Application of EGFR, Ki 67 and GFAP as a panel for the diagnosis and grading of astrocytomas

Aliaa Atef and Mohamed El-Rashidy
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I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

Mohamed L. Salem,
Editor in Chief
Application of EGFR, ki 67 and GFAP as a panel for the diagnosis and grading of astrocytomas

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ABSTRACT

Background: Astrocytic tumors are the most common primary central nervous system tumors. WHO grading scheme is based on the presence or absence of the following four histological parameters using haematoxylin and eosin (H&E) staining: cellular atypia (WHO grade II), mitotic figures (WHO grade III), endothelial proliferation, and/or necrosis (WHO grade IV). Immunohistochemistry (IHC) has become an essential tool in diagnosis of brain tumors and helps in predicting the prognosis of certain brain including astrocytomas. Aim: Study the immunohistochemical expressions of (Epidermal growth factor receptor (EGFR), Ki67 and Glial fibrillary acidic protein (GFAP)) in different grades of astrocytomas.

Materials and Methods: This study included 44 cases of astrocytomas classified as: 3 pilocytic astrocytomas, 6 diffuse low grade astrocytomas, 15 anaplastic astrocytomas and 20 glioblastoma multiformes. Immunohistochemical staining of all cases using EGFR, Ki67 and GFAP was done.

Results: EGFR showed positive membranous and cytoplasmic expression in 33 (75%) cases, GFAP positive staining was detected in 38 (86.4%) of studied cases while all astrocytomas of different grades showed ki67 positivity with variable degrees with the mean ± SEM of Ki67 LI 17.76% ± 2.46. There was statistically significant relation between EGFR, Ki 67 and GFAP expression and tumor grade.

Conclusions: EGFR, Ki 67 and GFAP should be used in combination as a panel side by side with established histological criteria of malignancy and WHO grading system for accurate diagnosis and grading of astrocytic tumors.

INTRODUCTION

Central nervous system tumors (CNS) constitute 1-2% of adult neoplasms. According to cell type, WHO (2016) classification divided gliomas into astrocytomas, oligodendrogliomas, and ependymomas. Astrocytomas are the most common primary central nervous system tumors. According to the 2016 WHO classification, the astrocytic neoplasms were grouped into two major categories; diffusely invasive astrocytoma (diffuse astrocytoma, anaplastic astrocytoma, glioblastoma) and the relatively more circumscribed tumors (pilocytic, pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma and subependymal giant cell astrocytoma). The neuropathological grading of infiltrating astrocytomas consisted of a three grades (II, III and IV) while grade I represents non-infiltrating astrocytomas (Louis et al., 2016). WHO grading scheme is based on the presence or absence of four histological parameters: depending on the assessment of features such as cellular atypia (WHO grade II), mitotic figures (WHO grade III), endothelial proliferation, and/or necrosis (WHO grade IV), while WHO grade I showing only increased cellular proliferation with lacking of all the previously mentioned criteria. All these histological parameters can be detected by haematoxylin and eosin (H&E) staining (Popova et al., 2014). Based on morphological criteria, this classification remains unsatisfactory with variable rates of diagnostic pitfalls. Therefore, a well-founded diagnostic evaluation has become a necessity for security of the upcoming management and therapy decisions (Belghali et al., 2017).

Immunohistochemistry (IHC) has been essential in the diagnosis of brain tumors. Despite the
conventional H&E staining is the mainstay for histopathologic diagnosis, IHC played a great role in differential diagnosis and in improving the diagnostic accuracy of CNS tumors. The application of a panel of selected immunostains is very helpful in diagnostically challenging cases. IHC was also found to be of great help in predicting the prognosis of certain brain tumors including astrocytomas (Goyal et al, 2015).

Many growth factors and their receptors play vital roles in cell division, proliferation and differentiation. These receptors are expressed on the surface of cancer cells. One of these receptors is the epidermal growth factor receptor (EGFR), which is the first member of ErbB family of receptor tyrosine kinases (RTK) important in cell development and proliferation. EGFR is expressed in a large number of tumor types, including brain tumors. EGFR can be one of the promising molecular immunohistochemical markers for the prognosis and grading of astrocytomas (Carvalho et al., 2014). The EGFR protein has an extracellular ligand-binding domain, a transmembrane region and an intracellular domain with intrinsic protein-tyrosine kinase activity. Ligand binding of the EGFR activates the EGFR tyrosine kinase which phosphorylates proteins in the signal transduction pathway that leads to activation of genes that regulate cell proliferation, angiogenesis, motility, invasion and metastasis (Abdulghani et al., 2019).

The proliferative index is a potent biological factor that estimates the growth of neoplasms quantitatively and thus will aid in predicting the prognosis of different tumors. One of the most potent methods for assessing the proliferative index in brain tumors is the Ki-67 labeling index (Ki-67 LI). Ki-67 is a nuclear antigen expressed in the G1, S, G2, and M phases of the cell cycle but absent in the resting phase and its expression is assessed using Ki-67 LI which represents the percentage of Ki-67 positive cells (Das et al., 2018).

In addition to detection and measurement of the proliferative activity of astrocytomas by EGFR and Ki67 respectively, Glial fibrillary acidic protein (GFAP) can be applied along with the previous two markers for assessing the prognosis and diagnosis of astrocytic tumors. GFAP is one of the major cytoplasmic intermediate filaments and is the main cytoskeletal component of astrocytes. GFAP is widely known as a diagnostic marker for astrocytic tumors with its expression seen in normal, reactive, and neoplastic astrocytes. Most of the astrocytic tumors show GFAP positivity except protoplasmic astrocytoma (WHO Grade II) where GFAP immunoreactivity is either scanty or absent (Jaiswal, 2016).

This work aimed to study the immunohistochemical expressions of EGFR, Ki67 and GFAP in different grades of astrocytoma to assess the cellular proliferation degree and the cell lineage of studied cases and to correlate their expressions with the grade and other clinical and histopathological parameters to explore the prognostic value of these markers.

**MATERIALS AND METHODS**

This study was carried out retrospectively on 44 brain biopsies diagnosed as astrocytoma divided into 4 grades according to WHO grading (2016): 3 cases of pilocytic astrocytomas (Grade I), 6 diffuse low grade astrocytoma (Grade II), 15 anaplastic astrocytomas (Grade III) and 20 glioblastoma multiforms (Grade IV) (Louis et al., 2016). Samples were collected as formalin-fixed, paraffin-embedded tissue blocks, with H&E stained slides from the archives of the pathology department of faculty of medicine, Tanta University where this study took place) and private labs (36 cases) or received as fresh specimens (8 cases) during the period between February 2019 and July 2020. This study was approved by the research ethics committee of faculty of medicine of Tanta university.

**Immunohistochemistry**

It was performed using the immunoperoxidase method on 4µ-thick sections from the paraffin-embedded blocks. Tissue sections were deparaffinized in xylene and rehydrated in descending grades of alcohol (100, 95, 85 and 75% ethanol) and blocked with 3% hydrogen peroxide for 15 min at room temperature. Antigen retrieval was performed after heating in citrate buffer at 98°C for 10 min. Pretreated sections were incubated with rabbit polyclonal EGFR antibody (1:200, Thermo Scientific, Egypt
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Catalog # PA1-1110, Ki67 a rat monoclonal antibody (1:100, Thermo Scientific, Egypt, Catalog # 13-5698-82) and GFAP a mouse monoclonal antibody (1µg/ml, Thermo Scientific, Egypt, Catalog # MA5-12023) was also applied to the sections at 4°C overnight and incubated with secondary antibody (HRP-Rabbit/Mouse) for 30 min at 37°C. The signal was detected with 3,3'-diaminobenzidine solution. Using a light microscope. As a negative control, a section was processed in which the primary antibody was changed by PBS. Immunohistochemical staining was evaluated independently by two pathologists.

Interpretation of immunohistochemical Staining

Every immunohistochemically stained slide was scanned and the fields that reflect the best of the overall immunostaining were chosen and captured using light microscope attached with an imaging system (Lieca DM 2000, Lieca Microscopy and Scientific Instruments Group Germany).

EGFR positive cells showed membranous and / or cytoplasmic staining. A positive stain is indicated by a golden brown precipitate at the site of specific cellular antigen localization. Immunohistochemical staining for EGFR was graded according to percentage of positive tumor cells as follows: 0 (no cell stained), +1 (< 5% tumor cells), +2 (5- 50% cells), and +3 (> 50% cells). For statistical analysis, a score of 0 and 1 were considered negative and a score of 2 or 3 was considered positive (Abdulghani et al., 2019).

Ki67 positively stained cells showed brown diffuse or granular nuclear reactivity. Immunostaining was evaluated at the low-power examination. Fields from the area of maximal positivity were chosen for counting. In glioblastomas, areas free from necrosis or capillary endothelial proliferation were selected. The Ki-67 LI represents the number of Ki-67 positive tumor nuclei expressed as a percentage of a total number of tumor nuclei counted using high-power magnification (x400). A total of at least 1000 tumor nuclei were counted in each case. The mean of Ki67 LI was tabulated (Das et al., 2018).

GFAP positivity was qualitatively assessed by the intensity of the immunostaining: 0 (negative), +1 (weak staining), +2 (moderate staining) and +3 (intense staining) (Belghali et al., 2017).

Statistical analysis

Statistical analysis was done using statistical package for the social sciences (SPSS). The analysis of the relation between markers expression and the clinicopathological characteristics was performed using the Kruskal Wallis test, Fisher’s exact test or qui square (x2) test with Yates continuity correction. P-value < 0.05 was considered statistically significant.

RESULTS

The age of patients at the time of surgery ranged between 8 and 82 years; the mean age was 53.8 years. Gender distribution showed slight male predominance with 25 males and 19 females. Cases were divided into low grade (LG) astrocytomas (3 cases of pilocytic astrocytomas (Grade I) and 6 diffuse low grade astrocytoma (Grade II); and high grade (HG) astrocytoma (15 anaplastic astrocytomas (Grade III) and 20 glioblastoma multiforms (grade IV) (Fig. 1,2,3). Regarding tumor site, 22 cases were frontal, 8 cases were parietal, 5 cases were temporal while 9 cases were occipital.

Immunohistochemical staining results of EGFR

EGFR showed positive membranous and cytoplasmic expression in 33 (75%) cases out of 44 studied cases including: 4 LG astrocytomas (1 case of grade I and 3 cases of grade II) (Fig. 4); and 29 HG astrocytomas (11 cases of grade III astrocytoma and 18 cases of grade IV) (Fig. 5) (Table 1). The relation between the immunohistochemical staining results of EGFR and the clinicopathological characters were summerized in Table (2). There was a statistically significant relation between EGFR expression and tumor grade where EGFR expression increased with higher grades (p= 0.021). Also the percentage of staining was significantly increased in the group of HG astrocytomas than in LG astrocytomas (p=0.03).

Immunohistochemical staining results of Ki67

All astrocytomas of different grades showed ki67 positivity with variable degrees. The mean
Immunohistochemical staining results of GFAP

GFAP positive cytoplasmic expression was variable according to the tumor grade. Positive staining was detected in 38 cases (86.4%) of studied cases. Areas of necrosis in glioblastomas showed no immunostaining with the palisaded cells showing positive staining. The percentage of GFAP expression was higher in LG astrocytomas (all cases of grade I and grade II (100%) (Fig. 8); than in HG astrocytomas (13 cases (86.7%) of grade III astrocytoma and 16 cases (80%) of grade IV) (Fig. 9). The intensity of GFAP expression was inversely related to tumor grade with decreased intensity in higher grades ($p=0.015$) (Table 4). The relation between the immunohistochemical staining results of GFAP and the clinicopathological characters were summarized in Table (5). There was a statistically significant inverse relation between GFAP expression and tumor grade where GFAP expression decreased with higher grades ($p=0.026$).

DISCUSSION

Astrocytic tumors are the most common primary tumors of the brain that have rapid progression and invasiveness. The current WHO classification of astrocytic tumors has its limitations in predicting prognosis and diagnosis, hence the need for additional factors.

In spite of the advances in the prognosis, diagnosis and treatment modalities, the prognosis of these tumor patients remains poor. Therefore, in order to identify accurate prognostic factors for astrocytomas and evaluate the effectiveness of appropriate treatment, a proper investigation of the molecular mechanisms and carcinocinogenesis of these tumors with the discovery of new molecular markers has become mandatory (Hu et al., 2013).

In the present study, we intended to assess the efficacy of EGFR, Ki67 and GFAP in diagnosis and prognosis of astrocytomas especially their ability to detect the grade of astrocytoma side by side with the WHO grading system.

EGFR serves as an important and well-characterized mitogenic factor in several ectodermal tissues, including glial cells. EGFR activation is involved in cell differentiation, proliferation, and migration (Burel-Vandenbos et al., 2011). In our study, EGFR showed positive cytoplasmic and/or membranous expression in 75% of studied astrocytomas with statistically significant relation between EGFR expression and tumor grade. EGFR expression increased with higher grades ($p=0.021$) with significantly increased intensity of staining in HG astrocytomas ($p=0.03$).

Maiti et al. (2008) results were in approval with ours. They stated that the percentage of EGFR positive cells was 2.17 +/- 0.475 in Grade II astrocytoma, 12.63 +/- 1.79 in Grade III astrocytoma and 22.86 +/- 1.792 in glioblastoma. As well, a significant increase in EGFR percentage of expression from lower to higher grades was also detected. In 2013, Hu et al., similarly noted that EGFR expression was associated with glioma grade.

Table 1. The immunohistochemical staining results of EGFR in studied cases:

<table>
<thead>
<tr>
<th>Astrocytoma</th>
<th>Immunohistochemical expression of EGFR</th>
<th>$\chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Negative)</td>
<td>+1 (Negative)</td>
<td>+2 (Positive)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Low grade (n=9)</td>
<td>Grade I (n=3)</td>
<td>2</td>
<td>66.6%</td>
</tr>
<tr>
<td></td>
<td>Grade II (n=6)</td>
<td>2</td>
<td>33.3%</td>
</tr>
<tr>
<td>High grade (n=35)</td>
<td>Grade III (n=15)</td>
<td>1</td>
<td>6.7%</td>
</tr>
<tr>
<td></td>
<td>Grade IV (n=20)</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Statistically significant (P<0.05). $\chi^2$: Chi-square test.
EGFR, Ki67 and GFAP in astrocytomas...

Table 2. The association between EGFR expression and the clinicopathological characters of studied cases:

<table>
<thead>
<tr>
<th>Variables</th>
<th>Negative (0&amp;+1)</th>
<th>Positive (+2&amp;+3)</th>
<th>X²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;50 (20)</td>
<td>N = 11</td>
<td>6 14</td>
<td>0.5</td>
<td>0.47</td>
</tr>
<tr>
<td>&gt;50 (24)</td>
<td></td>
<td>5 19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex Male (25)</td>
<td>N = 4</td>
<td>7 18</td>
<td>1.66</td>
<td>0.19</td>
</tr>
<tr>
<td>Female (19)</td>
<td></td>
<td>4 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal (22)</td>
<td>N = 8</td>
<td>8 14</td>
<td>4.3</td>
<td>0.20</td>
</tr>
<tr>
<td>Parietal (8)</td>
<td></td>
<td>2 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temporal (5)</td>
<td>N = 0</td>
<td>0 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occipital (9)</td>
<td>N = 1</td>
<td>1 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I (3)</td>
<td>N = 2</td>
<td>2 1</td>
<td>5.3</td>
<td>0.021*</td>
</tr>
<tr>
<td>Grade II (6)</td>
<td></td>
<td>3 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade III (15)</td>
<td></td>
<td>4 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade IV (20)</td>
<td>N = 2</td>
<td>2 18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant (P<0.05). χ²: Chi-square test

Table 3. The association between Ki67 LI and the clinicopathological characters of studied cases.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Ki67 LI (Mean ± SEM)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt;50 (20)</td>
<td>17.25% ± 2.36</td>
<td>0.91</td>
</tr>
<tr>
<td>&gt;50 (24)</td>
<td>18.47% ± 2.38</td>
<td></td>
</tr>
<tr>
<td>Sex Male (25)</td>
<td>18.03% ± 3.10</td>
<td>0.90</td>
</tr>
<tr>
<td>Female (19)</td>
<td>17.46% ± 3.97</td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td>Frontal (22)</td>
<td>17.62% ± 4.07</td>
<td></td>
</tr>
<tr>
<td>Parietal (8)</td>
<td>17.14% ± 3.52</td>
<td></td>
</tr>
<tr>
<td>Temporal (5)</td>
<td>18.40% ± 3.65</td>
<td></td>
</tr>
<tr>
<td>Occipital (9)</td>
<td>17.65% ± 4.83</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td>&gt;0.001*</td>
</tr>
<tr>
<td>Grade I (3)</td>
<td>1.42% ± 0.64</td>
<td></td>
</tr>
<tr>
<td>Grade II (6)</td>
<td>6.75% ± 1.26</td>
<td></td>
</tr>
<tr>
<td>Grade III (15)</td>
<td></td>
<td>23.56% ± 2.25</td>
</tr>
<tr>
<td>Grade IV (20)</td>
<td>38.63% ± 3.45</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant (P<0.05). SEM: Standard error of the mean.

EGFR positive staining was observed in 22.2% of grade I, 44.6% of grade II, 71.2% of grade III and 88.6% of grade IV. EGFR positivity was significantly higher in the high grade gliomas (III and IV) than in the low grade gliomas (P=0.021). They added as well that marked EGFR staining, +2 or +3 was found in 40, 78.4 and 90.3% of grade II, III and IV gliomas respectively.

Similarly, Popova et al. (2014), They noticed that EGFR positivity was noted in all studied glioma subtypes with the percentage of EGFR positive cells different significantly, from a few scattered immunoreactive cells to staining almost all tumor cells. In contrast with our results, they noticed that despite 45% of low-grade gliomas, 57% of high-grade gliomas and 58% of GBMs were positive, strong EGFR expression was detected independently from WHO grades.

Another study by Carvalho et al. (2014), noticed cytoplasmic expression of EGFR protein in 75% of astrocytomas, and 24% of the astrocytomas showed nuclear localization. They explained EGF nuclear expression by the translocation of the EGFR protein from the cell surface to the nucleus, as other ErbB family member proteins, by endocytosis which was attributed to its different roles in cell proliferation, tumor progression, DNA repair, and replication.

Few other studies showed different results. An early study by Agosti et al. in 1992, observed EGFR positivity in 37% of glioblastomas while pilocytic astrocytoma grade I, grade II and anaplastic astrocytoma grade III were EGFR negative. Later in 2009, Guillaudeau et al. also found frequent EGFR expression in glioblastomas with no staining in GI, GII and GIII tumors.

Table 4. The immunohistochemical staining results of GFAP in studied cases

<table>
<thead>
<tr>
<th>Astrocytoma</th>
<th>Immunohistochemical expression of GFAP</th>
<th>X²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (Negative)</td>
<td>+1 (Weak)</td>
<td>+2 (Moderate)</td>
</tr>
<tr>
<td>Low grade (n=9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I (n=3)</td>
<td>0 0%</td>
<td>0 0%</td>
<td>1 33.3%</td>
</tr>
<tr>
<td>Grade II (n=6)</td>
<td>0 0%</td>
<td>0 0%</td>
<td>3 50%</td>
</tr>
<tr>
<td>High grade (n=35)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade III (n=15)</td>
<td>2 13.3%</td>
<td>4 26.6%</td>
<td>6 39.9%</td>
</tr>
<tr>
<td>Grade IV (n=20)</td>
<td>4 20%</td>
<td>10 50%</td>
<td>5 25%</td>
</tr>
</tbody>
</table>

*Statistically significant (P<0.05). χ²: Chi-square test.
Table 5. The association between GFAP expression and the clinicopathological characters of studied cases:

<table>
<thead>
<tr>
<th>Variables</th>
<th>Negative GFAP N = 6</th>
<th>Positive GFAP N = 38</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 (20)</td>
<td>2</td>
<td>18</td>
<td>3.9</td>
<td>0.28</td>
</tr>
<tr>
<td>&gt;50 (24)</td>
<td>4</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (25)</td>
<td>1</td>
<td>24</td>
<td>0.24</td>
<td>0.62</td>
</tr>
<tr>
<td>Female (19)</td>
<td>5</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frontal (22)</td>
<td>2</td>
<td>20</td>
<td>0.19</td>
<td>0.64</td>
</tr>
<tr>
<td>Parietal (8)</td>
<td>0</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temporal (5)</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occipital (9)</td>
<td>3</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I (3)</td>
<td>0</td>
<td>3</td>
<td>4.93</td>
<td>0.026*</td>
</tr>
<tr>
<td>Grade II (6)</td>
<td>0</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade III (15)</td>
<td>2</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade IV (20)</td>
<td>4</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant (P<0.05). $\chi^2$: Chi-square test.

Recently in 2019, Abdulghani et al. similarly discovered positive EGFR expression in 15.9% of grade IV astrocytoma whereas all cases of grade I, II, III were negative.

These divergent IHC results can be due to different applied antibodies in different studies, differences in tissue processing and interobserver discrepancies in the subjective assessment of results (Marquez et al., 2004).

On the other hand, one of the well-known immunohistochemical methods for assessing the proliferation rate is Ki67 LI, though previous studies that used it in grading gliomas and astrocytomas have shown conflicting results. The heterogeneity in Ki-67 LI between different specimens and different fields of the same specimen, made the average values of Ki67 LI more representative of the proliferative potential of the tumor more than the maximal Ki67 value (Chaloob et al., 2012).

The present study showed that all different grades of astrocytomas showed ki67 positivity with Ki67 LI 17.76% ± 2.46 with a range of 0% to 70%. In the main time, there was a statistically significant relation between Ki67 LI and tumor grade where Ki67 LI , and in turn tumor proliferation, increased with higher grades ($p>0.001$). There was no significant statistical relation between Ki67 LI and age, sex or site of astrocytoma.
In approval with our results, Das et al. (2018) found the mean Ki67 LI increasing with increased tumor grade, in Grade I astrocytomas was 4.66, in Grade II astrocytomas was 8.07, in Grade III astrocytomas was 13.5, in Grade IV astrocytomas was 22.93. Also, there was a highly significant relation between the histopathological grade of astrocytomas and Ki67 LI (p<0.05). This was close to the results found earlier in 2006, by Johnnessen et al. They reviewed the results of previous 16 studies. These studies showed an increase in Ki67 LI with the increasing tumor grade with a statistically significant difference between the indices of low grade II and high-grade tumors (grade III and IV) (P<0.05), but not between grade III and grade IV tumors (P>0.05).

In 2014, Thotakura et al. as well, stated that Ki67 LI increased with the tumor grade with significant difference between all grades of astrocytoma except between Grade I and Grade II tumors. On studying Ki67 expression in gliomas, both Hu et al. (2013) and Belghali et al. (2017) noted that Ki-67 is more expressed in the high grade gliomas with a very significant difference between the various glioma grades.

Interestingly, Shivaprasad et al. (2017) noticed that the mean Ki67 LI in Grades I, II, III, and IV was 0.02, 0.81, 9.14, and 17.81, respectively with a statistically significant difference between Ki67 LI of LG (Grade II) and HG astrocytomas (Grades III and IV). They also observed significant increase in Ki67 LI in 90% of high grade astrocytomas and non-significant increase in the remaining 10% of cases. Worth mentioning that there was an overlap of Ki67 LI between the different grades as the values of the glioblastomas could be as low as Grade II
tumors, indicating that Ki-67 LI cannot be used alone as a diagnostic measure.

These conflicted results can be attributed to the different fixatives used, immunohistochemical procedures, especially antigen retrieval, and variable interpretation of the immunostaining results. The low Ki67 LI value in high-grade astrocytoma could be the result of inadequate tissue sampling or using computer-assisted methods for assessment of Ki67 LI that may underestimate LI compared to manual methods (Das et al., 2018).

An accurate diagnosis of a neoplasm is a crucial factor in prognosis as well as in predicting response to therapy. Hundreds of antibodies have been suggested as potential diagnostic markers for gliomas including astrocytomas, but only a handful have turned out to be reliable. The most effective was GFAP for glial differentiation and demonstrating tumor astrocytic lineage (Paulus, 2009).

In this study, GFAP showed cytoplasmic expression 86.4% of studied cases with areas of necrosis in glioblastomas showing no immunostaining and the palisading cells showing positive staining. GFAP expression was higher in LG astrocytomas than in HG astrocytomas. The intensity of GFAP expression was inversely related to tumor grade with decreased intensity in higher grades \( (p=0.015) \). A statistically significant inverse relation between GFAP expression and tumor grade where GFAP expression decreased with higher grades \( (p=0.026) \).

Goyal et al. (2015) approved with our results by demonstrating that 81% of their studied glial tumors were widely reactive for GFAP. The staining pattern of GFAP in high grade astrocytomas was more variable. More anaplastic areas of the tumors showed less positivity. They suggested that GFAP immunostain helped in the objective assessment of the degree of differentiation in astrocytomas as astrocytic nature of the tumor was confirmed by demonstration of GFAP in less anaplastic foci. They added that positive GFAP staining helped overcoming histopathological diagnostic dilemmas in some undifferentiated tumors and to categorizing these tumors as glioblastomas.

Similarly, Belghali et al. (2017) showed that GFAP staining was variable according to the type and the grade of the tumor. They also noticed a lack of expression in almost half of the cases and that GFAP expression was relatively strong in the low grade compared with the high grade (31% and 6%, respectively) though, the difference was not statistically significant \( (p = 0.149) \).

A wider scale of positivity was observed earlier by Haah et al. in 1991. They noticed positive GFAP staining in all astrocytic tumor cases. Furthermore, they suggested that immunostaining patterns of glioblastomas is different from that of astrocytoma grade II and III. Compared to the more differentiated astrocytomas, grade II, grade III, grade IV showed focally unstained foci implying the decrease of staining with the decrease of degree of differentiation.

Many other studies as well noted GFAP positivity in all studied astrocytic tumors (Cosgrove et al., 1993, Oh et al., 1999 and Goswami et al., 2007). Recently in 2019, van Bodegraven et al. found that GFAP positive cells were present in all grades of malignancy with decreased GFAP levels with increasing astrocytoma grade. Interestingly, in their study, a significant correlation was not consistently found which may be attributed to intra- and inter-tumor heterogeneity of GFAP positive cell localization, morphology, and GFAP variants.

The variability of results between our study and some of the previously mentioned ones can be explained by the heterogeneity of GFAP expression within the same tumor and the application of different staining protocols (Jaiswal, 2016).

Because of these discrepancies and the small sample size in this study, we recommend further studies with larger number of studied cases to emphasize on the role of these markers in the grading and prognosis of astrocytic tumors. Also, the relation between the expression of these markers and disease recurrence and disease free survival are needed to be investigated for their great effect on the prognosis.
CONCLUSION
Assessment of the expression of EGFR, Ki67 and GFAP is sufficient for diagnosis and grading of astrocytomas, and can be recommended for application in clinical practice. Due to a greater spread of values of Ki67 between the various grades of astrocytoma and the heterogenicity of EGFR and GFAP expression within tumor tissue, these markers should be used in combination as a panel side by side with established criteria of histological malignancy and WHO grading system for accurate diagnosis and grading with subsequent close association to prediction of astrocytic tumors prognosis. Further studies should be carried out on larger scales for more confirmation of our results and approving the validity of this panel.

CONFLICTS OF INTEREST
All authors declared no conflicts of interest.

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No fund was received for this work.

REFERENCES


Egyptian Association for Cancer Research (EACR)
http://eacr.tanta.edu.eg/

EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbjournals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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