Original Article

# Glial Fibrillary Acidic Protein, CD34, Ki-67, and p53 Immunohistochemistry Expression Study to Estimate the Concordance between the Morphology and the Awarded Grades of the Brain Gliomas

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## Abstract

**Background:** The 2007 WHO grading system of gliomas recognizes four prognostic grades of the latter. Based exclusively on morphological patterns, this classification remains unsatisfactory with rates of diagnostic conflicts between pathologists ranging from 20% to 50%, particularly between Grades II and III. Misestimating the grade implies impertinent care decisions. We evaluated the concordance between grades and morphological characteristics of our series. **Methods:** Our study is a retrospective covering 3 years and wherein the biopsies of 32 formalin-fixed paraffin-embedded brain gliomas were explored. The histopathological diagnosis had been revised, and all cases were stained by immunohistochemical (IHC) technique with glial fibrillary acidic protein (GFAP), Ki-67, p53, and CD34 tumor markers. Comparisons were made between the grades and the IHC results and between the latter and the morphological characteristics of the studied gliomas. We used Kolmogorov–Smirnov test for normal distribution, Chi-square test of Pearson and Fisher test for qualitative variables, Mann–Whitney U-test, and Kruskal–Wallis test for continuous variables. The significance is retained for $P < 0.05$. **Results:** GFAP is expressed in all of the 32 studied tumors. Our markers are more expressive in high-grade gliomas (GFAP [$P = 0.149$], Ki-67 [$P = 0.001$], p53 [$P = 0.012$], and CD34 [$P = 0,004$]). Labeling index increases with the cellular density (Ki-67 [$P = 0.001$], p53 [$P = 0.031$]) and with the mitotic activity (Ki-67 [$P = 0.001$], p53 [$P = 0.056$]). CD34 is more expressive in the presence of endothelial-capillary proliferation ($P = 0.02$), of palisading necrosis ($P = 0.015$), and of nonpalisading necrosis ($P = 0.076$). Ki-67 is more specific and more sensitive than p53 ($P < 0.001$). The value of ideal expression of Ki-67 proposed for our sample is 8.5% ($P < 0.001$). It allows 93.7% of specificity and 75% of sensibility and separates the low grade (I, II) of the high grade (III, IV). **Conclusion:** The results prove the concordance of the established grades and gliomas morphology of our series.

**Keywords:** *CD34, concordance, glial fibrillary acidic protein, gliomas, grade, immunohistochemistry, Ki-67, morphology, p53*

## Introduction

Gliomas are tumors which arise from glial cells and correspond to the cells which gave them birth. They come in four categories: astrocytomas, oligodendrogliomas (ODGs), oligoastrocytomas, and ependymomas. Gliomas are the most frequent primitive tumors of the central nervous system (CNS). The anatomopathological examination of CNS tumors is decisive for the management of patients. According to the 2007 WHO recommendations,[1] the grading system of gliomas recognizes four prognostic grades for them. We distinguish four grades of malignancy: I, II (low-grade gliomas [LGGs]), III and IV (high-grade gliomas [HGGs]).[1] The diagnosis of glioblastomas (Grade VI) and Grade I gliomas is practically easy. However, the classification of other gliomas is much more difficult. Based only on morphological criteria (mitotic activity, cellular density, cytonuclear atypia, necrosis, and endothelial-capillary proliferation [ECP]), this classification remains unsatisfactory with rates of diagnostic conflicts between anatomopathologists varying from 20% to 50%, in particular in Grades II and III.[2]

A well-founded diagnostic evaluation means more security of the upcoming steps. The place of the anatomopathological examination is, then, essential, and the interest of the immunohistochemistry (IHC)

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in the histopathological orientation of the diagnosis as well as in the grading prognosis can no longer be demonstrated. Many IHC markers can be used in this perspective. In fact, glial fibrillary acidic protein (GFAP) is a lineage marker and has a diagnostic value.[3] The Ki-67 is a nuclear antigen whose expression is associated with the cellular proliferation and correlated with the grade.[4] Another useful marker to determine prognosis is the nuclear phosphoprotein p53 existing in cells in low content, generally below the threshold of detection of the immunohistochemical methods, because of its short half-life. Its IHC expression is considered positive when at least 10% of the tumoral cells present an intense nuclear marking. This expression is correlated to the grade.[4] Finally, the studies show that the ECP is increased with the progress of the glioma in the grade. This proliferation is revealed in IHC by the antibody anti-CD34, a transmembrane glycoprotein expressed in the vascular endothelium.[2] These IHC markers are particularly useful in cases where the histology reveals an LGG, whereas other parameters (clinical or radiological, etc.) indicate the opposite.

Misestimating the grade implies impertinent care decisions.[5] Thus, we studied the IHC expression of the tumoral markers GFAP, Ki-67, p53, and CD34 allowing, respectively, to rule on the cells lineage, cellular proliferation degree, TP53 gene mutations, and t ECP which are determining parameters in the statement of the glioma grade. The IHC results are confronted, at the same time, with the already established grades as well as with the morphological data having guided to their establishment. It is a way of verifying the degree of concordance between gliomas grades and morphological characteristics of the gliomas of our series.

## Methods

It is a retrospective study covering the period from January 2012 to December 2014. It explored 32 cases of gliomas biopsies of Moroccan patients. These biopsies were fixed in the formalin and included in the paraffin wax. The study took place in the Pathological Anatomy Department. The cases are randomly chosen at the rate of eight cases per grade.

The documentary analysis of the anatomopathological examination reports of the 32 chosen cases allowed us to collect the clinico-histopathological parameters. Blocks containing the tumor are split up into 5 μm slices by the Leica RM2255 microtome. Four blades (one per marker) were made to be used in IHC techniques.

The primary antibodies*:* GFAP Clones 6F2, CD34 ClasII Clone QBEnd10, Ki-67 Clone MIB-1, and p53 Clone DO-7 (Dako) were, respectively, applied on blades intended for the marking of the GFAP, CD34, Ki-67, and p53. The IHC procedure is realized using the automaton Dako-Link®. For the negative control, the primary antibody is omitted. The positive internal control is considered for GFAP and CD34 blades, colon IHC examination for Ki-67, and skin IHC examination for p53. Blades are dehydrated on alcohol baths, cleared up in toluene baths, and mounted afterward.

The Ki-67 and p53 expressions are quantitatively estimated by counting the percentage of marked cells in ten randomly chosen fields with strong magnification. The definitive percentage is the average of the percentages of these ten fields. Each brown tagged point is considered positive, without taking into account the intensity of the color. The percentage of colored cells is recorded as labeling index (Ki-67-LI) and labeling index (p53-LI), respectively. Endothelial cells are tagged by the CD34, and it is the ECP that is looked for under an aspect of endothelial focal glomerule-like groupings. Blades describing ECP are considered positive. For the GFAP, we appreciated qualitatively the intensity of the immunolabeling (weak labeling [$GFAP^{+}$], moderate labeling [$GFAP^{++}$], and intense labeling [$GFAP^{+++}$]). The analysis of immunolabeling was carried out in a blind way by the above-mentioned pathologist.

## Results

Our sample contains the four types of cerebral gliomas described by the WHO with 59.4% of astrocytomas, 9.4% of ODGs, 15.6% of oligoastrocytomes, and 15.6% of ependymomas. Fifty percent of the cases of our sample come from patients under 40. Sex-ratio of our patients is 1.13. Samples reach the Pathological Anatomy Department generally in the form of operating pieces (68.8%) or exeresis-biopsies (25%) and rarely in the form of simple biopsies (6.3%). 50% of the cases are sustentorial.

Our results showed that the immunoreaction in the GFAP is variable according to the type and the grade [Figure 1]. We also noticed a lack of expression in almost half of the cases and that the expression $GFAP^{+++}$ is considerably strong in the low grade compared with the high grade (31% and 6%, respectively). However, this difference is not statistically significant ($P = 0.149$). Concerning CD34, results showed that it is more expressed in the HGGs, ($P = 0.004$) with 80% of cases, whereas it is only 24.5% in the cases for the LGGs [Figure 2]. For both p53 and Ki-67, we observe

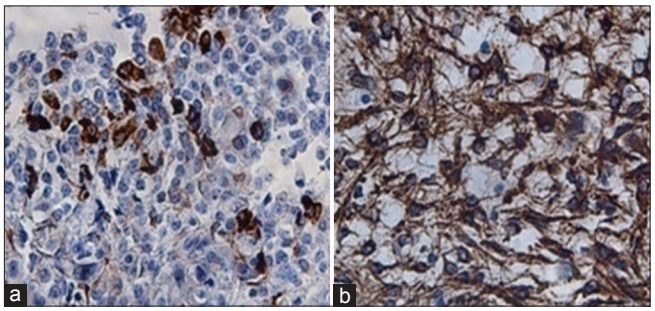



**Figure 1: Microphotography of slices showing glial fibrillary acidic protein immunoreactivity. (a) Weak tagging in anaplastic ependymoma III, (×400). (b) Intense tagging in pilocytic astrocytoma I (×400)**

that they are differently expressed in the studied cases [Figures 3 and 4]. It was noticed that Ki-67 is more expressed in the HGG with a very significant difference ($P < 0.001$). The same tendency is observed for p53 ($P = 0.012$) [Table 1].

The study of IHC results and the corresponding gliomas morphology showed that the CD34 is more expressed in the presence of the palisading necrosis, ($P = 0.015$), with positivity in 100% of the cases showing this morphological aspect. We notice that the same tendency for the nonpalisading necrosis, which is accompanied by a bigger expression of this marker, has a result that is close to the statistical meaning ($P = 0.076$). The CD34 is also more expressed in the presence of the ECP, ($P = 0.02$). Ki-67 is more expressed in the cases where the cellular density is higher ($P < 0.001$) [Table 2]. The same tendency is noticed for p53 ($P = 0.031$). Besides, Ki-67 is more expressed when the mitotic activity goes higher ($P < 0.001$). The same tendency is observed for p53 with a difference of averages close to the statistical meaning ($P = 0.056$) [Table 2]. Concerning the relation between IHC results with the cytonuclear atypia, the differences of average expressions are not statistically significant neither for Ki-67 ($P = 0.64$) nor for p53 ($P = 0.30$) [Table 2].

The confrontation of our markers' expressions showed that Ki-67-LI is higher in CD34-positive cases ($P = 0.007$) [Table 3]. The same tendency is observed for p53 ($P = 0.034$) [Table 3]. The test of correlation of Spearman allowed us to notice that p53-LI and Ki-67-LI are correlated ($P = 0.004$). The regression study between our markers showed that Ki-67 is the most reliable ($P = 0.033$), followed by CD34 ($P = 0.114$), GFAP ($P = 0.335$) then comes p53 ($P = 0.984$). Finally, the receiver operating characteristic (ROC) curve [Figure 5] showed that the area under the curve of Ki-67 ($P < 0.001$) is more important than that of p53 ($P = 0.013$), which asserts that the Ki-67 examination is more specific and more sensitive for gliomas.

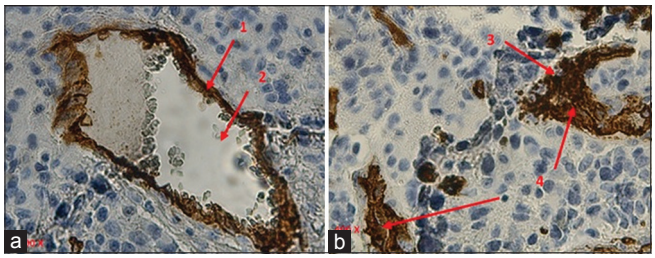



**Figure 2: Microphotography of slices showing CD34 immunoreactivity. (a) No endothelial-capillary proliferation in pilocytic astrocytoma I. 1: Fine vascular wall, 2: Free vessel lumen. (b) Endothelial-capillary proliferation in glioblastoma multiforme. 3: Vascular endothelium proliferation, 4: Blocked vessel lumen**

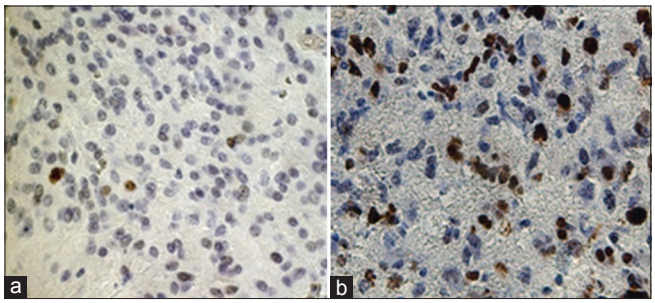



**Figure 3: Microphotography of slices showing p53 tagging immunoreactivity. (a) Weak expression (<1%) in pilocytic astrocytoma I (×400). (b) Very strong expression (>50%) in glioblastoma multiforme IV (×400)**

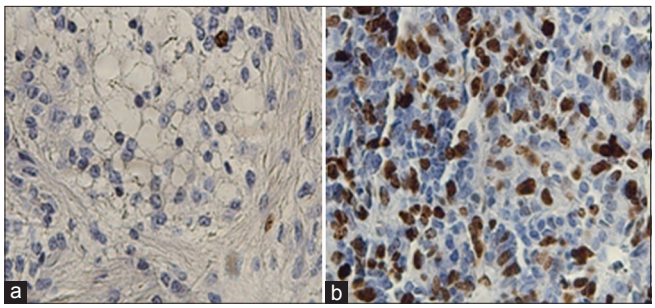



**Figure 4: Microphotography of slices showing Ki-67 tagging immunoreactivity. (a) Weak expression (<1%) in pilocytic astrocytoma I (×400). (b) Very strong expression (>50%) in glioblastoma multiforme IV**

**Table 1: Ki-67-labeling index and p53-labeling index according to glioma grade**

| | Ki-67-LI (%) | p53-LI (%) |
|---|---|---|
| Low grade | 2,88±2,3 | 3,38±18,7 |
| High grade | 18,81±13,9 | 15,19±9,9 |

LI: Labeling index

**Table 2: Ki-67-labeling index and p53-labeling index according to variations of histological variations**

| | Ki-67-LI (%) | p53-LI (%) |
|---|---|---|
| Cellular density | | |
| Weak | 2.00±0.8 | 0.25±0.50 |
| Moderate | 3.17±2.6 | 4.42±11.33 |
| Moderate to high | 4.00±2.6 | 2.00±2.64 |
| High | 22.23±13.1 | 18.23±19.56 |
| Mitotic activity | | |
| Absent | 2.00±1.3 | 5.00±13.30 |
| Weak | 6.00±8.2 | 3.00±3.20 |
| Moderate | 14.00±5.6 | 15.00±19.10 |
| High | 32.00±14.8 | 24.00±23.00 |
| Nuclear atypia | | |
| Absent | 10.00±13.8 | 1.00±1.00 |
| Present | 11.00±12.8 | 11.00±16.70 |

LI: Labeling index

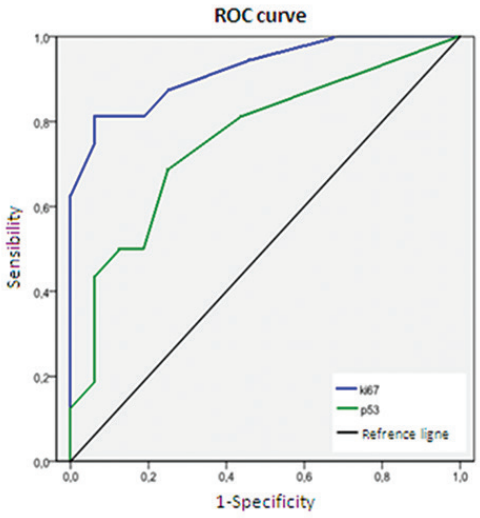
**Table 3: Ki-67-labeling index and p53-labeling index according to CD34 expression**

| | Ki-67-LI (%) | p53-LI (%) |
|---|---|---|
| $CD34^-$ | 7±12.1 | 3±9.6 |
| $CD34^+$ | 15±12.1 | 16±19.1 |

LI: Labeling index

**Figure 5: Receiver operating characteristic curve for Ki-67-labeling index and p53-labeling index**

| Variables | Zone | *P* | CI 95% asymptotic | |
|---|---|---|---|---|
| | | | Inf limit | Sup limit |
| Ki-67 | 0.922 | 0.000 | 0.830 | 1.000 |
| p53 | 0.758 | 0.013 | 0.587 | 0.928 |




a. in nonparametric hypothesis

| Curve coordinates | | | |
|---|---|---|---|
| Variables | Positive if ≥ than a | Sensibility | 1-specificity |
| Ki-67 | 0.00 | 1.000 | 1.000 |
| | 1.50 | 1.000 | 0.688 |
| | 2.50 | 0.938 | 0.438 |
| | 3.50 | 0.875 | 0.250 |
| | 4.50 | 0.813 | 0.188 |
| | 6.00 | 0.813 | 0.063 |
| | 8.50 | 0.750 | 0.063 |
| | 12.50 | 0.625 | 0.000 |
| | 17.50 | 0.438 | 0.000 |
| | 25.00 | 0.313 | 0.000 |
| | 35.00 | 0.125 | 0.000 |
| | 45.00 | 0.063 | 0.000 |
| | 51.00 | 0.000 | 0.000 |
| p53 | −1.00 | 1.000 | 1.000 |
| | 0.50 | 0.813 | 0.438 |
| | 1.50 | 0.688 | 0.250 |
| | 3.00 | 0.500 | 0.188 |
| | 4.50 | 0.500 | 0.125 |
| | 7.50 | 0.438 | 0.063 |
| | 15.00 | 0.375 | 0.063 |
| | 25.00 | 0.313 | 0.063 |
| | 35.00 | 0.188 | 0.063 |
| | 45.00 | 0.125 | 0.000 |
| | 51.00 | 0.000 | 0.000 |

b. null hypothesis/: Real zone = 0.5

CI: Confidence interval, ROC: Receiver operating characteristic

## Discussion

GFAP is expressed in the 32 studied tumors. It is a filamentous intracytoplasmic protein that is part of the glial cell cytosquelette. GFAP is present in astrocytes, oligodendrocytes,[6] and ependymocytes.[7] Besides, the GFAP labeling in ODGs shows an astrocytic reactional gliosis to this type of aggression and takes the aspect of checkerboard.[8] Concerning the mixed gliomas, the GFAP expression is systematically found in the astroglial component, compared with the oligodendroglial tumor cells.[1] GFAP is weakly expressed in 47% of the cases of our sample. Moreover, its strong expression ($GFAP^{+++}$) nullifies in the Grade IV. Certainly, this result is not statistically significant. However, it mimes the described tendency in the literature. Indeed, when astrocytes become malignant, a progressive loss of the production of GFAP is observed.[9] Nevertheless, the GFAP expression remains highly variable in the glioblastoma multiformes (GBMs) according to the differentiation of cancer cells since once the glial cells become undifferentiated, they produce no more GFAP.[3]

It is noticed that the Ki-67-LI increases in a statistically significant way with the grade. This result aligns with the data of the literature [Table 4].

This is explained by the relation between the Ki-67 expression and the cell proliferation which is related, in its turn to prognosis and thus to the grade. It is necessary to note that there are possible overlapping values between the diffuse astrocytomas and those of the GBM[16] and that the multiform GBM shows a wide regional variation.[16] Concerning the cellular density, it was observed that the Ki-67-LI correlates to the latter. A very significant difference is observed between every level of cellular density and the higher level which follows it. Indeed, the highest expressions of Ki-67 indicating a stronger cellular proliferation were associated with a more serious prognosis.[17] In addition, the analysis of a vast range of astrocytomas showed a clear correlation of proliferation (thus of density) with the clinical result.[18] In the same way, it was noticed that the Ki-67-LI is higher in the cases showing more important mitotic activity. This is explained by the presence of the protein Ki-67 during all the active phases of the cellular cycle, whereas it is absent during the G0 phase. Indeed, this protein is an excellent marker to determine the increasing proportion of the studied cellular population.[19] The small difference of the Ki-67-LI in the presence of cytonuclear atypias (11% ± 12.8%) and in their absence (10% ± 13.8%) was not statistically decisive ($P = 0.64$). However, it is necessary to underline that in numerous types of tumors, the strong expression of Ki-67 reflecting a high mitotic activity is accompanied with abnormal mitosis leading to an uneven separation

**Table 4: Ki-67-labeling index according to gliomas grades**

| Gliomas | Ki-67-LI (%) | Reference |
|---|---|---|
| Pilocytic astrocytoma I | <1 | [10] |
| Astrocytoma II | 3.8 | [11] |
| Astrocytoma III | 18.4 | [11] |
| Astrocytoma IV | 31.6 | [11] |
| Oligodendroglioma II | <5 | [7] |
| Oligodendroglioma III | >5 | [30] |
| Oligoastrocytoma II | 6 | [26] |
| Anaplastic oligoastrocytoma III | >10 | [14] |
| Ependymoma I | 1 | [15] |
| Ependymoma II | 3.6 | [15] |
| Ependymoma III | 9.5 | [15] |
| Multiform GBM | 20 | [8] |

GBM: Glioblastoma multiforme, LI: Labeling index

of chromosomes between the two new cells. This results in micronucleus cell and a macronucleus one with a conversely nucleo-cytoplasmic report.[20]

The difference between the p53-LI averages between the low grade and the high grade in our series proved to be significant. The p53 expression increases with the grade. In fact, the functional status of p53 is connected with the prognosis and with the progress of certain cancers.[21] Our results show an increase of p53-LI with the mitotic activity and with the cellular density. The obtained difference between p53-LI compared with the mitotic activity levels is close to the statistical significance ($P = 0.056$), while it is significant concerning p53-LI compared with the cellular density. The same result was reported by Kucuk *et al*.[4] showing a good correlation between p53 expression, mitotic activity, and cellular density.

The angiogenesis is an essential process in malignant tumor development. The intratumoral microvascular density is usually used in the evaluation of the angiogenesis. In our study, we scrutinized exclusively the ECP tagged by the CD34 which is a relevant sign of malignancy.[22] We have noticed that the CD34 tagging the ECP is more expressed in HGG. Indeed, this proliferation is generally seen in brain tumors,[23] essentially the most aggressive ones associated with an unfavorable prognosis.[24] A significant correlation was described between the CD34 expression and the astrocytomas clinicopathological characteristics.[25]

Besides, we noticed that CD34 is more expressed in the presence of the palisading necrosis. This result, close to the statistical meaning ($P = 0.076$), is in accordance with the literature where the zones of necrosis are present in the anaplastic astrocytoma III, the anaplastic ODG III, and the anaplastic oligoastrocytoma III.[1] These gliomas are all known for presenting a palisading necrosis.[22] On the other hand, the same authors suggested that the ECP (marked by CD34) would develop under the influence of angiogenic growth factors in answer to the hypoxico-ischemic infringements. In the GBMs, this proliferation is known to border the zones of necrosis.[26]

To verify the deductions, we established basing on chosen marker; we confronted them two by two. We ended up making a regression test of these markers where we noticed more Ki-67 expression in $CD34^+$ gliomas. As a matter of fact, the proliferation marker Ki-67 shows an important increase of the proliferative activity of the endotheliocapillar cells proliferation tagged by CD34.[26] Besides, it was noticed that p53 is more expressed in $CD34^+$ gliomas. It is suggested that the expression of the angiogenic antigen (CD34) was stimulated by the accumulation of p53 which is consecutive to the p53 gene mutation.[27] The mutated gene p53 can stimulate the vascular endothelial growth factor by the activation of the protein kinase C synthesis as well as the wild gene p53 that can settle the production of thrombospondin 1, angiogenesis inhibitor.[28] Our sample showed a strong correlation between the p53 expression and that of Ki-67 ($r = 0.96$). This result is in perfect agreement with the work of Sirvent *et al.,*[29] who described that the p53 expression evolves in the same sense as that of Ki-67. The p53 mutation can reflect a greater proliferation that is itself marked by Ki-67.[30]

The ROC curve shows that Ki-67 is more specific and sensitive than the p53. This summarizes that the capacity of Ki-67 to discriminate between HGG and LGG is bigger than p53. For Ki-67, the value of ideal expression proposed for our sample is 8.5% for Ki-67 allowing 93.7% of specificity (diagnosis), and 75% of sensibility (screening). Indeed, the threshold from which the forecast is modified varies according to the studies between 3% and 8%; however, the prognosis keeps getting worse with the increase of this index.[31] In the case of p53, the ideal expression value proposed for our sample is 7.5% allowing 93.7% of specificity (diagnosis), and 43.8% of sensibility (screening). However, the immunolabeling p53 is considered positive when at least 10% of the tumoral cells present an intense nuclear labeling.[2] These two proposed values separate the LGG (I and II) of the HGG (III and IV) and allow reconciling between sensibility and specificity of the tests.

The regression study between our markers showed that Ki-67 is the most susceptible to direct the grade. In fact, the presence of >6 mitoses by ten fields of strong magnification leads to a diagnosis of anaplasia.[32] The evaluation of the mitotic activity must be completed by the study of the index of the K-67-LI, particularly in Grade II and III gliomas.[2]

## Conclusion

At the end of the statistical tests which were made between the grades and the IHC results, on the one hand, and between the latter and the morphological characteristics of the studied gliomas, on the other hand, we noticed

significant relations on both sides except for the GFAP which did not show link with the grade. Knowing that the grade about which we speak was established on the basis of the morphological characteristics compared with the IHC results, we conclude that the already attributed grades suit the studied gliomas morphology.

The comparisons between the expressions of our markers are also statistically significant and concord along with the literature data. This gives us more confidence regarding the previous deductions and proves the validity of our IHC technique.

The specificity-sensibility test allowed us to conclude that Ki-67 is the antibody of choice for the screening (sensibility) as well as for the diagnosis (specificity) of gliomas and to propose a cutoff value of 8.5% marked cells to classify a glioma as being of Grade III.

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### Conflicts of interest

There are no conflicts of interest.

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