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Immunohistochemical Profile of Molecular Subgroups In Gliomas

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Abstract

Background & Objective: The consecutive revised-WHO classifications (2016 & 2021) incorporated new key molecular parameters into the classification and grading of CNS tumor entities. The present study was conducted to evaluate immunohistochemical (IHC) profile in gliomas, to correlate various subgroups with clinical parameters and to discuss challenges, faced in the implementation of WHO classification wherever possible.

Materials & Methods: The present analysis included 55 clinically suspicious or recurrent cases of gliomas. Additional sections of 2-3 microns were taken on Poly-L-lysine coated slides in each case and subjected to immunostaining for IDH1-R132H, ATRX, P53, and Ki-67 markers.

Results: Out of 55 included cases (09 patients with recurrent tumor); 40 cases (72.7%) were predominantly (55% of male patients) in 41-60 years age group. Most frequent tumor site was frontal lobe. 09 cases of WHO grade2 astrocytoma showed IDH1-R132H mutation (9/9), p53 mutation (5/9), loss of ATRX (7/9) and Ki-67 labeling index <5% in 8 cases (8/9). All 7 cases of WHO grade3 astrocytoma expressed mutant IDH-1. Of the 32 cases of glioblastomas NOS, 25 cases were negative for IDH1-R132H mutation by IHC, while retained ATRX and P53 mutational expression were seen in 22 and 23 cases respectively. All 4 cases of oligodendroglioma WHO grade2 were positive for IDH1-R132H mutation, wild type P53 expression and retained ATRX. 01 case of oligodendroglioma grade3 was found negative for IDH1-R132H mutation by IHC.

Conclusion: We conclude that histomorphology with surrogate IHC can no longer be enough for a valuable diagnosis of adult diffuse gliomas.

Keywords: ATRX, Glioma, IDH1, Ki-67, p53, WHO

Introduction

Approximately 2% of all adult malignancies are brain tumors, and about 70% of these are gliomas, regardless of gender or ethnicity.[1,2] WHO-2007 classification of tumors of central nervous system (CNS), had grouped the glial tumors according to their histopathological similarities, putative cells of origin and levels of differentiation. The consecutive revised-WHO classifications (2016&2021) incorporated new key molecular parameters into the classification and grading of CNS tumor entities. This integrated phenotypic and genotypic approach classifies tumor according to its biology, thus yielding more accurate prognostication and management. This changed diagnostic algorithm will result in more accurate diagnosis of entities like oligoastrocytomas and may also result in morphological and molecular discordance. Genotype triumphs in discordant situations. However, utility of histopathological evaluation is still important to make an initial diagnosis (glioma versus any other tumor), to grade tumors and to define rare tumors lacking genetic characteristics.

WHO 2021 classification has separated paediatric and adult diffuse gliomas because of their different molecular pathways; Adult gliomas have also been classified based on presence of IDH mutations (astrocytoma & oligodendroglioma) or its absence (glioblastoma). Moreover, grade 4 was assigned to an IDH mutant astrocytoma in presence of either the defining histomorphological features (necrosis and/or microvascular proliferation) or presence of homozygous loss of CDKN2A/2B. Glioblastomas have been redefined as astrocytic tumors without IDH mutations with either characteristic histomorphological features or presence of defining mutations (TERT promoter, EGFR, chromosome +7/ -10).

The present study was conducted with the following objectives:

1. To apply IHC markers (IDH-1, ATRX, p53 & Ki-67) in gliomas as recommended by WHO 2016 classification
2. To correlate various subgroups with clinical parameters wherever possible
3. To discuss challenges, faced in the implementation of WHO classification.

Materials & Methods

A retrospective as well as prospective analysis was done in 55 clinically suspicious or recurrent cases of gliomas after the commencement of approval from institutional ethical committee. Excision specimens of these cases were received at histopathology section of pathology department over a period of 40 months. Pilocytic astrocytoma and pleomorphic xanthoastrocytoma cases were excluded from the study. The included cases were analyzed for clinical and histopathological parameters with recommended IHC markers. Clinical parameters included age, gender, symptoms at onset, tumor localization, focality, recurrence or treatment. Radiological findings, intraoperative cytodiagnosis and histomorphological diagnoses were recorded.

Additional sections of 2-3 microns were taken on Poly-L-lysine coated slides in each case and were subjected to immunostaining for IDH1-R132H (monoclonal antibody clone DIA-H09, DIANOVA, 1:20 dilution), ATRX (monoclonal antibody clone BSB-108, BIO-SB, RTU), P53 (antibody clone DO7, CELL MARQUE, 1:300 dilution) and Ki-67 (antibody clone SP6, CELL MARQUE, 1:300 dilution) markers.

Cytoplasmic expression of IDH1-R132H and nuclear expression / loss of ATRX were used to categorize the cases. Further expression of p53 (taking 10% or more positive stained cells as mutant p53 expression) and Ki-67 labeling index (taking >5% as cut-off for increased proliferation index) were evaluated in each tumor.

Results

Table 1 summarized the clinicopathological findings of the included patients.

In the present study, 07 cases were diagnosed as WHO grade 2 astrocytoma (Figure1a-c) by histopathological evaluation (06 male patients & 03 female patients), which showed IDH1-R132H mutation (7/7), p53 mutation (5/7), loss of ATRX (7/7) and Ki-67 labeling index <5% in 7 cases (7/7). 02 cases with histomorphology suggestive of grade2 astrocytoma were found to be immunopositive for mutant IDH1 but had retained expression of ATRX and revised diagnosis of Glioma, NOS grade2 was rendered. These cases showed p53 expression in >10% of tumor cells. 05 cases of WHO grade3 astrocytoma by histomorphology expressed mutant IDH-1(evidenced by IHC), p53 mutant type expression, Ki-67 labeling index ranging 6-50% and loss of ATRX expression. Diagnosis of 2 cases with histomorphology suggestive of grade3 astrocytoma with retained ATRX retention was revised to Glioma, NOS grade3. Cytogenetics for 1p/19q codeletion was advised in all 04 cases of Glioma, NOS grade 2/3.

All 4 cases of histomorphologically diagnosed oligodendroglioma WHO grade2 (Figure1d-h) were positive for IDH1-R132H mutation by IHC. P53 expression was seen in <10% of tumor cells in all cases and all 4 cases had retained ATRX. Ki-67 labeling index was <5% in 02 cases and >5% in 2 cases. Of all the 3 cases histomorphologically suggestive of anaplastic oligodendroglioma, one case was negative for IDH1-R132H mutation by IHC with proliferation index of 10-15% for which further molecular testing was advised. Retained ATRX expression, wild type p53 expression and Ki-67 labeling index of >5% was observed in all the 03 cases. Cytogenetics for 1p/19q codeletion by FISH was also suggested.

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Of the 32 cases of glioblastomas by histolomorphology, 25 cases were negative for IDH1-R132H mutation by IHC (Figure2e-h) and hence by WHO classification 2021, confirmed to be glioblastoma……. 07 cases were positive for IDH1-R132H mutation and revised diagnosis of astrocytoma grade4 was offered as per 2021 who classification. Retained ATRX and P53 mutational expression were seen in 22 and 23 glioblastoma cases respectively. Proliferation index upto 50% was noticed in 28 cases.[Table 2]

Integrated diagnosis (combined histological & IHC findings) is represented in Table3.

Mutational analysis by molecular tests was not available or not performed in any of the included cases till the commencement of this study.

Discussion

In diffuse gliomas, cytosolic IDH1 or mitochondrial IDH2 mutations are considered as driver mutations; out of which most common (over 90%) is heterozygous point mutation at nucleotide position 395 of the IDH1 replacing guanine by adenine (G395A) resulting in replacement of arginine by histidine at amino acid residue 132 of the protein (R132H). Other hot spot mutations identified are R132C, R132S, R132G, R132L in IDH1 and R172K, R172M, R172W and R172G in IDH2. IDH mutant cells have decreased levels of NADPH and GSH, increased reactive oxygen species and D-2-HG production, leading to hyper-methylation at a number of gene loci forming glioma-CpG island methylation phenotype and oncogenic effects.[3] There is growing interest in the possibility of targeted molecular therapies for malignant tumors. Several laboratory techniques like IHC, fluorescence in situ hybridization (FISH), pyrosequencing, and direct sequencing with polymerase chain reaction can be employed to identify IDH-1 mutations.[4]

In the present study 22 of the 23 grade 2 & 3 gliomas expressed mutant R132H IDH-1 on IHC, thus 95.65% of cases could be confirmed as gliomas by HPE & IHC alone. However, for diagnosis of one case further molecular testing was required. This case could either be positive for a variant IDH-1 mutation or for molecular signature of gliobastoma. Further 7 of the 32 cases diagnosed as glioblastoma by HPE were positive for mutant R132H, IDH-1, which with revised 2021 classification would be recategorized as Astrocytoma grade 4.

Agarwal et al, in their evaluation of 50 gliomas, found concordance of 88% for IDH-1, R132H mutation by IHC & DNA sequencing. Of the 6 discordant cases in their study, 3 harbored IDH-1, R132L mutation. Further in 46.6% of IDH1-R132H mutant gliomas, IHC expression of the mutant protein was heterogeneous and focal.[5]

Incidence of IDH-1 mutations in grade 2/3 gliomas and secondary glioblastoma was found to be 80% in many studies while 12% in glioblastomas. IDH1-R132H mutations accounted for >85% of IDH mutant gliomas.[6] Relatively uncommon IDH2 mutations are mutually exclusive with IDH-1 mutation and are more common in oligodendrogliomas (0.9% versus 5.2%).[7] Thus it has been recommended that IDH2 mutation studies can be avoided in astrocytic morphology tumors. No IDH2 mutation has been reported from India.[8] IDH mutations are more common in diffuse astrocytoma grade 2 as compared to anaplastic astrocytoma grade 3. IDH mutations confer a better prognosis to both grades of astrocytic tumors. Since IDH mutant diffuse astrocytomas grade 2 do better than IDH mutant anaplastic astrocytomas grade 3, morphological grading is still relevant.[8]

Since IDH mutant glioblastomas have a survival benefit, these have been recategorized as Astrocytomas grade 4. Morphologically diffuse astrocytomas and anaplastic astrocytomas with wild type IDH are a heterogenous group, requiring further molecular characterization. Many of these have molecular signature of glioblastomas.[9]

Cho et al. examined frequency of IDH-1 and IDH-2 mutation by PNA mediated real time PCR in 87 gliomas, found infrequent IDH-2 mutation in gliomas (1/87), significant percentage of IDH wild type gliomas (frequency higher than reported) and this variation may be attributed to variations in region/testing methodologies.[10] WHO 2016 classification included the entity of IDH-1 wild type gliomas. However, in the revised 2022 classification, these could be IDH-1 wild type glioblastomas without the necrosis / microvascular proliferation but harboring glioblastoma specific mutations (EGFR mutation, TERT promoter mutation & +7 / -10 chr.).

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ATRX inactivation due to mutation or deletion or gene fusion, induces abnormal telomeres and has strong association with IDH1 and p53 mutations. ATRX mutation is mutually exclusive with 1p/19q codeletion, hallmark of oligodendrogliomas. ATRX mutation seen as loss of expression by IHC is a specific biomarker of astrocytomas.[11] Since 1p/19q codeletion and ATRX mutation are mutually exclusive, IHC for ATRX has found it's place in diagnostic algorithm based on WHO 2016 classification.

If an IDH mutant glioma has loss of ATRX by IHC, it is diagnosed as astrocytoma without testing for 1p/19q codeletion. However, retained ATRX expression cannot be a surrogate marker for 1p/19q codeletion. ATRX mutation is seen in 86% of astrocytomas and confers a better prognosis to IDH mutant astrocytomas.[11,12]

In our study, 12 of the 16 cases (75%) of grade 2 & 3 astrocytomas showed ATRX loss by IHC, FISH for 1p/19q codeletion was advised in the 4 cases with retained ATRX expression to rule out oligodendroglioma. All 7 cases of oligodendiogliomas in the present study, had retained ATRX and hence, were also advised 1p/19q codeletion. In cases of morphological and molecular discrepancy, molecular diagnosis supersedes the morphological diagnosis.

Of the 32 cases for GBM, 25 cases had wild type IDH-1 while 7 had mutant IDH-1, which were reclassified as astrocytoma grade 4 by WHO 2021 classification. All IDH mutant GBM / grade 4 astrocytomas had loss of ATRX expression. 22 of the IDH wild type glioblastomas had retained ATRX while 3 cases showed a loss of ATRX expression. ATRX expression in GBMs is variably reported. Liu et al. observed ATRX loss in IDH mutant GBMs (grade 4 astrocytomas), while Cai et al. reported a higher loss of ATRX in IDH wild type GBM & grade 3 gliomas as compared to grade 2 gliomas.[13,14]

TP53 mutation occurs early in astrocytomas while PTEN and EGFR mutations are restricted to high grade gliomas. 1p/19q codeletion is an early event in oligodendrogliomas. The majority of IDH-1 mutations are observed in combination with either TP53 mutations or co-deletion of 1p/19q chromosomes.[4] TP53 mutation is less frequently seen in oligodendrogliomas. 75% of TP53 mutations are missense leading to complete or partial loss of p53 and are detected as overexpression in cells by IHC. However, this is not true for non-missense mutations.

Variable cut offs have been used to interpret p53 mutation. Gillet et al. observed best sensitivity and specificity (77.4%&78.6% respectively) for TP53 mutation at a threshold level of 10%. In their study, p53 immunolabeling could detect 92% of mutated tumors.[15] However, IHC for p53 should be interpreted with morphology and ATRX mutation as inactivating ATRX mutations coexist with p53 mutations in IDH mutant astrocytomas. Takami et al. evaluated p53 mutation status by IHC on 157 diffuse gliomas and concluded that strong immunoreactivity in >10% of tumor cells showed a sensitivity of 78.8% and specificity of 96.7% for mutations. Thus p53 by IHC is a strongly specific predictor of TP53 mutation.[16] Nayak et al. in 2004 found that TP53 mutations were more common in astrocytic tumors than oligodendroglial tumors.[17]

Astrocytomas (grade2,3&4) are characterized by IDH, ATRX & TP53 mutations while oligodendrogliomas lack ATRX and TP53 mutations. Primary GBMs lack IDH mutations but do exhibit TP53 mutations also along with other mutations.[18] 80% of IDH mutant astrocytomas show TP53 mutations while IDH wild type glioblastomas show TP53 mutation in 23-28% cases. The prevalence of TP53 mutation in oligodendiogliomas is even lower.[19]

Glioma characterization requires a basic simple approach; correlating the histomorphology with the findings whether a tumor is IDH mutant or IDH wild type or exhibits 1p/19q codeletion by IHC or by FISH. Other molecular markers such as TERT promoter mutations, P53 or histone H3 mutations, EGFR amplification, or CDKN2A/B alterations, need to be assessed for specific diagnoses in cases of IDH wild type gliomas or discrepent cases.[20] IDH wild type astrocytomas can be called as GBM in the presence of microvascular proliferation and/or necrosis / TERT promoter mutation / EGFR gene amplification / +7/10 chromosome variation as per 5th edition of WHO classification of CNS tumor. CDKN2A and CDKN2B homozygous deletion in diffuse astrocytomas (IDH mutated) also confers a grade 4 even in absence of microvascular proliferation or necrosis.

In a study in 2018 by Pant et al, categorisation of GBM was based on IDH mutations as wild type or mutant. They found that mean survival of IDH mutant

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glioblastomas was better than GBM wild type and its incidence was at a lower age (mean age 41 versus 60). This study was in agreement with the inclusion of GBM, IDH mutant into category of Astrocytoma, IDH mutant, grade 4.[21]

Diffuse gliomas are traditionally graded by histomorphological evaluation of cellularity, atypia, mitosis, necrosis and microvascular proliferation. Ki-67 labelling index has been used as an ancillary technique to confer more objectivity. In the present study, 23.07% morphologically grade2, IDH mutant gliomas had a Ki-67 labeling index of >5%. Further 28.6% grade 3 astrocytomas and 3.1% of glioblastoma cases showed a proliferation index of <5%. Thus the mitotic activity, microvascular proliferation and necrosis, the standard criteria for grading, did not correlate completely with Ki-67 labeling index. Such patients with lower histomorphological grade and higher Ki-67 index should be followed-up for presence of CDKN2A homozygous loss and overall survival to assess the relevance. Single case of oligodendroglioma, NOS, grade 3 (IDH wild type by IHC) had a proliferation index of 10-15%. Patient was advised FISH for 1p/19q codeletion and testing of glioblastoma signature markers in case of negative FISH. In our observation, 84.37% of histomorphological cases of glioblastomas showed proliferation index ranging 6-50%. In contrast, Pant et al. reported 80% cases of GBM with proliferation index equal or less than 5% in their study.[21]

Cut-off values of Ki-67 index proposed by various authors are variable for distinguishing low and high grade gliomas.[22] Theresia et al. obtained Ki-67 labeling index cut point of 6.35% with significantly sensitive and specific for determining low- or high-grade gliomas ($p<0.001$).[23] Some authors had described shorter survival of patients with mitotic count cut-off with =>2 mitosis in entire specimen as compared to those with <2 mitosis, in pre-WHO2016 era. In case of very small biopsies, even single mitosis is important, however, higher mitotic activity is needed in larger tissue specimens.[24]

Conclusion:

1. IDH mutation is reliably detected by IHC in most cases, thus establishing a diagnosis of glioma with correlating morphology.
2. For all IDH wild type adult glial tumors without histomorphological features of glioblastoma, molecular testing for glioblastoma signature mutations will be required.
3. Retained ATRX in adult diffuse IDH mutant glial tumors will require testing for 1p/19q codeletion, irrespective of morphology.
4. Grading of IDH mutant glial tumors is incomplete without additional testing for CDKN2A homozygous loss.

Thus, though the present study is limited by small number of cases, we conclude that histomorphology with surrogate IHC can no longer be enough for a valuable diagnosis of adult diffuse gliomas and in-house facilities/outsourcing to accredited laboratories is mandatory.

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TABLE1. CLINICOPATHOLOGICAL FEATURES OF INCLUDED CASES:

VARIABLES		MALE			FEMALE		
AGE (yrs)		20-40	41-60	>60	20-40	41-60	>60
CLINICAL FEATURES	Headache	7	12	4	4	4	1
	Seizure	4	6	2	2	2	1
	Hemiplegia	2	4	2	1	1	-
	Altered sensorium	1	3	1	2	2	1
	H/O treatment	1	1	-	1	-	-
	Recurrence	3	4	1	1	-	-
SITE	Frontal	7	5	2	4	1	1
	Parietal	2	-	1	-	1	-
	Temporal	-	5	-	-	-	1
	Occipital	1	-	-	-	-	-
	Diffuse(> 1 lobe)	1	7	3	3	3	-
	Others	1	5	-	-	1	-
LATERALITY	Left	6	12	4	1	3	1
	Right	6	10	2	6	3	1
FOCALITY	Unifocal	11	20	5	5	6	2
	Multifocal	1	2	1	2	-	-
HPE DIAGNOSIS	AST G2	4	2	-	2	1	-
	AST G3	1	2	-	2	2	-
	OLG G2	2	1	1	-	-	-
	OLG G3	2	1	-	-	-	-
		3	16	5	3	3	2

	GBM NOS						

TABLE 2. IMMUNOHISTOCHEMICAL PANEL FINDINGS OF INCLUDED CASES:

HPE DX	No. of cases	IDH1(by IHC)		ATRX		P53		Ki-67 INDEX (%)		
		Mutant	Wild	Loss	Retained	Mutant	Wild	=<5	6-50	>50
Astrocytoma G2	09	9	-	7	2	05	04	8	1	-
Astrocytoma G3	07	7	-	5	2	05	02	2	5	-
OLG II	04	4	-	-	4	-	04	2	2	-
OLG III	03	2	1	-	3	-	03	-	3	-
GBM NOS	32	7	25	10	22	23	09	1	27	4

TABLE 3. INTEGRATED DIAGNOSIS OF INCLUDED CASES BASED ON HISTOMORPHOLOGY & IHC FINDINGS :

INTEGRATED DIAGNOSIS (HISTOLOGY + IHC)	**No. of cases**	**MOLECULAR ANALYSIS REQUIRED**
Astrocytoma G2, IDH mutant, p53 mutant with ATRX loss & Ki-67 <5%	03	CDKN2A homozygous loss for grade
Astrocytoma G2, IDH mutant, p53 wild type with ATRX loss & Ki-67 <5%	03	CDKN2A homozygous loss for grade
Astrocytoma G2, IDH mutant, p53 wild type with retained ATRX & Ki-67 <5%	01	1p/19q codeletion, CDKN2A homozygous loss for grade
Astrocytoma G2, IDH mutant, p53 mutant with retained ATRX & Ki-67 <5%	01	1p/19q codeletion, CDKN2A homozygous loss for grade
Astrocytoma G2, IDH mutant, p53 mutant with ATRX loss & Ki-67 >5%	01	CDKN2A for grade
Astrocytoma G3, IDH mutant, p53 mutant, with ATRX loss & Ki-67 >5%	03	CDKN2A homozygous loss for grade
Astrocytoma G3, IDH mutant, p53 mutant, with ATRX loss & Ki-67 <5%	02	CDKN2A homozygous loss for grade
Astrocytoma G3, IDH mutant, p53 wild type with retained ATRX & Ki-67 >5%	02	1p/19q codeletion, CDKN2A homozygous loss for grade
Astrocytoma G4, IDH mutant, p53 mutant with ATRX loss & Ki-67 >5%	06	Not required

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Astrocytoma G4, IDH mutant, p53 mutant with ATRX loss & Ki-67 <5%	01	Not required
Oligodendroglioma G2, IDH mutant, p53 wild type, retained ATRX & & Ki-67 <5%	02	1p/19q codeletion, CDKN2A homozygous loss for grade
Oligodendroglioma G2, IDH mutant, p53 wild type, retained ATRX & & Ki-67 >5%	02	1p/19q codeletion, CDKN2A homozygous loss for grade
Oligodendroglioma G3, IDH mutant, p53 wild type, retained ATRX & Ki-67 >5%	02	CDKN2A homozygous loss for grade
Oligodendroglioma G3, NOS (IDH wild type by IHC)	01	IDH1/2 mutation, 1p/19q codeletion, CDKN2A homozygous loss for grade
Glioblastoma, IDH wild type (>55 years of age)	13	Not required
Glioblastoma, IDH wild type by IHC (=<55 years of age)	08	IDH1/2 mutation & TERT promoter mutation / EGFR gene amplification / +7/10 chromosome variation
Glioblastoma, NOS	04	Not required

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Figure – 1

a - c. Photomicrograph showing Grade 2 astrocytoma exhibiting positive immunoexpression for mutant IDH1 & p53 mutant type immunoreaction.

d – h. Photomicrograph showing Grade 2 oligodendroglioma exhibiting positive immunoreaction for mutant IDH1, retained ATRX, Wildtype p53 immunoexpression & low Ki-67 index.

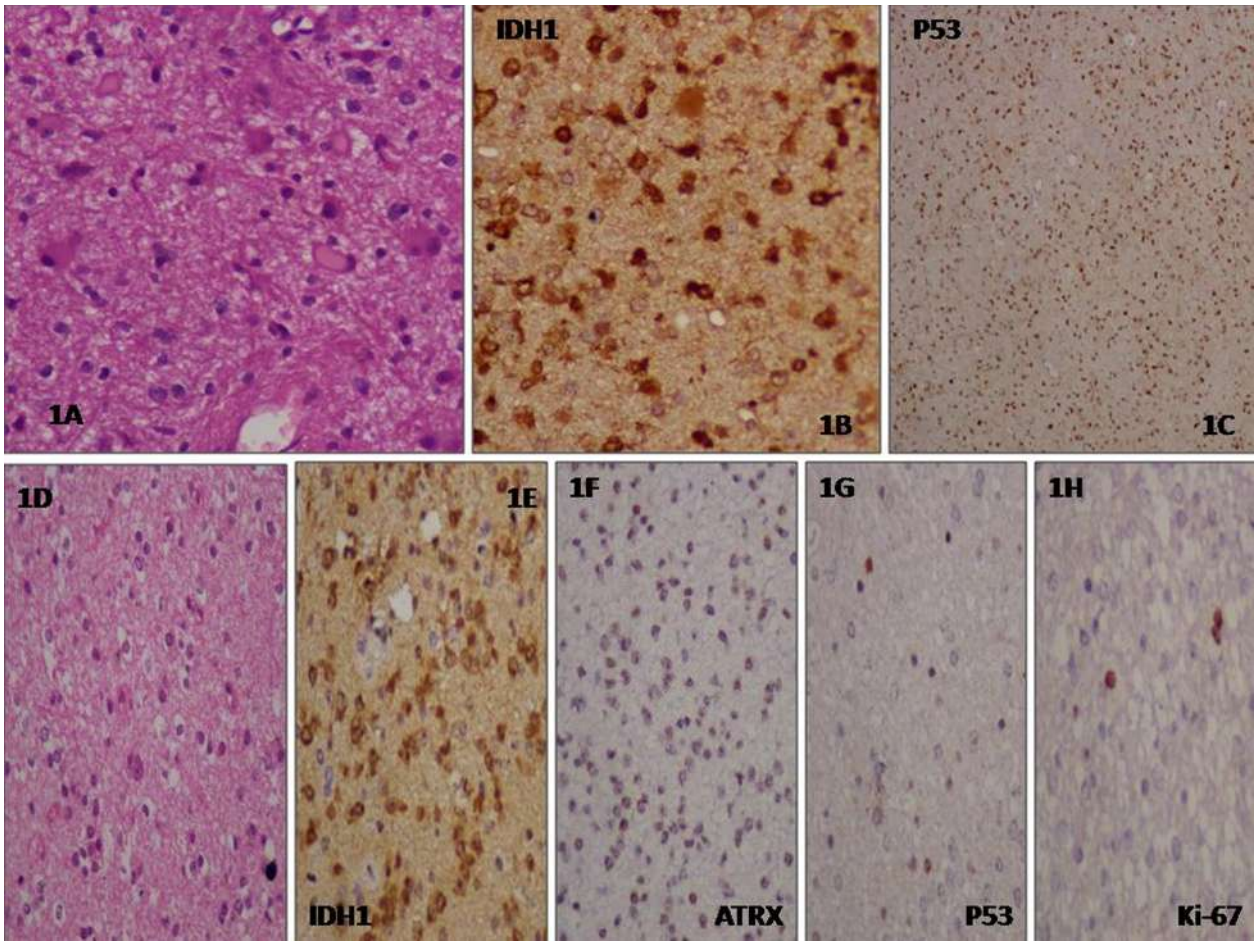

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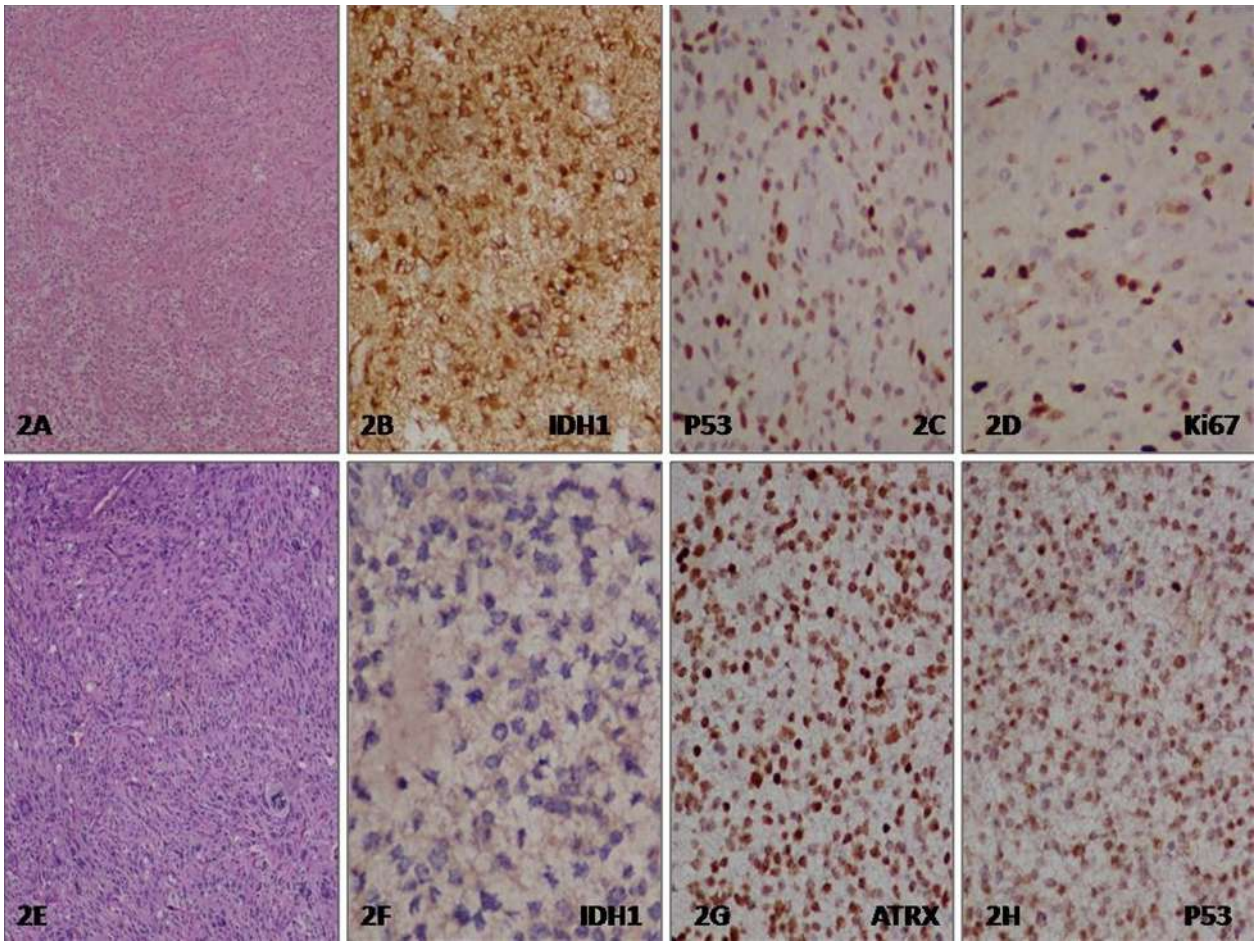
Figure 2

a - d. Photomicrograph showing Grade 4 astrocytoma exhibiting positive immunoexpression for mutant IDH1 & p53 mutant type immunoreactions with high Ki-67 index.

e – h. Photomicrograph showing case of glioblastoma exhibiting negative immunoexpression for IDH1, retained ATRX & mutant type p53 immunoreaction.



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