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Original Article

Algorithmic approach utilizing histology and immunohistochemistry for the current classification of diffuse glioma

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Abstract: Introduction: Diffuse glioma constitutes 28% of primary brain tumors. Until recently morphologic appearance was the only criterion for classifying these tumors. However, WHO 2016 incorporates molecular information in the primary diagnosis of gliomas such as Isocitrate dehydrogenase 1 (IDH1), Alpha thalassemia/mental retardation syndrome X inked (ATRX) as well as 1p/19q codeletion on FISH. In a resource-limited setup where FISH is not available, Alpha internexin (INA) has been suggested as a surrogate IHC marker. Material and methods: Cross-sectional study conducted in the Department of Pathology for two years. Tissue blocks and clinical as well as radiological details were obtained from departmental archives. After assessing the morphologic details, routine IHC markers such as GFAP, Ki67 and P53 along with molecular markers like IDH-1, ATRX, and INA were applied. Results: Out of 55 cases of diffuse glioma, 23 cases of astrocytoma and 32 cases of oligodendroglioma with an overall mean age of presentation of 41.49 ± 12.47 years. IDH-1 expression among diffuse glioma was 89.1% in our study. Alteration in the ATRX gene expression was observed in 95.7% of astrocytomas. 75% of oligodendrogliomas expressed INA with no significant difference in expression between the two grades. Based on the algorithmic approach using molecular surrogate markers, diffuse gliomas were categorized into six distinct groups. IDH-mutant, ATRX loss of expression astrocytoma and IDH-mutant, INA positive oligodendroglioma are two categories that do not require further molecular testing. This comprises 72.7% of the cases and these do not warrant further workup. Conclusion: Implementation of combined phenotypic-genotypic diagnosis with the use of histomorphology and immunohistochemical surrogates for molecular genetic alterations will yield more homogeneous and narrowly defined diagnostic entities which will provide better prognostication and definitive treatment. It also is cost-effective in a resource-limited setup.

Keywords: Diffuse glioma, IDH-1, alpha thalassemia/mental retardation syndrome X linked, alpha internexin

Introduction

Diffuse gliomas constitute diffuse proliferation of glial cells which infiltrate into the adjacent brain parenchyma and it constitutes a major proportion of primary brain tumors in adults as well as children [1]. It includes WHO grade II and III astrocytoma, oligodendroglioma, oligoastrocytoma, and grade IV glioblastoma.

Until recently, morphologic appearance was the only criterion for classifying these tumors. However, it is challenging to differentiate astrocytomas, oligodendrogliomas, and oligoastrocytomas as well as differentiation of low-grade glioma from reactive gliosis on morphology alone. To overcome this 150 neurooncological specialists had a consensus under the sponsorship of the International Society of Neuropathology and have given ISN-HAARLEM guidelines for nervous system tumor classification and grading, all of which have been included in the new 2016 WHO classification [2]. Here the histomorphologic findings are incorporated along with molecular information which defines the tumor entities to provide an integrated diagnosis. The reporting format is layered and includes - integrated diagnosis with histologic

subtype, WHO grading, and molecular information.

In recent years, extensive research has been done regarding these molecular alterations of which isocitrate dehydrogenase (IDH) is well established. It has been described as an early event in the diffuse variant of gliomagenesis. α-thalassemia mental retardation X-linked protein (ATRX) is a newer mutation identified in tumors of astrocytic lineage and forms the basis of the classification of glial tumors. In addition, oligodendrogliomas are characterized and diagnosed by the presence of 1p/19q codeletion. These molecular alterations not only have diagnostic significance but also pave the way for targeted therapy and prognosis. They help in distinguishing diffuse glioma from reactive gliosis which does not harbor IDH mutations.

With recommendations from the WHO, these pivotal molecular alterations are diagnosed by various modalities such as immunohistochemistry and next-generation sequencing (NGS) for IDH mutations, 1p/19q co-deletion by Fluorescent In-Situ Hybridization (FISH) [3]. In recent years the role of immunohistochemistry has been established to act as surrogate markers for molecular alterations. Therefore, in a resource-limited setup with financial constraints, it would be possible to utilize an algorithmic approach using IHC for categorizing glial tumors in the majority of the cases. It would reduce the number of cases further requiring NGS and FISH to give a complete diagnosis. Alpha-internexin is one such surrogate marker for 1p/19q co-deletion for oligodendroglioma [4-6].

The distinction between different subtypes of diffuse glioma has a critical impact on prognostic evaluation and treatment considerations but can be difficult to diagnose because of the subjective histopathologic criteria. Also, studies related to molecular alterations in diffuse gliomas are limited in the Indian population, hence this study helps determine the frequency of genetic alterations in our population as well.

The approach we propose here is to have an algorithm using histomorphology and IHC-Glial fibrillary acidic protein (GFAP), p53, Ki67% as well as new immunohistochemical markers which are surrogates for molecular alterations such as IDH-1, ATRX, and alpha Internexin (INA) to reclassify diffuse gliomas according to WHO classification 2016.

Materials and methods

A cross-sectional study was conducted in our department from January 2016 to February 2019. The study was conducted after obtaining clearance from the institutional ethics committee with a project number of JIP/IEC/2017/0269. All cases operated for glial tumors were included. Cases with prior treatment with radio/chemotherapy and pediatric glial tumors were excluded from the study. The biopsy specimen was obtained following surgery from the Department of Neurosurgery. Formalin-fixed paraffin-embedded tissue blocks were used to obtain hematoxylin and eosin-stained sections for assessing the morphology of the tumor. These were classified as grade II or III astrocytoma/Oligodendroglioma.

Inclusion criteria: All cases of diffuse glioma in the Department of Pathology during the study period.

Exclusion criteria: 1) Glioblastoma. 2) Midline glioma. 3) Paediatric glioma. 4) Post-radiotherapy, Post chemotherapy. 5) The nonavailability of tissue blocks in old cases.

Immunohistochemistry

This was followed by an IHC panel by manual staining technique which included IDH1 (Dianova H09), ATRX (Sigma Aldrich HPA001906) and Alpha internexin (Abcam ab7259), P53 (Dako), Ki67 (Thermo-scientific), IDH-1 mutation in the R132H region was interpreted as cytoplasmic positivity with >10% [7, 8] of the tumor show positivity. Mutation in the ATRX gene leads to the loss of expression in tumor cells, hence this is a negative marker. Loss of nuclear reactivity in >90% of cells is considered positive for the particular mutation (endothelial cells as controls). For INA tumor with ≥10% positivity in the cytoplasm as well as in the perinuclear region is considered positive [6].

Initially, glial tumors were classified based on the previous classification system into astrocytoma, oligodendroglioma and oligoastrocytoma. Few of the cases that had overlapping morphological features were reported as diffuse

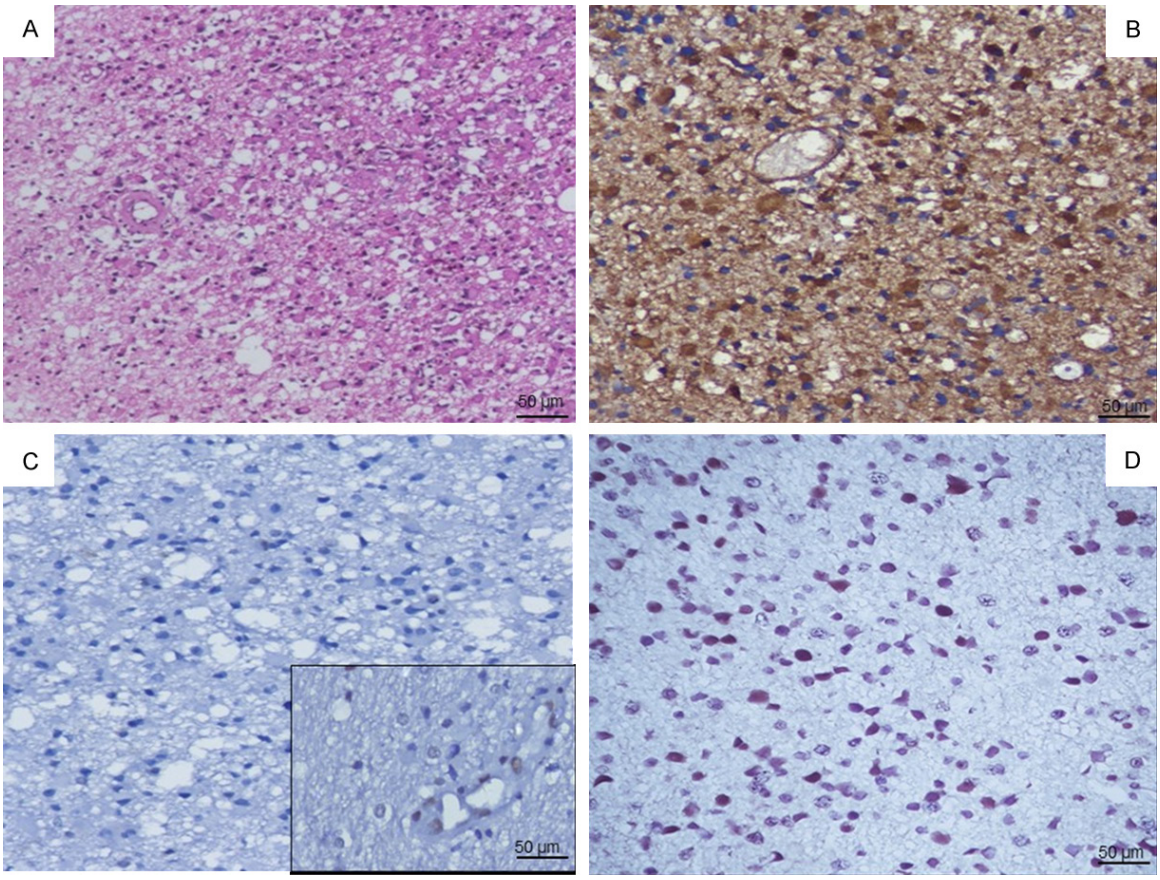

Figure 1. Case of gemistocytic astrocytoma: A. Biopsy showing >20% gemistocytes (H&E; ×100). B. IHC with IDH-1 showing cytoplasmic positivity (DAB; ×200). C. ATRX negative in tumor cells (DAB; ×400). The inset shows internal control with endothelial cells showing nuclear positivity. D. P53 positivity in >30% of tumor cells (DAB; ×400).

glioma grade II/III. Following our IHC approach grade II and grade III diffuse glioma could be categorized into 6 groups: 1) IDH-1 mutant, ATRX loss of expression & INA negative diffuse astrocytoma. 2) IDH-1 negative, ATRX loss of expression & INA negative astrocytoma. 3) IDH-1 mutant, ATRX retained expression & INA positive diffuse glioma. 4) IDH-1 mutant, ATRX retained expression, INA positive oligodendroglioma. 5) IDH-1 mutant, ATRX retained, INA negative oligodendroglioma. 6) Oligodendroglioma, NOS.

Statistics

The distribution of categorical variables such as gender, IHC markers and site of biopsy was expressed as frequency and percentage. The continuous variables such as age were expressed as mean with standard deviation. The comparison of IHC markers with other categorical variables and histomorphology was carried out by using the Fisher exact test. The diagnostic accuracy of the IHC marker for subtyping the glioma in comparison with histomorphology was carried out by estimating sensitivity and specificity along with predictive values. All the statistical analysis was carried out at a 5% level of significance and $P<0.05$ was considered significant.

Results

The study included 55 cases (**Figure 1**) with age ranging from 18 to 72 years and overall mean age of 41.49 ± 12.47 years. Astrocytomas had a male preponderance while oligodendrogliomas had an almost equal incidence in both genders. Most of the diffuse glioma was located supratentorial with the commonest location being the frontal lobe (41.8%). Astrocytomas had a higher occurrence in the temporal lobe (34.7%), while oligodendrogliomas were frequently found in the frontal lobe (53.1%) (**Table**

Table 1. Case distribution in the study

Tumor/Grade	WHO grade II	WHO grade III	Total cases
Astrocytoma	16	7	23
Oligodendroglioma	11	21	32

1). Patients with oligodendroglioma had seizures (40.6%) and headache (43.7%) as the most common presenting symptoms. Hemiparesis and seizures were common in astrocytomas (39.1% each).

Immunohistochemistry

The overall expression of IDH-1 in our study was 89.1%. The proportion of astrocytoma positive for IDH-1 is 87% and oligodendroglioma was 90.6%. Anaplastic astrocytoma had a lower proportion of IDH-1 expression in comparison to diffuse astrocytoma. ATRX loss of expression was seen in 95.7% of astrocytomas with a *p*-value of <0.001. Sensitivity and specificity for ATRX expression are 95.7% and 90.6% respectively. It has a positive predictive value of 88% and a negative predictive value of 96%. Only one case of astrocytoma in our study had ATRX retained expression. Nuclear localization of P53 had a significant association with tumors of astrocytic lineage. Sensitivity and specificity were 69.6% and 58.2% respectively. This was lower than ATRX for identifying astrocytoma. P53 had a better negative predictive value of 50.9%. INA had a sensitivity of 75% with an equal proportion of positivity among both tumor grades (**Table 2**).

Change in primary diagnosis applied to 10 cases due to change in the genetic profiling. Six of these ten cases had mixed morphology with two populations of cells having astrocytic and oligodendroglial morphology (**Figure 2**). Five of these cases turned out to be oligodendroglioma and one turned out as astrocytoma. 2 cases of astrocytoma had increased cellularity and minimal nuclear pleomorphism with negative IDH-1. This was further categorized as reactive gliosis (**Figure 3**). One case of oligodendroglioma was reclassified as astrocytoma based on loss of ATRX expression and negative INA expression. However, in such cases, further analysis with FISH is essential. One case which was reported as gliomatosis cerebri was reclassified as anaplastic astrocytoma.

Discussion

Integrated diagnosis for diffuse glioma was incorporated in the recent 2016 WHO by incorporating molecular findings along with routine histology. This has not only improved diagnostic accuracy but also revolutionized patient care. Diffuse gliomas are heterogeneous tumors with unique gene expression profiles and cytogenetic events defining them. It becomes important to stratify these tumors into subgroups to determine the treatment, prognosis, and overall survival. Various studies have shown molecular alterations such as IDH mutation and 1p/19q co-deletion which have a better prognosis compared to tumors with IDH wildtype and non-codeleted 1p/19q. There are several methods employed to categorize diffuse gliomas which comprise FISH, IHC, and NGS. Our study focuses on using an algorithm based only on IHC to categorize these tumors (Supplementary Figure 1). This approach is useful in our country where economic constraints are present for performing FISH and NGS [7, 8].

IDH is a crucial molecular alteration in tumorigenesis described in various solid and hematologic malignancies. Our understanding of IDH and its role in the development of glioma has drastically improved over the years. It is pivotal in the diagnosis and prognosis of grade II and grade III diffuse glioma and for its role as a therapeutic target for which drugs like AG-120 and AG-122 are in clinical trials [9]. In-depth genomic analysis is seldom available in all institutions; hence immunohistochemistry is the method of choice for the detection of IDH. The frequency of IDH mutation is variable ranging from 54% to 95% as described by different studies [10, 11]. Of these IDH-1 mutations, R132H is the most frequent and constitutes about 80-90%. We studied IDH-1 mutation by IHC in 55 cases. Overall positivity was 89.1% with astrocytoma showing 87% and oligodendroglioma with 90.6% positivity. It was also noted that as the tumor grade increased the positivity of IDH reduced. Similar findings were observed in grade II astrocytoma (93.7%) had higher positivity rates in comparison with grade III astrocytoma (71.4%). About 10.9% of the cases that were IDH-1 negative have to be further analyzed for other IDH mutations by IHC for IDH-2 and Sanger sequencing for other IDH1/2 mutations before labeling them as IDH wild-

Table 2. Final diagnosis with molecular information

	Molecular diagnosis N=55 (%)	
	Grade II	Grade III
IDH-1 mutant, ATRX loss of expression Astrocytoma	15/55 (27.3%)	4/55 (7.3%)
IDH-1 negative, ATRX loss of expression Astrocytoma	1/55 (1.8%)	2/55 (3.6%)
IDH-1 mutant, ATRX retained expression Astrocytoma	0	1/55 (1.8%)
IDH-1 mutant, INA positive Oligodendroglioma	7/55 (12.7%)	14/55 (25.5%)
IDH-1 mutant, INA negative Oligodendroglioma	3/55 (5.5%)	5/55 (9.1%)
Oligodendroglioma - NOS	1/55 (1.8%)	2/55 (3.6%)

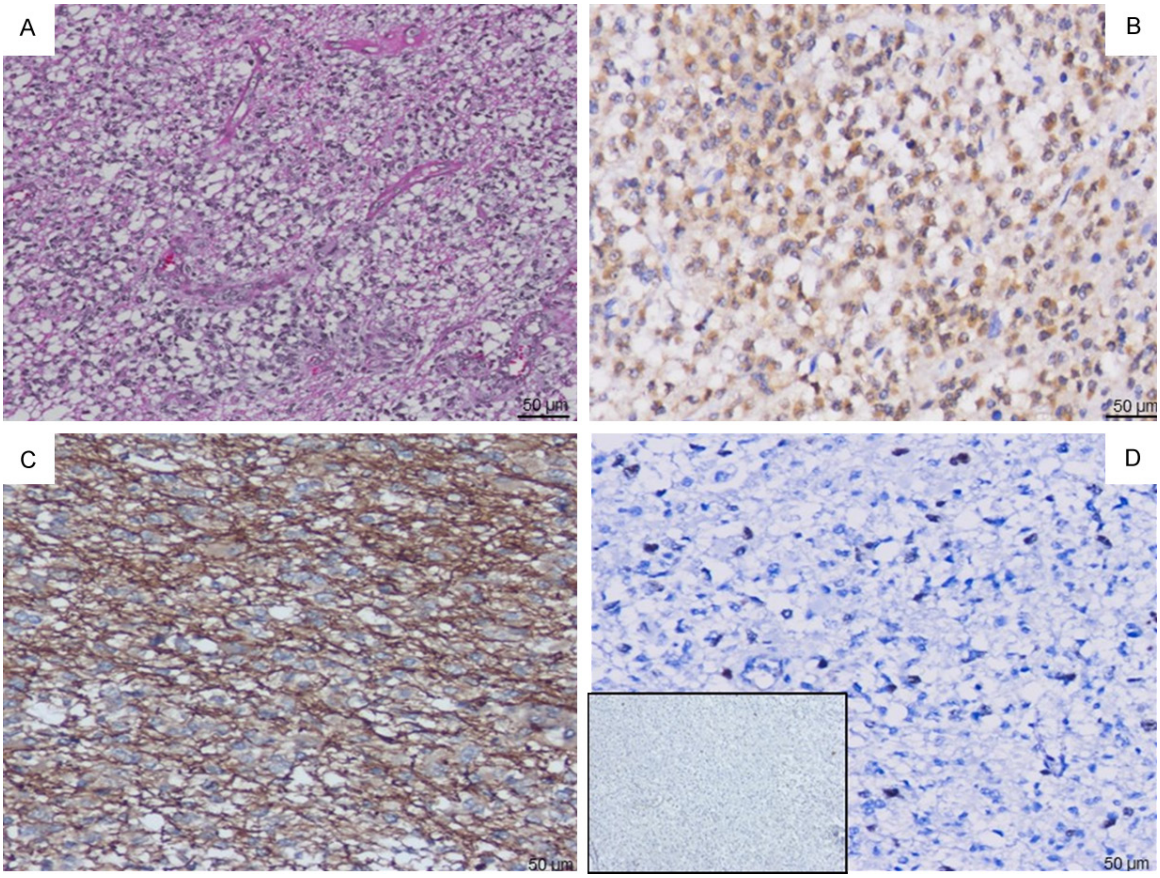

Figure 2. Case of oligodendroglioma grade 3: A. Biopsy showing tumor infiltrating normal brain parenchyma with oligodendroglial morphology (H&E; ×200). B. Cytoplasmic positivity of IDH-1 (DAB; ×200). C. INA showing cytoplasmic positivity (DAB; ×400). D. Increased Ki67~6%. Inset shows negative p53 expression.

type. As stated by Ruess et al, most of the IDH wildtype gliomas can be resolved into other tumor entities like glioblastoma [12]. This can also be linked to the fact that IDH wild-type tumors have a poor prognosis. In such cases, evaluation of TERT promoter mutation will solve the problem as they are exclusive to GBM.

The utility of IDH-1 is not limited only to the diagnosis of diffuse glioma. It also helps to differentiate gliomas from reactive gliosis and glioblastoma, especially in small biopsies. We had four cases of reactive gliosis, two of which were misdiagnosed as astrocytoma. The possible reason for this is the morphological similarities between low-grade astrocytoma and reactive gliosis. In a small biopsy, both have increased cellularity with mild nuclear atypia and absent mitotic figures. Even GFAP and Ki67 can have overlapping results and cannot distin-

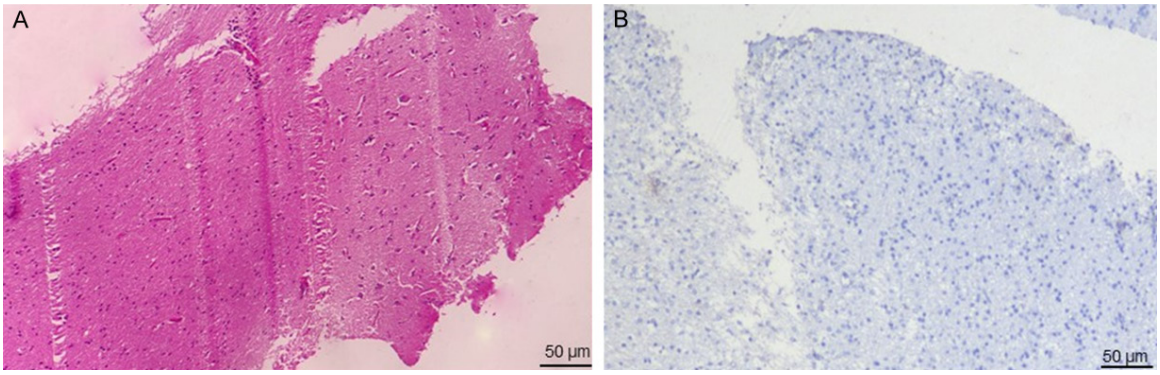

Figure 3. Case of reactive gliosis: A. Biopsy showing increased cellularity, absence of mitotic figures (H&E; ×100). B. IHC with IDH-1 is negative (DAB; ×200).

guish the two entities. Though radiologic findings give clues, IDH-1 is more important for a definitive opinion. However, this has to be aided by ATRX expression to rule out IDH-1 negative astrocytomas. All four of our cases had IDH-1 negativity with retained ATRX expression.

ATRX immunoreactivity follows all or none pattern and an intermediate category is uncommon. There is also a significant correlation between ATRX protein expression with ATRX gene mutation and its interpretation in the presence of internal control is fairly reliable. Another marker for astrocytic lineage is the p53 mutation, which is an overexpression type of mutation. However, there is heterogeneity in the staining of p53 which can lead to misinterpretation. While it is specific for astrocytic lineage, a significant percentage of oligodendroglioma can also show p53 overexpression. In the literature, we found various cut-offs being described for overexpression of p53 ranging from 10 to 50% [13, 14]. Taking this into account ATRX is a superior and more reliable marker than p53 to identify astrocytic lineage. Recent data suggest the scope for therapeutic applications of ATRX, as these mutations are associated with alternative lengthening of telomeres (ALT) [15]. Tumors with ALT respond to ATR inhibitors, nevertheless, more clinical trials need to be done to confirm it. The frequency of ATRX mutation varies among different studies ranging from 80-100% [16]. Our study showed >90% loss of reactivity in 95.7% of astrocytomas. This was significant with a *p*-value of <0.001. The sensitivity and specificity of ATRX were 95.7% and 90.6% respectively. Only 1 case of gemistocytic astrocytoma had retained expression of ATRX.

In the past decade, various researchers have brought to light the utility of alpha internexin, a proneuronal marker be a surrogate for 1p/19q co-del. There are considerable deletions that occur in oligodendrogliomas such as isolated 1p deletion or partial 19q deletion. However, loss of heterozygosity at 1p/19q is the only one that is clinically relevant because of its favorable outcome. Although it is not possible to replace molecular genetic analysis with FISH, IHC with INA helps in the selection of patients that necessitate genetic testing. Diagnosis of oligodendroglioma mainly depends on the classical histomorphology aided by the presence of 1p/19 co-deletion or INA expression. In cases where histomorphology favors oligodendroglioma with IDH1 positivity, retained ATRX along with INA positivity reflex, molecular testing would not be required. Overall INA positivity was 75% with no significant difference noted between the two grades. Although eight cases did not express INA, they were categorized as oligodendroglial tumors due to their histologic features. A negative correlation with p53 and ATRX was also taken into account before the final diagnosis.

Oligoastrocytoma is a tumor entity falling into the grey zone consisting of both astrocytic as well as oligodendroglial morphology. This overlapping feature in histology is diagnostically challenging and has been attributed to tumor heterogenicity. These mixed pattern diffuse gliomas had been classified as a separate entity until 2016. Following the advent of molecular profiling, these mixed tumors were reclassified into either astrocytic or oligodendroglial lineage based on their genotype. No overlapping of genetic profiles is seen even in the presence of

overlapping phenotypes. Among the various markers used to define the lineage 1p/19q, co-deletion is the most important criterion in the classification of diffuse glioma with a mixed pattern. In our study, five out of six cases of overlapping morphology turned out to be genotypically oligodendrogliomas while one case was astrocytoma.

Various algorithmic approaches have been tested over the past decade to effectively classify diffuse glioma. Our approach utilizes the basic IHC panel to provide necessary molecular information in a resource-limited setup. Five molecular markers used are IDH-1, ATRX, INA, p53, and Ki67. Following our IHC approach grade, II and grade III diffuse glioma could be categorized into 6 groups. Group 1 and Group 4 constituted 72.7% of all cases (n=40). They contain an integrated diagnosis with a comprehensive panel of molecular information and do not necessitate further molecular workup. Tumors that are IDH-1 negative have to be evaluated for other IDH mutations before labeling them as IDH-wild type. Astrocytic and oligodendroglial tumors which are IDH-wild type have a poorer prognosis. Detection of other IDH mutations can be done by IHC for IDH-2 and NGS for rarer mutations. Tumors that are negative for INA have to undergo FISH analysis for 1p/19q codeletion for confirmation. Also, it is recommended that astrocytoma with retained ATRX expression be tested for 1p/19q co-deletion to rule out a possibility of oligodendroglioma.

As neuro-oncology is not part of the curriculum for all neurology residents, it is important to provide an overview of the most important molecular features in glioma including their diagnostic, prognostic, and potential therapeutic roles [17]. Recently two diffuse glioma entities were discovered that have histone 3 (H3) alterations. Both are rare, IDH-wildtype, most often seen in children and young adults, with a prognosis similar to that of glioblastoma, IDH-wildtype [18]. MGMT promotor methylation status is a biomarker of both prognostic and therapeutic significance in glioblastoma, IDH wildtype.

Glioblastoma with MGMT promotor hypermethylation has a better prognosis and a more favorable response to chemotherapy [19]. This MGMT promoter methylation is more commonly seen in recurrent GBM [20] and it shows a better response to Temozolomide (TMZ) therapy [21].

The shortcoming of this study is that it would have been helpful to have done molecular testing for the IDH negative cases by Sanger sequencing.

Conclusion

Since the latest WHO Classification of CNS tumors incorporates molecular information in addition to histology to define tumor entities, it is essential to use economical methods to achieve relevant diagnosis in a resource-limited setup. This study utilized a minimum panel of IHC markers to classify the tumors algorithmically. With this approach, it was possible to arrive at a final diagnosis along with providing molecular information in 72.7% of cases. Although 1p/19q co-deletion plays a pivotal role in classifying oligodendrogliomas, the surrogate marker INA can form the initial tool to stratify the cases that will require molecular testing. Also, these molecular alterations are implicated in prognosis and are a focus of interest in developing targeted therapies that will ultimately improve patient care.

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Disclosure of conflict of interest

None.

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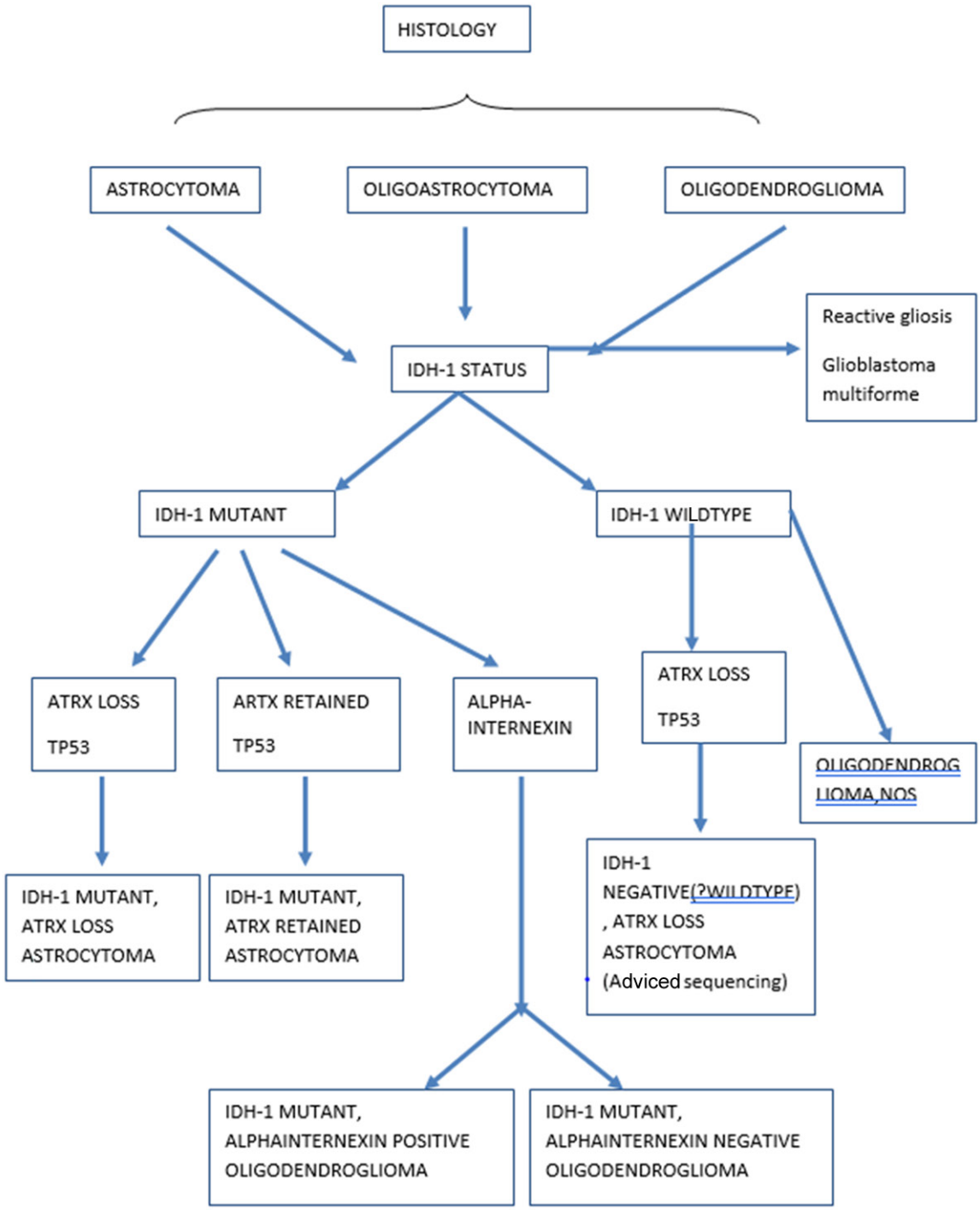

Supplementary Figure 1. Morphological and immuohistochemistry approach to diffuse gliomas.