Evaluation of Immunohistochemical Expression of IDH, ATRX and Ki-67 in the Diagnosis and Classification of CNS Gliomas

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Authors' contributions

This work was carried out in collaboration among all authors. Authors REE and AME contributed to the selection and diagnosis of cases. In addition, they contributed to writing the manuscript, performing histopathological examination, and assessment of the immunohistochemical results. Authors MME, MMM and MAA revised the diagnosis of the cases, interpretations of the markers and manuscript writing. All authors read and approved the final manuscript.

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ABSTRACT

Background: WHO blue book has emphasized on incorporating histopathological as well as molecular basis for accurately diagnosing and risk stratifying cases of brain gliomas. Using surrogate immunohistochemistry may substitute molecular testing especially in low-income countries.

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Aim: this retrospective cohort study was carried out at the Pathology department, Tanta university to evaluate the immunohistochemical expression of IDH, ATRX and KI-67 in diagnosis and classification of brain gliomas.

Materials and methods: Two hundred and fifty (250) formalin-fixed paraffin-embedded tissue blocks from cases with CNS tumors were evaluated for immunohistochemical expression using IDH, ATRX and KI-67.

Results: In cases of diffuse gliomas; tumor types showed significant difference as regard age of patients ($p<0.001$), as well as IDH1 and ATRX immunohistochemical expression ($p<0.001$). The proliferation index of Ki-67 also showed significant difference amongst groups ($p<0.001$). One hundred and ninety-six cases of diffuse gliomas were classified following immunohistochemical staining into 89 *IDH*-mutant tumors (45.4%) and 107 *IDH*-wild type tumors (Glioblastomas) (54.6%). Eighty-nine cases of *IDH*-mutant gliomas were further sub classified based on cell of origin and WHO grade into astrocytomas; grades 2, 3 and 4, as well as oligodendrogliomas; grades 2 and 3. In cases of localized gliomas; No significant data were detected as regard age of affected patients, or tumor location. All cases showed negative staining for IDH1 and retained nuclear expression for ATRX. The proliferation index showed variance amongst tumors ($p<0.001$). A comparison group of patients with reactive gliosis was also studied and showed negative staining for IDH1, retained ATRX expression with low Ki-67 proliferation index.

Conclusions: IDH, ATRX and Ki-67 may be beneficial in categorizing and properly diagnosing patients with brain tumors; which may serve not only as a surrogate for molecular testing, but also can guide therapeutic decisions.

ABBREVIATIONS

ATRX: *Alpha thalassemia/mental retardation syndrome X-linked*
CNS: *central nervous system*
DAB: *diaminobenzidine*
DNA: *deoxy ribonucleic acid*
G: *Grade*
IDH: *isocitrate dehydrogenase*
IHC: *immunohistochemistry/ immunohistochemical*
NOS: *non otherwise specified*
SWI2/SNF2: Switch/Sucrose Non-Fermentable
USA: *United States of America*
WHO: *World Health Organization*

1. INTRODUCTION

The diagnosis of gliomas has entirely relied on their histologic appearance with regard to potential cell of origin and degree of differentiation taking into consideration nuclear atypia, mitotic activity, vascular proliferation, necrosis, proliferative potential, and clinical course, as well as treatment outcome [1]. However, this approach showed significant interobserver variability and lacked precision in determining appropriate prognosis even for patients with the same diagnosis [2]. For the first time, the 2016 revised edition of World Health Organization (WHO) classification of brain tumors used molecular parameters in addition to traditional histology to achieve the so called "layered diagnosis" to diagnose central nervous system (CNS) tumors, resulting in major classification restructuring. Accordingly, nomenclature for selected entities now includes both histopathological diagnosis and defining molecular features [3]. Unfortunately, widespread availability of molecular testing is yet a dream to be achieved, which has led to the quest for alternative means to incorporate molecular testing. The availability and feasibility of the immunohistochemical (IHC) techniques has encouraged finding surrogates for molecular testing, and of the most eminent agents in this prospect are isocitrate dehydrogenase (IDH) and alpha thalassemia/mental retardation syndrome X-linked (ATRX) [4].

The isocitrate dehydrogenase (IDH) family of enzymes catalyzes reactions in the Kreb's cycle and in the cytoplasm. Somatic mutations in genes encoding the 2 isoforms of isocitrate dehydrogenase (IDH1 and IDH2) are present in a variety of tumors. In gliomas, *IDH* mutations were first reported in 2009. They are considered early and common events in the etiopathology of gliomas and are associated with increased DNA methylation [5].

Alpha thalassemia/mental retardation syndrome X-linked (ATRX) is a member

of the Switch/Sucrose Non-Fermentable (SWI2/SNF2) family of Deoxy ribonucleic acid (DNA) helicases that plays a role in chromatin modulation and maintenance of telomeres. Somatic ATRX mutations were first detected in pancreatic neuroendocrine tumors followed by pediatric glioblastomas in 2011 [6].

The present study aimed to study the immunohistochemical expression of IDH, ATRX and KI-67 in available glioma subtypes with assessment of their diagnostic significance; and to evaluate the potential role of such markers in the classification of gliomas.

2. MATERIALS AND METHODS

Patient cohort and tissue processing: 250 formalin-fixed paraffin-embedded tissue blocks from cases with brain tumors were retrieved during the period from January 2019 through December 2021. Approval from research ethics committee was taken antecedent to conduction of study (code 32288/04/18). Clinical data were obtained from requisition sheets enclosed with the specimens or from the final pathology reports including age and sex in addition to location of the tumor. Each paraffin block was re-cut by rotatory microtome at 4-5 microns' thickness, then mounted on glass slides and stained by hematoxylin and eosin for routine histopathological examination; or mounted on charged slides for immunostaining. The routine histopathological examination included assessment of the morphologic appearance of the tumor to identify the histological type and diagnosis as well as determining grade (G) of the lesion. Immunohistochemical staining was carried out using anti IDH (Mouse Monoclonal Primary Antibody clone R132H 132, GenomeMe, BC, CA), ATRX (Mouse Monoclonal Primary Antibody Clone D-5, Santa Cruz biotechnology, Tx, United States of America 'USA') and Ki-67 (Rabbit Monoclonal Primary Antibody Clone 30-9, Ventana, Roche, Az, USA) antibodies. Immunohistochemical staining was performed in an automated staining instrument (Ventana, BenchMark ULTRA). The antibody was detected using a Ventana ultraView Universal (diaminobenzidine) DAB Detection Kit, and hematoxylin was used as counterstain.

Interpretation of immunohistochemistry staining results: IDH positivity was detected as nuclear and cytoplasmic staining [7]. Cases with ≥10% overall positive tumor cells were rated as positive for IDH1 R132H mutation, while cases with less than 10% overall positive tumor cells were rated as negative for such mutation [8]. On the other contrary, positivity for ATRX antibody indicated absence of gene mutation; and detected as strong nuclear staining [9], a threshold of at least 10% of strong positive tumor nuclei was used to assign immunopositivity for ATRX [10]. This negative staining was considered specific if tumor cell nuclei were unstained while nuclei of non-neoplastic cells such as endothelia, microglia, lymphocytes and reactive astrocytes were strongly positive, which were served as internal positive control [11]. The Ki-67 index was calculated as the average percentage of positive nuclei out of the total number of nuclei in high power microscopic fields at 400x magnification [12].

Statistical analysis: was done using SPSS v27 (IBM©, Chicago, IL, USA). The Shapiro-Wilk test was used to evaluate normality of the distribution of data. Quantitative non-parametric data were presented as range and median and were analyzed by Kruskal-Wallis's test. Qualitative variables were presented as frequency and percentage (%) and were analyzed utilizing the Chi-square test. A two tailed P value < 0.05 was considered statistically significant.

3. RESULTS

The study included 250 cases divided into 3 main groups: diffuse gliomas representing 78.4 % (196 cases), localized gliomas representing 19.6% (49 cases) and reactive gliosis representing 2% (5 cases).

Group A: Diffuse gliomas (Fig. 1): Tumors groups and patient demographics: represented in Table 1 and Immunohistochemistry results: represented in Table 2.
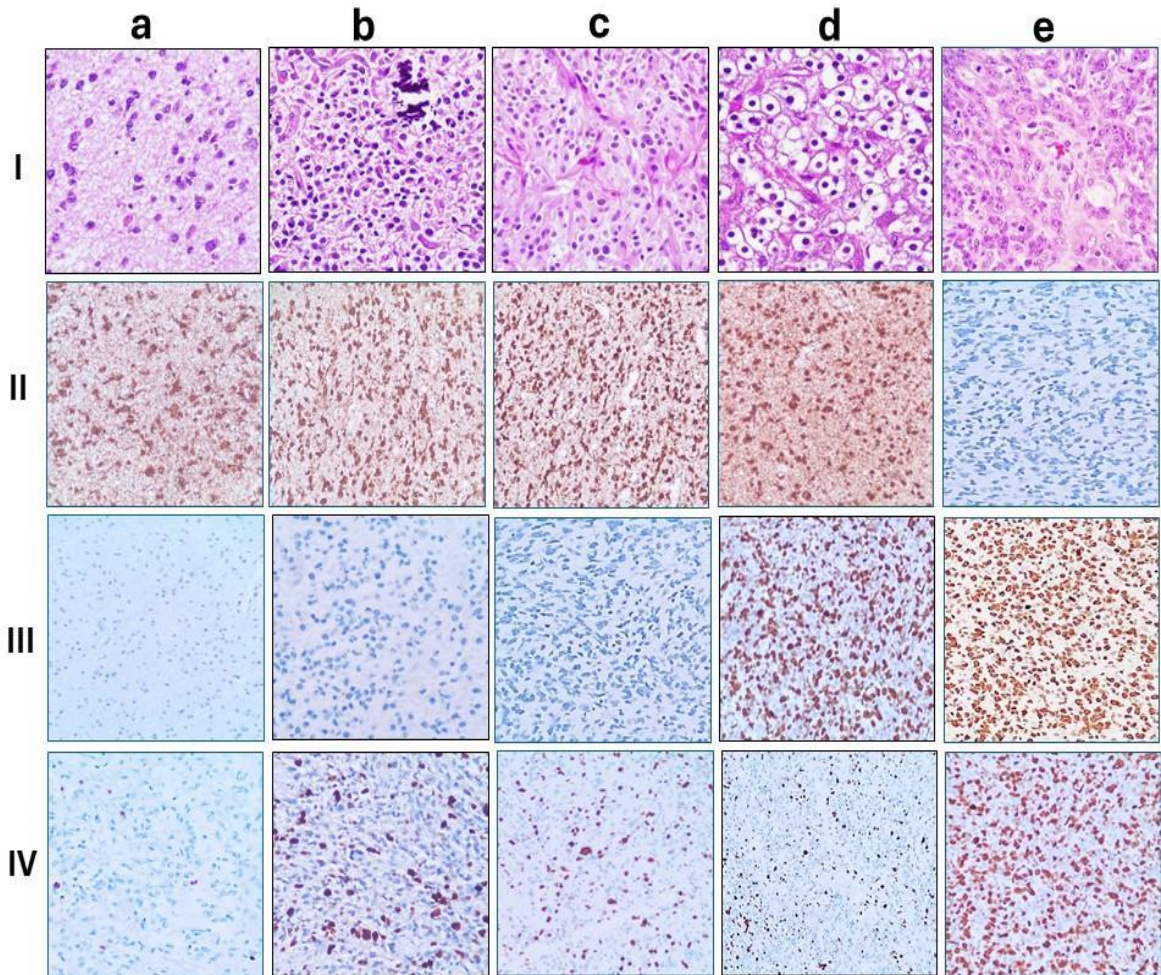
Summary of diffuse gliomas:

- Different tumor types showed significant difference as regard to age of affected patients (p<0.001), as well as difference in IDH1 and ATRX immunohistochemical expression (p<0.001).
- The proliferation index of Ki-67 also showed significant difference amongst groups (p<0.001).
- One hundred and ninety-six cases of diffuse gliomas were classified following immunohistochemical staining into 89 *IDH*-mutant tumors (45.4%) and 107 *IDH*-wild type tumors (Glioblastomas) (54.6%).
- Eighty-nine cases of IDH-mutant gliomas were further subclassified based on cell of

origin and grade into astrocytomas, grades 2, 3 and 4, as well as oligodendrogliomas grades 2 and 3.

Group B: Localized gliomas (Fig. 2): Tumors groups and patient demographics: represented in Table 3, and Immunohistochemistry results: represented in Table 4.

Summary of localized gliomas:

- No significant data were detected as regard to age of affected patients, tumor location or immunohistochemical expression results of IDH and ATRX.
- The proliferation index of Ki-67 showed significant difference amongst different groups (p<0.001).

Fig. 1. Morphology and immunohistochemical results of diffuse gliomas. Astrocytoma WHO grade 2 (column a) shows low cellularity and atypia when compared to astrocytoma grade 3 (column b) and grade 4 (column c) (H&E, x40). Oligodendroglioma (column d) shows the characteristic morphology with "fried egg" appearance and delicate vasculature (H&E, x40). Glioblastoma (column e) shows marked pleomorphism and frequent mitoses (H&E, x40). IDH1 (row II) positive nuclear and cytoplasmic expression is seen in all cases of diffuse gliomas except for glioblastoma (IHC, x20). ATRX nuclear staining (row I) is lost in astrocytoma grades 2, 3 and 4 (IDH-mutant astrocytomas), and retained oligodendroglioma in and glioblastoma (IHC, x20). The proliferation index of Ki-67 (row IV) is low in astrocytoma grade 2, and moderate to high in astrocytoma grade 3, 4, glioblastoma, and in one case of oligodendroglioma grade 3 (column d) (IHC, x20)

Table 1. Clinical data of the diffuse glioma group

| Tumor type and grade	Number and percentage of cases	Age (median and range)	Tumor location
Astrocytoma G2	33 (16.8%)	33 (range 19-72 years)	Frontal 70% Temporal 11% Parietal 7% Posterior fossa 7% Corpus callosum 4%
Astrocytoma G3	30 (15.3%)	41 (range 18-65 years)	Frontal 58% Parietal 16% Temporal 11% Occipital 5% Insular 5% Brain stem 5%
Astrocytoma G4	7 (3.6%)	31 (range 17-61)	Frontal 100%
Oligodendroglioma G2	8 (4.1%)	36 (range 31-42 years)	Frontal 71% Temporal 14% Occipital 14%
Oligodendroglioma G3	11 (5.6%)	42 (range 22-72 years)	Frontal 80% Temporal 20%
Glioblastoma G4	107 (54.6%)	58 (range 11-87)	Frontal 27% Parietal 20% Temporal 18% Spinal 3% Occipital 2% Cerebellar 2% Pons 1% Ventricular 1% N/A 26%
P value	N/A	p<0.001	p=0.39

Table 2. IHC results in the diffuse glioma group

Tumor type and grade	IDH1	ATRX	Ki-67 (Median)
Astrocytoma G2	Positive 100%	Negative 100%	2% (1%-4%)
Astrocytoma G3	Positive 100%	Negative 90%	7% (5%-40%)
Astrocytoma G4	Positive 100%	Negative 100%	10% (5-15%)
Oligodendroglioma G2	Positive 100%	Positive 100%	3% (2%-4%)
Oligodendroglioma G3	Positive 100%	Positive 100%	15% (10%-20%)
Glioblastoma G4	Negative 100%	Positive 98.1%	15% (10%-60%)
P value	p<0.001	p<0.001	p<0.001

Table 3. Clinical data of the localized glioma group

Tumor type and grade	Number and percentage of cases	Age (median)	Tumor location
Pilocytic Astrocytoma G1	10 (20.4%)	7.5 (range 3 to 29 years)	Posterior fossa 50% Ventricular 20% Frontal 10% Midbrain 10% Spinal 10%
Pleomorphic xanthoastrocytoma G2	5 (10.2%)	10 (range 9-27 years)	Temporal 60% Frontal 20% Parietal 20%
Pleomorphic xanthoastrocytoma G3	4 (8.2%)	36.5 (range 28-60 years)	Temporal 75% Parietal 25%
Subependymal giant cell astrocytoma G1	2 (4.1%)	15.5 (range 7-14 years)	Lateral ventricle 100%
Subependymoma G2	3 (6.1%)	34 (range 33-35)	Fourth ventricle 100%
Ependymoma G 2	10 (20.4%)	30.5 (range 3 to 65 years)	Posterior fossa 55.8% Cervical 22.2% Lumbar 11.1% Temporal 11.1%
Ependymoma G 3	11 (22.4%)	31 (range 2 to 64)	Posterior fossa 40% Parietal 20% Frontal 10% Temporal 10% Lumbar 10% Thoracic 10%
Myxopapillary Ependymoma G 2	4 (8.2%)	35 (range 18-48 years)	Lumbosacral 100%
P value	N/A	p=0.54	p=0.08

Table 4. IHC results of the localized glioma group

Tumor type and grade	IDH	ATRX	Ki-67 (Median)
Pilocytic Astrocytoma G1	Negative 100%	Positive 100%	2% (1% to 5%)
Pleomorphic xanthoastrocytoma G2	Negative 100%	Positive 100%	2% (1%-3%)
Pleomorphic xanthoastrocytoma G3	Negative 100%	Positive 100%	15% (10%-30%)
Subependymal giant cell astrocytoma G1	Negative 100%	Positive 100%	2% (1-2%)
Subependymoma G2	Negative 100%	Positive 100%	1% (1-2%)
Ependymoma G 2	Negative 100%	Positive 100%	3% (2%-4%)
Ependymoma G 3	Negative 100%	Positive 100%	25% (10%-40%)
Myxopapillary Ependymoma G 2	Negative 100%	Positive 100%	4% (3%-5%)
P value	N/A	N/A	$p < 0.001$
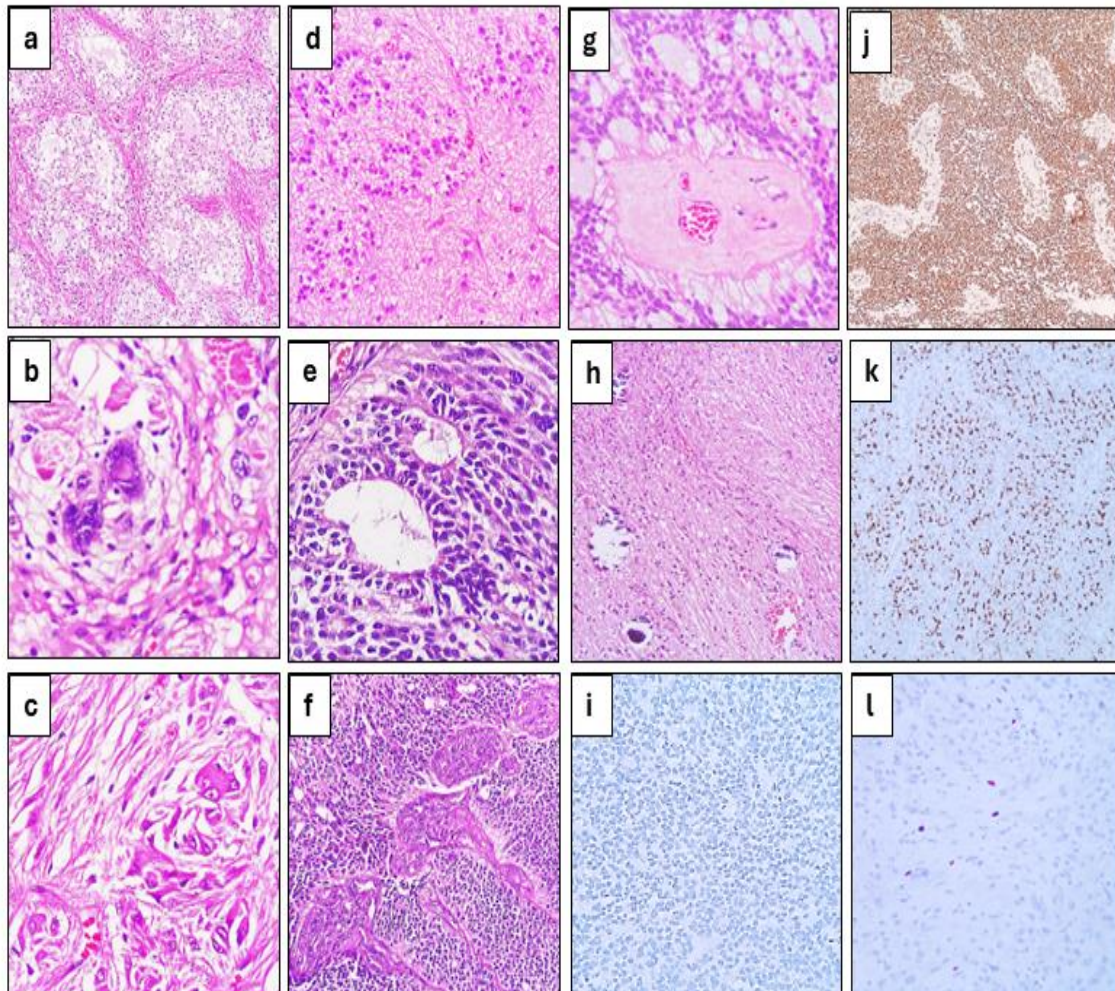
Fig. 2. The morphologic spectrum and immunohistochemical expression observed in localized gliomas and reactive gliosis. Panel (a) pilocytic astrocytoma WHO grade 1 exhibiting the characteristic biphasic pattern with fibrillary and microcystic areas (H&E, x10). Panel (b) high power view from pleomorphic xanthoastrocytoma showing spindle cells and bizarre tumor cells with intranuclear inclusions (H&E, x40). Panel (c) a case of subependymal giant cell astrocytoma showing large and multinucleated tumor giant cells admixed with smaller spindle cells (H&E, x40). Panel (d) displaying a case of subependymoma with monotonous tumor cells arranged in lobulated pattern (H&E, 20). Panel (e) showing the characteristic true ependymal rosettes in which tumor cells are arranged around empty lumina in a case of ependymoma WHO grade 2 (H&E, x40). Panel (f) showing extensive microvascular proliferation in ependymoma, WHO grade 3 (H&E, 20). Panel (g) high power field in a case of myxopapillary ependymoma, WHO grade 2 showing the characteristic arrangement of tumor cells around blood vessels with hyaline and myxoid degeneration of vessel walls (H&E, x40). Panel (h) section from reactive gliosis showing bland astrocytic cells dispersed in fibrillary background (H&E, x10). Panel (i) as shown in this case of ependymoma, all cases of localized glioma and reactive gliosis showed negative expression of IDH1 by immunohistochemistry (IHC, 20). Panel (j) the previous case stained by ATRX showing retained nuclear expression, a staining pattern that was similar to all other cases of localized glioma and reactive gliosis (IHC, x10). Panel (k) showing high proliferation index of Ki-67 inthe previous case, resembling the pattern observed in higher grade lesions in the localized glioma group (IHC, x10). Panel (1) a representative section for low Ki-67 expression, a pattern that was observed in low grade diffuse gliomas in addition to cases of reactive gliosis (IHC, x20)

Group C: Reactive gliosis: Summary of patient demographics, location, underlying etiology and Immunohistochemistry results:

- The study included five cases of reactive gliosis accounting for 2% of the total cases; with median age was 43 years (range 6 to 60 years), and the occipital lobe was more commonly affected (40%).
- Gliosis occurred following infection with subsequent brain abscess formation in 3 cases (60%), while in the remaining 2 cases, reactive gliosis was secondary to infarction.
- All cases showed negative staining for IDH1 by IHC, ATRX staining showed retained nuclear expression, while the proliferation index of Ki-67 was low (ranging from 1% to 3%) (Fig. 2).

4. DISCUSSION

The World Health Organization (WHO) classification of tumors of the CNS is the standard and universally used diagnostic system for the classification of brain tumors. It was originally built on the morphological appearance of tumor cells and their resemblance to normal brain cells, with a grading system based on the outcome of tumors [13]. Studies over the past two decades have clarified the genetic basis of tumorigenesis that may contribute to an enhanced classification of these tumors [3]. In 2014, a meeting held in Haarlem (the Netherlands) under supervision of the International Society of Neuropathology, established guidelines for how to incorporate molecular findings into brain tumor diagnoses, setting the stage for the 2016 CNS WHO classification [14]. However, this approach is still not standardized given unavailability and/or cost constraints of molecular testing; therefore, an economical working formula is essential to reach a meaningful diagnosis especially in resource-limited settings [15]. In this context, the current study was carried out to evaluate IHD1, ATRX and Ki-67 immunohistochemical stains in the diagnosis and classification of gliomas and to determine the potential role of immunohistochemistry as a surrogate for molecular testing.

IDH1 (isocitrate dehydrogenase 1) gene mutations were first identified in a single case of colorectal cancer as part of an early investigation for protein-coding mutations in human cancers [16]. Later on, they were frequently detected in cancers of various origins, including acute myeloid leukemia, cholangiocarcinoma, chondrosarcoma and glioma [17].

In the present study, *IDH1* mutation was detected through surrogate immunohistochemical staining in all cases of diffuse astrocytoma, WHO grades 2 and 3, therefore these cases were assigned the diagnosis of *IDH*-mutant astrocytoma, and these findings match the results of many studies stating that up to 80% of World Health Organization (WHO) classified grade 2 and 3 gliomas harbor the *IDH* disease-defining clonal driver mutations [7,18] .

IDH1 immunohistochemical staining of grade 4 astrocytomas revealed positive staining in only 7 cases (representing 6.1%). These cases were assigned as per the new WHO classification the nomenclature of *IDH*-mutant Astrocytoma, WHO grade 4, whereas the former name "secondary glioblastoma or *IDH*-mutant glioblastoma" is no longer being used [19]. These patients had a median age of 31 years, and all cases showed frontal lobe affection, which is compatible with previous reports that found out that patients with the formerly named "secondary glioblastoma" are usually younger than patients with primary glioblastoma, and that those tumors usually share a frontal lobe location similar to their precursors (astrocytomas, WHO grade 2 and grade 3) [20].

The majority of grade 4 astrocytoma cases (107/114 representing 93.9%) were negative for IDH1 by IHC thus exhibiting the IDH-wildtype phenotype, therefore were diagnosed as *IDH*-wildtype glioblastoma, WHO grade 4, and that corresponded to the general observation in prior literature that the majority glioblastomas (up to 90%) are *IDH*-wildtype tumors (primary glioblastoma) [21]. Similarly, all cases of glioblastoma subtypes (such as epithelioid glioblastoma and giant cell glioblastoma) were negative for *IDH1* mutation by immunohistochemistry which is consistent with previous reports documenting that histologic subtypes of glioblastoma are of *IDH*-wildtype genotype [22,23].

According to Louis et al., 2016 in the WHO blue book, for practical reasons, it is sufficient to rely on the negativity of IDH1 testing by immunohistochemistry alone in older patients (above 54 years) with histologically

classic glioblastoma provided that these patients have no history of a preexisting lower-grade glioma. They state that the designation of "*IDH*-wildtype" can therefore be safely applied in this setting, even in the absence of *IDH* gene sequencing. If we apply that concept to this study, absence of IDH1 staining by immunohistochemistry is sufficient to make the diagnosis of *IDH*-wildtype glioblastoma in 65.4% (70 cases of 107 glioblastoma cases) without the need for additional confirmation by molecular testing. The remaining cases (34.6%) would require confirmation of absence of *IDH* mutation and assessment of other mutations as well.

The incidence of IDH-positive diffuse gliomas in this study was 45.4% (89 cases of 196) emphasizing on the fact that *IDH* mutation evaluation in gliomas has become imperative to identify these tumors especially since secondary malignant transformation is almost inevitable in the many of WHO grade 2 or 3 astrocytomas necessitating close follow up of these patients [24]. Additionally, many of these tumors recur despite neurosurgical resection with adjuvant therapy leading to limited life expectancy of these patients. Therefore, it is of great significance to find new targets for diagnosis and treatment [25]. For all these reasons, *IDH* mutations have become one of the strongest prognostic factors in gliomas and detection of *IDH* mutations can act as a useful predictive biomarker foreseeing chemosensitivity to alkylating chemotherapies [4]. Patients with *IDH*-mutant tumors can also benefit from any *IDH*-targeted treatment modalities such as small-molecule inhibitors, immunotherapy or vaccines that can prevent progression of lower grade tumors to more aggressive forms [26].

Regarding IDH status in oligodendrogliomas, all cases in this study whether grade 2 or 3 tumors showed positive staining for IDH1 indicating presence of *IDH* mutation and acquiring the nomenclature of *IDH*-mutant oligodendroglioma. Findings of this study matched the general agreement of many studies that up to 90% of oligodendrogliomas harbour *IDH* mutations [27]. This situation also highlights another diagnostic value of IDH evaluation; in conditions where the diagnosis of oligodendroglioma is suspected, negativity of IDH testing can be useful in excluding the diagnosis of oligodendroglioma from the start.

On the other hand, all cases of localized gliomas in this study showed negative expression for IDH indicating absence of the *IDH* gene mutation, which goes along with previous literature that reported *IDH*-wildtype phenotype in localized gliomas, with the rarity of *IDH* mutations in these tumors categories [28,29,30].

In many circumstances, the diagnosis of one of the subtypes of localized glioma can be a challenging task, for example, in some cases of pilocytic astrocytoma, the pilocytic differentiation can be subtle and the histological appearance can resemble diffuse astrocytoma, grade 2. In other cases, pilocytic astrocytomas can show marked anaplasia with vascular proliferation and necrosis which are features suggestive of higher-grade glioma. Moreover, pleomorphic xanthoastrocytoma and ependymoma can pose a diagnostic dilemma especially in cases with marked anaplasia, necrosis and overlapping radiological appearance, and in these situations, IDH assessment can have a substantial diagnostic importance and positivity of IDH by immunohistochemistry can reliably exclude the diagnosis of any type of localized glioma and ensure the diagnosis of *IDH*-mutant diffuse glioma. In the same context, it is documented that *IDH* mutation is mutually exclusive with *BRAF* mutation and that it's exceptionally rare for a single tumor to have concomitant *BRAF* and *IDH* mutation emphasizing on the notion that the presence of *IDH* mutation excludes other tumors where *BRAF* mutations are a common occurrence such as pilocytic astrocytoma and pleomorphic xanthoastrocytoma [31]. This concept adds more proof that diffuse and localized gliomas are entirely different on the molecular level even if they share the same cell linage. Also, this highlights the role of *IDH* mutation status detection in predicting the genetic profile of different types of gliomas.

In the present study, IDH1 was negative in all cases of gliosis (3.8%) indicating absence of the mutation and highlighting the reactive nature of these lesions. These situations point to another fundamental diagnostic role for IDH1 assessment by immunohistochemistry in neuropathology which is accurate differentiation between diffuse astrocytoma grade 2 and one of its main histological mimickers (reactive gliosis), and in these situations, positivity for IDH confirms the neoplastic nature of these lesions.

IDH1 staining in the current study have proven tremendous application for this IHC marker which has led us to believe that immunohistochemistry can be used as an available cost-effective

alternative for molecular sequencing in wide range of patients and situations.

Similarly, in many other studies, sensitivity and specificity of IDH immunohistochemistry antibodies reached up to 100%. They recommend performing immunohistochemistry for IDH as an initial step in the diagnostic workup. Molecular sequencing is done as second step only when immunohistochemistry results are negative or equivocal [15] [32,33].

The second investigated IHC stain in this study was ATRX. The alpha thalassemia/mental retardation syndrome X-linked (ATRX) gene was first discovered in 1995 through a study assessing patients with the x-linked mental retardation (MR) syndrome presenting with α-thalassemia, severe psychomotor impairments, urogenital abnormalities, and patterns of characteristic facial dysmorphism [34]. The gene plays a crucial role in normal telomere homeostasis acting as a core component of chromatin remodeling complex and regulates incorporation of histone H3.3 into telomeric chromatin [35]. Loss of function of *ATRX* gene leads to alternative lengthening of telomeres resulting in cellular immortality and tumorigenesis. Mutations in *ATRX* gene have recently been described in at least 15 types of human tumors including neuroblastoma, osteosarcoma, pancreatic neuroendocrine tumors and gliomas [36].

In this study, ATRX showed lost nuclear expression in all diffuse astrocytomas, WHO grades 2 and 4 by immunohistochemistry indicating gene mutation, while grade 3 tumors showed loss of expression in the majority of cases (90%). These results were close to the findings of several other studies that reported high prevalence of *ATRX* mutations in *IDH*-mutant gliomas reaching up to 90% of cases. These studies state that *ATRX* and *IDH* mutations are common co-mutations occurring in a wide spectrum of adult gliomas [27] [37] [38]. On the other hand, 98.1% of *IDH*-wildtype glioblastoma cases showed intact nuclear expression by the antibody indicating absence of *ATRX* gene mutation which is compatible with the previous reports documenting the rarity of *ATRX* mutations in *IDH*-wildt*ype* glioblastomas [39].

Regarding oligodendroglioma all cases showed retained nuclear expression of ATRX indicating absence of gene mutation. A finding that could be explained by the notion that *ATRX* mutation and 1p 19q codeletion is mutually exclusive, meaning that a single tumor cannot harbor both genetic alterations and that *ATRX* mutation is strongly correlated with astrocytic lineage [40].

Even though the recent WHO guidelines necessitate assessment of 1p 19q codeletion status in cases with oligodendroglioma morphology and recommend that cases where molecular testing is not done to be labelled as oligodendroglioma, non-otherwise specified [NOS] [41], some authors rely, however, on the former fact and advocate that *ATRX* mutation excluded by immunohistochemistry can be used as a surrogate for molecular testing for 1p 19q codeletion [42]. In the previous setting ATRX testing will not only influence prognosis but will also act as an important pillar in the diagnostic workup of diffuse gliomas.

By contrast, all cases of localized gliomas in the present study showed preserved nuclear staining by the ATRX antibody indicating absence of the mutation. This finding matches results of other studies that concluded *ATRX* mutation is rarely encountered in pilocytic astrocytoma [9], pleomorphic xanthoastrocytoma [43] and ependymomas [44]. Cases diagnosed with reactive gliosis in this study also showed intact ATRX expression indicating a non-mutated gene. This finding can be explained by the fact that normal human cells as well as cells with reactive changes do not normally harbor *ATRX* mutations.

Similar to IDH1 in this study, and taking into consideration its accuracy and usefulness, IHC for ATRX has been recommended by other authors who also had satisfactory results with detection of *ATRX* mutation status by immunohistochemistry to use the commercially available antibodies as a surrogate for molecular testing [45,46]. These studies reported that ATRX protein expression correlated perfectly with *ATRX* gene mutation, and that ATRX immunoreactivity was associated significantly with histological subtypes and with other key molecular alterations known to occur in diffuse gliomas such as *IDH* mutations [47]. Some of these studies even recommended immunohistochemistry for ATRX as an early step in examining cases of gliomas suggesting that *ATRX* mutation status can help predict the *IDH* phenotype [10].

In general, assessment of *ATRX* mutations in gliomas has become a cornerstone in the diagnostic process. First, it plays a role in reaching the proper diagnosis in many circumstances. Second, tumors with *ATRX* mutations have unique features that drive them to grow faster but respond better to therapies that cause double strand DNA breaks. So, ATRX is an important prognostic indicator and patients with ATRX mutations have better outcome and prolonged survival than patients with ATRX wildtype genotype [48]. Finally, there is growing evidence that *ATRX* mutation can be used for personalized targeted therapeutic agents [49].

The last investigated IHC marker in this study was the proliferation index of Ki-67 which is a potent biomarker. It is the most reliable marker of cell proliferation, and it is commonly routinely performed as a part of the glioma diagnostic work up [50].

To simplify Ki-67 assessment in this study, we followed a common three-tiered expression segregation pattern of low, moderate and high labelling, in which low proliferation indicated expression in less than 4-5% of cells, moderate proliferation ranged from 5-10%, while high proliferation indices indicated that 10% or more of tumor cells showed nuclear expression of Ki-67.

Low grade tumors (grades 1 and 2) in both the diffuse and localized gliomas categories showed low proliferation index. Cases of gliosis also showed low proliferation which was compatible with the reactive nature of these lesions. In all these instances, the low proliferation index has helped to confirm the proper diagnosis and grade as well as avoid overdiagnosis of entities such as pleomorphic xanthoastrocytoma as malignant (given its bizarre morphological appearance). Many cases of diffuse astrocytoma, grade 3 showed moderate proliferation index of Ki-67, while some cases showed higher proliferation reaching up to 40%. Despite the elevated proliferation in these cases, they still didn't show necrosis or microvascular proliferation, so the morphological appearance was more conclusive than Ki-67 index, and the diagnosis was not changed to glioblastoma even after showing such high Ki-67 index.

The vast majority of glioblastoma and glioblastoma subtypes (98% of cases) showed proliferation index of 10% or higher in tumor cells. This finding closely correlates with the general understanding that higher levels of Ki-67 correlate with higher tumor grades [51].

Establishing a Ki-67 cut-off value to differentiate low- and high-grade lesions in the CNS has been the topic of interest in prior literature, and many studies have concluded a 10% positivity in tumor nuclei as a clinically relevant cut point for differentiation [52] [53]. In the present study, only 11.8% of grade 3 and 4 cases across the diffuse and localized glioma categories showed proliferation index of less than 10%, in keeping with the previous observation.

Although the grading process of gliomas relies primarily on histological appearance; in some instances, this approach can be difficult and even inaccurate. An example of such situations is small biopsy specimens which is a commonly encountered problem in the practice of neuropathology, and in such cases, depending on morphological appearance alone can be misleading. Another commonly encountered scenario is inadequate sampling of heterogenous tumors. For instance, a non-representative biopsy can fail to sample an area of necrosis leading to underdiagnosis of a case of glioblastoma as a lower grade astrocytoma. The value of Ki-67 staining in these cases is of utmost importance. High proliferation index in a small biopsy accompanied by the picture of radiologically aggressive looking tumors can help suggesting the diagnosis of higher-grade glioma in the setting of morphologically unequivocal tumors. The value of Ki-67 assessment for the prognostication process of gliomas is an agreed-upon fact. A very small subset of studies suggested that higher Ki-67 values in gliomas correlated with better survival [53]. On the other hand, the vast majority of studies indicated that higher values of Ki-67 correlated with higher tumor grades as well as worse overall survival. These studies reported that Ki-67 is an independent prognostic factor in glioma. Some studies attributed this to the ability of a cell to continue to proliferate after the time of tumor resection leading to higher incidences of recurrence [11] [54].

The results of one large metanalysis published in 2015 analyzed all the prior research related to assessment of Ki-67 in gliomas has also reached similar conclusion that linked higher Ki-67 levels with worse patient outcome and concluded that evaluating Ki-67 index by immunohistochemistry is reliable and can be used as a potential predictive factor in the prognosis of gliomas [55,56].

5. CONCLUSION

- In a resource-limited setup where molecular testing is lacking, immunohistochemical staining is a useful, affordable and widely available option.
- Staining brain tumors for IDH, ATRX and Ki-67 is beneficial in categorizing and diagnosing patients with brain tumors, which not only serves as the basis of therapeutic decisions, but may also help to predict outcome of these patients.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

DATA AVAILABILITY

The datasets used and/or analyzed during this study are available from the corresponding author upon request.

ETHICS APPROVAL AND CONSENT

The current study was conducted after obtaining the approval from research ethics committee, Faculty of Medicine, Tanta University, Egypt (32288/04/18). Informed written consent was obtained form involved participants in the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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