The role of immunohistochemical markers in diagnosis and prognosis of diffuse astrocytoma

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Abstract: Background: The astrocytoma is a tumor of the central nervous system derived from glial cells. Histopathologic diagnosis of diffuse astrocytoma is essential for optimal prognostic and treatment. Distinction between different tumor grades can be challenging despite criteria given by World Health Organization (WHO). Additional markers are needed to improve the diagnostic and prognostic accuracy. **Objective:** To investigate the roles of ki67, p53 and CD34 in the process of astrocytoma cell proliferation and progression and correlation of them with the malignant degree of astrocytoma, age sex and site of the tumor. **Methods** A total of 3o cases of diffuse astrocytomas (12 fibrillary WHO grade II, 8 anaplastic astrocytoma WHO grade III and 10 glioblastoma WHO grade IV) were immunolabeled using ki67, p53 and CD34 monoclonal antibodies and analyzed statistically with respect to grade and other relevant parameters. **Results:** There was a stepwise increase of Ki-67 Labeling index (LI) from low-grade to high-grade astrocytomas (6.23±1.79, 13.06±4.47, and 22.15±7.63 respectively) which is statistically significant (P – value <0.001). The microvessel density (MVD) count [assessed using anti-CD34 antibody) increased with the progression of the pathological grade of astrocytoma. The mean value of MVD expressed by CD34 were (23.74±0.69, 43.40±1.03 and 63.53±2.23 respectively). Both ki67 and microvascular density correlated with grading and age (p<0.005), but did not show significant difference among patient sex and tumor location. P53 had no significant relationship with grading (p=0.0 57), but p53 overexpression is an early event in astrocytic progression. **Conclusion,** Ki-67 as markers for proliferation, and MVD as a marker of angiogenesis, could be used as ancillary methods to assist tumor grading in astrocytomas and help us to predict their prognosis. P53 tumor suppressor gene is an important early genetic event in malignant transformation of astrocytomas.
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Key words: astrocytoma, tumor grades,Ki-67 Labeling index, CD34, microvessel density (MVD)

1.Introduction:

Astrocytic tumors are the most common primary tumors of the central nervous system. These tumors have an inherit tendency for progression and recurrence **(Mohammed et al 2012)**. Histopathologic diagnosis is essential for optimal prognostication and treatment. According to World Health Organization (WHO), diffuse astrocytomas can be divided into diffuse fibrillary astrocytoma grade II, anaplastic astrocytoma grade III, and glioblastoma grade IV **(Louis et al 2007).** Basically, these categories result from the recognition of anaplasia findings (nuclear atypia, cell pleomorphism, mitotic activity, endothelial hyperplasia and necrosis) through routine histological analysis by light microscopy **(Mário et al 2007).**

The histopathological examination still serves as the gold standard for making the diagnosis of astrocytic tumors. However, the current classification scheme is not optimal because of several parameters including tumor heterogeneity, sampling error, small tumor specimens, poor reproducibility, and that tumors with common histology can be genetically different. For these reasons, additional markers are needed to improve the diagnostic and prognostic accuracy (***Lind –landstrom et al., 2012).***

The number of mitoses is of paramount importance, but can be hard to identify in haematoxylin and eosin (H&E)-stained sections by several factors including squeezed cells in stereotatic biopsies, distortion and similarities to chromatin changes in apoptotic and pyknotic cells ***(Prayson, 2002).*** Since proliferative activity is reliable method to asses tumor biology. The monoclonal antibody Ki-67 is commonly used and has proven prognostic and diagnostic power in astrocytic tumors. It is an antigen expressed during all the active phases of the cell cycle ***(Lind-Landstrom et al 2012).***

One of the most frequent and earliest genetic alterations recognized to occur in these tumors is the p53 mutation. ***[Kraus et al 1994, Louis, 1994].*** Detection of p53 abnormalities may have diagnostic, prognostic, and therapeutic implications ***(Hollstein et al 1991).*** Some studies have shown that the frequency of p53 mutation is higher in tumors that progress to anaplastic astrocytoma and glioblastoma ***(Sarkar et al 2002)***.

Further, with respect to growth and progression of tumor, angiogenesis plays an important role. Microvessel density (MVD) is quite a useful parameter in the evaluation of angiogenesis. MVD is increased with the progression of the pathological

grade of astrocytoma. Thus, high MVD may be a contributing factor to the invasive growth of astrocytoma. It has also been suggested that tumor angiogenesis and vascular density can be used as determinants of radiosensitization, of response to anti-angiogenesis drugs and as reliable prognostic markers ***(Norden et al 2008).***

The aim of this study was to investigate the roles of ki67, p53 and CD34 in the process of the astrocytoma occurrence, cell proliferation, progression and correlation of them with the grades of astrocytoma,age,sex and site of the tumor.

2.Material and methods;

2.1.Materials:

2.1.1.Samples:

In this study, a total of 30 formalin-fixed paraffin-embedded brain excisional biopsies of diffuse astrocytic tumors covering the period from March 2010 to June 2012 were retrieved from the archival materials of the Histopathology Department of Al-Azhar University Hospital and private laboratory. All haematoxylin and eosin (H&E) stained sections were reviewed and tumor grading was based on the WHO 2007 criteria ***(Cavenee et al 2007),*** 12 tumors were classified as diffuse fibrillary astrocytomas (grade II), 8 tumors were graded as anaplastic astrocytomas (grade III) and 10 cases as glioblastomas(Grade IV). All the clinicopathological data were obtained from the available histopathological reports and included age, sex, site of tumor and tumor grade.

2.2.Methods:

2.2.1.Immunohistochemical study:

From each block, 4 sections of 4 μm thickness were taken. One section was stained with hematoxylin and eosin for revision of the histopathological diagnosis, and the other 3 sections were stained immunohistochemically using the streptavidin–biotin alkaline phosphatase method for monoclonal antibodies: (Ki-67-specific monoclonal antibody; DAKO, Denmark, Dilution-1:50), p53 (Dako, Denmark; Clone-DO7 Dilution-1:200) and CD34 (Biogenex, USA; Clone-QBEND/10, Dilution-1:40). Technical negative controls were obtained by omitting the primary antibody for the three markers under identical test condition, respectively. Sections from a lymph node with follicular lymphoid hyperplasia known to be immunoreactive for Ki-67, and CD34 were used as positive controls and For p53 a known case of colonic adenocarcinoma was used as positive controls (as recommended by the manufacturer).

The sections were deparaffinized in xylene, rehydrated in graded alcohol dilutions, washed in PBS, incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, washed in PBS again, and boiled in citrate buffer solution (pH 6.0) using a microwave for 10 min at 60 C° for antigen retrieval. After cooling at room temperature, the sections were incubated with the primary antibody overnight in a humidified chamber. After washing with PBS, the slides were incubated with the multilink secondary antibody (BioGenex; 20min), after which streptavidin horseradish peroxidase was applied (20 min). The sections were visualized using diaminobenzidine (15min) and hematoxylin as counterstains.

2.2.2 Evaluation of staining

* Assessment of Ki67 labelling index

Positive immunohistochemical staining for Ki-67 is brown nuclear with a diffuse pattern.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 (LI) **(Edilson et al 2010).**

***Assessment of p53 labelling index**

Positive immunohistochemical staining for p53 was brown nuclear with a granular pattern, The p53 labeling index (LI) were calculated as the percentage of positively-stained nuclei out at least 1000 cells in 10 high – power field as follows:

$$P53 = \frac{\text{Number of nuclei showing positive staining}}{\text{Total number of nuclei counted}} \times 100$$

The positive index (PI) represented the percentage of tumors positive for the antigens studied in each group (histological grade). Cases with p53 LI greater than 10% were taken as p53 positive and cases with less than 10% were taken as p53 negative ***(Abdulrauf et al 1998).*** Labeling index for glioblastomas was calculated from areas of sections that were free from necrosis or capillary endothelial proliferation. The infiltrative edge of the tumor where neoplastic cells surround normal neurons and glia was also avoided.

***Assessment of Microvessel density count using anti-CD34 antibody**

The positive expression of CD34 was mainly confined to the cytoplasm of vascular endothelial cells as brownish yellow granules. Microvessels were represented by brownish yellow capillaries, Single cells or small cell clusters. Microvessel density (MVD) was evaluated according to the method described by ***Ding et al, 2003.*** The entire tumor section was scanned at low magnification to identify the area of highest vessel density (hot spot). The five most vascular areas within the tumor mass were chosen. Individual microvessels in each hot spot were counted in a single 200x field and the average counts of the 5 fields were recorded and defined as MVD. A vessel lumen was not required for identification of a microvessel. Single cells or cell clusters were counted.

Large vessels with thick muscular walls or with lumina greater than 50 um were excluded from the count.

2.2.3. Statistical analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (SPSS) program version 17. The qualitative data were presented as numbers and percentages while the quantitative data were presented as mean, standard deviations and ranges. The comparison between groups with qualitative data were done by using Chi-square test and Fisher exact test was used only when the expected count in any cell found less than 5. Also the comparison between two groups with quantitative data was done by using Independent t-test and the comparison between three groups with quantitative data was done by using One Way ANOVA. Pearson correlation coefficient was used to assess the relations between the studied parameters.

3-Results:

The median age of the studied cases (30 cases) was 51.1±19.5 with range 19.5 – 78 years and male to female ratio was 6: 4. The majority of the tumors 13|30 (43.3%) were located in frontal region; Clinical data are summarized in (table 1).

3-1-Classification of astrocytomas regarding the grades: They were classified as 12(40%) cases were fibrillary astrocytomas (WHO grade II) (5 males and 7 females), the mean age of patients was 37.35±15.5 years (range, 19.5–63 years), Five tumors were located in the frontal, three in the cerebellar, three in the parietal and one temporal regions. Eight (26.7%) cases were anaplastic astrocytomas (WHO grade III) (6 male and 2 females), the mean age of patient was 53.25±15.82 (range 22.5 - 70.5), four tumors were located in the frontal, two in the temporal and two in the cerebellar regions. The remaining 10 (33.3 %) cases were glioblastomas multiform (WHO grade IV) (5 males and 5females), the mean age of patients was 65.80±15.6 years (range, 25 – 78), four tumors were located in the frontal, four in the temporal and two in the cerebellar regions. Based on the histologic grading, the strongest positive correlation was found between histologic grade and age among diffuse astrocytomas, however there was no significant correlation between histologic grade and sex or site of the tumor. Comparison between different (grades) types of astrocytomas regarding sex, site and age are shown in table (2).

Table (1): Clinical data

Clinical Variables	NO. (%)
Age	
Mean±SD	51.1±19.5
Range	19.5 – 78
Gender	
Male	18 (60%)
Female	12 (40%)
Site	
Frontal	13 (43.3%)
Temporal	7 (23.3%)
Cerebellar	7 (23%)
Parietal	3 (10%)
Grade	
Grade II	12 (40%)
Grade III	8 (26.7%)
Grade IV	10 (33.3%)

Table (2) Comparison between different types of astrocytomas regarding sex, site and age

		Fibrillary astrocytomas		Anaplastic astrocytomas		Glioblastomas		P-value
		No.	%	No.	%	No.	%	
Sex	Female	5	41.70%	2	25.00%	5	50.00%	0.554
	Male	7	58.30%	6	75.00%	5	50.00%	
Site	Frontal	5	41.70%	4	50.00%	4	40.00%	0.317
	Temporal	1	8.30%	2	25.00%	4	40.00%	
	Cerebellar	3	25.00%	2	25.00%	2	20.00%	
	Parietal	3	25.00%	0	0.00%	0	0.00%	
Age	Mean ±SD	37.35±15.5		53.25±15.82		65.80±15.6		0.001
	Range	19.5 - 62		22.5 - 70.5		25 – 78		

3.2. Ki67, p53 and CD34 expressions and its correlation with clinicopathologic factors:

Based on the histopathologic grading of astrocytoma, both ki67 and microvascular density correlated with grading (P – value <0.005), but no significant correlation was found in p53 (P – value 0.075) as shown in table (3).

The general Positive index of Ki-67 proteins expression was 96.6 % (29/30) (fig., 1A &1B),all cases of CD34 were positive (fig., 2A,2B), while Positive index of p53 in these study showed 80% of grade IV (fig.,3A), 75 % of grade III (fig.,3B) and 83.3% of grade II patients, With labeling indices greater than 10% taken as p53 positive.

Table 3: ***Correlation of Ki-67 LI, p53 LI and CD34 (MVD)with the histopathological grade of the cases.***

		Mean±SD	P-value
Ki67 LI	Fibrillary	6.23±1.79	<0.001
	Anaplastic	13.06±4.47	
	Glioblastoma	22.15±7.63	
P53 LI	Fibrillary	18.02±8.1	0.075
	Anaplastic	26.92±12.18	
	Glioblastoma	24.06±4.98	
CD34 (MVD)	Fibrillary	23.74±0.69	<0.001
	Anaplastic	43.40±1.03	
	Glioblastoma	63.53±2.23	

There was a significant correlation between the age of cases studied and ki67 LI and CD34 (r=0.683,*p*=0.000,r=0.576,0.001 respectively), but no significant correlation of age with p53 (r=0.297,p =0.111)as shown in table (4).

Table (4): Correlation of Ki-67 labeling index, p53 labeling index and CD34 (MVD) with the age of cases

	Age	
	r	p-value
Ki67	0.683**	0.000
CD34	0.576**	0.001
P53	0.297	0.111

There was no significant correlation between gender and site of the tumor, and Ki-67 LI, p53 LI and CD34 (MVD). Statistically, there was a positive correlation between Ki67 LI and CD34 (MVD) in all types of astrocytoma, this correlation was highly significant (p-0.000) (table 5 , fig.,4,, but no significant correlation was found between both ki67,CD34 and p53 (P-0.205, 0.214 respectively) .

Table (5) relationships between the Ki67 and CD34 marker in all types of astrocytoma

	Ki67	
	r	p-value
CD34	0.688**	0.000

Table (6) relationships between the Ki67, CD34 and p53 markers

	P53	
	r	p-value
CD34	0.234	0.214
Ki67	0.238	0.205

Tumor growth depends upon multiple factors including cell proliferation, genetic alterations or deletion of negative regulatory elements, oncogene amplification and angiogenesis. In the present study, three parameters were used, Ki-67, p53 and CD34 in diffuse astrocytomas to investigate their roles in the proccess of the astrocytoma occurrence,cell proliferation and progression and correlation of them with the malignant degree of astrocytoma,age, gender and site of the tumor.

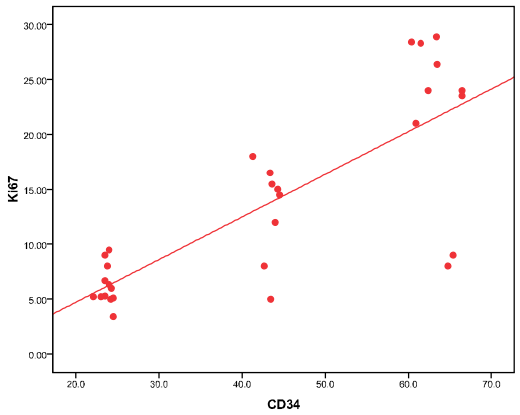

Fig (4): relationships between the Ki67 and CD34 marker

4.Discussion

One of the best and well known immunohistochemical methods for evaluating the proliferation rate is quantitation of Ki-67. A technique which shows all phases of the cell cycle except cells in G0 phase ***(Mahzouni et al 2007).***

There was a stepwise increase of Ki-67 LI from low-grade to high-grade astrocytomas which is statistically significant. our result agrees with the studies done by ***(Abdelaziz et al 2010, Huang.,2010 and Scott et al.,2005).*** There was a significant correlation between the age and the Ki-67 LI. This result is supported by study done by ***Mohammed et al 2012***, and by a study done by **Rodriquez-*Pereira et al. (2000)***, While ***Isolan et al.(2005)***, revealed no correlation between age and Ki-67 LI. The current work showed that Ki-67 LI was not significantly affected by gender. This result is in agreement with the results of the studies done by ***Mohammed et al (2012***), ***Abdelaziz et al. 2010 and Liwei and Xiuzhen (2010***). However, a study done by ***Yong-hua et al. (2003),*** demonstrated a significant correlation between gender and immunohistochemical expression of Ki-67. This discordance could be attributed to environmental, racial and geographical differences, in addition to the difference in the sample size and antibodies used for the detection of Ki-67 antigen. The current work showed also that Ki-67 LI was not significantly affected by the site of astrocytomas (p = 0.230). This observation agrees with study done by ***Liwei and Xiuzhen (2010).***

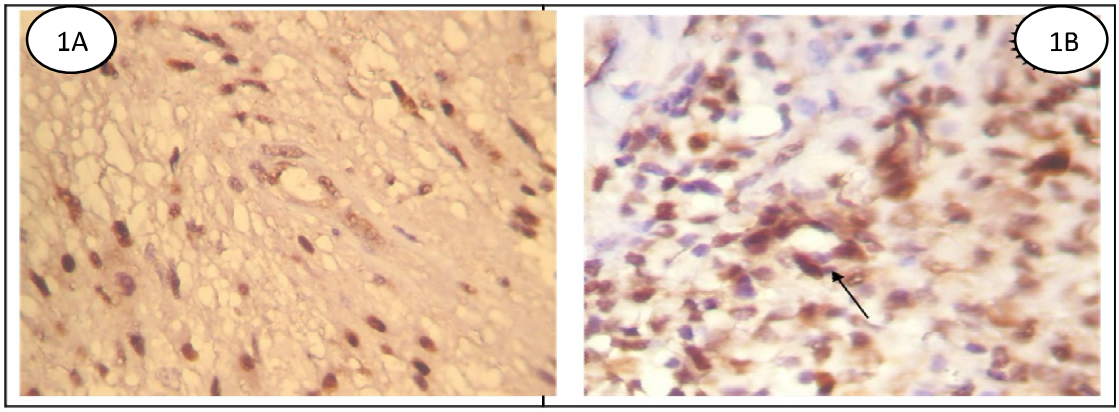

FIGURE 2 - A. fibrillary astrocytoma (grade II) stained immunohistochemically with ki67 antibody showing scattered positive brown nuclear expression of ki67 stained nuclei . B . Glioblastoma multiforme (grade IV) stained immunohistochemically with anti-Ki-67 showing positive brown nuclear expression of Ki-67 with large number of stained nuclei reflecting active cellular proliferation (arrows).. (original magnification (A) At power (×40); (B) At power (×10).).

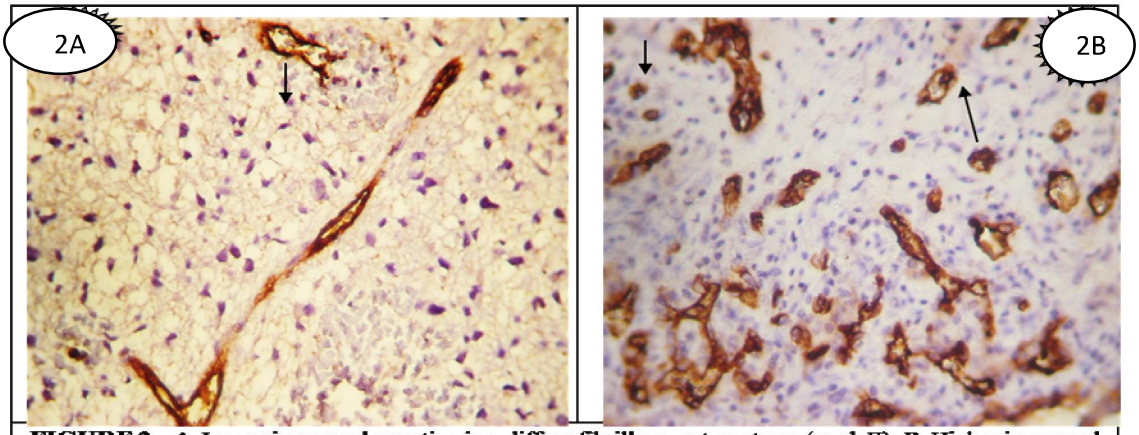

FIGURE 2 - A. Low microvessel counting in a diffuse fibrillary astrocytoma (gradeII). B. High microvessel counting and marked microvessels proliferation (arrows) in an anaplastic astrocytoma (grade III) (original magnification (A) At power (×10); (B) At power (×40).).

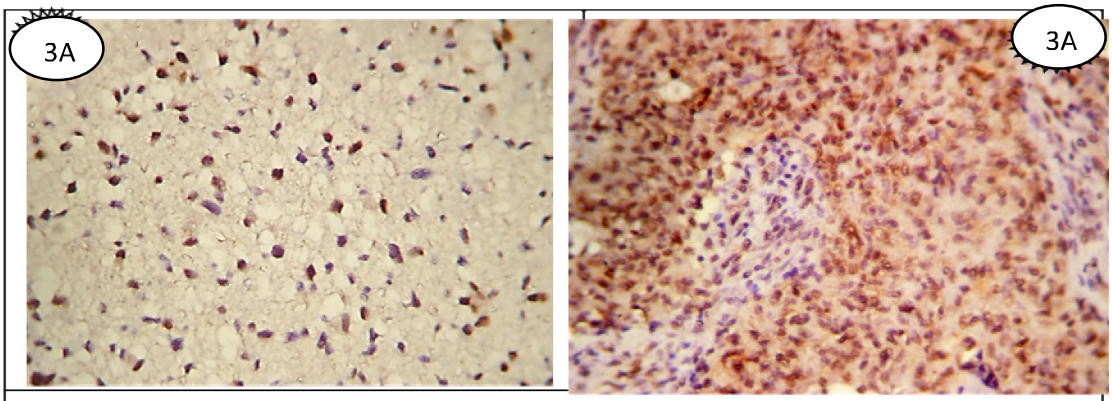

Fig. 3 – A. fibrillary astrocytoma (grade II) stained immunohistochemically with anti-p53 showing positive brown nuclear expression of p53 in less than 25% of cells . B. Glioblastoma multiforme (grade IV) stained immunohistochemically with anti-p53 showing positive brown nuclear expression of p53 in more than 75% of cells (original magnification (A) and (B) At power (×10)).

Some genetic changes related to malignant progression can be used to improve diagnosis on limited samples where histology alone may not reveal all the features. P53 protein immunohistochemical findings have been proposed as such markers ***(Ganju et al 1994)***. The present study documented p53 immunopositivity in 83.3.0% of diffuse fibrillary astrocytomas, 75% of anaplastic astrocytomas and 80 % of GBM cases. Thus p53 immunopositivity was demonstrated in more than tree fourth of tumors of all the three categories. Mean labeling indices of p53 for the three grades were 18.02, 26.92, and 24.6 respectively. Our study showed no correlation between p53 and malignancy grade. A previous studies also indicated absence of correlation between p53 LI and the tumor grade ***(Moses et al., 2010, Nayak et al., 2004).*** The frequencies of p53 immunopositivity and p53 labeling indices in diffuse fibillary and high-grade glioblastoma multiform astrocytomas in our study were similar, as well as mean p53 LI in grade III anaplastic astrocytomas was higher when compared to diffuse fibillary and Over expression of p53 was more after malignant transformation. Thus our study confirms that inactivation of p53 tumor suppressor gene is an important early genetic event in malignant transformation of astrocytomas as proved by earlier studies ***(Moses et al 2010.Nayak et al., 2004).*** *But p53 expression does show positive correlation with grade of malignancy, as well as* There was no clear association between p53 expression and age, sex or site of the tumor in our study.

Microvascular density (MVD) represents the degree of tumor neovascularization, and it has been found to be an important indicator of malignant behavior in many human neoplasms. The relationship of tumor microvascular density to patient prognosis has been shown in diverse brain tumors of varied grades***. (Li VW et al 1994,Leon et al 1996).*** It is likely that MVD may become an integral part in tumor grading system and routine prognostic evaluation,as well as It is widely assumed that tumors with high MVD are good candidates for clinical trials of antiangiogenic therapies, whereas tumors that typically have low MVD are thought to be poor candidates for such clinical trials (***Zhong and Bowen, 2006; Laquente et al., 2007).***

The present study recorded that MVD detected by CD34 antibody is increased with the progression of the pathological grade of astrocytoma. Significant differences of MVD were found among astrocytomas of different grades (p<0.001). Thus, high MVD may be a contributing factor to the invasive growth of astrocytoma. This result in agreement with the results of study by ***Mohammed et al 2012, El-Sayed and Taha 2011.***

The present study recorded a significant correlation between the age of patients and MVD (p<0.001). This result is supported by studies conducted ***Mohammed et al 2012***, while ***Lebelt et al.2008*** revealed no significant difference between age and MVD (detected by CD31 and anti-von Willebrand factor). This difference is attributed to many factors, including differences in sample size, markers used to assess MVD, and population.

There was no clear association between CD34 expression and sex or site of tumor (p= 0.720, 0. 111 respectively) in our study. This result goes with studies done by others ***(Mohammed et al 2012, El-Sayed and Taha 2011, Chu et al 2005).***

Conclusion

- Determination of proliferative activity in astrocytomas using antibodies against Ki-67 antigen may assist in the histopathological grading, especially because mitoses can be hard to detect.
- High MVD may be a contributing factor to the invasive growth of astrocytoma.
- Among the clinical variables, age is a well-established prognostic factor in diffuse astrocytoma.
- p53 tumor suppressor gene is an important early genetic event in formation of astrocytomas

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