Use of an Orthotopic Xenograft Model for Assessing the Effect of Epidermal Growth Factor Receptor Amplification on Glioblastoma Radiation Response

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Abstract

Purpose: The influence of epidermal growth factor receptor (*EGFR*) amplification on glioblastoma patient prognosis following definitive radiotherapy has been extensively investigated in clinical studies, and yet the relationship between EGFR status and radiation response remains unclear. The intent of the current study was to address this relationship using several EGFR-amplified glioblastoma xenografts in an orthotopic athymic mouse model.

Experimental Design: We examined the effect of radiation on the survival of nude mice with intracranial xenografts derived from 13 distinct patient tumors, 7 of which have amplified EGFR. Mice with established intracranial tumors were randomized to sham treatment or 12-Gy radiation in six fractions delivered over 12 days.

Results: For six of the xenografts, radiation of mice with intracranial tumor significantly extended survival, and four of these xenografts had *EGFR* amplification. For seven other xenografts, radiation treatment did not significantly extend survival, and three of these, including GBM12, had *EGFR* amplification. Similar to *EGFR,* the tumor genetic status of *p53* or *PTEN* did not show preferential association with radiation-sensitive or radiation-resistant xenografts whereas hyperphosphorylation of Akt on Ser^{473} was associated with increased radioresistance. To specifically investigate whether inhibition of EGFR kinase activity influences radiation response, we examined combined radiation and EGFR inhibitor treatment in mice with intracranial GBM12. The combination of oral erlotinib administered concurrently with radiation resulted only in additive survival benefit relative to either agent alone.

Conclusions: Our results indicate that *EGFR* amplification, as a biomarker, is not singularly predictive of glioblastoma response to radiation therapy, nor does the inhibition of EGFR enhance the intrinsic radiation responsiveness of glioblastoma tumors. However, efficacious EGFR inhibitor and radiation monotherapy regimens can be used in combination to achieve additive antitumor effect against a subset of glioblastoma.

Glioblastoma is the most common and malignant form of central nervous system tumors and is fatal in nearly all instances. Following optimal surgical debulking, radiation therapy is a mainstay of treatment that doubles the median survival from 6 to 12 months (1–3). Despite aggressive radiation treatment, the vast majority of patients suffer tumor recurrence within or immediately adjacent to the high-dose radiation field (4, 5), even with dose escalation up to 90 Gy (6). This clinical observation of radiation resistance in a substantial subset of glioblastoma has motivated extensive research aimed at defining the molecular features of this cancer that contribute to tumor radioresistance (7–9). Among the various biomarkers examined, tumor epidermal growth factor receptor (*EGFR*) gene amplification status has attracted significant attention (10–12). Unfortunately, a straightforward assessment of potential relationships between *EGFR* amplification and tumor response to radiation therapy is made difficult by combinations of clinical characteristics that are unique to each patient and influence survival.

The importance of EGFR in glioblastoma radiation response also has been studied *in vitro* using established glioblastoma cell lines (13–15) or short-term primary glioblastoma cell cultures (16). Through such investigations, radiation-induced EGFR activation has been shown to stimulate downstream signaling effectors that include phosphatidylinositol 3-kinase, Akt, Ras, and mitogen-activated protein kinase (MAPK), and the constitutive activation of these effectors likely contributes to enhanced tumor cell survival following radiation. However,

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EGFR amplification in glioblastoma is progressively diminished and ultimately lost with prolonged cell culture (17, 18) and, consequently, *in vitro* EGFR signaling studies provide only a limited understanding of how *EGFR* amplification might affect radiation response in a clinical setting.

We have previously described a panel of glioblastoma xenografts established directly from patient surgical specimens and that are maintained as s.c. xenografts through serial passaging in immune-deficient mice (18). This approach to glioblastoma propagation is the only means that has been shown to preserve tumor *EGFR* amplification status (18, 19). Consequently, the xenograft panel we have established offers a unique resource with which to address questions on *EGFR* amplification–associated biology and therapeutic response in glioblastoma. Here we have used several xenografts in an orthotopic model of glioblastoma (20) to determine whether tumors with *EGFR* amplification and attendant high-level EGFR expression differ in their radiation treatment responsiveness relative to tumors that lack *EGFR* amplification. To further investigate a potential role of EGFR signaling in glioblastoma response to radiation therapy, we have examined singular and combined effects of radiation with the EGFR small-molecule inhibitor erlotinib (21). In total, our results indicate that glioblastoma *EGFR* amplification does not correlate with responsiveness of glioblastoma xenografts to radiation therapy but that EGFR kinase inhibition in combination with radiation has additive antitumor activity against a subset of glioblastoma.

Materials and Methods

Patient tissues and clinical information. The xenografts used in this study were established with tumor tissue from patients undergoing surgical treatment at Toronto Western Hospital, Toronto, Ontario, Canada (GBM15 only) or the Mayo Clinic, Rochester, Minnesota. These studies were approved by the respective Institutional Review Boards and only samples from patients who had provided prior consent for use of their tissues in research were included. WHO criteria were used for tumor classification (22) and all tumors used for xenograft establishment were diagnosed as glioblastoma, with the exception of GS28, of which the diagnosis was gliosarcoma. Patient clinical data were obtained retrospectively through a review of the patient charts.

Orthotopic xenograft model and therapy response experiments. All xenograft therapy evaluations were done using an orthotopic tumor model for glioblastoma on a protocol approved by the Mayo Institutional Animal Care and Use Committee (20). In brief, flank tumor xenografts were harvested, mechanically disaggregated, and grown in short-term cell culture (5-14 days) in DMEM supplemented with 2.5% fetal bovine serum, 1% penicillin, and 1% streptomycin. Cells were harvested by trypsinization and injected (3×10^5 or 1×10^6 cells per mouse, suspended in 10 μL) into the right basal ganglia of anesthetized athymic nude mice (athymic Ncr-*nu*/*nu*, National Cancer Institute, Frederick, MD) using a small animal stereotactic frame (ASI Instruments, Houston, TX). Just before treatment initiation, animals were randomized to treatment groups of 5 to 10 mice each. Radiation and/or EGFR small-molecule inhibitor therapy was initiated 2 weeks before the time mice were expected to become moribund, as established through preliminary studies with each xenograft line. Radiation was delivered to the entire head of unanesthetized mice, immobilized in a plastic restraint, through a single right lateral beam from a ^{137}Cs source. The remainder of the body was shielded with a lead block. Three different radiation schedules were used during the course of this study: 2 Gy M-W-F for 2 weeks (12 Gy total administered over 12 days), 2 Gy thrice daily for 2 days (12 Gy total), or 2 Gy twice daily for 5 days (20 Gy total). Erlotinib (courtesy of Dr. Ken Iwata, OSI Pharmaceuticals, Mellville, NY) was administered by oral gavage (100 or 150 mg/kg) either for 2 weeks or until moribund (daily, Monday through Friday). All mice used for therapy response evaluations were euthanized at the time of reaching a moribund condition.

Fluorescence in situ hybridization. Formalin-fixed, paraffin-embedded intracranial xenograft tissues were examined by fluorescence *in situ* hybridization for *EGFR* amplification. *EGFR* and chromosome 7 centromeric probes were labeled with Spectrum orange and Spectrum green (Vysis, Downers Grove, IL), respectively, and hybridized to tissue sections as previously described (18).

Western blotting. Flank tumor specimens were lysed in buffer A [20 mmol/L Tris HCL (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EGTA, 1.5 mmol/L $MgCl_2$, 10% glycerol, 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 10 μg/mL leupeptin, 20 nmol/L microcystin, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate]. Lysates were cleared of insoluble material by centrifugation. Samples were boiled in SDS sample buffer, equal amounts of protein were loaded and electrophoresed through SDS-PAGE gels, and resolved proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked with 5% milk dissolved in TBS containing 0.02% Tween 20 and then incubated with primary antibody diluted in the same buffer. After washing, membranes were incubated with either goat anti-rabbit (Cell Signaling, Danvers, MA) or goat anti-mouse (Pierce, Rockford, IL) antibodies conjugated to horseradish peroxidase. Blots were developed with Super Signal Chemiluminescence reagent (Pierce). Immunoblotting was done with phosphospecific antibodies first and then membranes were stripped and reprobed with the relevant nonphosphospecific antibodies. Antibodies used in this study were specific for total MAPK (Cell Signaling), phospho-T202/Y204 MAPK (Cell Signaling), total Akt (Cell Signaling), and phospho-S473 Akt (Cell Signaling).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were immunostained for total EGFR protein using mouse monoclonal antibody 528 (Oncogene Research Products, San Diego, CA) as previously described (23), with trypsin pretreatment and a primary antibody dilution of 1:50. Staining was visualized by use of the Dako Envision kit (Carpinteria, CA) according to the instructions of the manufacturer.

p53 and PTEN genetic analysis. High molecular weight DNAs isolated from flank tumor xenografts were used in preparatory PCRs to generate products for subsequent phosphatase and tensin homologue (*PTEN*) and *TP53* sequence analysis (all exons examined for *PTEN* and exons 5-8 examined for *TP53*; ref. 24). Tumor homozygous PTEN deletion was determined based on lack of PTEN PCR product from preparatory PCRs.

Statistical analysis. The Kaplan-Meier estimator (25) was used to generate the survival curves and to estimate the median survival values. Differences between survival curves were compared using a log-rank test (26). Two-way categorical comparisons (i.e., *EGFR* amplification versus radiation treatment sensitivity, PTEN status versus radiation treatment sensitivity, and p53 status versus radiation treatment sensitivity) were done using Fisher's exact test. All tests were two-sided and $P < 0.05$ was considered to be statistically significant.

Results

Patient characteristics. Each of the 13 serially passaged xenografts used in this study was derived from a different patient. For 12 of these patients, the tumor diagnosis was glioblastoma whereas in one case, #28, the diagnosis was that of gliosarcoma (Table 1). Information about radiation treatment was obtained for all patients, 11 of which received 6,000 to 6,480 cGy high-dose external beam radiation therapy following initial tumor resection. Patient #43 had radiation treatment discontinued at 2,880 cGy of a planned 6,000-cGy regimen because of tumor progression. Patient #44 did not

receive radiation therapy due to advanced age and poor performance status. For the 11 patients who completed high-dose radiation treatment, the time from completion of radiotherapy to clinical or radiographic progression was 2 to 9 months (Table 1). Overall survival for all 13 patients, measured from the date of initial tumor resection, was 3 to 31 months. Ten of these patients succumbed to progressive tumor; one patient (GBM12) died without evidence of recurrent cancer. Two of the patients were alive at most recent follow-up (GBM38 at 11 months and GBM39 at 20 months) but with recurrent, progressive tumor. The tissues used for xenograft establishment were obtained at the time of initial resection in 10 instances whereas in 3 instances (patients #10, #14, and #46), the tumor tissue was obtained at resection for recurrent disease after radiation therapy and salvage chemotherapy (Table 1).

Xenograft genotypes and intracranial radiation response. The radiation response of each of the 13 xenografts was evaluated using an orthotopic model (20). Tumor cell suspensions derived from short-term cultures of s.c. flank tumors were injected into the basal ganglia of nude mice, and mice were subsequently randomized to radiation (2 Gy × 6 fractions over 12 days) or sham treatment. On the basis of trial growth rate analyses conducted with each xenograft line, radiation therapy was initiated 2 weeks before the time at which mice were expected to become symptomatic of tumor burden (20). Mice were observed daily following tumor implantation and were euthanized on reaching a moribund state. Treatment with this relatively low-intensity regimen was effective in distinguishing radiation response among the 13 xenografts and allowed their classification as sensitive or resistant to this radiotherapy regimen on the basis of Kaplan-Meier statistical analysis (Table 2). Tumor *EGFR* amplification status (Table 2), of which the association with patient prognosis following radiation has been controversial, showed no association with radiation sensitivity or resistance (Fig. 1A). Four of six radiation-sensitive tumors were amplified for EGFR whereas three of seven radiation-resistant tumors had *EGFR* amplification ($P = 0.59$; Fig. 1B). The EGFR-amplified and radiation-sensitive tumors included two tumors that express the common activated truncation mutant, EGFR variant III (GBM3 and GBM39); the only other EGFR mutant tumor in the panel, EGFR variant II (27, 28), was among the xenografts resistant to the indicated radiation regimen (GBM36). Tumor p53 and PTEN status similarly showed no association with tumor sensitivity to this radiation regimen ($P = 1.00$ in both cases; Table 2).

Effects of alternative radiation regimens. To examine the potential influence of tumor cell repopulation on the response to the initial radiation schedule, we used four of the xenograft lines to compare survival effects of the original 12 Gy/12 day schedule (2 Gy, M-W-F × 2 weeks) versus an accelerated regimen of 12 Gy administered over 2 days (2 Gy thrice daily for 2 days, 6-hour intervals between fractions). For one of the EGFR-amplified xenografts, GBM12, the accelerated fractionation schedule resulted in a significant increase in survival of treated mice relative to the control group ($P = 0.05$; Table 3) whereas administration of the original 12-day radiation regimen once again produced no significant survival benefit ($P = 0.43$). A second EGFR-amplified xenograft, GBM15, showed similar levels of sensitivity to both 12- and 2-day administration regimens ($P < 0.01$, relative to control for each regimen). Nonamplified xenografts GBM10 and GBM14 both showed relative resistance to the accelerated radiation regimen.

To determine effects of a more intensive radiation treatment, we applied an accelerated and higher total dose regimen (2 Gy twice daily for 5 days, 20 Gy total) to six of the xenografts. GBM14, which had shown sensitivity to the initial regimen, also benefited from the more intensive regimen with a 38-day prolongation in median survival as compared with control, versus a 27 to 42 day prolongation in survival with the 12 Gy/12 day regimen relative to control. The more intensive radiation

Table 1. Patient characteristics

Xenograft	Age (y)/sex	Tumor location	Diagnosis	TTP (mo)	Survival (mo)
6	65/M	Frontal	Glioblastoma	5	13
8	74/F	Frontal	Glioblastoma	5	16
10*	41/M	Temporoparietal	Glioblastoma	7	26
12	68/M	Occipital	Glioblastoma	NA	3†
14*	57/M	Temporal	Glioblastoma	4	11
15	63/M	Frontal	Glioblastoma	9	25
28	67/M	Temporal	Gliosarcoma	4	8
36	53/M	Cerebellum	Glioblastoma	2	10
38	71/F	Temporal	Glioblastoma	3	11‖
39	51/M	Frontal	Glioblastoma	5	20‖
43	69/M	Temporal	Glioblastoma	0	3§
44	79/F	Frontal	Glioblastoma	NA	17‡
46	55/M	Frontoparietal	Glioblastoma	4	19

Abbreviation: TTP, time-to-progression.
*Xenograft line derived from recurrent tumor.
†Died from a pulmonary embolus with no evidence of recurrent disease.
‡Treated with supportive care only following resection.
§Disease progression during radiation therapy.
‖Alive with disease.

Table 2. Xenograft gene alterations and radiation sensitivities

Xenograft	Control survival*	RT survival*	$P^{\dagger}$	EGFR status‡	p53 status§	PTEN status§	p16 status§
6	65	116	0.01	$+^{v\,III}$	Mutant	wt	Null
14	62	89	<0.01	–	wt	Null	Null
15	52	99	<0.01	$+^{wt}$	wt	wt	Null
28	41	53	0.04	–	Mutant	Mutant	wt
38	61	68	0.01	$+^{wt}$	Mutant	wt	Null
39	88	>135	<0.01	$+^{v\,III}$	wt	wt	Null
8	82	90	0.49	$+^{wt}$	wt	Null	Null
10	41	39	0.95	–	wt	Null	Null
12	36	41	0.35	$+^{wt}$	Null	wt	Null
36	67	62	0.57	–	Mutant	Null	Null
43	24	24	0.19	–	Mutant	wt	Null
44	65	62	0.52	–	wt	wt	Null
46	51	53	0.40	$+^{v\,II}$	ND	ND	Null

*Control/placebo and radiation treatment (RT) survivals represent median values in days.
†P values indicated are based on Kaplan-Meier survival analysis.
‡Tumors with EGFR amplification are indicated with "+" and amplified tumors are either indicated as wild-type (wt), vIII mutant, or vII mutant.
§p53, PTEN, and p16 status are either normal/wild-type (wt), missense mutant (mutant), or mutant with lack of detectable protein (null).

regimen significantly enhanced the survival of mice with intracranial GBM12 or GBM43, which were resistant to the 12 Gy/12 day regimen: 20-Gy treatment increased survival by 15 days ($P < 0.01$) and 16 days ($P = 0.02$), respectively, as compared with control. In contrast, xenografts GBM8, GBM10, and GBM44 continued to display radiation resistance irrespective of the regimen used (Table 3).

Analysis of EGFR signaling mediators. Because signaling through phosphatidylinositol 3-kinase/Akt and Ras/MAPK pathways have been implicated in modulating the response of tumor cells to radiation, we examined the basal activation status of Akt and MAPK in xenograft tissues in an effort to identify potential associations with tumor radiation sensitivity (Fig. 2). There was no apparent correlation between MAPK activation and radiation response because phospho-MAPK was readily detectable in all but one radiation-sensitive and one radiation-resistant xenograft. In contrast, increased phosphorylation of Akt was associated with radiation resistance: each of the three flank tumors with low/undetectable phospho-Akt were among the radiation-sensitive tumors whereas all of the radiation-resistant tumors showed significant levels of Akt phosphorylation. There was no readily apparent association between the presence of detectable phospho-Akt and corresponding xenograft EGFR and/or PTEN status (Fig. 2; Table 2).

Effects of EGFR inhibition on radiation response. As an additional approach to examining potential relationships between EGFR signaling and glioblastoma radiation response, we evaluated the efficacy of erlotinib, a highly specific, Food and Drug Administration–approved EGFR kinase inhibitor, administered singularly and in combination with radiation. Erlotinib alone (100 mg/kg/d by oral gavage, 5×/wk for 2 weeks) showed antitumor activity against GBM12 ($P = 0.04$; Fig. 3A) but there was no indication of additive survival benefit of erlotinib given concurrently with the 12 Gy/12 day radiation regimen, relative to erlotinib alone, for mice with intracranial GBM12 ($P = 0.98$; Fig. 3A). Results from the analysis of intracranial tumor phospho-EGFR content in erlotinib-treated versus untreated mice showed the effective inhibition of xenograft EGFR signaling through oral administration of this small-molecule inhibitor.[5]

Because the more aggressive radiation regimens had produced survival benefit for mice with intracranial GBM12 (Table 3), we also tested erlotinib in combination with the increased and accelerated radiation treatment (20 Gy/5 day) to determine whether a more efficacious radiation regimen would benefit from concomitant EGFR inhibition. For this experiment, a slightly higher dose of erlotinib (150 mg/kg daily PO M-F) was used and dosing was extended until death in the relevant arms to mimic more closely how erlotinib might be used in patients. As before, oral administration of erlotinib provided significant survival benefit to mice with intracranial GBM12 ($P < 0.01$) and, as previously noted, the 20 Gy/5 day radiation regimen proved the most effective of the three radiation treatments used with this xenograft (Table 3). The most extensive survival benefit, however, was experienced as a result of combination therapy, which increased median survival by 22, 14.5, and 7 days relative to untreated control, erlotinib only, and radiation only treatment groups, respectively (Fig. 3B).

Discussion

The identification of molecular features that predict for radiation response potentially could be used to individualize therapy for patients with glioblastoma multiforme. One potential glioblastoma molecular classification scheme is based on the presence or absence of signature gene alterations, and among these genetic changes, loss of PTEN function and

[5] In preparation.

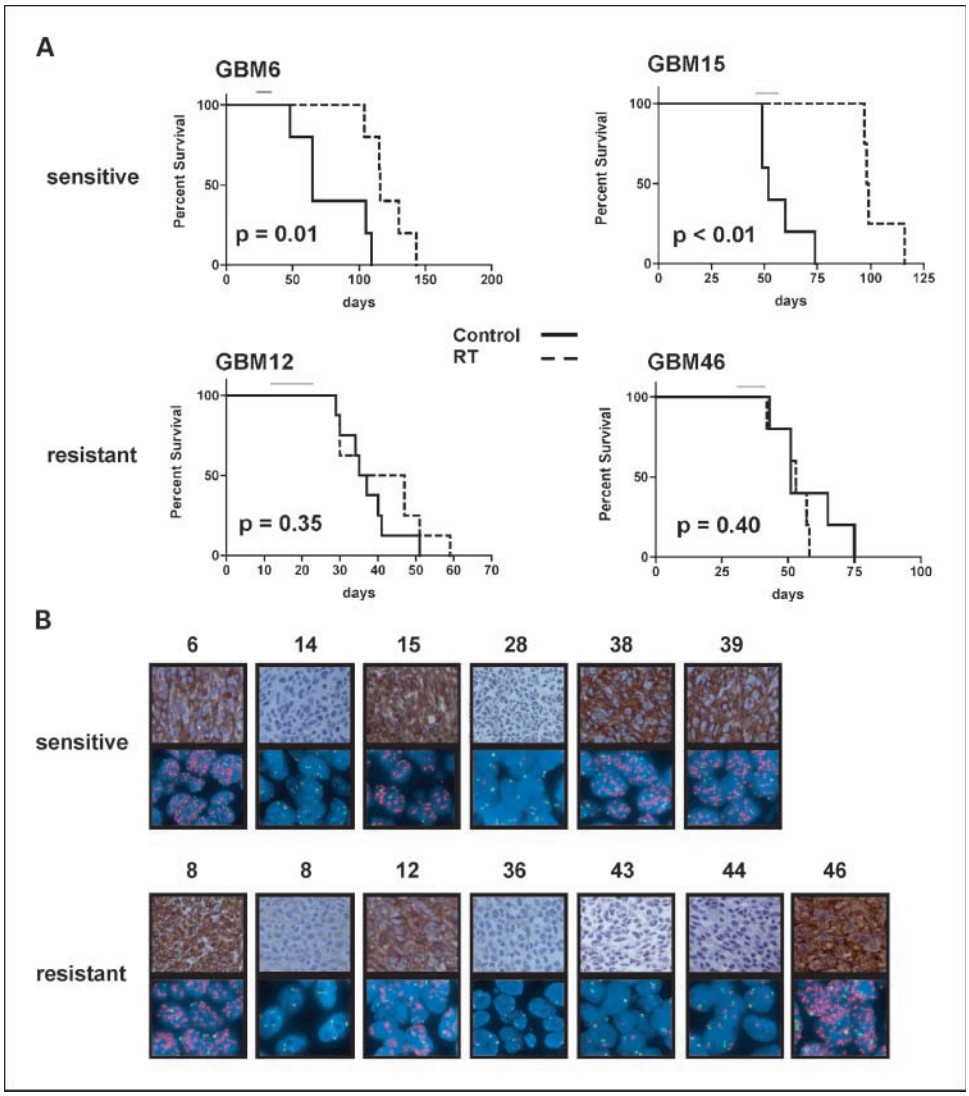

Fig. 1. *A,* Kaplan-Meier survival analysis of intracranial glioblastoma xenograft radiation treatment response. As detailed in Materials and Methods, cells from glioblastoma xenografts were injected intracranially into nude mice that were subsequently randomized into sham (control) versus radiation treatment groups. Radiation treatment was initiated 2 weeks before expected presentation of symptoms indicative of tumor burden and was administered to provide a total radiation dose of 12 Gy (2 Gy M-W-F × 2 weeks: period of treatment indicated by the gray bar above each survival plot). Results shown are for four EGFR-amplified xenografts: mice with intracranial GBM6 and GBM15 experienced significant survival benefit from the indicated radiation regimen whereas mice with intracranial GBM12 and GBM46 did not experience significant survival benefit. *B,* results from EGFR immunohistochemical (*top*) and fluorescence *in situ* hybridization (*bottom*) analyses for each xenograft line. Results are from intracranial tumor specimens derived from the sham/control groups of the survival experiments reported in Table 2. The intense brown immunohistochemical staining in glioblastomas 6, 8, 12, 15, 38, 39, and 46 is indicative of high-level EGFR protein expression that accompanies *EGFR* amplification, which is indicated by the fluorescence *in situ* hybridization results showing increased red (EGFR probe) to green (chromosome 7 centromere marker) signal in the same specimens.

amplification of *EGFR* have been associated with a worse prognosis in patients treated with radiation therapy (29, 30). Moreover, constitutive signaling within the pathways affected by these gene alterations has been linked with increased radiation resistance in cell culture studies. Unfortunately, the influence of *EGFR* amplification and the corresponding high-level expression of EGFR cannot be directly assessed with established cell culture approaches because sustained *in vitro* propagation of glioblastoma cells selects against *EGFR* amplification (17, 18). However, amplified EGFR can be maintained in glioblastoma tumors that are propagated as xenografts in immune-deficient mice, and through the use of this approach, we have developed a number glioblastoma xenograft lines with sustainable *EGFR* amplification that can be used for addressing *EGFR* amplification–associated hypotheses.

In this study, we have used a panel of glioblastoma xenografts to evaluate the relationship between signature molecular features of glioblastoma and radiation responsiveness, and our data specifically show that *EGFR* amplification is not predictive for glioblastoma response to a low-dose radiation regimen (Fig. 1; Table 2). The clinical outcomes for patients whose tumors were used to establish these xenograft lines also support this idea, with mean overall survival for the *EGFR* amplification group somewhat greater (17.3 months, $n = 6$) than that of patients with amplification-negative glioblastoma (12.5 months, $n = 6$). Thus, both from the xenograft and clinical data, there is no indication of amplified EGFR conferring an unfavorable prognosis in glioblastoma. Whereas the patient and corresponding xenograft cohort we have examined is small, our conclusion is nonetheless consistent with results recently published in association with the retrospective analysis of a large series of glioblastoma patients treated with conventional radiation therapy regimens (12). We would point out, however, that another retrospective clinical investigation has suggested that *EGFR* amplification was associated with an adverse prognosis (11), and it is precisely because of the discordant conclusions of such studies that we were motivated to apply an alternative approach to investigating potential EGFR-glioblastoma radiation response relationships. In combination with the clinical data, our results support the idea that *EGFR* amplification will not be an effective biomarker predictive of radiation responsiveness.

Laboratory studies with glioblastoma tumor models suggest that persistent signaling from phosphatidylinositol 3-kinase

through Akt is associated with radiation resistance (31–33), and an analysis of glioblastoma tumor specimens has shown an association between increased phosphorylation of Akt on Ser^{473} and decreased survival in patients receiving radiation therapy (34). Consistent with these studies, Western blot analysis of the flank tumor specimens used for establishing the orthotopic xenografts evaluated in the current radiation sensitivity analysis has revealed that all of the radiation-resistant tumors have easily detectable levels of phospho-Akt whereas three of the radiation-sensitive tumors have no readily detectable Akt phosphorylation (Fig. 2). Interestingly, neither the radiation responsiveness nor extent of Akt phosphorylation correlated with corresponding xenograft genetic status of *PTEN*, which encodes a lipid phosphatase that normally dampens phosphatidylinositol 3-kinase–dependent signaling (34). Consequently, our observations suggest that Akt hyperactivation in glioblastomas is influenced by EGFR-independent as well as PTEN-independent mechanisms, and that Akt hyperactivation may be related to a radiation-resistant phenotype. Given the results in Fig. 2, it would be of interest to examine the efficacy of Akt signal transduction inhibitors in combination with radiation in the current panel of xenografts.

Table 3. Radiosensitivity effects associated with regimen modifications

Xenograft	Radiation regimen	Survival differential* (d)	*P*
8	12 Gy/12 d	8†	0.78†
8	20 Gy/5 d	0	0.40
10	12 Gy/12 d	−2†,‡	0.95†
10	12 Gy/12 d	−2‡,§	0.10§
10	12 Gy/2 d	2§	0.64§
10	20 Gy/5 d	3	0.07
12	12 Gy/12 d	5†	0.35†
12	12 Gy/12 d	1§	0.43§
12	12 Gy/2 d	6§	0.05§
12	20 Gy/5 d	15	<0.01
14	12 Gy/12 d	27†	<0.01†
14	12 Gy/12 d	42§	<0.01§
14	12 Gy/2 d	22§	<0.01§
14	20 Gy/5 d	38	<0.01
15	12 Gy/12 d	47†	<0.01†
15	12 Gy/12 d	147§	<0.01§
15	12 Gy/2 d	156§	<0.01§
43	12 Gy/12 d	0†	0.19†
43	20 Gy/5 d	16	0.02
44	12 Gy/12 d	−3†,‡	0.52†
44	20 Gy/5 d	−3‡	0.92

*Survival differentials represent the difference in median survival for control versus radiation treatment arms. All experiments had between 7 and 10 animals per group.
†Denotes results from the initial 12 Gy/12 day survival comparison (Table 2), which are shown here to facilitate comparison with results from subsequent experiments.
‡Negative numbers denote instances in which the median survival of the radiation treatment group was less than that of the control group.
§Denotes results from experiments in which there were both 12 Gy/12 day and 12 Gy/2 day.

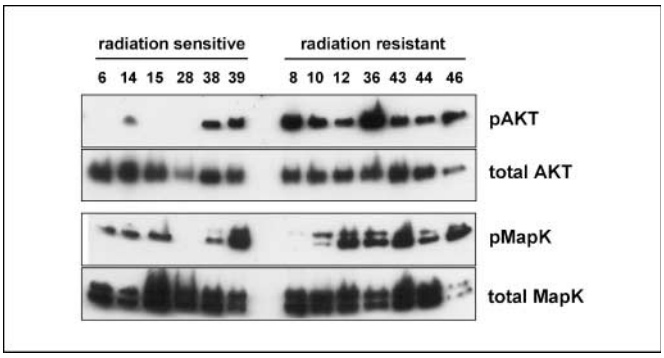

Fig. 2. Western blot analysis of MAPK and Akt phosphorylation status in the flank tumors that were used to prepare short-term cell cultures for the intracranial injection and analysis of xenograft radiation response (Table 2). Note the lack of detectable phospho-Ser^{473}-Akt in three of the radiation-sensitive xenografts (6, 15, and 28).

A comparison of xenograft radiation response and patient tumor time-to-progression (Table 1) suggests that the orthotopic xenograft model may be a reasonable predictor of clinical radiation responsiveness. For those instances in which xenografts were established from initial surgical (pretreatment) specimens and from patients who received radiation therapy subsequent to surgery, the average time-to-progression for patients from which radiation-sensitive xenografts were derived was 5.2 months (glioblastomas 6, 15, 28, 38, and 39: range, 3-9 months) whereas the average time-to-progression for patients from which radiation-resistant xenografts were derived was 2.3 months (glioblastomas 8, 36, and 43: range, 0-5 months; $P = 0.18$, Wilcoxon rank-sum test). Although indicative of a trend towards longer time-to-progression for patient tumors that gave rise to radiation-sensitive xenografts, the limited number of patients available for this analysis and the adjuvant therapies received by some patients limit the conclusions that can be drawn from these data.

Tumor proliferation during a fractionated course of radiotherapy (tumor repopulation) can contribute to apparent radiation resistance, and constitutive EGFR signaling is associated with increased tumor proliferation. Therefore, to adequately study the potential influences of *EGFR* amplification on radiation, we tested all of the xenograft lines with a protracted radiation regimen of 12 Gy in 12 days. On the basis of previous experience with similar treatment regimens using xenografts from established glioma cell lines,[6] this treatment regimen was anticipated to distinguish radiation-sensitive from moderate to highly resistant tumors. To address whether radiation resistance in the protracted regimen might be associated with repopulation (reviewed in ref. 35), a subset of tumor lines were treated with an accelerated regimen: 12 Gy delivered over 2 days (2 Gy thrice daily for 2 days). Of the xenograft lines assessed using the two 12-Gy regimens, the highly sensitive GBM15 line and the radioresistant GBM10 line did not significantly benefit from the accelerated regimen. Whereas the accelerated treatment regimen was associated with an improved survival as compared with placebo in GBM12, there was not a statistically significant difference when the survival results were compared directly between the two 12-Gy treatment regimens ($P = 0.14$). Interestingly, the accelerated treatment regimen (12 Gy/2 day)

[6] Unpublished data.

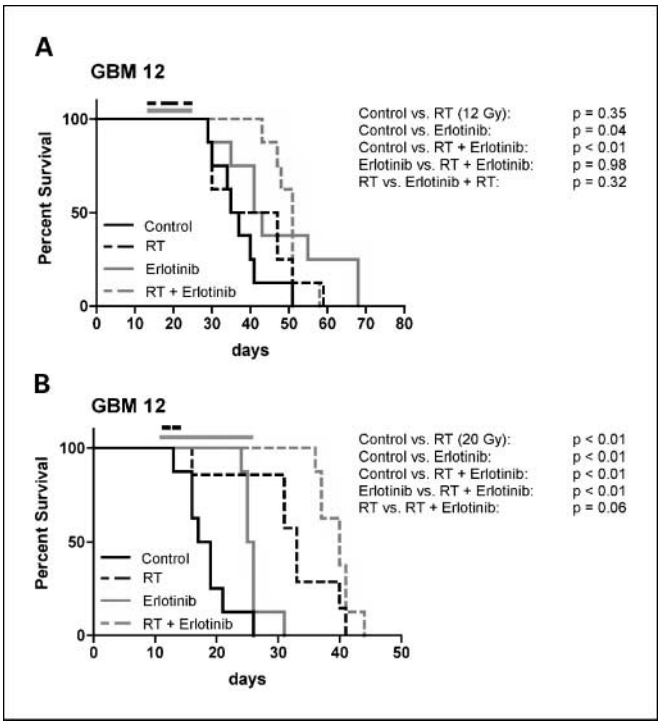

Fig. 3. Effect of EGFR small-molecule inhibition on intracranial xenograft radiation response. *A,* mice with intracranial GBM12 were randomized to one of four treatment groups: sham (control), radiation treatment (*RT*; 2 Gy M-W-F for 2 weeks, 12 Gy total), erlotinib (100 mg/kg administered orally, M-F for 2 weeks), and radiation treatment + erlotinib administered concurrently according to the same protocols used to administer each as a monotherapy (*broken black line* and *solid gray line,* period of time over which radiation and erlotinib were administered, respectively). Kaplan-Meier survival analysis shows significant survival benefit from erlotinib administered alone or in combination with radiation treatment as compared with control ($P = 0.04$ and 0.01, respectively). *B,* the experiment in (*A*) was repeated but with a radiation treatment regimen of 2 Gy delivered 2×/d for 5 consecutive days (20 Gy total) and erlotinib administered at 150 mg/kg M-F until mice were removed from the study as a result of becoming moribund. In this experiment, oral administration of erlotinib again shows antitumor effect by significantly extending the survival of treated mice relative to control. In addition, the accelerated and higher-dose radiation treatment confers survival benefit to treated mice and the combined therapeutic regimen shows survival benefit beyond that associated with either agent administered as a monotherapy.

in GBM14 was significantly less effective than the 12 Gy/12 day regimen ($P = 0.03$). Thus, tumor repopulation does not seem to be a significant factor influencing the responsiveness of the xenograft lines examined. A more intensive and accelerated radiation regimen (2 Gy twice daily for 5 days, 20 Gy total) was also evaluated in a subset of xenograft lines. When applied to the radiation-resistant xenograft lines, as defined by the 12 Gy/12 day regimen, one of two EGFR-amplified and one of three nonamplified xenografts showed significant response to this more intensive regimen (Table 3). Collectively, these data highlight the spectrum of radiation responsiveness seen in the glioblastoma xenograft model from marked radioresistance (GBM44) to significant radiosensitivity (GBM15).

The EGFR has not only been considered as a biomarker for radiation response but has also been examined as a pharmacologic target for enhancing the efficacy of radiation therapy. Several studies in flank xenograft models have shown that inhibition of EGFR signaling with either small-molecule kinase inhibitors or anti-EGFR antibodies can significantly enhance the efficacy of radiation therapy (29, 30). Moreover, in a randomized clinical trial, an anti-EGFR antibody significantly improved tumor control and survival when administered concurrently with radiation therapy in head and neck squamous cell cancers (36). In the current report, we have included results from a single xenograft tested with erlotinib (GBM12; Fig. 3) in attempt to address whether disruption of EGFR signaling acts to radiosensitize glioblastoma. Interestingly, concurrent inhibition of EGFR signaling by erlotinib combined with the low-dose radiation regimen (12 Gy in 12 days) was associated with a modest but not significant improvement in survival as compared with either treatment alone. When erlotinib therapy was given during a more intensive radiation regimen (20 Gy in 5 days) and extended until mice became symptomatic of tumor burden, combination therapy provided additive survival benefit as compared with either treatment administered alone. In conjunction with the data showing no correlation between radiation responsiveness and *EGFR* amplification status in the xenograft panel, these combination therapy data suggest that constitutive EGFR signaling is not a major determinant of radiation responsiveness in glioblastoma. However, the data do suggest that continuous suppression of EGFR signaling during and after radiotherapy may be of benefit in treating some glioblastoma tumors, and this strategy is currently being tested in a North Central Cancer Treatment Group Phase II clinical trial in newly diagnosed glioblastoma patients.

In total, the results of this study indicate that common gene alterations in glioblastoma, including *EGFR* amplification, are not singularly predictive of tumor radiation response. However, the distribution of radiation response among the panel of xenografts we have examined suggests their utility in a more extensive molecular profiling that could lead to the identification of patterns predictive of tumor radiation sensitivity and may prove useful in developing novel therapeutic treatment strategies.

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