Research Article

High-Grade Glioma Formation Results from Postnatal Pten Loss or Mutant Epidermal Growth Factor Receptor Expression in a Transgenic Mouse Glioma Model

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Abstract

High-grade gliomas are devastating brain tumors associated with a mean survival of <50 weeks. Two of the most common genetic changes observed in these tumors are overexpression/mutation of the epidermal growth factor receptor (EGFR) vIII and loss of PTEN/MMAC1 expression. To determine whether somatically acquired EGFRvIII expression or Pten loss accelerates high-grade glioma development, we used a previously characterized RasB8 glioma-prone mouse strain, in which these specific genetic changes were focally introduced at 4 weeks of age. We show that both postnatal EGFRvIII expression and *Pten* inactivation in RasB8 mice potentiate high-grade glioma development. Moreover, we observe a concordant loss of Pten and EGFR overexpression in nearly all high-grade gliomas induced by either EGFRvIII introduction or *Pten* inactivation. This novel preclinical model of high-grade glioma will be useful in evaluating brain tumor therapies targeted to the pathways specifically dysregulated by EGFR expression or Pten loss. (Cancer Res 2006; 66(15): 7429-37)

Introduction

High-grade gliomas are the most common brain tumors in adults, accounting for ~60% of all gliomas (1). The most malignant grade 4 astrocytoma (glioblastoma multiforme) is composed of poorly differentiated neoplastic astrocytes (a subtype of glial cell), developing from a preexisting low-grade astrocytoma (LGA) or arising *de novo* in an individual without a previously identified brain tumor. Those tumors that form *de novo* are termed primary glioblastoma multiforme, whereas those that progress from LGAs are termed secondary glioblastoma multiforme. Irrespective of their developmental origin, glioblastoma multiformes are clinically indistinguishable and associated with a dismal prognosis, with a mean survival of <1 year. Two of the most commonly observed single genetic changes in both primary and secondary glioblastoma multiformes are overexpression and amplification of the epidermal growth factor receptor (EGFR) with or without an activating mutation (2–5) and loss of PTEN expression (6–10). The most common EGFR mutation consists of an aberrantly spliced form that lacks exons 3 to 6 (EGFRvIII), resulting in a constitutively active receptor (11, 12). Because of the frequency of EGFR amplification/mutation in glioblastoma multiformes, small-molecule EGFR inhibitors and EGFRvIII antibodies are currently being studied as glioblastoma multiforme therapies (13, 14). Loss of PTEN expression, resulting in aberrant activation of the phosphatidylinositol 3-kinase signaling pathway in glioblastoma multiformes, has similarly led to therapeutic interest in targeting this signaling pathway. The importance of these two genetic alterations toward modulating the therapeutic response of glioblastoma multiformes is highlighted by recent data, suggesting that EGFR inhibitors are more effective in glioblastoma multiformes where EGFRvIII and PTEN are both expressed but are not effective when PTEN expression is lost (15). The frequency of these genetic events and the interest in developing biologically based therapies underscore the need to develop robust preclinical models of high-grade gliomas harboring these genetic changes.

Several preclinical mouse glioma models have shown that aberrant expression of EGFRvIII or loss of Pten expression in embryonic and/or adult glial cells by themselves do not lead to glioma formation (16–19). However, mouse glial cells harboring additional glioma predisposing genetic alterations, in conjunction with increased EGFRvIII or decreased Pten expression, develop high-grade gliomas, supportive of their role in glioma progression. For example, glioma formation induced by a truncated version of the SV40 large T antigen (T_{121}) is markedly accelerated by breeding to $Pten^{+/-}$ mice (20). Similarly, two studies with introduction of EGFRvIII in glial cells, which are null for *Ink4a* inactivation, led to high-grade glioma formation (16, 17). Our previously described glioma-prone mice (RasB8 mice), in which activated oncogenic Ha-Ras (V^{12}Ha-Ras) is expressed under regulation of the human glial fibrillary acidic protein (GFAP; hGFAP) promoter commencing at embryonic days E14 to E16, are also supportive of these findings (21, 22). RasB8 pups are viable but commence development of progressive diffuse astroglial hyperproliferation early after birth, with subsequent development of low-grade gliomas (30% of the mice at 2 months of age) and then high-grade gliomas between 4 to 6 months, with majority of the gliomas being of astrocytic lineage. In addition to pathologic progression to higher-grade gliomas, there is molecular progression with acquisition of additional genetic alterations, reminiscent of "secondary" human glioblastoma multiforme. These include *Tp53* mutations in the low-grade gliomas and overexpression of EGFR and loss of Pten in the high-grade gliomas (21, 22). In contrast to V^{12}Ha-Ras, hGFAP-regulated

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expression of wild-type (WT) or mutant EGFRvIII by itself did not lead to glioma formation. However, double transgenics created from EGFRvIII (not WT EGFR) and RasB8-bred mice developed high-grade gliomas at a significantly earlier age than RasB8 mice alone (18).

Although the preclinical models to date are supportive to directly evaluate the contribution of EGFRvIII expression and PTEN loss to glioma malignant progression, these genetic changes should be introduced as somatically acquired and temporally distinct events in genetically engineered mice prone to glioma formation. In this regard, one recent study showed that high-grade glioma formation was accelerated when somatic viral-mediated *Pten* inactivation was introduced in 5- to 8-week-old GFAP-T_{121} glioma-prone mice (23). However, analogous data for EGFR is not available. In this report, we provide the first study, in which EGFRvIII expression and Pten loss acquired as somatic "second hits" postnatally in a single glioma-prone model system greatly potentiates high-grade glioma formation. The availability of this novel preclinical model of high-grade glioma formation will be invaluable for analyzing therapies specifically targeted to the pathways deregulated by EGFRvIII expression or Pten loss in glioblastoma multiforme.

Materials and Methods

Mice. All mice were maintained at the University of Toronto (Toronto, Ontario, Canada) in accordance with institutional animal care protocols. *hGFAP:V^{12}Ha-Ras:IRES:LacZ* (RasB8) mice were maintained as described previously (21). Mice expressing a conditional *Pten* allele (*$Pten^{f/f}$*) were generously provided by Dr. Tak Mak (Ontario Cancer Institute, Toronto, Ontario, Canada; ref. 24). The *$Pten^{f/f}$* mice were mated with mice expressing Cre recombinase under the control of the hGFAP promoter (25). *$Pten^{f/f}$*;hGFAP-Cre (*$Pten^{hGFAP}$*CKO) mice were compared with control *$Pten^{f/wt}$*;hGFAP-Cre littermates.

Cell culture. Primary astrocyte cultures from CD1-ICR mouse pups were prepared as described (21). HEK293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and antibiotics.

Adenovirus generation. Rat EGFRvIII cDNA was constructed using stepwise PCR to delete an 801 nt sequence of the extracellular domain of rat EGFR cDNA template (a kind gift from S. Earp, University of North Carolina, Durham, NC). Rat EGFRvIII was cloned into pcDNA3 (Invitrogen, Carlsbad, CA) and verified by direct sequencing. To generate Ad5-EGFRvIII virus (Ad:EGFRvIII), the rat EGFRvIII was subcloned into the pAdCMV-Link 1 plasmid (Fig. 1*A*), and virus was produced by homologous recombination in human embryonic kidney (HEK293) cells as described previously (26). We tested and made sure that the recombinant Ad:EGFRvIII vector was replication incompetent by making sure it lacked the WT adenoviral E1A and E1B transcripts by PCR, which was only present in the helper 293 cells. The resulting recombinant virus was grown and titrated in HEK293 cells infected with Ad:GFP or Ad:EGFRvIII virus [4×10^9, 4×10^8, 4×10^7, 4×10^6, or 4×10^5 plaque-forming units (pfu)]. Two days after infection, green fluorescent protein (GFP) or EGFRvIII expression was determined by Western blotting and immunofluorescence as described previously (18, 21). EGFRvIII expression was verified using an antibody specific for human and rat EGFRvIII (13).

Adenoviral Cre-recombinase (Ad:Cre; Ad:CMV-Cre-IRES-EGFP) was constructed using the Transpose-Ad Adenoviral Vector System (Q-Biogene, Montreal, Quebec, Canada). An EGFP tag was added to Cre recombinase (gift from Dr. A. Nagy, Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada) and subcloned into pCR259, a transfer vector containing the human cytomegalovirus (CMV) promoter/enhancer (Q-Biogene). The adenovirus (E1 and E3 deletion) was generated by homologous recombination of pCR259-Cre-IRES-EGFP with the Transpose-Ad 294 plasmid. An identical strategy, without Cre recombinase, was used to manufacture the control Ad:GFP virus. Recombinant virus was grown and titrated in human embryonic kidney (HEK293) cells. Expression of Cre was confirmed using Western analysis (antibody from Novagen, Madison, WI; data not shown), and then the adenoviral constructs were sent to Aegera (Iles-des-soeurs, Quebec, Canada) for amplification and purification.

Intracranial injection of adenovirus. Four-week-old *hGFAP:V^{12}Ha-Ras:IRES:LacZ* transgenic mice (RasB8; refs. 21, 22) and CD1-ICR control littermates were used in accordance with institutional animal care policies. Mice were anesthetized with ketamine (60 mg/kg) and xylazine (7.5 mg/kg). A small burr hole was drilled into the skull anterior to the coronal and lateral to the sagittal suture under sterile conditions. The appropriate adenovirus was injected slowly ($\sim$15 seconds/μL) at a concentration of 4×10^7 pfu in 5 μL DMEM supplemented with 5% FBS. To minimize backflow, the needle was left in place for 30 seconds following completion of the injection. The mice were observed for any neurologic symptoms or failure to thrive at which point they were euthanized as per institutional animal care guidelines. The brain was fixed in formalin for 48 hours. Coronal sections (2 mm) were embedded in paraffin blocks for serial sectioning.

Immunohistochemical analysis. Paraffin-embedded brains were cut in 5-μm serial sections for immunohistochemical analysis using microwave antigen retrieval. Primary antibodies included GFAP (1:3,000; DAKO, Glostrup, Denmark), Nestin (1:1,000; Chemicon International, Temecula, CA), EGFRvIII (1:10; Novocastra, Newcastle upon Tyne, United Kingdom; recognizes human, rat, but not mouse EGFRvIII), Olig2 (1:10,000; gift from Dr. C. Stiles, Dana-Farber Cancer Institute, Boston, MA), mouse anti-adenovirus antibody (1:100; Chemicon International.), and β-galactosidase (1:10,000; Promega, Madison, WI). LacZ staining was used to detect expression of the *hGFAP:V^{12}Ha-Ras:IRES:LacZ* transgene (data not shown; ref. 21). Secondary biotinylated antibodies (Vector Laboratories, Burlingame, CA) and the avidin-biotin-peroxidase complex method were used for detection with diaminobenzidine tetrachloride (Vector Laboratories). For immunofluorescence staining, FITC-conjugated secondary antibodies and, for double-labeling experiments, a rhodamine trimethylrhodamine isothiocyanate (TRITC)–conjugated secondary antibody were used (Jackson Immunoresearch, West Grove, PA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). In some experiments, the total number of astrocytes was quantitated by counting cells that were positive for both GFAP and DAPI staining.

Bromodeoxyuridine labeling. Mice were injected with 50 mg/kg bromodeoxyuridine (BrdUrd) 2 hours before euthanasia. Plane-matched sections were stained by double labeling with BrdUrd (1:1,000; Caltag, Burlingame, CA) and GFAP (1:3,000), and the number of BrdUrd-expressing cells were counted.

Magnetic resonance imaging. Magnetic resonance imaging (MRI) was done on a 7-Tesla MRI scanner (Varian, Palo Alto, CA) specifically outfitted for multiple-mouse MRI (27). Mice were injected with 20 mg/kg $MnCl_2$ i.p. and imaged 48 hours later. Mice were anesthetized with 1% isofluorane in O_2, and body temperature was maintained at 37°C with flowing warm air during imaging. Mice were imaged with a three-dimensional spin-echo pulse sequence in 2 hours and 45 minutes (TR = 300 ms, TE = 10 ms, readout $\times$ phase $\times$ phase = $256 \times 128 \times 128$, FOV = 4 cm $\times$ 2 cm $\times$ 2 cm, NEX = 2) to produce 156-μm isotropic imaging voxel. Following imaging, brains were removed and sectioned for histopathology.

Statistical analysis. Mean values and SDs were analyzed by ANOVA followed by Bonferoni t test with significance set at $P < 0.05$.

Results

Somatic expression of EGFRvIII in the RasB8 brain results in high-grade glioma formation *in vivo*. The results from the previously reported EGFRvIII-RasB8 double transgenics showed that simultaneous embryonic activation of Ras and EGFRvIII were associated with high-grade gliomagenesis (18). However, these experiments did not specifically address the role of mutant EGFRvIII in high-grade glioma progression when acquired as a temporally distinct secondary somatic genetic alteration. To directly examine the relationship between EGFRvIII expression

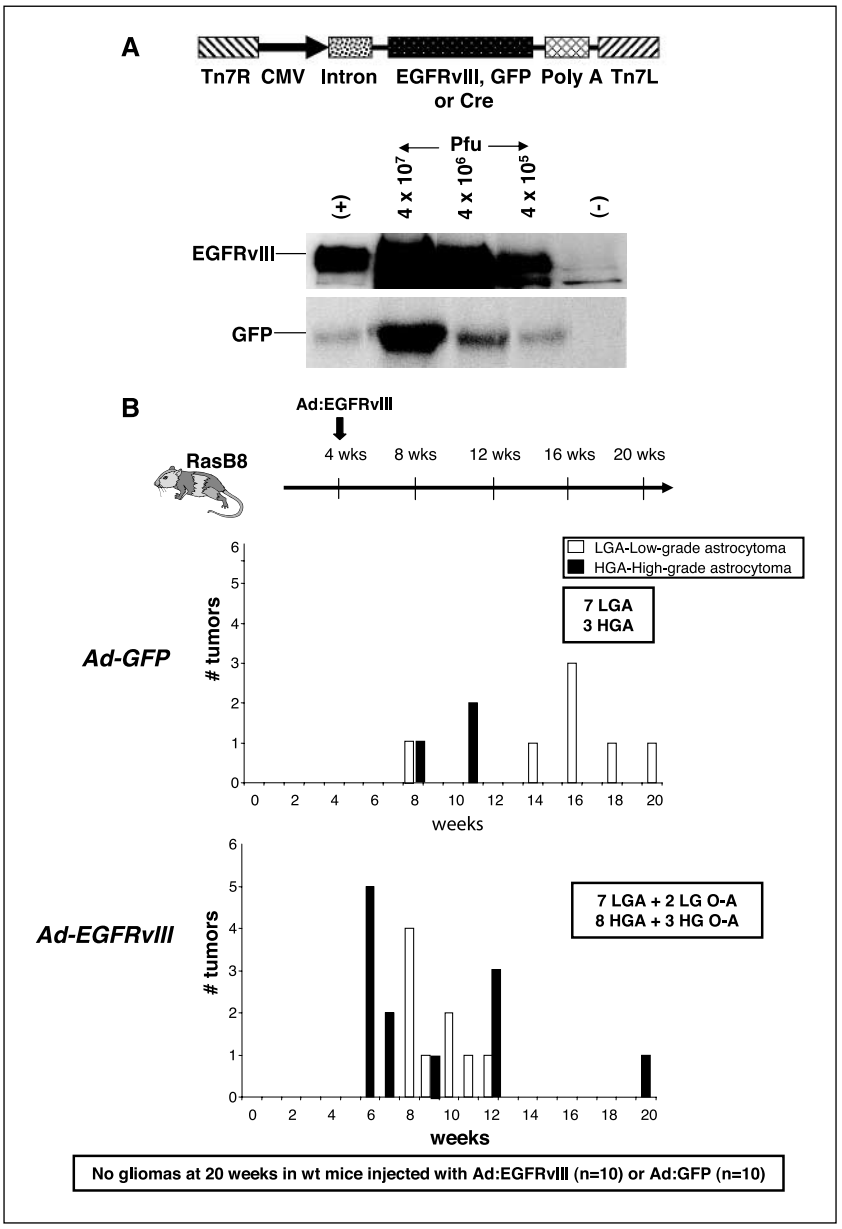


Figure 1. Postnatal CNS expression of EGFRvIII. *A, top,* pAdCMV-Link 1 plasmid construct used to generate Ad:EGFRvIII or Ad:GFP. Recombinant adenoviruses, made replication deficient by removal of the WT adenoviral E1A and E1B regions, were generated by homologous recombination in HEK293 cells, with gene expression driven by the human CMV immediate early promoter/enhancer. *Bottom,* expression of EGFRvIII and GFP was confirmed by infecting 2×10^5 to 3×10^5 astrocytes derived from newborn CD1-ICR mice with Ad:EGFRvIII and Ad:GFP (4×10^7, 4×10^6, and 4×10^5 pfu). Two days after infection, Western blot analysis for EGFRvIII and GFP (Clontech) was undertaken. Ad:EGFRvIII (4×10^7 pfu) and Ad:GFP (4×10^7 pfu) were used for the intracranial mice injections. *B, top,* stereotactic adenoviral injections into the frontal lobes of 4-week-old RasB8 and age-matched CD1-ICR control WT mice. Injection of either Ad:EGFRvIII or Ad:GFP into WT mice did not lead to glioma formation. *Middle,* Ad:GFP-injected RasB8 mice mainly harbored LGAs (7 of 10 tumors), which were not localized around the injection site and occurred predominantly after 12 weeks of age (6 of 10 tumors). This pattern of glioma formation is similar to that previously documented for the RasB8 model. *Bottom,* Ad:EGFRvIII injections into RasB8 mice resulted in mainly high-grade gliomas [HGA or HG O-A, 11 of 20 tumors], which commenced from the injection site but infiltrated the surrounding white matter tracks (see Figs. 2 and 3) and occurred early (19 of 20 tumors before 12 weeks) compared with Ad:GFP-injected or RasB8 mice.

and glioma progression, we sought to determine if expression of EGFRvIII postnatally in the RasB8 glioma-prone mice resulted in high-grade gliomas. For these experiments, we generated adenovirus expressing EGFRvIII and GFP (Ad:EGFRvIII; Ad:GFP; Fig. 1*A*) and determined that infection of primary astrocytes with 4×10^7 pfu resulted in robust GFP and EGFRvIII expression with no associated cellular toxicity. This concentration of virus was used for subsequent *in vivo* experiments.

Somatic EGFRvIII expression in the postnatal brain was achieved by adenovirus injections into the frontal lobes of 4-week-old RasB8 transgenic mice or control CD1-ICR littermates. Previous studies have shown that by 4 weeks of age, RasB8 mice exhibit widespread hyperplasia consisting of nontransformed, $GFAP^+$ astrocytes (22). Consistent with our previous studies, 70% of RasB8 mice injected with the control Ad:GFP developed LGAs, and the majority of these developed after 13 weeks of age (Fig. 1*B*). Less commonly, tumors with pathologic features of high-grade glioma were observed. In contrast, RasB8 mice injected with Ad:EGFRvIII began to develop tumors earlier, with 19 of 20 mice developing gliomas by 12 weeks of age (8 weeks after adenoviral injection). Furthermore, unlike the low-grade gliomas in Ad:GFP-injected mice, the majority of tumors in Ad:EGFRvIII-treated RasB8 mice (11 of 20) exhibited pathologic features of high-grade glioma. These included nuclear pleiomorphism, pseudopalisading necrosis of the tumor core, and invasion into surrounding normal brain parenchyma similar to human glioblastoma multiformes (Fig. 2*A*). The majority of the gliomas (low- or high-grade) were astrocytic, with strong expression of the astrocyte marker GFAP and weak expression of the oligodendrocyte marker Olig2 in the tumor cells. A subset of gliomas in the Ad:EGFRvIII-treated mice (5 of 20) exhibited a mixed oligo-astrocyte morphology, with both $GFAP^+$ and $Olig2^+$ glioma cells. Like the pure astrocytic tumors, the majority of the mixed oligo-astrocyte gliomas (3 of 5) were high grade and developed within 4 weeks of injection (Fig. 2*A*). Importantly, WT littermates

injected with Ad:EGFRvIII or Ad:GFP did not develop gliomas after 20 weeks of age (data not shown). This is consistent with our previous studies showing that EGFRvIII expression alone is insufficient for astrocytoma formation (18). Taken together, these data show that postnatal expression of EGFRvIII in RasB8 glioma-prone mice results in high-grade glioma formation *in vivo*.

High-grade glioma formation correlates with EGFRvIII expression. To determine if EGFRvIII expression is specifically associated with high-grade glioma formation or simply accelerates gliomagenesis, we did immunohistochemistry on tumor sections using an antibody that recognizes rat EGFRvIII. We evaluated two LGA and high-grade astrocytoma (HGA) from the 12-week cohort. We reasoned that if EGFRvIII expression was required for high-grade glioma formation, all resulting high-grade tumors would harbor this genetic change. Consistent with our hypothesis, only the high-grade gliomas, irrespective of whether they were astrocytic (7) or mixed [high-grade oligo-astrocytoma (HG O-A)], expressed EGFRvIII (Fig. 2*A*). Furthermore, immunofluorescent detection of adenoviral protein was not found in low-grade but present in high-grade gliomas, such as the LGA and HGA from 12-week-old Ad:EGFRvIII-injected mice shown in Fig. 2*B*. Expression of adenoviral protein and corresponding EGFRvIII in these 12-week-old high-grade gliomas (8 weeks after inoculation; Fig. 1*B*) does not imply adenoviral replication in the dividing glioma cells because the extra chromosomal recombinant Ad:EGFRvIII is rendered replication incompetent. However, continued adenoviral expression may be present for long periods in the initial transfected cells, and transfer of extra chromosomal adenoviral elements to daughter cells can occur, depending on initial number of viral particles infected per cell. Together, these data suggest that

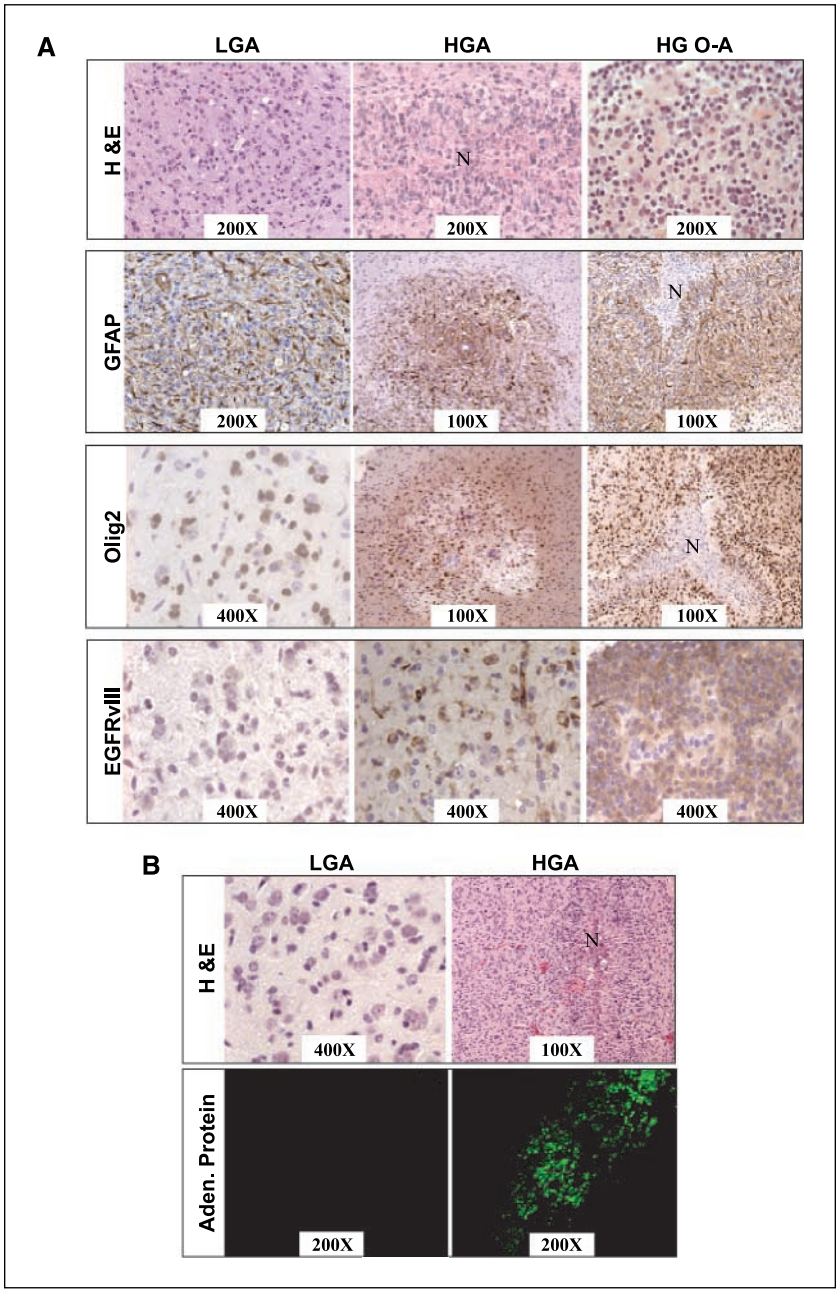


Figure 2. Gliomas in the Ad:EGFRvIII-injected RasB8 mice. *A,* LGA (7 of 20), HGA (8 of 20), and HG A-O (3 of 20). LGAs were GFAP$^+$ invasive tumors, with scant Olig2 expression without detectable EGFRvIII expression. HGAs were GFAP$^+$ invasive tumors with pathologic similar characteristics of high-grade human astrocytomas, including pseudopalisading necrosis (*N*). Some of the GFAP$^+$ astrocytoma cells were also Olig2$^+$, with most expressing EGFRvIII. HG O-A had both transformed astrocytes and oligodendrocytes (GFAP$^+$ and Olig2$^+$), with regions of necrosis. Like HGA, HG O-A also expressed EGFRvIII. *B,* LGA and HGA in 12-week-old Ad:EGFRvIII RasB8 mice showed continued adenoviral core protein expression (antibody courtesy of Dr. T-J. Liu, M.D. Anderson Cancer Center Houston, TX) only in the HGA, consistent with expression of EGFRvIII in the high-grade and not low-grade gliomas (*A*).

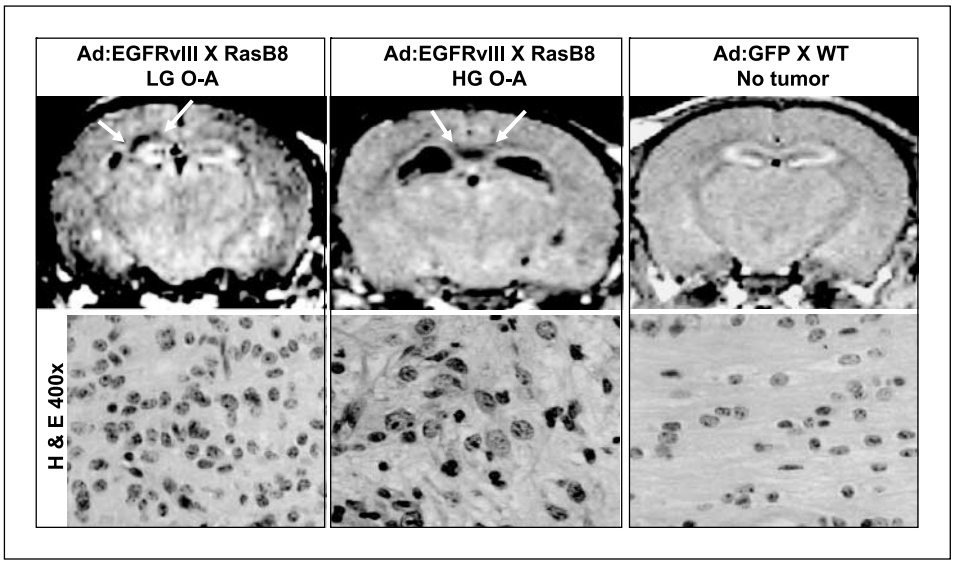


Figure 3. Examples of gliomas detected by small animal MRI after $MnCl_2$ contrast enhancement in Ad:EGFRvIII-injected RasB8 mice at 12 weeks of age. Invasive low-grade oligo-astrocytoma and HG O-A into the white matter tracks of the corpus collosum are seen as dark lesions (*white arrows*). H&E sections corresponding to MRI lesions confirm presence of the gliomas.

EGFRvIII expression, independent of glioma histologic subtype, is specifically associated with high-grade glioma formation.

Gliomas in RasB8;Ad:EGFRvIII can be detected by MRI using a Mn^{2+} contrast agent. Previous attempts to visualize brain tumors in our RasB8 glioma model have been unsuccessful, most likely due to the multifocal and small tumors that develop in this model.[6] Because human gliomas are radiographically detected and followed by MRI, we sought to determine if we could improve our ability to visualize gliomas in the RasB8;Ad:EGFRvIII mice as an initial step toward using these mice as a preclinical high-grade glioma small animal model. We took advantage of a novel Mn^{2+} contrast agent that acts as an internalized Ca^{2+} analogue in neurons and astrocytes and results in a bright signal on T1-weighed magnetic resonance images in normal mouse brains (28). Recent studies have shown that this method allows for fine anatomic studies during brain development (29) as well as tumor detection in mice (30). We hypothesized that the normal influx of Mn^{2+} cations in glioma-associated cells would be disrupted, resulting in a hypointense signal in Mn^{2+}-enhanced images of tumor-bearing mice. In three of four RasB8;Ad:EGFRvIII mice imaged, we observed dark lesions by magnetic resonance analysis, whereas no such abnormal signal was detected in control mice injected with Ad:GFP. The presence of glioma in each of the three mice with abnormal neuroimaging was confirmed by pathologic analysis (Fig. 3), with no tumor detected in the fourth MRI^- mouse. These preliminary imaging data indicates the potential use of this model of high-grade glioma for preclinical studies analyzing tumor response to therapies that target pathways deregulated by EGFRvIII expression.

Glial *Pten* inactivation *in vivo* results in postnatal lethality. To directly test the role of Pten in gliomagenesis, we also used hGFAP-regulated trangenesis and our RasB8 glioma model. We first generated double transgenics, in which *Pten* was conditionally inactivated in astrocytes by crossing mice expressing conditional *Pten* alleles ($Pten^{f/f}$) to mice that express Cre recombinase under the control of the hGFAP promoter (hGFAP-Cre; ref. 25). The resulting *Pten* conditional knockout mice ($Pten^{hGFAP}$CKO) were indistinguishable from their control littermates at birth. However, by 2 weeks of age, $Pten^{hGFAP}$CKO mice were less active, assumed a retracted posture, and showed abnormal movements, characteristic of generalized seizures. At 2 weeks of age, 2 of 17 $Pten^{hGFAP}$CKO mice were dead following recurrent seizure-like activity, and all were dead by 6 weeks of age (Fig. 4*A*).

Consistent with previous report (19), brains from $Pten^{hGFAP}$CKO mice showed a progressive increase in mass relative to body size (Fig. 4*B*), although no gliomas were observed. By 4 weeks of age, the brains of $Pten^{hGFAP}$CKO mice weighed ~30% more than that of control littermates. Immunohistochemical analysis showed loss of Pten expression and high levels of active, phosphorylated Akt in $GFAP^+$ cells in the $Pten^{hGFAP}$CKO mice (Fig. 4*C*). To determine if the increased brain weight in the $Pten^{hGFAP}$CKO mice was due to increased astroglial cell proliferation, we counted $GFAP^+$ cells in plane-matched sections of the cortex and hippocampus from $Pten^{hGFAP}$CKO and $Pten^{f/wt}$;h*GFAP*-Cre control mice. In both cortex and hippocampus, there was a 3-fold increase in the number of astrocytes in $Pten^{hGFAP}$CKO mice (Fig. 4*D*). Using BrdUrd labeling in 2-week-old $Pten^{hGFAP}$CKO and control mice, we found a 10-fold increase in BrdUrd labeling in all brain regions examined (Fig. 4*E*). In contrast, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining, to identify apoptotic cells, did not show any significant differences between $Pten^{hGFAP}$CKO mice and controls (Fig. 4*E*). These data indicate that, although inactivation of *Pten* in astrocytes increases astrocyte proliferation and promotes an overall increase in brain size, it is insufficient for glioma formation within the life span of the mice. Furthermore, because the $Pten^{hGFAP}$CKO mice die before 6 weeks of age, a transgenic approach, in which *Pten* inactivation and oncogenic Ha-Ras expression are both controlled by the hGFAP promoter, as had been used in our previous studies of the EGFRvIII (18) could not be done.

Somatic inactivation of Pten in the RasB8 brain results in high-grade glioma formation *in vivo*. Having ascertained that embryonic excision of Pten, even restricted to $GFAP^+$ glial cells, was vital for normal central nervous system (CNS) development, we elected to use Ad:Cre injections to delete Pten in brains of 4-week-old $Pten^{f/f}$ and $Pten^{f/wt}$ mice. Excision of Pten by itself did not lead to any tumor formation, similar to expression of EGFRvIII in normal mouse brains (Fig. 5*C*). We then proceeded to determine

[6] Garbow, D.H. Gutmann, and A. Guha, unpublished data.

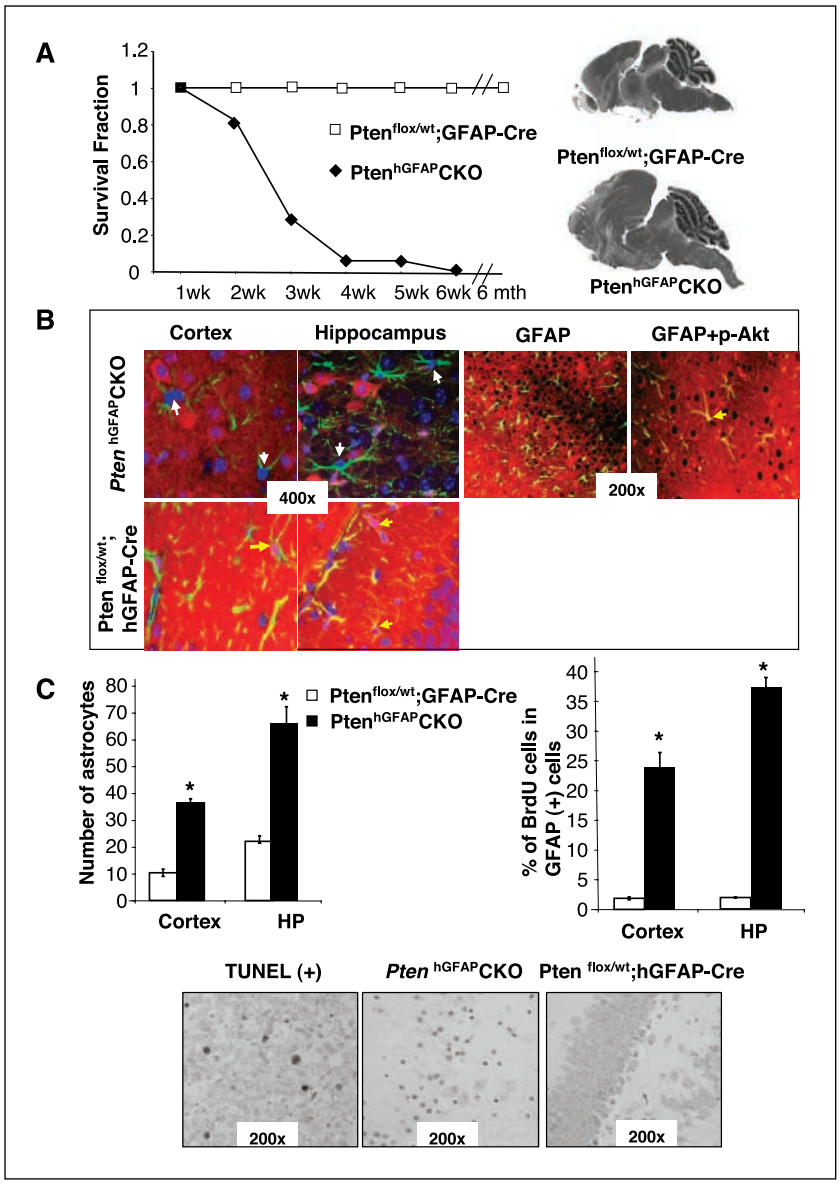


Figure 4. Embryonic GFAP-regulated knockout of *Pten*. *A, left,* survival of *Pten*^{hGFAP}CKO mice (*solid box*; $n = 17$) was dramatically decreased relative to that of controls (*open box*; $n = 16$). All *Pten*^{hGFAP}CKO mice died between 2 to 6 weeks after birth, whereas the *Pten*$^{f/wt}$;h*GFAP-Cre* control mice were normal up to 6 months of observation. *Right,* sagittal H&E sections of same magnification from 2-week-old mouse brain of *Pten*^{hGFAP}CKO and *Pten*$^{f/wt}$;hGFAP-*Cre* control littermates, showing predominant enlargement of cerebellum and cortex in *Pten*^{hGFAP}CKO mice. *B,* excision of *Pten* and activation of Akt in *Pten*^{hGFAP}CKO mice: colocalization of GFAP$^+$ [*GFAP*(+)] astrocytes [FITC-labeled anti-GFAP (*green*)], Pten (*red*) with nuclear DAPI (*blue*) staining of 2-week-old *Pten*^{hGFAP}CKO mouse cortex and hippocampus (*HP*) compared with age-matched *Pten*$^{f/wt}$;h*GFAP-Cre* controls. Pten expression is lost in most of astrocytes of *Pten*^{hGFAP}CKO mice (cells with green cytoplasm and blue nuclei; *white arrows*) in contrast to astrocytes in the *Pten*$^{f/wt}$;h*GFAP-Cre* control mice, where Pten expression is maintained (cells with blue-pink nuclei; *yellow arrows*). Double immunofluorescence staining with phosphorylated Akt (TRITC; *red*) and GFAP (FITC; *green*) indicates phosphorylated Akt colocalizing with GFAP$^+$ cells (*yellow arrows*) in the brains of *Pten*^{hGFAP}CKO mice. Bar, 100 μm. *C, left,* number of GFAP$^+$ and DAPI$^+$ astrocytes, in plane-matched sections from *Pten*^{hGFAP}CKO mice (*black columns*) and *Pten*$^{f/wt}$;h*GFAP*-Cre controls (*white columns*), in the cortex and hippocampus. Number of astrocytes were significantly increased in all regions of the *Pten*^{hGFAP}CKO mice compared with *Pten*$^{f/wt}$;h*GFAP*-Cre controls (*, $P < 0.0001$). *Right,* percentage of BrdUrd$^+$ astrocytes in brains of 2-week-old *Pten*^{hGFAP}CKO mice (*black columns*) and *Pten*$^{f/wt}$;h*GFAP*-Cre controls (*white columns*) in the cortex and hippocampus. Proliferating BrdUrd$^+$ astrocytes were significantly increased in all regions of the *Pten*^{hGFAP}CKO mice compared with *Pten*$^{f/wt}$;h*GFAP*-Cre controls (*, $P < 0.0001$). *Bottom,* TUNEL staining did not reveal any differences in number of apoptotic cells in *Pten*^{hGFAP}CKO and *Pten*$^{f/wt}$;h*GFAP*-Cre control mice in all regions examined [cortex, subcortical white matter and hippocampus (shown) and cerebellar white matter]. Breast carcinoma section was used as a positive control. Bar, 100 μm.

whether *Pten* inactivation in the context of a glioma-predisposed mouse, such as RasB8, would result in glioma progression. Double transgenics of RasB8;*Pten*$^{f/f}$ and RasB8;*Pten*$^{f/wt}$ mice were created, genotyped, and confirmed to have the same glioma incidence as previously reported RasB8 mice (data not shown; refs. 21, 22). Intracranial Ad:Cre injections were undertaken in these 4-week-old double transgenics and sacrificed on failure to thrive as per institutional animal care guidelines (Fig. 5*A*). Six of the nine RasB8;*Pten*$^{f/wt}$;Ad:Cre-injected mice developed gliomas by 15 weeks of age, with majority (four of six) being LGAs (Figs. 5*C* and 6*A*). In contrast, 11 of 14 of RasB8;*Pten*$^{f/f}$;Ad:Cre mice developed high-grade gliomas (astrocytomas) with 8 of 11 of these tumors developing within 4 weeks of injection (8 weeks of age; Fig. 5*B*). Histopathologically, these GFAP$^+$ tumors exhibited characteristic features of HGAs, including hypercellularity, increased nuclear to cytoplasmic ratio, pleiomorphic nuclei, hypervascularity, and occasional regions of necrosis (Fig. 6*A*). Taken together, these data support a role for *Pten* loss in progression of gliomas to increased malignancy but not initiation.

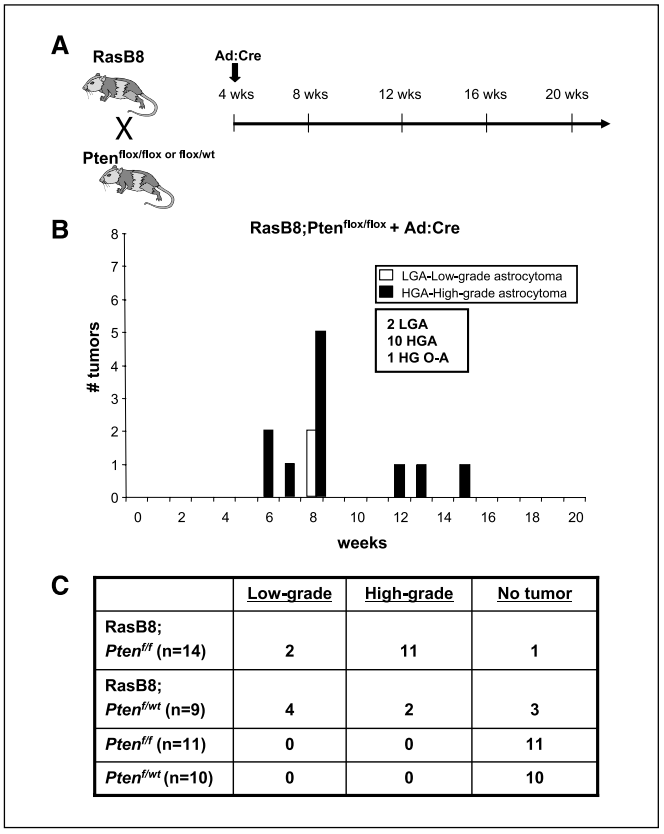
Molecular epidemiologic studies suggest that 20% to 30% of human glioblastoma multiformes harbor both EGFR amplification/mutation and *PTEN* inactivation, with implications for the response to biologically based tumor therapy (15). We therefore sought to determine if high-grade gliomas that resulted from somatic expression of EGFRvIII or loss of Pten expression in RasB8 model exhibited evidence of the reciprocal genetic event (Fig. 6*B*). Because available EGFR antibodies do not recognize mouse EGFRvIII, we examined high-grade gliomas for increased total EGFR expression. In all six RasB8;*Pten*$^{f/f}$;Ad:Cre HGAs analyzed, EGFR was overexpressed. Conversely, in four of six RasB8;Ad:EGFRvIII high-grade gliomas, Pten expression was lost. These data indicate that EGFR mutation or amplification and *Pten* inactivation frequently occur concordantly in high-grade gliomas, regardless of the initiating genetic change.

Discussion

Although multiple genetic changes have been identified in human gliomas, a few of these changes have been implicated in the

progression from low-grade to high-grade glioma (1, 31). In one large study of 240 glioblastoma multiformes (7), the most common genetic changes observed included loss of heterozygosity at chromosome 10q (69%), EGFR amplification (34%), *Tp53* mutation (31%), *CDKN2A* homozygous deletion (31%), and *PTEN* mutation (24%). Similarly, a series of 174 newly diagnosed gliomas (32) showed that EGFR amplification, *PTEN* mutation, and loss of 10q occur more commonly in glioblastoma multiformes than in the next lower grade 3 anaplastic astrocytomas, suggesting that these genetic events may contribute to the formation of glioblastoma multiformes. Although these molecular epidemiologic studies confirm that EGFR overexpression and/or *PTEN* mutation occurs frequently in glioblastoma multiformes, it remains unclear if these genetic changes have prognostic significance or are directly involved in malignant progression of gliomas.

Several studies suggest that EGFR overexpression is associated with poorer patient survival (33–35), whereas other studies have found that EGFR amplification has no independent prognostic significance (7, 36–38) and may even be associated with longer survival in some patient populations (7, 32, 38–41). The prognostic implications for the subset of glioblastoma multiformes, which express mutant EGFRvIII, are also not clear. In a cohort of 50 glioblastoma multiforme patients followed at our institution, EGFRvIII expression overall was not a significant determinant of prognosis; however, in younger patients (<50 years of age), detection of EGFRvIII was a significant negative survival prognosticator.[7] Similarly, although EGFRvIII expression lacked overall prognostic value in glioblastoma multiformes in a cohort of 44 patients, expression of the mutant receptor helped differentiate a subgroup of anaplastic astrocytomas whose clinical course was as virulent as glioblastoma multiforme (42). Similarly, loss of PTEN expression has been associated with poor patient prognosis in some studies (38, 39) but not in others (7, 40, 41).



	Low-grade	High-grade	No tumor
RasB8; *Pten*$^{f/f}$ (n=14)	2	11	1
RasB8; *Pten*$^{f/wt}$ (n=9)	4	2	3
Pten$^{f/f}$ (n=11)	0	0	11
Pten$^{f/wt}$ (n=10)	0	0	10

Figure 5. Postnatal CNS knockout of *Pten*. *A,* four-week-old *Pten*$^{f/f}$ or *Pten*$^{f/wt}$ mice or double transgenics created by mating these to RasB8 mice, which underwent stereotactic adenoviral injections into the frontal lobe with Ad:Cre or Ad:GFP control. *B,* glioma formation in RasB8;*Pten*$^{f/f}$ double transgenics injected with Ad:Cre at 4 weeks of age. There were increased numbers of predominantly astrocytic gliomas, which were of higher grade compared with RasB8;*Pten*$^{f/wt}$; Ad:Cre-injected mice. *C,* glioma formation with somatic (4-week-old) knockout of *Pten* by Ad:Cre injection. Excision of both (*Pten*$^{f/f}$) or single (*Pten*$^{f/wt}$) copy of *Pten* by itself does not initiate glioma formation. Glioma formation was potentiated by excision of *Pten* in the RasB8 glioma prone mice. Excision of both copies of *Pten* in RasB8;*Pten*$^{f/f}$ mice by Ad:Cre markedly accelerated high-grade glioma formation (see *B*) compared with excision of one copy of *Pten* in RasB8;*Pten*$^{f/wt}$ mice.

To directly test whether EGFRvIII and/or loss of Pten expression is involved in malignant progression of gliomas, genetically engineered murine (GEM) glioma models are an attractive strategy to develop robust preclinical models to test novel therapies. Using the same initiating event (V^{12}Ha-Ras overexpression) in the glioma-prone RasB8 mouse strain, we directly analyzed the role of somatic events (EGFRvIII expression or *Pten* inactivation) on astrocytoma malignant progression. Our studies show that glial expression of EGFRvIII or *Pten* inactivation by itself does not initiate gliomagenesis but greatly accelerates glioma malignant progression in the glioma-prone RasB8 mouse. Adding to our previous report of glioma progression with germ-line expression of EGFRvIII in RasB8 mice (18), here, we show that somatic expression of EGFRvIII in RasB8 mice leads to development of gliomas at an earlier age (19 of 20 gliomas, RasB8;Ad:EGFRvIII versus 4 of 10 gliomas, RasB8 mice before 12 weeks). Second, the majority of these gliomas (11 of 20 RasB8;Ad:EGFRvIII versus 3 of 10 RasB8) exhibited pathologic features associated with high-grade human gliomas. Lastly, expression of adenoviral-mediated rat EGFRvIII, in only the high-grade gliomas in RasB8;Ad:EGFRvIII mice, is another strong indication of the direct role of EGFRvIII in glioma progression.

Of additional interest, in contrast to mainly oligodendroglial lineage gliomas promoted by germ-line expression of EGFRvIII in RasB8 mice (18), the majority of high-grade gliomas in the RasB8;Ad:EGFRvIII mice were predominantly astrocytic tumors (eight HGA and three HG O-A). This data and those of others using other GEM glioma models are consistent with the hypothesis that the specific genetic change and the developmental stage of the target cell (cell of origin) are both important determinants of tumor lineage (17, 43).

Similar to somatic EGFRvIII expression (18), *Pten* inactivation in astrocytes as a single genetic change does not promote glioma formation *in vivo*. However, embryonic loss of Pten expression has profound effects on brain development (19, 44). Consistent with previous studies, *Pten*^{hGFAP}CKO mice exhibit decreased survival, increased brain mass, and an increased number of proliferating astrocytes (19). However, during the short life span of these mice, no gliomas were detected. Similarly, focal inactivation of *Pten* by injection of adenovirus encoding Cre recombinase into the brains of *Pten*$^{f/f}$ mice in our experiments did not develop gliomas. Taken together, these data indicate that *Pten* inactivation is not critical for glioma initiation.

In contrast to focal somatic *Pten* inactivation in a glioma-prone GEM model (RasB8), we observed a dramatic acceleration of

[7] A. Guha, unpublished data.

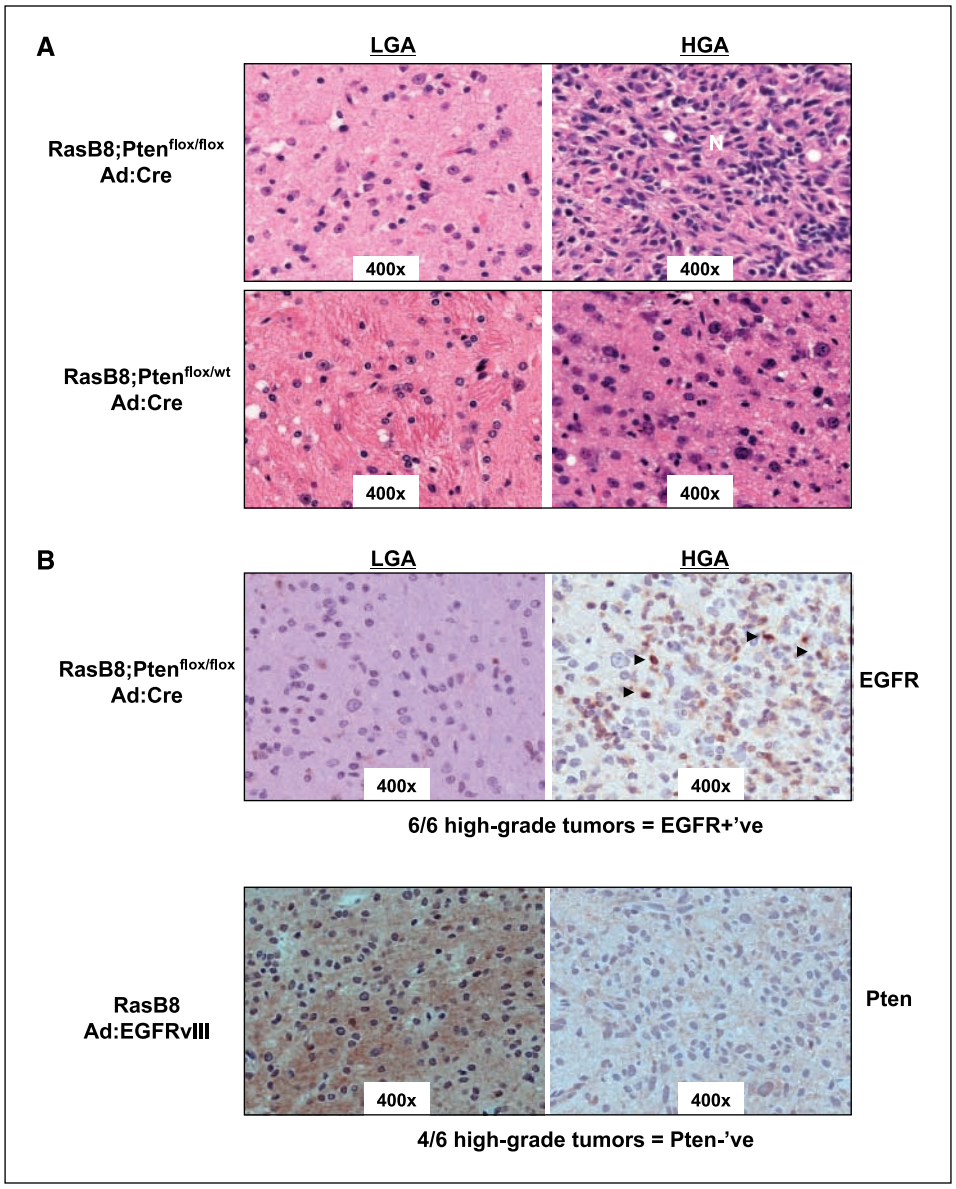


Figure 6. Gliomas in *Pten*-deleted RasB8 mice. *A,* LGA and HGA in the Ad:Cre-injected RasB8;*Pten*$^{f/f}$ and RasB8;*Pten*$^{f/wt}$ double transgenics. The predominant GFAP^{+} astrocytomas were invasive, with the HGAs harboring pleomorphic astrocytes with regions of necrosis. *B,* concordant aberrations in EGFR and Pten in high-grade gliomas. *Top,* increased EGFR (WT) expression in HGAs (*black arrowheads*), but not LGAs, which developed after excision of Pten by Ad:Cre in 4-week-old RasB8;*Pten*$^{f/f}$ mice; *bottom,* loss of Pten expression in HGAs, but not LGAs, which developed after Ad:EGFRvIII injection in 4-week-old RasB8 mice.

high-grade glioma formation. These data provide *in vivo* evidence of the role of *Pten* in astrocytoma progression and are consistent with a previous study showing that *Pten* activation in mice with Rb and p53 pathway inactivation (T_{121} GEM glioma model) promoted astrocytoma malignant progression (23). Interestingly, *Pten* heterozygosity also promoted gliomagenesis, as seven of nine RasB8;*Pten*$^{f/wt}$;Ad:Cre mice developed gliomas, although these tumors were more likely to be low-grade neoplasms. This is consistent with previous studies showing that *Pten* haploinsufficiency promotes tumor formation in other tissues (20, 45).

Based on our observations that EGFRvIII expression or *Pten* inactivation promotes malignant progression of gliomas, we sought to determine if reciprocal loss of these two progression factors occurs in high-grade gliomas. In all of the high-grade gliomas resulting from *Pten* inactivation, EGFR was overexpressed as determined by immunohistochemical analysis. We were unable to determine if the mutated receptor EGFRvIII was responsible for this overexpression, as available antibodies to EGFRvIII only detect the human, rat, and not mouse EGFRvIII. Similar to our results examining EGFR overexpression, in four of six high-grade gliomas resulting from EGFRvIII overexpression, Pten expression was absent. These data are intriguing in light of a recent report examining tumor response to EGFR kinase inhibitors (15). In this study, although 7 of 13 patients with coexpression of EGFRvIII and PTEN responded to erlotinib therapy, none of the 13 patients with EGFRvIII expression and PTEN loss responded. The high rate of coordinate EGFR amplification/mutation with *Pten* loss in high-grade glioma-prone mice provides a preclinical model for validating this finding *in vivo* as well as examining the use of alternative therapies for high-grade gliomas that fail to respond to this biologically based therapy.

In this report, we provide the first study to directly examine the contribution of EGFRvIII expression and *Pten* inactivation toward glioma malignant progression when these changes are acquired as somatic second hits in a single glioma-prone GEM model system. These data strongly suggest that both EGFR amplification/mutation and *Pten* loss are important genetic changes, which directly influence progression of low-grade to high-grade glioma.

Moreover, we show that these small high-grade tumors can be detected *in vivo* using magnetic resonance–based imaging techniques, thereby providing a rapid method for detecting and following sequentially these small tumors in large numbers of intact animals. The adaptation of this method provides a rapid method for detecting these small tumors in the intact animal and facilitates the use of this GEM glioma model for preclinical therapeutic trials using biologically based agents directed against intracellular signaling pathways deregulated by EGFR activation and *PTEN* loss.

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