Cancer Therapy: Preclinical

Rapid and Robust Transgenic High-Grade Glioma Mouse Models for Therapy Intervention Studies

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Abstract

Purpose: To develop a transgenic mouse model of glioma that can be conveniently used for testing therapy intervention strategies. High-grade glioma is a devastating and uniformly fatal disease for which better therapy is urgently needed. Typical for high-grade glioma is that glioma cells infiltrate extensively into surrounding pivotal brain structures, thereby rendering current treatments largely ineffective. Evaluation of novel therapies requires the availability of appropriate glioma mouse models.

Experimental Design: High-grade gliomas were induced by stereotactic intracranial injection of lentiviral GFAP-Cre or CMV-Cre vectors into compound LoxP-conditional mice, resulting in K-RasV12 expression and loss of p16Ink4a/p19Arf with or without concomitant loss of p53 or Pten.

Results: Tumors reproduced many of the features that are characteristic for human high-grade gliomas, including invasiveness and blood-brain barrier functionality. Especially, CMV-Cre injection into p53; Ink4a/Arf;K-RasV12 mice resulted in high-grade glioma with a short tumor latency (2-3 weeks) and full penetrance. Early detection and follow-up was accomplished by noninvasive bioluminescence imaging, and the practical utility for therapy intervention was shown in a study with temozolomide.

Conclusion: We have developed a realistic high-grade glioma model that can be used with almost the same convenience as traditional xenograft models, thus allowing its implementation at the forefront of preclinical evaluation of new treatments. Clin Cancer Res; 16(13); 3431–41. ©2010 AACR.

High-grade gliomas, in particular glioblastoma multiforme (GBM), are the most common primary brain tumors in adults and among the deadliest of human cancers (1). Their location and the extensive infiltration of tumor cells into surrounding pivotal brain structures are an impediment for all therapeutic interventions (2). The real challenge in improving the survival of GBM patients is to design therapies against the numerous malignant cells that cannot be removed by surgical resection, as they have migrated (deeply) into the normal surrounding brain parenchyma. Radiation therapy shows too many neurotoxic side effects to enlarge the high-dose target volume to include these glioma cells. Furthermore, most chemotherapeutic agents cannot reach the invasive tumor cells because these reside in regions where the vasculature is largely intact with functional blood-brain barrier (BBB) properties. Appropriate mouse models of glioma may support the search for better therapies. We have therefore developed a glioma mouse model that is practically applicable for use in intervention studies.

Gliomas are classified from grade 1 (more benign) to grade 4 (highly malignant; GBM) according to the WHO system (2), which is based on histologic features (e.g., mitotic activity, microvascular proliferation, and pseudopalisading necrosis). GBMs can be distinguished into primary GBMs, which rapidly arise without clinical evidence of a previously existing lower-grade lesion, and secondary GBMs, which develop through progression from low-grade astrocytomas or anaplastic astrocytomas. Although primary and secondary GBMs are histologically indistinguishable, there are differences in their molecular pathology (3).

During recent years, genomic analyses of tumor material have provided better insight into the genetic alterations associated with high-grade gliomas. Common alterations include amplification or activating mutations of EGFR, PDGFR, and CDK4 and inactivation by mutation or deletion of the tumor suppressor genes INK4A-ARF, p53, PTEN, and RB (for review, see refs. 4, 5). These alterations cause increased activation of growth factor signaling...
pathways [PI3K/Akt/mammalian target of rapamycin (mTOR) and Ras/mitogen-activated protein kinase (MAPK)] and disrupted cell cycle regulatory pathways (INK4A/CDK4/RB and Arf/MDM2/p53). Understanding the molecular pathology underlying GBM now permits targeted therapeutic intervention strategies (6), which need to be evaluated in relevant GBM models.

Xenograft models using glioma-derived cell lines have been most commonly used for intervention studies, but their clinical relevance is questionable because they do not recapitulate many of the features that characterize high-grade gliomas. Recently, more sophisticated models that might be useful for preclinical testing of anticancer agents are being developed by direct grafting of patient-derived surgical specimens without cell culturing (7, 8) and by engraftment of GBM-derived stem cell cultures (9, 10). Also, genetically engineered mouse (GEM) models of glioma have been generated (11), which have been helpful in unequivocally identifying the driver mutations in these tumors. Unfortunately, however, few of the glioma GEM models have been practical for use in intervention studies due to their stochastic and late onset and the inherent difficulty to diagnose tumors in a timely fashion.

We here describe a glioma GEM model that can be conveniently used for intervention studies. High-grade gliomas were induced in adult mice carrying conditional alleles for distinct combinations of p16\(^{−/−}\)/p19\(^{−/−}\), K-Ras\(^{v12}\), and Pten or p53. Following stereotactic injection of a self-deleting lentiviral vector that mediates Cre expression under the glial fibrillary acidic protein (GFAP) or the cytomegalovirus (CMV) promoter, high-grade gliomas were generated (12) with a 256-bp DNA sequence of the lentiviral packaging construct, pBob-iCre-SD. Virus titers of human high-grade gliomas. The conditional Luciferase reporter (LucR) gene allowed timely detection and the convenience of this model permits its implementation at an early stage of preclinical evaluation.

**Materials and Methods**

**Plasmid design and production of lentivirus**

A self-deleting GFAP-iCre lentivirus was constructed by replacing the CAGG promoter from a pBob-CAGG-iCre-SD lentiviral vector (12) with a 256-bp DNA sequence of the GFAP-promoter region, which was previously reported to be the minimal element for efficient and astrocyte-specific expression (13). Primers were designed to amplify the 256-bp region of the GFAP promoter, using DNA from wild-type FVB mice as a template. The resulting PCR product was then cloned into topo v2.1 (Invitrogen Corp.). Incorporated into the primers were ClaI restriction sites (5\(^′\): GTAATCGATAGCTGTTTCCTCGGCCC; 3\(^′\): CTGACCAACGCTTCGGAGCAT) used for subcloning this fragment from the topo v2.1 into the ClaI-XbaI sites of the lentiviral packaging construct, pBob-iCre-SD. Virus was generated using a third-generation, tat-free packaging system. Virus particles were concentrated more than 1,000-fold using ultracentrifugation and the pellet was resuspended in 300 μL of sterile 1× HBSS (Invitrogen). Virus concentrations were determined using anti-p24 immunoassay (Alliance HIV-1 Elisa Kit, Perkin-Elmer).

**Mice**

p16\(^{−/−}\)/p19\(^{−/−}\), K-Ras\(^{v12}\), Pten, p53, and LucR Cre-LoxP-based conditional mice (14–17) were cross-bred to generate different combinations of compound conditional mice: Ink4a/Arf;K-Ras\(^{v12}\);LucR, pten;Ink4a/Arf;K-Ras\(^{v12}\);LucR, and p53;Ink4a/Arf;K-Ras\(^{v12}\);LucR. Animals were housed and handled according to institutional guidelines complying with Dutch law. The mice were housed in a temperature-controlled environment (22-25°C) with a 12-hour light/12-hour dark cycle and were given a standard diet (AM-II, Hope Farms B.V.) and acidified water ad libitum. All animal experiments have been approved by the animal ethics committee of our institute.

**Stereotactic intracranial injections**

For lentiviral injections, 2 μL of virus suspension (p24 concentration of 4.5 μg/mL for GFAP-Cre and 7.6 μg/mL for CMV-Cre) were injected intracranially into Ink4a/Arf;K-Ras\(^{v12}\);LucR, Pten;Ink4a/Arf;K-Ras\(^{v12}\);LucR, and p53;Ink4a/Arf;K-Ras\(^{v12}\);LucR conditional mice ages between 8 and 16 weeks. Stereotactic injections were done as described in detail previously (18).

**In vivo bioluminescence imaging of intracerebral gliomas**

Mice were monitored for the development of tumors by i.p. injection of 150 mg/kg luciferin in PBS. Bioluminescence was measured using the Xenogen IVIS 200 Imaging system (Xenogen Corporation) as described in detail previously (18).

**Translational Relevance**

We here present a robust method to induce high-grade gliomas in mice that shows many similarities with high-grade glioma in patients. This transgenic mouse model combines high tumor take rate and rapid speed of induction (2-3 weeks) with the capacity of noninvasive assessment of tumor load using bioluminescence imaging. As shown in a study with temozolomide, it can be used for intervention studies with almost the same convenience as with the classic cell line–based intracranial xenograft models. Transgenic models may provide a more accurate prediction of the efficacy of therapeutics than xenograft models, and the convenience of this model permits its implementation at an early stage of preclinical evaluation.
Magnetic resonance imaging

Animals were given temozolomide (100 mg/kg/d orally) for 5 or 6 days and were transported to Nijmegen for magnetic resonance imaging (MRI). Imaging was done on a 7T/30cm horizontal-bore magnet interfaced to a clinical console (ClinScan, Bruker BioSpin). A circular polarized receive-only coil optimized for mouse brain was used in combination with the circular polarized birdcage body coil. A coil was made in the three orthogonal directions, followed by a T2 weighted fat-suppressed image series with slice sets in the three orthogonal directions (TSE: TE 43 ms, TR 3880 ms, echo spacing 14.2 ms, turbo factor 7, voxel size 0.1 × 0.1 × 0.7 mm). Subsequently, a three-dimensional T1 weighted CSF-suppressed image series (MPRAGE: TE 3.09 ms, TR 2000 ms, TI 1000 ms, voxel size 0.1 × 0.1 × 0.5 mm) was acquired before and immediately after i.v. injection of 0.2-mL Gd-DTPA (20 mmol/L; Magnevist, Schering).

Histologic and immunohistochemical analyses

Mice were sacrificed at 15% to 20% weight loss or after they developed symptoms of neurologic illness. Whole heads (without skin) or isolated brains were fixed in a 4% formaldehyde. Decalcified heads or isolated brains were embedded in paraffin and cut into 4-μm-thick coronal slices. Next, sections were stained with H&E and analyzed microscopically. Immunohistochemical stainings were done using primary antibodies: BCRP (BXP53, 1:400; Abcam), GFAP (1:400; DAKO), Glut-1 (DAKO), nestin (1:300; BD Pharmingen), β-tubulin III (1:1,000; Sigma), phospho-p44/42 mitogen-activated protein kinase (1:50; Cell Signaling), proliferating cell nuclear antigen (1:500; Santa Cruz), and Pten (1:100; Cell Signaling). For all stainings, a negative control was included in which primary antibodies were omitted.

Results

Loss of p16Ink4a/p19Arf and activation of K-Ras<sup>v12</sup> with or without Pten or p53 loss lead to grade 3 and 4 high-grade gliomas

We successfully induced brain tumors in adult mice following focal intracranial switching of genes that are frequently deleted or mutated in human high-grade glioma, such as p16<sup>Ink4a</sup>/p19<sup>Arf</sup>, Pten, and p53 (Supplementary Fig. S1A and B). Furthermore, Ras pathway activation was achieved by using a conditional K-Ras<sup>v12</sup> transgene. The time lapse between GFAP-Cre lentivirus injection and lethal tumor development differed between the genotypes. Tumors in p53<sup>−/−</sup> mice developed with a higher incidence and more rapidly than the tumors in Pten<sup>−/−</sup> mice and Ink4a/Arf<sup>−/−</sup> mice (Fig. 1; Table 1). As a control, we also injected similar K-Ras<sup>v12</sup> transgene–negative animals and found that none of these mice developed tumors during follow-up for more than a year. Furthermore, we injected animals with a similar replication-defective, self-deleting Cre lentivirus containing the CMV instead of the GFAP promoter sequence. CMV-Cre lentivirus resulted in tumor development in 7 of 7 (100%) Ink4a/Arf<sup>−/−</sup>;K-Ras<sup>v12</sup>;LucR mice and 6 of 6 (100%) p53<sup>−/−</sup> mice after a very short latency period (Fig. 1; Table 1). Injection of CMV-Cre lentivirus in K-Ras<sup>v12</sup>;LucR mice did not result in tumor development.

Tumors were independently assessed by three pathologists and graded according to the WHO criteria (Table 1). High-grade gliomas developed in 8 of 23 (35%) Ink4a/Arf; K-Ras<sup>v12</sup>;LucR mice that were injected with GFAP-Cre lentivirus, with 6 of 8 tumors graded as anaplastic astrocytomas (WHO grade 3). Tumors were highly invasive and showed increased cell density with nuclear atypia and mitosis (Fig. 2A). Moreover, 2 of 8 tumors induced in this genetic background were assessed as GBM (WHO grade 4) because they additionally showed areas of pseudopalisading necrosis (Fig. 2C). Tumors generated in Pten<sup>−/−</sup> mice had progression to grade 4 gliomas (Fig. 2H). In p53<sup>−/−</sup> mice, malignant gliomas were found in 12 of 15 (80%) cases, with 4 of 12 tumors being assessed as anaplastic astrocytomas and 8 of 12 as GBM because they showed typical histologic characteristics, such nuclear atypia, mitosis, giant cells (Fig. 2B), and pseudopalisading necrosis.
necrosis (Fig. 2D). Another GBM hallmark seen in patients is vascular proliferation that can occur in two pathologically distinct forms: a diffuse increase in vascular density resulting from more densely arrayed small vessels, and microvascular proliferation (19). High-grade gliomas from all genotypes displayed increased vascular and enlarged blood vessels in compact tumor areas (Fig. 2E), but not in diffuse invasive areas. Furthermore, tumor cells migrated distant from the tumor center by co-option of existing host vessels (Fig. 2F and I). Several grade 4 astrocytomas showed vessels with endothelial hyperplasia (Fig. 2G) or with incipient microvascular proliferation (Fig. 2H). However, we did not observe the overt features of microvascular proliferation that are seen in patients.

Invasion throughout the brain parenchyma is a key feature in high-grade gliomas and a major reason for the poor treatment response. Besides progression via perivascular growth (Fig. 2F and I), tumors also displayed diffuse infiltrative growth along white matter tracts in the brain parenchyma (Fig. 2J). CMV-Cre–induced tumors showed very similar histologic features of high-grade gliomas; however, occasionally, areas resembling meningioma (n = 1 of 13) or gliosarcomas were found (n = 2 of 13; data not shown).

**Lentiviral-induced gliomas are highly proliferative**

Immunohistochemical stainings were done to show the “multiforme” and highly proliferative nature of the derived gliomas. All tumors of the three different backgrounds were highly positive for nestin (Fig. 3). Although tumors were also GFAP positive, most GFAP-positive cells within the tumors seem to be reactive astrocytes, especially in the rim around the tumor. Furthermore, tumors induced in all genotypes were highly proliferative, as shown by the proliferating cell nuclear antigen stainings. Additionally, immunohistochemical stainings for Pten and pAkt confirmed that injection of GFAP-Cre lentivirus into the different strains results in specific molecular alterations. Gliomas induced in a Pten;Ink4a/Arf;K-Ras<sup>12/12</sup>;LucR background were Pten negative and showed increased pAkt levels, whereas gliomas from a Ink4a/Arf;K-Ras<sup>12/12</sup>;LucR background were Pten positive and did not show increased pAkt levels, compared with the surrounding normal brain parenchyma.

### Table 1. Incidence of high-grade gliomas

<table>
<thead>
<tr>
<th>Mouse strains</th>
<th>GFAP-Cre lentivirus</th>
<th>CMV-Cre lentivirus</th>
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</thead>
<tbody>
<tr>
<td>Tumor incidence (%)</td>
<td>Grade (WHO)</td>
<td>Tumor incidence (%)</td>
</tr>
<tr>
<td>Ink4a/Arf;K-Ras&lt;sup&gt;12&lt;/sup&gt;</td>
<td>8/23 (35)</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>Pten;Ink4a/Arf;K-Ras&lt;sup&gt;12&lt;/sup&gt;</td>
<td>6/8 grade 3; 2/8 grade 4</td>
<td>6/7 grade 3; 1/7 grade 4</td>
</tr>
<tr>
<td>p53;Ink4a/Arf;K-Ras&lt;sup&gt;12&lt;/sup&gt;</td>
<td>4/13 (31)</td>
<td>2/4 grade 3; 2/4 grade 4</td>
</tr>
<tr>
<td></td>
<td>12/15 (80)</td>
<td>4/15 grade 3; 8/15 grade 4</td>
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<td>4/6 (100)</td>
<td>6/6 (100)</td>
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### Tumor vessels express BBB markers

The BBB plays an important role in the brain penetration of chemotherapeutic agents. We first investigated the status of the BBB by immunostaining for three markers: GLUT1, a membrane glucose transporter, which is specifically expressed on brain capillary endothelial cells (20); BCRP, which is a drug efflux transporter that, together with P-glycoprotein, plays an important role in the BBB in restricting the entry of many agents, and mouse immunoglobulin G (IgG), a marker for more chronic passive leakiness of the BBB (21). In all genotypes, the majority of the tumors exhibited GLUT1-positive vessels (Fig. 4A and B), indicating that tumor cells propagate around existing vessels that express BBB markers. Also, in some tumors, areas with GLUT1-negative vessels were observed, which is suggestive of neovascularization (Fig. 4B and C). Furthermore, besides GLUT1 expression in the brain vasculature, areas with GLUT1-expressing tumor cells were also observed to disperse through the tumor, indicative of hypoxia (Fig. 4C). Specifically, hypoxia-related GLUT1 expression is induced in tumor cells adjacent to necrosis (ref. 22; Fig. 4C, asterisk). Interestingly, within the same genetic background, heterogeneity was observed between the tumors with respect to vessel permeability (IgG staining; data not shown). Similarly, BCRP is also expressed heterogeneously in the tumor vessels. Most tumors showed areas with clearly positive endothelial cells that often stained even more intensely than vessels in the surrounding normal brain parenchyma, although there were also areas where BCRP-negative vessels were observed (Fig. 4D and E). Overall, these results show that the majority of lentivirus-induced gliomas express BBB markers and exhibit variable vessel permeability, which is also seen in human malignant gliomas.

### Applicability of in vivo bioluminescent, spontaneous high-grade gliomas for therapy intervention studies

A major goal of this work was to create GEM models of high-grade glioma that would also be practically applicable for preclinical testing of novel therapies. Our strategy of creating gliomas by concurrent (in)activation of specific oncogenes and tumor suppressor genes via intracranial injection of lentiviral Cre in compound loxP mice results in rapid tumor induction, in particular in
p53;Ink4a/Arf;K-Ras	extsuperscript{V12} mice. All of these animals that were injected with CMV-Cre lentivirus developed tumors with a time window comparable to that of traditional orthotopic xenograft models of glioma (18). Furthermore, the LucR gene allowed tumor mass quantification by noninvasive bioluminescence monitoring. To show the applicability of our model for therapy intervention studies, we first injected a small cohort of p53;
Ink4a/Arf;K-Ras<sup>v12</sup> mice with CMV-Cre lentivirus. The presence of a growing tumor mass was assumed when the bioluminescence signal increased at two consecutive occasions, which occurred between days 15 and 25 after lentivirus injection (Supplementary Fig. S2). Animal #1 was not treated, whereas the other mice were treated with six daily doses of 100 mg/kg temozolomide, currently the chemotherapy of choice for GBM (23). Detection and follow-up of tumors by bioluminescence were convenient, and based on these results, the animals were scheduled for MRI before showing any signs of illness (weight loss or neurologic defects). Tumors were clearly visible on T2 weighted scans, whereas they were hardly visualized by T1 weighted MRI (Fig. 5A). Moreover, there was no enhancement using Gd-DTPA MRI, indicating that these tumors are not (very) leaky. Histology confirmed the presence of these lesions in all animals at the time of sacrifice (Fig. 5B). Notably, whereas animal #2 showed tumor progression based on bioluminescence images, histology showed areas of cell damage within the tumor that were most likely due to the temozolomide treatment.

We next injected a second cohort of p53;Ink4a/Arf;K-Ras<sup>v12</sup> mice (n = 8) with CMV-Cre lentivirus, which had all developed a tumor by day 14. Animals were stratified based on their bioluminescence signal into a control (n = 4) and a treatment group (n = 4), and treatment involved daily temozolomide for 9 consecutive days. Treatment was well tolerated and resulted in a significant reduction in tumor progression, which was evident from the bioluminescence results as well as from the overall survival data (Fig. 6).

**Discussion**

We here describe the generation of a high-grade glioma mouse model that is very suitable for intervention studies. This was achieved by intracranial injection of Cre lentiviral vectors into Ink4a/Arf;K-Ras<sup>v12</sup>, Pten;Ink4a/Arf;K-Ras<sup>v12</sup> and

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![Fig. 3. Lentiviral-induced high-grade gliomas are highly proliferative. Representative nestin, GFAP, proliferating cell nuclear antigen (PCNA), Pten, and pAkt stainings in grade 3 and 4 gliomas.](image-url)
p53;ink4a/Arf;K-Ras<sup>12LoxP</sup> conditional adult mice. Tumor incidence and grade of malignancy were highest in the latter genotype. Tumors reproduced many of the histologic features that are characteristic of human gliomas, including tumor cell heterogeneity, invasiveness, vessel permeability, and BBB characteristics. Crossing in of the conditional LucR gene allowed monitoring of tumor growth for a sufficiently long period of time to have a time window for intervention. In particular, the short latency time and the very high incidence seen in p53;ink4a/Arf;K-Ras<sup>12LoxP</sup> mice when using lentiviral CMV-Cre make this a practical model for use in therapy intervention studies, which was shown in an efficacy study using temozolomide.

Lentiviral vectors are considered attractive CNS gene transfer tools due to their capacity to transduce dividing or nondividing cells in the brain (24). To achieve targeted expression of Cre recombinase in glial cells, we equipped the lentivirus with a mouse GFAP promoter. Previously, it has been shown that, in this way, one can target transgene expression to glial cells in the brain (25). Although we occasionally observed meningioma or sarcoma-like lesions after intracranial injections with CMV-Cre, the majority of the tumors were high-grade gliomas as induced by GFAP-Cre (Fig. 5B). With CMV-Cre, tumor incidence was 100% and the latency period was significantly shorter than that with GFAP-Cre. This may be due to a difference in infection efficiency between the two lentiviruses or the fact

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**Fig. 4.** Lentiviral-induced high-grade gliomas express BBB markers. We here show results after intracranial injection of GFAP-Cre lentivirus into p53;ink4a/Arf;K-Ras<sup>12LoxP</sup> mice, but similar results were obtained with the other genotypes (data not shown) as well as with CMV-Cre lentivirus. A, the majority of the tumor vessels were positive for GLUT1, indicative of preexisting vasculature (magnification, ×10). B, besides GLUT1-positive vessels (asterisk), also GLUT1-negative vessels (arrows) were observed in some tumors. C, moreover, proliferating tumor cells around the border of necrotic areas (asterisk) express GLUT1. Frequently, areas with GLUT1-negative vessels were associated with GLUT1-expressing tumor cells (see arrows). D and E, all genotypes stained positive for the drug transporter BCRP in both tumor vessels (V) and tumor cells (T), which were often stronger compared with the blood vessels from the normal surrounding brain tissue (indicated by asterisk). Furthermore, a marked heterogeneity of BCRP expression within tumor areas is frequently found.
Fig. 5. MRI and histology of nontreated and temozolomide-treated gliomas induced in p53;Ink4a/Arf;K-Ras^{V12};LucR mice. A, animal #1 was untreated; animals #2 and #3 received daily 100 mg/kg temozolomide, qd×9, when they developed a growing tumor mass (increasing bioluminescence imaging signal). Animal #2 was killed after 8 d of treatment, whereas animal #3 first responded and was killed 21 d after the end of treatment when tumor progression led to symptomatic disease. Tumors are visible on T2 weighted (T2w) images, but not or hardly on T1 weighted images before (T1 pre) and after Gd-DTPA enhancement (T1-Post-Gd-DTPA). Gd-DTPA–enhanced MRI results indicate an intact BBB. Hemorrhage was observed (confirmed by histology) within the tumor on the T2 weighted image of temozolomide-treated animal #2. B, immunohistochemical stainings show GLUT1-positive tumor vessels, whereas staining for IgG also reveals minimal vessel leakage in mouse #1 (untreated). H&E of temozolomide-treated animal #2 showed a mixed response. Tumor cells in area (*) show extensive hydropic swelling and contain fragmented DNA, indicative of cell damage, whereas other areas of this tumor present diffuse infiltrative growth (#) with highly proliferating tumor cells (Ki67 staining). Such effects were not seen in animal #3, whose tumor had progressed for some time after treatment. Caspase-3 expression was seen in the responsive tumor area of animal #2, whereas animal #1 (untreated) and animal #3, apart from a single positive cell, were negative.
that CMV-Cre, but not GFAP-Cre, can switch the conditional alleles in cell types that give rise to gliomas more efficiently. Recently, it has been reported that lentiviral vectors encoding floxed H-Ras\(^{v12}\) and AKT oncogenes gave rise to tumors in GFAP-Cre;p53\(^{+/−}\) transgenic mice with a latency of 80+ days (26). In this study, it was shown that following intracranial injection of these lentiviruses, only a very small number (<60) of GFAP-positive cells were transduced. In line with this finding, we have also found very few LacZ-positive cells when injecting lentiviral Cre into Rosa-LacZ reporter mice (data not shown), indicating that tumors arose from a small number of infected cells.

Although human gliomas rarely show RAS mutations (27), virtually all malignant human gliomas show elevated levels of activated RAS (28) as a result of other lesions such as receptor tyrosine kinase mutations (29) or inactivation of NF1 (30) and Rig (31). Indeed, several glioma mouse models have shown the important role of RAS (32,33). In this study, we used a conditional K-Ras\(^{v12}\) transgene to activate this commonly affected pathway in glioblastoma. A combination of continuous expression of mutant K-Ras and loss of p16\(^{ink4a}\)/p19\(^{arf}\) was sufficient to give rise to high-grade gliomas in about 35% of the animals injected with GFAP-Cre. Additionally, if combined with loss of function of p53, the tumor latency time was significantly reduced. Moreover, the incidence increased and tumors were mostly grade 4 (8 of 12), which is in agreement with previously studies (26,34). Interestingly, the implementation of p53 inactivation for developing mouse models of primary GBM has gained significance because recently published studies have shown that abrogation of the p53 pathway is also a key event in primary GBM (35,36) and not, as was previously assumed, predominantly involved in initiation of low-grade astrocytomas that progress later into secondary GBMs. In contrast to the deletion of p53, deletion of Pten does not seem to shorten the latency, although larger cohorts might have revealed subtle differences. The conditional K-Ras\(^{v12}\) transgene used in this study showed some spontaneous background of mammary tumors in females, making tumor incidence curves less reliable. In this respect, use of the conditional mutant K-Ras knock-in allele (37) might be a preferred alternative for future studies.

Many publications describing GEM models for glioma have discussed their potential usefulness for therapeutic intervention screens. However, only few reports describe the application of glioma GEM models for therapy intervention studies (38, 39). This application requires that the tumor incidence is high and the lag time of tumor onset sufficiently short (e.g., less than a few months), with tumors arising within a fairly narrow time window. Furthermore, a relatively simple, noninvasive method for measuring tumor burden is mandatory for handling larger cohorts of animals. We have designed our model to meet these criteria. First, the strategy of localized injection of lentiviral-Cre permits simultaneous targeting of multiple oncogenes and tumor suppressor genes, resulting in rapid tumor induction. Second, the LucR gene allows bioluminescence imaging as a relative simple means for monitoring tumor growth. These features make our model suitable for intervention studies, as shown in an efficacy study with temozolomide. Moreover, this model can serve as a basis of further studies using additional or different genetic modifications than the ones described here. In addition, by analogy to a recent study (26), one might use a combination of lentiviral Cre and a lentivirus carrying an oncogene such as EGFR\(^{vIII}\). Although their applicability for therapeutic intervention studies has not yet been established, two other recently reported glioma models also seem to meet the criteria on tumor latency time, incidence, and monitoring (40, 41). Importantly, these developments will allow the more generalized use of relevant glioma GEM models for preclinical efficacy screening.

An important aspect of a potentially predictive model for glioma is the status of the BBB within the tumor. Most agents are developed for other (major) tumor types than
brain tumors and considered for testing against GBM when they share aberrant molecular pathways. Because these agents may not have the optimal molecular qualities to penetrate the BBB, they may not reach all tumor cells at therapeutically effective concentrations. High-grade gliomas in humans consist of a more central, highly angiogenic region that harbors leaky blood vessels as visualized by contrast-enhanced MRI (42, 43). However, there are also many invasive glioma cells that use the preexisting vasculature, which has an intact BBB (44). Eradicating this cell population, which is left behind after resection of the bulk tumor, is the real therapeutic challenge. Based on histologic analyses and supported by MRI data, it seems that most of our tumors do not show a profound angiogenic phenotype. Although one might argue that this does not conform to human GBM, we consider this as an advantage for the use of intervention studies, as these tumors make use of the existing vasculature with intact BBB properties and thus may better reflect the residual tumor cells that are typically left after surgery.

We have investigated the expression of typical BBB features by immunohistostaining staining. It is generally believed that GLUT1-positive vessels are preexisting vessels with more intact BBB properties, whereas newly formed vessels by angiogenesis are GLUT1 negative and potentially more leaky. Indeed, in our model, newly formed leaky vessels were predominantly seen in areas of compact tumor growth, where tumor cells were expressing GLUT1, likely as a consequence of hypoxia. When tumor cells become hypoxic, HIF-1α proteasomal degradation is diminished and, consequently, HIF-1 accumulation in the cell triggers a cascade of events including enhanced GLUT1 (45) and vascular endothelial growth factor expression (46). Most of the tumor vasculature, however, exhibited GLUT1 expression, indicative of intact BBB functionality. Moreover, in agreement with a previous study in glioma patients (47), capillaries in the tumor express the drug efflux transporter BCRP, often at increased levels, indicating that these tumor vessels may restrict drug uptake.

In line with previous reports (10), it was possible to establish tumorigenic neurosphere cultures that recapitulate the characteristics of the primary tumor closer than do the currently available cell lines (48) when injected into immunodeficient mice (see Supplementary Fig. S1C). We expect that these cell cultures may also be useful for preclinical testing of new therapeutics.

In conclusion, we have established a versatile mouse model for high-grade gliomas. Tumors exhibited many similarities to human malignant glioma with respect to invasive growth, histologic characteristics, and BBB features. Advantages of this model are high incidence, short latency period, and the possibility to monitor tumor burden by high-throughput noninvasive bioluminescence imaging, which allows for practical application in intervention studies. This makes this model particularly attractive for preclinical efficacy studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. E. Bindels for his advice on lentivirus production; Dr. J. Westera; Prof. Dr. P. Wesseling, and Dr. S. van Duinen for histologic analysis; and Dr. J.P. Medema, J.C. Verhoeef, and Dr. M.R. Sprick for their discussions on this project.

Grant Support

Hersenstichting grant 13F05.24 (A.J.M. Berns). Magnetic resonance measurements were made possible by Investment grants from NWO (VISTA grant) and ZONMW (911-06-021) to Dr. A. Heerschap. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 01/03/2010; revised 04/08/2010; accepted 05/10/2010; published OnlineFirst 06/29/2010.

References


Clinical Cancer Research

Rapid and Robust Transgenic High-Grade Glioma Mouse Models for Therapy Intervention Studies

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Clin Cancer Res  Published OnlineFirst May 14, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-3414

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2010/05/17/1078-0432.CCR-09-3414.DC1

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