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.

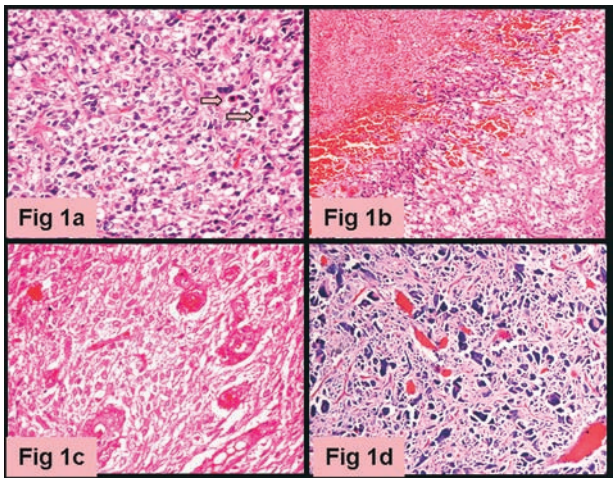

Fig. 1. Hematoxylin and eosin staining. (a) Photomicrograph of a glioblastoma showing brisk mitotic activity. (b) Typical pallisading necrosis. (c) Glioblastoma with endothelial proliferation. (d) A case showing bizarre multinucleated tumor giant cells. Original magnification, ×200 for a–d.

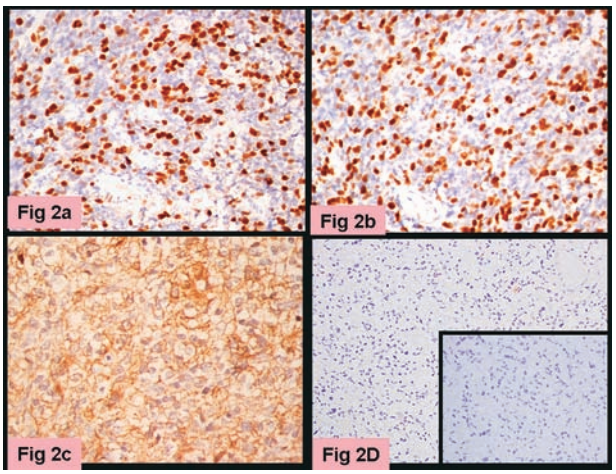

Fig. 2. Immunohistochemical staining. (a) A case showing very high proliferation activity as demonstrated by immunoreactivity to MIB-1. (b) Immunohistochemistry for p53 showing strong (4+) nuclear immunoreactivity. (c) Diffuse membranous expression of epidermal growth factor receptor in tumor cells. (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

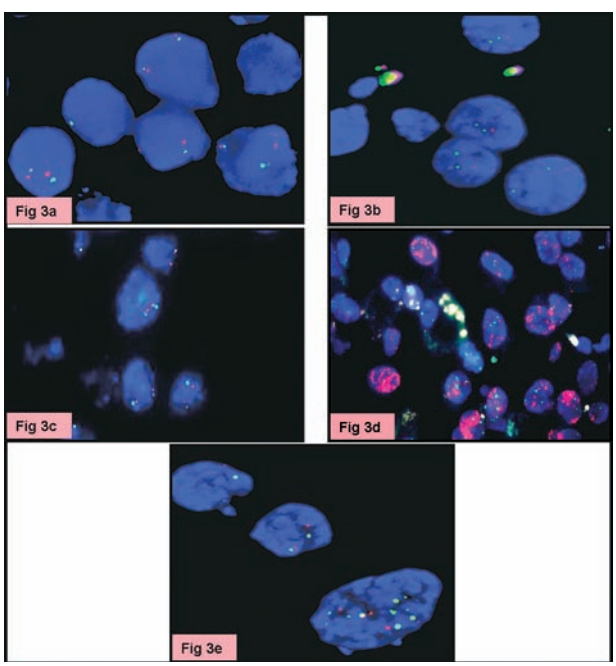

Fig. 3. Fluorescence in situ hybridization analysis. (a) A case with tumor cells expressing normal phosphate and tensin homolog protein (PTEN; two red signals per cell) and CEP10 (two green signals per cell). (b) Photomicrograph of a tumor showing hemizygous deletion of 10q23/PTEN locus: a single red 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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Molecular profile of oligodendrogliomas in young patients

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Several studies on molecular profiling of oligodendrogliomas (OGs) in adults have shown a distinctive genetic pattern characterized by combined deletions of chromosome arms 1p and 19q, O6-methylguanine-methyltransferase (MGMT) methylation, and isocitrate dehydrogenase 1 (IDH1) mutation, which have potential diagnostic, prognostic, and even therapeutic relevance. OGs in pediatric and young adult patients are rare and have been poorly characterized on a molecular and biological basis, and it remains uncertain whether markers with prognostic significance in adults also have predictive value in these patients. Fourteen cases of OGs in young patients (age, ≤25 years) who received a diagnosis over 7 years were selected (7 pediatric patients age ≤18 years and 7 young adults aged 19–25 years). The cases were evaluated for 1p/19q status, MGMT promoter methylation, p53 mutation, and IDH1 mutation. None of the pediatric cases showed 1p/19q deletion. In young adults, combined 1p/19q loss was observed in 57% and isolated 1p loss in 14% of cases. The majority of cases in both subgroups (71% in each) harbored MGMT gene promoter methylation. TP53 and IDH1 mutations were not seen in any of the cases in both the groups. To our knowledge, this is the first study to show that molecular profile of OGs in pediatric and young adult patients is distinct. Further large-scale studies are required to identify additional clinically relevant genetic alterations in this group of patients.

Keywords: adolescent, IDH1, MGMT, oligodendrogliomas, 1p/19q, pediatric, p53, TP53, young adults.

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Over the past decade, a plethora of cytogenetic and molecular genetic alterations in gliomas have been intensely studied and exploited to facilitate glioma classification, prognostication, and therapeutic monitoring. Oligodendroglial tumors have attracted great interest in both basic and clinical neuro-oncology, because they have a better overall prognosis and longer survival times than the diffuse astrocytic tumors.

Combined loss of 1p and 19q, seen in 40%–80% of oligodendrogliomas (OGs), has been established as a molecular signature of oligodendroglial tumors.[1–3] Assessment of allelic status of chromosomes 1p and 19q plays a crucial role in the diagnosis, treatment, and prognostic assessment of adult patients with OGs.[4–6] Thus, unambiguous classification of oligodendroglial tumors has become vital for improved clinical management in the field of neuro-oncology.

O6-methylguanine-methyltransferase (MGMT) promoter methylation has been established as an independent predictor for therapeutic response to chemotherapy in high-grade astrocytic tumors treated with temozolomide.[7,8] A few recent studies have reported MGMT promoter methylation in majority of adult OGs and also documented its significant positive correlation with 1p/19q loss.[9–11] These authors have also suggested that MGMT promoter methylation in oligodendroglial tumors could possibly be associated with chemosensitivity and prolonged survival.[9,12]

In 2008, in a genome-wide sequencing study by Parsons et al, mutations in a gene-encoding isocitrate dehydrogenase (IDH) were identified in 12% of glioblastomas (GBMs).[13] Subsequent studies have shown these mutations in a significant proportion of diffuse gliomas (astrocytic, oligodendroglial, and oligoastrocytic) and secondary GBMs.[14,15] The frequency in astrocytic and oligodendroglial tumors is almost similar.[16–18] Various studies have shown a strong association of isocitrate

dehydrogenase 1 (IDH1) mutation with p53 mutations in astrocytomas and 1p/19q deletion in OGs.[17–19] Recent clinical trials have recognized IDH1 mutation as an independent diagnostic and prognostic marker in both astrocytic and oligodendroglial tumors and have shown a strong association of IDH1 mutation with better overall survival (OS) and progression-free survival (PFS).[13,20–22]

Another molecular alteration that is seen in a majority of astrocytic tumors but extremely rare in OGs is TP53 mutation.[3,23,24] Various studies have shown an inverse correlation between 1p/19q deletion and TP53 gene mutation.[25,26]

Thus, the molecular profile of adult OGs has been extensively studied, and various studies have given an insight into the molecular pathogenesis of these tumors.

On the other hand, OGs in younger age group are extremely rare and comprise only 1%–3% of pediatric central nervous system neoplasms.[27,28] Their molecular profile has not been well characterized.

There are only a few studies available in the English literature that have suggested that molecular alterations in pediatric OGs are distinctly different from their adult counterparts.[29,30] There is no study to date on MGMT promoter methylation and IDH1 mutation status in pediatric cases. Therefore, this study was undertaken with the aim of comprehensive analysis of frequency of various molecular alterations in OGs in pediatric and young adult patients. On careful search of the neuropathology record of the past 7 years, we found 14 cases of OGs in patients aged ≤25 years. We analyzed these tumors for frequency of 1p/19q loss, MGMT promoter methylation status, TP53, and IDH1 mutations.

Materials and Methods

Clinical Data

One hundred eighty cases of OGs diagnosed from January 2003 through December 2009 were identified from a detailed review of the neuropathology records of the All India Institute of Medical Sciences (New Delhi, India). Age and sex of all patients were noted. Approximately 8% of these (14 cases) were reported in young patients (age, ≤25 years). The patients were divided into 2 subgroups: pediatric (age, ≤18 years) and young adults (age, 19–25 years).

Histopathological Examination

The original hematoxylin and eosin (H&E) slides were re-evaluated independently by 3 neuropathologists (C.S., M.C.S., and V.S.). The diagnosis was reconfirmed in accordance with the recent World Health Organization classification.[31] Age- and sex-matched normal cortical brain tissue samples from patients with epilepsy surgery were chosen as controls.

Fluorescence In Situ Hybridization Analysis of 1p and 19q

Dual-probe fluorescence in situ hybridization (FISH) assay was performed on paraffin-embedded sections, with locus-specific probes for 1p36 and 19q13 paired, respectively, with the reference probes for 1q25 and 19p13 (Vysis). Deparaffinization of the sections was performed with 3 10-minute immersions in xylene, followed by 2 3-minute immersions in 100% ethanol. After 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 exposed to 0.04% pepsin (P-7000; Sigma- Aldrich) digestion for 30 min at 37°C, fixed, and dehydrated. Probe mixture (10 μL per slide) was applied on each section. 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) buffer (2 min at 73°C), followed by 0.5× SSC (2 min at room temperature); 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 form nonneoplastic cortical tissue obtained from epilepsy surgery specimens were used as a control for each probe pair. At least 50% nuclei had to show a ratio of 1:2 for test versus reference probes to be scored as deletion. The cutoff value was estimated by calculating the mean plus 3 standard deviations (SDs) of deletions shown by nonneoplastic brain.

Methylation-Specific Polymerase Chain Reaction for MGMT Promoter

Genomic DNA was isolated from formalin-fixed paraffin-embedded (FFPE) sections after conformation of histology. One section of each paraffin block was stained with H&E, and areas with maximum amount of tumor tissue without necrosis were marked out. Subsequently, 4 20-μm sections were serially cut from the same block and taken on separate sterile slides. Tumor area matched from H&E sections was dissected with a disposable scalpel to ensure that all the sections contained >80% tumor tissue. All these sections were then pooled in a sterile eppendorf vial. DNA was extracted using genomic DNA extraction kit (Real Genomics).

DNA methylation pattern of the MGMT gene promoter was determined using methylation-specific polymerase chain reaction (PCR). This procedure involves chemical modification of unmethylated, cytosine to uracil, followed by a nested, 2-stage PCR. Genomic DNA (~500 ng) isolated from paraffin blocks of each sample was modified by sodium bisufite treatment (EZ Gold DNA methylation kit; Zymo Research). Enzymatically methylated DNA was used as positive methylation control, whereas normal lymphocytic DNA served as unmethylation control. Methylation-specific primers were used for PCR. First-stage primer

recognizes the bisulfite-modified template flanking the MGMT gene but does not discriminate between methylated and unmethylated alleles. The PCR amplification procedure for stage 1 was as follows: an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C, and a final elongation step of 7 min at 72°C in DNA Engine Peltier Thermal Cycler (Bio Rad) with use of recombinant Taq DNA polymerase (Fermentas Life Sciences). A 25-μL volume was used in all PCRs. The stage-1 PCR product was diluted 20-fold, and 2 μL of this dilution was subjected to a stage-2 PCR. Methylation and unmethylation primers were used separately for each test. The PCR protocol for stage 2 was as follows: an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 62°C, and 15 s at 72°C, and final elongation step of 7 min at 72°C. Methylation-specific PCR was performed to amplify 83 base pair fragment of the methylated MGMT gene promoter and 91 base pair fragment of unmethylated product. Amplified products were separated on 4% agarose gel, ethidium bromide stained, and visualized under UV illumination.

Mutation Analysis of TP53 and IDH1 by Sequencing

For Frozen Samples—In 6 cases, fresh frozen tissue was available in the tumor bank. For DNA isolation, the frozen tumor tissue was cryosectioned at 5 μm and stained H&E. These sections of each collected specimen were reviewed to verify adequacy of the tumor area (ie, minimal contamination with nonneoplastic elements) and to assess the extent of tumoral necrosis and cellularity. The subsequent 15 serial sections of 40 μm were taken separately and stored in liquid nitrogen–chilled vials. The sections were used for DNA isolation by Genelute Mammalian DNA Miniprep Kit (Sigma Aldrich) according to the manufacturer's instructions. In them, TP53 and IDH1 mutational analysis was performed.

All TP53 coding region from exon 5–8 were investigated by direct sequencing protocol, as described by IARC p53 database.[32] The following primer sequences were used for exon 5–6: forward 5′ TGTTCACTT GTGCCCTGACT 3′ and reverse 5′ TTAACCCCTCC TCCCAGAGA 3′; exon 7: forward 5′ CTTGCCA CAGGTCTCCCCAA 3′ and reverse 5′ AGGGGTC AGCGGCAAGCAGA 3′; and exon 8: forward 5′ TTG GGAGTAGATGGAGCCT 3′ and reverse 5′ AGGC ATAACTGCACCCTTGG 3′.

Mutations in exon 4 of IDH1 were determined using direct sequencing protocol. Primer sequences used were forward 5′ AATGAGCTCTATATGCCATCACTG 3′ and reverse 5′ TTCATACCTTGCTTAATGGGTGT 3′. PCR amplification was performed in a total of 10 μL of reaction mixture containing 50 ng of tumor DNA, 1 μL of 10× PCR buffer, 0.8 μL of 10 mM dNTPs, 0.25 μL of each forward and reverse primers, and 0.2 μL of AmpliTaq Gold PCR Master Mix (Applied Biosystems). The reaction mixture was subjected to an initial denaturation of 95°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 95°C for 1 min, annealing at 57°C for 45 s, and extension 72°C for 2 min. Bidirectional sequencing was performed using ABI 3730 sequencer (Applied Biosystems).

For FFPE Samples—In 8 cases in which only formalin fixed paraffin embedded (FFPE) blocks were available, DNA was isolated as described earlier for MGMT gene promoter methylation assay. TP53 and IDH1 sequencing on FFPE DNA was performed using comparatively smaller product length.

TP53 sequencing was performed as described by IARC p53 database.[32] The primers used were exon-5: forward 5′ TTCAACTCTGTCTCCTTCCT 3′ and reverse 5′ CAGCCCTGTCGTCTCTCCAG 3′; exon-6: forward 5′ GCCTCTGATTCCTCACTGAT 3′, and reverse 5′ TTAACCCCTCCTCCCAGAGA 3′; exon-7: forward 5′ AGGCGCACTGGCCTCATCTT 3′ and reverse 5′ TGTGCAGGGTGGCAAGTGGC 3′; and exon-8: forward 5′ TTCCTTACTGCCTCTTGCTT 3′ and reverse 5′ AGGCATAACTGCACCCTTGG 3′.

For IDH1 mutational analysis, a fragment of 129 base pairs corresponding to exon 4 of IDH1 was amplified using the following primer sequence: forward 5′ CGGTCTTCAGAGAAGCCATT 3′ and reverse 5′ GCAAAATCACATTATTGCCAAC 3′. The composition of the 10× PCR buffer used was 100 mM Tris-HCl (pH, 8.3), 15 mM MgCl2, and 500 mM KCl. PCR was performed in 50-μL volume containing 50 ng of genomic DNA, 1× PCR buffer, 250 μM dNTPs (NEB), 100 nM of each primer (Sigma), and 1.5 U of Taq DNA polymerase (NEB). Reactions were performed in a thermal cycler (MyCycler; Biorad) as follows: 95°C for 10 min, 37 cycles (95°C for 30 s, 56°C for 30 s, and 72°C for 30 s), and 72°C for 10 min. Sequencing was performed using the forward primer used for PCR on ABI 3730 sequencer (Applied Biosystems).

Results

A summary of the clinical features and results of each of the 14 cases (7 pediatric and 7 young adults) is shown in Table 1. The age of the patients ranged from 3 to 25 years, with a mean of 16.4 years. There were 9 male and 5 female patients. On histopathological analysis, 7 of the 14 cases were diagnosed as OG, World Health Organization grade II, whereas the remaining 7 were classified as anaplastic OG, World Health Organization grade III. All the cases showed classical histological features, similar to those of adults. These included cells with round to oval nuclei, perinuclear halos, fine branching capillary network, and presence of microgemistocytes. Anaplastic OGs showed prominent mitotic activity and focal endothelial cell proliferation. Necrosis was seen in 2 cases.

1p/19q Status

None of the pediatric cases showed 1p and/or 19q deletions. Of interest, combined 1p/19q loss (4 [57%]

Table 1. Details of the oligodendrogliomas (OGs) in pediatric and young adult patients

Case no.	Diagnosis	Age/ Sex	1p deletion (FISH)	19q deletion (FISH)	MGMT methylation status (PCR)	TP53 mutation	IDH1 mutation
1	OG II	4/M	−	−	Methylated	−	−
2	OG II	15/F	−	−	Methylated	−	−
3	OG III	18/F	−	−	Methylated	−	−
4	OG III	20/F	−	−	Unmethylated	−	−
5	OG II	20/M	+	+	Methylated	−	−
6	OG II	19/M	+	+	Methylated	−	−
7	OG III	9/M	−	−	Methylated	−	−
8	OG III	11/M	−	−	Unmethylated	−	−
9	OG III	18/M	−	−	Methylated	−	−
10	OG II	3/M	−	−	Unmethylated	−	−
11	OG III	25/F	+	+	Methylated	−	−
12	OG III	23/M	+	−	Methylated	−	−
13	OG II	25/M	+	+	Methylated	−	−
14	OG II	20/F	−	−	Unmethylated	−	−

Abbreviations: FISH, fluorescence in situ hybridization; IDH1, isocitrate dihydrogenase 1; PCR, polymerase chain reaction.

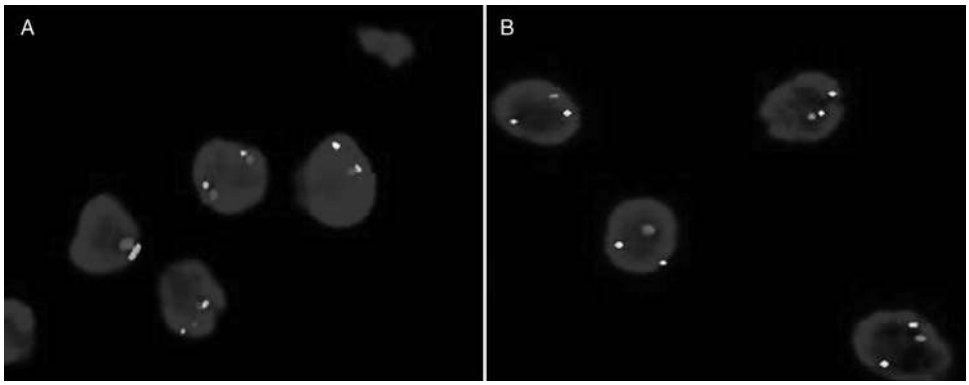

Fig. 1. Hetrozygosity status of 1p chromosome, as determined by FISH). (A) A case showing 2 red (test: 1p36) and 2 green (control: 1q 25) signals, implying no 1p deletion. (B) A case showing 1 red (test) and 2 green (control) signals, implying 1p deletion.

of 7 cases) was observed in majority of young adults. Isolated 1p deletion was noted in 1 case (14%) (Fig 1B).

IDH1 Mutation

None of the cases in either of the subgroups showed this alteration.

TP53 Mutation

TP53 mutation was also not seen in any of the 14 cases analyzed.

MGMT Promoter Methylation

The majority of cases in both subgroups (5 [71%] of 7 in each) showed methylation of MGMT promoter (Fig 2).

Discussion

The pathological evaluation and subtyping of gliomas is sometimes very challenging. Although morphologically classical OGs are easy to diagnose, there are a small fraction of grade II and III diffuse gliomas that show ambiguous histology.[33,34] In addition, dysembryoplastic neuroepithelial tumor, extraventricular neurocytoma, clear cell ependymoma, and pilocytic astrocytomas with focal oligodendroglial differentiation can sometimes pose diagnostic dilemma.[33]

In this regard, assessment of 1p/19q has been shown to represent a potentially useful marker in establishing diagnosis in small biopsy specimens or diagnostically challenging cases.[35] Assessment for 1p/19q status has objectivized the diagnosis of oligodendroglial tumors in adults.[34] Furthermore, association of this molecular signature with better prognosis and chemosensitivity in adult OGs has generated a lot of interest in use of this marker in routine neuropathology practice.[33,34]

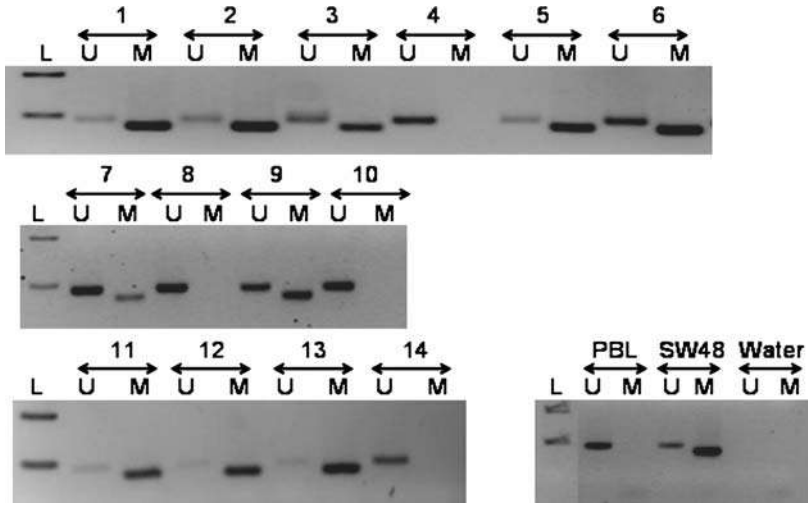

Fig. 2. Methylation status of the MGMT promoter, as determined by methylation-specific PCR assay. DNA from normal peripheral blood lymphocytes (PBL) was used as a control for the unmethylated MGMT promoter (U), enzymatically methylated lymphocytic DNA (SW48) served as a positive control for the methylated MGMT promoter (M), and water was used as a negative control for the PCR. A 100-bp marker ladder (L) was loaded to estimate molecular size. M: PCR product amplified by methylation-specific primers; U: PCR product amplified by unmethylated-specific primers; L: ladder; SW48: methylated control DNA; PBL: unmethylated control DNA. Samples 1, 2, 3, 5, 6, 7, 9, 11, 12, and 13 were methylated with presence of band in methylated "M" lane. Band in "U" lane is because of lymphocytes and normal tissue present with tumor tissue. Cases 4, 8, 10, and 14 were unmethylated with no band in "M" lane. In control samples, PBL had a band only in "U" lane, whereas SW48 had a band only in "M" lane.

Table 2. Comparison of results of 1p/19q chromosomal status in oligodendrogliomas (OGs) involving pediatric and young adult patients with the available literature.

Reference, year [No.]	No. of cases	Age range (years)	Age <10 years	Isolated 1p deletion	Isolated 19q deletion	Combined 1p/19q deletion	Method used
Raghavan et al, 2003 [29]**	26	2–18	15	2/26 (7.7%)	0/25	3/25 (12%)	FISH
Myal et al, 2003 [36]**	3	2–17	1	None	None	None	PCR
Kreiger et al, 2005 [30]**	13	0–19	4	1/13 (7.7%)	None	None	FISH
Hergersberg et al, 2006 [37]	4*	<25	–	–	–	1/4 (25%)*	PCR
Hyder et al, 2007 [38]**	7	0.3–16.8	5	1/7 (14.3%)	1/7 (14.3%)	None	PCR
0ur series	14	3–25	3	1/14 (7.1%)***	None	4/14 (28.6%)***	FISH
TOTAL	67	–	28	5/67 (7.5%)	1/66 (1.5%)	8/66 (12.1%)	–

Abbreviations: FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction.
*Derived from figure in the paper.
**No case with 1p 19q deletion in first decade.
***All the cases with 1p &/or 19q loss were young adults.

There are only a few isolated studies on 1p/19q status in oligodendroglial tumors in young patients (Table 2). The largest series is by Raghavan et al on 23 pediatric cases. The authors showed combined 1p/19q deletion in 12% and isolated 1p deletion in 7.7% of cases.[29] In another series of 13 cases, only 1 case showed isolated 1p loss.[30] In our study, none of the cases in the pediatric age group showed 1p/19q loss. However, a significant number of young adults (4 [57%] of 7) showed combined 1p and 19q deletion. In a previous study, we observed a frequency of 63% in adult patients with OG.[3]

Raghavan et al raised a possibility that a high-definition technique, such as loss of heterozygosity analysis, should be performed in pediatric tumors to rule out small interstitial losses that may not be apparent by FISH assay.[29] However, 3 studies in which PCR assay was used found that the frequency of this alteration is equally rare even with this technique (Table 2).[36–38]

After compiling data from all the series, a total of 67 pediatric and young patients with oligodendroglial tumors were assessed for 1p/19q status. Combined 1p/19q loss was observed in 12.1%, isolated 1p loss in 7.5%, and isolated 19q loss in 1.5%. There were 28 cases in the first decade of life, and none of these had this alteration (Table 2). Although the number of cases analyzed is very small, these observations suggest that this molecular alteration does not occur in the very young patients (first decade) and is infrequent until the age of 18.

MGMT hypermethylation is another molecular alteration that has been established as an independent predictor of response to chemotherapy with alkylating agents in malignant astrocytomas.[7,8,39] Few recent studies have documented MGMT promoter methylation in 60%–90% of adult OGs.[9–11,40] We have also observed a frequency of 84% in adult OGs.[41]

A significant positive correlation of this alteration with 1p and 19q loss has also been reported.[9–11] Some authors have suggested that MGMT promoter methylation is associated with frequent sensitivity of oligodendroglial tumors to alkylating agents and better prognosis.[9,11,40] In the Neuro-Oncology Working Group-04 trial, on anaplastic gliomas, hypermethylation of MGMT promoter was associated with prolonged PFS in both radiotherapy and chemotherapy arms.[42]

In the present series, the majority of pediatric and young adult patients (71% each) showed MGMT promoter methylation. There is no previous study on methylation status of pediatric OGs. In a study by Möllemann et al on assessment of hypermethylation of MGMT gene in oligodendroglial tumors, there were 3 pediatric cases, 2 of which showed promoter methylation.[9] Although the frequency of this alteration in pediatric cases seems to be similar to that reported in adults,[9,12] rarity of 1p/19q loss observed in pediatric population suggests that events involving 1p/19q loss and MGMT promoter methylation are independent.

In a genome-wide sequencing analysis by Parsons et al in 2008, somatic mutations in the gene encoding IDH were identified in 12% of GBMs at position 395 (amino acid 132) of the IDH1 transcript, and most were a G-A change with amino acid substitution of arginine to histidine.[13] This revolutionary discovery led to an increase of studies on various aspects of IDH1 mutation in gliomas of all types and grades. These studies have documented a very high frequency of IDH1 mutations in grade II and grade III gliomas (astrocytic, oligodendroglial, and oligoastrocytic) and secondary GBMs.[16–18,43]

A positive correlation of IDH mutation has been noted with p53 mutation in astrocytic tumors and 1p/19q loss in oligo/oligoastrocytic tumors.[17–19] The presence of mutations was shown in most of the studies to be associated with younger age of the patients and longer survival.[13,20] Van den Bent et al determined the effect of IDH1 mutations on PFS and OS and its correlation with other clinical and molecular features in the prospective randomized European Organisation for Research and Treatment of Cancer study 26951 on adjuvant procarbazine, lomustine, and vincristine (PCV) therapy in anaplastic OGs.[22] The presence of IDH1 mutation was found to carry a very strong prognostic significance for PFS and OS, but no evidence was found of a predictive significance for outcome to PCV chemotherapy. A few studies have also shown mutations of enzyme isoform 2 (IDH2) in a small fraction of gliomas.[17,22,43]

In the present study, none of the pediatric or young adult cases showed IDH1 mutation. In a recent study on GBMs, we did not find IDH1 mutation in any of the 15 pediatric cases. Only 1 (age, 28 years) of 4 patients in the age group of 19–30 years showed this alteration.[44] Even Yan et al in 2009 (15 patients with GBM aged <21 years) and Antonelli et al in 2010 (27 pediatric high-grade gliomas) did not find IDH1 mutation in any of the cases.[17,45] Thus, it appears that IDH1 mutation is conspicuously absent in pediatric gliomas.

In contrast, in a recent study of 100 gliomas, we observed IDH1 mutation in 18 (66.7%) of 27 adult cases with oligodendroglial and mixed oligoastrocytic phenotype.[46]

Two recent studies have suggested that IDH1 mutations are very early events in gliomagenesis, which occur before p53 mutations or 1p/19q loss, and may affect a common stem cell population that can cause both oligodendrocytes and astrocytes.[17,47] On the contrary, presence of 1p/19q deletion along with complete absence of IDH1 mutation in young adults observed in the present study implies that the sequence of molecular events in this age group may not be similar to that in their adult counterparts.

TP53 gene mutation and p53 protein expression is more common in astrocytic tumors than in oligodendroglial tumors.[3,23,24] Various studies have shown an inverse correlation between 1p/19q deletion and TP53 gene mutation.[25,26]

In the present series, none of the pediatric or young adult cases showed p53 mutation, suggesting that this alteration is extremely rare even in these age groups.

To conclude, molecular pathogenesis of OGs arising in pediatric and young adult patients appears to be noticeably distinct from that in adults. The 1p/19q deletion, the molecular signature of adult OGs, is extremely rare in pediatric patients and virtually absent in the first decade. However, the majority of young adults show this alteration. The IDH1 mutation, which occurs at very high frequency in adult OGs, seems to be virtually absent in patients <25 years of age. High frequency of MGMT promoter methylation and absence of TP53 mutation are the only alterations shared by OGs of all age groups. Thus, the results of studies on prognostication and clinical management in adult OGs cannot be translated to pediatric cases. Multicentric studies with genome-wide expression profiling and therapeutic response monitoring should be performed to delineate the unique molecular characteristics of OGs arising in young patients.

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Conflict of interest statement. None declared.

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