Role of CDKN2A deletion in grade 2/3 IDH-mutant astrocytomas: need for selective approach in resource-constrained settings

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OBJECTIVE The authors aimed to assess the frequency of homozygous CDKN2A deletion in isocitrate dehydrogenase (IDH)–mutant diffuse astrocytomas (grade 2/3) and to narrow down the clinicopathological indications in which the CDKN2A fluorescence in situ hybridization (FISH) assay is cost-effective in resource-constrained settings.

METHODS IDH-mutant astrocytomas were analyzed for ATRX, p53, MIB1-LI, and p16 expression using immunohistochemistry. The FISH assay was used to evaluate CDKN2A deletion and 1p/19q codeletion. Survival outcomes were assessed according to the different molecular markers.

RESULTS A total of 150 adult patients with IDH-mutant grade 2 (n = 95) and grade 3 (n = 55) astrocytomas (145 primary and 5 recurrent) were analyzed. Using a cutoff value of 30% for defining significant homozygous CDKN2A deletion, none of the grade 2 and 10.9% (6/55) of grade 3 astrocytomas showed this deletion (4 primary and 2 recurrent grade 3 tumors) and were reclassified as grade 4. This mutation was more frequent in recurrent (40%, 2/5) than primary (2.76%, 4/145) gliomas. Half (3/6, 50%) of the CDKN2A-deleted cases demonstrated poor outcomes; 2 of these cases experienced recurrence at 12 and 36 months after surgery, and 1 died at 5 months. The majority of CDKN2A-deleted cases showed marked cellularity (100%), pleomorphism (100%), brisk mitosis (83.3%), and tumor giant cell formation (83.4%). None of the cases with retained p16 expression harbored this deletion. Both overall survival (p = 0.039) and progression-free survival (p = 0.0045) were found to be worse in cases with p16 loss. Selectively performing CDKN2A FISH only in high-risk cases with histomorphological features of anaplasia, p16 loss, or recurrent tumors achieved a sensitivity and negative predictive value of 100%. This approach would have resulted in saving 41.1% of the original expenditure ($6900 US per 150 samples) and 27.6 person-minutes per sample without compromising the identification of deleted cases.

CONCLUSIONS Homozygous CDKN2A deletion is conspicuously absent in grade 2 and rare in primary grade 3 IDH-mutant astrocytomas. The authors propose that restricting use of the FISH assay to cases showing histomorphological features of anaplasia, p16 loss, or recurrent tumors will help this platform to be utilized in the most cost-effective manner in resource-constrained settings.

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KEYWORDS CDKN2A deletion; p16 loss; IDH-mutant astrocytoma; WHO 2021 classification; glioma

Gliomas are the most common primary CNS tumors,1 accounting for approximately 30% of all CNS tumors and 80% of all malignant brain tumors.2 Prior to 2016, the histological classification served as the gold standard for glioma diagnostics and therapeutic decision-making.3,4 However, this classification is associated with considerable interobserver variability and does not provide insights into underlying tumor biology.5 Therefore, it cannot be relied upon completely for precise individualized patient treatment.3,5 Over the last few decades, genome-wide molecular-profiling studies have revealed the characteristic genetic alterations and epigenetic profiles associated with different types of gliomas. Thus, there has been a paradigm shift in diagnostic criteria integrating both morphological and molecular features initiating an era of personalized neuro-oncology.6–10

ABBREVIATIONS FFPE = formalin-fixed paraffin-embedded; FISH = fluorescence in situ hybridization; IDH = isocitrate dehydrogenase; IHC = immunohistochemistry; NPV = negative predictive value; OS = overall survival; PFS = progression-free survival.


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In the current WHO 2021 classification, all isocitrate dehydrogenase (IDH)–mutant diffuse astrocytomas are considered a single type (astrocytoma, IDH-mutant) and are then graded as CNS WHO grade 2, 3, or 4. The presence of homozygous CDKN2A deletion results in a CNS WHO grade of 4, even in the absence of microvascular proliferation or necrosis. Although CDKN2A deletion has emerged as a marker of poor prognosis, the literature is scarce on the frequency of its occurrence in grade 2/3 gliomas worldwide. Furthermore, the available data regarding use of the fluorescence in situ hybridization (FISH) assay are highly variable, as various studies have used different cutoff values for reporting positive homozygous CDKN2A deletion.

This study aims to assess the frequency of homozygous CDKN2A deletion in IDH-mutant diffuse astrocytomas (grade 2/3) and to narrow down clinicopathological indications in which the FISH assay for CDKN2A deletion is extremely useful. Additionally, we assess the utility of these indications for risk stratification of IDH-mutant astrocytomas to increase the cost-effectiveness of CDKN2A testing in resource-constrained settings.

Methods

Study Population and Data Collection

This was an ambispective study in which all consecutively diagnosed WHO grade 2 and 3 astrocytomas over a period of 7 years (2014–2021) were retrieved from the archives of our Neuropathology Laboratory. The study included only IDH-mutant astrocytomas that were diagnosed over this period. The histopathological features were reviewed by three independent neuropathologists (V.S., M.C.S., and S.S.), and grading was performed according to the current WHO CNS 5 classification. The neuropathologists reviewing the histopathological features were blinded to the radiological imaging and molecular profiling of the cases. Patient demographics, tumor location and size, radiological findings, histopathological findings, and surgical outcome were noted. The follow-up was performed using serial MRI after surgery. Survival outcome was documented in the form of overall survival (OS) and progression-free survival (PFS). Pediatric patients (age < 18 years), cases having inadequate formalin-fixed paraffin-embedded (FFPE) tissue, and cases with incomplete clinicoradiological follow-up were excluded from the study. The study was approved by the institute’s ethics committee.

Immunohistochemical Staining for IDH-R132H, ATRX, p53, and MIB-L1

Immunohistochemical staining was performed on 5-µm-thick FFPE tumor sections using an automated immunostainer (Benchmark XT, Ventana). Immunohistochemistry (IHC) was conducted using antibodies against IDH-R132H (H09, Dianova, mouse monoclonal, 1:50), ATRX (Sigma-Aldrich, dilution 1:400), p53 (Santa Cruz Biotechnology Inc., dilution 1:200), and Ki-67/MIB-L1 (DAKO, dilution 1:200). For IDH, combined cytoplasmic and nuclear staining was interpreted as immunopositive; for ATRX, complete absence of nuclear staining with retained expression in endothelial cells serving as internal controls was taken as negative. Strong nuclear staining of p53 (> 10% of tumor cells) was considered positive. IDH1-R132H IHC-negative cases that showed ATRX loss were subjected to Sanger sequencing for IDH1 and IDH2 genes (ABI 3500xL, Applied Biosystems). Astrocytoma cases harboring an IDH mutation by sequencing were reclassified as IDH-mutant astrocytomas; the rest were excluded from the study. All IDH-mutant grade 2 and 3 astrocytomas were selected and further analyzed for p16 IHC (Ventana, mouse monoclonal, clone E6H4). Tumor cells with either nuclear immunoreactivity or both nuclear and cytoplasmic immunoreactivity were considered positive. Complete absence of staining in tumor cells was classified as p16-negative.

FISH for Homozygous CDKN2A Deletion and 1p/19q Codeletion

The following commercial sets of fluorochrome-labeled probes were applied (all produced by Vysis Inc., Abbott Laboratories SA). Locus-specific probes for 1p36 and 19q13 paired, respectively, with reference probes for 1q25 and 19p13 for 1p/19q codeletion; and the locus-specific probe for CDKN2A (9p21) paired with a corresponding reference centromeric probe for chromosome 9. 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. Homozygous CDKN2A deletion was identified by the simultaneous lack of both signals of the locus-targeted probe and the presence of a signal of the reference probe. Based on previous studies, a cutoff of 30% was used to define significant deletion. To qualify for 1p/19q codeletion, a minimum of 40% of nuclei must show a ratio of 1:2 for test versus reference probe signals.

Statistical Analysis

The study data were recorded using Microsoft Excel 2019. All statistical analyses were performed using RStudio using ggplot, survminer, and ggstatsplot as additional packages. Associations among clinicopathological factors were tested using the chi-square test for categorical variables or the nonparametric t-test (Mann-Whitney U-test) for continuous variables. Survival analysis was performed using the Kaplan-Meier method and the log-rank test. All statistical tests were two-sided and p values < 0.05 were considered statistically significant.

Results

A total of 150 adult patients with diffuse infiltrating WHO grade 2 (n = 95) or grade 3 (n = 55) astrocytic tumors were analyzed. The mean patient age was 32.9 years and 42.2 years for grades 2 and 3, respectively (range 21–70 years). Of 150 cases analyzed, 149 cases were positive for IDH1-R132H expression. For the 1 case with negative IDH expression on IHC, Sanger sequencing demonstrated an IDH1-R132C mutation. Loss of ATRX expression was observed in 93.6% (89/95) of grade 2 and 87.2% (48/55) of grade 3 tumors (Figs. 1 and 2). The majority of grade 2 (67.4%, 64/95) as well as grade 3 (83.6%, 46/55) tumors

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FIG. 1. Study flowchart showing the distribution of patients according to the WHO CNS 5 classification. H&E, IDH, and ATRX stains, original magnification ×200; FISH images, original magnification ×1000.
were p53-positive. None of the cases with retained ATRX expression showed 1p/19q codeletion. The mean MIB-LI values of grade 2 and grade 3 tumors were 4% and 13%, respectively.

### Homozygous CDKN2A Deletion

Using a cutoff at 10%, 7.4% (7/95) and 72.7% (40/55) cases of grade 2 and 3 astrocytomas, respectively, were positive for homozygous CDKN2A deletion. After increasing the cutoff to 20%, none of the grade 2 and 30.9% (17/55) of grade 3 astrocytomas demonstrated homozygous deletion. At a cutoff value of 30% (significant homozygous deletion), none of the grade 2 and only 10.9% (6/55) of grade 3 astrocytomas showed CDKN2A deletion (4 primary and 2 recurrent grade 3 tumors). Therefore, in the entire cohort of 150 astrocytomas, only 4% of cases (6/150) harbored this deletion and were thus reclassified as astrocytoma IDH-mutant CNS WHO grade 4. The clinical, radiological, and histomorphological characteristics of these 6 cases have been summarized in Fig. 3.

**FIG. 3.** Clinical, radiological, and histomorphological characteristics of cases with homozygous CDKN2A deletion. All radiological images are axial MR images. HPF = high-power fields; S. No. = serial number.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Age</th>
<th>Sex</th>
<th>Primary / Recurrent</th>
<th>Radiology</th>
<th>Histomorphology</th>
<th>p16 IHC</th>
<th>CDKN2A Deletion (% Cells)</th>
<th>Follow up Status</th>
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<tr>
<td>1</td>
<td>50</td>
<td>M</td>
<td>Primary</td>
<td>Moderate</td>
<td>Marked</td>
<td>Absent</td>
<td>4-5</td>
<td>Loss 30%</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>Alive with no deficits</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>M</td>
<td>Primary</td>
<td>Marked</td>
<td>Marked</td>
<td>Present</td>
<td>3-4</td>
<td>Loss 49%</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Died 5 months post surgery</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>M</td>
<td>Primary</td>
<td>Marked</td>
<td>Marked</td>
<td>Present</td>
<td>4-5</td>
<td>Loss 61%</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Alive with no deficits</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>M</td>
<td>Recurrent</td>
<td>Marked</td>
<td>Moderate</td>
<td>Present</td>
<td>2-3</td>
<td>Loss 33%</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td>Alive with cognitive loss</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>M</td>
<td>Recurrent</td>
<td>Marked</td>
<td>Marked</td>
<td>Present</td>
<td>5-6</td>
<td>Loss 38%</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Alive with motor weakness</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>M</td>
<td>Primary</td>
<td>Moderate</td>
<td>Marked</td>
<td>Present</td>
<td>3-4</td>
<td>Loss 34%</td>
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<td>Alive with no deficits</td>
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**FIG. 2.** Alluvial plot showing the recurrence status, histomorphological grade, p16 IHC, and CDKN2A FISH results according to the WHO CNS 5 grade.
Comparison of Histomorphological Features in CDKN2A-Deleted Cases

Most of the reclassified grade 4 astrocytomas showed marked cellularity (100%), pleomorphism (100%), brisk mitosis (83.3%), and tumor giant cell formation (83.4%). In comparison with the histomorphological features of the nondeleted cases, these features in CDKN2A-deleted cases were significantly higher (Fig. 4).

Correlation of CDKN2A Deletion With ATRX, p53 Expression, and MIB-L1

The majority of cases showed loss of ATRX expression (91.3%) and p53 positivity (72.7%), but no correlation was observed with CDKN2A deletion (p = 0.477 and 0.736, respectively). However, high MIB-LI was found to be higher in patients with CDKN2A deletion (mean 11% ± 4.7%) compared with those without deletion (mean 7% ± 8.4%), but this difference did not reach statistical significance (p = 0.146).

Correlation of CDKN2A Deletion With p16 Expression

Loss of p16 expression was noted in only 15.8% of cases (15/95) of grade 2 and 26.5% (13/49) of grade 3 astrocytomas. All cases with CDKN2A deletion (6/6) that were reclassified as grade 4 demonstrated loss of p16 expression. None of the cases with positive p16 expression harbored CDKN2A deletion, while 17.6% of cases (6/34) with p16 loss demonstrated this deletion. The p16 IHC test showed sensitivity and negative predictive value (NPV) of 100% for evaluating CDKN2A deletion status, making it a very good screening criterion (Fig. 5A).

CDKN2A Deletion in Recurrent Cases

In the present study, 5 cases were analyzed with both parent and recurrent samples (paired cases). Among the parent samples, 4 were grade 2 tumors and 1 was a grade 3 tumor. None of the parent samples demonstrated homozygous CDKN2A deletion. On recurrence, all 5 tumors showed histomorphological features of grade 3 astrocytoma. Forty percent of cases (2/5) harbored this deletion and were reclassified as grade 4 tumors (Figs. 1 and 2). The detailed comparison of histomorphological features and molecular profile of these cases is summarized in Table 1.

FIG. 4. Comparison of various histomorphological features across grade 2, grade 3, and reclassified grade 4 gliomas.
Selective Approach for CDKN2A FISH Assay

We propose that the administration of FISH for CDKN2A deletion may be limited to a subset of cases showing either any two histomorphological features (increased cellularity, moderate to marked pleomorphism, tumor giant cells, brisk mitosis) or loss of p16 expression, or tumor recurrence that portends a high risk of this alteration. Modeling this strategy for the present study, only 81 cases of 150 samples in the primary cohort showed one or more of these clinicopathological indications warranting CDKN2A FISH assay. The remaining 69 cases that did not have any of these indications were considered to be at low risk for having homozygous CDKN2A deletion. This selective approach for using the FISH assay achieved excellent sensitivity (100%) and NPV (100%) and thus could have resulted in the identification of all 6 cases (Fig. 5B).

Validation of Selective Approach for CDKN2A FISH Assay

We validated the proposed selective approach for the FISH assay in an independent sample of 40 cases reviewed prospectively (January–June 2022). In this cohort, only one recurrent grade 3 IDH-mutant astrocytoma (1/40, 2.5%) showed homozygous CDKN2A deletion and was thus reclassified as grade 4. There were 15 high-risk and 25 low-risk cases according to our proposed selection criteria. Similar to the primary cohort of 150 cases, the validation cohort also demonstrated excellent sensitivity (100%) and NPV (100%; Fig. 5C).

Analysis of Cost and Workforce Requirement in Primary Cohort

Considering a cost of approximately $2 (US) for standard histopathology, $10 for p16 IHC, and $100 per sample for FISH, the resulting total expenditure was $112 × 150 = $16,800 for 150 samples. If the proposed selective FISH assay strategy was used, it would have resulted in a total expenditure of $12 × 150 = $1800 and $100 × 81 = $8100. Thus, the proposed strategy would result in a savings of $6900 per 150 samples (41.1% of the original expenditure). Similarly, a CDKN2A FISH assay requires 1 person-hour per sample. Selective testing would thus result in saving 69 person-hours per 150 samples, which amounts to 27.6 person-minutes per sample (Fig. 6).

Survival Analysis

The loss of p16 expression was significantly associated with worse OS (3-year survival = 76% in p16-positive vs 54% in p16-negative, p = 0.039) and PFS (3-year survival = 76% in p16-positive vs 43% in p16-negative, p = 0.0045). Half of the cases (3/6, 50%) with CDKN2A deletion demonstrated poor outcome; 2 of these cases experienced a recurrence (12 and 36 months after surgery) and 1 died at 5 months. On survival analysis, we observed a worse PFS of the CDKN2A-deleted cohort as compared with the nondeleted cases at 3 years (41.7% vs 72.0%), but this did not reach statistical significance (log-rank p value = 0.18). The difference in OS also failed to reach significance (p = 0.69) due to the very small number of cases (n = 6) with this deletion (Fig. 7).

Discussion

With the advent of the WHO 2021 classification, there has been a paradigm shift toward integrated diagnosis with the introduction of several new molecular markers as part...
Multiple previous studies have suggested the poor prognostic role of $CDKN2A$ in adult gliomas (Supplementary Table 1). In this study, the patients with $CDKN2A$ deletion showed a trend toward a worse PFS compared with the cases lacking this alteration, which did not reach statistical significance due to the rarity of this alteration. With a growing body of literature suggesting an association of $CDKN2A$ with poor prognosis, the latest edition of the WHO classification has included homozygous $CDKN2A$ deletion as a diagnostic criterion for $IDH$-mutant astrocytoma. However, a few concerns need to be addressed regarding this change in guidelines to include $CDKN2A$ deletion assessment in routine clinical neuro-oncology.

$CDKN2A$ deletion can be evaluated using multiple modalities, such as FISH, copy number variation, multi-
plex ligation-dependent probe amplification,\textsuperscript{19} or comparative genomic hybridization arrays.\textsuperscript{18} However, the testing platforms or cutoff values have not been specified in the WHO 2021 classification.\textsuperscript{11} Various studies have used different cutoffs for FISH assays, leading to highly variable data (frequency of 0\%–45\% in grade 2 and 3\%–51\% in grade 3; Supplementary Table 1).\textsuperscript{12–18} Additionally, a scarcity of literature on the frequency of occurrence of this deletion in grade 2 and 3 IDH-mutant astrocytomas raises the concern about the practical application of this marker in routine practice. In the current study, we used FISH assays with a stringent cutoff value of 30\%, and only 4\% of cases (6/150) showed CDKN2A deletion. Most of the recurrent gliomas (40\%, 2/5) harbored this alteration, compared with only 2.76\% (4/145) of primary gliomas. In our experience, a cutoff value of 30\% or higher should be used to avoid false-positive reporting and to identify cases that are expected to have an aggressive clinical course, despite being histologically grade 2/3. Furthermore, FISH is a laborious and time-intensive procedure and requires technical expertise.\textsuperscript{20} These characteristics highlight the need to establish selective indications for evaluating this deletion in IDH-mutant astrocytomas to efficiently utilize this molecular marker in a cost-effective manner (Fig. 6).

In the present study, p16 IHC proved to be a useful surrogate with a sensitivity and NPV of 100\%. Most cases retained p16 expression (77.3\%, 116/150). Interestingly, none of the cases with retained p16 expression showed CDKN2A deletion, while a substantial proportion of cases with p16 loss (17.7\%) harbored CDKN2A deletion. Additionally, the loss of p16 expression was found to be significantly associated with worse OS and PFS. These findings suggest that p16 IHC can be used as a surrogate marker with excellent sensitivity to screen cases for a CDKN2A FISH assay (Fig. 5A). There is a need for strict histomorphological evaluation, which might also help to narrow down aggressive cases. In our study, we found that all 6 cases that were reclassified to grade 4 based on CDKN2A deletion had soft morphological hallmarks suggestive of aggressive behavior (Figs. 3 and 4). In our analysis of paired cases, we found that the recurrent gliomas warrant this evaluation as the frequency of CDKN2A deletion was relatively higher in recurrent cases (Table 1). Combining the histomorphological features, p16 loss, and recurrence status to guide a selective approach for performing a FISH assay is expected to have a meaningful impact in terms of cost and workforce, especially in resource-constrained settings (Fig. 6). This selective approach can be an excellent risk-stratification strategy for identifying high-risk cases warranting a CDKN2A FISH assay (Fig. 5B). We have validated this selective approach in an independent cohort prospectively with excellent sensitivity and NPV (Fig. 5C).

This study is the first of its kind to suggest clinicopathological indications for narrowing the utility of the FISH assay for the assessment of CDKN2A deletion in resource-limited settings. The introduction of multiple molecular markers in the CNS 5 classification might actually widen the gap in the implementation of these guidelines globally, with economic constraints in using these recommended molecular markers in routine clinical practice. The present study will help to bridge this gap and help these newly recommended molecular markers to be utilized more efficiently and economically in clinical practice. The validity of the proposed selection criteria must be evaluated in future multinstitutional studies across the globe. Future studies will help in tailoring the recommendations of the CNS 5 classification for lower- and middle-income countries and make it more relevant globally.

Conclusions
Homozygous CDKN2A deletion is absent in grade 2 and is rare in primary grade 3 IDH-mutant astrocytomas. The FISH assay could be restricted to cases showing histomorphological features of anaplasia, loss of p16 expression, or in recurrent tumors. Using this strategy, it is possible to utilize this platform in the most cost-effective manner, especially in resource-constrained settings.

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References

Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

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