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Loss of GFAP expression in high-grade astrocytomas does not contribute to tumor development or progression

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In astrocytic neoplasms, the number of cells expressing glial fibrillary acidic protein (GFAP) is inversely proportional to the extent of anaplasia. The loss of GFAP expression, the principal marker of astroglial cells, in these tumors has been proposed to constitute a step in their development and progression. To test this hypothesis, we crossed p53-negative ($p53^{-/-}$) mice, which frequently develop astrocytomas after intrauterine exposure to ethylnitrosourea, with GFAP-negative ($GFAP^{-/-}$) mice or $GFAP^{+/+}$ controls. Brain tumors of glial origin were found in 12 of 35 $GFAP^{+/+}$ $p53^{-/-}$ mice (34%) and in 11 of 27 $GFAP^{-/-}$ $p53^{-/-}$ mice (41%). The two groups did not differ in the age at which tumors were detected or in tumor histology or progression. Thus, the loss of GFAP expression frequently seen in high-grade astrocytomas does not constitute a step in tumor development. Rather, it may represent the undifferentiated state of these cells.
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Brain tumors are the third most common cause of death among 18- to 35-year olds, and their incidence is increasing (Steck *et al*., 1997). Glioblastoma multiforme and anaplastic astrocytoma arise from astroglial cells and are the most frequent primary brain tumors in adults (Bigner *et al*., 1998). Despite the recent advances in molecular genetics of brain tumors (for review, see Weiss, 2000; Zhu and Parada, 2002), the current understanding of the tumor pathogenesis does not yet allow specific and successful therapeutic intervention in patients with high-grade astrocytomas. The most widely used marker of astroglial cells is the glial fibrillary acidic protein (GFAP), which was first identified in the brains of patients with multiple sclerosis (Eng *et al*., 1971). In astrocytes, GFAP is the principal component of intermediate filaments, a part of the cytoskeleton (for review, see Pekny, 2001). Even though, in most situations, GFAP seems to be substituted by other intermediate filament proteins, the absence of intermediate filaments in astrocytes was shown to lead to impaired and more prolonged healing following injury of the central nervous system (Eliasson *et al*., 1999; Pekny *et al*., 1999). A role for GFAP in the development of astrocytoma has been proposed, but this issue has never been directly addressed owing to the lack of a suitable experimental model.

Several *in vitro* studies have suggested a strong negative correlation between the level of GFAP expression and the dedifferentiation and transformation of astrocytes. In U-251 human astrocytoma cells, suppression of GFAP expression by stable transfection with an antisense GFAP construct led to decreased cell differentiation and loss of the ability to extend processes in response to neurons (Westermark, 1973; Weinstein *et al*., 1991). Restoration of GFAP expression reversed the phenotype (Chen and Liem, 1994). In another study, U-251 cells transfected with GFAP antisense cDNA showed increased saturation densities, enhanced proliferative potential, and improved anchorage-independent growth in soft agar (Rutka *et al*., 1994). In SF-126 human astrocytoma cells, which are normally GFAP negative, overexpression of GFAP slowed proliferation, increased the size of cellular processes, and reduced the number and growth of colonies in soft agar (Rutka and Smith, 1993). Similarly, stable transfection of rat astrocytoma C6 cells with GFAP cDNA suppressed cell growth and increased the extension of cellular processes (Toda *et al*., 1994) and reduced tumorigenicity *in vivo* (Toda *et al*., 1999). We have shown that primary astrocytes from $GFAP^{-/-}$ mice grow more quickly in culture and reach higher saturation densities than wild-type cells, while retaining their ability to extend processes in response to neurons (Pekny *et al*., 1998).

Malignant astrocytic tumors are often GFAP negative, and many high-grade gliomas seem to lose GFAP expression (Jacque *et al*., 1978; van der Meulen *et al*., 1978; Jacque *et al*., 1979; Velasco *et al*., 1980; Tascos *et al*., 1982). In addition, GFAP-negative cells proliferate more rapidly than GFAP-positive cells in the same tumor (Hara *et al*., 1991; Kajiwara *et al*., 1992). These *in vivo* findings allow two interpretations. The loss of GFAP expression could represent secondary loss of a differentiation marker. Alternatively, it could be a step in tumor development.

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The proliferative advantage, signs of transformation *in vitro*, and increased tumorigenicity *in vivo* associated with the absence of GFAP in astrocytes might indicate that the focal loss of GFAP expression allows tumor progression toward a more rapidly growing and malignant phenotype. $GFAP^{-/-}$ mice would be an ideal experimental model for addressing this issue. However, in mice, the incidence of spontaneous glial tumors is negligible, and the induction of glial tumors by chemical carcinogens is not effective (Wechsler *et al*., 1979). Recently, Oda *et al*. (1997) generated glial tumors in mice by combining prenatal exposure to ethylnitrosourea (ENU) with genetic ablation of the p53 tumor suppressor gene. The absence of p53 expression is associated with high-grade astrocytomas in both mice and humans (Sidransky *et al*., 1992; Yahanda *et al*., 1995). In this study, we crossed $GFAP^{-/-}$ mice onto the p53-null background and studied the incidence, histology, and progression of glial tumors induced with ENU.

The incidence of brain tumors after prenatal exposure to ENU was similar in $GFAP^{-/-}p53^{-/-}$ mice and $GFAP^{+/+}p53^{-/-}$ controls (41 and 34%, respectively; Table 1, Figure 1). In the control group, clinical signs of the tumors appeared between postnatal day 51 (P51) and P78, consistent with previous findings (Oda *et al*., 1997). In the $GFAP^{-/-}p53^{-/-}$ mice, the tumors became manifest over a longer period, between P59 and P127. However, the median day of tumor appearance was not significantly different in the two groups (P62 versus P65, respectively), neither was it sex-dependent (data not shown).

Mice in which we did not detect brain tumors showed clinical signs of extracranial tumors, which appeared between P50 and P78 in $GFAP^{+/+}p53^{-/-}$ mice (median P61) and between P53 and P180 (median P63.5) in $GFAP^{-/-}p53^{-/-}$ mice. Most of these tumors were quickly growing lymphomas of the thymus. Some mice with brain tumors also had extracranial tumors. The incidence and types of extracranial tumors were similar in $GFAP^{+/+}p53^{-/-}$ and $GFAP^{-/-}p53^{-/-}$ mice (data not shown).

Previous studies (Oda *et al*., 1997; Leonard *et al*., 2001) showed somewhat higher incidence of glial tumors in $p53^{-/-}$ mice prenatally exposed to ENU (60–70% compared to 34–41% in this study). While we killed the mice immediately after they showed first clinical signs of a growing tumor, Oda *et al*. allowed at least some of them to progress until they died, which could have accounted for the increased incidence of glial tumors in the mice primarily affected by lymphomas and other tumors.

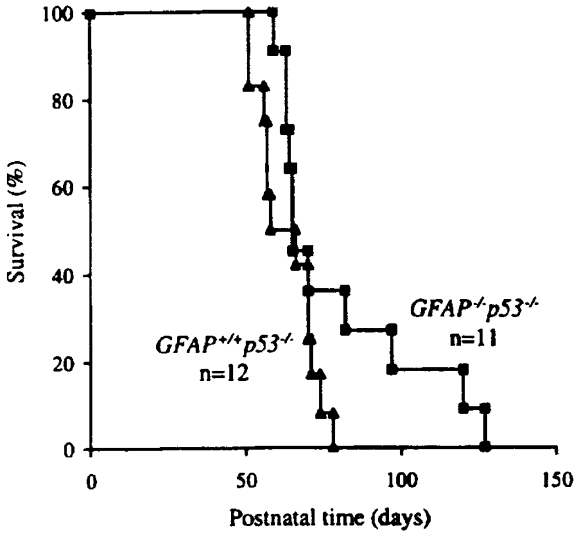

Figure 1 Survival of mice with glial tumors. Two-tailed Student's *t*-test was used to compare the survival of mice in the two groups, $P > 0.05$. $GFAP^{-/-}$ mice (Pekny *et al*., 1995) were crossed with $p53^{+/-}$ mice (Livingstone *et al*., 1992) from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained on a mixed C57Bl/129 genetic background and housed in a barrier animal facility with food and water *ad libitum*. $GFAP^{-/-}$ $p53^{+/-}$ and $GFAP^{+/+}p53^{+/-}$ females were mated with males of the same genotypes and checked for vaginal plugs the day after mating. At embryonic day 14.5 (E14.5), pregnant females were injected intraperitoneally with ENU (Sigma-Aldrich, St Louis, MO, USA; 25 mg/kg in 0.9% NaCl solution) as described (Oda *et al*., 1997). The newborn mice were housed with their mothers until weaning at 3–4 weeks of age. The genotype of the offspring was determined by PCR (for p53) and Southern blot (for GFAP) analyses of DNA from tail biopsies (Livingstone *et al*., 1992; Pekny *et al*., 1995). The mice were examined daily and killed as soon as they showed any clinical signs of a growing tumor

Histological examination demonstrated that all of the brain tumors were of glial origin. The location, size, and histology of the tumors were comparable in the two groups. Of 12 brain tumors, 11 in $GFAP^{+/+}p53^{-/-}$ mice contained an abundance of GFAP-positive cells (Figure 2). In both groups, tumors developed most commonly in the cerebrum close to the hippocampus,

Table 1 Incidence of glial tumors, proliferative index, and age at tumor detection

Group	*Mice with glial tumors (n)*	*Proliferative index (%)*	*Tumor incidence (%)*	*Day of tumor detection*	
				Range	*Median*
$GFAP^{+/+}p53^{-/-}$ ($n=35$)	12	22 ± 5.7	34	51–78	62
$GFAP^{-/-}p53^{-/-}$ ($n=27$)	11	28 ± 6.2*	41**	59–127	65*

To visualize $Ki67^{pos}$ cells, we used anti-Ki67 rat monoclonal antibodies (DAKO, Glostrup, Denmark; dilution 1:25) and Cy3-conjugated anti-rat antibodies (Jackson Immunoresearch, West Grove, PA, USA) as described in Figure 2. Cell nuclei were visualized by TO PRO-3 (Molecular Probes, Eugene, OR, USA). Proliferative index was calculated as a ratio of $Ki67^{pos}$ cell nuclei and the total number of nuclei in a 4 μm thick superimposed image obtained by using a laser-scanning confocal microscope (TCS NT, Leica, Heidelberg, Germany). Proliferative index is expressed as mean $\pm$ s.e.m. *$P > 0.05$ versus value in $GFAP^{+/+}$ controls (two-tailed *t*-test). **$P > 0.05$ versus value in $GFAP^{+/+}$ controls (two-tailed χ^2 test).

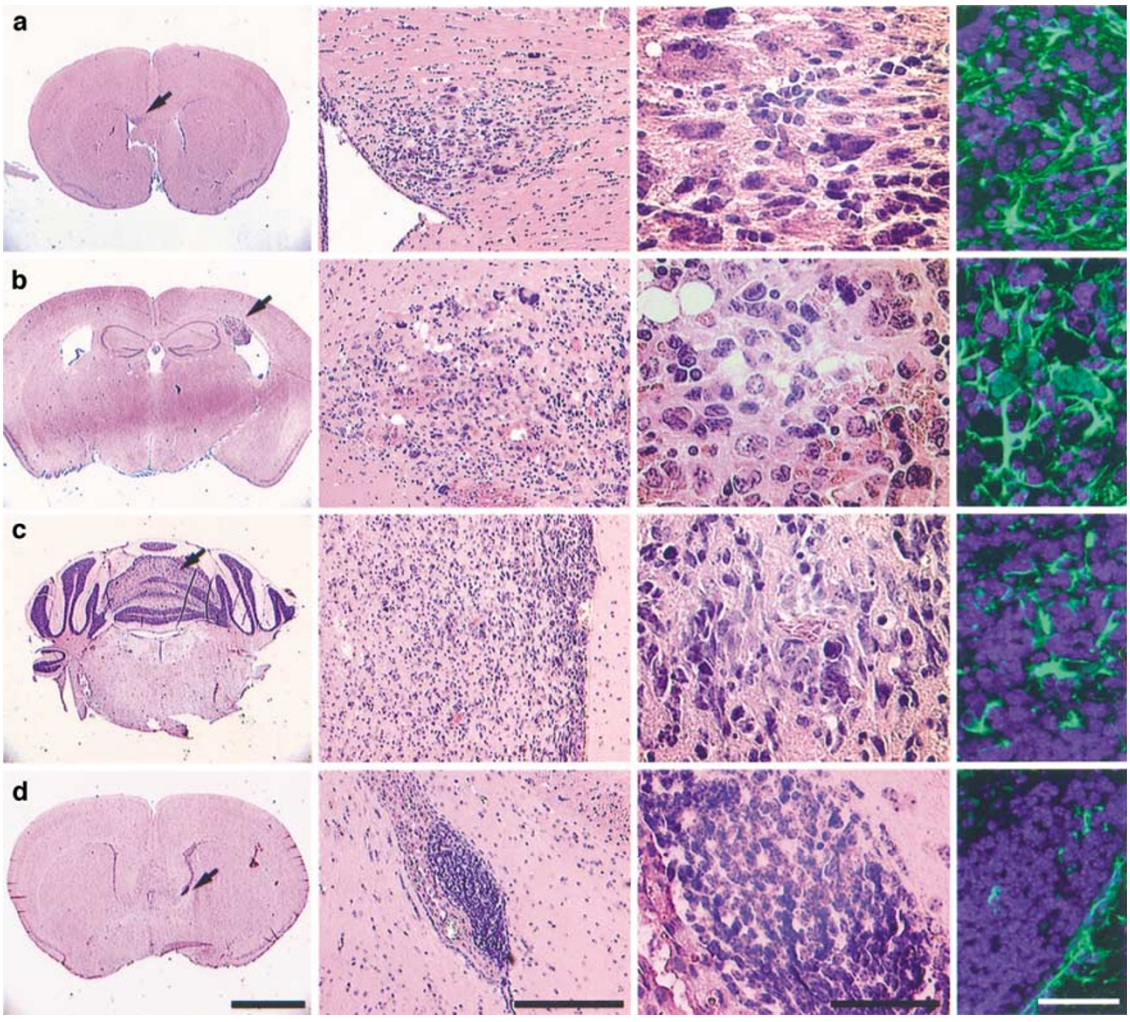

Figure 2 Glial tumors in $GFAP^{+/+}p53^{-/-}$ and $GFAP^{-/-}p53^{-/-}$ mice. The tumors in $GFAP^{+/+}p53^{-/-}$ mice (**a**–**d**) were most commonly found in the vicinity of the lateral ventricle and corpus callosum (**a**, **b**), or in the cerebellum (**c**). The location and histological appearance of the tumors in $GFAP^{-/-}$ $p53^{-/-}$ mice (**e**–**h**) were similar to those in $GFAP^{+/+}p53^{-/-}$ mice. Three panels on the left show sections stained with hematoxylin and erythrosine. Scale bars: left, 2000 μm; center-left, 200 μm; center-right, 50 μm. The right panel (**a**–**d**) shows GFAP-immunoreactivity in $GFAP^{+/+}p53^{-/-}$ tumors: green indicates GFAP-positive cells; blue indicates nuclei. Scale bar, 50 μm. After necropsy, the brains were fixed overnight in 4% phosphate-buffered formaldehyde, dehydrated, and embedded in paraffin. The brains were serially sectioned in the frontal plane, and 10 adjacent sections (5 μm thick) were collected every 250 μm. In the anterior forebrain and in the cerebellum, the regions where the tumors most commonly occurred, this interval was decreased to 150 μm so that even minute tumors would not escape detection. One slide from each series was deparaffinized, rehydrated, stained with hematoxylin and erythrosine, and examined by light microscopy. The presence and the character of a tumor was verified by a mouse pathologist. For GFAP immunohistochemistry, the sections were deparaffinized, rehydrated, placed in 0.01 M citrate buffer (pH 6.0), and heated for 15 min in a microwave oven for antigen retrieval. Next, the sections were incubated for 30 min with 1% bovine serum albumin (BSA) and 0.05% Triton X-100 in PBS to block nonspecific background and then for 1 h with rabbit GFAP antibodies (DAKO, Glostrup, Denmark) diluted 1:100 in 0.1% BSA and 0.05% Triton X-100 in PBS. After washes in PBS, the sections were incubated with secondary antibody (Alexa 488-conjugated anti-rabbit; Molecular Probes) and TO PRO-3 (Molecular Probes) for 1 h. All incubations were performed at room temperature. The sections were mounted in Mowiol (Clariant GmbH, Frankfurt am Main, Germany) and examined and photographed with a laser-scanning confocal microscope (TCS NT, Leica)

often adjacent to the lateral ventricle (Figure 2a, b, e, f), and in the cerebellum (Figure 2c, g). The tumors ranged from small to large. Small tumors of high cellularity were found compressing the brain ventricle (Figure 2d, h). Larger tumors were found throughout the brain, in some cases close to the ventricles, and often contained large cells and a high proportion of dividing cells (Figure 2a, b, e, f). None of these tumor characteristics showed any difference between the $GFAP^{+/+}p53^{-/-}$ and $GFAP^{-/-}p53^{-/-}$ groups.

Proliferative index was determined for all the tumors and was comparable between $GFAP^{+/+}p53^{-/-}$ and $GFAP^{-/-}p53^{-/-}$ mice (22 ± 6 and $28\pm6\%$, respectively, $P>0.05$; Table 1), and the values were similar to those obtained in human glioma xenografts transplanted into nude rat brains (Engebraaten *et al*., 1999).

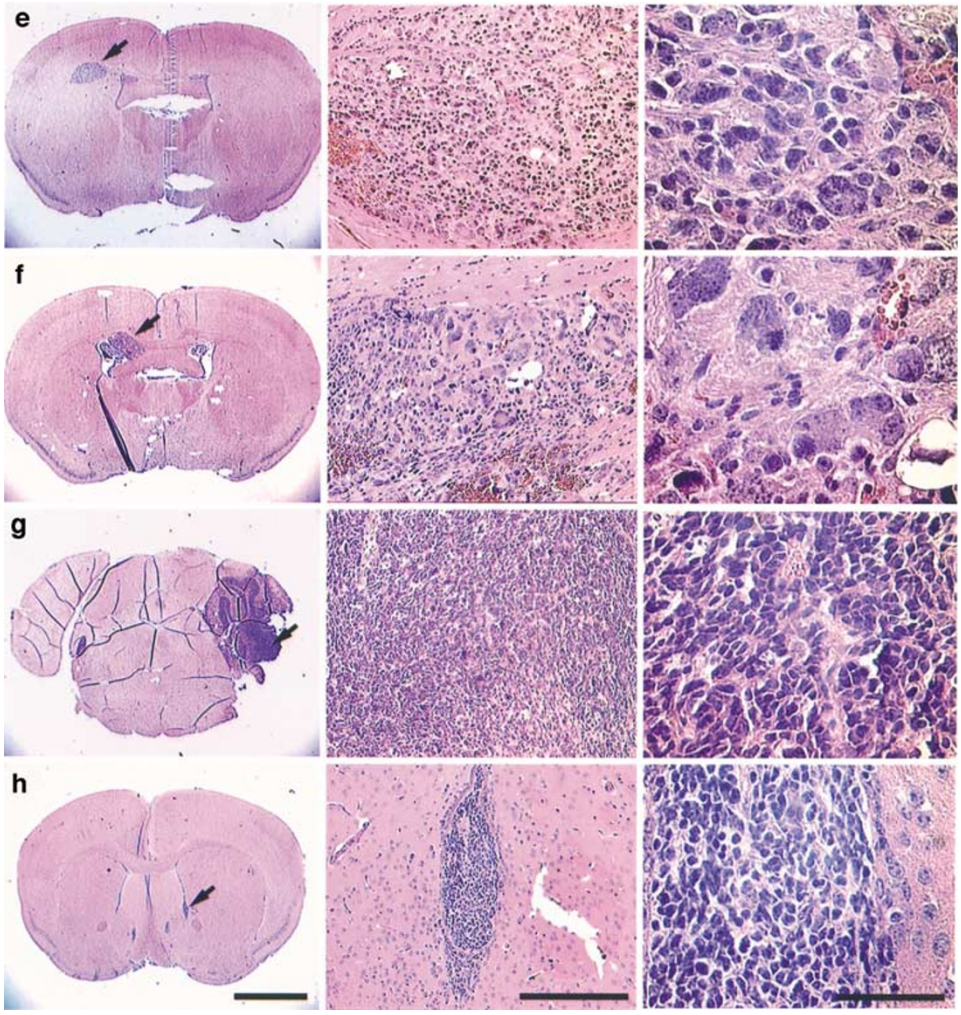

Figure 2 *Continued*

The possible role of GFAP in the development of astrocytomas has been discussed in dozens of articles spanning more than a quarter of a century. The generation of $GFAP^{-/-}$ mice on the p53-null background provided a model to test the concept that *GFAP* might be a tumor suppressor gene. Utilizing this model, we found no difference in the incidence, growth, or histological characteristics of brain tumors between $GFAP^{-/-}$ mice and $GFAP^{+/+}$ controls.

Although the number of GFAP-positive cells within a glioma is inversely proportional to the extent of anaplasia (Deck *et al*., 1978; Eng and Rubinstein, 1978), the distribution of GFAP expression within glioblastomas varies considerably. Astrocyte-like tumor cells, especially gemistocytes, are often strongly GFAP positive, whereas small undifferentiated cells within the same tumor are often negative. Our findings in this study demonstrate that the GFAP expression does not contribute to the development or progression of astrocytomas.

How can our findings be reconciled with numerous observations showing that cells lacking GFAP have a proliferative advantage over their GFAP-positive counterparts? In astrocytes, GFAP is the principal component of cytoskeletal intermediate filaments (Eng *et al*., 2000; Pekny, 2001). Before cell division, intermediate filaments are depolymerized, and phosphorylation plays a key role in this process (Ku *et al*., 1996). Some kinases, such as protein kinase C and cdc2, that are involved in cell-cycle regulation also participate in the phosphorylation of intermediate filaments (Inagaki *et al*., 1990; Dalton, 1992; Yong, 1992; Tsujimura *et al*., 1994). Consequently, the absence of intermediate filaments may increase the accessibility of such kinases to substrates involved in the cell cycle. As a result, cultured cells devoid of GFAP (Weinstein *et al*., 1991; Rutka *et al*., 1994; Pekny *et al*., 1998) and the GFAP-negative fraction of cells within astrocytoma would proliferate more rapidly than GFAP-positive cells. Nevertheless, the absence of GFAP does not seem to increase the transformability of such astrocytes *in vivo*. Thus, the loss of GFAP expression frequently seen in high-grade astrocytomas does not constitute a step in tumor development.

Rather, it may reflect the undifferentiated state of these cells.

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