# Pediatric glioblastomas: A histopathological and molecular genetic study

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**Glioblastoma multiforme (GBM) occurs rarely in children. Relatively few studies have been performed on molecular properties of pediatric GBMs. Our objective in this study was to evaluate the genetic alterations in pediatric GBM (age ⩽ 18 years) with special reference to p53, p16, and p27 protein expression, alterations of the epidermal growth factor receptor (EGFR), and deletion of the phosphate and tensin homolog gene (*PTEN*). Thirty cases of childhood GBMs reported between January 2002 and June 2007 were selected, and slides stained with hematoxylin and eosin were reviewed. Immunohistochemical staining was performed for EGFR, p53, p16, and p27, and tumor proliferation was assessed by calculating the MIB-1 labeling index (LI). Fluorescence in situ hybridization analysis was performed to evaluate for *EGFR* amplification and *PTEN* deletion. Histopathological features and MIB-1 LI were similar to adult GBMs. p53 protein expression was observed in 63%. Although EGFR protein overexpression was noted in 23% of cases, corresponding amplification of the *EGFR* gene was rare (5.5%). Deletion of the *PTEN* gene was also equally rare (5.5%). One case showed polysomy (chromosomal gains) of chromosomes 7 and 10. Loss of p16 and p27 immunoexpression was observed in 68% and 54% of cases, respectively. In pediatric de novo/primary GBMs, deletion of *PTEN* and *EGFR* amplification are rare, while p53 alterations are more frequent compared to primary adult GBMs. Frequency of loss of p16 and p27 immunoexpression is similar to their adult counterparts. This suggests that pediatric malignant gliomas are distinctly different from adult GBMs, highlighting the need for identification of molecular targets that may be adopted for future novel therapeutic strategies.**
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Malignant gliomas are much rarer in children than in adults, comprising only 5%–10% of childhood intracranial neoplasms.[1] In adults, distinct molecular pathways have been described for the development of these tumors. The primary/de novo glioblastomas (GBMs), which typically affect older patients, are characterized by amplification of the epidermal growth factor receptor gene (*EGFR*) along with deletion or mutation of phosphate and tensin homolog tumor suppressor gene (*PTEN*), a negative regulator of the phosphatidyl inositol 3 kinase/Akt signaling pathway. In contrast, secondary GBMs, which evolve from low-grade lesions and occur in younger individuals, often have mutations of the tumor suppressor gene *TP53* but only infrequently have amplification of *EGFR* or alterations of *PTEN*.[2–5]

Compared with the extensive work that has been performed to characterize the molecular features of adult malignant gliomas, relatively little has been reported on characterization of these features in pediatric gliomas.[6] A few studies have suggested some differences in the genetic pathways leading to the formation of de novo GBMs in children compared to adults.[7–9] In view of the ongoing efforts to apply therapeutic strategies directed at EGFR and Akt signaling pathways to the treatment of malignant glioma in adults, and the recent interest in translating such approaches to childhood tumors, we

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need a more precise understanding of the cellular and molecular basis of the disease in childhood.

Hence, we undertook this study to assess the molecular profile of pediatric primary GBMs with special reference to EGFR alterations (protein expression and gene amplification), *PTEN* deletion, and p53, p16, and p27 protein expression. Immunohistochemical staining was used to evaluate protein expression and to determine tumor proliferation by calculating the MIB-1 labeling index (LI). For molecular profiling of these tumors, fluorescence in situ hybridization (FISH) technique was applied on paraffin-embedded sections, using EGFR/CEP7 (chromosome 7 centromere probe) and PTEN/CEP10 paired commercial probes. To the best of our knowledge, this is the first FISH analysis of a representative cohort of pediatric GBMs from India.

## Materials and Methods

### *Clinical Patient Data*

Forty-five children (≤18 years of age) diagnosed with glioblastomas between January 2002 and June 2007 were identified from a detailed review of the neuropathology records of the All India Institute of Medical Sciences. Age and sex of all patients were noted.

### *Histopathological Examination*

Thirty cases of supratentorial pediatric glioblastoma with sufficient material available in paraffin blocks were selected for further analysis. The original hematoxylin and eosin slides were reevaluated independently by two neuropathologists (C.S. and V.S.). Detailed histopathological features were noted: cellularity, pleomorphism, presence of giant cells, mitotic activity, endothelial proliferation including glomeruloid formation, and necrosis (confluent/palisading). The diagnosis was reconfirmed per the recent WHO classification.[10]

### *Immunohistochemical Staining for p53, p16, p27, EGFR, and MIB-1*

Monoclonal antibodies for p53-DO1 (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), p16 (1:50; Neomarkers, Fremont, CA, USA), p27 (1:25; Dako, Glostrup, Denmark), EGFR-NCL (1:50; Dako), and MIB-1 (1:200; Dako) were used. Universal-labeled streptavidin biotin kit was used as the detection system (Dako).

Briefly, 5-μm sections were cut from paraffin-embedded blocks and baked for 2 h. After deparaffinization and rehydration in descending grades of alcohol, the sections were brought to water. Sections for EGFR immunostaining were subjected to protease digestion (Dako S 3020) for 1 h. For p53, p16, p27, and MIB-1 staining, antigen retrieval was performed by transferring the sections into 0.01 M citrate buffer (pH 6.0) previously heated in a microwave oven. After washing in Tris (pH 7.6) and blocking with 3% $H_2O_2$ in methanol for 30 min at room temperature (RT), the sections were incubated overnight at 4°C with the primary antibodies. The sections were washed in Tris, treated with the biotin-labeled secondary antibody for 60 min at RT, and then washed in Tris. The sections were then incubated with tertiary antibody for 60 min at RT and washed in Tris. Sections were then stained with diaminobenzidine for 10 min, washed with distilled water, counterstained in hematoxylin for 1 min, and mounted.

Tumor cell staining for p53 and EGFR was graded as 0 if no cells stained, 1+ if 1%–10% stained, 2+ if 11%–25% stained, 3+ if 26%–50% stained, and 4+ if 51%–100% stained.[8] The MIB-1 LI was calculated in the highest proliferating area as percentage of labeled nuclei per 1,000 cells. Expression for p16 and p27 was evaluated as either positive or negative.

### *FISH Analysis of PTEN and EGFR*

In 18 of 30 cases, where sufficient material was available in the blocks, a dual-probe FISH assay was performed on paraffin-embedded sections, with locus-specific probes for EGFR and PTEN paired with centromere probes for chromosomes 7 (CEP7) and 10 (CEP10) (Vysis, Downers Grove, IL, USA), respectively. Deparaffinization of the sections was carried out with three 10-min immersions in xylene followed by two 3-min immersions in 100% ethanol. Following rinsing in water, target retrieval was achieved by immersing the slides in citrate buffer (pH 6.0) and boiling in a microwave for 20 min. Slides were digested in 0.04% pepsin (P-7000; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C, fixed, and dehydrated. Probe mixture (10 μl per slide) was applied on each section. Paired probe mixture for PTEN/CEP10 was diluted from stock with hybridization buffer and distilled water to a concentration of 1:7:2, respectively. The probe for EGFR/CEP7 was available prediluted. Simultaneous probe/specimen denaturation at 73°C for 5 min with subsequent overnight incubation at 37°C was performed in a Thermobrite hybridization chamber (Vysis). The sections were washed the next day in 2× saline–sodium citrate (SSC; 2 min at 73°C) followed by 0.5× SSC (2 min at RT), counterstained with 4,6-diamidino-2-phenylindole (Vysis), and visualized under a fluorescent microscope.

Signals were scored in at least 200 nonoverlapping, intact nuclei. Sections from nonneoplastic cortical tissue obtained from epilepsy surgery specimens were used as a control for each probe pair. *EGFR* amplification was considered when more than 10% of tumor cells exhibited either a EGFR:CEP7 ratio > 2 or innumerable tight clusters of signals of the locus probe. Hemizygous deletions were defined as >50% nuclei containing either one signal of locus-targeted probe and two or more signals of reference probe (absolute deletions) or two signals of locus-targeted probe and more than four signals of reference probe (relative deletions). Homozygous deletions were identified by the simultaneous lack of both signals of locus-targeted probe and the presence of reference probe signals in more than 30% of cells. Monosomy for chromosome 10 was defined by the presence of one CEP signal per cell in >50%. Polysomies (chromosomal

gains) were defined as >10% of nuclei containing three or more signals for locus or CEP.[7]

## Results

Of 528 glioblastomas diagnosed in our neuropathology laboratory between January 1, 2002, and June 30, 2007, 45 occurred in children, constituting 8.5% of all GBMs. Ages ranged from 9 months to 18 years, with a mean of 11.2 years. There was a marked male preponderance (M:F ~ 2:1; Table 1).

The histological features of the childhood GBMs (Fig. 1a–d) were similar to those of adult GBMs in that they showed marked pleomorphism, giant cells, brisk mitotic activity, endothelial proliferation, and necrosis (confluent and pseudopalisading). The mean MIB-1 LI (Fig. 2a) was 38.6% (range, 8%–65%). p53 protein expression (Fig. 2b) was observed in 63% (19 of 30) of cases. Approximately 70% of these showed 3+ or 4+ expression. EGFR protein expression (Fig. 2c) was seen in seven (23%) cases. In approximately 85% of the EGFR-positive cases, 3+ or 4+ expression was observed. Approximately 20% ($n$ = 6) of cases were double negative; that is, they did not show either p53 or EGFR staining. Positivity for both EGFR and p53 was noted in 7% of cases ($n$ = 2). Loss of p16 and p27 immunoexpression was observed in 68% (20) and 54% (16) of cases, respectively (Fig. 2d).

Representative results of FISH analysis are presented in Fig. 3. Of the 18 cases analyzed, *EGFR* amplification was seen in only one case (5.5%). A comparative analysis of EGFR protein expression in these 18 cases revealed overexpression in seven cases (39%). Thus, only 15% of the childhood GBM cases showing EGFR protein overexpression had concomitant *EGFR* amplification, further highlighting the fact that protein expression cannot be translated to gene amplification and thus cannot be used for molecular profiling.

*PTEN* deletion was equally rare and observed in only one case. This was a hemizygous deletion, because most cells showed only one signal for PTEN and two signals for the CEP probe. One case showed polysomies (chromosomal gains) of chromosomes 7 and 10.

**Table 1.** Summary of immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) results for each patient

| Patient No. | Age (Years) | Sex | EGFR IHC | *EGFR* FISH | p53 | p16 | p27 | *PTEN* |
|---|---|---|---|---|---|---|---|---|
| 1 | 17 | M | 0 | No Ampl | 4+ | P | P | No Del |
| 2 | 5 | M | 0 | NA | 3+ | P | P | NA |
| 3 | 16 | M | 0 | NA | 0 | N | N | NA |
| 4 | 2 | M | 0 | No Ampl | 4+ | N | P | No Del |
| 5 | 15 | F | 0 | NA | 0 | P | P | NA |
| 6 | 10 | F | 0 | NA | 3+ | P | N | No Del |
| 7 | 13 | F | 0 | Ampl 1 | 0 | N | P | No Del |
| 8 | 10 | M | 2+ | No Ampl | 1+ | N | P | No Del |
| 9 | 11 | M | 4+ | No Ampl | 0 | N | P | NA |
| 10 | 6 | F | 0 | NA | 4+ | N | P | NA |
| 11 | 3 | M | 0 | No Ampl | 0 | N | N | No Del |
| 12 | 5 | M | 0 | No Ampl | 2+ | N | N | No Del |
| 13 | 14 | F | 0 | NA | 1+ | N | N | No Del |
| 14 | 3 | M | 3+ | No Ampl | 4+ | P | P | No Del |
| 15 | 15 | M | 3+ | No Ampl | 0 | N | P | NA |
| 16 | 14 | M | 0 | NA | 4+ | P | P | NA |
| 17 | 14 | F | 0 | NA | 4+ | P | N | NA |
| 18 | 13 | F | 0 | NA | 3+ | P | N | NA |
| 19 | 11 | M | 0 | NA | 4+ | P | P | NA |
| 20 | 8 | F | 0 | NA | 3+ | N | N | NA |
| 21 | 12 | M | 0 | NA | 4+ | N | N | NA |
| 22 | 3 | M | 0 | No Ampl | 0 | N | P | Del+ |
| 23 | 17 | M | 0 | No Ampl | 2+ | N | N | No Del |
| 24 | 15 | M | 0 | No Ampl | 3+ | N | N | No Del |
| 25 | 18 | M | 4+ | No Ampl | 0 | N | P | No Del |
| 26 | 0.75 (9 months) | M | 0 | No Ampl | 0 | N | N | No Del |
| 27 | 17 | M | 0 | No Ampl | 4+ | P | N | No Del |
| 28 | 5 | F | 4+ | No Ampl | 0 | N | N | No Del |
| 29 | 16 | M | 0 | No Ampl | 1+ | N | N | No Del |
| 30 | 12 | M | 4+ | Aneuploid | 0 | N | N | Aneuploid |

Abbreviations: EGFR, epidermal growth factor receptor; *PTEN*, phosphate and tensin homolog gene; M, male; Ampl, amplification; P, positive; Del, deletion; NA, not available; N, negative; F, female.

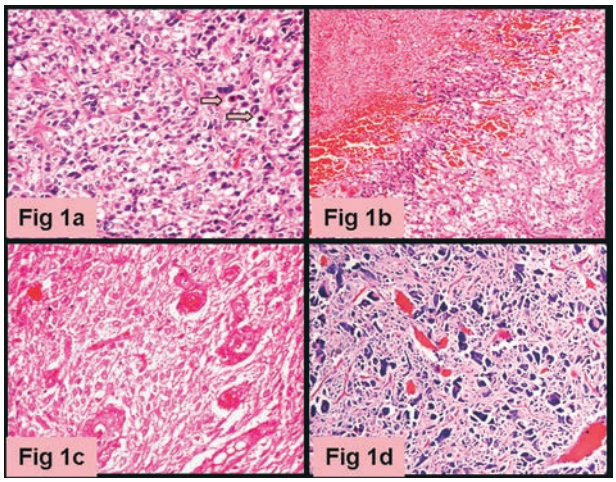


Fig. 1. Hematoxylin and eosin staining. (a) Photomicrograph of a glioblastoma showing brisk mitotic activity. (b) Typical pallisading necrosis. (c) Glioblastoma with endothelial proliferation. (d) A case showing bizarre multinucleated tumor giant cells. Original magnification, ×200 for a–d.

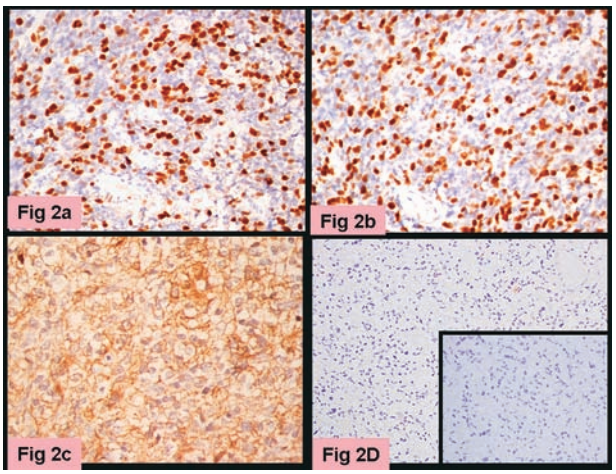


Fig. 2. Immunohistochemical staining. (a) A case showing very high proliferation activity as demonstrated by immunoreactivity to MIB-1. (b) Immunohistochemistry for p53 showing strong (4+) nuclear immunoreactivity. (c) Diffuse membranous expression of epidermal growth factor receptor in tumor cells. (d) A case showing loss of expression (negativity) of p16 protein. (Inset) Same case showing negativity for p27 protein. Original magnification: ×200 for a–c and d inset; ×100 for d.

## Discussion

Childhood glioblastomas are extremely rare compared with their adult counterparts.[10] These tumors comprised 8.52% of all glioblastomas diagnosed in our department in the last 5.5 years.

The molecular genetics of adult GBMs has been intensely studied over the past years.[10] They commonly demonstrate amplification of *EGFR*,[2–5] which encodes a tyrosine kinase involved in cell replication, and inactivation by mutation or deletion of *PTEN*, a negative regulator of phosphatidyl inositol 3 kinase/Akt signaling.[11–14] In contrast, secondary adult malignant gliomas that evolve from low-grade lesions generally affect young adults and often have mutations in *TP53*, which encodes the p53 protein, but only infrequently have amplification of *EGFR* or alterations of *PTEN*.[2–5] The identification of high-frequency gene alterations in these tumors has motivated substantial efforts toward evaluating related hypotheses involving targeted therapies.

In adult malignant gliomas, various therapeutic strategies have been developed to interfere with Akt-mediated signal transduction, which is derepressed by the loss of *PTEN*, and to block the EGFR tyrosine kinase and diminish downstream signaling from this receptor. In addition, antibody- and ligand-mediated therapeutic agents, such as immunotoxins and radioimmunoconjugates, are being targeted to tumor cells that overexpress EGFR.[15] Given the potential of these new strategies, there has been significant interest in assessing their efficacy in treating malignant gliomas in children. However, the extent of similarity between genetic pathways of malignant gliomas in children and adults is largely unknown, and the rationale for applying therapeutic approaches used for the treatment of malignant gliomas in adults to corresponding tumors in pediatric patients therefore lacks justification.

Only a few studies have addressed the chromosomal and genetic alteration in childhood glioblastomas

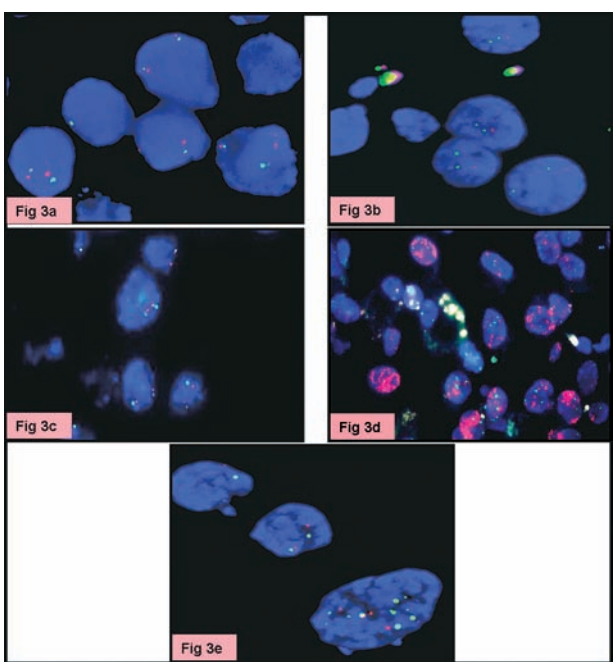


Fig. 3. Fluorescence in situ hybridization analysis. (a) A case with tumor cells expressing normal phosphate and tensin homolog protein (PTEN; two red signals per cell) and CEP10 (two green signals per cell). (b) Photomicrograph of a tumor showing hemizygous deletion of 10q23/PTEN locus: a single red signal in most of the tumor nuclei (PTEN) and paired green signals for CEP10. (c) Normal EGFR/CEP7 expression (two red and two green signals in most of the tumor cells). (d) Section from a tumor showing *EGFR* amplification. Innumerable red signals (EGFR) are seen compared to few green signals (CEP7). (e) Section of a tumor showing polysomies (chromosomal gain). Original magnification, ×1,000 for a–e.

because of low incidence. Recent institutional pilot studies and multiinstitutional analyses have indicated that malignant gliomas arising in children have molecular features and prognostic correlates that are distinct from those in adults.[16–20] These observations have important implications for the translation of therapies from adult to pediatric gliomas.

In the present study, we analyzed the molecular profile of childhood GBMs in terms of EGFR, p53, p16, and p27 protein expression and assessed the status of *EGFR* amplification and *PTEN* deletion. MIB-1 LI was also evaluated.

It has been postulated previously that *EGFR* amplification is much less common in pediatric glioblastomas compared to adult primary glioblastomas. *EGFR* amplification occurs in approximately 35%–50% of adult primary glioblastomas.[21,22] Only one case in the present study showed *EGFR* amplification. This case also showed a very high EGFR protein expression (4+). Some other studies found no *EGFR* amplifications among pediatric glioblastomas,[23–26] and others found amplification in very few cases.[27,28] On the other hand, the reports on EGFR protein expression have been quite varied. Sure et al.[29] reported EGFR overexpression in only 2 of 20 cases (10%). Similar observations were made by Cheng et al.,[23] who found only very small foci of tumor cells showing EGFR immunopositivity in 4 of 13 glioblastomas. On the other hand, Bredel et al.[28] reported elevated immunoreactivity for EGFR in 80% of high-grade nonbrainstem gliomas. Such a large disparity could be due to variation in types of antibodies used, antigen retrieval timings, protocols followed, and methods of fixation.

In the present series, 23% of cases showed EGFR protein expression. Despite the infrequency of EGFR amplification, the overexpression of EGFR protein observed could be due to upregulation of receptor expression by mechanisms other than amplification.

*PTEN* mutations have been detected by different techniques in 15% to more than 40% of GBMs from adult patients.[13,30,31] The data reported on *PTEN* alterations in pediatric malignant gliomas are variable. Cheng et al.[23] found *PTEN* mutations in only 8% of pediatric high-grade gliomas, while Rasheed et al.[32] did not detect any *PTEN* mutation in 22 childhood gliomas of all grades. Raffel et al.[33] detected *PTEN* mutations in 3 of 15 pediatric GBMs (20%), which was the only alteration significantly associated with decreased survival in their study group. Sung et al.[34] reported on homozygous *PTEN* deletions in 2 of 28 pediatric high-grade astrocytomas. Kraus et al.[35] found *PTEN* mutation in one of six cases. We observed *PTEN* deletion in only 5.5% of cases.

Regulation of proliferative activity and cell cycle progression depends on sequential activation of a set of cyclin-dependent kinases (CDKs).[36] Two families of tumor suppressor genes, Cip/Kip (*p21*, *p27*, and *p57*) and INK4 (*p15*, *p16*, *p18*, and *p19*), regulate cell proliferation and neoplastic transformation. It has been well established that in adult astrocytomas the expression of CDK inhibitors p16 and p27 decreases with increasing tumor grades. Loss of p16 immunoexpression was seen in approximately 57% of adult glioblastomas in a study by Ranuncolo et al.[37] Further, lack of p16 protein expression due to homozygous gene deletion or mutation is associated with poor survival.[38] Regulation of p27 occurs primarily at the posttranslational level by ubiquitin-mediated degradation.[39] Decreased levels of p27 are associated with a poor prognosis and short survival.[40,41] Very few studies have evaluated CDK inhibitors in pediatric GBMs. Sure et al.[29] detected loss of p16 expression in 61% of pediatric glioblastomas. In the present study, loss of p16 and p27 immunoexpression was observed in 68% and 54% of cases, respectively, which is similar to that observed in adult GBMs.

Pediatric high-grade gliomas resemble the pattern seen in adult secondary GBMs with regard to p53 overexpression, which is often used as a surrogate indicator of alterations on *TP53* gene functional status. *TP53* mutations are the genetic hallmark of secondary glioblastoma and are significantly less frequent. In a study by Watanabe et al.,[5] the incidence of p53 protein accumulation (nuclear immunoreactivity to PAb 1801 polyclonal antibody) was lower in primary (~25%) than in secondary (>65%) glioblastomas. Similar trends have been observed in recent studies.[5,42,43] p53 protein expression was seen in 63% of our cases. Similar observations have been made by previous authors. Cheng et al.[23] documented p53 protein accumulation in 75% of high-grade childhood gliomas, and p53 mutation in 38%. Pollack et al.[18] documented increased expression of p53 in about 35% of high-grade gliomas in children, which increased to 58% when only glioblastomas were considered. The authors determined that this feature was an adverse prognostic factor in a cohort of children who were treated uniformly with surgery, radiotherapy, and chemotherapy. The frequency of p53 mutations was 33%, which is more in line with the frequency of such mutations in the secondary malignant gliomas that affect young adults.[5,24,44]

To conclude, childhood de novo/primary GBMs resemble their adult counterparts with regard to clinical history: they show no evidence of previous low-grade astrocytoma, and they share similar histomorphological features. However, the patterns of genetic alterations in pediatric primary GBMs appear to be distinct from those in adult GBMs. In our cases, alterations of *PTEN* and amplification of *EGFR* were uncommon. A large majority of cases showed p53 protein expression. Loss of p16 and p27 expression was observed in a significant number of cases. More such studies with proper follow-up data are needed in deciding if extrapolations from the study of adult astrocytomas can be implemented for glioma therapies in children. Further, determination of both the similarities and differences between pediatric and adult astrocytomas will aid in the development of targeted, individualized therapies that will be of benefit to all individuals afflicted with this type of cancer.

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