IMMUNOHISTOCHEMICAL EXPRESSION OF CLUSTER OF DIFFERENTIATION-68(CD-68) AND Ki-67 IN ASTROCYTOMAS

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ABSTRACT

Background: Astrocytoma is common primary malignant brain tumors which arise from immortalized astrocytes. In astrocytoma microglia density is higher than in normal brain, and microglia increase in number according to the grade of malignancy. Intense CD68 staining was marginally significant prognosticator for shorter survival. Since proliferative activity is a reliable method to assess tumour biology, Ki-67/MIB-1 has proven to be of prognostic and diagnostic power in astrocytic tumours and shown to correlate with tumour grade.

Aim: This study was conducted to detect immunohistochemical expression of CD-68 and Ki-67 in astrocytoma and assessing their relation to proliferative activity and prognosis of such tumors.

Methods: Thirty representative cases; 30 cases of astrocytoma examined immunohistochemically using antibodies against CD-68 and Ki-67.

Results: CD-68 was positive in 73.3% of astrocytoma cases and it was negative in 26.7%. Low Ki-67 LI was found in low grade astrocytoma and high Ki-67 LI was found in high grade astrocytoma.

Conclusion: CD-68 and Ki-67 were the most useful IHC markers in the discrimination of different grades of astrocytoma and predict proliferative activity and prognosis of such tumors.

Key words: Astrocytoma, Prognosis, CD-68, Ki-67.

INTRODUCTION

Astrocytoma is the most common primary tumor in the CNS. The diagnosis is based primarily on histo-pathological criteria defined by the World Health Organization (WHO) that grades astrocytoma as pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma (grade IV) [1].

CD68 is a trans-membrane glycoprotein, which is mainly located in lysosomes expressed by monocye/macrophage lineages and serves as a marker for microglia [2].

In human glioma, intra-tumoral microglia density is higher than in peri-tumoral and normal brain, and microglia increase in number according to the grade of malignancy [3].

CD68 was clearly reactive in neoplastic astrocytes, whereas astrocytes in normal brain specimens were not reactive, [4].

Intense CD68 staining was marginally significant prognosticator for shorter survival. There was a significant prognostic value of CD68 tumor staining in the group of patients with anaplastic astrocytoma, which may be important for the management of patients with longer survival than these with glioblastoma. Further studies are necessary to investigate the possible mechanisms and consequence of macrophage phenotype expression of malignant astrocytoma, as well as possible role of microglia for tumor progression and patient prognosis. [5].

Some studies did not find positive correlations between mitoses and most of the proliferative markers. Hence, this observation suggests that antibodies against proliferation-associated antigens are useful to obtain an optimal profile of the proliferative activity, especially in small brain tumour samples [6].

SUBJECT AND METHODS

Cases:

This is a retrospective study. 30 cases of astrocytoma biopsy specimens received from department of pathology faculty of medicine Zagazig University, referred paraffin blocks to national cancer institute and some private laboratories in the period from September 2009 to September 2012. Specimen was obtained by stereotactic and open biopsy fixed immediately in 10% formalin for 18-24 hours.

The paraffin embedded tissue blocks were previously diagnosed as follows: 2 cases of pilocytic astrocytoma WHO grade I, 8 cases of diffuse astrocytoma WHO grade II, 12 cases of anaplastic astrocytoma WHO grade III, and 8 cases of glioblastoma- multiform WHO grade IV.

The clinical data of the patients were obtained from the medical files.

Immunohistochemical Study:

The immunohistochemical reactions were carried out using streptavidin-biotin immunoperoxidase staining technique ( Dako-Cytomation, Glostrup, Denmark) Paraffin sections were cut at 3-4 μ thickness, deparaffinized by incubating them in the oven at 56°C for 15 minutes, inserted in xylene for 30 minutes and rehydrated in descending grades of alcohol at room temperature. Thereafter, blocking of endogenous peroxidase activity with 0.3 %...
hydrogen peroxide in water for 30 minutes at room temperature was carried out. Antigen retrieval was performed using Dako target retrieval solution (PH 6.0) (EZ retrieval system, Biogenex, Fremont, CA, USA); boiled in a microwave. Slides were incubated with primary antibodies for 30 minutes at room temperature; antibodies used were; anti Standard CD68 (CD68 Std mouse monoclonal antibody, from Thermo Scientific/Lab Vision Corporation, Fremont, USA, and clone: 156-3c11. 0.09% sodium azide. Dilution 1:100).

Ki-67 (Ki-67 Rabbit Polyclonal antibody, from Thermo Scientific/Lab Vision Corporation, Fremont, USA, 0.09% sodium azide. Dilution 1:300) Incubation with secondary antibody were performed then product visualization by incubated with diaminobenzidine (DAB) substrate as a chromagen (Dako-Cytomation, Glostrup, Denmark); for 5-10 minutes. Sections were counterstained with Mayer's hematoxyl-

3-Interpretation and evaluation of immunostaining

1- CD 68 immunostaining:

Standard CD68 immunostaining was cytoplasmic. Negative: No stained cells. Weak (+) when less than 5% of cells labeled, moderate (+++) when between 5% and 50% of cells labeled, and strong (++++) when more than 50% of cells labeled. [8]

2- Ki-67 immunostaining:

Ki-67 labeling index was calculated as percentage of positively stained nuclei per thousand cells, 1000 cells (X400 magnifications) were counted in several areas of the tumor where positively stained nuclei are evenly distributed. But in areas where no even distribution of positive nuclei the tumor cells were counted in areas of with highest density of positive nuclei. The immunoreactivity of Ki-67 was classified as low expression (<10%) and high expression (≥10%). [9]

Spleen and normal tonsil section was used as positive controls for cd 68 and Ki-67 respectively. Their negative controls were obtained by omission of the primary antibody.

RESULTS

Table (1): CD-68 expression in all the studied cases:

<table>
<thead>
<tr>
<th></th>
<th>Cases No.=30</th>
<th>Negative Interpretation No.=8</th>
<th>Positive Interpretation No.=22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----</td>
<td>---</td>
<td>-----</td>
</tr>
<tr>
<td>astrocytoma grade I</td>
<td>2</td>
<td>6.6</td>
<td>1</td>
</tr>
<tr>
<td>astrocytoma grade II</td>
<td>8</td>
<td>26.7</td>
<td>4</td>
</tr>
<tr>
<td>astrocytoma grade III</td>
<td>12</td>
<td>40.0</td>
<td>2</td>
</tr>
<tr>
<td>Glioblastomamultiforme grade IV</td>
<td>8</td>
<td>26.7</td>
<td>1</td>
</tr>
</tbody>
</table>
The CD-68 expression in studied cases of astrocytoma grade I was statistically insignificant (p. value>0.05) but was statistically significant in astrocytoma grades II, III and IV. (p. values were = 0.001,0.004 and 0.006 respectively)

KI -67 expression analysis:
Immuno-histochemical reactivity pattern of KI -67 in astrocytoma:
  The estimates for mean values of proliferative marker Ki-67, in grade I, II, III and IV tumors were 1.5, 3.5, 11.5 and 16.2, respectively

Table (2): KI -67 expressions in studied astrocytoma cases:

<table>
<thead>
<tr>
<th>Astrocytoma</th>
<th>Cases No.=30</th>
<th>Low No.=10</th>
<th>High No.=20</th>
<th>X²</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>astrocytoma grade I</td>
<td>2 No. 6.6 %</td>
<td>0 No. 0.0 %</td>
<td>2 No. 0.0 %</td>
<td>4.29</td>
<td>0.038*</td>
</tr>
<tr>
<td>astrocytoma grade II</td>
<td>8 No. 26.7 %</td>
<td>8 No. 80.0 %</td>
<td>0 No. 0.0 %</td>
<td>21.82</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>astrocytoma Grade III</td>
<td>12 No. 40.0 %</td>
<td>0 No. 0.0 %</td>
<td>12 No. 60.0 %</td>
<td>10</td>
<td>0.002*</td>
</tr>
<tr>
<td>Glioblastoma-multiform grade IV</td>
<td>8 No. 26.7 %</td>
<td>0 No. 0.0 %</td>
<td>8 No. 40.0 %</td>
<td>5.45</td>
<td>0.019*</td>
</tr>
</tbody>
</table>

There were statistically significant values of KI -67expressions in studied grade I, II, III and IV cases (p. values were =0.038, <0.001, =0.002 and =0.019 respectively)

Table (3): The correlation between CD-68 immunohistochemical expression and KI-67 labeling index in different grades of astrocytoma

<table>
<thead>
<tr>
<th>Astrocytoma</th>
<th>CD-68 positive cases</th>
<th>KI-67 labeling index</th>
<th>X²</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.=30</td>
<td>No.=30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-ve No.=8</td>
<td>low(+) No.=5</td>
<td>moderate(++) No.=6</td>
<td>high(+++) No.=11</td>
</tr>
<tr>
<td>pilocytic astrocytoma WHO grade I</td>
<td>1 No. 12.5 %</td>
<td>0 No. 0.0 %</td>
<td>0 No. 0.0 %</td>
<td>2 No. 20.0 %</td>
</tr>
<tr>
<td>Diffuse fibrillary astrocytoma WHO grade II</td>
<td>4 No. 50.0 %</td>
<td>0 No. 0.0 %</td>
<td>0 No. 0.0 %</td>
<td>8 No. 80.0 %</td>
</tr>
<tr>
<td>Anaplastic astrocytoma WHO Grade III</td>
<td>2 No. 25.0 %</td>
<td>0 No. 0.0 %</td>
<td>6 No. 100.0 %</td>
<td>4 No. 36.4 %</td>
</tr>
<tr>
<td>Glioblastoma multiform WHO grade IV</td>
<td>1 No. 12.5 %</td>
<td>0 No. 0.0 %</td>
<td>7 No. 63.6 %</td>
<td>8 No. 0.0 %</td>
</tr>
</tbody>
</table>

The correlation between CD-68 immunohistochemical expression and KI-67 labeling index in astrocytoma grade I cases was statistically in- significant. (p. value>0.05), but was statistically significant in astrocytoma grade II,III,IV cases (p. value were <0.001, <0.001, = 0.003 respectively)
Fig. (1): **Pilocytic astrocytoma WHO grade I** shows focal nuclear Ki-67 immunoreactivity (ki-67 LI<2%). (Ki-67 immunostaining, DAB chromogen. Mayer's hematoxylin M.H counter stain X 400).

Fig. (2): **Pilocytic astrocytoma WHO grade I** shows mild granular and diffuse cytoplasmic positivity for CD-68(+) in less than 5% of tumor cells (CD-68 immunostaining, DAB chromogen. Mayer's hematoxylin M.H counter stain X 400).
Fig. (3): Fibrillary astrocytoma WHO grade II shows focal nuclear Ki-67 immunoreactivity (Ki-67 LI<5%). (Ki-67 immunostaining, DAB chromogen. Mayer’s hematoxylin M.H counter stain X 400).

Fig. (4): Fibrillary astrocytoma WHO grade II shows mild granular and diffuse cytoplasmic positivity for CD-68(+) in less than 5% of tumor cells (CD-68 immunostaining, DABchromogen. Mayer's hematoxylin M.H counter stain X 400).
Fig. (5): **Anaplastic astrocytoma WHO grade III** shows marked nuclear Ki-67 immunoreactivity (Ki-67 LI >10%). (Ki-67 immunostaining, DAB chromogen. Mayer's hematoxylin M.H counter stain X400)

Fig. (6): **Anaplastic astrocytoma WHO grade III** show moderate granular and diffuse cytoplasmic positivity for CD-68(++) staining from 5% to 30% of tumor cells (CD-68 immunostaining, DAB chromogen. Mayer's hematoxylin M.H counter stain X 400).
**Fig. (7):** Glioblastoma multiform WHO grade IV show marked nuclear Ki-67 immunoreactivity (Ki-67 LI>20%). (Ki-67 immunostaining, DAB chromogen. Mayer’s hematoxylin M.H counter stain X 400).

**Fig. (8):** Glioblastoma multiform WHO grade IV show marked granular and diffuse cytoplasmic positivity for CD-68(+++) staining more than 30% of tumor cells (CD-68 immunostaining, DAB chromogen. Mayer’s hematoxylin M.H counter stain X 400).

**DISCUSSION**

The current study revealed that 22/30 (73.3%) cases of astrocytoma showed positive immune-histochemical expression of this antibody with variable intensity and negative expression was found in 8/30 (26.7%) cases.

Negative results was found in 1/2 (50%) cases of astrocytoma grade I, 4/8 (50%) cases of astrocytoma grade II, 2/12 (16.6%) cases of
astrocytoma grade III and 1/8(12.5%) cases of astrocytoma grade IV. The negative immunohistochemical results were statistically insignificant (p. value >0.05).

Weak positive expression (+) was found in 1/2(50%) cases of astrocytoma grade I, 4/8(50%) cases of astrocytoma grade II. Weak expression was detected in 5/10(50%) cases of low grade astrocytoma (grade I&II). That immunohistochemical results were statistically significant. (P. value =0.002).

There were no statistically significant difference was found between immunohistochemical expression of CD-68 in grade I and grade II astrocytoma (p. value >0.05).

Moderate positive expression (++) was found in 6/12(50%) cases of astrocytoma grade III. That immunohistochemical results were statistically significant (p. value=0.007)

Strong positive expression (+++) was found in 4/12(33.3%) cases of astrocytoma grade III and in 7/8(87.5%) cases of Glioblastoma-mutilform grade IV. That immunohistochemical results were statistically significant (p. value=0.012).

There was no statistically significant difference in immunohistochemical expression of CD-68 between astrocytoma grade III & grade IV (p. value >0.05). There was statistically significant difference in immunohistochemical expression of CD-68 between astrocytoma grade I & III (p. value =0.0326) There was statistically significant difference in immunohistochemical expression of CD-68 between astrocytoma grade II & III (p. value =0.006). There was statistically significant correlation between increasing grade of the astrocytoma and the immune-histochemical expression of CD-68 (p. value<0.05).

So the study revealed that immunohistochemical expression of CD-68 is increasing in ascending grades of astrocytoma and helpful in differentiating low grade astrocytoma from high grade astrocytoma but of no value in differentiating anaplastic astrocytoma from glioblastoma- mutilform.

That result of the current study were in agreement with Stanca et al., 2012 study of the CD68 immune-expression revealed that Low intensity (+) was showed by 1/2(50%) cases of diffuse astrocytoma study of the CD68 immune expression revealed that 3/4 (75%) cases of anaplastic astrocytoma express that marker to variable intensity moderate intensity (++) was showed by 2/4(50%) cases of anaplastic astrocytoma, strong (+++) was found in 1/4(25%) of cases and all 5/5(100%) cases of glioblastoma had expressed CD68 with maximum intensity [8].

The results of that study were in agreement with Hewdi et al., 2013 that stated that The density of CD68-positive cells was noted in 85.7% compared with 42.9% of anaplastic astrocytoma (P = 0.004), 38.5% of diffuse astrocytoma (P = 0.08), and 33.3% of pilocytic astrocytoma (P = 0.001).[11]

The results of the current study were in agreement with Strojnik et al., 2010 Results that stated that, the majority (about 85%) of the studied cases expressed CD68 to various extents. (+) staining was found in 64% in low grade astrocytoma (grade I and grade II). They stated that staining for CD68 in high grade astrocytoma (grade III & IV) was significantly more pronounced than in the low grade astrocytoma (grade I & II). High (+++) CD68 IHC expression from the high grade astrocytoma were observed in 80% of the cases. [12]

The results of the current study were slightly different from Yang et al., 2011 that stated that all glioblastoma exhibited CD68-positive cells compared with 86% of pilocytic astrocytoma (p = 0.0014). These differences may be due to the larger number of his cases as it was 91 cases of pilocytic astrocytoma and glioblastoma.[13]

The result of the current study was totally different from results of Klein and Roggendorf 2001 found the highest indices of CD68-positive cells in pilocytic astrocytoma with an average rate of 32% of all proliferating cells. In contrast, the proliferation indices of microglia were lowest in fibrillary astrocytoma with 8.6% of all proliferating cells. In anaplastic astrocytoma and glioblastoma the percentage of CD68-positive cells showed a slight increase to 8.8% and 13.4%, respectively.

These differences in results may be differences in number of cases, different technique of staining, the use of different type of primary monoclonal antibodies, the different method of calculating the IHC scores, or due to use of other markers that proved more immune response of pilocytic astrocytoma as it has good prognosis and decrease the immunity in ascending grades of astrocytoma that explain their virulent nature and aggressive behavior. [2]

The current study revealed that KI-67 labeling index was low <10% in low grade astrocytoma (grade I & grade II) (p. value =0.038 and<0.001) respectively and high >10% in high grade astrocytoma (grade III & grade IV) (p. value =0.002 and0.019) respectively with The
estimates for mean values of proliferative marker Ki-67, in grade I, II, III and IV tumors were 1.5, 3, 13 and 19, respectively.

There were statistically significant values of KI-67 expressions in studied grade I, II, III and IV cases (p. values were =0.038, <0.001, =0.002 and =0.019 respectively).

The comparison of KI-67 expression in studied astrocytoma grade I & astrocytoma grade II cases showed no statistical difference between both grades (P. value<0.05).

The comparison of KI-67 expression in studied astrocytoma grade III & astrocytoma grade IV cases showed no statistical difference between both grades (P. value<0.05).

The comparison of KI-67 expression in studied astrocytoma grade I & astrocytoma grade III cases showed highly significant statistical difference between both grades (P. value<0.001).

So the current study revealed that KI-67 labeling index is of significant value in differentiating low grade from high grade astrocytoma.

The results of the current study were in agreement Stanca et al., 2012 that stated that mean values for proliferative marker Ki-67 in grade II, III and IV tumors were for the whole material 3.5, 11.5 and 16.2, respectively. There was a significant difference between the indices of low- (grade II) and high-grade tumors (grade III and IV) (P<0.05), and that there was a significant difference between the indices of low- (grade II) and high-grade tumors (grade III and IV).[8]

The results of that study were in agreement Suren & Ozen, 2013 Ki-67 labeling index In the DA cases there was a significant increase in the Ki-67 labeling index with increasing grade of disease (p=0.001). The average Ki-67 labeling index was 3.7% in DA, 5.4% in AA, and 25.5% in GBM. [9].

The results of that study were like Sengupta et al., 2012 who stated that Ki67 labeling index (LI) in pilocytic astrocytoma and diffuse astrocytoma was significantly lower than that of high-grade astrocytoma. [14]

The results of that study were in agreement Ambroise et al., 2011 that stated that The difference in the mean MIB-1 LI between pilocytic astrocytoma and diffuse astrocytoma was not significant statistically. However, the mean MIB-1 LI of pilocytic astrocytoma was significantly lower when compared to that of anaplastic astrocytoma (P value, .016) and glioblastoma (GBMs) (P value, <.0001). The mean MIB-1 LI of diffuse astrocytoma was also significantly lower when compared to that of anaplastic astrocytoma (P value, .004) and GBMs (P value, <.0001). [15]

The results of the current study were different from Klein and Roggendorf, 2001 They found that high KI67 labeling index in pilocytic astrocytoma. However, because the proliferation rate does not solely reflect the proliferation of tumor cells demonstrated that proliferation rates in astrocytoma not only reflect proliferation of tumor cells but also that of microglial, especially in pilocytic astrocytoma., authors stressed on a double labeling study [using the antibodies MIB-1 (Ki-67) as proliferation marker and Ki-M1P (CD68) as microglia marker] for final diagnosis. [2]

The results of the current study were different from Uematsu et al., 2005 that proved that The MIB-1 index gave overlapping results and found that in grade II and III, the difference in survival times between the high and low MIB-1 indexes was not significant so MIB 1 index was not sensitive marker in differentiating different grades of astrocytoma or predicting prognosis and overall survival These differences may be due to the use of larger sample size, different technique of staining or different type of monoclonal antibody used. [16]

The current study made a correlation between immune histochemical expression of CD-68 and KI-67 labeling index and the results were as follows.

KI-67 expressions in CD-68 negative astrocytoma cases was statistically in-significant. (p. value >0.05)
KI-67 expressions in CD-68 positive astrocytoma grade I cases was statistically in-significant. (p. value >0.05), but was statistically significant in astrocytoma grade II, III, IV cases (p. value were <0.001, = 0.007, 0.006 respectively).

The correlation between CD-68 immune-histochemical expression and KI-67 labeling index in astrocytoma grade I cases was statistically in- significant. (P. value >0.05), but was statistically significant in astrocytoma grade II, III, IV cases (p. value were <0.001, <0.001, = 0.003 respectively).

So the current study stated that there were direct correlation between both markers and both were increasing with ascending grades of astrocytoma, and may be of benefit in differentiating low grade from high grade astrocytoma. That with the routine hematoxylin& eosin staining and with diagnostic radiography especially MRI can help the neurosurgeon in
planning therapy for patients with different grades of astrocytoma whether surgery alone in case of low grade astrocytoma (grade I&II) or use adjuvant chemotherapy or radiotherapy in high grade astrocytoma (grade III&IV).

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القيمة التعبيرية لعوامل التقييم المناعي النسيجي سي دي-8 وكي-86 في أورام الخلايا النجمية

الملخص العربي

خلفية: تعتبر الأورام الدبقية هي الأكثر أهمية وشيوعا في أورام الدماغ الأولية، وتكون أورام الخلايا النجمية المنتظمة هي الأكثر شيوعا بين الأورام الدبقية. يعتبر تميل سي دي-86 في هذه الأورام يعبر عن المسار الالتهابي لحياة المرضى حيث فكرا ازداد تمثيله ازداد نمو الورم. أما بالنسبة لعامل التقييم المناعي كي-86 هو يعبر عن نمو خليا الورم.

هدف هذه الرسالة هو توضيح القيمة الصناعية المناعية لـ(سي دي-86) و(كي-86) في أورام الخلايا النجمية الممختة. أظهرت الدراسة أن معدل العمر لجميع الحالات تراوحت من (2-73) سنوات. معظمهمن الذكور. وقد أظهرت هذه الدراسة (سي-دي-86) كان إيجابيا في 73.5% من حالات أورام الخلايا النجمية ذات الدرجات المختلفة كان سلبيا تماما في 26.2%. من حالات أورام الخلايا النجمية ذات الدرجات المختلفة. ويعتبر (سي-دي-86) إذا كانت إيجابية بالغة في التمييز بين أورام الخلايا النجمية المنخفضة الدرجة وأورام الخلايا النجمية المرتفعة الدرجة حيث أن (قيمة الاحتمالية م)<0.001. وقد كان مؤشر (كي-86) منخفض في حالات أورام الخلايا النجمية المنخفضة الدرجة إلا أنه كان مرتفعا في أورام الخلايا النجمية المرتفعة الدرجة. ولقد وجد أن (كي-86) كان بالغ الامتعه في التمييز بين حالات أورام الخلايا النجمية المنخفضة الدرجة وأورام الخلايا النجمية المرتفعة الدرجة (قيمة الاحتمالية م)<0.001.

المستنتاج: توصلت هذه الدراسة إلى أن (سي-دي-86) و(كي-86) هما الأكثر أهمية واستخداما للتمييز بين أورام الخلايا النجمية ذات الدرجات المختلفة وخاصة مع الخزعات الصغيرة. وخلصنا إلى أن تطبيق الصباغة المناعية الكيميائية (سي-دي-86) و(كي-86) جنب إلى جنب مع اقتران النتائج بالتشخيص البتولوجي الروتيني هي الطريقة المثلى والدقيقة للتفريق بين أورام الخلايا النجمية ذات الدرجات المختلفة، والذي يعتبر رائدة تشخيصية في بعض الحالات.