Proliferative features of diffuse astrocytic tumors Grade III-IV and their impact on the prognosis

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Anaplastic astrocytoma and glioblastoma are malignant brain tumors with a poor prognosis. The aim of our study was the complex investigation of the factors, those influence their aggressive behavior and proliferation (Ki-67, EGFR, MMP-9, PR, p53, vascular network formation), and the evaluation of their prognostic impact. These data could be used for optimization of target therapy in different groups of patients with diffuse astrocytic tumors Grade III-IV. Our study included 30 patients who were put through brain surgery for the first time and were divided into two equal groups: 15 patients who experienced a recurrence within 1 year after the surgery (1st group) and 15 patients without recurrence within 1 year after the surgery (2nd group). Postoperative tumor materials were obtained in formalin-fixed, paraffin-embedded blocks. In addition, we investigated the case histories of these patients. The expression of Ki-67, EGFR, MMP-9, PR, p53, VEGF, and CD34 was evaluated with immunohistochemical testing. Chi-squared test, Mann-Whitney U and Kruskal-Wallis H tests were performed for comparison of quantitative parameters between groups. Spearman’s rank correlation coefficient was used for the measure of rank correlation between quantitative variables. Results of our study showed, that there was a tendency ($U_{emp}=75.00; p>0.05$) to higher proliferative index in the 1st group ($18.29\pm3.44$) compared to the 2nd group ($16.57\pm3.09$). EGFR expression was significantly higher in the 1st group ($U_{emp}=70.50; p<0.05$). Moreover, the higher EGFR expression was associated with higher MMP-9 expression ($U_{emp}=7.500; p<0.01$) and lower p53 expression ($r_s =-0.62, p<0.001$). The higher MMP-9 expression was also associated with higher vascularization index (MVD(VEGF)/MVD(CD34)) ($r_s=0.43; p<0.05$). Our data confirm the close connections of different factors of tumor aggressiveness and the presence of molecule-biological discrepancy in homogenous histologically, but heterogenous prognostically groups of tumors. This evidence may be used in future for better-personalized therapy of patients with diffuse astrocytic tumors Grade III-IV.

Keywords: glioblastoma, anaplastic astrocytoma, proliferation, tumor aggressiveness, prognosis, EGFR.

Introduction

Diffuse astrocytic tumors Grade III-IV is the most common malignant neoplasms of the brain [15, 17]. Glioblastoma accounted for 46,6% of all malignant tumors of the central nervous system (CNS) [17]. Due to a numerous mutations in anaplastic astrocytomas and glioblastomas the individual prognosis of relapse-free survival and overall survival for different patients can vary significantly and the study of these mutations will allow selecting the most effective targeted therapy for each patient [24]. According to the 2016 WHO classification of tumors of the CNS, it was proved, that patients with IDH1 mutant glioblastomas have better overall survival than patients with glioblastoma IDH-wild type (31 and 15 months respectively) [14, 17]. The immunohistochemical markers, that give information about activity of oncogenesis, are promising in terms of prognosis and open up broad possibilities for the selection of targeted therapy. The family of tyrosine kinase receptors (RTKs) is a good example of such promising markers. The most important members of this family are the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGF) [19, 20]. EGFR belongs to the crucial receptors influencing to the tumor progression [3, 18]. VEGF
is an angiogenic protein and malignant transformation has been shown to induce its expression [5, 6, 19, 22]. According to some scientific reports the VEGF expression in tumor cells of diffuse astrocytic neoplasms is a poor prognostic factor [6, 9, 13]. Glial tumor cells rarely show the progesterone receptor (RP) expression in cytoplasm, nuclei and also in endothelium of blood vessels [11, 25]. But the causes of this phenomenon are still unclear and insufficiently studied.

There is data, that RP can increase EGFR and VEGF expression in tumor cells [8]. On the other hand, the tumor with no or low EGFR expression show the higher levels of p53 tumor protein, that respond of apoptosis [6, 13]. The numerous reports show that anaplastic astrocytomas and glioblastomas with p53 expression grow slower and give a good response to temozolomide and as a result the patients with such tumors have better overall survival prognosis [13, 16].

Matrix metalloproteinase-9 (MMP-9) expression plays an important role in facilitating cancer invasion. According to different research reports, the high MMP-9 expression is a sign of aggressive tumor behavior [1, 4, 6], so the level of MMP-9 expression theoretically can be an important predictive factor of tumor progression.

The gold standard in investigation of proliferative activity of any tumor is the evaluation of Ki-67 proliferative index (PI). Nevertheless, there are no exact levels of Ki-67 PI, those can definitely confirm the worse prognosis for patients with anaplastic astrocytoma or glioblastoma [13, 23].

Contemporary data about the role of expression of each of above-mentioned markers in glioblastomas are inconsistent and need to be specified [10, 21, 27, 28]. There is no complex investigation of the role of Ki-67, EGFR, MMP-9, p53, PR expression and the vascular network development in diffuse astrocytic tumors Grade III-IV, which would be aimed to find the optimal criteria for prognosis. It is caused the short lifespan the patients with diffuse astrocytic tumors Grade III-IV and numerous difficulties due to selection good-quality observation groups.

The aim of our study is the clarification the correlations between expression a number of markers, that describe the oncogenesis (Ki-67, EGFR, MMP-9, p53) and the development of tumor vascular network (VEGF, CD34) in anaplastic astrocytomas and glioblastomas. Moreover, we are tried to find the prognostic value of the expression of these markers by the patients with high-grade diffuse astrocytomas.

Materials and methods
We formed two groups of patients to investigate the proliferative features, aggressive behavior of diffuse astrocytic tumors Grade III-IV and also to find predictive criteria of long-term survival of patients with these tumors. The first group included 15 tumor samples collected from 15 patients, who were primarily diagnosed with a high-grade astrocytic tumor, were treated with surgery and had a recurrence for a year after surgery. The second group included 15 tumor samples collected from 15 patients, who were primarily diagnosed with a high-grade astrocytic tumor, were treated with surgery and did not have a recurrence for a year after surgery. Postoperative tumor material was obtained from pathology departments of Kharkiv Regional Clinical Hospital and Kharkiv City Clinical Hospital №7. All surgical resections were performed between 2011 and 2016. Parts of the tumors were formalin fixed and paraffin embedded (FFPE). All tumors had the supratentorial location and were resected as much as possible (at least 95% of tumor tissue were resected). All patients had a standard course of chemo and radiotherapy. Eligibility criteria included the availability of follow-up data at least for a year after surgical resection, good quality and sufficient quantity of tumor material for immunohistochemical analysis. The details are presented in Table 1.

The histological investigation performed with the light microscope Primo Star (Carl Zeiss), magnification x40, 100, 400, 1000. Results were photographed with ZEISS AxioCam ERC5. The immunohistochemical study was performed with 7 primary antibodies, those are presented in Table 2.

We used a semiquantitative approach for assessment of the results. The expression of EGFR, VEGF, RP scored in such a scale: "-" - no expression; "+" - low expression; "++" - high expression; "+++" - very high expression.

Table 1. The clinical and morphological features of observed tumor samples in two groups.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Age</td>
<td>(M±m)</td>
<td>50.13±10.86</td>
</tr>
<tr>
<td>Tumor location</td>
<td>Frontal lobe</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>Other supratentorial location</td>
<td>10 (67%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Grade</td>
<td>III</td>
<td>1 (7%)</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>9 (60%)</td>
</tr>
</tbody>
</table>

Table 2. The primary antibodies panel.

<table>
<thead>
<tr>
<th>Name</th>
<th>Clon</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>SP84</td>
<td>1:100</td>
<td>ThermoScientific, USA</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Ab-1 GE-213</td>
<td>1:200</td>
<td>ThermoScientific, USA</td>
</tr>
<tr>
<td>Ki-67</td>
<td>SP6</td>
<td>1:400</td>
<td>ThermoScientific, USA</td>
</tr>
<tr>
<td>P53</td>
<td>SP5</td>
<td>1:200</td>
<td>ThermoScientific, USA</td>
</tr>
<tr>
<td>VEGF</td>
<td>JH121</td>
<td>1:20</td>
<td>ThermoScientific, USA</td>
</tr>
<tr>
<td>anti-CD34 antibody</td>
<td>QBEnd 10</td>
<td>1:50</td>
<td>Dako, Denmark</td>
</tr>
<tr>
<td>progesterone receptor (RP) antibody</td>
<td>polyclon</td>
<td>1:200</td>
<td>ThermoScientific, USA</td>
</tr>
</tbody>
</table>
"++" - moderate expression; "++++" - high expression.

For MMP-9 evaluation we counted the percentage of tumor cells that show positive expression. The evaluation was performed in hot spot areas (the foci, where the MMP-9 expression was very prominent).

The proliferative index was evaluated due to the count of the percentage of tumor cells that show positive Ki-67 expression. We considered such levels as 0-4%, under 15% and more than 15% of tumor cells [13].

The index of p53 expression was counted as the ratio of tumor cells with p53 expression multiplied by 100% to the total number of tumor cells in field of view.

The vascularization features were studied due to evaluation of CD34 expression in endothelial cells. VEGF expression helped to value the neoangiogenesis. For the complex investigation of the vascular network in studied tumors, we used such a term as a vascularization index, that can be defined as a ratio of microvascular density (MVD) by VEGF to MVD by CD34.

Vascularization index = MVD(VEGF)/MVD(CD34).

All statistical analyses were performed with the "Microsoft Excel 2010" and the "Statistica 10.0". The significant result was p<0.05. We were used methods of parametric and non-parametric statistics for analysis of immunohistochemical data. In our study we used such criteria as Mann-Whitney U-test, Kruskal-Wallis test, Pearson's chi-squared test ($\chi^2$). The correlation was evaluated with Spearman's rank correlation coefficient. Cheddok scale correlation was also used.

Results

We had evaluated proliferative activity of tumors that relapsed during a year and tumors that didn't. In order to do this, we determined PI based on evaluation of a nuclear expression of Ki-67 in tumor tissue (an example of expression of a marker is shown on figure 1). The average value of PI was higher in a group of tumors which have relapsed during a year (18,29±3,44), against a group of tumors that didn't relapse (16,57±3,09). Observations reveal that average IP of a first group was higher than second's even if anaplastic astrocytomas are excluded from comparison (18,79±2,94) versus (18,00±0,98), although evaluating significance of this difference by Mann-Whitney test did not show reliable difference of IP value between two groups ($U_{emp}=75,00$; p>0,05).

We had also evaluated expression of p53 marker in a group of tumors that relapsed during a year after the surgery and in a group without such relapse. An average percentage of tumor cells that expressed p53 was insignificantly higher in a second group (13,85±4,08) than in a first one (12,73±3,84). Verifying significance of observed differences by Mann-Whitney test did not show reliable difference in p53 expression values between the tested groups ($U_{emp}=88,50$; p=0,336). Some scientists say about direct link between IP and p53 expression by tumor cells but in our research such link was not observed ($r_s=0,08$, p>0,05).

Expression of EGFR was also evaluated. For this evaluation tumors were classified by 2 groups: 1) ones that have no or have weak expression of EGFR by tumor tissue; 2) ones that have moderate or high expression. 46,7% of patients that had relapse during a year appeared in a group with moderate or high expression of EGFR against only 20% of patients that had no relapse. Mann-Whitney test confirms statistically significant difference of EGFR expression between the groups ($U_{emp}=70,50$; p<0.05) (see fig. 2).

Data from literature suggests that EGFR marker is one of the main markers that point to aggressive potential of a tumor so it was important to establish if there is a connection of this marker with clinical and morphological characteristics of tumors being researched. As can be seen from table 3, statistically significant connections were only established with MMP-9 and p53 markers as well as with vascularization index. Further in our study we researched these connections in deeper detail.

Evaluating expression of MMP-9 as a possible...
independent factor that affects prognosis did not reveal statistically significant differences between groups (p>0.05), although tumors that didn’t relapse during a year have in general shown a tendency towards lower MMP-9 that those that did relapse (see fig. 3).

It was revealed that in many cases when expression of EGFR was absent an expression of MMP 9 was also absent. Comparing groups with low and moderate or high EGFR expression had revealed that higher MMP-9 expression is more common for the latter group (Mann-Whitney test: $U_{emp}=7,500; U_{cr}=16; p<0.01$).

A connection has been revealed between EGFR expression level and vascularization index (Kruskal-Wallis test $H (3, N=30) = 10,66 p<0.05$) and a moderately strong link between MMP-9 expression and vascularization index ($r_s=0,43; p<0.05$) (see fig. 4).

In addition we have verified a theory about presence of an inverse relationship between expression level of EGFR and expression of p53. It is believed that EGFR expression is often related with more aggressive tumors and is more common among primary glioblastomas while p53 expression is more common in secondary glioblastomas and is related to slower development of a tumor. Kruskal-Wallis test in our research shows significance of p53 expression in groups with various EGFR expression indicators ($H (3, N=30) = 12.46 p<0.01$). In order to find presence of a correlation we have computed a Spearman rank correlation coefficient which appeared to be $r_s=-0.43; p<0.05$, which points to moderately strong inverse relationship between expression level of EGFR and expression of p53 by tumor cells: higher the expression of EGFR, the lower is the expression of p53 (see fig. 5).

In three cases of tumors that have relapsed during a year there was an expression of progesterone in tissues of a tumor (fig. 6). Cells that expressed progesterone were

<table>
<thead>
<tr>
<th>Feature</th>
<th>Number of cases</th>
<th>EGFR (-)</th>
<th>EGFR (+)</th>
<th>EGFR (++/+++)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>The total number of patients</td>
<td>30</td>
<td>100</td>
<td>11</td>
<td>36,70</td>
<td>9</td>
</tr>
<tr>
<td>MMP-9 &lt;33%</td>
<td>24</td>
<td>80</td>
<td>11</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>MMP-9 &gt;33%</td>
<td>6</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ki 67 &lt;17,4</td>
<td>12</td>
<td>40</td>
<td>6</td>
<td>54,50</td>
<td>5</td>
</tr>
<tr>
<td>Ki 67 &gt;17,4</td>
<td>18</td>
<td>60</td>
<td>5</td>
<td>45,50</td>
<td>4</td>
</tr>
<tr>
<td>Vascularization index &lt;0.67</td>
<td>18</td>
<td>60</td>
<td>9</td>
<td>81,800</td>
<td>6</td>
</tr>
<tr>
<td>Vascularization index &gt;0.67</td>
<td>12</td>
<td>40</td>
<td>2</td>
<td>18,20</td>
<td>3</td>
</tr>
<tr>
<td>P53 &lt;13,29</td>
<td>14</td>
<td>46,70</td>
<td>2</td>
<td>18,20</td>
<td>5</td>
</tr>
<tr>
<td>P53 &gt;13,29</td>
<td>16</td>
<td>53,30</td>
<td>9</td>
<td>81,80</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3. The correlation of main immunohistochemical features with the level of EGFR expression.
located close to necrosis zones. All three tumors had quite high vascularization index (1,170±0,610). It should be noted that these tumors had moderate or high expression of EGFR and a high expression of MMP-9 (the number of tumor cells that express MMP-9, was 41% and higher). This phenomenon was not statistically analyzed due to low number of observations. In biopsies taken from peripheral areas of some tumors with high expression of EGFR there was a clearly observed expression of MMP-9 and VEGF markers in tumor cells.

Discussion

The levels of proliferative index by Ki-67 in high grade astrocytic tumors are the same to WHO data for these tumor histotypes [13,14]. It was not found any influence of PI to the prognosis (p>0.05). Although, there was found a tendency to higher PI in a group with a recurrence within 1 year after the surgery in comparison to group without a recurrence.

The investigation of aggressive behavior of tumors showed that tumors with a recurrence within 1year after the surgery had moderate or high EGFR expression in 46.7% of cases compared to 20% of cases in group of tumors without a recurrence. Mann-Whitney U-test confirmed the significant difference between EGFR expression in these two groups (U=70,50; p<0.05). Our data about the prevalence of tumors with high EGFR expression in the group with adverse prognosis are similar to data of meta-analysis, that was based on 17 research reports and included 1458 tumor samples. According to the data of meta-analysis, the high EGFR expression in the tumor tissue can be used as a significant predictive factor of adverse prognosis and shorter relapse-free survival for patients [12].

There are a lot of data about the presence of some associations between different features of aggressive tumor behavior. The immunohistochemical study presents the activity of different oncogenetical processes due to several criteria, for instance, the level of EGFR expression, the number of MMP-9-positive tumor cells and the level of angiogenesis activity (vascularization index (MVD(VEGF)/MVD(CD34); VEGF expression in tumor cells. It was caused the fact of the stimulating effect of EGFR to the MMP-9 and VEGF synthesis [6,13,19,20]. Our research also confirmed the presence of such association. We found the significant correlation between EGFR expression (++/++++) and the higher percentage of MMP9-positive tumor cells (U_emp=7,50; U critical=16; p<0.01), and the correlation between EGFR expression (++/++++) and higher value of vascularization index (Kruskal-Wallis H-test (2, N=45)=34,19 (p<0.0001)). There is also a direct moderate correlation between vascularization index and the percentage of MMP9-positive tumor cells (rs=0,43; p<0,05).

It should be also mentioned the VEGF and MMP9 expression in the samples obtained from the peripheral tumor regions. The high EGFR expression was also observed in these areas. According to the data of WHO and numerous studies, the VEGF expression in tumor cells is a sign of adverse prognosis [6,9,13]. The fact that this expression was better observed in the peripheral tumor regions pointed out the active tumor growth, so pathologists should carefully investigate besides central tumor regions also its peripheral zones to understand totally the level of tumor aggressive potential [5,6,9]. The high VEGF expression in tumor cells may serve as a direct indication for bevacizumab prescription [5,7].

It was found the significant difference among p53 expression in groups with different levels of EGFR expression (neg, +, ++, ++++) (Kruskal-Wallis H-test (3, N=30) = 12.46, p<0.01) and the presence of the moderate inverse correlation between EGFR expression and p53 expression (rs = -0.62, p<0.001). Our data are similar to scientific data, that confirmed that the level of EGFR expression was opposite to the level of p53 expression. It is explained by the fact that tumors with high EGFR expression usually belong to primary glioblastomas and tend to progress faster than the tumors with high p53 expression that grow slower and usually associated with secondary glioblastomas [2,6,13,26].

Worth to be mentioned also such an observation as 3
cases of PR expression in tumors with a recurrence within 1 year after the surgery. These observations were not statistically analyzed, because the number of cases was insufficient, but some researchers mentioned, that RP was able to influence to the proliferation due to increasing the EGFR and VEGF synthesis in tumor cells. This mechanism is caused by Ser345-phosphorylation of RP-β, that in its turn is associated with Sp-1 transcription factor, that regulated EGFR expression [8].

In the future, it will be planned to investigate the proliferative features of high grade diffuse astrocytic tumors and their aggressive potential in larger groups of patients with not only immunohistochemical, but also molecular-genetical methods. In addition, it will be tracked the 3-year and 5-year survival among groups of patients with initially different prognosis and to figure out if the trends that we found in this research are constant with the length of time. We are also going to deeply investigate the molecular features of early and remote relapses of high grade diffuse astrocytic tumors. The obtained result may be used for development of the more effective chemotherapy and may be interesting for oncologists when they choosing the most effective treatment strategy for patients.

References
Яковцова І. І., Гаврилюк А. О., Чертенко Т. М.

Анапластичні астроцитоми та гліобластоми є високо злоякісними пухлинами з поганим прогнозом. Метою нашого дослідження було комплексне вивчення факторів, що сприяють формуванню агресивного потенціалу цих пухлин та впливають на їх проліферативну активність (Ki-67, EGFR, MMP-9, PR, р53, розвиненість судинного русла), а також визначення вірогідного прогностичного значення цих факторів та взаємозв'язків між ними, що в майбутньому може бути корисно для оптимізації таргетної терапії хворих на дифузні астроцитарні пухлини ІІІ-ІВ ступенів злоякісності. Для дослідження була відібрана післяопераційна матеріал від 30 вперше прооперованих з приводу пухлини пацієнтів, що були розподілені на 2 рівні групи: 15 пацієнтів, які мали рецидив протягом року після операції та 15 пацієнтів без рецидиву протягом року.

Інформація наступних маркерів: Ki-67, EGFR, MMP-9, PR, р53, VEGF та CD34. Для оцінки статистично значущих відмінностей між однорідними за гістологією, але різними за прогнозом групами дифузних астроцитарних пухлин ІІІ-ІВ ступенів злоякісності, використовували наступні показники: Кількість ембріональних клеток (Ki-67), експресія EGFR, MMP-9, PR, р53, розвиненість судинного русла.

Більш висока експресія EGFR була пов'язана з більш високою експресією MMP-9 (p<0,001), більш висока експресія EGFR була пов'язана з більш високою експресією MMP-9 (U=75,00; p<0,05) та більш низькою експресією p53 (U=7,500; p>0,05). Більш висока експресія EGFR була пов'язана з більш високою експресією MMP-9 (U=7,500; p=0,01) та більш низькою експресією p53 (r = -0,62, p<0,001). Більш висока експресія MMP-9 також була пов'язана з більш високою експресією MMP-9 (r = 0,43; p<0,005).

Таким чином, дослідження показало, що вплив факторів EGFR та MMP-9 на прогностичні показники пухлин впливає на вплив факторів агресивності пухлин, а також вказує на наявність молекулярно-біохімічних відмінностей між однорідними за гістологією, але різними за прогнозом групами дифузних астроцитарних пухлин ІІІ-IV ступенів злоякісності, що в подальшому може бути використано для оптимізації стратегії лікування хворих на ці пухлини.

Ключові слова: гліобластома, анапластична астроцитома, особливості проліферації, агресивний потенціал, прогноз, EGFR.
Проводился анализ экспрессии таких маркеров: Ki-67, EGFR, MMP-9, PR, р53, VEGF и CD34. Для оценки статистически значимых различий между группами использовали следующие критерии: χ² Пирсона, критерий Манна-Уитни, критерий Краскера-Уоллиса. Корреляционная связь между количественными показателями оценивали с помощью коэффициента корреляции Спирмена. Выявлена тенденция (Uэмп=75,00; р>0,05) к более высокому индексу пролиферации в группе опухолей, давших рецидив в течение года (18,29±3,44) по сравнению с группой без рецидивов (16,57±3,09). Экспрессия EGFR была достоверно выше в группе опухолей с рецидивами в течение года (Uэмп=70,50; p<0,05). Более высокая экспрессия EGFR была связана с более высокой экспрессией MMP-9 (Uэмп=7,50; p<0,01) и более низкой экспрессией р53 (r = -0,62, p<0,001). Более высокая экспрессия MMP-9 также была связана с более высоким индексом васкуляризации (ПМР/VEGF/ПМР/CD34) (r = 0,43; p<0,05). Полученные результаты свидетельствуют о тесной взаимосвязи различных проявлений агрессивного потенциала опухолей, а также наличии молекулярно-биологических различий между однородными по гистологии, но различными по прогнозу группами опухолей, что в дальнейшем может быть использовано для оптимизации стратегии лечения пациентов с диффузными астроцитарными опухолями III-IV степени злокачественности.

Ключевые слова: глиобластома, анапластическая астроцитома, особенности пролиферации, агрессивный потенциал, прогноз, EGFR.