Clinical, Morphological, and Molecular Study of Diffuse WHO Grade II and III Astrocytomas: A Retrospective Analysis from a Single Tertiary Care Institute

Ramya Lakshmi Veduruvada1 Megha S. Uppin1 Meher Lakshmi Konatam2 Rajesh Alugolu3 Vamsi Krishna Yeramneni3 Suchanda Bhattacharjee3 Mudumba Vijaya Saradhi3 Monica Malik Irukulla4 Madhumohan Rao5 Nagaraj Velugonda6

1 Department of Pathology, Nizam’s Institute of Medical Sciences, Punjagutta, Hyderabad, Telangana, India
2 Department of Medical Oncology, Nizam’s Institute of Medical Sciences, Punjagutta, Hyderabad, Telangana, India
3 Department of Neurosurgery, Nizam’s Institute of Medical Sciences, Punjagutta, Hyderabad, Telangana, India
4 Department of Radiation Oncology, Nizam’s Institute of Medical Sciences, Punjagutta, Hyderabad, Telangana, India
5 Stem Cell Facility and Regenerative Medicine, Nizam’s Institute of Medical Sciences, Hyderabad, Telangana, India
6 Department of Medical Oncology, Nizam’s Institute of Medical Sciences, Hyderabad, Telangana, India

Address for correspondence Megha S. Uppin, MD, Additional Professor, Department of Pathology, Nizam’s Institute of Medical Sciences, Punjagutta, Hyderabad, Telangana-500082, India (e-mail: megha_harke@yahoo.co.in).

Abstract

Introduction  Astrocytomas are the most common gliomas, classified on the basis of grade and IDH mutation status according to the World Health Organization (WHO) 2016 update. IDH mutations are seen in 70 to 80% of diffuse grade II and III astrocytomas and are associated with better outcome. They serve as predictive biomarker in IDH-targeted therapies such as small-molecule inhibitors or vaccines.

Objective  The aim of this study was to analyze the clinical, morphological, immunohistochemical, and molecular genetic characteristics of diffuse astrocytoma (DA: grades II and III). The IDH mutant and wild-type tumors are compared and contrasted with survival analysis on follow-up.

Materials and Methods  This was a retrospective study conducted on surgically resected tumor specimens. The hematoxylin and eosin-stained slides were examined for histologic features. Immunohistochemistry (IHC) was performed using IDH1R132H, ATRX, p53, and Ki67. All cases of negative immunohistochemical expression of IDH1R132H were subjected to IDH1 mutation analysis by Sanger sequencing. Overall survival was estimated by the Kaplan-Meier method using the log-rank (Mantel–Cox) test.

Keywords
► glioma
► diffuse astrocytoma
► immunohistochemistry
► IDH1/2 mutations
► Sanger sequencing
► survival

Results  The study included 51 cases of DA in the age of 17 to 66 years, mean ± standard deviation was 35.5 ± 9.7 years, and male:female ratio was 2:1. The \( IDH1R132H \) cytoplasmic immunopositivity was seen in 36 cases (70.5%), of which 63.6% were of grade II and 72.5% were of grade III. \( ATRX \) showed loss of expression in 50 cases (98%), and \( p53 \) showed diffuse strong immunohistochemical expression in all the cases of \( IDH \) mutant tumors. The difference in the age at presentation for \( IDH \) mutant (32.5 years) and wild type tumors (38 years) was statistically significant. Median survival was 55.3 months and 22.2 months in of \( IDH \) mutant and wild type cases, respectively.

Conclusion  IHC and sequencing for \( IDH \) mutations is helpful in making an integrated diagnosis and classifying definite molecular subgroups of astrocytic tumors. Mutations in \( IDH \) core-olate with survival. \( IDH \) mutant tumors showed longer survival duration and are good prognostic indicators.

Introduction

The World Health Organization (WHO) 2016 update brought in paradigm changes in understanding and classification of central nervous system neoplasms bringing in the molecular biology of these tumors.\(^1\) The adult diffuse gliomas are essentially classified on the basis of \( IDH \) mutations, which have been observed to be an important prognostic and predictive biomarker.\(^1\) Other studies have reported \( IDH \) mutations in \( >80\% \) of WHO grade II/III gliomas.\(^4\) Other studies have reported \( IDH \) mutations in 72% of grade II and 64% of grade III astrocytomas.\(^5,6\) Diffuse gliomas that do not harbor \( IDH \) mutations exhibit aggressive behavior irrespective of the grade of the tumor. \( IDH \) wild-type diffuse astrocytoma (DA) and anaplastic astrocytoma (AA) have a survival similar to or only slightly longer than \( IDH \) wild-type glioblastoma.\(^2-4,7,8\) Presence of \( EGFR \) amplification and a genotype of \( 7q \) gain and \( 10q \) loss have been associated with worse outcome.\(^9\) Study of \( IDH \) mutations alone in diffuse glioma is an important research tool for targeted therapies and possible vaccines.

In this article, we share our experience with diffuse astrocytic tumors, WHO grades II and III, with respect to clinical features, histopathology, and molecular studies. The survival was compared between two molecular subgroups.

Materials and Methods

Study Design:  This was a retrospective evaluation of the clinical, histopathological, and molecular markers of WHO grade II and III astrocytomas. The \( IDH \) mutant and wild-type tumors have been compared with respect to survival.

Study Setting:  The study was performed in a tertiary care center. The study period was from January 2015 to June 2017.

Participants:  All patients diagnosed on histopathology as grade II or III astrocytoma were included in all age groups. Other types of gliomas were excluded.

Variables:  The astrocytomas were classified into \( IDH \) mutant and wild-type gliomas. The two molecular types were compared with respect to demographic features, location, survival, and outcome.

Primary Outcome:  Comparison of the longest survival period between \( IDH \) mutant and wild-type tumors.

Secondary Outcome:  Comparison of other variables like age, location, and histopathology between \( IDH \) mutant and wild-type tumors.

Data Collection:  The patient details including demographic features, clinical presentation, and radio imaging findings were obtained from the request forms. The treatment details were obtained from medical records. The duration of the follow-up was calculated on the basis of last follow-up date or death of the patient, whichever was earlier.

Histopathology

The tumor specimens were fixed in formalin and processed routinely. The hematoxylin and eosin-stained slides were examined for histologic features of DA and categorized into grade II or III as per the WHO 2016 Classification.\(^1\)

Immunohistochemistry (IHC)

This was performed using \( IDH1R132H \) (clone D09, Dianova, dilution 1:200), \( ATRX \) (HPAR01906, Sigma Aldrich, dilution 1:500), \( p53 \) (Clone D07, Bio-Genex), and \( Ki67 \) (Sigma Aldrich, Ready to Use). Cytoplasmic staining for \( IDH1R132H \) and nuclear staining for \( ATRX, p53 \), and \( Ki67 \) were interpreted as positive

Sequencing

All cases of negative \( IDH1R132H \) immunoeexpression were subjected to \( IDH \) mutation analysis by Sanger sequencing.

DNA Extraction FFPE Tissue

Formalin-fixed, paraffin-embedded (FFPE) blocks were cut into 12 serial sections of 18 µm each. Areas consisting of at least 80% tumor cells were marked on the unstained sections that were then scraped into polypropylene tubes and deparaffinized using xylene. The collected tumor tissue was subjected to deoxyribonucleic acid (DNA) extraction with the QIAamp DNA FFPE Tissue kit following manufacturer’s protocol. Isolated DNA concentration was quantified by spectrophotometry using Nano drop 2000 instrument.
Fluorescence In Situ Hybridization
Fluorescence in situ hybridization (FISH) was performed on FFPE sections using dual color locus specific probes for 1p36 and 19q13, respectively, with the reference probes for 1q25 and 19p13 (Vysis). This test was performed in the study group in IDH mutant gliomas if the ATRX immunopositivity was retained. Following pretreatment, probes were added to the sections and the slides were subjected to denaturation at 78°C for 5 minutes and hybridization at 37°C for 16 hours in thermobrite chamber. After counter staining with 4′,6-diamidino-2-phenylindole, slides were examined under fluorescence microscope. The hybridization signals were scored in at least 200 nonoverlapping nuclei. The ratio of 1p1q and 19q19p was calculated by taking the number of test and control signals. Also, a 1:2 ratio of signals in >50% cells was taken as a criteria for codeletion.

Sample Size: The study included 51 patients. All consecutive cases diagnosed as DA WHO grades II and III were included in the study period.

Statistical Analysis
Demographic and other relevant clinical data were presented as mean or proportioned appropriately. Statistical analysis was performed using Graph Pad Prism 8.4.3. Data were analyzed and correlation was done between IDH mutation status and grading of astrocytoma. Overall survival (OS) was defined as the interval between primary surgery and death or last follow-up. OS was estimated by the Kaplan-Meier method using the log-rank (Mantel–Cox) test. A p-value less than 0.05 was considered to be of statistical significance.

Interpretation of Testing (Table 1)
DA, IDH Mutant: Positive IDH1R132H IHC or mutation analysis with loss of ATRX expression and diffuse p53 immunopositivity.
DA Wild type: Negative IDH1R132H IHC and mutation analysis with loss of ATRX expression or absence of 1p19q codeletion if the ATRX was retained.

Ethics
The study was approved by Nizam’s Institute of Medical Sciences (EC/NIMS/2316/2019, dated 01.04.2019). Waiver of consent was granted since the study was retrospective and was performed on archival tissue blocks. Confidentiality was maintained by the deidentification of data. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1964, as revised in 2013.

Results
Demographic, Clinical, and Radiological Data
Total 51 cases were diagnosed as astrocytoma (WHO grades II and III) in the study period. The patients were in the age group of 17 to 66 years with mean ± standard deviation (SD) age being 35.5 ± 9.7 years, with a male:female (M:F) ratio of 2:1. Of the 51 cases, 11 were WHO grade II and 40 were WHO grade III. The mean age was 31.2 ± 8.6 years (median 29 years) and 36.45 ± 9.7 years (median 33 years) for grade II and III tumors, respectively. Only two patients were less than 18 years of age with one each of grade II and grade III tumors. Graph 1 depicts the distribution of grades within various age groups.

Most common clinical presentation of the patients was headache (33, 70%) followed by seizures (25, 53%) and

| Table 1 Molecular subgroups of different astrocytomas based on IDH mutation and grade |
|-------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                   | IDH mutant     | IDH wild type   | IDH mutant      | IDH wild type   |
| Age group (years)                 | astrocytoma,   | astrocytoma,    | astrocytoma,    | astrocytoma,    |
|                                   | WHO grade II   | WHO grade II    | WHO grade III   | WHO grade III   |
| (N = 7)                           | (N = 4)        | (N = 31)        | (N = 9)         |
| Mean age                          | 29             | 38              | 36              | 38              |
| Overall survival (months)         | 63             | N/A             | 60              | 90              |
| Median survival (months)          | 60             | N/A             | 56              | 38              |

Abbreviations: N/A, not available; WHO, World Health Organization.

IDH Mutation Analysis
Polymerase chain reaction (PCR) amplification of the target region was performed by mixing 50 ng of extracted tumor DNA as template, 10 μL of HotStar Taq 2X Mastermix (DSS Takara), 200 nM of the respective forward and reverse primers, and high-purity water to a final volume of 20 μL (IDH1 PCR primers: forward, AATGAGGCTCTATATGCCATCACTG; reverse, TTCATACCTGGTAAATGGGTGT). The PCR cycling conditions included an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, concluding with a final elongation step at 72°C for 10 minutes. The PCR products were paired, respectively, with the reference probes for 1p19q codeletion.

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weakness of limbs, and the most common location of tumor was frontal lobe (14, 32%).

The magnetic resonance imaging findings were reviewed and correlated with final histopathology. Thirteen cases of grade III astrocytoma were considered to be low-grade glioma due to absence of perilesional edema and contrast enhancement, whereas three cases of grade II astrocytoma were considered to be of high grade due to extensive perilesional edema and mass effect. Apart from these, there was correlation of radio imaging and histopathological features. Two cases, one each of grades II and III, had pattern of gliomatosis cerebri.

The surgical notes mentioned that 62% of the tumors had gross total resection.

**Histomorphology**

WHO grade II astrocytoma constituted 11/51 (21.6%) cases. These tumors showed increased cellularity and pleomorphism without atypical mitosis. WHO grade III astrocytoma constituted 40/51 (78.4%) cases. These tumors showed hypercellularity, pleomorphism, and atypical mitosis. Atypical mitosis was more than one per section in grade III tumors. Microcystic spaces were identified in 11/51 (21.5%) tumors. Focal oligodendrogial pattern was observed in 2/51 (3.9%) grade III tumors and these resembled the erstwhile oligoastrocytomas. Tumor calcification was identified in only one (1.9%) grade III tumor. Gemistocytes were observed in 15/51 (29.4%) cases, of which 13 belonged to grade III. Necrosis and microvascular proliferation, which are typical features of grade IV tumors, were absent in all cases.

**Immunohistochemistry**

**IDHR132H**: The cytoplasmic immunopositivity was seen in 36/51 (70.5%) cases, of which 63.6% were grade II and 72.5% were grade III.

**ATRX**: The ATRX showed loss of expression in 50 cases whereas one IDH mutant AA (grade III) showed retained ATRX nuclear expression, but this case did not show 1p19q codeletion on FISH analysis. Of the cases that were negative for IDH1R132H, five had loss of ATRX expression.

**p53**: There was diffuse strong immunohistochemical expression of p53 in all the cases of IDH mutant tumors. Among 15 tumors that were negative for IDH1R132H, 6 showed diffuse p53 expression whereas the rest 9 were negative.

**Ki67**: The mean ± SD Ki67 for grade II tumors was 2.36 ± 1.0 (range: 1–5) and grade III tumors was 15.6 ± 11.3 (range: 6–64).

**Sequencing**

Out of the 15 tumors that were negative for IDH1R132H IHC, only 2 cases (both belonging to grade III) showed mutations in Sanger sequencing as depicted in Fig. 1. These mutations were also identified in IDH1R132H locus. None of the cases showed any other IDH1 mutations.

Applying integrated diagnosis, 74.5% tumors in this study were IDH mutant.

**Follow-Up Period and Survival**

Follow-up data were available in 37/51 patients. All these patients were given 54 Gy of intensity modulated radiotherapy (IMRT) and chemotherapy with temozolomide for a period ranging from 31 to 1,488 days and a mean of 374 ± 269.4 days. All the patients were followed up for a period of 2 days to 90 months. The follow-up and survival analysis were done separately for IDH mutant and IDH wild-type astrocytomas. The OS was 48.6%, and 51.4% patients died at the end of follow-up period. Eighteen of the patients are alive at the longest follow-up including 14 IDH mutant and 4 IDH wild-type patients. The OS differed in grades with 57.2% in grade II and 46.7% in grade III astrocytomas.
Recurrence of tumors occurred in 11 (29.7%) cases at the mean ± SD interval of 85 ± 68.4 days. In two of these patients, the tumors upgraded from grade II to grade III. The IDH mutant and wild-type patients were compared for age, M:F ratio, location, and median survival by calculating p-value using chi-square test. The p-value was statistically significant (p < 0.001) for the age distribution showing difference in age at diagnosis between IDH mutant (32.5 years) and wild-type patients (38 years). There was no statistical significance for the gender distribution and location between the groups.

Fig. 1 (Top) Microscopic images of a grade III anaplastic astrocytoma. (A) Cellular tumor with marked pleomorphism and atypia showing many gemistocytes (H&E ×40). (B) Cytoplasmic positivity for IDH1R132H; HRP-polymer ×100. (C) Tumor cells showing diffuse strong expression of p53; HRP-polymer ×40. (D) Loss of ATRX expression; HRP-polymer ×100. (E) IDH1 polymerase chain reaction products (500 bp length) run on 2% agarose gel; ethidium bromide used as deoxyribonucleic acid (DNA) staining dye. (Bottom) Detection of DNA changes at R132 locus (R132H mutation, G > A) in two cases of IDH1R132H immunohistochemistry negative astrocytoma.

The patient survival period was calculated by Kaplan-Meier survival analysis. Median survival was 55.3 months and 22.2 months in IDH mutant and IDH wild-type cases, respectively. From the survival analysis, it was evident that patients with IDH mutations had better survival. Log-rank (Mantel–Cox) test was done for comparison of survival curves, which did not show any significance between these groups. This is shown in Graphs 2 and 3.

The survival analysis showed that IDH wild-type grade III tumors had least median survival period with poor prognosis.
Discussion

DA is a common brain tumor mainly affecting young adults, and encompasses ~11 to 15% of all astrocytic brain tumors. In older adults over 40, the age-adjusted annual incidence rate of astrocytic tumors is 6.8/100,000 people.6,11,12 DA and AA are defined as tumors that lack the 1p/19q codeletion and usually demonstrate loss of expression of ATRX and express mutant p53 protein.

IDH mutant and wild-type astrocytomas are clinically different tumors despite overlapping histological appearances. IDH mutations are the driving mutations in gliogenesis, identified in 70 to 80% of DA. Majority of these mutations are IDH1 type, commonest being IDH1R132H.13 IHC for IDH1R132H is a surrogate for the mutation, and DNA sequencing is advocated only for the rare IDH1/2 mutations whenever diffuse gliomas are immunonegative for IDH1R132H.6

In this study, DA constituted 21.7% (51/234) of all the primary brain tumors in the study period. This is comparable to the study by Watanabe et al6 (29.5%), however is lesser than that reported by Rajeswarie et al14 (47.5%). The distribution of grades is similar to these two studies.

The mean age at presentation in our patients was 35.5 ± 9.7 years, which is comparable to that reported in other studies.11,12,14 IDH mutant gliomas have been found to have earliest age at diagnosis.

The most common presenting complaint was headache (70%) followed by seizures (53%), which is comparable to the study by Mu et al.11 Most common location in this study was frontal lobe (32%) consistent with findings by Rajeswarie et al14 (41% in grade II and 46.4% in grade III), Dong et al.15 and Mu et al.11

IHC with IDH1R132H was positive in 70.5% cases, of which 19.4% belonged to grade II and 80.5% belonged to grade III. Study by Mu et al11 observed IDH1R132H positivity in 77% of DA (19% of grade II and 81% of grade III). Median age of IDH1R132H mutant astrocytoma was 32 years, which is comparable to other studies.

ATRX mutations have been reported either by sequencing or IHC in 45 to 67% of DA and 57 to 73% of AA.16–19 The mutations in ATRX are supposed to follow IDH mutations for astrocytic phenotype in a diffuse glioma. These are mutually exclusive of 1p19q codeletion. A surrogate IHC with loss of nuclear staining for ATRX in neoplastic cells, with retention in nonneoplastic cells serving as an internal positive control, is a reliable indicator of ATRX mutations.20 Inactivation of the adenosine triphosphate–dependent helicase ATRX has been linked to recombination-driven alternative telomere maintenance mechanisms and may provide glioma cells with unlimited proliferative capacity.21–23 In this study, 97.5% of IDH mutant astrocytoma showed loss of ATRX.

The WHO 2016 eliminated oligoastrocytoma as a distinct entity, since nearly all histologically defined oligoastrocytoma can be recategorized as oligodendroglioma or astrocytoma based on molecular features.24 We observed two cases of oligoastrocytoma that could be reliably classified as IDH mutant astrocytoma due to loss of ATRX and diffuse p53 positivity. Another case of IDH mutant grade III astrocytoma with retained ATRX did not show 1p19q co deletion hence was classified as astrocytoma.

Tumorigenic Tp53 mutations have been reported to be present in >50% of gliomas with astrocytic features, including 59 to 74% of DA and 53 to 65% of AA.4 In this study, diffuse and strong positivity for p53 was observed in all cases of IDH mutant astrocytoma. Grade II and III astrocytomas, which showed diffuse and strong positivity for p53, constituted 72.8 and 91.6%, respectively.

Pediatric DA is distinct clinically and molecularly from their adult counterparts. Clinical implications of BRAF alterations in these tumors and behavior of tumors with MYB/MYBL1/FGFR alterations are less clear.25 Although extremely common in adult diffuse glioma, IDH1/2 mutations are much less prevalent in pediatric gliomas. Previous reports suggested extremely low numbers of IDH mutants in pediatric gliomas ranging from 0 to 17%.25 In this study we noticed two pediatric patients with diffuse IDH mutant astrocytoma accounting for 2/40 (5%) of cases. These tumors are known to behave like adult IDH mutant astrocytoma. We did not identify any case of IDH wild type pediatric DA in our series.

Loss of the CDKN2A gene or p16 protein (the CDKN2A product) appears an ideal candidate for distinguishing the molecular phenotypes of WHO grade II and III IDH mutant gliomas. Studies showed that CDKN2A loss is associated with worse survival in astrocytoma.26 Available evidence from retrospective studies suggests that homozygous deletion of
CDKN2A/B is associated with shorter survival in patients with IDH mutant astrocytoma and that its presence corresponds to WHO grade IV clinical behavior.

However, the molecular study was limited to identification of IDH mutation status, and other mutations that cause astrocytoma were not identified in this study.

Survival patterns for lower-grade astrocytoma remain poorly characterized. Several independent projects have demonstrated that histologic grading standards probably cannot distinguish prognoses for patients with IDH mutant astrocytoma in the WHO grade II and III entities. On the contrary, some studies have concluded that traditional grading system is still able to stratify prognoses for these patients.

In this study, all grade II and III DA patients with available follow-up received combined 54 Gy of IMRT and chemother-apy with temozolomide for a period ranging from 31 to 1,488 days and a mean of 374 days, while the study by Mu et al observed that 48.6% received both chemotherapy and radiotherapy and 51.4% received only radiotherapy. Study by Dong et al observed 52.69% of grade II and 77.35% of grade III tumors receiving radiation.

The prognostic importance of IDH mutation is independent of other known prognostic factors, including age, grade, and MGMT methylation status. IDH mutations exhibit G-CIMP signature in glioma and the gliomagenesis has been attributed to the oncometabolite, 2-hydroxylutarate (2-HG). This results in DNA and histone methylation. 2-HG is a part of DNA repair pathway and serves as an inhibitor of DNA repair enzymes as well as inhibits the homologous recombination DNA repair process. This helps in targeting the DNA repair enzymes by chemoradiotherapy giving a therapeutic benefit. IDH mutant tumors are also common in surgically amenable sites and gross total resection gives additional survival advantage. The second mutant protein, ATRX, is a chromatin-binding protein (SNF family) and the mutations result in telomere dysfunction. ATRX deficiency is associated with genomic instability that can induce p53-dependent cell death. So p53 mutations in DA may enable tumor cell survival in the setting of ATRX loss.

Studies showed that median survival of grade II and grade III astrocytomas is 7 to 10 years and 3.5 years, respectively. Pekmezci et al observed median OS is better in patients with IDH mutant astrocytoma (9.3 years) than IDH wild-type astrocytoma (1.2 years). Another study identified that the median survival of IDH wild-type gliomas was 1.7 years as against 6.3 years for IDH mutant gliomas without 1p19q codeletion. The similar was observed in our study with a median survival of 55.3 months (4.6 years) and 22.2 months (1.9 years) in IDH mutant and wild-type astrocytomas, respectively. However, this difference was not found to be statistically significant. This could perhaps be related to less number of IDH wild-type cases.

In a study by Shirahata et al, it was identified that there was difference in survival of IDH mutant tumors. On follow-up, survival of 31.7% of IDH mutant glioblastomas and 42.6% of IDH mutant AA was similar and this was associated with CDKN2A/B homozygous deletion. However, we did a similar comparison of median survival of IDH mutant AA with IDH mutant glioblastomas in the same period. We identified 61.7% of IDH mutant AA with median survival of 55.3 months and 7.3% of IDH mutant glioblastomas with median survival of 12 to 15 months. However, we need to confirm these findings further by performing CDKN2A/B deletion studies and expanding the sample size.

Limitations: The limitations of this study are retrospective study design. The follow-up details of all the patients were not available. Limited sample size of 51 patients is also a limitation for a complete statistical analysis.

Future Research Directions: There are advances in molecular genetics of diffuse gliomas including astrocytoma. The IDH mutant tumors are further classified based on CDKN2A/B homozygous deletions. The tumors with these deletions are shown to have aggressive behavior even in IDH mutant tumors. We plan to perform this deletion analysis and correlate with patient survival.

Conclusion

In conclusion, we observed that IHC with IDH1R132H, ATRX, and p53 is helpful in making an integrated diagnosis of astrocytic tumors as per the updated 2016 WHO classification of tumors. Other studies have also shown utility of IHC in subtyping the gliomas and our results are similar to these studies. Classification into definite molecular subgroups by sequencing to find out other variant IDH1 or IDH2 mutations is necessary according to WHO guidelines. IDH mutations correlate with survival and are good prognostic indicators in DA.

Source of Funding
Nil.

Conflict of Interest
Nil.

References
Morphologic and Survival Analysis of Diffuse Astrocytomas

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