Original Paper

Immunohistochemical Expression of Ki 67 &CD31 in Iraqi Patients with Astrocytomas, Clinicopathological Studies.

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Abstract

Background: Astrocytomas are the most common primary tumor of the central nervous system, the diagnosis absolutely based on histology has a high interobserver variations and remains problematic even for an experienced neurological pathologist.
The objective of this study: To assess the immunohistochemical expression of Ki67 as proliferative markers and CD31 as an endothelial cell marker in astrocytomas interrelated with some clinicopathological parameters (age, gender, site of the tumor, and tumor grade) in Iraqi patients.
Materials and methods: In this retrospective study, 41 formaline–fixed paraffin –embedded tissue blocks represent cases of Astrocytomas, The histopathologic diagnosis had been revised and all cases were stained by immunohistochemical technique with Ki 67 & CD 31 antibodies and assessed independently by three pathologists. Values were considered statistically significant when $p<0.05$.
Results: Fibrillary astrocytoma (WHO grade II) was found to be the most common type among astrocytic tumors with the peak age incidence in the second and sixth decades of life, and a slight male predominance had been identified. Parietal location of tumor consider most common site ,There was a significant correlation between the age of the patients and the grade of the tumor on one side & Ki-67 labeling indix, and microvessel density (MVD) detected by CD31 on other side ($p<0.05$). While no significant correlation with the site and gender.
Conclusion: A significant correlation was found between Ki67 labeling indices, and MVD (microvessel density) in one side, and the clinicopathological variables of astrocytomas in other side, Could be used as ancillary methods in the differentiation of borderline grades of astrocytomas.

Keywords: Astrocytoma, immunohistochemistry Ki‑67, immunohistochemistry CD 31.

Introduction

Astrocytic tumors are the most frequent primary tumors of the central nervous system[(1)]. The yearly occurrence of CNS tumors spans from 10 to 17 per 100,000 persons for intracranial tumors and 1 to 2 per 100,000 persons for intraspinal tumors, In Iraq, CNS malignant tumors are the fourth most common ten malignant tumors[(2)]. Various grading systems are used to grade astrocytomas. The most commonly used system is the World Health Organization (WHO) classification (1979, 1993, 2000,2007 and 2016) that grades astrocytomas (I-IV) build on cytological atypia, mitotic activity, vascular proliferation, and necrosis: pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma (grade IV) [(3)]. With an important consideration for the 2016 WHO classification was the implementation of genotypic analysis in addition to the phenotypic patterns in diagnosis of astrocytic tumors[(4)].
This Histological grading is consider as gold standard for making the diagnosis of

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astrocytoma and thus estimation the prognosis of these tumors. However, the morphologic criteria are not always reliable prognostic indicators of individual case & the current classification is not optimal due to several parameters like tumor heterogeneity, sampling errors, poor reproducibility, and those tumors with shared histology but ,can be different genetically [5]. Thus, an additional markers are needed to enhance the diagnostic and prognostic validity. Measurement of proliferative activity is important in dectating the grade, recurrence range, and malignancy of astrocytoma [6, 7].

Ki-67 is a nuclear marker used to demonstrate the proliferative phase of cell cycle ,the Ki‑67 labeling index (Ki67 LI) is one of the most potent methods for estimation the proliferative index of central nervous system[8], It serves as an important supplemental implement in the diagnostic and prognostic evaluations of human astrocytomas. Ki-67 alone cannot be used as a diagnostic factor but should be used in synergy with traditional criteria of histological malignancy, due to a greater spread of values between the various grades of astrocytoma. Thus, immunohisto-chemical evaluation may prove to be a vital supplementary tool for diagnostic and prognostic purposes.[9]

Angiogenesis and tumor cell invasion play a critical role in astrocytoma development and growth [10]. And it is a feature of a more aggressive tumor such as a glioblastoma multiforme, and histopathologic methods based on the number of vessels, degree of glomeruloid vascular formation [11]. Microvascular density analysis with panendothelial staining techniques has been used to quantify angiogenesis and has been found to act as an independent predictor of prognosis [12,13]. MVD is increased with the advancement of the pathological grade of astrocytoma. Significant differences of MVD were found among astrocytomas of different grades. Thus, high MVD may be a be partly responsible for the invasive growth of astrocytoma[14]. This study is designed to assess the immunohistochemical of Ki 67 &CD 31in correlation with the World Health Organization (WHO) histological grades of astrocytomas & with some clinicopathological parameters to predict the biological behavior, which may give a guidance for using Ki‑67 & CD 31 as adjuncts to the routine histology in grading astrocytomas precisely & help in prediction the prognosis of these tumors.

Materials and methods

In this retrospective study during the period from January 2016 to November 2017, formaline –fixed paraffin –embedded tissue blocks with initial diagnosis of Astrocytomas were collected from the archived materials in the histopathology department of Gazy Al Hariri teaching hospital for surgical specializations at Bagdad ,These parraffin blocks represent (41) cases of excisional brain biopsies, diagnosed as astorocytoma including 7 cases of pilocytic astrocytomas,14 cases of diffuse fibrillary astrocytomas,8 cases of anaplastic astrocytomas & 12 cases of glioblastomas. Clinicopathological parameters such as age, gender patient, site of tumor and histological/morphological grades were obtained from the available histopathologic reports.

Three sections of 5 Mm thickness were taken from each blocks, the first one was stained by hematoxylin and eosin (H&E) for histopathologic estimation, the other two sections were put in a positively charged slide and stained immunohistochemically for ki 67 & CD31. The H&E stained sections were reestimate for the histologic grades of the tumor by three pathologists. For recognition of CD 31 we used monoclonal mouse antibody (clone JC70A) manufactured by DAKO with a dilusion of (2 ug/ml), sections from the soft tissue mass diagnosed as hemangioma and known to be immunoreactive for CD 31 was used as a positive control, while the recognition of ki

67 is performed by the application of a commercially available ki67 immunohistochemical kit (Clone MIB-1, mouse monoclonal antibody, dilusion 1:75-1:50, Dako), A section from tonsil regarded as a positive control, The negative control slides for both markers were obtained by omitting the primary antibody. All sections were stained using an autostainer equipment (X biogenex i6000), In order to do immunohistochemical staining bimanually on each block, 5 micrometer sections were made and applied the section on a positive charge dewaxing paraffin-embedded sections. After antigen retrieval was accomplished, the tissue sections were covered with peroxide block solution for 5 minutes followed by incubation of Primary antibody for 30 minutes, then the tissue sections covered with PolyExcel poly HRP and incubate for 30 minutes at room temperature, working solution and incubate for 5 minutes at room temperature, and finally Hematoxyline stain was applied Immunohistochemical stained sections were evaluated under a light microscope independently by three pathologists using a microscope (leica DM750).

Pictures taken in this study using the camera (Leica Icc 50E), The results described as Positive immunohisto-chemical staining for Ki-67 is brown nuclear with a diffuse pattern. Ki-67 expression was evaluated semi-quantitatively. It was scored by counting at least 1000 cells in 10 high-power fields. Every brown stained nucleus was considered positive, irrespective of intensity. The percentage of positive stained cells was recorded as Ki-67 labeling index (Ki-67 LI) [15].

microvessel count (MVC) was performed after the areas of interest was determind by counting the individual stained blood microvessels (at power ×20) representing a field size of 0.74 mm2. Three hot spots were chosen, and MVD was calculated by dividing the mean of the MVC of the examined fields on the high-power field area which is 0.74 mm2. [16,17 &18]

Statistical analysis: was performed using SPSS V. 25 (statistical package for social sciences), using t-test for two continuous variables & one way ANOVA with post hoc test. Results were expressed as mean± slandered deviation. The differences were considered statistically significant when P value less than 0.05.

Results

The study included 41 cases of Astrocytomas, 7 (17.1%) cases of pilocytic (who grade I), 14 (34.1%) cases of diffuse fibrillary astrocytioma (who grade II), 8(19.5%) cases of Anaplastic astrocytoma (who grade III) & 12 (29.3%) cases of Glioblastoma (who grade IV) (figure 1&2) GII, The mean ± SEM of age for cases enrolled in the present study was 40.61 years with a range of 12-65 years.

Gender distribution of brain astrocytoma cases showed slight male predominance with 24 (58.5%) cases compared to female with 17 (41.5%) cases; male to female ratio was 1.41:1, When the tumor site is taken into consideration 10 (24.4%) cases were frontal, 4 (9.8%) cases were cerebellar, 16 (39 %) cases were parietal and 11 (26.8%) cases were temporal.

In the studied cases, the mean ± SEM of Ki-67 LI was 14.9 % ± 3 with a range of 0.4% to 75%, Regarding the CD31 antibody, the mean ± SEM of MVD was 33.85 ± 3.43 with a range from 11-73.

There was a significant correlation between the age of cases studied, and Ki-67 LI ($p<0.05$), also, the age is significant correlated with MVD ($p<0.05$).

There was no significant correlation between gender and Ki-67 LI, and MVD ($p=0.643$, $p=0.691$), respectively.

Regarding the site of the tumor and Ki-67 LI &CD 31 there was no significant correlation ($p=0.448$, $p=0.081$) respectively. There is significant increases in Ki-67 LI & MVD among astrocytoma grades (figure 2 &3), (table 1 &2)

By Comparison of Ki-67 labeling index & MVD between the different grades of astrocytomas, there is a significant correlation between different grades of astrocytomas except in between third & fourth grades in which it is insignificant. (table3&4)

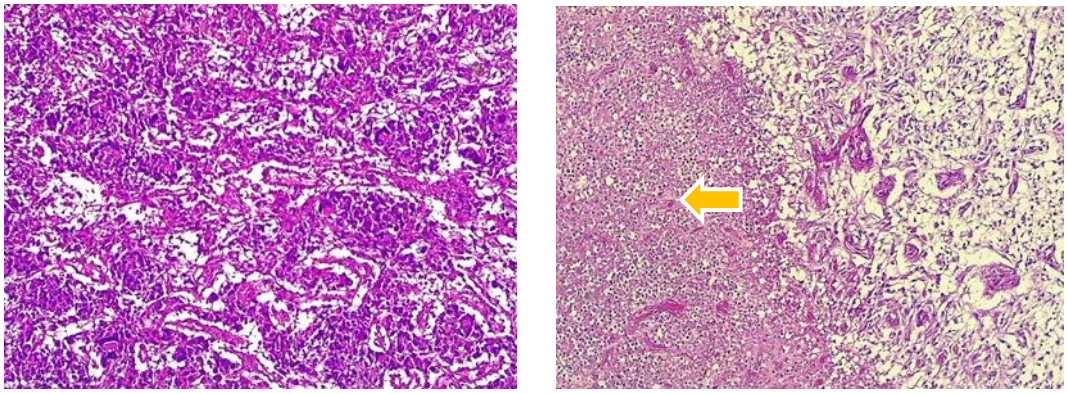

Figure 1. Grade IV astrocytoma showing poorly differentiated pleomorphic cells with nuclear atypia, Note the prominent vascularity as well as the area of necrosis (arrow). (H&E, X20).

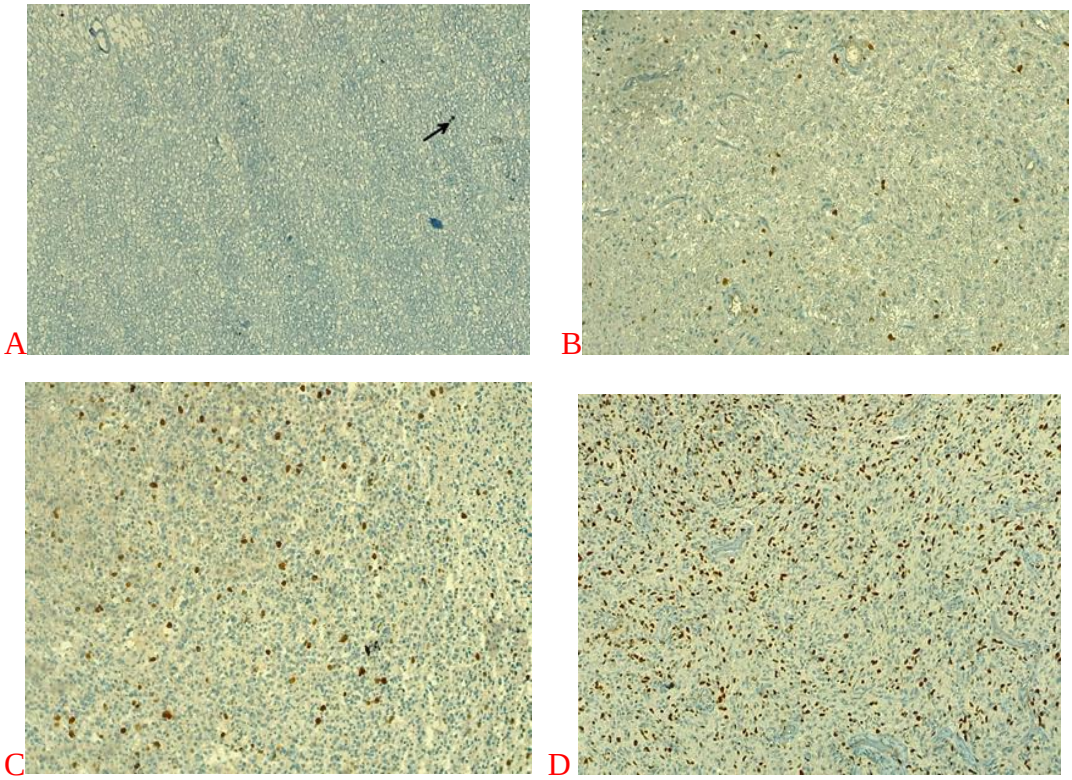

Figure 2. Astrocytomas (A: grade I,B grade II,C: grade III,D: grade IV) stained immunohistochemically with anti-Ki-67 showing positive brown nuclear expression of Ki-67, increase the nuclear staining with increasing the grade of astrocytomas (×20).

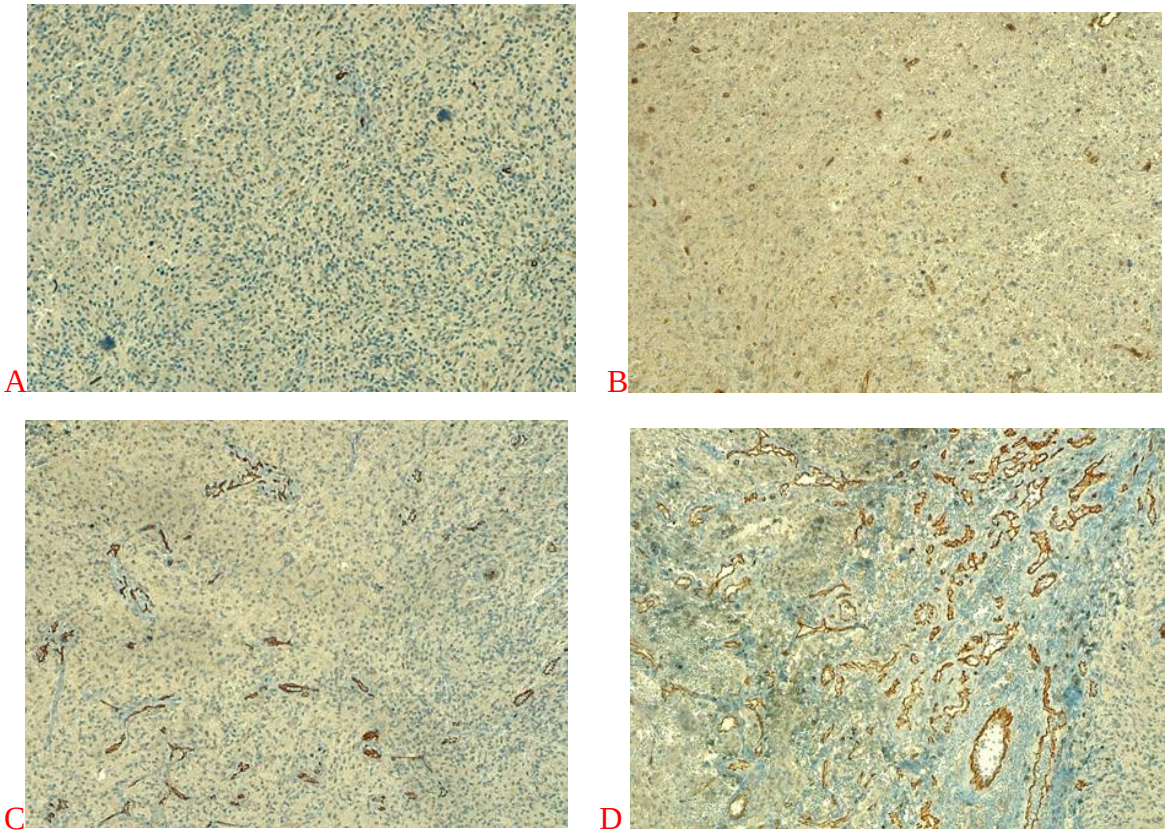

Figure 3. Astrocytomas (A:grade I,B gradeII,C:grade III,D:grade IV) stained immunohistochemically with anti-CD31 showing positive brown cytoplasmic staining of endothelial cells and marked microvessels proliferation(arrows) (×20).

Table 1. Correlation of Ki-67 labeling index (Ki-67 LI), with the histopathological grades of the cases

Grade of astrocytic tumors	KI 67 mean ± SD	Range	P Value
Grade I Tumors (pilocytic astrocytoma)	1.06 ± 0.57	0.40 – 2.00	<0.05
Grade II Tumors (diffuse astrocytoma)	4.36 ± 1.39	2.00 – 7.00	
Grade III Tumors (anaplastic astrocytoma)	21.38 ± 14.26	11.00 – 55.00	
Grade IV Tumors (Glioblastoma)	34.67 ± 22.33	15.00 -75.00	
SD = Standard deviation			

Table 2. Correlation of MVD, with the histopathological grades of the cases.

Grade of astrocytic tumors	MVD mean ± SD	Range	P Value
Grade I Tumors (pilocytic astrocytoma)	13.86 ± 3.132	11 -20	<0.001
Grade II Tumors (diffuse astrocytoma)	23.86 ± 7.584	11 -35	
Grade III Tumors (anaplastic astrocytoma)	39.00 ± 10.156	26 -50	
Grade IV Tumors (Glioblastoma)	53.75 ± 17.525	30 - 73	
SD = Standard deviation			

Table 3. Comparison of Ki-67 labeling index between the grades of astrocytoma

Grades of Astrocytic Tumors KI 67	P Value
Grade I and Grade II	<0.001
Grade I and Grade III	0.02
Grade I and Grade IV	0.001
Grade II and Grade III	0.046
Grade II and Grade IV	0.003
Grade III and Grade IV	0.391
P Value < 0.05 is significant	

Table 4. Comparison of MVD between different grades of astrocytomas

Grades of Astrocytic Tumors	P Value
Grade I and Grade II	0.002
Grade I and Grade III	0.001
Grade I and Grade IV	<0.001
Grade II and Grade III	0.015
Grade II and Grade IV	<0.001
Grade III and Grade IV	0.118
P Value < 0.05 is significant	

Discussion

Astrocytic tumors comprise a wide range of neoplasms that vary in their growth potential, extent of invasiveness, morphological features and tendency for progression.

Ki-67 LI is one of the most useful markers for evaluating cellular proliferation in various intracranial tumors [19,20], In this study clinical benefits of evaluation proliferative activity and microvessel density in astrocytomas in which they were assessed by Ki 67 LI & MVD.

The data analysis in this present study, showed slightly male predominance; as in a study by Ganju et al. [21] & Chaloob et al[22] Thotakura M et al.[23] ,There are significant variations in age ranges as studies done by Whitton and Bloom and Jaskɑlsky et al. [24,25],The distribution of astrocytomas differs by anatomic site between different grades & between adults and children. [26,27,28,29].

In our study there was no significant correlation between the gender and Ki-67 LI (p>0.05). This is agree with the studies done by Mahzouni et al.1 Abdelaziz et al.[30] and Liwei and Xiuzhen;[31] but, a study done by Yonghua et al.[32] illistrate a significant correlation between gender and immunohistochemical expression of Ki-67. This dissonance might be contributed to variations in the environments, race and geographical distribution, also might be to the size of the population and differences in antibodies used for Ki-67 antigen detection. [16,30,33].

The current study showed there was significant correlation between the age and the Ki-67 LI (p<0.05). This result is in agreement with study done by Abdelaziz et al. [30], Rodriquez-Pereira et al.[33]and Chaloob et al [22]. However study done by Isolan et al.[34] demostrated no correlation between age and the expression of Ki-67. This differnces may be due to different antibodies used for immunohistochemical detection of Ki-67 antigen (Ki-67 antibody was used in the study of Isolan et al. while MIB-1 was used in this study. MIB-1 antibody can be used on formalin-fixed paraffin-embedded section, and this is its advantage over the original Ki-67 antibody), adding to variations in sample size and ethnic groups.

This study illistrat that Ki-67 LI was not significantly influenced by the site of astrocytomas (p>0.05). This observation agrees with study done by Liwei and Xiuzhen.[31] chaloob et el[22].

There was a highly significant correlation between the histopathological grade of astrocytomas and Ki-67 LI (p<0.001). This

result agrees with the studies done by Abdelaziz et al., [30] Mahzouni et al. [1], Atik et al. [35], Kayaselcuk et al[36], Liwei and Xiuzhen. [31] ,Huang.[37], Rodriquez-Pereira et al. [33], and Scott et al. [38] ,Thotakura, et al. [23] & Das B et al. [39], Ambroise et al [40] & Wakimoto et al [8]

In a study by Montine et al. the Ki-67 LI was found to be a significant prognostic indicator for the entire group of astrocytomas and was significantly related to survival rate of astrocytomas.[41], studies by Wakimoto et al. and Rathi et al. demonstrated significant relation between tumor grade and Ki-67 LI. They establish a significant difference between low and high grade astrocytomas. [8,42].

Like to the present study, some studies showed a very low LI for pilocytic astrocytomas and there was significant difference in the distribution of Ki-67 LI between pilocytic and diffuse infiltrating astrocytomas ($p<0.001$)[43,44], in contrast to other studies that don't reveal any significant prognostic role for Ki-67 labeling index in pilocytic astrocytomas and diffuse astrocytomas like Thotakura M et al and Ambroise et al.[23,40], But similar to them when comparing Ki-67 LI between pilocytic astrocytomas and other grades (Grade III and Grade IV) of astrocytomas that there is significant statistical correlation was found.

Klein and Roggendorf elucidate that proliferation rates in astrocytomas not only reflect proliferation of tumor cells but also that of microglial cells, especially in pilocytic astrocytomas. So, using this marker to differentiate pilocytic astrocytoma from gliosis should be done with care and this marker is not reliable for definitive diagnosis.[45] Suggesting restricted role of Ki-67 LI in pilocytic astrocytomas like observations were made by Ambroise et al. [40].

In the present study, diffuse astrocytoma (Grade II astrocytoma) showed mean Ki-67 LI of 4.36 with a range of 2.00 – 7.00

Statistically significant P values (<0.05) were obtained on comparing diffuse astrocytomas with anaplastic astrocytoma and glioblastoma, similar to the most studies showed similar statistically significant differences in Ki-67 LI between high grade (Grade III and Grade IV) and low-grade (Grade II) astrocytomas. [46]

in the present study, P value on comparing Grade III and Grade IV tumors was 0.39; which is a statistically not significant value (>0.05), Like the other studies that did not show a significant difference between anaplastic astrocytoma and glioblastoma. [38,46] while Wakimoto et al. and Rathi et al. showed a significant difference of Ki-67 LI between anaplastic astrocytoma and glioblastoma.[8,42], if we match the Ki-67 LIs done by other authors, it is obvious that wide differences exist among Ki-67 LI values of various studies. Such differences may be explained partially by technical issues with different sensitivity of detection methods like peroxidase anti-peroxidase, avidin, streptavidin, various dilutions of antibodies used and time of incubation, different fixation protocols and most importantly the antigen retrieval methods applied by different workers.

The wide range of ki 67 LI may be due to interobserver counting variability , in the present study the counting was carried out in areas of highest densely labeled nuclei,like some other studies [42], Our study confirm that Ki-67 LI is not dependent on factors like, sex and site but dependent on histological grade. Though the average level of Ki-67 LI varies considerably in the different grades of astrocytomas, considerable overlap can be observed between them and Ki-67 LI has certain limitations in cases of pilocytic astrocytoma. So, Ki-67 LI should be wisely used in combination of the histopathological features.

The present study revealed significant correlation between the age of patients and MVD ($p<0.05$). This result is supported by studies conducted by Lebelt et al.[47] (detected by CD31 and anti-von Willebrand factor), while Leon et al.[48] and Izycka-Swieszewska et al. [49] recorded there is a

significant correlation between age and MVD in glioblastomas had been identified, This difference is attributed to many factors, including differences in sample size, technical issues used to detect MVD, and population.

There was no significant correlation between gender and MVD ($p>0.05$). This result concide with other studies done by El-Sayed and Taha, [50] and Chu et al.[51] & challoob et al. [22],also there was no significant correlation between the site of astrocytomas and MVD ($p>0.05$). This result is in agree with that gained by El-Sayed and Taha.[50] Chu et al.,[51] and Lebelt et al. [47], The present study demonstrated a highly significant correlation between MVD and the grade of astrocytoma ($p<0.001$). This result goes with studies done by others,[50,51,52,53] but disagrees with an Iranian study done by Mahzouni et al.[54] who used factor VIII-related antigen (von Willebrand factor) to reveal the MVD. This discordance may be due to different sample size and different markers used to assess angiogenesis.

Conclusion

A significant correlation was found between Ki67 labeling indices, and MVD (microvessel density) detected by CD31, and between the clinicopathological variables of astrocytomas (age and grade of tumor), Changing the classification to include diagnostic categories that depend on genotype may create certain challenges with respect to testing and reporting. These challenges include the availability and choice of genotyping and surrogate genotyping assays, that are not available in some centers ,Iraqi Centers are examples of these centers ,So we recommend to a more simple and available means like KI 67 LI as a marker for proliferation &CD 31 as a marker of angiogenesis as ancillary methods and as adjuncts with the morphologic features for diagnosis and differentiation of borderline grades of astrocytomas and help in prediction the prognosis of astrocytic tumors especilally in centers where there are difficulty in providing the molecular analysis .

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