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ORIGINAL PAPER

Osteopontin Expression Correlates with Angiogenesis and Survival in Malignant Astrocytoma

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Abstract Osteopontin (OPN) is a phosphorylated glycoprotein with diverse functions including angiogenesis, cancer development, invasion and metastasis. The aim of the study was to analyze the expression of OPN in human astrocytomas and to correlate it with angiogenesis and patients' outcome. Seventy-six human astrocytomas including eight pilocytic astrocytomas (grade I), 10 diffuse astrocytomas (grade II), 8 anaplastic astrocytomas (grade III) and 50 glioblastomas (grade IV) were immunohistochemically stained for OPN protein. The distribution of OPN staining (cytoplasmic and/or interstitial) was assessed and compared to microvessel number and patients' survival. In normal brain tissue some glial and neuronal cells showed weak cytoplasmic staining, while interstitium was negative. Astrocytomas were heterogeneous regarding the OPN expression. High cytoplasmic OPN expression in glioblastomas was associated with poor patients' survival (p=0.012). Also, we found the association of interstitial OPN expression and angiogenesis (p=0.033), i.e. the number of newly formed blood vessels was higher in tumors showing high interstitial OPN expression. Our results indicate the overexpression of OPN protein in astrocytoma cells and suggest the role of OPN in astrocytoma progression and angiogenesis.

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Abbreviations

ECM	extracellular matrix
MVD	microvascular density
OPN	osteopontin
VEGF	vascular endothelial growth factor

Introduction

The hallmark of astrocytomas, the most common primary brain tumors, is the ability to diffusely invade the normal brain tissue. Local invasion increases with histological grade and represents one of the most important determinants of poor prognosis associated with those tumors. It is well known that tumor cell motility and invasion are mediated by cell adhesion molecules that transduce signals from extracellular matrix to the cell. Among the most important mediators of cell migration and invasion in glioma biology are integrins, alpha-beta heterodimers that bind noncovalently to RGD (Arg-Gly-Asp) sequence present in many extracellular matrix proteins, like fibronectin, laminin, collagen, and osteopontin [1]. Osteopontin (OPN) is a phosphorylated glycoprotein with diverse functions including angiogenesis, cancer development, invasion and metastasis [2, 3]. As an RGD-containing protein, OPN exists both as an immobilized extracellular matrix (ECM) molecule in mineralized tissue and as cytokine in body fluids. It can engage a number of receptors, including integrins and CD44 molecule. These receptors have been shown to cooperate with other receptor molecules, including various growth factors, resulting in

complex molecular cross-talk initiating signal-transduction cascades. Cell and tissue properties influenced by OPN include migration and invasion [4], tumor angiogenesis [5], cell proliferation [6, 7], and cell survival through inhibition of apoptosis [8, 9]. OPN is found to be overexpressed in many human tumours [10–14]. However, it has not been extensively studied in human astrocytomas.

The aim of our study was to analyze the expression of OPN in normal brain tissue and in various grades of human astrocytomas, as well as the association of OPN expression with disease outcome in patients with glioblastoma. Since OPN has been implicated in angiogenesis, and it has been shown that OPN overproduced by tumor cells acts as a potent angiogenic factor contributing to tumor growth [5], we also analyzed the correlation between OPN expression and microvessel density in glioblastomas. To the best of our knowledge, this is the first work aimed at analyzing clinicopathological correlates of OPN expression in human astrocytomas.

Materials and Methods

Tumor Samples

A total of 76 astrocytomas were obtained from patients treated at the Department of Neurosurgery, Rijeka University Hospital. Tumor samples were fixed in 4% buffered formalin, embedded in paraffin and routinely stained with haematoxylin and eosin. All cases were reviewed by two pathologists and classified according to the WHO classification [15] as follows: 8 pilocytic astrocytomas (grade I), 10 diffuse astrocytomas (grade II), 8 anaplastic astrocytomas (grade III) and 50 glioblastomas (grade IV). Follow up information was obtained from patients' medical records and from files of the Croatian Cancer Registry. Normal brain tissue was obtained from autopsy of patients without brain pathology.

Immunohistochemistry

Tumor samples were processed for immunohistological analysis on paraffin-embedded sections to determinate microvascular density (MVD) and OPN expression. Indirect immunoperoxidase staining was performed using LSAB2 HRP system on the automatic immunostainer (DAKO, TechMate, Glostrup, Denmark), according to the manufacturer's protocol. Antigen retrieval was achieved as follows: OPN retrieval by immersing slides in 10 mM citrate buffer (pH 6.0) and boiling for 10 minutes in a pressure cooker; endoglin retrieval by predigestion with proteinase K (DAKO, TechMate, Glostrup, Denmark) at room temperature for 10 min. OPN protein was detected by goat anti-human monoclonal antibody (clone K-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:100), followed by donkey anti-goat IgG as secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:250). For microvessel visualization, anti-endoglin (CD105) antibody (clone SN6h, DAKO, TechMate, Glostrup, Denmark, dilution 1:25) was used, followed by goat anti-mouse Ig (ChemMate Link, DAKO, Glostrup, Denmark). For a negative control, an irrelevant goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse IgG (DAKO, Glostrup, Denmark), when appropriate, was used.

Evaluation of Immunohistochemistry

The immunohistochemical staining results were examined independently by two pathologists in a semi-quantitative manner by assessing the percentage of positive tumor cells and the intensity of staining, according to modified Allred score [16]. For each sample, we analyzed tumor cell proportion score and intensity score. Proportion score included the fraction of tumor cells positively stained for OPN as follows: 0=none, 1=<1%, 2=1–10%, 3=11–33%, 4=34–75%, 5=>75%. The staining intensity of tumor cells was expressed either as low (1) or high (2) and was multiplied by proportion score. Cytoplasmic OPN expression in tumors scored as 0–5 was considered low, while tumors scored by 6–10 were considered as having high OPN expression. We also assessed the intercellular distribution of staining, which was categorized as focal or diffuse, with the staining intensity expressed either as low or high. Tumors with either a diffuse or strong expression were considered positive in interstitial distribution of OPN staining.

For the assessment of microvessel density, most vascular areas (so-called hot spots) were located at low magnification (×40) and than counted at high magnification (×400). Each positive endothelial cell or group of cells was counted as an individual vessel. The mean vessel count from three fields was used as a number of microvessels.

Statistical Analysis

Statistical analysis was performed using Statistica 6.1 software (StatSoft, Inc., Tulsa, OK, USA). Pearson's χ^2-test was used to assess the significance of correlation between categorical data. The measures of central tendency for continuous data were compared by Student's *t*-test and Mann–Whitney *U*-test, when appropriated. Survival probabilities were estimated by the univariate Kaplan–Meier method, and survival curves were compared by the log-rank test. The correlation of immunohistochemical staining for OPN with patients' survival was evaluated using Kaplan–

Meier method, and differences between groups were tested by the log-rank test. Statistical differences with p value less than 0.05 were considered significant.

Results

Immunohistochemical Staining for OPN

In normal brain tissue, obtained from autopsy of patients without brain pathology, the expression of OPN was present in neuronal cells in grey matter and in some glial cells in the form of weak cytoplasmic granular staining. (Fig. 1a). Astrocytomas were heterogeneous regarding the OPN expression (Fig. 1b–f). OPN was present mainly in tumor cells in the form of cytoplasmic granular staining of various intensities (Fig. 1c and d). Strong cytoplasmic OPN expression was observed in 3 grade I (37.5%), 4 grade II (40%), 2 grade III (25%) and 21 grade IV (42%) astrocytomas. In some tumors OPN was also present interstitially, in the neuropil between tumor cells (Fig. 1d). Interstitial distribution of OPN was observed in 1 pylocytic astrocytoma (12.5%), 2 low grade astrocytomas (20%), 2 anaplastic astrocytomas (25%) and 25 glioblastomas (50%) i.e. it was more often present in glioblastomas, compared to lower astrocytoma grades (p=0.086). However, when tumors with any interstitial staining were considered positive, compared to those that were completely negative, the association of OPN expression and higher WHO astrocytoma grade was statistically significant (p=0.012). Interstitial staining pattern was usually accentuated around necrosis, blood vessels, as well as at the infiltrative tumor margins, at the border with normal brain tissue. In 35 astrocytoma samples of various grades of malignancy OPN was also expressed in endothelial cells, either focally or diffusely throughout the tumor (Fig. 1b and c). Microglial cells and histiocytes around tumor necrosis were also positive (Fig. 1e).

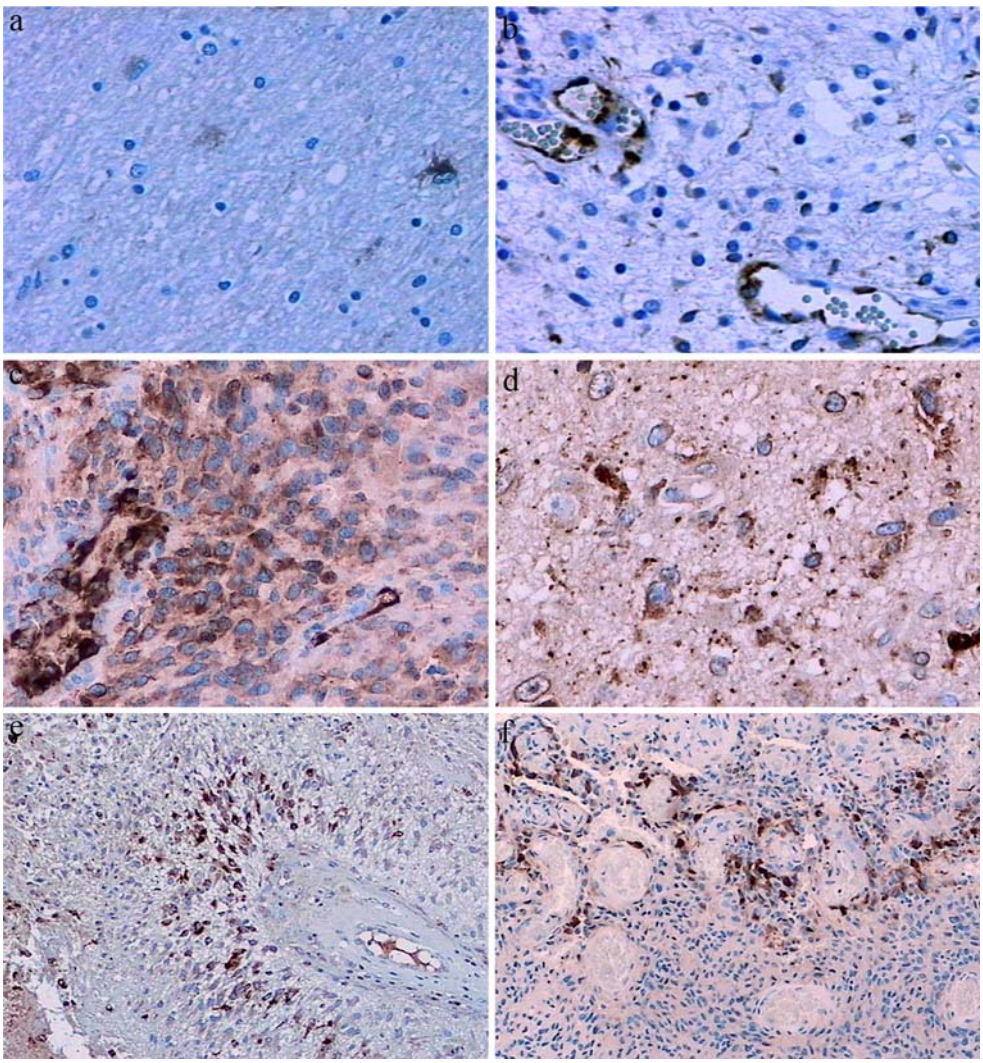

Fig. 1 Immunohistochemical staining of osteopontin (*OPN*) in normal brain tissue (**a**) and in astrocytoma tissue (**b**–**f**). In normal white matter there is a weak granular staining in some glial cells, while interstitium is negative (**a**, ×400). Pilocytic astrocytoma showing weak OPN expression in a few tumor cells and strong OPN expression in endothelial cells (**b**, ×400). Strong cytoplasmic staining for OPN in tumor cells and endothelial cells in glioblastoma (**c**, ×400). Interstitial OPN expression showing granular staining pattern, along with cytoplasmic positivity of some tumor cells in glioblastoma (**d**, ×400). OPN expression is accentuated in microglial cells and histiocytes around necrosis (**e**, ×200) and in tumor cells and interstitium around blood vessels (**f**, ×200)

Association of OPN and Microvessel Density

We found the association of interstitial OPN expression and angiogenesis in glioblastomas (p=0.033; Table 1). Namely, microvessel density was higher (median 57.5; range, 30–98.3) in tumors with positive, compared to those with negative interstitium (median 43.7; range, 27.3 – 99.3). However, there was no association between microvessel density and the level of cytoplasmic OPN expression (p=0.233).

Association of OPN and Patients' Survival

The association of immunohistochemical positivity for OPN in glioblastomas and the cumulative proportion of patients surviving during the follow up are shown in Fig. 2. The 1-year survival rate was 14.3% for 21 patients whose tumors showed high cytoplasmic OPN expression, compared to 48.3% for 29 patients with low or absent OPN expression. Over the time period of 35 months, the overall survival was significantly shorter in patients whose tumors showed high intracellular OPN expression (p=0.012). There was no association between interstitial OPN expression and patients' survival (p=0.358; Table 1).

In two diffuse astrocytomas interstitial OPN expression was associated with poor patient outcome. Namely, these two patients died during the follow up period, while other patients with diffuse astrocytomas, whose tumors show only cytoplasmic OPN expression, stayed alive.

Discussion

In several human tumors OPN has been shown to be overexpressed in comparison with normal tissues and associated with parameters of poor prognosis [11–13]. However, there are only a few reports on the OPN expression in brain

Table 1 Association of OPN expression with MVD and survival in glioblastomas

		MVD (median, range)	Survival (months; mean±SD)
Intracytoplasmic OPN	Low	45 (27.3–99.3)	12.5±11.1
	High	57.2 (30.3–89)	5.3±5.9
P value		0.233[a]	0.01[b]
Interstitial OPN	Negative	43.7 (27.3–99.3)	10.8±10.4
	Positive	57.5 (30–98.3)	8.2±9.3
P value		0.033[a]	0.358[b]

OPN osteopontin, *MVD* microvessel density
[a] Mann–Whitney *U*-test
[b] Student's *t*-test

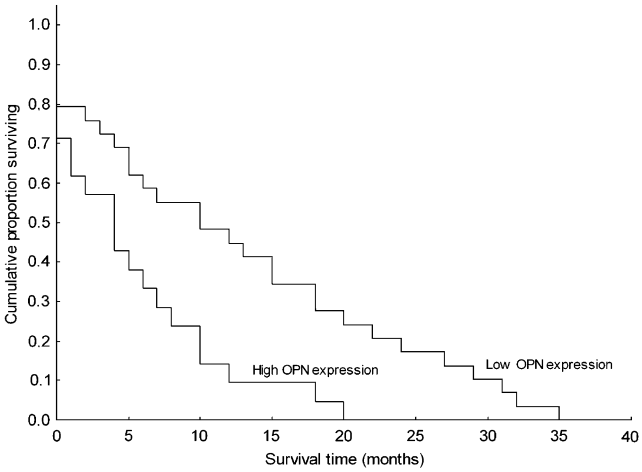

Fig. 2 Kaplan–Meier cumulative survival analysis according to staining for osteopontin (*OPN*) in glioblastomas. The log-rank test showed significantly shorter survival in patients with tumors showing high cytoplasmic OPN expression; p=0.012

tissue, mainly obtained in vitro or in animal models. In central nervous system OPN was found to be upregulated during the certain forms of injury [17–19] and in multiple sclerosis [20]. Data obtained in vivo on human tumor tissue are rare and somewhat contradictory. Ding et al. analyzed the OPN expression in normal adult brain by immunohistochemistry and western blot analysis [21]. They found OPN in cortical grey and white matter, with the level of OPN expression being equivalent to those in malignant astrocytic tumor biopsies, as assessed by Western blot analysis. Also, they showed that OPN expressed in normal brain has the potential to promote malignant astrocytoma cell invasion in vitro. In opposite to this observation, Saitoh et al found ten times higher OPN mRNA in human glioblastomas compared to lower-grade astrocytomas (grade I–III) and non-neoplastic tissue [22]. By immunohistochemical staining of 13 astrocytomas of various grades of malignancy, they showed strong OPN staining in malignant astrocytomas and slight staining in benign astrocytomas. Similarly, Said et al. also found the upregulation of OPN in glioblastomas, compared to normal brain and low grade astrocytomas, at mRNA and protein level [23]. Our results also show week OPN expression in normal brain tissue, mainly in neuronal and glial cell cytoplasms, while there was an overexpression of OPN in some astrocytic tumors of various grades of malignancy, whether cytoplasmic, interstitial, or both. Strong cytoplasmic OPN expression in glioblastomas was associated with poor patient outcome, as has been shown in some other human malignancies [12, 13].

The upregulation of OPN gene has also been shown in some in vitro studies on brain tumor-derived cell lines [24, 25]. By microarray analysis Jang et al. identified OPN as the most up-regulated gene in rat glioma. By immunohistochemistry, they confirmed OPN expression in tumors,

while no immunostaining was seen in normal brain [24]. Also, OPN gene expression pattern was identified as one of the invasion-promoting genes that could distinguish highly invasive glioblastoma from non-invasive pilocytic astrocytoma [26]. The association of OPN overexpression with malignancy grade and poor prognosis, as observed by in vivo and in vitro tumor systems, could be related to well known attachment and invasion-promotive function of OPN, which could be exerted by ligation of integrins and CD44 molecule. Infiltrative behavior of glioma cells, as one of the most important determinants of the poor prognosis, is a function of two phenotypes: migration and invasion; both of them have been shown to be mediated by integrins, cell surface receptors that mediate the physical and functional interactions between a cell and its ECM [1]. In opposite to laminin, collagen and fibronectin, the three principal stroma-derived ECM proteins which are localized to the perivascular space, some ECM proteins have been shown to be expressed directly by glioma cells themselves, including OPN. These ECM proteins may thus function as autocrine factors that promote glioma migration. In this regard, recombinant OPN was found to promote attachment of U-251MG human malignant astrocytoma cells in a process that was inhibited by anti-αvβ3, anti-αvβ5 and anti-α5 integrin antibody [21]. Mariani et al. [27] used the microarray analysis to uncover the genes associated with the motility of glioma cell line G112. Among others, OPN was found to be upregulated when the motility behavior was engaged.

In our work we showed the association of strong interstitial OPN expression and angiogenesis in glioblastomas. The cooperative role of OPN protein in angiogenesis is one of proposed mechanisms through which OPN can mediate tumor progression and metastasis [5, 28]. OPN augments endothelial cell migration induced by vascular endothelial growth factor (VEGF) in an αvβ3 integrin-dependent manner [28] and enhances survival of endothelial cells [29]. Interaction between the αvβ3 integrin and the extracellular matrix is crucial for the sprouting of endothelial cells from capillaries and for angiogenesis. Furthermore, integrin-mediated outside-in signals cooperate with growth factor receptors to promote cell proliferation and motility. Besides being the most important survival system for nascent vessels by regulating cell adhesion to matrix such as OPN and vitronectin [29], the αvβ3 integrin participates in the full activation of VEGFR-2 triggered by VEGF. VEGF stimulation on endothelial cells in vitro induces expression of several molecules, including OPN [28]. In the work of Takano et al. [30] OPN and αvβ3 were predominantly observed in the microvasculature of glioblastomas associated with VEGF expression. A very few clinical investigations analyzed interaction between VEGF and OPN in human malignancies. Shijubo et al. [14] have shown that coexpression of VEGF and OPN is associated with angiogenesis and poor outcome in patients with stage I lung carcinoma. Our results also support the role of OPN in angiogenesis in human glioblastoma.

In conclusion, we showed the overexpression of OPN protein in human astrocytomas compared to normal brain tissue, and the association of OPN expression with angiogenesis and poor patients' outcome in glioblastoma. Due to its invasive and angiogenesis—regulating properties OPN promises to become a novel target for the experimental therapy of human astrocytomas.

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