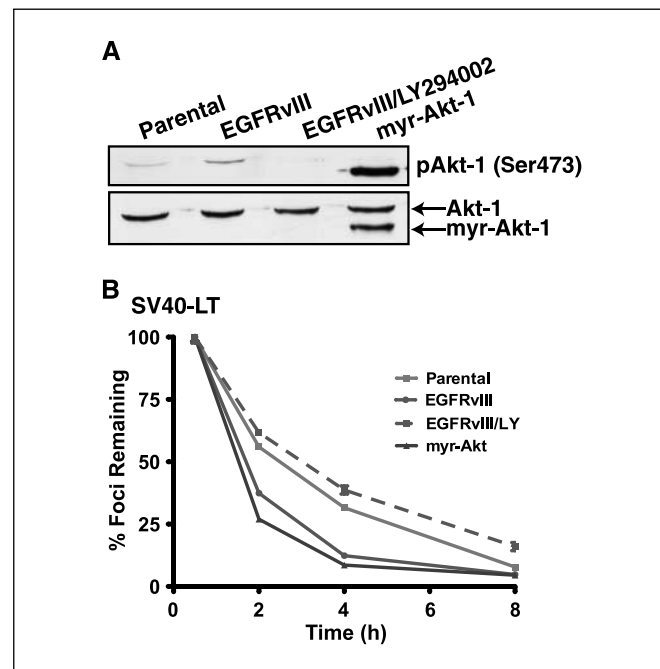


Figure 4. Akt-1 activation in mouse astrocytes mimics the effects of EGFRvIII expression on DSB repair. *A*, phosphorylation of Akt-1 (at Ser473) was assayed by Western blotting of SV40-LT-expressing mouse astrocytes (parental), SV40-LT astrocytes with EGFRvIII overexpression, EGFRvIII-expressing astrocytes pretreated with a PI3K inhibitor (LY294002), or SV40-LT astrocytes expressing myristylated Akt-1. *B*, astrocytes were irradiated (1 Gy) and immunostained for 53BP1 foci to obtain DSB repair kinetics. Bars, SE. Note proficient repair in cells expressing myr-Akt-1 (blue line), whereas repair was abrogated in EGFRvIII-expressing cells treated with LY294002 (dashed red line).

response to IR. Activation of DNA-PKcs in response to IR involves its autophosphorylation at defined serine/threonine residues (18). The extent of phosphorylation can be quantified by Western blotting with phospho-specific antibodies and provides an accurate measure of DNA-PKcs activation *in vivo* (38). Because these phospho-specific antibodies recognize only human DNA-PKcs, we used a human glioma cell line, U87-MG, to examine the effects of EGFRvIII overexpression on DNA-PKcs activation. Expression of EGFRvIII, EGFRwt, and EGFRkd in these lines has been described (39) and was confirmed by Western blotting (Fig. 5A). Expression of EGFRvIII, but not EGFRwt, in U87 cells significantly increased radiation survival (Fig. 5B). As with mouse astrocytes, U87 cells expressing EGFRvIII were significantly sensitized to IR by pretreatment with the DNA-PKcs inhibitor NU7026. Interestingly, expression of EGFRkd also resulted in a certain degree of radiosensitization as reported (13), possibly due to a dominant-negative effect. Most importantly, EGFRvIII expression resulted in faster and improved DSB repair kinetics compared with parental U87 lines, recapitulating results obtained with mouse astrocytes (Fig. 5C).

Having established that EGFRvIII overexpression in U87-MG cells recapitulates results obtained with mouse astrocytes, we examined the extent of DNA-PKcs activation after IR in U87 cells. Phosphorylation of DNA-PKcs was quantified by Western blotting of nuclear extracts from mock-irradiated or γ -irradiated cells with a phospho-DNA-PKcs antibody (pT2647; refs. 18, 23; Fig. 5D). Although total levels of DNA-PKcs (*bottom*) were the same in all four cell lines, DNA-PKcs activation, as evidenced by its phosphorylation at T2647, was remarkably higher in cells expressing EGFRvIII (*top*), providing a mechanistic link between EGFRvIII overexpression and improved DSB repair in these cells.

EGFRvIII enhances DSB repair in a mouse orthotopic glioma model. Given that cell culture conditions *in vitro* may not necessarily recapitulate the complex microenvironment within a tumor *in vivo*, it is possible that the efficient DSB repair observed on EGFRvIII expression may not hold true in the context of GBMs. Therefore, we used, for the first time, a mouse orthotopic glioma model to visualize DSBs and quantify DNA repair *in vivo*. U87 parental and U87-EGFRvIII cells (both expressing luciferase reporters) were stereotactically injected into the striatum of a cohort of nude mice (19). Intracranial tumor growth was monitored by serial luciferase imaging. Once tumors reached 50% maximal tolerated size (established in pilot experiments and corresponding to approximately 11–13 days postimplantation for U87-EGFRvIII cells and 16–18 days for U87 parental cells), mice were anesthetized and received cranial irradiation (total dose, 2 Gy). Mice were anesthetized and cardiac perfused with fixative at 0.5, 2, 4, or 8 hours postirradiation. Brains were paraffin embedded for routine immunohistochemistry and all tumor sections were H&E stained to determine the size and location of tumors (Fig. 6A). Tumor sections were then stained with α -53BP1 antibody to visualize DSBs and quantify repair kinetics (Fig. 6A). DSB repair kinetics indicated significantly faster DSB repair rates in tumors derived from U87-EGFRvIII cells (Fig. 6B). These results, showing *in vivo* tumor cell repair kinetics for the first time, suggest that EGFRvIII overexpression in glioma-relevant orthotopic tumors significantly augments the repair of radiation-induced DNA damage.

Mouse orthotopic tumors expressing EGFRvIII are refractory to radiation therapy. To test whether proficient DNA repair mechanism(s) observed in EGFRvIII-expressing tumors translates

into *in vivo* radioresistance, we exposed nude mice bearing intracranial U87 parental or U87-EGFRvIII tumors to whole-brain radiotherapy. As reported previously (40), the rate of U87-EGFRvIII intracranial tumor growth is significantly higher than that of U87 parental tumors. Due to the faster growth rate of U87-EGFRvIII glioma cells, the timing of whole-brain radiotherapy was adjusted such that IR (4 Gy, one time dose) was delivered when the tumors were approximately of similar size (at day 6 postimplantation for U87-EGFRvIII tumors and at day 10 for U87 parental tumors;

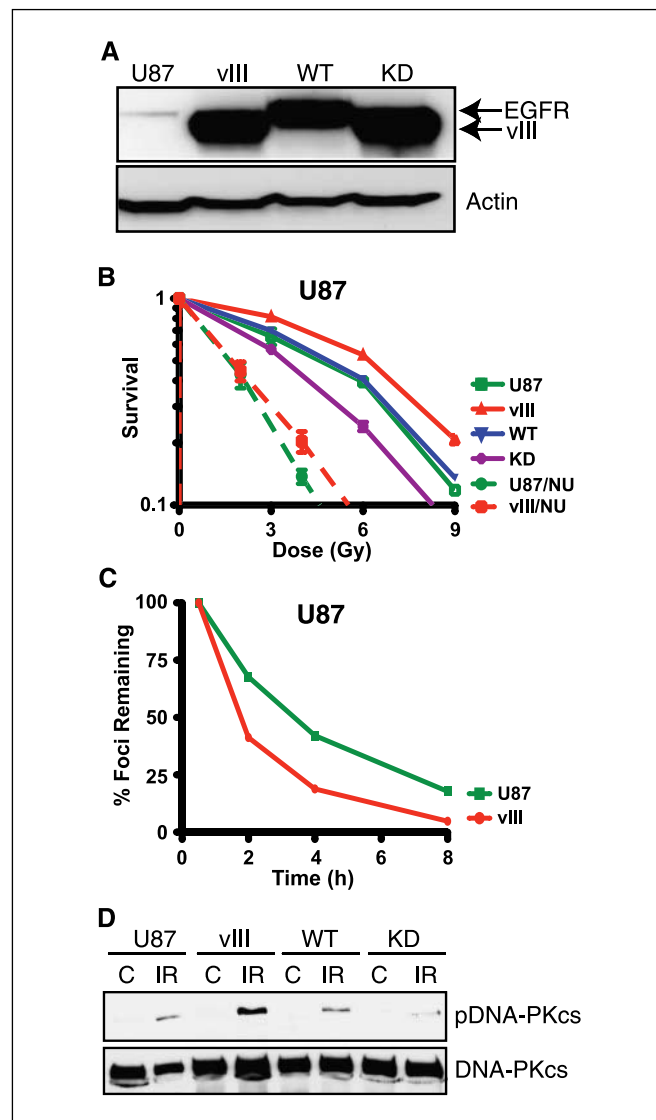


Figure 5. EGFRvIII overexpression results in hyperactivation of DNA-PKcs in response to IR. **A**, expression of EGFRvIII, EGFRwt, or EGFRkd was assessed in human U87 glioma cells by Western blotting with α -EGFR and α -actin (loading control) antibodies. **B**, radiation survival of U87 cells was quantified by colony formation assay. Where indicated, cells were pretreated with the DNA-PKcs inhibitor NU7026. The fraction of surviving colonies (*y axis*) was plotted against the corresponding radiation dose (*x axis*). Bars, SE of experiments done three or more times. Note increased survival of EGFRvIII-expressing cells (*solid red line*) and radiosensitization with NU7026 (*dashed red line*). **C**, U87 parental or U87-EGFRvIII cells were irradiated (1 Gy) and immunostained for 53BP1 foci at various time points post-IR to obtain DSB repair kinetics. Note proficient repair in cells expressing EGFRvIII (*red line*). Bars, SE. **D**, activation of DNA-PKcs in irradiated U87 cells was assayed by Western blotting with α -phospho-DNA-PKcs (pT2647) and α -DNA-PKcs antibodies. Note hyperphosphorylation of DNA-PKcs in cells expressing EGFRvIII.

Figure 6. EGFRvIII enhances DSB repair in a mouse orthotopic glioma model *in vivo*. **A**, orthotopic U87 parental and U87-EGFRvIII tumors were generated in nude mice. Once tumors reached 50% maximal tolerated size, mice received cranial irradiation (2 Gy) and were sacrificed at 0.5, 2, 4, and 8 h post-IR to obtain DNA repair kinetics. Intracranial tumors were identified in coronal brain sections by H&E staining. Brain regions occupied by tumor mass were stained with α -53BP1 antibody to visualize radiation-induced DSBs. **B**, 53BP1 foci were scored to obtain DSB repair kinetics. Bars, SE. Note proficient repair in EGFRvIII-expressing tumors (red line). **C**, growth of orthotopic U87 parental and U87-EGFRvIII tumors was monitored by serial luciferase imaging (representative images are shown). Nude mice with intracranial U87 parental or U87-EGFRvIII tumors were either mock irradiated or γ -irradiated (4 Gy; $n = 5$ per cohort) and luciferase intensities were quantified over a period of 20 d. The plot represents average signal intensity (photons per second $\times 10^4$) for each cohort (y axis) plotted versus time postimplantation (x axis). Arrows, time of radiation. Bars, SE. Note marked decrease in the rate of U87 parental tumor growth (dashed green line) following IR ($P < 0.01$), whereas U87-EGFRvIII tumors (dashed red line) show no significant difference in rate of tumor growth ($P > 0.05$). **D**, Kaplan-Meier analyses of mice with intracranial U87 parental or U87-EGFRvIII tumors ($n = 6$ per cohort). Note no significant increase in postirradiation (4 Gy) survival of mice harboring U87-EGFRvIII tumors (dashed red line; $P > 0.05$) in contrast to marked increase in survival of mice bearing U87 parental tumors (dashed green line; $P < 0.01$).

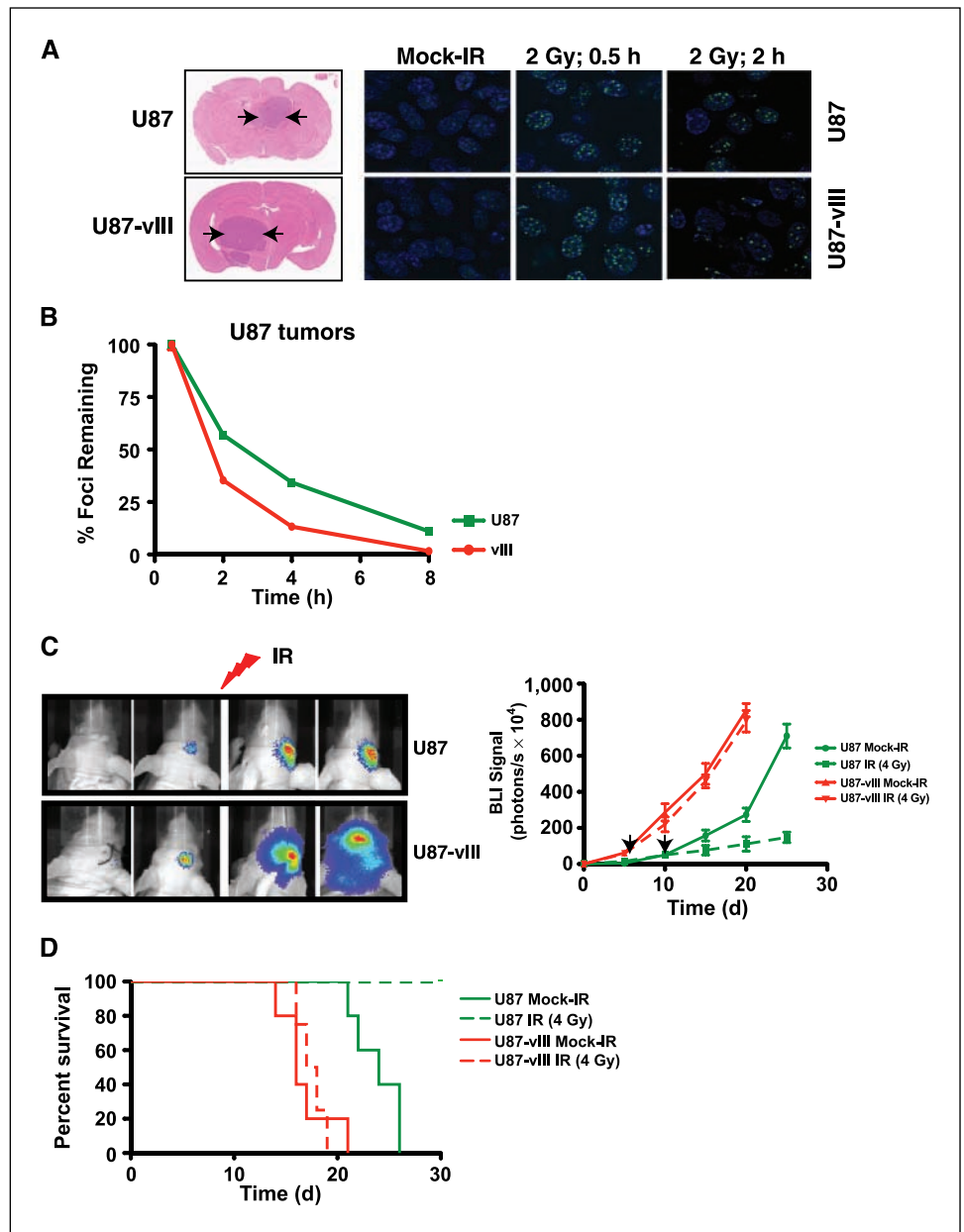


Fig. 6C). Mice were sacrificed when they became moribund or at day 30 postimplantation. Following whole-brain radiotherapy, the growth rate of U87 parental tumors was significantly reduced compared with the growth of mock-irradiated tumors (Fig. 6C). In contrast, there was no difference in the rate of U87-EGFRvIII tumor growth with or without radiation. Correspondingly, following whole-brain radiotherapy, there was little improvement in overall survival (Kaplan-Meier analyses) of mice bearing U87-EGFRvIII tumors compared with mock-irradiated mice (Fig. 6D). In contrast, all mice with U87 parental intracranial tumors that were irradiated were alive at time of sacrifice. These data indicate that proficient DSB repair in EGFRvIII-expressing tumors contributes to tumor radioresistance. We speculate that the extremely rapid and complete DSB repair observed in EGFRvIII-expressing cells may prevent the initiation of programmed cell death on IR, resulting in tumors that are refractory to radiation.

Discussion

Our data show that EGFRvIII overexpression results in increased resistance to IR in primary mouse astrocytes that either lack Ink4a/Arf tumor suppressor genes or have functionally suppressed p53/Rb due to SV40 large T-antigen expression. Similar results are also observed when EGFRvIII is expressed in human U87-MG glioma cells. Increased radioresistance is specific to the constitutively active EGFRvIII receptor and is not seen with equivalent levels of EGFRwt or EGFRkd expression. EGFRvIII-mediated radioresistance is associated with hyperactivation of DNA-PKcs and enhanced DSB repair kinetics, which is possibly transduced via the PI3K/Akt-1 pathway. These observations may provide an important mechanistic basis for the radioresistance of GBMs, which poses a major obstacle to effective treatment of these tumors. In addition to identifying accelerated DSB repair in EGFRvIII-expressing cells *in vitro*, we also provide *in vivo* evidence, using an orthotopic glioma model, that

corroborates these findings and shows these mechanisms to be operative in the context of the complex tumor microenvironment.

Amplification of the constitutively active EGFRvIII receptor is one of the most significant genetic alterations in GBM (1, 3, 4). Expression of EGFRvIII correlates with poor prognosis in patients (41) and higher tumorigenic capacity in both orthotopic and *de novo* mouse glioma models (9, 11). Our work and that of Golding and colleagues (42) indicate that a major contributing factor to GBM radioresistance is augmented DSB repair. Our finding that EGFRvIII stimulates the repair of radiation-induced DNA damage is not completely unexpected given that EGFR is activated by radiation (43–46) and EGFRvIII displays even higher levels of radiation-induced activation compared with the wild-type receptor (12). Our observation that EGFRvIII promotes the repair of DSBs via DNA-PKcs is consistent with previous reports showing that, in human bronchial carcinoma cells, radiation induces EGFR nuclear import and direct physical association with, and activation of, DNA-PKcs (24, 47, 48). However, we see no evidence of nuclear translocation of EGFRvIII under basal conditions or under graded doses of IR in primary murine astrocytes and in U87 glioma cells or orthotopic tumors. It is, therefore, plausible that EGFRvIII expression in GBM tumor cells might enhance DSB repair via the canonical PI3K/Akt-1 pathway rather than by direct physical association with DNA-PKcs.

Hyperactivation of the PI3K/Akt-1 pathway due to EGFR amplification (and concomitant PTEN loss) is one of the hallmarks of GBMs (1, 3, 4). Moreover, whereas ligand-activated EGFR stimulates both the Ras/Raf/MAPK and PI3K/Akt-1 pathways (7), EGFRvIII preferentially activates PI3K/Akt-1 (29, 30). Recent reports have suggested that activated Akt-1 may play a role in the repair of DSBs. Blocking PI3K/Akt-1 signaling using small-molecule inhibitors impairs DSB repair in GBM (33) and breast cancer (31) cells, resulting in radiation sensitivity. Conversely, hyperactivation of PI3K/Akt-1 due to PTEN deletion promotes DSB repair and radioresistance (33). We find that a PI3K inhibitor, LY294002 (37), can abrogate the accelerated DSB repair conferred by EGFRvIII overexpression and that hyperactivation of Akt-1 mimics the effects of EGFRvIII expression on DSB repair in astrocytes. A close physical association between Akt-1 and DNA-PKcs at sites of DSBs on IR has been shown (34–36). Activation of DNA-PKcs by IR involves its phosphorylation at several serine/threonine residues (17, 18). Because Akt-1 is a serine/threonine

kinase, it is conceivable that the hyperphosphorylation of DNA-PKcs observed by us in EGFRvIII-expressing cells may be potentiated by activated Akt-1. Indeed, small interfering RNA-mediated knockdown of Akt-1 has been recently shown to attenuate DNA-PKcs autophosphorylation on IR (49). The presence of major components of the PI3K/Akt-1 pathway in the nucleus (50) presages a more intimate link between RTK signaling and nuclear processes like DNA repair than has previously been postulated. In future studies, it will be important to elucidate the precise mechanism by which PI3K/Akt-1 activation by EGFRvIII might promote DNA-PKcs hyperactivation in GBM tumor cells.

Our finding that gefitinib (Iressa) can abrogate the proficient DSB repair and radiation resistance due to EGFRvIII is consistent with data from GBM clinical trials in which 15% to 20% of GBM patients experience significant tumor regression in response to EGFR small-molecule inhibitors (51, 52). Our results with a small-molecule inhibitor of DNA-PKcs, showing significant radiosensitization of murine astrocytes and human glioma cells expressing EGFRvIII, are important from a therapeutic standpoint. Several DNA-PKcs inhibitors are currently being developed as radiosensitizers (53), and it may be worthwhile in the future to develop formulations of DNA-PKcs inhibitors that are compatible with *in vivo* testing in preclinical GBM models. In aggregate, our results show that DNA-PKcs hyperactivation and proficient DSB repair in EGFRvIII-expressing cells and tumors provide a mechanistic basis for the marked radioresistance of GBMs with EGFR amplification and PTEN loss (52). These results suggest that DNA-PKcs and/or EGFR inhibition concurrent with radiation might be an effective strategy for radiosensitizing high-grade gliomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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