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Prognostic value of CD3, CD4, CD8 and CD 68 in high-grade diffuse astrocytomas, the transformation of an immune profile in recurrent tumors

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Abstract

Introduction and purpose. Immunotherapy has shown good results in the treatment of other cancers. There is a lack of data about the role of the immune system in high-grade glioma development. Moreover, the available data is ambiguous. Our recent study was aimed to clarify the prognostic value of T-cell and macrophage infiltration in high grade diffuse astrocytic tumors. In addition, we also investigated the changes in immune cell infiltration in paired primary and recurrent tumors. **Methods.** This study included 45 tumor samples that were collected from 15 patients without recurrence within 1 year after the surgery and from 15 patients who experienced a recurrence within 1 year after the surgery (30 paired samples). Immunohistochemistry with primary antibody CD3, CD4, CD8 and CD68 was used in our study. We marked lack of immune cell infiltration or single cell infiltration as "1" in our study and moderate or high infiltration as "2". **Results.** CD68 "2" expression in tumor tissue is a significant independent factor of the worse prognosis (Fisher's exact p-value=0,037). The significantly better prognosis was given to patients with such immune patterns as CD3 "1"/CD4 "2"/CD8 "2" and CD3 "1"/CD4 "2"/CD68 "2" (Fisher's exact p-value=0,01869 and 0,01392 respectively). We also found that solid tumors with CD3 "2"/CD8 "1" and CD8

"1"/CD68 "2" immune had a significantly worse prognosis (Fisher's exact p-value= 0,04981 in both groups). In relapsed tumors, we observed a significant increase of CD8-cell infiltration (McNemar test p-value=0,01343;p <0,05) that probably may be a result of treatment with temozolomide. **Conclusion.** Our findings confirmed the fact that immune cell infiltration may play an important role in brain tumor development. Further study in larger groups may lead to a better understanding of different immune profiles of glial tumors and to development of their targeted immunotherapy.
Keywords: glioblastoma; T cells; CD68; paired tumors; immunohistochemistry; prognostic markers.

Introduction

Anaplastic astrocytoma(AA) and glioblastoma (GBM) are the high grades diffuse astrocytic tumors. GBM is the most frequent primary brain tumor [14, 17]. According to CBTRUS data, glioblastoma accounted for 14,9% of all primary brain and other tumors of the central nervous system (CNS) in the USA [17] and WHO reported that 12-15% of all intracranial neoplasms are GBMs [14]. Despite progress in the therapy of brain tumors the overall survival of patients with high grade diffuse astrocytic tumors remains poor. Surgical resection is rarely performed at recurrence that leads to a lack of information regarding the pathological and molecular features of relapsed AAs and GBMs [5]. AA progressed to GBM for 2 years on average. [14]. In retrospective population-based studies from Canada and Switzerland, less than 20% of patients with GBM survived more than 1 year and less than 3% lived longer than 3 years [14].

The immunotherapeutic approach has shown great benefit in different types of cancers. The CNS, at the same time, was accepted, traditionally, as an immune-privileged organ, because of the presence of the blood-brain barrier (BBB) and absence of lymphatic vessels. [11] The recent research has shown that activated immune cells are able to cross the BBB, which is often disrupted at the tumor site [2, 11, 19, 24]. Thus, the role of the immune system in brain tumor progression is still ambiguous. On the one hand, GBM can release of soluble immunosuppressive factors and some immune cell subpopulations promote tumor progression.[7, 16] On the other hand, several research reports pointed at the fact that patients with activated NK cells in addition to T cell receptor (TCR) α/β-positive and CD8-positive lymphocytes displayed a strong correlation with increased survival in GBM. [11, 15] Furthermore, it was confirmed that microglial cells may play the role of antigen-presenting cells. Intratumoral microglia density is higher than in the peritumoral and normal brain, and

microglia increase in number according to the grade of malignancy [1, 20], some authors reported that more than 30% of the GBM tissue consisted of tumor-associated microglia/macrophages (TAMs). [20, 21]

In the present study, we tried to concentrate on the T cell lymphocytic (CD3, CD4, CD8-positive lymphocytes) and macrophage (CD68-positive) infiltration and their complex impact on the tumor progression. We knew that approximately 80% of GBM patients die or have a recurrence in the first year after surgical treatment. Hence, we supposed that patients, which are still alive and do not have a recurrence, will have a better prognosis. We compared data from 2 groups of patients, who had a relapse and did not have a relapse in the first year after operation. It gave us an opportunity to detect several immune profiles of tumors that are able to influence on prognosis. Better prognosis should have patients with such combinations of immune cell infiltrations: low or no CD3-positivity/moderate or high CD4-positivity/ moderate or high CD8-positivity and low or no CD3-positivity/moderate or high CD4-positivity/moderate or high CD68-positivity (Fisher's exact p-value=0,01869 and 0,01392 respectively). The worse prognosis was observed in patients with independent moderate or high CD68 infiltration of tumor tissue (Fisher's exact p-value=0,037) and in patients with a solid structure of tumors and such immune profiles as moderate or high CD3-positivity/ low or no CD8 -positivity and low or no CD8 –positivity/ moderate or high CD68-positivity (Fisher's exact p-value= 0,04981 in both groups). We also analyzed paired primary and recurrent tumors, because one of our goals was the investigation of tumor transformation before and after standard treatment. The current study has shown the significant increase of CD8 lymphocytic infiltration in relapsed tumors (p-value=0,01343;$p < 0,05$).

Materials and methods

Patients and tumor material

Tumor samples for our study were taken from 2 groups of patients. 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 did not have a recurrence for a year after surgery. The second group 30 paired primary and recurrent tumor samples collected from 15 patients diagnosed with a high-grade astrocytic tumor. All these patients had tumor recurrence less than 1 year after primary surgical resection. They were treated with radiotherapy and chemotherapy with temozolomide (TMZ). The general characteristics of both groups were given in Tab.1.

Table 1. General characteristics of 2 groups of patients, who were primarily diagnosed with a high-grade astrocytic tumors

Groups / Feature	1	2
Sex		
m	9 (60%)	9 (60%)
f	6 (40%)	6 (40%)
Age		
M±σ	56,2±12,295	50,13±10,86
Intratumoral cysts		
Yes	6 (40%)	7 (47%)
No	9 (60%)	8 (53%)
Grade		
III	3 (20%)	1 (7%)
IV	12 (80%)	14 (93%)

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 2017. Parts of the tumors were formalin fixed and paraffin embedded (FFPE). 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.

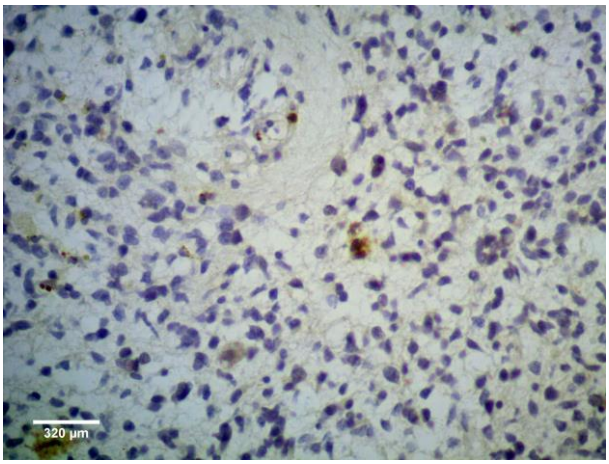
Immunohistochemistry

FFPE tissue was subjected to immunohistochemistry (IHC) using the indirect peroxidase anti-peroxidase (PAP) complex method according to the Dako protocol for manual IHC staining. FFPE sections were immunolabeled with rabbit anti-human CD3 early T-cell marker, 1:150 (ThermoFisher Scientific, USA); mouse anti-human CD4 clone 4B12, ready-to-use (Dako, Denmark); rabbit anti-human CD8 clone SP16, 1:50 (ThermoFisher Scientific, USA) and mouse anti-human CD8 cloneKP1, ready-to-use (Dako, Denmark) primary monoclonal antibodies. Counterstaining was performed with Mayer's hematoxylin. Human tonsil tissue was used for external positive control. Results were visualized and photographed under a light microscope (ZEISS Primo Star, ZEISS Axiocam ERc5).

We used a semiquantitative approach for assessment of the results. It means, that we evaluated the intense of protein expression and percentage of immune-positive cells. The evaluation was performed by examing each section using at least 5 different high-power fields of view (magnification 400x) in which the maximum number of immune-positive cells were observed (Fig.1) As the result of our research, we decided that the most convenient approach is the division of tumor samples into 2 groups. The first group included samples with no

expression or weak expression of a marker and single immune-positive cells. We were labeled this group with tag “1” (CD3 “1”, CD4 “1”, CD8 “1”, CD68 “1”). The second group included samples with moderated or strong expression of a marker and numerous immune-positive cells. We were labeled this group with tag “2” (CD3 “2”, CD4 “2”, CD8 “2”, CD68 “2”).

a



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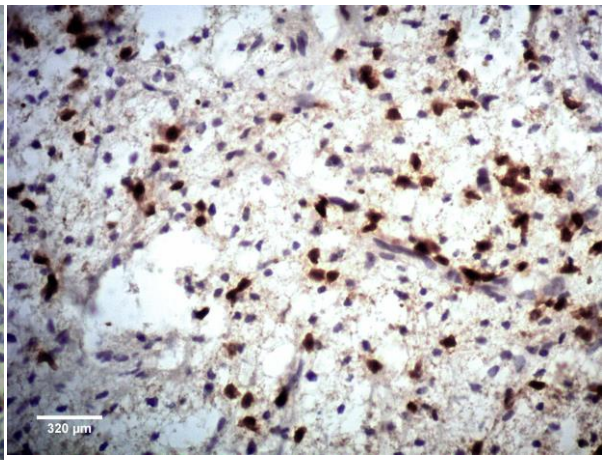

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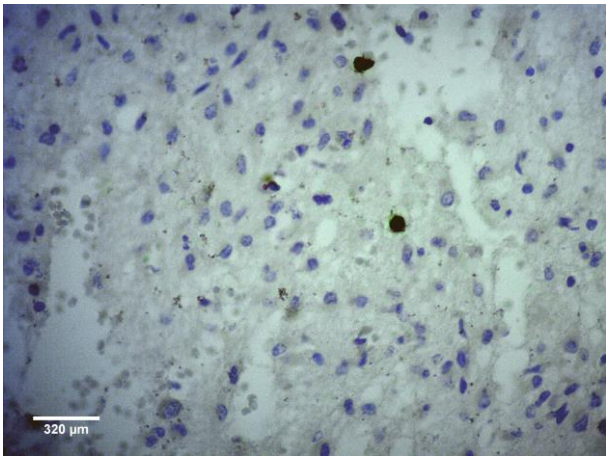

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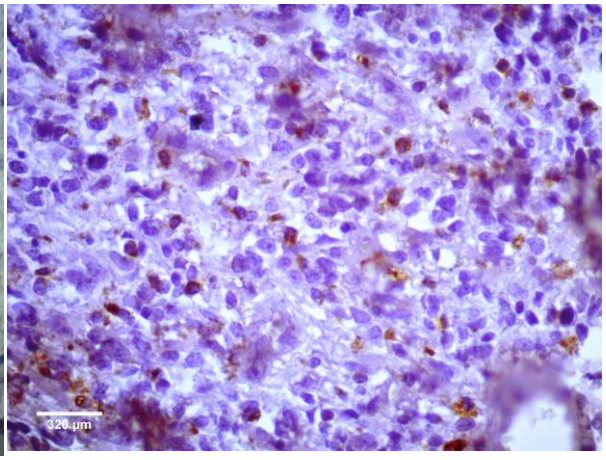

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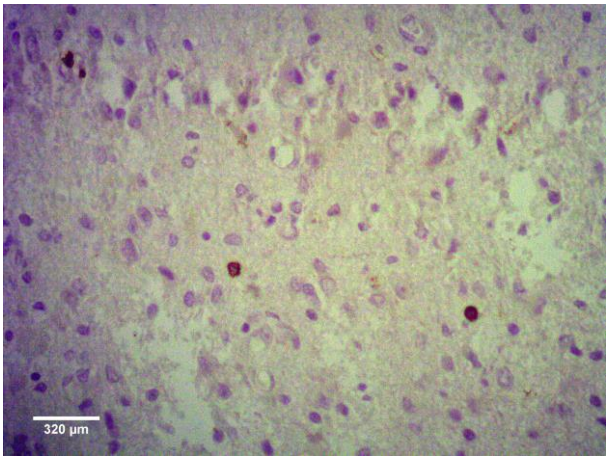

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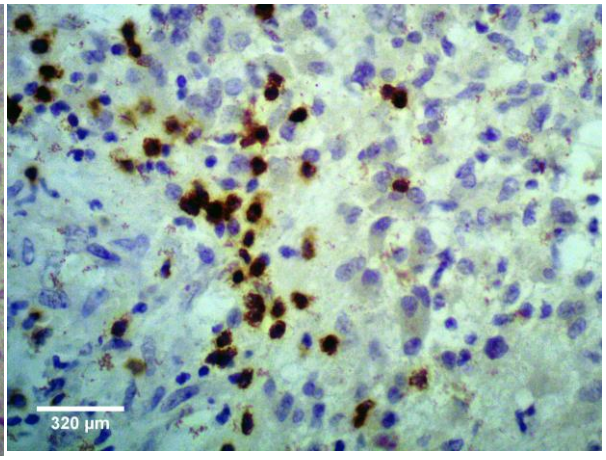

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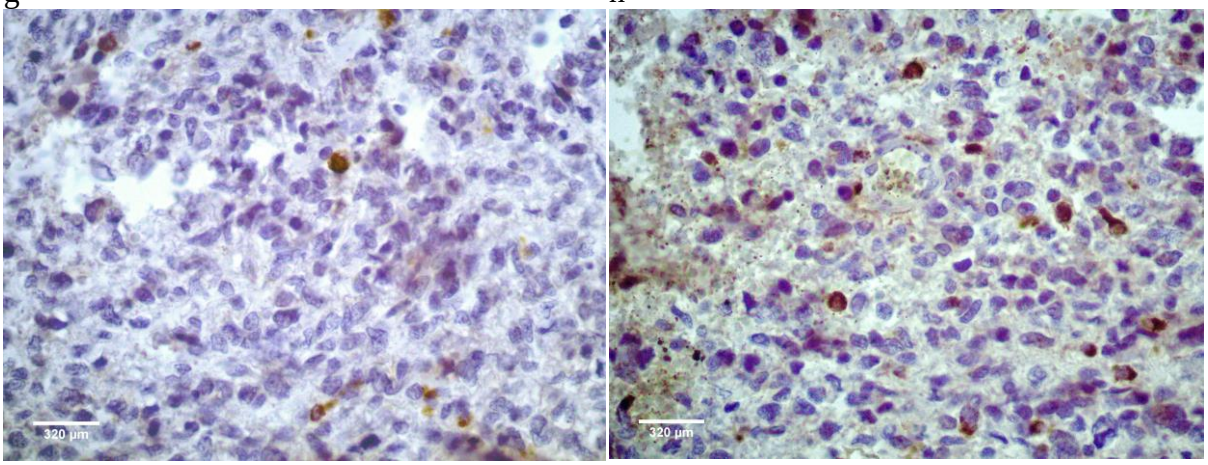

Fig. 1 Immune cell infiltration in tumors, representative IHC staining of tumor samples, magnification – 400x; **a**: CD3 staining – low number of CD3 positive cells, **b**: CD3 staining – high number of CD3 positive cells, **c**: CD4 staining – low number of CD4 positive cells, **d**: CD4 staining – moderate number of CD4 positive cells, **e**: CD8 staining – low number of CD8 positive cells, **f**: CD8 staining – high number of CD8 positive cells, **g**: : CD68 staining – low number of CD68 positive cells, **h**: moderate number of CD68 positive cells.

Statistical analysis

The relationship between different features of tumors and their prognostic impact were estimated using the Fisher's exact test (Fisher's exact p-value<0,05). The McNemar test was used to compare CD3, CD4, CD8 and CD68 expression in paired samples. The mean (m) and standard deviation (σ) were also calculated. An extensive parameter (%) was used to describe qualitative characteristics. All statistical analyses were performed with the "Microsoft Exel 2010" and the "Statistica 10.0".

Results

Prognostic significance of CD3, CD4, CD8, and CD 68 infiltration

We analyzed infiltration by CD3, CD4, CD8 and CD68 immune cells in 2 groups of samples (Tab.2). The first group consisted from 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. The second group included 15 tumor samples collected from 15 patients primary diagnosed with a high-grade astrocytic tumor, were treated with surgery and had a recurrence of less than 1 year after primary surgical resection.

Table 2. Demographics, clinical and immunohistochemical data about primarily diagnosed tumors analyzed in the study

Patient	Group	Sex	Age	Cysts[1]	Diagnose[2]	CD3[3]	CD4[3]	CD8[3]	CD68[3]
A	2	f	59	1	GBM	2	2	1	1
B	2	f	48	1	GBM	2	2	2	1
C	2	m	62	0	GBM	2	2	1	2
D	2	f	48	1	GBM	2	2	1	1
E	2	m	59	0	GBM	2	2	1	2
F	2	f	60	1	GBM	2	2	1	2
G	2	m	53	0	AA	2	1	2	2
H	2	m	37	0	GBM	2	1	2	2
L	2	m	31	1	GBM	2	2	1	2
M	2	f	29	1	GBM	1	1	2	2
N	2	m	57	0	GBM	2	1	2	2
O	2	m	56	0	GBM	2	2	1	2
P	2	f	43	0	GBM	2	2	1	1
Q	2	m	49	0	GBM	1	2	1	2
R	2	m	61	1	GBM	2	2	1	2
AM	1	m	74	0	GBM	2	2	1	1
AW	1	m	62	0	GBM	1	2	2	2
AX	1	m	65	0	GBM	2	2	2	1
AZ	1	f	59	1	GBM	2	1	2	2
J	1	f	43	1	AA	1	2	1	1
OM	1	m	50	0	GBM	2	2	2	1
PR	1	f	65	0	GBM	2	1	2	2
S	1	f	27	0	AA	1	2	1	2
T	1	m	60	1	GBM	1	2	2	2
U	1	m	61	1	GBM	2	2	2	1
V	1	f	63	1	GBM	2	2	1	1
W	1	m	44	1	AA	2	2	2	1
X	1	f	67	0	GBM	2	2	1	1
Y	1	m	43	0	GBM	2	2	2	1
Z	1	m	60	0	GBM	2	1	2	2

[1] 1- intratumoral cysts, 0 – solid structure
[2] GBM – glioblastoma, AA- anaplastic astrocytoma
[3] 1- no or single cell infiltration, 2- moderate or high infiltration

Independent significant prognostic impact had only CD68 infiltration. Tumors with CD68 "2" expression had a higher recurrence risk for a year after primary surgical resection than tumors with CD68 "1" expression. (Fisher's exact p-value=0,037).

We also observed in some samples expression of CD68 by tumor cells (Fig.2). 2 tumors, which recurred, showed CD3 expression by tumor cells (Fig.3).

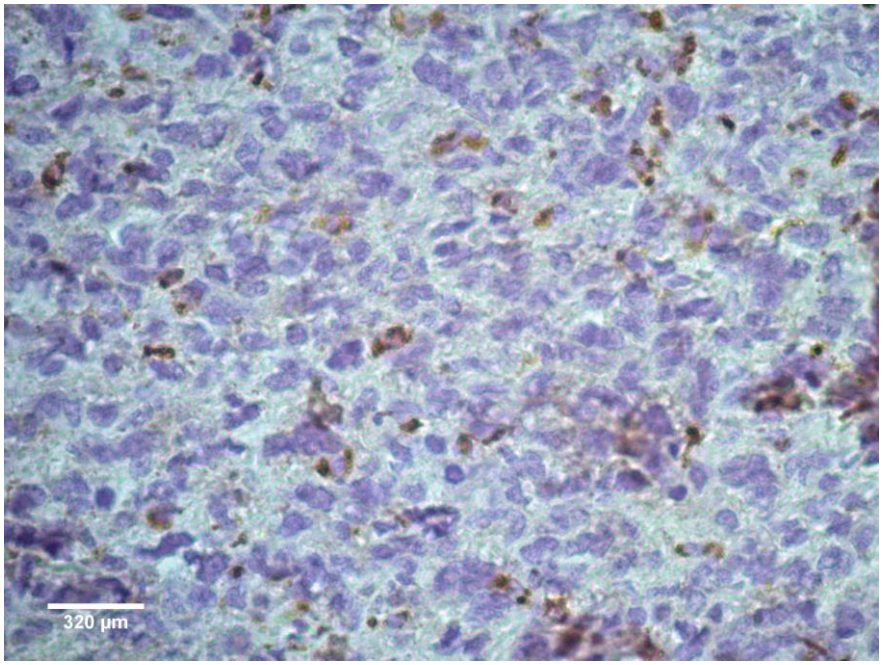

Fig. 2 Expression of CD68 in tumor cells. Glioblastoma sample, IHC staining, magnification – 400x.

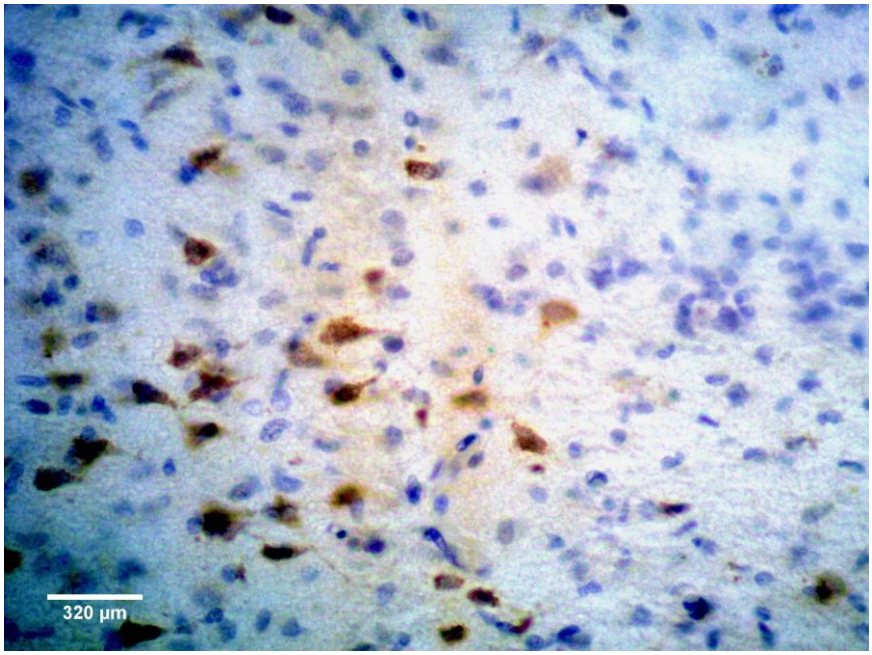

Fig. 3 Expression of CD3 in tumor cells. Anaplastic astrocytoma sample, IHC staining, magnification – 400x.

We took into account the complexity of immune defense mechanisms and analyzed combined lymphocytic and macrophage infiltration status. Two important combinations were found. The combined infiltration by CD3 “1”/CD4 “2”/CD8 “2” as also the combined infiltration by

CD3 “1”/CD4 “2”/CD68 “2” were the significant factors for lower risk of for a year after primary surgical resection (Fisher’s exact p-value=0,01869 and 0,01392 respectively). We assumed that CD8 infiltration did not play here crucial role, because in our study we have 2 patterns of tumor infiltration: CD3 “1”/CD4 “2”/ CD8 “1”/CD68 “1” and CD3 “1”/CD4 “2”/ CD8 “2”/CD68 “1”. We had 71,4% of no recurrent tumors in the first case and 83,3% of no recurrent tumors in the second case, but Fisher’s exact p-value were > 0,05 in both cases. When we excluded CD8 infiltration and consolidated these group we became Fisher’s exact p-value=0,01392.

It was also evaluated the role of sex, age and tumor cysts in prognosis. No one of these factors played an independent role in tumor progression. But in our study, we found that such combinations as solid structure/CD3 “2”/CD8 “1” and solid structure/CD8 “1”/CD68 “2” showed higher risk of recurrence for a year after primary surgical resection (Fisher’s exact p-value= 0,04981 in both groups).

Tumor transformation

We analyzed changes in infiltration by CD3, CD4, CD8 and CD68 in paired primary and recurrent tumor samples. The results were collected in table 3 and illustrated in the diagram (Fig.4).

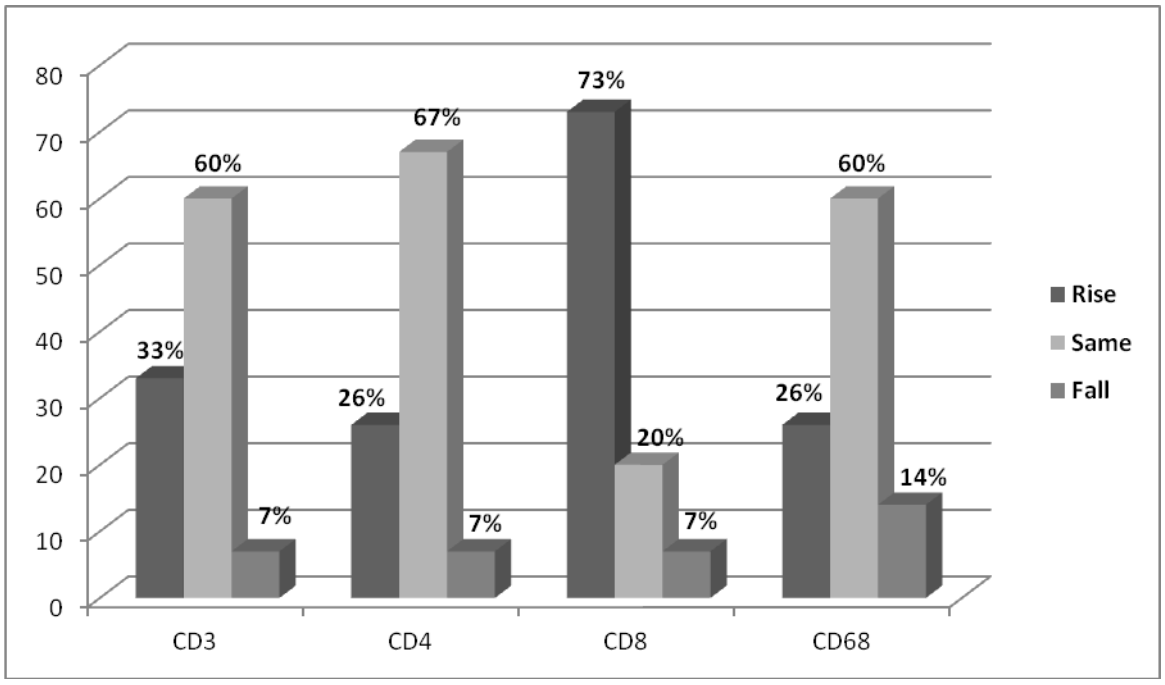

Fig. 4 The changes of CD3, CD4, CD8 and CD68 intratumoral infiltration in paired cases (before and after standard treatment) in percentage (%)

Table 3. The changes of CD3, CD4, CD8 and CD68 intratumoral infiltration in paired cases (before and after standard treatment)

	CD3		**CD4**		**CD8**		**CD68**	
	Number	%	Number	%	Number	%	Number	%
Rise	5	**33**	4	**26**	11	**73**	4	**26**
Same	9	**60**	10	**67**	3	**20**	9	**60**
Fall	1	**7**	1	**7**	1	**7**	2	**14**
Total	15	**100**	15	**100**	15	**100**	15	**100**

It is well seen from the diagram that the biggest changes were in the expression of CD8. 73% of patients showed the increase of CD8 expression. This data was statistically proved with the McNemar test (p-value=0,01343; p <0,05). It means that there is a significant increase in CD8 expression in recurrent tumor samples.

Discussion

There are contradictory research reports about the impact of different populations of immune cells on high grade diffuse astrocytic tumor progression. For instance, some authors in their reports showed, that significant increase of CD3- T-cell infiltration in GBMs was independent factors of better survival prognosis [11]. In our study, we found only the influence of increased CD68 cells infiltration on prognosis. Patients with CD68 "2" infiltration had a worse prognosis than patients with CD68 "1" infiltration (Fisher's exact p-value=0,037). Moreover, we figured out that some tumor cells were able to adopt a macrophage phenotype and express CD68 marker. Our data confirmed results of the research report of Strojnik T. and co., that said about a role of high CD68 infiltration as an independent factor of worse prognosis [22]. Some authors also described the expression of CD68 by human malignant astrocytes as a sign of more aggressive tumor behavior [13, 22].

Our study showed also that such immune patterns as CD3 "1"/CD4 "2"/CD8 "2" and CD3 "1"/CD4 "2"/CD68 "2" may be a factor of a better prognosis (Fisher's exact p-value=0,01869 and 0,01392 respectively). In the first case, we were uncertain, if the level of the main T-cell marker (CD3) may be lower than the level of its subsets: CD4 and CD8 lymphocytes. But then we found that lymphocytes in some stage of differentiation may have such combinations of CD receptors.[6] The most useful for us was the report of Jenny N. Penttila and co., who found the cell line with pattern $CD3^-/CD4^+/CD8^+$ in mice, that were infected with *Chlamydia pneumonia*. These cells were able to proliferate but did not secrete gamma interferon. [18].

Furthermore, in other scientific reports, we found information about the ability of gamma-interferon to elevate the number of program death ligand1 (PD-L1) in glioblastoma cells [12, 25]. PD-L1 is a subset of program death 1 (PD-1) protein that helps glioma cells to escape from immune recognition and injury and finally leads to worse survival prognosis. Moreover, some reports confirmed that PD-L1 expression was associated with CD-3 positive T-cell infiltration [3, 12]. This difficult chain of reactions gave us a clue to suppose that our finding of CD-3 expression in some GBM cells may be a result of immune evasion mechanism of the tumor. But taking into account the small number of objects of study, we cannot but exclude false-positive reaction on CD3 marker in tumor cells hence this data needs more careful investigation in larger groups.

Our study showed also a bend of the immune profile with tumor structure, scilicet a relationship of such combination as solid structure/CD3 “2”/CD8 “1” and solid structure/CD8 “1”/CD68 “2” with a worse prognosis (Fisher’s exact p-value= 0,04981 in both groups). The data of numerous research reports provide us with the information that most GBMs have a solid structure. [4, 23] In addition, the cysts in gliomas are more typical for lower grade diffuse astrocytomas and associated with better survival prognosis [8, 23]. We tend to think that in association with low activity of cytotoxic ($CD8^+$) T cells and high or moderate CD68 or CD3 infiltration, which, as we had already described, could be associated with worse prognosis, these combinations may be a strong predictive factor of worse survival.

Our study of tumor transformation showed us a significant increase in CD8 T cells infiltration in tumor samples (p-value=0,01343;p <0,05). Different research reports confirmed the ability of chemotherapy with TMZ to elevate the number of CD8 cells [9, 10] All our patients took TMZ, based on this fact, we supposed that significant increase of CD8 T cells infiltration may be a result of chemotherapy with TMZ. Moreover, as we supposed in our study, an independent increase of CD8 T cell infiltration did not play a crucial role in prognosis, hence only elevated levels of CD8 T cell infiltration were not able to protect patients from recurrence. All this information may explain why we did not find any other significant changes in recurrent tumors.

Acknowledgments

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samples. We are also grateful to the Departments of Neurosurgery of these hospitals for provided access to patients' case histories and follow-up data about their treatment.

Author contribution

TCh and IY designed research. TCh collected all tumor samples and analyzed clinical data. TCh, OD, and SD performed research. SD prepared tumor slices and carried out immunohistochemistry. IY and TCh provided antibodies. TCh and OD analyzed and interpreted the immunohistochemical data. TCh wrote the manuscript and generated the figures.

Compliance with ethical standards

Conflict of interest. The authors have declared that no conflict of interest exists.

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