Tumorigenesis and Neoplastic Progression

Pathological and Molecular Progression of Astrocytomas in a GFAP:¹²V-Ha-Ras Mouse Astrocytoma Model

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We previously characterized a genetically engineered mouse astrocytoma model with embryonic astrocyte-specific, activated ¹²V-Ha-RAS (GFAP-RAS) transgenesis. The GFAP-RAS line Ras-B8 appears normal at birth, but 50% of mice die by 4 months from low- and high-grade astrocytomas. We examined the development and progression of astrocytomas in the Ras-B8 genetically engineered mouse. At embryonic day 16.5 (E16.5), there were no pathological differences compared to control littermates, aside from transgene expression. Diffuse astroglial hyperplasia was the first distinguishing feature in the 1-week-old Ras-B8 mice; however, these astrocytes were not transformed *in vitro* or *in vivo*. From 3 to 8 weeks the incidence of low-grade astrocytomas progressively increased with 85% of 12-week-old mice harboring low- or high-grade astrocytomas, the latter characterized by increased proliferation, nuclear atypia, and angiogenesis. *Tp53* mutations were detected in both astrocytoma grades, with high-grade astrocytomas expressing elevated levels of epidermal growth factor receptor and vascular endothelial growth factor, plus decreased levels of PTEN and p16, similar to human astrocytomas. We postulate that expression of ¹²V-Ha-RAS in astroglial precursors induces astroglial hyperplasia, but transformation and subsequent progression requires additional molecular alterations resulting from aberrant activated p21-RAS. Of interest, many of these acquired alterations occur in human

astrocytomas, further validating GFAP-RAS as a useful model for studying astrocytoma development and progression. (Am J Pathol 2005, 167:859–867)

The molecular progression of human primary central nervous system tumors are typically inferred from the examination of cohorts containing large numbers of different patient specimens with varying tumor grades.¹ In the case of astrocytomas, the most common primary brain tumor in adults, this form of analysis has some limitations, due to the pathological heterogeneity, which is further compounded by the limited number of samples typically available for molecular studies. Spontaneous genetically engineered mouse (GEM) glioma models,^{2,3} based on loss or increased expression of relevant genes implicated in human glioma pathogenesis, provide a unique opportunity to examine the pathological and associated molecular alterations associated with tumor development and progression. We previously developed and characterized a multifocal invasive mouse astrocytoma model, generated by embryonic stem cell transgenesis of an activated ¹²V-Ha-RAS gene, under regulation of the human glial fibrillary acidic protein (GFAP) promoter.⁴ The premise for the choice of the transgene was based on our initial and subsequently other researchers' observations^{5–7} that human astrocytomas, although not harboring oncogenic p21-RAS mutations, exhibit elevated levels of activated p21-RAS. This increase in p21-RAS activity is functionally relevant, as genetic or pharmacological inhibition of p21-RAS activity with dominant-negative mutants or farnesyl-transferase-inhibitors, attenuates the growth of high-grade astrocytomas in preclinical xeno-

Supported by National Cancer Institute of Canada (to A.G.), the Cleveland Clinic Foundation (to A.G.), and the National Institutes of Health (grant NS41097 to D.H.G. and A.G.).

Accepted for publication May 19, 2005.

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graft models.^{6,7} Based on these and other findings, clinical trials in recurrent high-grade astrocytomas with farnesyl-transferase-inhibitors are presently underway.

Several transgenic GFAP-RAS lines were created, demonstrating a dose-dependent increasing incidence and grade of astrocytomas, evaluated at autopsy. In the Ras-D7 chimeric mice, containing several ¹²V-*Ha-RAS* transgene integration sites and high levels of activated p21-RAS expression, death occurred between 4 to 6 weeks after birth.⁴ Examination of the brains of these mice demonstrated diffuse glial hyperplasia with multiple foci of astrocytomas, with regions of necrosis, hemorrhage, and hypervascularity, pathological features associated with malignant astrocytomas.¹ In contrast, mice from the Ras-B8 line, with a single ¹²V-*Ha-RAS* integration site and moderate levels of p21-RAS transgene expression, went on to germline transmission. These mice were normal at birth, but succumbed to low- and high-grade infiltrating astrocytomas by 3 to 9 months.⁴

Derivative high-grade astrocytoma cell lines, from both the Ras-D7 and Ras-B8 GFAP-RAS lines, demonstrated tumorigenic growth in syngeneic and immunocompromised mice.⁴ Cytogenetic analysis of these derivative mouse glioma cell lines demonstrated several clonal aberrations, such as trisomy of mouse chromosome 10, which is syntenic to a region on human chromosome 12q, commonly amplified in human malignant astrocytomas.⁸ Western blot and immunohistochemical analysis of the derivative astrocytoma lines and tumors demonstrated additional genetic alterations also associated with human high-grade astrocytomas. These include loss of cell-cycle growth regulator (p16, p19, and Tp53) expression, overexpression of MDM2 or cyclin-dependent kinase-4 (CDK4), aberrant increased expression of the epidermal growth factor receptor (EGFR), loss of the PTEN/MMAC1 tumor suppressor gene, and increased expression of proangiogenic molecules, such as vascular endothelial growth factor (VEGF) and several angiopoietin proteins.^{4,9}

One of the limitations of the molecular genetic studies of human astrocytomas is the inability to readily correlate the molecular changes with the histopathological development of the tumors due to the lack of available tumor tissue in the early phases of tumorigenesis. Spontaneous astrocytoma-prone GEM models, especially ones that demonstrate pathological and molecular characteristics similar to human astrocytomas, provide an opportunity to track the molecular and pathological changes as a function of time. In this study, we used the Ras-B8 GEM, to study the order of specific genetic changes relative to the pathological appearance of GFAP-RAS mouse astrocytomas. Our findings suggest that expression of activated p21-RAS in glial progenitor cells leads to astroglial hyperplasia, which are not transformed but are genetically unstable. Accumulation of additional specific molecular alterations, many of which are also commonly found in human astrocytomas, facilitates transformation and subsequent astrocytoma progression in these mice.

Materials and Methods

Generation of Transgenic Mice

Embryonic stem cell transgenesis, with retinoic acid differentiation and GFAP promoter-regulated expression of ¹²V-*Ha-RAS:IRES-LacZ* in ICR mice, has been previously described.^{3,4,10} In brief, after electroporation of R1 embryonic stem cells with the linearized plasmid, G418 (200 µg/ml; Life Technologies, Long Island, NY) selection was undertaken along with retinoic acid differentiation to induce the GFAP promoter as previously described.^{3,4,10} LacZ(+) embryonic stem clones were selected, characterized *in vitro* for transgene expression, and then sent for aggregation to generate the mice. Male Ras-B8 mice, with a single copy transgene integration site and germline transmission,⁴ were bred to wild-type ICR females to generate the mice for this study. Genotyping was undertaken with polymerase chain reaction (PCR) amplification of genomic DNA obtained from tail biopsies, with primers located in the GFAP promoter and 3' region of the ¹²V-*Ha-RAS* transgene as previously described.⁴ Southern blot analysis was also undertaken with α³²P-dCTP labeled *Ha-RAS* and *LacZ* cDNA probes on the genomic DNA.

Histological Preparation

Ten Ras-B8 transgenic mice and five age-matched ICR control littermates were euthanized at representative times, including E16.5 and postnatal weeks 1, 3, 4, and 8. Seven transgenic mice at 12 weeks and age-matched littermates were examined. E16.5 embryos were immersion-fixed in neutral buffered formalin and the entire head embedded *en bloc* in paraffin for sectioning. For the postnatal mice, 50 mg/kg of BrdU was injected 2 hours before sacrifice, the brains extracted, immersion-fixed in formalin for 24 hours, cut at 2-mm intervals, and paraffin-embedded for sectioning. Screening for tumors was undertaken with hematoxylin and eosin (H&E), LacZ, GFAP, nestin, and BrdU staining of multiple 6-µm brain sections.

Immunohistochemistry

Immunohistochemistry was performed on 6-µm sections after microwave retrieval and blocking with either 20% goat (Life Technologies) or 10% rabbit (Zymed, South San Francisco, CA) serum in phosphate-buffered saline (PBS). After incubation with the primary antibody at 4°C overnight, standard protocols incorporating the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) with 3'3' diaminobenzidine tetrachloride as the chromophore, were used for visualization. All cases were stained with rabbit polyclonal anti-GFAP (1:3000, pepsin; DAKO, Carpinteria, CA), mouse monoclonal anti-BrdU (1:80; Boehringer-Mannheim, Indianapolis, IN), mouse monoclonal anti-LacZ (1:10,000; Promega, Madison, WI) and mouse monoclonal anti-nestin (1:200; Pharmingen, La Jolla, CA). In the slides where astrocytomas were identified, additional immunostaining was

performed using antibodies that recognize proteins implicated in human astrocytoma development and progression, including rabbit polyclonal anti-PTEN (1:3500, trypsin pretreatment; gift of late Peter Steck, MD Anderson, Houston, TX), rabbit polyclonal anti-p16 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-phospho-Akt (1:200; BioLab), mouse monoclonal anti-phospho-MAPK (1:2000, BioLab), rabbit polyclonal anti-Tp53 (1:2000, Santa Cruz Biotechnology), rabbit polyclonal anti-EGFR (1:3500, trypsin pretreatment; Santa Cruz Biotechnology), and mouse monoclonal anti-VEGF (1:200, UBI) (see Figures 2B, 3A, and 4).

Semiquantitative Immunohistochemistry Analysis

Computer-assisted image analysis was used with the MicroComputer Image Device (MCID-Imaging Research, Inc.) linked to a color charge-coupled device camera (Sony DXC 970 MD) mounted on a transmitted-light microscope (Axiophot; Zeiss, Thornwood, NY). The number of GFAP(+) astrocytes and LacZ(+) transgene-expressing cells in 3-, 8-, and 12-week-old mice brains, representing time points after completion of cerebral maturation, were analyzed (Figure 1, B and C). The mean number of astrocyte nuclei, unequivocally associated with GFAP(+) cytoplasm and processes, in the cerebral convexity subpial region in 10 contiguous high-power fields, were calculated. Similarly, the mean number of LacZ(+) and LacZ(−) nuclei per 10 high-power fields, were also calculated in the cerebral convexity. One-way analysis of variance was used to determine whether with increasing age of the mice, there were increasing numbers of GFAP(+) astrocytes and LacZ(+) or LacZ(−) transgene-expressing cells. Analysis was also performed to calculate the BrdU labeling index, by counting 200 cell nuclei within regions of glial hyperplasia, low- and high-grade astrocytomas and controls. Nuclear morphometry was performed on 6- μ m Feulgen-stained sections, with determination of the glial cell nuclear cross-sectional area and perimeter in five representative $\times 1000$ fields.

Transformation Assays on Derivative Astrocyte Cultures

Astrocyte cultures were established from 1- and 12-week-old Ras-B8 transgenics and 1-week-old wild-type ICR mice brains, by standard methods.¹¹ The GFAP(+) astrocytes were grown in Primaria plates (BD Biosciences) in the presence of Dulbecco's modified Eagle's medium with L-glutamine, 4.5 g/L glucose with Na-pyruvate (Wisent), plus 10% fetal calf and horse serum (Wisent), and 12.5 ng/ml of EGF (Clonetics, San Diego, CA). Changes in the media were performed every 3 to 4 days, with passage 4 astrocytes used for further analyses. To confirm that the isolated astrocytes were expressing the transgene, ~500 astrocytes were grown overnight in eight-well chamber slides (Nalge-Nunc Labtek, Naperville, IL) in the presence of astrocyte medium described

Table 1. Anchorage-Independent Growth of Derivative Astrocytes

Type of astrocyte	Age of isolation (weeks)	Percentage of transformation
Wild-type (ICR)	1	0
GFAP-RAS (B8)	1	0
GFAP-RAS (B8)	12	5 to 10

above. The cells were washed two times with PBS, fixed in PBS/0.05% glutaraldehyde for 5 minutes at room temperature, washed two times with PBS, and incubated in 1 mmol/L $MgCl_2$, 5 mmol/L $K_4[Fe^{II}(CN)_6]$, 5 mmol/L $K_3[Fe^{III}(CN)_6]$ plus 1 mg/ml X-gal for 2 hours at room temperature. X-gal staining for LacZ activity was documented with a Leica DM IRE2 deconvolution microscope (data not shown).

The astrocytes were harvested and filtered several times with 80- μ m nylon meshes to put into single cell suspension. After performing a cell count, $\sim 1 \times 10^5$ cells were added to a mixture of 0.5% agarose and astrocyte medium to a final concentration of $\sim 0.35\%$ agarose. The cells suspended in agarose were plated on 150-mm tissue culture plates coated with 0.5% agarose. After hardening of the upper agarose layer, the astrocytes were grown in the presence of astrocyte medium with changes every 4 to 7 days for 4 to 5 weeks. A total of 1.2×10^6 cells were analyzed by soft agarose assays. The percentage of cells that led to transformed anchorage-independent growing colonies was documented with a Leica DM IRE2 deconvolution microscope at $\times 10$ magnification (Table 1).

Laser Capture Microdissection (LCM) and Tp53 Mutation Analysis

One of each low- and high-grade astrocytoma from 4- and 12-week mice, respectively, with immunohistochemical Tp53 accumulation (Figure 3A) were chosen for detection of Tp53 mutations. Tissue was fixed in formalin for 48 hours, then processed and paraffin-embedded. Five- μ m sections were mounted on noncoated slides (Knittel Glaser, Germany) dewaxed with xylenes, hydrated with alcohols, and stained with hematoxylin (DAKO). After staining, the tissue was dehydrated and dried in a dessicator for LCM. A thermoplastic, transparent film was placed on the top of the tissue section and astrocytoma cells were captured using LCM (Arcturus, Mountain View, CA) into high sensitivity caps to avoid contamination from adjoining cells. The film was activated with a pulse (7.5 μ m) from a focused laser beam for accurate and specific cell procurement (Figure 3B). Approximately 200 astrocytoma cells and equivalent numbers of normal astrocytes from the same samples were collected and DNA isolated using the PicoPure DNA isolation kit (Arcturus), designed precisely to extract DNA with high fidelity from few cells. After incubation overnight at 65°C, proteinase K was inactivated at 95°C for 5 minutes.

Table 2. Primer Sets and Conditions for Tp53 Mutational Analysis

Exon	Forward and reverse primer	Annealing temperature (°C)	Fragment size (bp)
5	5'-TCTCTTCCAGTACTCTCCTC-3' (sense) 5'-AGGCGGTGTTGAGGGCTTAC-3' (anti-sense)	58	250
6	5'-GGCTTCTGACTTATTCTTGC-3' (sense) 5'-CAACTGTCTCTAAGACGCAC-3' (anti-sense)	56	230
7	5'-TCACCTGGATCCTGTGTCTT-3' (sense) 5'-CAGGCTAACCTAACCTACCA-3' (anti-sense)	56	200
8	5'-AGT TTA CAC ACA GTC AGG ATG G-3' (sense) 5'-CCT GGT TCC TCC GCC TCC TCG G-3' (anti-sense)	64	250

Primer sequence for exons 5, 6, and 7 of the mouse *Tp53* gene was used, as previously reported.¹² The primers for exon 8 were designed using the Primer Express Software and purchased from DNA Integrated Technology. Primers were designed intronic to cover each exon for sequencing purposes. Primer sequences and PCR conditions are shown in Table 2. The PCR for exons 5 to 8 was performed in two rounds. Round one was done for total of 30 cycles and round two for 25 cycles. A 5- μ l aliquot of DNA was amplified in first and second round in a final volume of 25 μ l containing the following: 1 $\times$ Platinum High Fidelity buffer (Invitrogen, Carlsbad, CA), 0.2 mmol/L dNTP mix (Fermentas), 2 mmol/L MgSO₄ (Invitrogen), primers 4 ng/ μ l, Platinum High Fidelity *Taq* 0.1 U (Invitrogen). For second round PCR, 5 μ l from the first PCR was added to a final reaction volume (as above) of 25 μ l. Amplification was performed in an MJ Research Thermocycler (model PTC-200). First-round reactions were amplified with the following PCR conditions: 94°C for 2 minutes; 30 cycles of 94°C for 1 minute, 56°C or 58°C or 64°C (depending on the primers used) for 1 minute, 72°C for 2 minutes; and a final extension at 72°C for 5 minutes. Second-round PCR was performed with the following conditions: 94°C for 2 minutes; 25 cycles of 94°C for 30 seconds, 56°C or 58°C or 64°C (depending on the primers used) for 30 seconds, 72°C for 1 minute, and a final extension at 72°C for 5 minutes.

The quantity and quality of PCR was assessed by analyzing the second-round reactions on 2% agarose gels and visualized with ethidium bromide. Bands were excised and DNA was purified using the QIA purification kit (Qiagen, Valencia, CA). The nucleotide sequence of each PCR product was determined in both directions using the same PCR primers shown above. Sequencing was done using the Applied Biosystems sequencer (model 3100) at the automated sequencing facility at Hospital for Sick Children's Research Institute, Toronto, Canada (Figure 3C).

Results and Discussion

Transgene Expression and Astroglial Hyperplasia

Previous studies from our laboratories have shown that the *¹²V-Ha-RAS:IRES-LacZ* transgene is first detected by

E14.5, using LacZ as a surrogate marker for *¹²V-Ha-RAS* expression (Apicelli AJ, Gutmann DH; unpublished observations). By E16.5, there was obvious LacZ expression in the white matter regions of Ras-B8 mice, but without any significant increase in GFAP(+) astrocytes or any other detectable histological central nervous system abnormalities, compared to age-matched control embryos (Figure 1A). At 1 week, there was an increase in GFAP(+) astrocytes in the Ras-B8 mice compared to controls, but this did not reach statistical significance and glial hyperplasia was not noted (Table 3). Thereafter from 3 to 12 weeks, a statistically significant increase in the number of both LacZ(+) and GFAP(+) astrocytes were noted in the Ras-B8 mice, compared to control littermates (Figure 1, B and C). One-way analysis of variance revealed no influence of age on the number of LacZ(−) nuclei ($F = 3.3$, $P > 0.05$) but a strong and significant effect of age on the number of LacZ(+) nuclei ($F = 51.04$, $P < 0.001$). Similarly, control 3- to 12-week mice did not experience any change in the number of subpial GFAP(+) astrocytes ($F = 2.287$, $P > 0.05$), in contrast to their age-matched Ras-B8 littermates ($F = 5.8$, $P < 0.05$). The astroglial hyperplasia in the Ras-B8 mice was most evident in the cerebrum and subcortical white matter and less marked in the brainstem and cerebellum. The diffuse astroglial hyperplasia was comprised of GFAP(+)/nestin(−) cells, the latter being a reliable marker to distinguish astroglial hyperplasia from the GFAP(+)/nestin(+) astrocytomas of varying grades (Figure 2A).

Analysis was primarily guided by the World Health Organization pathological classification of human astrocytomas¹ in evaluating the Ras-B8 mice brains. Diffuse increased number of LacZ(+) and GFAP(+) astrocytes, which were not an infiltrative and expansile mass with nuclear atypia, as evaluated on the basis of serial H&E sections were termed astroglial hyperplasia. These hyperplastic astroglial cells were not transformed as suggested by the following experimental evidence: 1) astrocyte cultures from 1-week-old Ras-B8 and wild-type ICR mice both failed to form colonies in soft-agar, compared to 5 to 10% of the 12-week-old astrocyte cultures from the Ras-B8 mice (Table 1). 2) Hyperplastic astroglial cells stained for the differentiated astrocyte marker GFAP, but not for the glial precursor marker nestin, which were positive in the astrocytomas, as discussed below (Figure 2A). 3) The BrdU labeling index in the astroglial hyper-

Figure 1. Progressive postnatal astroglial hyperplasia in Ras-B8 mice. **A:** At E16.5 there was no difference in the embryonic central nervous system architecture between the Ras-B8 (right) and control littermates (left), including the number of GFAP(+) cells. ^{12}V -Ha-RAS:IRES-LacZ transgene was expressed at E16.5, as detected by LacZ-positive nuclei (bottom right and inset, arrowheads). **B** and **C:** Significant increase in LacZ(+) (**B**) and GFAP(+) (**C**) cells in the Ras-B8 mice from 3 to 12 weeks compared to control age-matched littermates, as per one-way analysis of variance ($P < 0.05$). The number of LacZ(-) cells in the Ras-B8 mice, indicative of cells without transgene integration, did not statistically change ($P > 0.05$) during this time interval. This suggests that expression of the ^{12}V -Ha-RAS:IRES-LacZ transgene in the GFAP(+) cells provided a proliferative stimuli leading to astroglial hyperplasia.

plastic regions of 3-week-old Ras-B8 mice was $<0.01\%$ ($n = 29$), which was no different from age-matched controls. In contrast, the nine low-grade and three high-grade astrocytomas evaluated as part of this study, ex-

hibited BrdU labeling indices of $2.8 \pm 1.7\%$ and $6.0 \pm 2.8\%$, respectively. 4) Feulgen staining demonstrated that the nuclei of the hyperplastic astroglial cells, other than being increased in numbers, were no different in terms of size or gross morphology compared to those observed in age-matched controls (Figure 2A). In contrast, the nuclei of the astrocytes in the low-grade and especially the high-grade astrocytomas were larger and pleomorphic in shape. 5) Additional genetic alterations, which were examined and are known to be associated with low- and high-grade human astrocytomas, as described below, were not found in the hyperplastic astroglial cells, except for expression of VEGF (Figure 2B). Expression of VEGF is consistent with previous reports that VEGF, although not expressed by quiescent astrocytes, is abundantly expressed by either reactive or transformed astrocytes.^{13–15}

Multifocal Astrocytoma Formation

Screening of the 6- μ m Ras-B8 brain sections from the various time points with H&E, LacZ, and GFAP staining yielded foci of suspected astrocytomas, evaluation of which were further supplemented by analyzing for nestin, Feulgen nuclear staining, and BrdU labeling (Figure 2A). These astrocytomas were locally infiltrative and expansile, but in this cohort of animals we did not find any mice that had multiple discrete astrocytomas in different separated geographic regions of the brain. An astrocytoma was classified as low grade if it lacked mitosis, necrosis, or endothelial proliferation, as per World Health Organization criteria, with the presence of any one of these criteria was sufficient to raise the diagnosis to a high-grade astrocytoma (Figure 2A, Table 3).

As per Table 3, we noted a progressive increase in the incidence of astrocytomas detected in the cohort of Ras-B8 mice analyzed, as a function of age. Astrocytomas were not detected in mice at E16.5 or 1 week of age, however, in the cohort of 10 mice each evaluated at 3, 4, and 8 weeks, we detected one, two, and three low-grade astrocytomas, respectively. At 12 weeks, six of seven mice ($\sim 85\%$) developed one astrocytoma each, with three having low-grade and another three mice having high-grade astrocytomas. Both low- and high-grade astrocytomas were histologically characterized as nests of GFAP(+)/nestin(+)-expressing cells, which infiltrated the subcortical white matter. Nestin immunoreactivity has been interpreted as suggestive of a stem cell phenotype in tumors,^{16,17} but can also accompany reactive proliferative gliosis adjacent to experimental stab wounds.¹⁸ Collectively, these results suggest that the nestin reactivity in the Ras-B8 tumors is part of an acquired stem cell-like phenotype, which may accompany a variety of pathological conditions. High-grade astrocytomas had presence of mitotic figures (Figure 2A, inset), with significantly higher BrdU labeling indices ($6.0 \pm 2.8\%$ versus $2.8 \pm 1.7\%$ for low grade; $P < 0.05$) and increased vascularity (data not shown). However, frank endothelial hyperproliferation with glomerular tufting, as found in human malignant astrocytomas,¹ was not appreciated in

Table 3. Pathology of Brains from Ras-B8 GEMs at Varying Time Points

Age	1 Week	3 Weeks	4 Weeks	8 Weeks	12 Weeks
Total mice sacrificed	10	10	10	10	7
Glial hyperplasia only	0	9	8	7	1
Low-grade astrocytoma	0	1	2	3	3
High-grade astrocytoma	0	0	0	0	3

this cohort or in our initial report.⁴ Furthermore, in this cohort we did not have any tumors associated with necrosis, the main criteria for the diagnosis of the most malignant grade of human astrocytoma, also known as glioblastoma multiforme (GBM). However, necrosis is found in astrocytomas in the Ras-B8 and Ras-D7 mice, as denoted by our initial report on this GEM on a separate cohort of mice that were analyzed at the point when they failed to survive,⁴ rather than at fixed time points of this progression study.

The location of the Ras-B8 astrocytomas were in the cerebral hemispheres ($n = 6$), basal ganglia ($n = 1$), septum ($n = 1$), or ventral brainstem ($n = 1$). Studies of astrocytoma formation associated with nitrosamide administration^{19–21} demonstrated that one target population for tumorigenesis is a poorly differentiated subependymal population, which initially proliferate in small clusters and then develop into morphologically primitive tumors or high-grade gliomas. A second pattern of nitrosamide-induced tumorigenesis results in the proliferation of subcortical and cerebellar glial cells, and results in anaplastic oligodendroglial tumors. In the Ras-B8 GEMs, we observed no strong predilection for periventricular growth and an early subependymal stage was not appreciated.

Molecular Aberrations Associated with Astrocytoma Progression

Several molecular aberrations commonly associated with human low- and high-grade astrocytomas were analyzed. Gain-of-function alterations found in the astrocytomas, but not in regions of astroglial hyperplasia in the young mice or surrounding peritumoral regions in older mice harboring astrocytomas, included increased expression of EGFR (wild type) and associated activated downstream signaling pathways, as demonstrated by increased phospho-MAPK and phospho-Akt immunoreactivity (Figure 2B). VEGF expression was noted in the hyperplastic astroglial cells and was also strong in the astrocytomas.

Several loss-of-function alterations, including Tp53 mutations, p16, and PTEN loss, were also noted. We used immunohistochemistry and LCM-coupled mutational analysis in a few specimens to identify changes in Tp53 expression or *TP53* mutations (Figure 3). Abnormal Tp53 expression or *TP53* mutations were not detected in control or astroglial hyperplastic cells, but was observed in all of the low- and high-grade astrocytomas (Figure 3A). LCM performed on one of the low-grade astrocytomas (4-week cohort), strongly positive for Tp53 expres-

sion, revealed a CAT(His)-CCT(Pro) mutation in mouse codon 190 (human codon 193), within exon 6 of the DNA binding region (Figure 3B). In one of the three high-grade astrocytomas with increased Tp53 expression (12-week cohort), there was a AAG(Lys)-ACG(Thr) mutation in mouse codon 289 (human codon 292), within exon 8 of the oligomerization domain (Figure 3B). Of interest, *Tp53* mutational analysis of the high-grade Ras-B8-derivative astrocytoma cell line previously reported,⁴ also demonstrated a mutation in exon 8 (AAG(Lys)-ATG(Met)) in mouse codon 302 (human codon 305). Human homologues of these *TP53* mutations have been previously reported in human cancers, including astrocytomas.^{22–27}

Semiquantitative analysis of the high-grade astrocytomas demonstrated decreased PTEN and p16 immunoreactivity (Figure 4). Percentage of low-grade astrocytoma cell nuclei in five high-power fields, which were positive for p16 was ~8%, similar to 7% of the nuclei of age-matched control mice or in the regions of astroglial hyperplasia. In contrast, only ~3% of the high-grade astrocytoma nuclei were positive for p16. Similarly, very few PTEN-positive astrocytes (less than 1%) were observed in the high-grade astrocytoma cells, with increased phospho-Akt in both the low- and high-grade astrocytomas (Figure 2B).

Conclusions

This study represents the first such analysis of brain tumor formation and progression from the stage of astroglial hyperplasia to overt low-grade astrocytoma formation and malignant progression, in a GEM glioma model. In summary, using the Ras-B8 GEM, we observed consistent patterns of tumor progression, beginning with astrocyte hyperplasia and culminating in low- and high-grade astrocytoma formation. At E16.5, expression of the *¹²V-Ha-RAS:IRES-LacZ* transgene did not result in any detectable phenotype, with minimal astroglial hyperplasia without tumor formation observed by 1 week of age. Increase in astroglial hyperplasia with GFAP(+)/nestin(–) nontransformed astrocytes continued during the 12-week evaluation period of this study (Figure 1, B and C). Beginning at 3 week of age, we first observed low-grade astrocytoma formation, evidenced by focal expression of nestin within the tumor cells, the presence of nuclear atypia, aberrant EGFR expression, and increased (mutant) Tp53 expression (Figures 2 and 3). The progression from low- to high-grade astrocytomas is accompanied by additional genetic changes, including loss of PTEN and p16 expression (Figure 4).

Figure 2. A: Nestin immunoreactivity and nuclear atypia accompany the transition to neoplasia. Panels of control littermates from 12-week-old mice (similar to controls from earlier time points), astroglial hyperplasia, low- and high-grade astrocytomas with H&E, GFAP, nestin, and Feulgen staining. The increased number of GFAP(+)nestin(-) astroglial cells is demonstrated in the 3-week-old mice. Nestin(+) was a clear marker of transformation in both low- and high-grade astrocytoma foci (low-power insets in the low-grade astrocytoma panels). Mitotic figures were abundant in the high-grade astrocytomas, as seen in the H&E high-power inset. Feulgen nuclear staining demonstrates that the astrocytoma foci were hypercellular, comprised of cells with nuclear atypia with larger nuclear cross-sectional diameter (average increase in cross-sectional diameter of high-grade astrocytomas was $\sim 2.7\times$, which was significantly different from hyperplastic astrocyte by paired *t*-test; $P < 0.001$). **B:** Characterization of gain-of-function alterations in Ras-B8 astrocytomas. Photomicrographs illustrating the expression of EGFR (wild type), activated P-MAPK, and P-Akt plus VEGF in the various histological stages of Ras-B8 GEM progression. The photographs are taken near infiltrating margins of the low- and high-grade astrocytomas, to emphasize cytological detail and do not reflect the overall cellularity of the tumors demonstrated in **A**. Wild-type EGFR (not EGFRvIII; data not shown) expression was increased in the astrocytomas, with highest levels in high-grade tumors, as denoted by semiquantitative computer-assisted image analysis. A similar pattern of expression was also noted in P-MAPK and P-Akt. In contrast, VEGF expression was present in astroglial hyperplastic regions, comprised of nontransformed astrocytes, at 3 weeks. Increased VEGF expression continued in the astrocytoma cells of both low- and high-grade astrocytomas. Original magnifications, $\times 1000$.

Figure 3. Ras-B8 glioma development is associated with aberrant expression of *Tp53* and the presence of *TP53* mutations. **A:** *Tp53* nuclear immunoreactivity was not seen in control or astroglial hyperplastic cells, but was present in a subset of cells within the low- or high-grade astrocytoma foci (arrowheads), with increased prevalence in the high-grade astrocytomas. **B:** LCM was performed to isolate tumor cells for the detection of *Tp53* mutations. In both low-grade and high-grade mouse astrocytomas, we observed mutations in the *Tp53* gene, compared to normal mouse astrocytes (NMA). The low-grade astrocytoma cells exhibited a codon 190 His to Pro (CAT to CCT) mutation, high-grade astrocytoma cells a codon 289 Lys to Thr (AAG to ACG) mutation, whereas derivative Ras-B8 high-grade astrocytoma cells demonstrated a codon 302 Lys to Met (AAG to ATG) mutation. Human equivalents of these *Tp53* mutations have been noted in a variety of human cancers, including astrocytomas.

These molecular alterations in the Ras-B8 mice astrocytomas have similarities to those associated with human low- and high-grade astrocytomas, as reviewed in Kleihues and Cavanee.¹ *Tp53* mutations are well documented in a subset of human astrocytomas of all grades (~33%) and acts as a molecular signature of secondary GBMs, which are thought to arise via progression from lower-grade astrocytomas.¹ In comparison, the so called primary GBMs that arise *de novo*, harbor *Tp53* mutations in less than 10% of the cases.¹ We postulate the progression of Ras-B8 GEM astrocytomas to high-grade lesions is akin to the human secondary GBM pathway. In support is our finding of *Tp53* overexpression and mutations in both low- and high-grade Ras-B8 astrocytomas (Figure 3). Furthermore, the diffuse astroglial hyperplasia, which is the first phenotype of the Ras-B8 mice, represented astrocytes that were not transformed, but with acquisition of additional genetic alterations gave rise to Ras-B8 astrocytomas. High-grade Ras-B8 astrocytomas, delineated by increased EGFR and activation of both P-MAPK and P-Akt, as well as decreased expression of PTEN and

Figure 4. Decreased PTEN and p16 expression accompanies progression from low- to high-grade astrocytomas. In the control mouse brain, PTEN expression was minimal in the astrocytes, although detected in the endothelial cells. PTEN was detected in the astroglial hyperplastic cells and many of the infiltrating cells in the low-grade astrocytomas of the Ras-B8 mice, but was detected in less than 1% of the astrocytoma cells in the Ras-B8 high-grade astrocytoma cells. As an internal control, pyramidal cortical neurons consistently stained for PTEN (*), whereas infiltrating astrocytoma cells did not (arrowheads). p16-expressing cells were detected in a few cells from 3-week-old controls. In 3-week-old Ras-B8 astroglial hyperplastic regions and low-grade astrocytomas, there were similar levels (~7 to 8%) of p16-positive nuclei. In high-grade Ras-B8 astrocytomas (12 weeks), p16-positive nuclei were detected in only ~3% of the nuclei. As an internal control, p16 staining was also consistently present in pyramidal neurons (one demonstrated), whereas infiltrating astrocytoma cells did not. Original magnifications, X1000.

p16, are well recognized molecular markers of human GBMs. It should be noted that amplifications of EGFR and associated mutant EGFRs such as EGFRvIII, prevalent in ~40% and ~15 to 20% of human GBMs,¹ was not found in the Ras-B8 high-grade tumors as previously documented in the original description of this GEM.⁴ With the initial characterization of the GFAP-RAS GEM, demonstrating pathological and molecular similarities⁴ and with this study demonstrating similarities in pathological and molecular progression features to human astrocytomas, we are using this GEM glioma model to determine the role of specific tumor-associated genetic alterations in the production of a glial tumor phenotype.²⁸

We hypothesize that ¹²⁵I-Ha-RAS expression by itself is sufficient to provide a significant astrocyte proliferative advantage leading to astroglial hyperplasia, but tumorigenesis requires genomic instability and accumulation of additional genetic mutations, such as *Tp53* loss. The thesis that ¹²⁵I-Ha-RAS can induce genomic instability and predispose to transformation is supported by studies in murine fibroblasts and thyroid carcinoma cells.^{29,30}

Genomic instability was also denoted by multiple karyotype abnormalities detected by SKY in the Ras-B8 malignant astrocytoma-derived cell lines, which were not present in normal murine astrocytes, in our original description of the GEM.⁴ Furthermore, specific genetic alterations in addition to Tp53, such as loss of Pten, p16, p19, and overexpression of CDK4, MDM2, and EGFR, were also detected in the derivative Ras-B8 astrocytoma cell lines and tumors, but not in normal murine astrocytes or brain.⁴ Similarities of these additional genetic alterations to those known to be prevalent in human malignant astrocytomas and the pathological and molecular progression described in the current study, justifies use of this GEM as a potential reagent to discover novel genetic alterations in human gliomagenesis. Toward this objective, are our current experiments using gene-trapping and gene-expression array strategies using this GEM of gliomagenesis.

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