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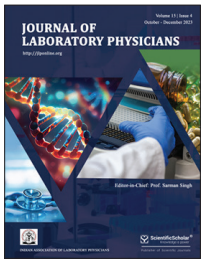

Original Article

Surrogate immunohistochemistry markers in adult gliomas – Experience in a tertiary care hospital

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Received: 16 July 2024
Accepted: 01 October 2024
EPub Ahead of Print: 09 November 2024
Published: 08 April 2025

DOI
10.25259/JLP_152_2024

Quick Response Code:

ABSTRACT

Objectives: The recent World Health Organization classification has recommended the usage of surrogate immunohistochemical markers for molecular classification of gliomas. However, only a few studies have attempted to study the expression of the entire panel of markers. The current study was undertaken to study the association of recognized surrogate immunohistochemical markers (isocitrate dehydrogenase 1 [IDH-1], alpha-thalassemia mental retardation X-linked [ATRX] and p53) in different histological lineages and grades of adult gliomas.

Materials and Methods: This study was conducted on 118 cases of adult gliomas diagnosed on histopathology over a 2-year duration in a tertiary care hospital. The expression of surrogate immunohistochemistry markers (IDH-1, ATRX, and p53) in these cases was studied.

Statistical analysis: Descriptive statistical analysis with the Statistical Package for the Social Science system version 17.0.

Results: The frequency of IDH-1 positivity was significantly higher in oligodendrogliomas (OG: 76.5%; oligoastrocytoma: 100%) versus astrocytoma (AS) (grades 2 and 3: 48.1%). It was also significantly higher in diffuse gliomas (grades 2 and 3) versus glioblastomas (64% vs. 16.9%). Among IDH-mutant diffuse gliomas, ATRX loss was significantly higher in AS versus OGs (84.6% vs. 7.7%). P53 overexpression correlated significantly with histological subtype (AS 2,3: 55.6% vs. OG: 5.9%).

Conclusions: The surrogate immunohistochemical panel of IDH-1, ATRX, and p53 showed significant association with distinct histopathological subtypes and is helpful in molecular stratification. Cut-offs of ≥ 10% nuclear positivity for p53 and 50% loss of nuclear ATRX expression showed a good correlation.

Keywords: Gliomas, Immunohistochemistry, Molecular classification, Surrogate

INTRODUCTION

Identification of molecular subtypes in gliomas has improved the overall diagnosis and prognosis of gliomas. The World Health Organization (WHO) 2016 and 2021 classification of central nervous system tumors requires both histological and molecular parameters to provide an integrated diagnosis. [1,2] This has superseded the previous classification based on histology alone. Isocitrate dehydrogenase (IDH) and alpha-thalassemia mental retardation X-linked (ATRX) mutations confer a better prognosis. The WHO diagnostic algorithm involves histologic phenotyping to identify a diffuse glioma and histological grade, followed by IDH mutation status and subsequent molecular testing for 1p19q co-deletion for only grade 2 and 3 tumors.[1,2] Oligodendrogliomas

(OGs) are IDH-mutant and 1p/19q-codeleted while astrocytomas (ASs) are IDH-mutant and 1p19q non-codeleted among grade 2 and 3 tumors. Molecular studies are, however, cumbersome with limited availability. Surrogate immunohistochemistry appears to be a more feasible and practical approach. The signature molecular characteristics of the IDH-1 can be demonstrated immunohistochemically.[3,4] There is no immunohistochemical marker available for 1p19q co-deletion.[5] Conversely, immunohistochemistry for ATRX and p53 can be used as a substitute for 1p19q co-deletion.[1,2] IDH-mutant ASs are characterized by mutations of ATRX and TP53, which are mutually exclusive with 1p19q co-deletion. Immunohistochemistry for ATRX mutation has been used as a surrogate marker in various studies.[6-10] Recent data support a diagnostic algorithm with histopathology followed by IDH-1 immunohistochemistry, ATRX, and p53.[1,10-12] This is followed by testing for 1p19q co-deletion to delineate OGs from ASs, and IDH sequencing is done where immunohistochemical testing is negative.[12,13] Only a few recent studies have attempted to study the entire surrogate marker panel for molecular subgrouping in diffuse gliomas.[6-10] The aim of our study was to study the expression of the entire panel of recognized surrogate immunohistochemical marker panels in adult gliomas and their association with histology and grades.

MATERIALS AND METHODS

This study consisted of 118 cases of adult gliomas obtained from routinely received neurosurgical specimens in the Department of Histopathology of a tertiary care hospital over a two-year duration. Cases with insufficient representative tissue for immunostaining, age <18 years, spinal tumors, and ependymomas were excluded. In addition, twenty non-neoplastic cases of reactive gliosis/normal glial tissue formed the negative control group for comparison.

Available clinical data, including patient age, gender, and clinic-radiological findings, were recorded. All the cases were analyzed by two pathologists for the WHO histological parameters and immunohistochemical expression of IDH-1, ATRX, p53, and Ki67. The tissue specimens were fixed in 10% buffered neutral formalin. Tissue sections of 3–4 μm thick were made from paraffin blocks and stained with hematoxylin and eosin for routine histomorphology and studied by light microscopy. Consecutive 3–4 μm sections were cut from paraffin blocks for immunohistochemistry against IDH1 R132H (mouse monoclonal, cloneH09, 1;500; Dianova, Germany), ATRX (rabbit polyclonal, 1:500; Sigma Aldrich, USA), p53 (ready to use, D07, mouse monoclonal; Biogenex, Fremont, CA), and Ki67 (ready to use, mouse monoclonal, clone MIB-1, Biogenex Fremont, CA) using peroxidase-antiperoxidase method.

Moderate to strong diffuse cytoplasmic immunoreactions of IDH-1 in tumor cells were considered positive.[1] A weak staining or staining of macrophages was considered negative. Expression of p53 and ATRX was determined semi-quantitatively by assessing the proportion of positively stained tumor cells in an entire section of the representative area. The cut-offs used for ATRX and p53 expression were based on those proposed by Takano *et al.* and Tanboon *et al.*[10,11] Only cells with intense nuclear staining were considered positive for p53. The cut-off for p53 was taken as ≥ 10% nuclear positivity in tumor cells. [1,10,11] Colon carcinoma was used as a positive control for p53. For ATRX, retained/loss of nuclear expression was studied. Nuclear loss of expression in more than 50% of tumor cells accompanied by positive staining in internal control (endothelial and microglial cells) was taken as ATRX loss.[10,11] The Ki-67 labeling index was calculated as a mean percentage by counting the stained nuclei of tumor cells in at least 1000 tumor cells in areas of the highest density of positive nuclei.[1]

The expression of established surrogate immunohistochemistry marker panel (IDH-1, ATRX, and p53) and their association with histological subtypes and grades were studied.

Statistical methods

Descriptive statistical analysis was conducted with the Statistical Package for the Social Science system version 17.0. Continuous variables were expressed as Mean ± Standard deviation and categorical variables were presented as absolute numbers and percentages. Nominal categorical data between the groups were compared using the Chi-square test or Fisher exact test as appropriate. Probability (P) < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

There was a male predominance (males: 63.56% and females: 36.44%). Age ranged from 20 to 80 years (mean, 46.84 years). The most common tumor locations were the frontal lobe, followed by the parietal and temporal lobe. Based on the WHO histomorphological criteria, the spectrum of cases is summarized in Table 1.

For simplification, IHC expression was studied in three subgroups: Diffuse gliomas (grades 2 and 3), glioblastomas (GBMs), and pilocytic ASs. Immunohistochemical findings were compared between GBMs and diffuse gliomas and also between primary (pGBM) and secondary GBM (sGBM).

Immunohistochemical expression

Results of immunohistochemistry in various grades and phenotypic types of diffuse gliomas (grade 2–3) are shown in Figure 1, Tables 2 and 3.

IDH-1

Table 1: Distribution of cases according to histomorphology.

Histological diagnosis	Grades	Groups	Total cases	Frequency (%)
AS *n*=30 (25.42%)	AS 1	Group 1 (Pilocytic AS)	3	2.54
	AS 2	Group 2 (Diffuse gliomas)	15	12.71
	AS 3		12	10.17
OG *n*=17 (14.41%)	OG 2		12	10.17
	OG 3		5	4.24
OA *n*=6 (5.09%)	OA 2		1	0.85
	OA 3		5	4.24
GBM/AS 4 *n*=65 (55.08%)	pGBM	Group 3 (GBMs)	56	47.46
	sGBM		9	7.62
Total			118	100

AS: Astrocytoma, OG: Oligodendroglioma, OA: Oligoastrocytoma, GBM: Glioblastoma, pGBM: Primary glioblastoma, sGBM: Secondary glioblastoma

IDH1 positivity was detected in 36.44% of gliomas. All the IDH-1-positive cases showed cytoplasmic staining of moderate-to-strong intensity. The frequency varied in glioma subtypes and different grades with Astrocytoma (AS) (2): 60%; AS (3): 33.3%; Oligodendroglioma (OG) (2): 83.3%; OG (3) 60%; oligoastrocytoma (OA) (2): 100%; OA (3): 100%, and GBM (4):16.9%, respectively. Majority of grade 4 ASs lacked IDH-1 (83.08%). All pilocytic ASs were negative.

ATRX

Loss of nuclear expression on immunohistochemistry was associated with ATRX mutation Heterogeneous staining pattern was noted in some cases. Overall, ATRX loss was seen in 50.8% of gliomas. ATRX loss was detected in 80% of diffuse ASs (grade 2), 83.3% of anaplastic ASs (grade 3), 8.3% of OGs (grade 2), 0% of OGs (grade 3), 0% of OAs (grade 2 and 3), and 56.9% in GBM. The frequency of ATRX loss was higher in AS grades 2 and 3 (80%; 83.3%) compared to GBMs (56.9%) and was statistically significant (P = 0.0001). Diffuse AS (grade 2 and 3) showed loss of nuclear expression of ATRX in 81.5% of cases, and retained nuclear expression was seen in 18.5%. Whereas 94.1% of OGs (2 and 3) showed retained ATRX expression, only 1 case (histology OG grade 2) showed loss of ATRX. All the 6 cases of grade 2 and 3 OAs showed retained ATRX expression. Among IDH-1-mutant ASs (grades 2 and 3), 84.6% showed ATRX loss, whereas 15.4% showed ATRX retained expression. Among IDH-1-mutant OG, 92.3% showed ATRX retained expression, whereas 7.7% showed loss of ATRX.

P53

p53 overexpression was seen in 33.1% of gliomas with frequency of 60% in diffuse AS (grade 2), 50% in anaplastic ASs (grade 3), 8.3% in OG (grade 2), 0% in OG (grade 3), 100% in OA (grade 2), 20% in OA (grade 3), and 32.3% in GBMs (32.1% in primary: 33.33% in sGBM).

Among GBMs, 72.73% of IDH-1-mutant GBMs showed ATRX loss, and the association was statistically significant (P = 0.002). 63.6% of IDH-mutant GBMs (7/11) showed p53 overexpression and the association was statistically significant (P = 0.021).

All 20 cases of reactive gliosis and all case of pilocytic ASs were negative for IDH-1 and p53 overexpression. ATRX retained nuclear expression was seen only in the endothelial cells and microglial cells.

DISCUSSION

The recent WHO classification has shown a paradigm shift in the diagnosis and treatment of gliomas by promoting the integration of molecular information with histopathology diagnosis. The key molecules for molecular subtyping of diffuse gliomas are IDH mutation and 1p/19q co-deletion.[1,2] 1p19q codeletion is detected only by molecular methods but has been found to be mutually exclusive with ATRX and p53 mutation in IDH-mutant diffuse gliomas.[1,11] IDH1 mutations have been identified as early and frequent genetic alterations in ASs, OGs, and OAs, as well as sGBMs, whereas pGBMs rarely contain IDH1 mutations.[14-17] Diffuse gliomas that do not harbor IDH mutations, regardless of their grade, tend to exhibit more aggressive clinical behavior.[18,19] All diffusely infiltrating gliomas are classified into IDH mutant, IDH wild type, and not otherwise specified.[20] IDH1 R132H mutation, accounting for about 90% of IDH mutations, can be detected using a mutation-specific antibody.[1,3-5] IDH immunohistochemistry has shown high reproducibility and strong concordance between immunohistochemistry for IDH1R132H (clone H09) and sequencing ranging from 88% to 100%.[21-25] Detection of IDH-1 mutation using immunohistochemistry has been recommended in the WHO 2016 and WHO 2021 classification. Sequencing is, however, recommended in all IDH-1 negative cases, particularly in age <55 years, to identify other minor IDH1/IDH2 mutations.[1,26] In this study, the frequency of IDH1 was found to be highest in grade 2 gliomas. It was significantly higher in oligodendroglial (OG: 76.5%; OA:100%) versus astrocytic phenotype (AS 2 and 3:48.1%) (P= 0.024). It was also higher in diffuse gliomas (grade 2 and 3) versus GBMs (64% vs. 16.9%, respectively; P = 0.0001). A significant difference was observed between secondary and pGBMs (44.4% vs. 12.5%; P = 0.0376). Our findings are similar to previous studies.[14-17] IDH mutations exist in a significant percentage of diffuse gliomas, particularly grade 2 and 3ASs, OGs, and ICBMs. Rajeswarie *et al.* [6], in their study on 449

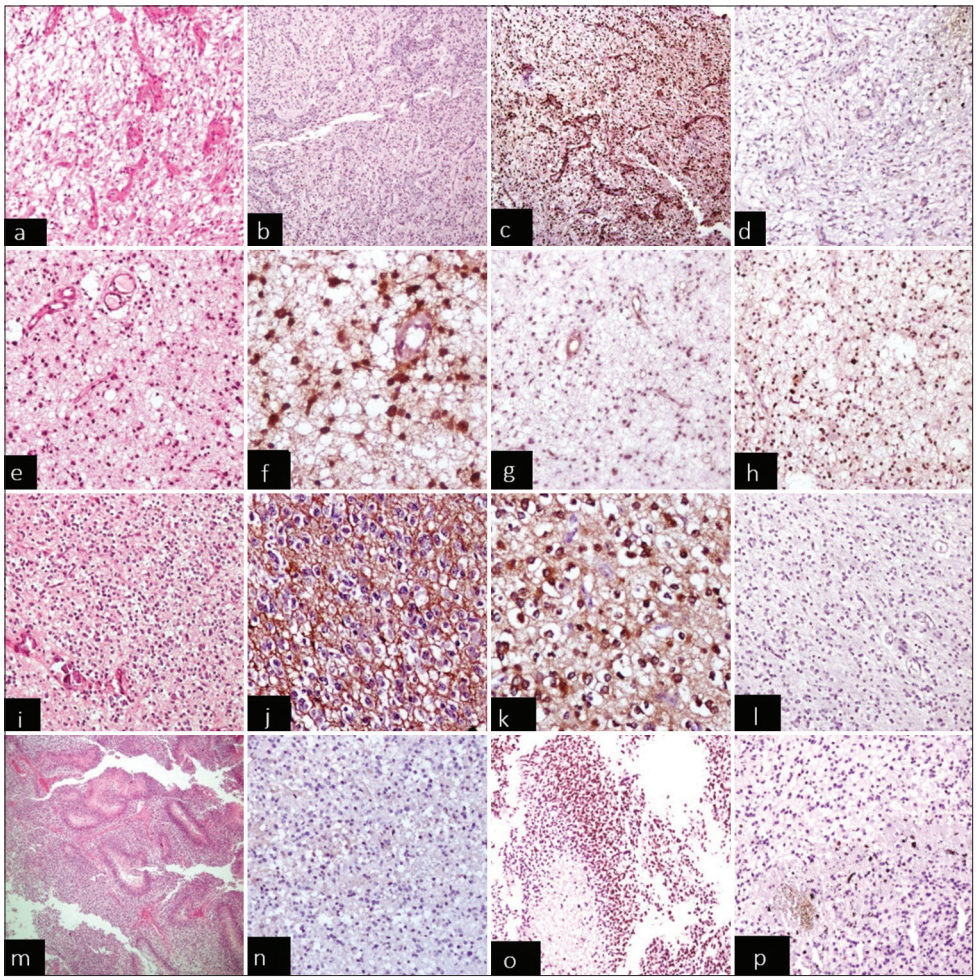

Figure 1: Surrogate immunohistochemistry (isocitrate dehydrogenase 1 [IDH-1], alpha-thalassemia mental retardation X-linked [ATRX], and P53) in gliomas. (a-d x40) Pilocytic astrocytoma: (a) H&E (b) absent IDH-1 (c) normal retained ATRX expression (d) absent P53. (e-h x40) Case of astrocytic phenotype grade 2: (e) H&E (f) positive immunostaining for IDH-1/ IDH mutant (g) loss of nuclear ATRX expression while endothelial cells of blood vessels as internal control are positive (h) p53 overexpression. (i-l x40) Oligodendroglioma grade2: (i) H&E (j) IDH-1 positive/ mutant (k) retained nuclear ATRX expression/no loss (l) absent p53. (m-p x20) Primary glioblastoma: (m) H&E (n) Absent IDH-1 (o) retained nuclear ATRX expression (p) p53 negative. H&E: Hematoxylin and Eosin.

diffuse gliomas, found higher IDH1 immunohistochemical positivity in grade 2 than grade 3 tumors and also in OGs than diffuse ASs. Similarly, Mellai *et al.*[15] observed that IDH-1 mutations were more frequent in OGs than ASs. IDH mutations are reported uncommonly in GBMs and are seen in approximately 10% of cases.[26]

Similar to previous studies,[7,11,27-29] loss of nuclear ATRX expression was frequent in the astrocytic phenotype (81.5%), while retained expression was seen in the oligodendroglial phenotype (94.1%) (P = 0.0001). Only 1 case of OG showed loss of ATRX expression. On reviewing this case, focal weak positivity was seen. However, confirmation by molecular testing for 1p19q co-deletion could not be done. Cai *et al.*[8] and Reuss *et al.*[28] found that the frequency of ATRX loss was higher in diffuse AS, anaplastic AS, and sGBM (76.56%, 77.78%, and 76.27%, respectively) and lower in OG, anaplastic OG, and in pGBM. In this study, the heterogeneous pattern of ATRX staining was observed in some cases. Previous studies on ATRX using similar polyclonal antibodies have shown somewhat contradictory results. [7,9,30] Ikemura *et al.* and Hewer *et al.*[7,9] demonstrated diffuse loss or complete retention of ATRX immunostaining in diffuse gliomas. Reuss *et al.*[28] noted that heterogeneous

Table 2: Summary of immunohistochemistry expressions in diffuse gliomas grade 2,3 (*n*=50).

Histological subtype	IDH-1		ATRX		P53		IDH 1 positive with ATRX and p53			
	+	−	−	+	+	−	ATRX+P53+	ATRX-P53+	ATRX+P53−	ATRX-P53−
AS II (15)	9	6	12	3	9	6	2	4	0	3
AS III (12)	4	8	10	2	6	6	0	2	0	2
n=27	13 (48.1%)	14 (51.9%)	22 (81.5%)	5 (18.5%)	15 (55.6%)	12 (44.4%)	2 (7.4%)	6 (22.2%)	0 (0%)	5 (18.5%)
OG II (12)	10	2	1	11	1	11	1	0	8	1
OG III (5)	3	2	0	5	0	5	3	0	0	0
n=17	13 (76.5%)	4 (23.5%)	1 (5.9%)	16 (94.1%)	1 (5.9%)	16 (94.1%)	4 (23.5%)	0 (0%)	8 (47.1%)	1 (5.9%)
OA II	1	0	0	1	1	0	1	0	0	0
OA III	5	0	0	5	1	4	1	0	4	0
n=6	6 (100%)	0 (0%)	0 (0%)	6 (100%)	2 (33.3%)	4 (66.7%)	2 (33.3%)	0 (0%)	4 (66.7%)	0 (0%)
Total (*n*)=50	32 (64%)	18 (36%)	23 (46%)	27 (54%)	18 (36%)	32 (64%)	8 (16%)	6 (12%)	12 (24%)	6 (12%)

IDH-1: Isocitrate dehydrogenase 1, ATRX (Alpha-Thalassemia/mental Retardation Syndrome X-linked) and IDH1 (Isocitrate Dehydrogenase 1) (+): IDH-1 positive/mutant, IDH-1 (−): IDH-1 negative/absent, ATRX (+): ATRX retained, ATRX (−): ATRX loss/mutant, P53 (+): P53 mutant/overexpressiom, P53 (−): P53 negative, AS: Astrocytoma, OG: Oligodendroglioma, OA: Oligoastrocytoma

Table 3: Summary of immunohistochemistry expression of IDH-1, ATRX, and p53 in GBMs (*n*=65).

Clinical subtypes	IDH-1		ATRX		P53		IDH-1, ATRX and P53			
	+	−	−	+	+	−	ATRX+P53−	ATRX-P53−	ATRX+P53+	ATRX-P53+
pGBM (56)	7	49	31	25	18	38	2	0	0	5
sGBM (9)	4	5	6	3	3	6	0	2	1	1
Total (*n*=65)	11 (16.9%)	54 (83.1%)	37 (56.9%)	28 (43.1%)	21 (32.3%)	44 (67.7%)	2 (3.1%)	2 (3.1%)	1 (1.5%	6 (9.2%)

IDH-1: Isocitrate dehydrogenase 1, ATRX (Alpha-Thalassemia/mental Retardation Syndrome X-linked) and IDH1 (Isocitrate Dehydrogenase 1) (+): IDH-1 positive/mutant, IDH-1 (−): IDH-1 negative/absent, ATRX (+): ATRX retained, ATRX (−): ATRX loss/mutant, P53 (+): P53 mutant/overexpressiom, P53 (−): P53 negative, GBM: Glioblastoma, pGBM: Primary glioblastoma, sGBM: Secondary glioblastoma

ATRX staining is exceptionally rare. Nguyen *et al*.[30] demonstrated that partial ATRX loss was common and was attributable to the quality of the antibody used. The varied results may have arisen due to different cut-offs used in different studies. Some studies have used single, strict criteria, that is, no staining of any tumor nuclei, while others have used varying cut-offs of ≤10%, ≤15%, or even ≤50% of stained tumor nuclei.[7,31-34] This emphasizes the need for standardization of cut-off for ATRX expression. Using a cut-off of 50% loss/retained nuclear ATRX expression, we found a good correlation with histological subtypes and, in analyzing cases, showed heterogeneous expression. ATRX mutation has been reported in a range of 45–67% of diffuse ASs, 57–73% of anaplastic AS, and 33–57% of secondary glioblastoma and is uncommon in pGBMs (4–7%).[27 33,34] p53 mutations occur early in glioma genesis and, like ATRX, are mutually exclusive with 1p19q co-deletion. Intense nuclear staining for p53 by immunohistochemistry has long been used as a surrogate marker for TP53 mutations.[11] Gillet *et al*.[35] found that TP53 mutational status correlated with p53 overexpression and tumor type. The presence of p53 nuclear immunoexpression supports the astrocytic subtype in IDH-mutant gliomas. Strong p53 nuclear positivity ≥10% of the tumor cells is the most accurate predictor for TP53 mutations in gliomas.[1,2,11] We found p53 overexpression in 55.6% of cases of diffuse AS (grade 2 and 3), whereas it was absent in all OGs except one case. TP53 mutations have been reported to be present in >50% of diffuse ASs and sGBM.[36,37] We found that p53 overexpression correlated significantly with histological subtypes astrocytic versus oligodendroglial tumors (55.6% vs. 5.9%; $P = 0.0001$). One case of oligodendroglial phenotype showed p53 overexpression. Ikemura *et al*.[7] also found a higher frequency of p53 expression in diffuse ASs compared to OGs (43.2% vs. 0%, respectively). In this study, 44.4% of diffuse ASs lacked p53 overexpression. This may be due to a lack of mutation or the presence of non-sense mutations that impair the expression of detectable protein, giving false-negative results. Although TP53 mutational studies are considered superior to immunohistochemistry, it is a moderately sensitive and highly specific marker in diffuse low-grade gliomas. [11,35,38]

The frequency of both ATRX loss and p53 mutations was found to be significantly higher in diffuse ASs (81.5%; 55.6%, respectively) compared to GBMs (56.9%; 32.3%, respectively) ($P = 0.021$; 0.033). sGBMs showed a higher frequency of ATRX loss as compared to pGBMs (66.7% vs. 55.4%). These findings are similar to previous studies.[7,8,14,39] However, in contrast to previous studies, no significant difference in the frequency of p53 overexpression was found between pGBM and sGBMs (32.1% vs. 33.3%, respectively). This may be due to the low number of sGBMs in this study.

Hence, among the IDH-mutant cases, those showing astrocytic phenotype along with ATRX loss or p53 overexpression can reliably be put into the astrocytic subgroup, while oligodendroglial phenotype with ATRX retained and negative p53 overexpression can reliably be put into an oligodendroglial subgroup. In mixed histology, the two antibodies can support, but for confirmation, molecular studies are needed. The main limitation of this study was that no correlation with molecular methods was done. However, an attempt to follow up for molecular confirmation was done for a subset of cases reported as OGs (grades 2 and 3). Only 5 cases out of 17 reported OGs (grade 2/3) were tested for 1p19q codeletion using the FISH technique and were positively confirmed. However, due to the limitations in several cases, molecular correlation was not possible.

CONCLUSIONS

To conclude, IDH immunohistochemistry can identify mutations in a significant number of gliomas. Cut-offs of ≥ 10% nuclear positivity for p53 and of 50% loss/retained nuclear ATRX expression showed a good correlation. A surrogate immunohistochemical panel of IDH-1, ATRX, and p53 showed significant association with distinct histopathological subtypes and is helpful in molecular stratification of diffuse gliomas, thereby significantly reducing the need for molecular studies. These data will be helpful to the practicing pathologists in a resource-limited setup. Molecular methods still remain superior as the latter can help in resolving discrepant cases and for confirmation.

Acknowledgment: I am extending my thanks to all my seniors, consultants, friends, technical staff, and statisticians who have supported me in completing the research work directly or indirectly.

Ethical approval: The study/research was approved by the Institutional Ethics Committee, number EC/11/17/1291, dated 24th November 2017.

Declaration of patient consent: The authors certify that they have obtained all appropriate patient consent.

Financial support and sponsorship: Nil.

Conflicts of interest: There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation: The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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How to cite this article: Shrivastava K, Jain S. Surrogate immunohistochemistry markers in adult gliomas – Experience in a tertiary care hospital. J Lab Physicians. 2025;17:18-25. doi: 10.25259/JLP_152_2024