ORIGINAL PAPERS

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Expression and Interactions Between Cell Adhesion Molecules CD44v6 and E-Cadherin in Human Gliomas*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation;
D – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Gliomas are a heterogenous group of tumors that show the same histological features but differ in their behavior. Gliomas are characterized by biological aggressiveness and extensive infiltrative growth into surrounding healthy brain tissue.
Objectives. In this study we estimated CD44v6 and E-cadherin expression and correlation between CD44v6 and E-cadherin in relation to glioma malignancy. We also analyzed simultaneous expression of CD44v6 and E-cadherin in the same tumor sample in order to determine the biological tumor behavior.
Material and Methods. Expression of CD44v6 and E-cadherin was evaluated on ninety-two formalin-fixed paraffin-embedded glioma tissue blocks using immunohistochemistry (IHC).
Results. CD44v6 expression was found in 71.6% of gliomas. There was a statistically significant difference between the frequency of positive cases for CD44v6 expression in low (grade I) vs. high (grade IV) as well as in grade I vs. grade II of glioma malignancy (p = 0.001). E-cadherin membrane staining was observed in 28.8% of gliomas. No significant differences were observed between E-cadherin expression and grade of gliomas (p > 0.05). However, re-expression of E-cadherin was found in grade II gliomas. In this group, E-cadherin expression was revealed in 43.3% of the cases. In order to define the relationship between CD44v6 expression and E-cadherin, we analyzed the simultaneous expression of CD44v6 and E-cadherin in the same glioma sample in the whole group and in respect to the degree of glioma malignancy. A positive correlation between studied biomarkers was observed in the analyzed gliomas (p = 0.004) but a simultaneous expression of CD44v6 and E-cadherin revealed no significant differences in respect to glioma malignancy.
Conclusions. Our results showed that the level of E-cadherin might reflect different biological features of gliomas, whereas CD44v6 is associated with tumor cell malignancy. The simultaneous presence of CD44v6 and E-cadherin in a set of low-grade gliomas indicates that both these molecules might strengthen cell migration and may be a hallmark of glioma invasive growth (**Adv Clin Exp Med 2014, 23, 5, 827–834**).

Key words: gliomas, CD44v6, E-cadherin, immunohistochemistry.

Gliomas comprise a heterogenous group of tumors that are histologically similar but different in biological behavior [1]. Gliomas show different stages of malignancy but the most aggressive form of brain tumors is glioblastoma [1]. Nevertheless, most low-grade brain tumors display extensive infiltrative growth into the surrounding normal brain tissue [2]. The infiltration pathway of glioma cells into the normal brain parenchyma is a complex process, including detachment of glioma cells from the original site, adhesion to the ECM (extracellular matrix), remodeling of the ECM, and cell migration [3–5].

Many factors are involved in each step of glioma invasion [5]. Up to now, the mechanisms as well as the factors that facilitate glioma cell migration form the primary tumor mass have been unknown [6].

Recent studies focusing on the function and relationship between different proteins involved in progressive growth of human brain tumors revealed that surface receptors and adhesion molecules might be associated with glioma cell migration [4, 6]. The pattern of glioma cell invasion is related to unique biological features, such as up-regulation of the transmembrane surface receptor and down-regulation of the molecules that regulate cell-cell adhesion [4, 6].

CD44 is a transmembrane glycoprotein expressed in different types of tumors and normal tissues [7]. CD44 exists as multiple isoforms and these isoforms are generated by alternative splicing of up to 20 exons [8]. It has been revealed that CD44 isoforms containing exon v6 can promote cell motility, inhibit apoptosis and promote tumor progression [9]. CD44v6 are expressed in cancers of different origin and are associated with aggressive tumor behavior [10]. Overexpression of CD44v6 was observed in breast and gastric cancers [11, 12]. In brain tumors, expression of CD44 and its isoforms was observed [13]. The overexpression of CD44 variant isoforms in gliomas mediates migration and invasion of glioma cells into normal brain parenchyma [13]. Recently, a study showed CD44v6 expression in glioblastomas, whereas CD44v6 was not detected in normal mouse brain or neural progenitors [8]. Jijiwa et al. [8] suggest that CD44v6 expression is associated with brain tumor stem cells and CD44v6-targeted therapy may reduce the growth of brain tumor stem cells and non-tumor stem cells with different potency. Cadherins are a superfamily of cell surface glycoproteins involved in cell-cell adhesion during a variety of biological processes occurring in normal and tumor tissues involving morphogenesis, cell movement, proliferation, and tumor invasion [4, 14]. Cadherins mediate cell-cell adhesion and induce cadherin signaling with β-catanin, p120-catenin (p120), and receptor tyrosine kinases (RTK) [14]. Disruption of normal cell-cell adhesion and loss of E-cadherin play a prominent role in malignant transformation enhancing migration, which occurs during epithelial-to-mesenchymal transition (EMT), leading to invasion and metastasis [14]. Several studies revealed that poorly differentiated and advanced carcinomas commonly have low or undetectable E-cadherin levels [14]. Expression of E-cadherin protein is a rare event in tumor and normal brain tissue [4, 15]. There are studies which showed that E-cadherin expression decreases with tumor grade malignancy when compared to normal brain tissue

and the authors pointed out that epithelial-to-mesenchymal transition might be involved in glioma progression [16, 17]. Hence, in epithelial tumors down-regulation of E-cadherin leads to a decrease or loss of cell adhesion to other cells as well as detachment from the basement membrane, thereby facilitating cells motility [14]. It seems to be interesting to study whether E-cadherin is responsible for cells adhesion in gliomas, whether the loss of E-cadherin expression reflects the damage of structural integrity of brain tissue. So far, the balance between cell-cell adhesion molecule members of family (CAM) and cell motility in primary brain tumors has not been fully analyzed [1, 18]. Moreover, the cooperation between these cell-cell adhesion molecules and their role in glioma progression has not been studied. In this study we estimated CD44v6 and E-cadherin immunohistochemical expression and correlation between CD44v6 and E-cadherin in relation to glioma malignancy. We also analyzed simultaneous expression of both molecules in the same tumor sample in order to determine the biological tumor behavior.

Material and Methods

Tissue Specimens

The study was performed on tissue sections from ninety-two patients diagnosed with primary gliomas, hospitalized at the Department of Neurosurgery of the Wroclaw Medical University, Wrocław, Poland, between 2006 and 2013. There were 53 male patients and 39 females, with ages ranging from 4 to 81 years (average 42.7). The patients' mean age was 21.1 years for grade I gliomas, 44.5 years for grade II gliomas, and 62.6 years for grade IV gliomas. Tumor tissues were obtained at initial surgery. None of the patients received any treatment before the operation. The sections underwent routine histopathological examination. All tumors were histologically verified to confirm the diagnosis, histological type, and tumor grade according to earlier established criteria established by the WHO classification of the central nervous system tumors [19]. According to the criteria of the WHO classification of gliomas, we subdivided gliomas into the following groups: grade I – 23 cases (23 cases of pilocytic astrocytoma), grade II – 30 cases (23 cases of fibrillary astrocytoma, 7 cases of oligodendroglioma), and grade IV – 39 cases (all glioblastoma). To establish the biological differences between low and high grade of tumor, malignancy tumors graded as GIII (anaplastic astrocytoma) were excluded from this study. All cases were reexamined by prof. dr hab. Michał Jeleń.

Immunohistochemical Staining

Immunohistochemical staining (IHC) for the analyzed proteins was performed on paraffin-embedded tissue using the Universal Dako LSAB + Peroxidase kit procedure (LSAB+ Kit, HRP, Dako, Copenhagen, Denmark) and 2 primary monoclonal antibodies – anti-CD44v6 and anti-E-cadherin.

Four-micrometer sections forming 1 selected block from each lesion were deparaffinized and boiled in citrate buffer (pH = 6.0) at 700 W in a microwave oven for 3×5 min for each antibody. After microwave treatment, the tissue sections were slowly cooled for 20 min. Endogenous peroxidase reactivity was blocked with 3% H_2O_2 and nonspecific tissue reactions were blocked with 10% BSA (bovine serum albumin). Tissue specimens were incubated with primary antibodies against an epitope encoded by a CD44 exon v6 (clone VFF-7) (Novocastra, United Kingdom) and human E-cadherin (clone 3685) (Novocastra) overnight at 4˚C. Following washing with 0.1 M Tris-buffer, pH = 7.4 (TBS), the tissue specimens were incubated with secondary biotinylated rabbit anti-mouse IgG antibody (Dako) and with streptavidin-horseradish peroxidase-conjugated antibody (Dako), both for 15 min at room temperature. After washing with TBS, the antigen-antibody reaction was visualized by DAB (3,3'-Diaminobenzidine) (Dako) as a chromogen (8 min, room temperature). The sections were counterstained with hematoxylin and mounted. The incubation buffer (TBS) without primary antibody was used as negative control. The internal positive controls were performed according to the manufacturer's protocol.

Interpretation of Immunostaining Results

The preparations were evaluated under a BX-51 Olympus light microscope. The localizations, distributions, and intensity of immunostaining were evaluated in the tissue sections. For CD44v6 and E-cadherin, membrane immunostaining was calculated as the percentage of positive tumor cells in relation to the total number of cells using semiquantative scale as follows: 0 = no staining to 10% of positive cells, 1 = above 10–40% of cells, 2 = 41–60% of cells, 3 = 61–100% of cells.

The intensity of staining was scored as absent (= 0), weak (1+), moderate (2+), and strong (3+). A final score was obtained by multiplying the score for extent and the score for intensity. The immunohistochemical analyses were interpreted without prior knowledge of the clinical information.

Statistical Analysis

Correlations between CD44v6, E-cadherin expression, and glioma grade malignancy were statistically studied using the Chi-square test. The relationships between CD44v6 and E-cadherin expression were analyzed by the Chi-square test using STATISTICA 10 PL statSoft program (Cracow, Poland), too. Differences were considered as significant when $p \leq 0.05$.

Results

Immunohistochemical staining revealed CD44v6 expression in 71.6% of gliomas. The pattern of CD44v6 immunoreactivity was heterogenous and was observed in 10 to 80% of tumor tissue. The specimens immunostaining positively for CD44v6 showed the membrane staining of glioma cells. In gliomas of low malignancy (pilocytic and fibrillary astrocytomas), CD44v6 immunohistochemical staining was limited to a small range of glioma cells (10–20% of positive tumor cells) distributed in tumor tissue (Fig. 1A). In glioblastomas, CD44v6 expression was strong and found in a high percentage of glioma cells (40–90% of positive cells). The CD44v6-positive glioma cells formed nest-like structures in the center and the edge of the tumor tissue (Fig. 1B). The heterogenous staining for CD44v6 molecule was observed not only between different glioblastomas cases but also in individual cases (Fig. 1B, C). The differences between the number of positive cells for CD44v6 in low and high glioma malignancy were not statistically significant. Taking into account the grade of glioma malignancy, CD44v6 was detected in 8/23 (34.7%) and in 21/30 (70.0%) of grade I and grade II gliomas, respectively. CD44v6 was observed in 31/39 (79.4%) of glioblastomas. There was a statistically significant difference between the frequency of positive cases for CD44v6 expression in low (grade I) vs. high (grade IV) glioma malignancy as well as in grade I and grade II glioma malignancy (p = 0.001) (Table 1). E-cadherin membrane staining was observed in 28.8% of gliomas. The positive immunostaining for E-cadherin was limited to a small surface of gliomas tissue (Fig. 2A). The majority of E-cadherin positive gliomas, graded as GI or GII, exhibited diffuse pattern of E-cadherin immunoreactivity in the center of tumor tissue. In glioblastomas many groups of E-cadherin positive cells were observed in the edge of tumor tissues. In individual cases, membrane location of E-cadherin – bound with glioma cell nest (Fig. 2B). No significant differences between E-cadherin expression
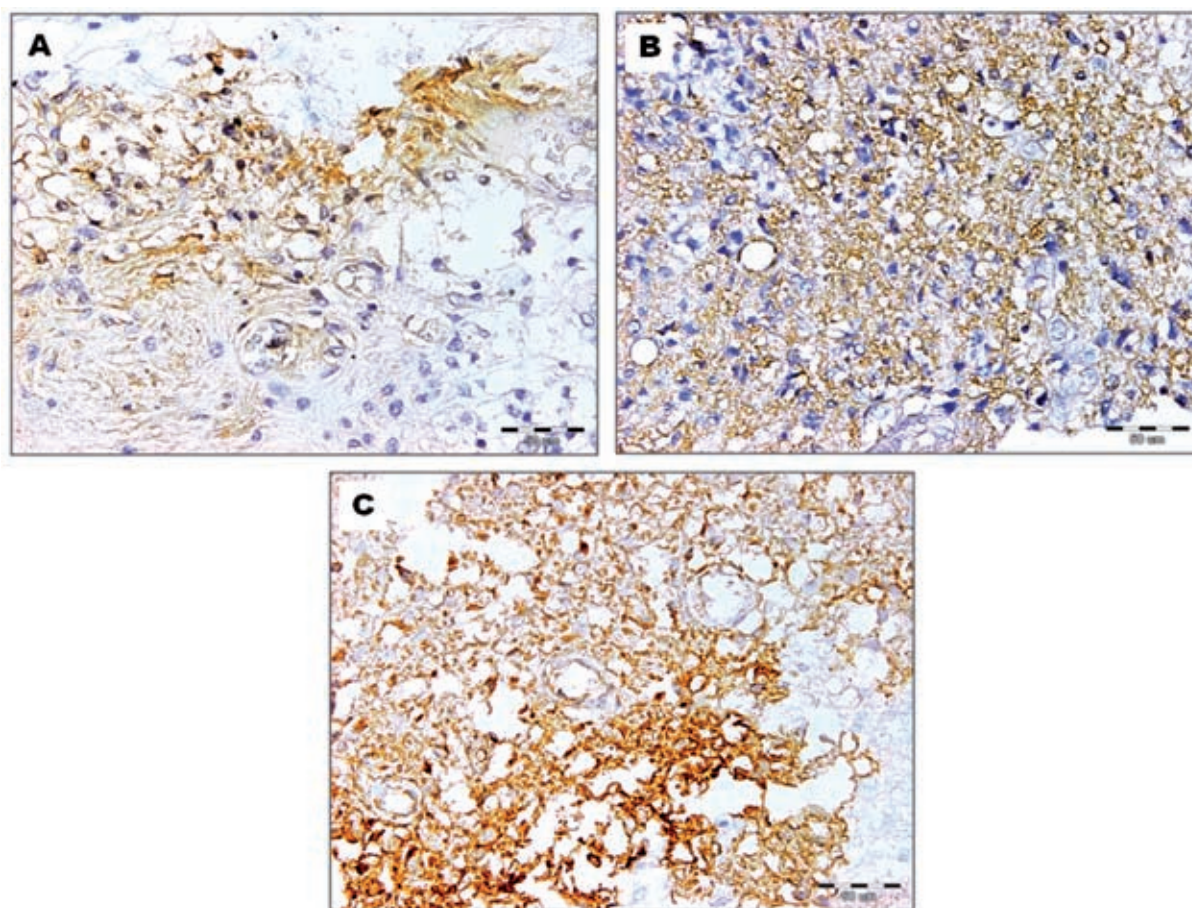
Fig. 1. Immunohistochemical staining for CD44v6 in gliomas: A – CD44v6 expression limited to a small percentage of the tissue of gliomas classified as grade II according to the WHO; B – strong membrane CD44v6 expression in glioblastoma C – many tumor cells display heterogenous CD44v6 immunostaining (avidin-biotin (ABC) staining ×400).

Table 1. CD44v6 and E-cadherin expression in gliomas

Parameters		Immunopositivity			
		CD44v6 expression		E-cadherin expression	
Gliomas	n	positive cases (%)	p-value	positive cases (%)	p-value
grade I	23	8(34.7%)*		6(26.0%)	
grade II	30	21(70.0%)*	0.001	13(43.3%)	> 0.05
grade IV	39	31(79.4%)**		9(23.0%)	

n – number of cases.
* statistically significant differences between gliomas in graded as GI vs. GII.
** statistically significant differences between gliomas graded as GI vs. GIV.
p – statistical differences.

and grade of gliomas were observed ($p > 0.05$). However, re-expression of E-cadherin was found in grade II gliomas. In this group, E-cadherin expression was revealed in 43.3% of cases. In order to define the relationship between CD44v6 expression and E-cadherin, we analyzed the simultaneous expression of CD44v6 and E-cadherin in the same glioma samples in the whole group (n = 92 cases) and in respect to the degree of glioma malignancy. A positive correlation was observed between studied biomarkers in the analyzed gliomas ($p = 0.004$) (Fig. 3). With respect to the degree of glioma malignancy, simultaneous expression of CD44v6 and E-cadherin did not show significant differences and was found in 3 of 23 (13.0%) gliomas in grade I, 12 of 30 (40.0%) gliomas in grade II, and in 9 of 39 (23.0%) gliomas in grade IV ($p > 0.05$).
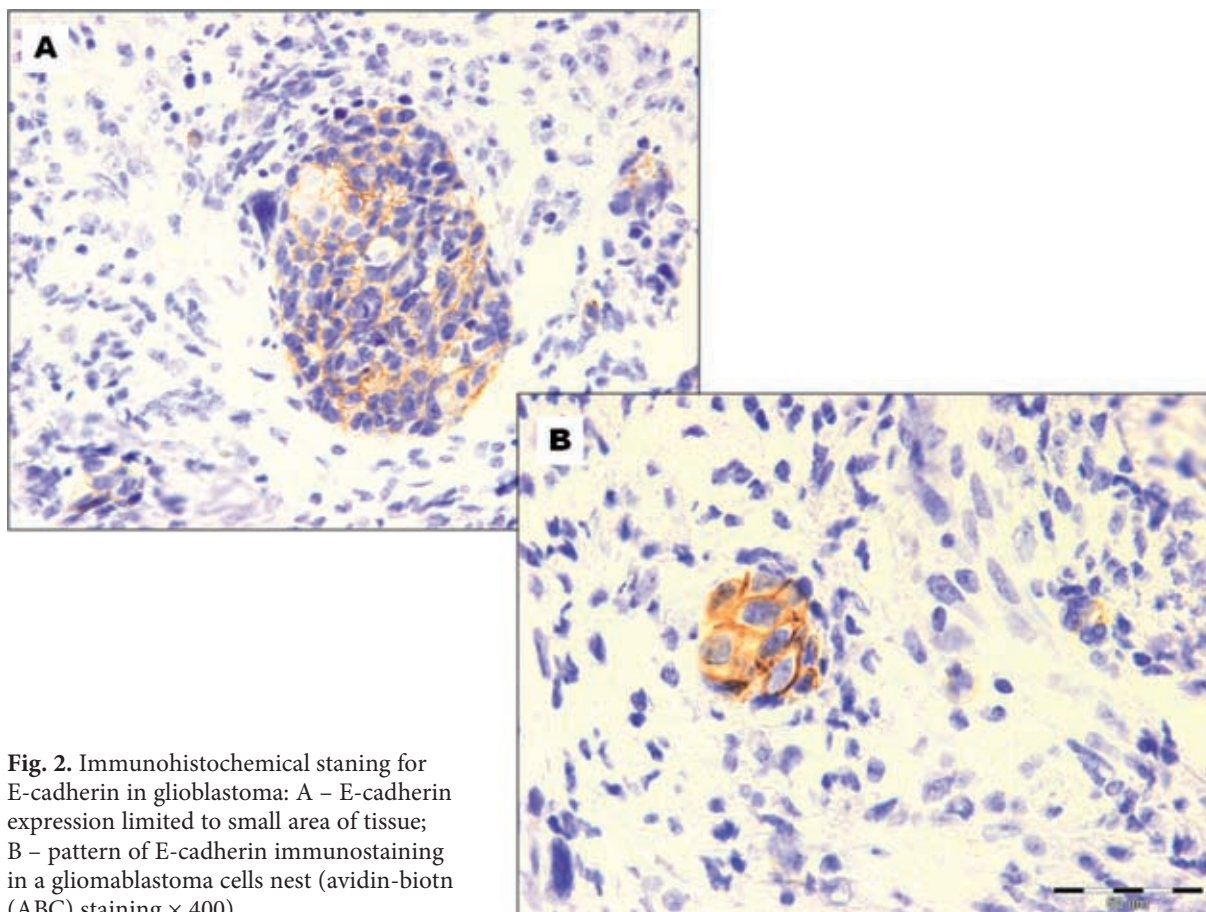
Fig. 2. Immunohistochemical staning for
E-cadherin in glioblastoma: A – E-cadherin
expression limited to small area of tissue;
B – pattern of E-cadherin immunostaining
in a gliomablastoma cells nest (avidin-biotn
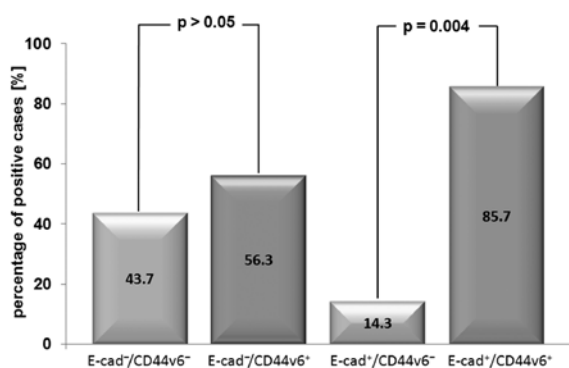(ABC) staining × 400)

Fig. 3. Simultaneous CD44v6/E-cadherin expression
in gliomas. Significant differences between immino-
phenotyp E-cadherin +/CD446- and E-cadherin +/
CD44v6+ were found (p = 0.004)

Discussion

Despite growing evidence showing the roles of
the family of cell adhesion molecules (CAM) in the
progressive growth of gliomas, their clinical signif-
icance is still unknown. Infiltrative nature of gli-
omas is a complex process, and the cell adhesion
molecules may play a crucial role in the early stage
of brain tumor progression [4, 20, 21]. Holland
et al. [3] found that grade II gliomas contain indi-
vidual cells with high infiltration capacity over long
distances. The authors [2, 3] suggest that despite
complete low-grade tumor resection, these tumors
contribute to recurrence but the mechanism of this
process is poorly documented. To confirm our hy-
pothesis that CD44v6 and E-cadherin are poten-
tial biomarkers of aggressive behavior of gliomas,
their protein expression and relation between each
other, including the degree of glioma malignancy,
were evaluated. Our results showed that CD44v6
expression was observed in 71.6% of gliomas and
increased with the malignancy grade of gliomas.
These findings are consistent with earlier pub-
lished data demonstrating that the CD44v6 ex-
pression was associated with progression of brain
tumors [8]. Our own and other results indicate
that CD44v6 might determine aggressive behavior
of glioma cells [8, 13]. Heterogeneous pattern of
CD44v6 immunostaining in gliomas observed in
this paper was also reported by other authors in
the case of endometrial carcinoma and osteosarco-
ma [22, 23]. In this paper, the differences in the in-
tensity and extent of immunostaining for CD44v6
were clearly visible in gliomas presenting different
grade of malignancy. In gliomas classified as grade
I and II tumors, the immunoreactivity for CD44v6
was observed in tumor core and was limited to
20–30% of brain tumor tissue. By comparison,
glioblastomas revealed CD44v6 overexpression in

a high percentage of tumor tissue (50–80% positive tissue) and CD44v6 expression was higher in invaded cells than in the tumor core. Taking into account the role of CD44v6 molecule, our results suggest that tumor cells located at the low-grade tumor margin might possess increased cell motility, which means the spread of cells from the tumor mass could be facilitated [8, 23]. We may also speculate that in this subgroup of low-grade gliomas, the interaction between tumor cells and the environment is important for the initial stage of invasive tumor growth. On the other hand, in glioblastomas, the alteration in adhesion junctions might be important in the formation of tumor structures [8]. This observation is in agreement with the data presented by Kim et al. [6] and Jijiwa et al. [8], who pointed out the role of CD44v6 in glioma invasion. Based on earlier published data, it is worth underlining that strong CD44v6 expression observed in individual cells of grade II gliomas might induce cell proliferation and migration [20, 23, 24]. On the other hand, this data raises a possibility that CD44v6-positive cells in gliomas with low grade of malignancy might be involved in malignant progression from low-grade astrocytomas to glioblastomas [20]. We could consider such mechanism in our subgroup of CD44v6--positive gliomas with low malignancy grade. This suggestion might be supported by recent results showing that a knockdown of CD44v6 molecule resulted in suppressed growth of human brain tumor stem-like cells in an animal model [8]. Interestingly, we found that, irrespective of tumor grade malignancy, in the case of extended CD44v6 expression, individual glioma cells located at the marginal tumor tissue showed strong CD44v6 expression compared to the remaining brain tumor tissue. Jijiwa et al. [8] found that brain tumor stem cells showed a high level of CD44v6 expression. The findings from this paper suggest that cells with strong CD44v6 immunopositivity in glioma specimens might reflect cancer stem cell phenotype. However, this observation should be confirmed by identification of cancer stem cell markers [25].

Down-regulation of E-cadherin is considered as one of the main molecular alterations involved in the invasive and aggressive tumor maintenance [14, 21, 26]. In gliomas, the role of down- or up-regulation of E-cadherin expression is still controversial [4, 16, 26]. Consistent with previous studies which found that E-cadherin expression is a rare event in gliomas [4, 16], in the current study the E-cadherin expression was limited to a small number of analyzed cases and a low percentage of positive brain tumor tissue. The relationship between the decreasing expression of E-cadherin and the increasing grade of malignancy observed by other authors [16] was not observed in our study. Similarly to other studies [4, 16], the low expression of E-cadherin in glioblastomas was revealed in the present study. Some authors indicate that tumor progression is accompanied by cadherin switching, resulting in down-regulation of E-cadherin and up-regulation of N-cadherin and cadherin 11 expression [14, 18, 26]. Our data on increased E-cadherin expression in gliomas classified as grade II according to the WHO and decreased expression in glioblastomas suggests that E-cadherin may play a crucial role in the biology of a subset of neoplastic astrocytomas and indicates that tumor cell malignancy is related to E-cadherin levels in tumors [16]. The results from this study are consistent with observations of Lewis-Tuffin et al. [4], who found that E-cadherin expression correlates with increased invasiveness of glioma xenograft cell line in a mouse model of invasion. On the basis of their results, the authors suggest an atypical role for E-cadherin in brain tumor biology [4]. A decreased level of E-cadherin found in low-grade gliomas (grade I) in the current study has been reported in an early stage of gastric cancer development [27]. The obtained data indicates that low expression of E-cadherin may be associated with increased cell motility [18]+Thiery JP and Seleeman JP [28] described the formation of the neural tube, allowing N-cadherin expressing cells in the developing neuroepithelium to separate and migrate away from surrounding E-cadherin expressing cells. The different level of E-cadherin expression observed in the analyzed subgroup of gliomas indicates that the level of this molecule might be important for causing changes in adhesive pathway in gliomas [28]. In our paper such a mechanism can be discussed in low-grade gliomas with loss or low E-cadherin expression. There is no data describing the relationship between CD44v6 and E-cadherin expression in gliomas. In this study simultaneous expression of CD44v6 and E-cadherin found in gliomas suggests that cascade activations might be considered in some gliomas. This suggestion may be supported by the findings that CD44s interact with the epidermal growth factor receptor (EGFR), epidermal growth factor receptor-2 (HER2), promoting growth through mitogen activated protein MAP/ERK kinase (MEK), extracellular signal-regulated kinase 1 (ERK1), and β-catenin [23, 25]. Interaction between CD44v6 and AKT was observed in glioblastomas [8]. Up to now, it has not been clarified why E-cadherin inhibits EGFR signaling in some cells but activates ligand-independent EGFR signaling in other cells [26]. It was found that E-cadherin may induce ligand-independent activation of tyrosine kinase receptors (RTK), such as EGFR and HER2 forming complexes, with both receptors and activate signaling to enhance proliferation and

migration [21, 26]. This data indirectly indicates that cooperation between CD44v6 and E-cadherin exists, and thereby may promote tumor cells dissemination [21]. In conclusion, our results clearly showed that the level of E-cadherin might reflect different biological features of gliomas, whereas CD44v6 is associated with tumor cell malignancy.

The simultaneous presence of CD44v6 and E-cadherin in a set of low-grade gliomas indicates that both these molecules might strengthen cell migration, which may be a hallmark of glioma cell dissemination. Moreover, further studies are needed to clarify the network of cell-cell adhesive molecules in gliomas.

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