Inhibition of the Type III Epidermal Growth Factor Receptor Variant Mutant Receptor by Dominant-Negative EGFR-CD533 Enhances Malignant Glioma Cell Radiosensitivity

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ABSTRACT

Purpose: **The commonly expressed variant epidermal growth factor receptor (EGFR), the type III EGFR variant (EGFRvIII), functions as an oncoprotein promoting neoplastic transformation and tumorigenicity. The role of EGFRvIII in cellular responses to genotoxic stress, such as ionizing radiation, is only minimally defined. Thus, we have investigated EGFRvIII as a potential modulator of cellular radiation responses and explored the feasibility of adenovirus (Ad)-mediated expression of dominant-negative EGFR-CD533 as a gene therapeutic approach for inhibiting EGFRvIII function *in vitro* and *in vivo*.**

Experimental Design and Results: **EGFR-CD533 and EGFRvIII were expressed *in vitro* and *in vivo* in malignant U-373 MG glioma cells through transduction with an Ad vector, Ad-EGFR-CD533 and Ad-EGFRvIII, respectively. *In vivo* studies defined the importance of EGFRvIII as a modulator of radiation responses, demonstrating a 2.6-fold activation of EGFRvIII in U-373 malignant glioma tumors. Concomitant expression of EGFR-CD533 inhibited the radiation-induced activation of EGFRvIII *in vitro* and completely abolished the enhanced clonogenic survival conferred by EGFRvIII. The ability of EGFR-CD533 to inhibit EGFRvIII function was further confirmed *in vivo* through complete inhibition of EGFRvIII-mediated increased tumorigenicity and radiation-induced activation of EGFRvIII. Growth delay assays with U-373 xenograft tumors demonstrated that the expression of EGFR-CD533 significantly enhanced radiosensitivity of tumor cells under conditions of intrinsic and Ad-mediated EGFRvIII expression.**

Conclusions: **We conclude that EGFRvIII confers significant radioresistance to tumor cells through enhanced cytoprotective responses, and we have demonstrated that dominant-negative EGFR-CD533 effectively inhibits EGFRvIII function. These data affirm the broad potential of EGFR-CD533 to radiosensitize human malignant glioma cells.**

INTRODUCTION

Growth factors and their receptors play a central role in regulating both developmental and neoplastic progresses. As part of neoplastic progression, overexpression of the epidermal growth factor receptor (EGFR), one of the ERBB receptors extensively implicated in tumorigenesis, is associated with the expression of naturally occurring variant species (1–6). Of these variant forms, the type III EGFR variant (EGFRvIII) is most commonly expressed in human tumors (3). *EGFRvIII* is characterized by deletion of exons 2 to 7, involving nucleotides 275 to 1075. Thus, EGFRvIII lacks a major portion of the Cys-rich ligand-binding domain, near the NH_2 terminus of the extracellular portion of the molecule (7). EGFRvIII is constitutively active and is not further activated by EGFR ligands (8). In contrast to the well-established functions of EGFRvIII as an oncoprotein (6, 8–11), the role of EGFRvIII in cellular responses to genotoxic stresses, such as ionizing radiation or other cytotoxic cancer therapeutic agents, has been only minimally investigated.

Ionizing radiation in the therapeutic dose range of 1 to 5 Gy activates EGFR wild-type (EGFRwt) and other ERBB receptors, initiating a major cytoprotective response through stimulation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3′-kinase, and AKT and consequential radiation dose-dependent proliferation and antiapoptotic responses (12–14). We have shown that a genetic approach of overexpressing EGFR-CD533, a dominant-negative (DN) variant of EGFR lacking 533 amino acids of the cytoplasmic domain (15, 16), disrupts this cytoprotective signaling by EGFRwt and its downstream effectors (17, 18). Furthermore, expression of EGFR-CD533 inhibits basal and radiation-induced EGFR activation and radiosensitizes both *in vitro* and *in vivo* (19). An important next step in the mechanistic analyses is to investigate whether expression of EGFR-CD533 also modulates EGFRvIII activity because EGFRvIII is commonly expressed in tumors, and previous findings suggest

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the inability of EGFRvIII to interact with other ERBB receptors (3, 8).

The constitutively active EGFRvIII is prominently activated by radiation, but not by growth factors (20). It is widely expressed *in vivo* when human tumor cells are propagated as xenograft tumors, but expression is quickly lost on *in vitro* cultivation of tumor cells. In the present report, the consequences of endogenous EGFRvIII expression on acute tumor cell radiation responses were examined in malignant glioma xenograft tumors. These studies were extended to include functional modulation of EGFRvIII by concomitant expression of EGFR-CD533 in tumor cells transduced by adenovirus (Ad) vectors Ad-EGFRvIII and Ad-EGFR-CD533. This system defined the effects of EGFR-CD533 and EGFRvIII at similar expression levels on radiosensitivity of U-373 MG cells *in vitro* and tumor xenografts.

We confirm that EGFRvIII is expressed in experimental U-373 MG xenograft tumors and that expression of EGFRvIII is down-regulated on *in vitro* cell culture. The basal expression of EGFRvIII in U-373 cells is markedly enhanced on transduction of cells with Ad-EGFRvIII; cells transduced *in vitro*, as a consequence, demonstrate accelerated growth rates *in vivo*, supporting the oncogenic activity of this constitutively active mutant receptor. Using an experimental gene therapy approach, *in vivo* delivery into U-373 xenograft tumors of Ad-EGFRvIII and Ad-EGFR-CD533 achieved high expression of one or both receptors. Importantly, the expression of DN EGFR-CD533 inhibited EGFRvIII activity with and without the combined use of radiation.

MATERIALS AND METHODS

Reagents and Cell Lines. Protease and phosphatase inhibitors and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). All electrophoresis reagents were from Bio-Rad (Hercules, CA). Media and antibiotics were from GIBCO-BRL (Rockville, MD), fetal bovine serum (FBS) was from Intergen (Purchase, NY), and tetracycline-free FBS was from Clontech Laboratories (Palo Alto, CA). The following monoclonal antibodies (mAbs) were used for Western blotting: anti-EGFR mAb mixture (Ab14), reacting with NH_2-terminal domains of the EGFR protein (Neo Markers, Fremont, CA); anti-EGFRvIII mAb DH8.3 reacting with the NH_2-terminal truncation of EGFR (AbCam Limited, Cambridge, United Kingdom); anti-phospho-Tyr mAb Ab2 and the secondary peroxidase-conjugated goat antimouse antibody (Oncogene Science, Cambridge, MA). For immunoprecipation EGFR mAb Ab5 (NeoMarkers) was used. Other reagents included protein G plus/protein A-agarose from Oncogene Science. The cDNA of EGFR-CD533 lacking the COOH-terminal 533 amino acids (15, 16) and the phβAc.EGFRvIII expression plasmid were kindly provided by A. Ullrich (Max Planck Institute for Biochemistry, Martinsried, Germany) and by D. Bigner (8), respectively. The following cell lines were obtained from American Type Culture Collection (Manassas, VA): U-373 malignant glioma and Chinese hamster ovary (CHO) cell lines. All cells were maintained at 37°C in 95% air/5% CO_2. CHO cell lines were maintained in RPMI 1640 containing 5% FBS with antibiotics (penicillin/streptomycin), whereas U-373 cells were maintained in minimum essential medium α containing 10% FBS and antibiotics.

Mice and Tumors. Athymic female nu/nu mice were obtained from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). Animals used in this study were maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institutes of Health. Solitary tumors were produced by subcutaneous inoculation of 10^7 U-373 cells into the right hind leg of 4- to 5-week-old mice. Tumor cell suspensions were prepared from cells grown as monolayers *in vitro*. Experiments were initiated when tumor sizes reached 8 to 10 mm in diameter.

Transient Transfection of Chinese Hamster Ovary Cells. CHO cells were routinely seeded at 1.5×10^5 cells per 60-mm dish. On day 3, transient transfections were performed at >80% efficiency with LipofectAMINE plus (GIBCO-BRL, Gaithersburg, MD) and 0.5 μg of the phβAc.EGFRvIII expression plasmid and 1.5 μg of an empty vector DNA (20). Western blot analyses verified detectable expression of the phβAc.EGFRvIII plasmid 48 hours after transfection. CHO cells transfected with phβAc.EGFRvIII, referred to henceforth as CHO.EGFRvIII, were used as positive controls for EGFRvIII in Western blot analyses.

Recombinant Adenoviruses and *In vitro* Transduction Conditions. Replication-incompetent Ad was produced as described previously and successfully used to generate the Ad-EGFR-CD533 vector (21, 22). Ad-EGFRvIII was constructed by transferring a *Sap*I-*Xba*I fragment, spanning the coding sequence of *EGFRvIII*, into pZeroTGCMV, followed by recombination in bacteria (21, 23). The pSL 1180-EGFRM was kindly provided by S. Batra (Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE). An Ad expressing the bacterial *lacZ* reporter gene (Ad-LacZ) was used as control virus (21, 23). Briefly, Ad-LacZ, Ad-EGFRvIII, and Ad-EGFR-CD533 were produced in 293 cells as described previously (21) and purified by double CsCl gradient centrifugation followed by dialysis against 13% glycerol in PBS (21, 23). The Ad vectors were frozen in liquid nitrogen and stored at −70°C until further use. Titration by standard plaque assay produced typical titers of 2 to 4×10^{11} plaque-forming units per mL.

Transduction of the U-373 cell line with Ad was carried out by diluting viral stocks with PBS, adding Ad preparations to cell monolayers, and placing dishes on a rocker with gentle continuous agitation in a tissue culture incubator for 4 hours at 37°C, followed by media change. A multiplicity of infection (MOI) of 3 was found to be optimal, as described previously (24). Mock transductions were carried out under conditions identical to those used for the Ad transduction, except for the addition of Ad preparations.

Cell Treatments and Irradiation Protocols. Cell treatments and irradiation experiments *in vitro* were performed on day 5, 48 hours after transduction. Cells were exposed to epidermal growth factor (EGF) at 10 ng/mL for 5 minutes. For all irradiation experiments, cells were exposed to single doses of

ionizing radiation at a dose rate of 1.8 Gy/minute using a ^{60}Co source. In time course experiments, cells were irradiated and incubated at 37°C for the times specified. Thereafter, media were removed, and cells were washed once in ice-cold PBS, frozen rapidly on dry ice, and stored at −80°C until further processing.

U-373 tumors of the specified sizes were irradiated *in vivo*. For this, animals were fully anesthetized, and irradiation was focused on the right posterior leg bearing the tumor. Controls included mock irradiation of tumors under identical conditions. For immunochemical verification of radiation-induced EGFRvIII activation, as assessed by the extent of EGFRvIII tyrosine phosphorylation, tumors were exposed to a single radiation dose of 4 Gy and excised 10 minutes after irradiation or mock irradiation, frozen immediately in liquid nitrogen, and processed as described below.

Immunoprecipitation and Immunoblotting Assays. Lysis and immunoprecipitation of cells were performed according to methods standard in our laboratory (17, 18, 25). For each experiment, equal amounts of protein were used for immunoprecipitation or Western blot analysis. EGFRwt, EGFRvIII, and EGFR-CD533 analyses in tumor tissue were performed in extracts from tumors frozen instantly in liquid nitrogen after excision. Tumor tissue was pulverized in liquid nitrogen using a mortar and pestle (26) and lysed immediately in ice-cold lysis buffer [25 mmol/L β-glycerophosphate, 25 mmol/L Tris (pH 7.4), 10% (v/v) glycerol, 1.5 mmol/L EGTA, 0.5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 1 mmol/L Na PP_i, 15 μg/mL aprotinin, 15 μg/mL leupeptin, 2 μmol/L benzamidine, and 150 μg/mL phenylmethylsulfonyl fluoride]. The cell suspension was passed sequentially through 16- to 21-gauge needles to facilitate cell lysis. Cell debris was removed by centrifugation at 14,000 × *g* at 4°C for 10 minutes, and the supernatants were processed for immunoprecipitation or Western blot analysis for EGFRwt, EGFRvIII, and EGFR-CD533 protein expression and for EGFR wt and EGFRvIII tyrosine phosphorylation. After electrophoresis on 6% SDS-polyacrylamide gels, proteins were transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). For visualization, bands were developed with the CDP-Star kit (Tropix, Inc., Bedford, MA). Autoradiograms were quantified using Sigma Scan software (Jandel Scientific, San Rafael, CA).

Colony Formation Assay. Forty-eight hours after transduction with 3 MOI of Ad-LacZ or Ad-EGFRvIII or the combination of Ad-LacZ + Ad-EGFR-CD533 or Ad-EGFRvIII + Ad-EGFR-CD533 at 3 MOI for each Ad vector, cells were harvested, and 500 cells were plated for each group into 4-well 60-mm dishes for clonogenic survival. Cells were incubated under standard culture conditions for 14 days and stained with crystal violet, and colonies containing ≥50 cells were counted to determine the plating efficiency (24, 27).

Tumorigenicity and Tumor Growth. Before subcutaneous inoculation, U-373 cells were transduced *in vitro* with 3 MOI for each Ad vector with Ad-EGFRvIII, Ad-LacZ, Ad-EGFRvIII + Ad-EGFR-CD533, or Ad-LacZ + Ad-EGFR-CD53 and suspended in 0.1 mL of PBS, and 8×10^6 cells per mouse and site were injected into the right flanks of female nude mice. The growth of palpable tumors was measured with a caliper every 3 days, and tumor volumes were calculated using width (*a*) and length (*b*) measurements [$a^2b/2.5$, where $a < b$ (28)]. Only tumors with a width or length of ≥4 mm were defined as palpable tumors. The end point for tumor growth assay was prospectively applied as evidence for measurable tumor growth within 65 days with a minimum mean tumor volume of 0.3 cm^3.

Tumor Growth Delay. For the production of U-373 xenograft tumors, 10^7 cells were injected into the right hind leg of mice approximately 23 days before Ad infusion. Tumors at an average size of 0.2 to 0.3 cm^3 were infused with the respective Ad vector or a combination of two Ad vectors; the combinations included Ad-LacZ/Ad-EGFR-CD533, Ad-LacZ/Ad-EGFRvIII, and Ad-EGFRvIII/EGFR-CD533. A single six-track infusion was performed as described previously (19, 24)on day −3 using 1.2×10^9 plaque-forming units/Ad vector/tumor. Irradiation with 3-Gy fractions on 3 consecutive days, as described above, was initiated 72 hours after Ad infusion on day 0. Control experiments in three mice confirmed that Ad-LacZ resulted in an average 70 ± 5% transduction rate *in vivo* within 72 hours. Tumor growth was measured with calipers as described above for the conditions studied (see also the Fig. 5 legend). The end point for tumor growth delay assays was applied as quadrupling of the initial tumor volume on day 0.

Statistical Analyses. All data are presented as means ± SE, unless otherwise specified. Statistical comparisons were carried out with the Student's *t* test, except for statistical comparisons between tumor growth curves, which were carried out using the F-test with the SAS software package (version 8.0; SAS Institute, Inc., Cary, NC). *P* values of <0.05 were considered statistically significant.

RESULTS

Expression of EGFRvIII in Malignant Glioma Cells and Xenograft Tumors. EGFRvIII, a common mutant form of EGFR, is expressed in a variety of human tumors including carcinomas and malignant gliomas (29). To study the functional consequences of EGFRvIII expression on cellular radiation responses, we defined EGFRvIII expression *in vivo* and *in vitro* in our experimental systems. We first quantified the levels of EGFRvIII protein expressed in xenograft tumors of U-373 cells relative to cells maintained *in vitro*. EGFRvIII expression was quantified in extracts from freshly excised tumors, thus reflecting the expression levels *in vivo*.

As demonstrated in Fig. 1*A*, *top panel*, EGFRvIII was expressed at measurable levels in established U-373 tumors but was undetectable in U-373 cells maintained *in vitro*. EGFRvIII protein expression was also not detected in cells that were isolated from EGFRvIII-expressing tumors and then cultured *in vitro* (data not shown). Relative to EGFRwt, the EGFRvIII protein expression varied widely from 1.2- to 2.3-fold (Fig. 1*A*, *top panel*), as demonstrated by Western blotting using a mAb with broad reactivity to the EGFR protein. As described previously, EGFRvIII was identified as a 140-kDa/155-kDa doublet band (6) with the smaller-sized component typically expressed at higher levels. Similar results were obtained with an EGFRvIII-specific mAb, confirming the absence of EGFRvIII in cultured cells and again demonstrating the prominence of the 140-kDa component in xenograft tumors. A similar 140-kDa

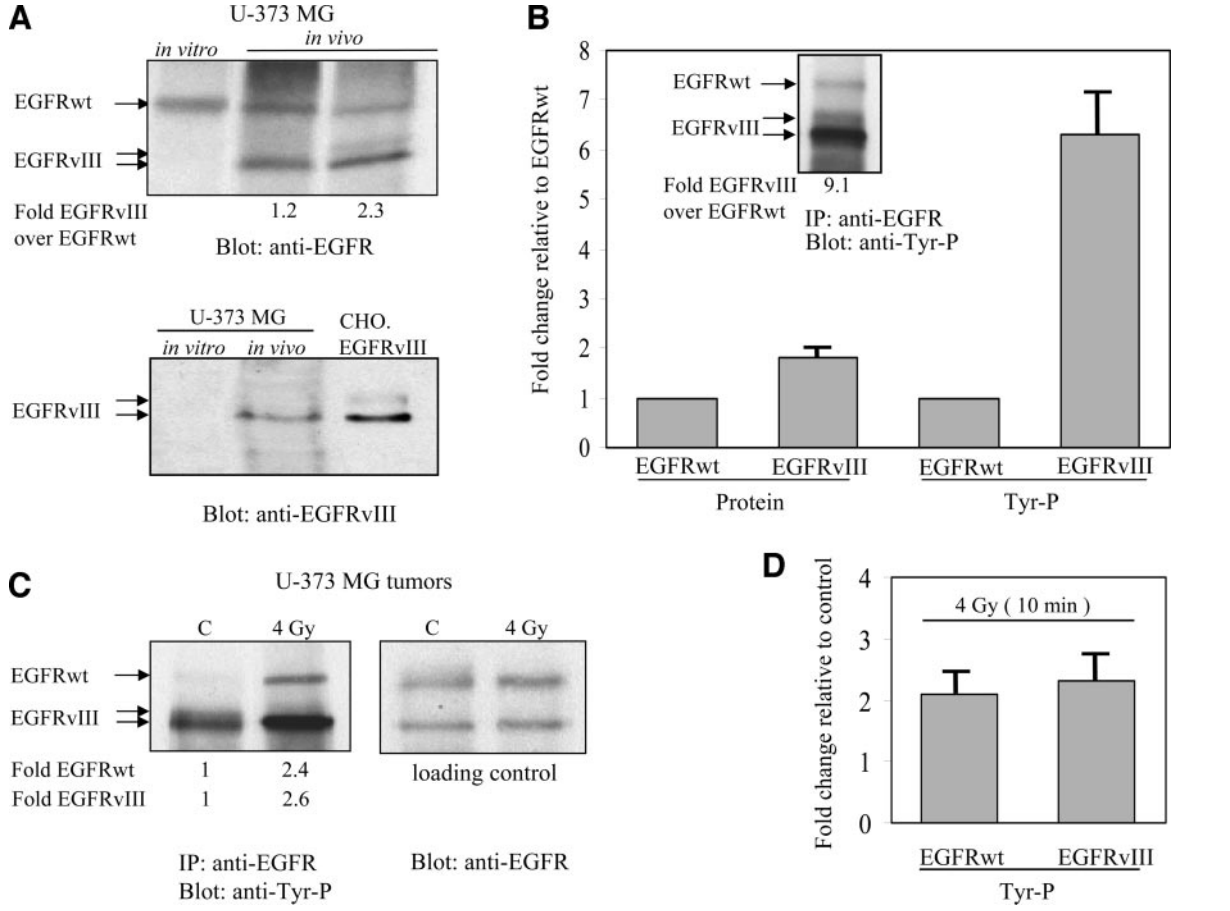


Fig. 1 Expression, tyrosine phosphorylation, and radiation-induced activation of EGFRvIII in U-373 malignant glioma (MG) cells and xenograft tumors derived thereof. *A*, EGFRvIII expression in U-373 tumors (*in vivo*) and the corresponding cells maintained *in vitro* (*in vitro*). CHO.EGFRvIII cell lysates were used as positive control (*bottom panel*). Autoradiograms were quantified by densitometry, and EGFRvIII expression levels relative to EGFRwt are shown below each lane and expressed as fold difference (*top panel*). *B*, summary of results of the densitometry analyses of EGFRvIII expression and tyrosine phosphorylation levels relative to EGFRwt in U-373 tumors. The autoradiogram shown of EGFRwt and EGFRvIII tyrosine phosphorylation (*inset*) is representative of four tumors analyzed. EGFRvIII tyrosine phosphorylation relative to that of EGFRwt is shown below and calculated as fold difference after correction for differences in protein expression. *Bar graphs* represent the fold difference of EGFRvIII protein expression and phospho-tyrosine levels relative to EGFRwt in tumors. *Error bars* represent mean ± SD. *C*, radiation-induced activation of EGFRvIII in established U-373 xenograft tumors. Tyrosine phosphorylation levels of EGFRwt and EGFRvIII were detected in tumors 10 minutes after mock irradiation (control, *Lane C*) or irradiation with a single exposure of 4 Gy and quantified by densitometry (*left panel*). Western blotting for EGFRwt and EGFRvIII protein verified equal loading (*right panel*; loading control). The data shown are representative of three independent experiments. *D*, summary of the densitometry analyses of EGFRwt and EGFRvIII tyrosine phosphorylation levels after administration of 4 Gy to U-373 xenograft tumors. Results are shown as fold changes of EGFRwt and EGFRvIII tyrosine phosphorylation relative to mock-irradiated tumors derived from three independent experiments. *Error bars* represent the mean ± SE.

EGFRvIII protein band was seen on Western blots with extracts from CHO.EGFRvIII cells (Fig. 1*A*, *bottom panel*).

Having demonstrated the presence of EGFRvIII in human xenograft tumors, we next quantified the protein expression levels of EGFRvIII relative to EGFRwt *in vivo* and compared basal tyrosine phosphorylation levels of both receptors after immunoprecipitation from tumor lysates before probing with anti-phospho-Tyr antibody on Western blots. As shown in Fig. 1*B*, there was an average 1.8-fold higher expression of EGFRvIII relative to EGFRwt. Most likely reflecting the state of high constitutive activity of EGFRvIII, the tyrosine phosphorylation of EGFRvIII *in vivo* was, on average, 6.3-fold higher ($P < 0.001$) than that of EGFRwt (Fig. 1*B*, *inset*). Similar results were obtained from MDA-MB-231 mammary carcinoma xenograft tumors (data not shown).

Ionizing Radiation Induces Tyrosine Phosphorylation of EGFRvIII *In vivo*. We have demonstrated previously (19) that ionizing radiation induced an immediate activation of EGFRwt in xenograft tumors, which showed a maximum activation within 10 minutes after a single 4-Gy radiation exposure. Thus, we investigated further the effects of ionizing radiation on EGFRvIII tyrosine phosphorylation *in vivo*. To examine radiation-induced activation of EGFRvIII, established U-373 xenograft tumors were irradiated with 4 Gy, harvested 10 minutes later, and processed for immunochemical analyses. We found that EGFRvIII and EGFRwt tyrosine phosphorylation increased

2.6- and 2.4-fold, respectively, whereas protein levels of both receptors remained unchanged (Fig. 1*C*). Thus, averaging at least three experiments, ionizing radiation induced a significant overall 2.3-fold (range, 1.5–2.8-fold) activation of EGFRvIII in U-373 tumors (Fig. 1*D*; $P < 0.01$), a response similar to the average 2.1-fold radiation-induced activation of EGFRwt. Considering the high constitutive activity of EGFRvIII (20), the mutant receptor, in addition to EGFRwt, is likely to represent an important modulator of radiation responses when tumor xenografts are irradiated.

Transduction of U-373 Cells with EGFRvIII and EGFR-CD533 Affects Receptor Tyrosine Phosphorylation. Our previous studies (19) have shown that expression of EGFR-CD533 reduced the basal and radiation-stimulated tyrosine phosphorylation of EGFRwt. In the present study, we examined whether expression of EGFR-CD533 could also modulate EGFRvIII activity. Because EGFRvIII was not expressed in cultured cells (Fig. 1*A*), these studies were performed in U-373 cells *in vitro* transduced with Ad-EGFRvIII alone or in combination with Ad-EGFR-CD533. MOIs between 1 and 10 produced a dose-dependent increase in EGFRvIII expression (Fig. 2*A*). Importantly, the expression of EGFRvIII was independent of the simultaneous expression of EGFR-CD533. The finding that a MOI of 3 produced similar expression levels of both mutant receptors demonstrates that there was no interference in the expression of the two receptors. In addition, within the time frame of the experiments, there was also no effect on the protein expression levels of EGFRwt (Fig. 2*A*, *top panel*).

The functional status of the EGFRvIII and EGFR-CD533 mutant receptors in U-373 cells was studied next (Fig. 2*B*) using conditions of equal protein expression (MOI = 3) as demonstrated in Fig. 2*A*. EGFRwt and EGFRvIII phosphorylation levels were then determined after expression of EGFR-CD533. Relative to untreated controls, EGF treatment of U-373 cells

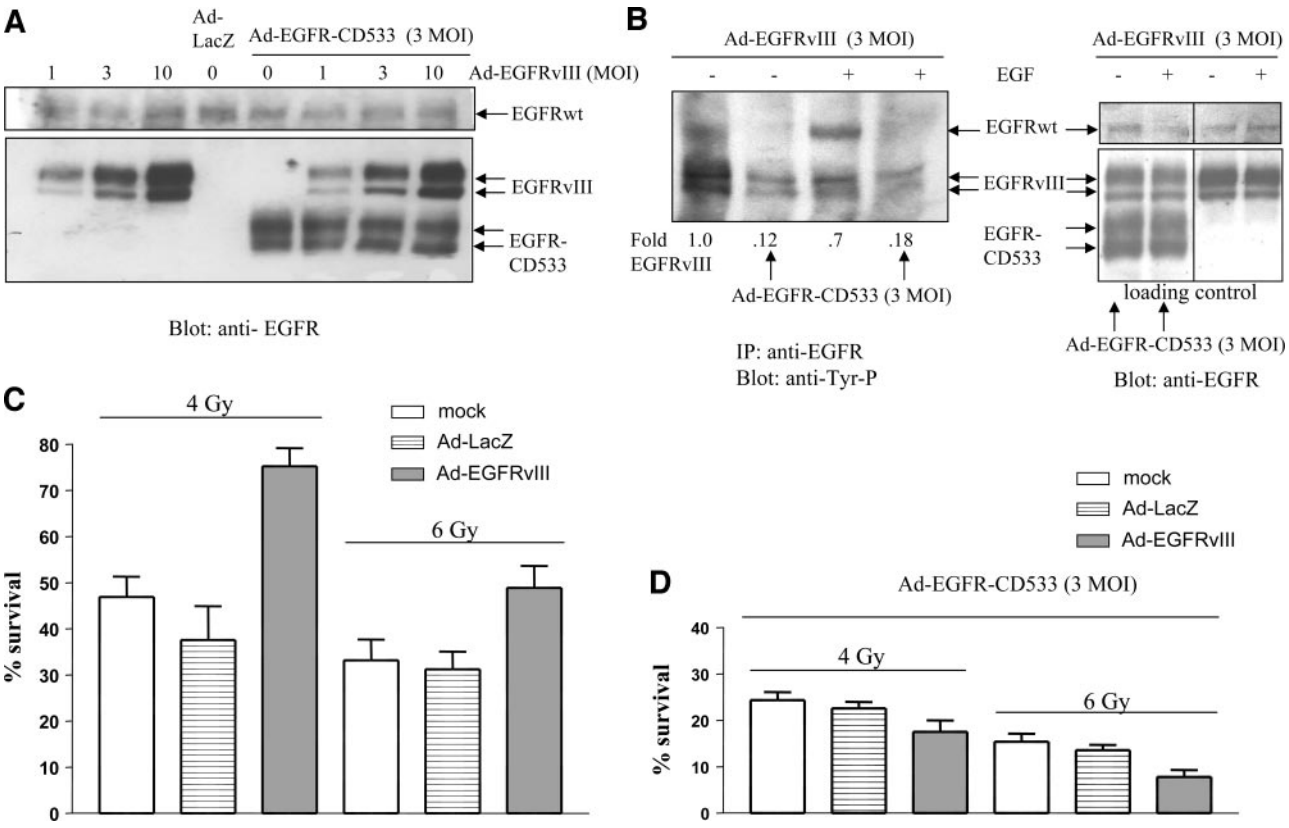


Fig. 2 Ad-mediated expression of EGFRvIII and EGFR-CD533, effects of EGFR-CD533 expression on EGFRvIII activity, and radiosensitivity of U-373 cells. *A*, Ad-mediated expression of EGFRvIII with and without concomitant expression of EGFR-CD533. The Ad-EGFRvIII-mediated, dose-dependent expression of EGFRvIII protein (varying MOIs; *Lanes 1–3*) was unaffected by the simultaneous transduction of cells with Ad-EGFR-CD533 at a constant MOI (*Lanes 5–8*); the same applied to the expression of EGFRwt (*top panel*; a longer exposure was required). *B*, modulation of EGFRvIII tyrosine phosphorylation by concomitant expression of EGFR-CD533. EGFRvIII tyrosine phosphorylation was quantified after transduction with Ad-EGFRvIII alone or concomitant transduction with Ad-EGFR-CD533 (*lanes* labeled by *arrows*) and with or without additional EGF treatment. Western blotting for EGFRwt and EGFRvIII protein verified equal loading (*right panel*; loading control; a longer exposure was required for EGFRwt). EGFRvIII tyrosine phosphorylation levels are shown below each lane expressed as fold difference relative to control (*Lane 1*). Data are representative of three independent experiments. *C*, modulation of the radiosensitivity of U-373 cells expressing EGFRvIII. The radiosensitivity for the different transduced cell groups irradiated with 4 or 6 Gy was measured by colony formation assays. *D*, radiosensitization of U-373 cells through EGFR-CD533 expression regardless of concomitant EGFRvIII expression. Cells were transduced, irradiated, and assayed for clonogenic survival under conditions identical to those in *C* with concomitant Ad expression of EGFR-CD533. Data presented in *C* and *D* are mean values ± SD from two independent experiments performed in quadruplicate.

induced a 2.2-fold activation of EGFRwt. At the same time, EGFRvIII did not respond to EGF, affirming that this receptor was not activated (3, 20). In contrast, the expression of EGFR-CD533 reduced the basal and EGF-induced tyrosine phosphorylation of EGFRvIII to 12% and 18% of the control conditions, respectively. This is in line with our previous observation that expression of EGFR-CD533 reduced EGFRwt tyrosine phosphorylation by >90% (see Fig. 2*B*).

EGFR-CD533 Counteracts the Cytoprotective Radiation Response of EGFRvIII. To correlate radiation-induced activation of EGFRvIII with the radiosensitivity of U-373 cells, we examined cell survival after single radiation doses using colony formation assays and comparing cells expressing EGFRvIII with control (Ad-LacZ) or mock-transduced cells (Fig. 2*C*). As assessed by colony formation assays, U-373 cells transduced with Ad-EGFRvIII exhibited reduced radiosensitivity compared with cells subjected to Ad-LacZ or mock transduction, a finding consistent with our previous results linking EGFRvIII expression to an enhanced cytoprotective response (20) and increased relative radioresistance (Fig. 2*C*). The different average 47% and 33% reductions ($P < 0.01$) in cell survival after 4 and 6 Gy, respectively, suggest a radiation dose response. This response was minimally modified by infection of U-373 cells with Ad-LacZ (Fig. 2*C*), yielding survival rates of 38% and 31% for 4 and 6 Gy, respectively. In contrast to control conditions, the transduction of cells through Ad-EGFRvIII yielded significantly ($P < 0.001$) enhanced survival rates of 75% and 49% for 4 and 6 Gy, respectively. The survival levels were also significantly different ($P = 0.019$) for the two doses used, again suggesting a cellular radiation dose response (Fig. 2*C*). In comparison, under all three conditions, the concomitant transduction with Ad-EGFR-CD533 not only significantly ($P < 0.01$) increased radiosensitivity of U-373 cells as described previously (17–19) but also completely reversed the enhanced clonogenic survival mediated by EGFRvIII (Fig. 2*D*). These results demonstrate that the radiation-induced activation of EGFRvIII conferred relative radioresistance through an enhanced cytoprotective response and that this response was completely inhibited by concomitant expression of EGFR-CD533 (20, 30).

EGFR-CD533 Inhibits the Enhanced Tumorigenic Effects of EGFRvIII Expressed in Xenograft Tumors. The next set of experiments examined the effects of EGFRvIII expression on U-373 xenograft radiosensitivity *in vivo*. These experiments are important because the high constitutive activity of EGFRvIII is one likely reason for decreased radiosensitivity and enhanced tumorigenicity of malignant glioma cells [expressing this mutated receptor (3)]. Hence, we extended our studies to EGFRvIII and EGFR-CD533 interactions *in vivo* and examined tumor growth as a function of EGFRvIII expression with and without the concomitant expression of EGFR-CD533. In the first set of experiments, the effects of enhanced EGFRvIII expression on xenograft tumor growth were examined relative to the LacZ control vector using cells that were grown *in vivo* after transduction *in vitro* (see Materials and Methods).

In addition, we defined the relative effects of EGFRvIII and EGFR-CD533 on xenograft growth in U-373 cells (Fig. 3*A* and *B*). U-373 cells were transduced *in vitro* through infections with Ad-EGFRvIII or Ad-LacZ and with Ad-EGFRvIII + Ad-EGFR-CD533 or Ad-LacZ + Ad-EGFR-CD533; 48 hours after infection, 8×10^6 transduced cells were inoculated into mice. Independent immunoblotting analyses for EGFRvIII and EGFR-CD533 verified similar Ad-mediated expression of EGFRvIII with and without concomitant expression of EGFR-CD533, and EGFRwt expression remaining unchanged (Fig. 2*A*). Colony formation assays for the four different cell groups, plated at the time of subcutaneous inoculation of cells, revealed plating efficiencies between 35% and 45%, indicating only minor differences in cell viability (Fig. 3*A*). U-373 cells expressing EGFRvIII demonstrated significantly accelerated growth ($P < 0.001$), generating tumors of 0.3 cm^3 within 20 days compared with 30 days for U-373 cells transduced with Ad-LacZ (Fig. 3*B*). This growth advantage was completely eliminated in cells concomitantly expressing both EGFR-CD533 and EGFRvIII; these U-373 cells did not generate tumors during the 65-day observation period. The expression of EGFR-CD533 had a similar growth inhibitory effect on U-373 cells transduced with Ad-LacZ, suggesting inhibition of both endogenous EGFRwt and EGFRvIII receptors by EGFR-CD533 as the likely mechanism.

This conclusion was further supported by immunochemical analyses (Fig. 3*C*) demonstrating the qualitative and quantitative expression of EGFRvIII *in vivo* 24 to 30 days after inoculation of tumor cells transduced with EGFRvIII or LacZ, respectively. Consistent with the data presented in Fig. 1, the basal expression of EGFRvIII in tumors favors the expression of the 140-kDa component of EGFRvIII, whereas transfection with the EGFRvIII plasmid or transduction with the Ad-EGFRvIII viral vector resulted in a relatively higher expression of the 155-kDa species (Fig. 3*C*).

Modulation of Radiation-Induced EGFRvIII Activation by EGFR-CD533 in Xenograft Tumors. We next examined the functional consequences of EGFR-CD533 expression on radiation-induced EGFRvIII activation in established U-373 xenograft tumors infused with Ad-LacZ or Ad-EGFR-CD533 using our previously established techniques (7). The radiation-induced activation of both EGFRwt and EGFRvIII in U-373 xenograft tumors was inhibited >90% when tumors were infused with Ad-EGFR-CD533 before irradiation; tumors infused with the Ad-LacZ control vector demonstrated radiation-induced tyrosine phosphorylation levels similar to untreated tumors (Fig. 4 *A*, *Lanes 1* and *2*; Fig. 1*C*, *left panel*). As shown in Fig. 4*A* (*Lanes 3* and *4*), EGFRwt and EGFRvIII protein expression was unaffected by infusion with Ad-EGFR-CD533 or Ad-LacZ. The protein expression data (Fig. 4*B*) demonstrated similar relative expression levels of EGFR-CD533 and EGFRwt *in vitro* and in tumors and showed no change in basal protein expression of EGFRwt and EGFRvIII in tumors after infusion with Ad-EGFR-CD533 or Ad-LacZ (see Fig. 5*A*).

Fig. 4*C* demonstrates, as controls, the expression patterns and levels for U-373 cells transduced with Ad-EGFR-CD533 and CHO cells transfected with the EGFRvIII plasmid. Combined intratumoral infusion of Ad-EGFRvIII and Ad-EGFR-CD533 resulted in similar receptor protein levels (Fig. 4*C*, *Lane 3*), relative to those seen after infusion of Ad-EGFR-CD533 and Ad-LacZ, conditions under which the EGFR-CD533 expression levels far exceeded the basal expression levels of EGFRwt and EGFRvIII (Fig. 4*B* and *C*; see also Fig. 5*B*).

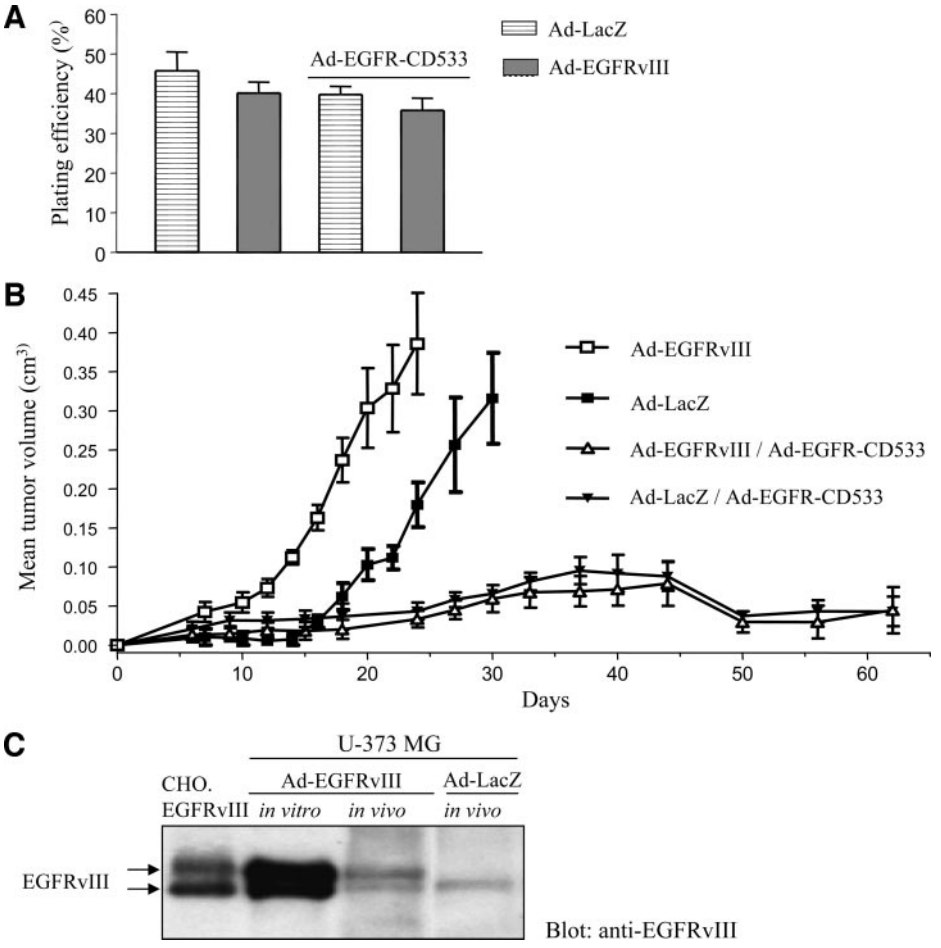


Fig. 3 U-373 malignant glioma (MG) xenograft growth in nude mice of cells infected *in vitro* with Ad-EGFR-CD533 or Ad-EGFRvIII and a combination of the two relative to Ad-LacZ control. *A*, colony formation efficiency of U-373 cells transduced *in vitro* with Ad-LacZ, Ad-EGFRvIII, Ad-LacZ + Ad-EGFR-CD533, or Ad-EGFRvIII + Ad-EGFR-CD533 before inoculation. Transduced U-373 cells were plated for colony formation assays at the time that same batch of cells was inoculated into nude mice. *B*, tumorigenic activity of U-373 cells expressing EGFRvIII compared with coexpression of EGFRvIII with EGFR-CD533. *Error bars* represent mean ± SE for five animals per group. *C*, Western blot analyses of Ad-mediated expression of EGFRvIII in established U-373 xenograft tumors at the same time at which growth curves under *B* were terminated. U-373 cells transduced with Ad-EGFRvIII *in vitro* (*in vitro lane*) and lysate of CHO.EGFRvIII cells served as positive controls for EGFRvIII.

EGFR-CD533 Radiosensitizes Tumor Xenografts in Growth Delay Assays Independent of EGFRvIII Expression Levels. The radiosensitizing effects of EGFR-CD533 were tested in U-373 xenograft tumors under conditions of basal EGFRvIII expression levels and overexpression after infusion of Ad-EGFRvIII into tumors (leading to high EGFRvIII expression levels as shown in Fig. 4*C*). Tumor growth delay assays, using three 3-Gy radiation exposures and the different transduction conditions after intratumoral infusion of each Ad vector, were used to quantify the relative effects of EGFRvIII and EGFR-CD533 on tumor radiosensitivity. Western blot analyses of extracts from infused U-373 tumors verified continued high expression levels of EGFR-CD533 even 32 days after Ad vector infusion (Fig. 4*B*, *Lane 3*); this affirmed the prolonged transgene expression after Ad transduction. The EGFR mutant protein expression levels resulting from intratumoral infusion with either Ad-EGFR-CD533 + Ad-EGFRvIII or Ad-LacZ + Ad-EGFR-CD533 are shown in Fig. 4*C*.

Following established procedures in our laboratory (19, 24), three daily exposures of 3 Gy were used starting 3 days after the single six-track intratumoral infusion of the Ad vectors. As end points for the tumor growth experiments, we defined the time in days required for two tumor volume doublings. Untreated U-373 tumors quadrupled in volume within 14 days (Fig. 5*A*), requiring sacrifice of the animals between days 20 and 25. This growth rate was only minimally retarded by the infusion of control Ad-LacZ vector (16 days), which was also used to quantify the *in vivo* transduction rates of 70 ± 5% on day 3 after infusion, the time of the first radiation exposure. Ad-EGFR-CD533 infusion alone resulted in a marginal growth delay of two tumor doublings to 21 days. Irradiation alone at three 3-Gy radiation exposures delayed tumor growth to approximately 34 days (Fig. 5*A*). Importantly, there was a significant additional growth delay when irradiation was combined with the infusion of Ad-EGFR-CD533 ($P = 0.014$); these tumors required 60 days to quadruple in volume (Fig. 5*A*), representing a 2.2-fold enhancement in growth delay (31). These data demonstrate the lack of toxicity of Ad infusion into tumors and confirm a greater than additive radiosensitization when EGFR-CD533 expression is combined with irradiation. Because the tumors express both EGFRvIII and EGFR-CD533 throughout the growth delay experiments (Fig. 4*B*), *in vivo* expression of intrinsic EGFRvIII was apparently neutralized by EGFR-CD533.

To quantify the inhibition of EGFRvIII by EGFR-CD533 further, experiments shown in Fig. 5*A* were repeated under conditions of high EGFRvIII expression. In these growth delay experiments (see Fig. 5*B*), tumors were infused with Ad-EGFRvIII + Ad-LacZ, Ad-EGFRvIII + Ad-EGFR-CD533, or Ad-

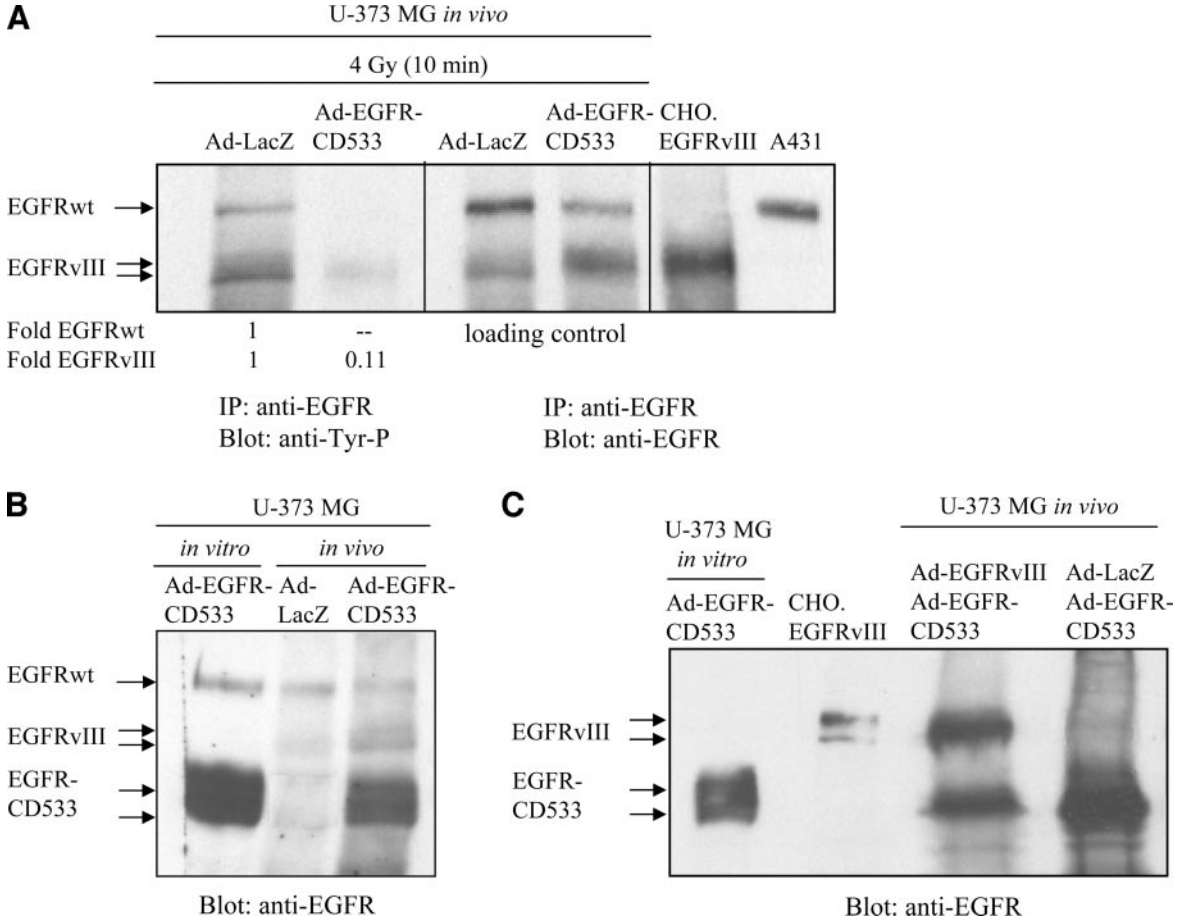


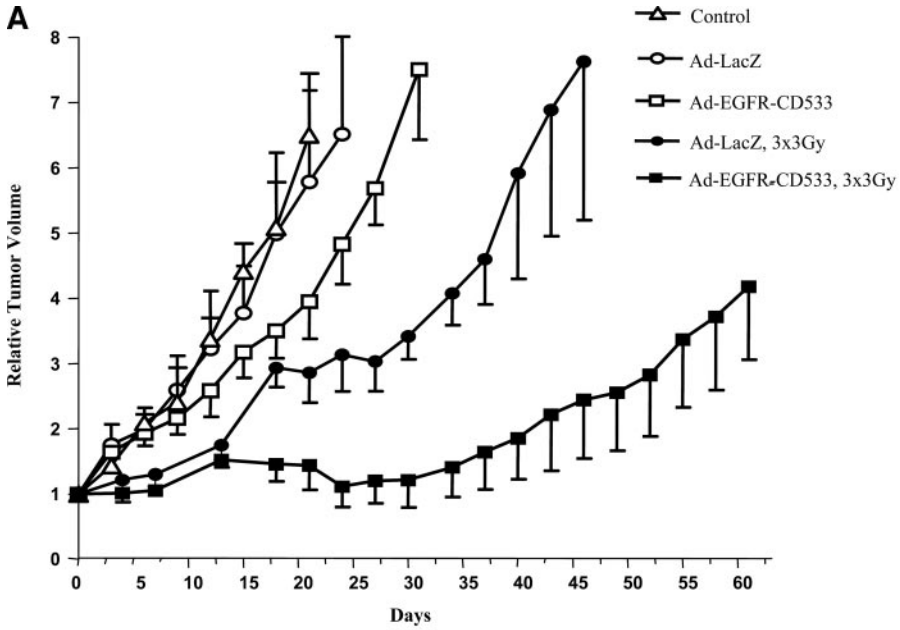
Fig. 4 Functional consequences of EGFR-CD533 expression on radiation-induced activation of intrinsic EGFRvIII in U-373 malignant glioma (MG) tumor xenografts and expression levels of EGFRvIII and EGFR-CD533 in established tumors intratumorally infused with different combinations of Ad vectors. *A*, decreases in EGFRvIII tyrosine phosphorylation in U-373 MG tumors intratumorally infused with Ad-EGFR-CD533 10 minutes after irradiation with 4 Gy. EGFRwt and EGFRvIII tyrosine phosphorylation levels were quantified by immunoblotting (*Lanes 1* and *2*), assuring identical protein loading (loading controls; *Lanes 3* and *4*). EGFRwt and EGFRvIII tyrosine phosphorylation levels are shown below and calculated as fold difference to LacZ. A431 and CHO.EGFRvIII cell lysates were used as positive controls. *B*, Western blot analyses of intrinsic EGFRvIII expression and Ad-mediated EGFR-CD533 expression in U-373 MG tumors (*in vivo lanes*). Established tumors were infused with either Ad-LacZ or Ad-EGFR-CD533 and examined 24 and 32 days later for EGFR expression, respectively. Lysates of cells transduced *in vitro* with Ad-EGFR-CD533 were used as positive control. These protein expression data apply to *in vivo* conditions of tumor growth assays shown in Fig. 5*A*. *C*, Western blot analyses of Ad-mediated concomitant expression of either EGFRvIII and EGFR-CD533 or LacZ and EGFR-CD533 in U-373 tumors (*in vivo lanes*). Established tumors were analyzed 3 days after intratumoral Ad infusion for EGFRvIII and EGFR-CD533 expression. Lysates of cells transduced *in vitro* with Ad-EGFR-CD533 and CHO.EGFRvIII cell lysates were used as positive controls. These protein expression data apply to *in vivo* conditions of tumor growth assays shown in Fig. 5*B*.

LacZ + Ad-EGFR-CD533 (for protein expression of the two mutant receptors, see Fig. 4*C*). All tumors were subjected to the same three 3-Gy irradiation schedule. Tumors infused with Ad-LacZ + Ad-EGFRvIII underwent two tumor volume doublings within 17 days (Fig. 5*B*). This tumor growth pattern replicated the growth of untreated U-373 tumors (see Fig. 5*A*). The combined infusion of Ad-EGFR-CD533 + Ad-EGFRvIII significantly delayed tumor growth to approximately 48 days ($P = 0.003$; Fig. 5*B*). This growth rate was further delayed to 74 days by expression of EGFR-CD533 after combined Ad-LacZ/Ad-EGFR-CD533 infusion, conditions under which tumors only express basal levels of EGFRvIII; however, this difference did not reach significance ($P = 0.147$). Importantly, the significant growth delay observed under simultaneous high expression of EGFR-CD533 and EGFRvIII affirmed the potent inhibitory effect of EGFR-CD533 on EGFRvIII (Fig. 5*B*).

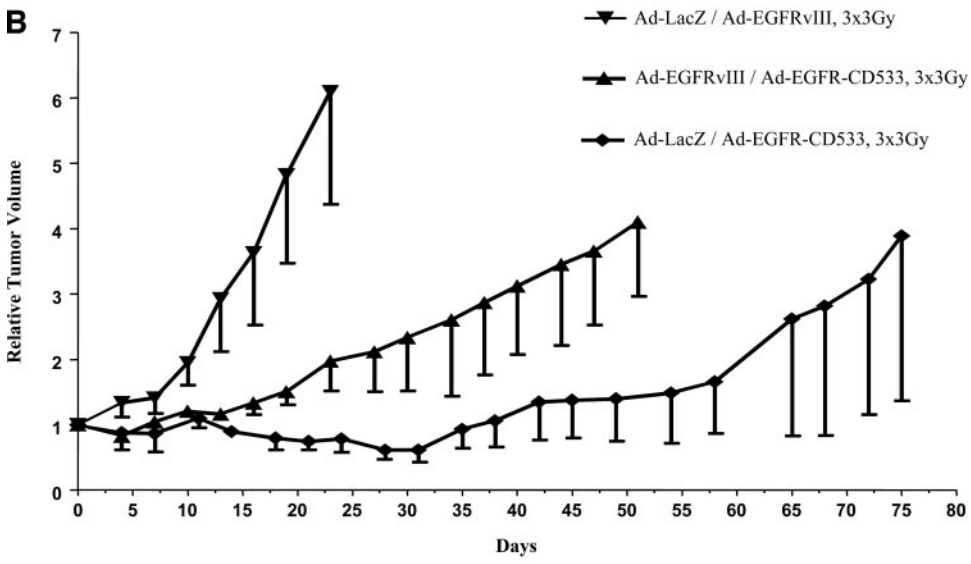
In summary, our results demonstrate that the radiosensitization of U-373 tumors mediated by EGFR-CD533 is independent of EGFRvIII expression levels, thus suggesting a functional dominance of EGFR-CD533. These data lend additional support to the effectiveness and feasibility of using Ad-EGFR-CD533–mediated gene therapy to counteract EGFRwt- and EGFRvIII-mediated radioprotective effects.

DISCUSSION

The data presented support previous findings that the most common mutant variant of EGFR, EGFRvIII, is widely ex-



Treatment:	Ad-EGFR-CD533	3 x 3 Gy	Ad-EGFR-CD533 + 3 x 3 Gy
Growth delay time (in days) relative to Ad-LacZ alone (to reach rel. tumor vol. 4)	5	18	44



Treatment:	Ad-EGFRvIII + Ad-EGFR-CD533 + 3 x 3 Gy	Ad-LacZ + Ad-EGFR-CD533 + 3 x 3 Gy
Growth delay time (in days) relative to Ad-EGFRvIII + 3x 3 Gy (to reach rel. tumor vol. 4)	31	57

Fig. 5 Effect of EGFR-CD533 expression on U-373 tumor xenograft radiosensitivity. *A,* tumor growth delay assays of tumor xenograft alone (control) or after intratumoral infusion with either Ad-LacZ or Ad-EGFR-CD533 and mock irradiation or repeated irradiation (three 3-Gy radiation exposures). *Error bars* represent the mean relative tumor volume ± SE for 4 to 6 animals per group. *B,* tumor growth delay assays of U-373 tumor xenografts after intratumoral infusion with Ad-LacZ + Ad-EGFR-CD533, Ad-EGFRvIII + Ad-EGFR-CD533, or Ad-LacZ + Ad-EGFR-CD533 and repeated irradiation (three 3-Gy radiation exposures). *Error bars* represent the mean relative tumor volume ± SE for a minimum of 4 animals per group. The tumor size on day 0 immediately before start of mock irradiation or irradiation under *A* and *B* was set as 1.0. The tables at the *bottom* of *A* and *B* summarize the calculated specific growth delay times for the various treatments.

pressed in xenograft tumors, as shown here for the malignant U-373 MG glioma cells, and that the receptor is rapidly downregulated when tumor cells are maintained under tissue culture conditions. The high level of EGFRvIII basal tyrosine phosphorylation, relative to that of EGFR wt, is reflective of the constitutive activity of this mutant receptor and is the likely reason for its pronounced 2- to 3-fold stimulation after radiation exposure of cells. Previous findings suggesting the inability of

EGFRvIII to interact with other ERBB receptors prompted our *in vitro* and *in vivo* studies to investigate the activity of EGFR-CD533 in inhibiting basal and radiation-induced tyrosine phosphorylation of EGFRvIII. Using most stringent conditions of EGFR-CD533 and EGFRvIII overexpression in U-373 MG xenograft tumors, EGFR-CD533 quantitatively counteracted the tumorigenic activity of EGFRvIII, inhibited radiation-induced activation of ERGFvIII, and, additionally, eliminated tumor radioresistance. These results extend the potential therapeutic inhibition of radiation-induced ERBB receptor activation (and consequential radioresistance) to also include the constitutively active EGFRvIII.

Expression of mutant forms of the EGFR has been implicated in the progression of many human tumors (32, 33). Previous reports indicate that EGFRvIII is expressed in a high proportion of human tumors, including malignant gliomas and breast and ovarian carcinomas (29). We have tested two different tumor types for the presence of EGFRvIII and demonstrated the consistent expression of this common EGFR variant at protein levels similar to or exceeding those of EGFRwt. Therefore, EGFRvIII represents a potentially powerful modulator of cellular radiation responses (4, 10, 32, 34).

Few mechanistic studies defining the function of EGFRvIII have been reported because EGFRvIII expression *in vivo* is limited to human spontaneous or xenograft tumors. Rapid loss of EGFRvIII expression on transfer of cells to *in vitro* conditions (4) was affirmed by our data, demonstrating that EGFRvIII expression was lost when cells were cultured (see Fig. 1; data not shown). These findings are presented for malignant glioma cells but also apply to human A431 squamous and MDA-MB-231 mammary carcinoma cells.[1] The mechanisms of growth environment-dependent expression of EGFRvIII are currently unknown (35). Consequently, the investigation of the truncated receptor described in this study concentrates on analyses of human xenograft tumors, which also represent a more meaningful model for functional studies on the role of EGFRvIII and EGFR-directed biological therapies. The high constitutive activity levels of EGFRvIII relative to EGFRwt found in this study are consistent with other data demonstrating the oncogenic activity of EGFRvIII including neoplastic transformation and enhanced tumorigenicity (3, 4, 36).

Our studies also reveal that malignant glioma cells expressing EGFRvIII exhibit enhanced tumorigenic capacity when implanted into nude mice subcutaneously (see Fig. 3*A* and *B*). Because this accelerated tumor cell growth is only observed *in vivo* and not *in vitro*, these results suggest that EGFRvIII might play a role in the interaction of tumor cells with their environment rather than directly affecting cell growth. Tumors at this early point of growth are dependent on neovascularization, and perhaps the signals transduced by EGFRvIII might enhance the ability of the tumor cells to induce this process (3, 6). Activation of EGFR has been shown to stimulate expression of angiogenic factors, *e.g.*, vascular endothelial growth factor, in numerous tumor cell types including malignant gliomas (37). The antitumor effects of EGFR-CD533 expression *in vivo* may thus also include an antiangiogenic component not seen *in vitro*. Furthermore, EGFR-CD533 expression in tumor endothelial cells will also be antiangiogenic (38). Thus, based on our data, we cannot exclude the possibility that the antitumor effects of EGFR-CD533 *in vivo* are independent of EGFRvIII.

We have shown that the endogenous, constitutively active EGFRvIII in human xenograft tumors is activated by ionizing radiation to a similar extent as EGFRwt (Fig. 1*C*). Based on its higher constitutive activity, EGFRvIII can be expected to initiate a stronger cytoprotective response in irradiated cells than we have previously demonstrated for EGFRwt (14, 17, 19, 24, 27, 30) and other ErbB receptors (39). Thus, EGFRvIII will significantly contribute to cellular radioresistance (20, 40) that is mediated by radiation-induced proliferation (17), antiapoptosis (20, 40), and enhanced DNA repair (17, 24, 27, 41). These cellular responses have been linked through EGFRwt, and now EGFRvIII, to amplified signals at the levels of MAPK (42), phosphatidylinositol 3′-kinase (43), c-Jun-NH_2-terminal kinase (36), and transcription factors (44). The prominent, radiation-induced activation of EGFRvIII over EGFRwt with amplified signals of MAPK and AKT has also been demonstrated in a more defined system using CHO cells (20). The functional link between cytoprotective prosurvival responses of the ERBB/MAPK/AKT cascades and increased radioresistance (17, 24, 27) emphasizes the importance of inhibiting EGFRvIII for tumor cell radiosensitization. Our findings that the expression of EGFR-CD533 can reduce basal and abrogate radiation-induced tyrosine phosphorylation of both EGFRwt and EGFRvIII suggest that the interference of DN EGFR-CD533 with ERBB receptor activation includes EGFRvIII (43) and may be due to protein–protein interactions between the two receptors (4).

In summary, we have demonstrated that EGFR-CD533 acts as a potent inhibitor of EGFRvIII, a constitutively active mutant receptor expressed in many human tumors that enhances tumor cell radioresistance. Ionizing radiation produces a significant activation of EGFRvIII *in vivo* and induces a more powerful cytoprotective response in tumors than has been demonstrated previously for EGFRwt (14, 24, 27). The highly effective inhibition of EGFRvIII by EGFR-CD533 likely occurs through receptor–protein interactions, as demonstrated for EGFRwt, because EGFR-CD533 eliminated the radiation-induced increase in tyrosine phosphorylation of both EGFRvIII and EGFRwt. Importantly, EGFR-CD533 abrogated the tumorigenic capacity and relative radioresistance conferred by EGFRvIII when expressed at endogenous levels or on Ad-mediated overexpression. Thus, the disruption of EGFRvIII function through the genetic approach of EGFR-CD533 overexpression represents a potentially powerful tool of mutant receptor inhibition and affirms the broad inhibitory activity of EGFR-CD533 toward ERBB receptors and ERBB variants.

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[1] Unpublished results.

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