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ORIGINAL PAPER

Correliation Between Osteopontin Protein Expression and Histological Grade of Astrocytomas

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Abstract Osteopontin is a ligand for the integrin proteins, which are cell surface receptors that mediate the physical and functional interactions between a cell and the extracellular matrix. The expression of osteopontin is reportedly increased in a number of transformed cell lines and tumor tissues. Furthermore, increased expression of osteopontin results in some infiltrative features of tumors. The aim of the study is to demonstrate that expression of osteopontin in human astrocytomas correlates with histological tumor grade. The expression of osteopontin in human astrocytomas was determined with immunohistochemistry. Median osteopontin expression levels were 1%, 7.5%, 60%, and 50% in grade I, II, III, and IV tumors, respectively. Osteopontin staining was significantly higher in high grade (grade III–IV) than low grade (grade I–II) tumors. These findings indicate that osteopontin immunoreactivity in human astrocytomas may correlate with the grade of a tumor.

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Introduction

Osteopontin (OPN), also known as Eta-1, is a highly phosphorylated and glycosylated protein secreted into the extracellular matrix (ECM) by a variety of cell types [1, 2]. OPN exerts its effects through interaction with integrin proteins like CD44 and alphavbeta3, which are cell surface receptors that mediate the physical and functional interactions between a cell and the ECM [3, 4]. OPN is believed to be involved in physiological cellular functions, including bone and vascular remodeling as well as cell-mediated immunity [1, 2]. It has also ability to act as a cytokine in cell signaling to promote cell proliferation and/or survival, and it can also act as cell-attachment protein [1, 3]. OPN also has been shown to play an important role in tumorigenesis, tumor invasion, and metastasis in variety of malignant tumors [3].

The role of OPN in tumor progression was first analyzed in experimental animal models, where it was identified as phosphoprotein secreted by transformed cells [5] and associated with increased metastatic potential in rodents. Following this observation, OPN mRNA and protein were also analyzed in several types of human cancers [6]. First, although both tumor cells and macrophages are immunohistochemically positive for OPN proteins, OPN mRNA was found to be produced primarily by tumor-associated macrophages rather than tumor cells themselves [6]. Authors suggested that OPN secreted by macrophages might bind to tumor cells through the RGD-binding domain in OPN. Later on, tumor cells were also identified as a source of OPN. The role of OPN in human tumorigenesis, both as a marker of malignancy as well as a candidate for testing as a prognostic

factor, began to be investigated [7–11]. OPN positivity specifically in tumor cells correlates with both shortened patient survival and later stages of several kinds of cancers, including breast, lung, colon, and kidney [7–11].

Astrocytomas, the most common subtype of primary brain tumors, are aggressive, highly invasive, and neurologically destructive tumors and considered to be among the deadliest of human cancers. The ability of glioma cells to infiltrate brain structures that are adjacent to or distant from the primary tumor site is one of the most important determinants of the poor prognosis associated with these tumors [12]. This infiltrative behavior of glioma cells is a function of two phenotypes: migration and infiltration. For both functions, there is a direct correlation with tumor grade: high grade gliomas demonstrate more extensive migratory and invasive capacities [12]. For this reason, we suggest that there may be a direct correlation between grade of gliomas and OPN protein expression since OPN is the most highly up-regulated gene during glial tumor development. It is believed that OPN is produced and secreted by tumor cells via modulation of the interaction between tumor cells and the ECM in this period in order to make tumors more invasive [12].

Materials and Methods

A total of 29 astrocytoma specimens were obtained from patients surgically treated between 1999 and 2003 at the Selcuk University, Meram Medical School. Tumor samples were fixed in 10% formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin. Tumors were classified and graded according to the WHO criteria (Grade I: pilocytic astrocytoma, Grade II: diffuse astrocytoma, Grade III: anaplastic astrocytoma, and grade IV: glioblastoma).

Immunohistochemistry

Anti-OPN rabbit monoclonal antibody (dilution at 1:1,000) was applied to 3 μM sections from formalin-fixed paraffin embedded tissue specimens using the avidin-biotin-peroxidase complex method following the manufacturer's instructions. In brief, the immunostaining was performed manually at room temperature. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with 3% aqueous hydrogen peroxide for 10 min. After washing with PBS for 5 min, slides were blocked with normal serum for 20 min and incubated with the anti-OPN primary antibody at the given dilution for 60 min. After rinsing with PBS for 5 min, sections were incubated with a biotinylated secondary antibody for 20 min. After washing with PBS for 5 min, slides were incubated with an avidin-biotin complex for 30 min and washed again. Chromogen was developed with 10 mg of 3,3'-diaminobenzidine tetrahydrochloride for 2 min. All samples were counterstained with hematoxylin for 30 s before dehydration and mounting.

Histological and immunohistochemical evaluations were performed by the same experienced pathologist. Every tumor was given a score, in which the percentage of stained epithelial cells was taken into consideration.

The approval of the Ethics Committee was obtained for this study

Statistical Analysis

All calculations were carried out with the SPSS program (version 13.0, SPSS, Chicago, Illinois, USA). All data are presented as medians and interquartile ranges (25–75%). Statistical comparison of quantitative data was performed by Kruskal Wallis and Mann Whitney U tests. P-values of <0.05 were considered to be statistically significant.

Results

Evaluation of Immunohistochemistry

In normal brain parenchyma, no cells were positive for OPN. The assessment of staining was made only on tumor cells, however, and positive staining was also present on stromal macrophages (mainly around the necrotic areas) and plasma cells. In all grades of tumor, the stain was preferentially cytoplasmic and localized to the core of the tumor. There was no staining at the periphery. OPN expression levels changed with tumor grade, but all gemistocytic tumor cells were positive.

Images of OPN expression in astrocytomas (Grade I–IV) are presented in Fig. 1. Median OPN expression levels and the variation of OPN expression levels in each tumor grade group are shown in Fig. 2. Median OPN expression levels were 1%, 7.5%, 60%, and 50% in tumors with grades I, II, III, and IV, respectively. Statistical comparison of OPN expression levels of the astrocytomas according to tumor grade (Grade I–IV) via a Kruskal Wallis test revealed a significant difference ($P<0.005$, Table 1).

Then, we performed a post hoc analysis to determine where the significant differences were localized by comparing two tumor grade groups via the Mann Whitney U test. According to our results (Table 2), there were no differences in OPN expression levels between grade 1 and 2 tumors or grade 3 and 4 tumors.

Therefore, we grouped tumors as low (Grade I–II) and high (III–IV) grade astrocytomas and compared OPN expression levels via the Mann Whitney U test. Significantly higher OPN expression levels were found in high grade astrocytomas than low grade astrocytomas (Table 3).

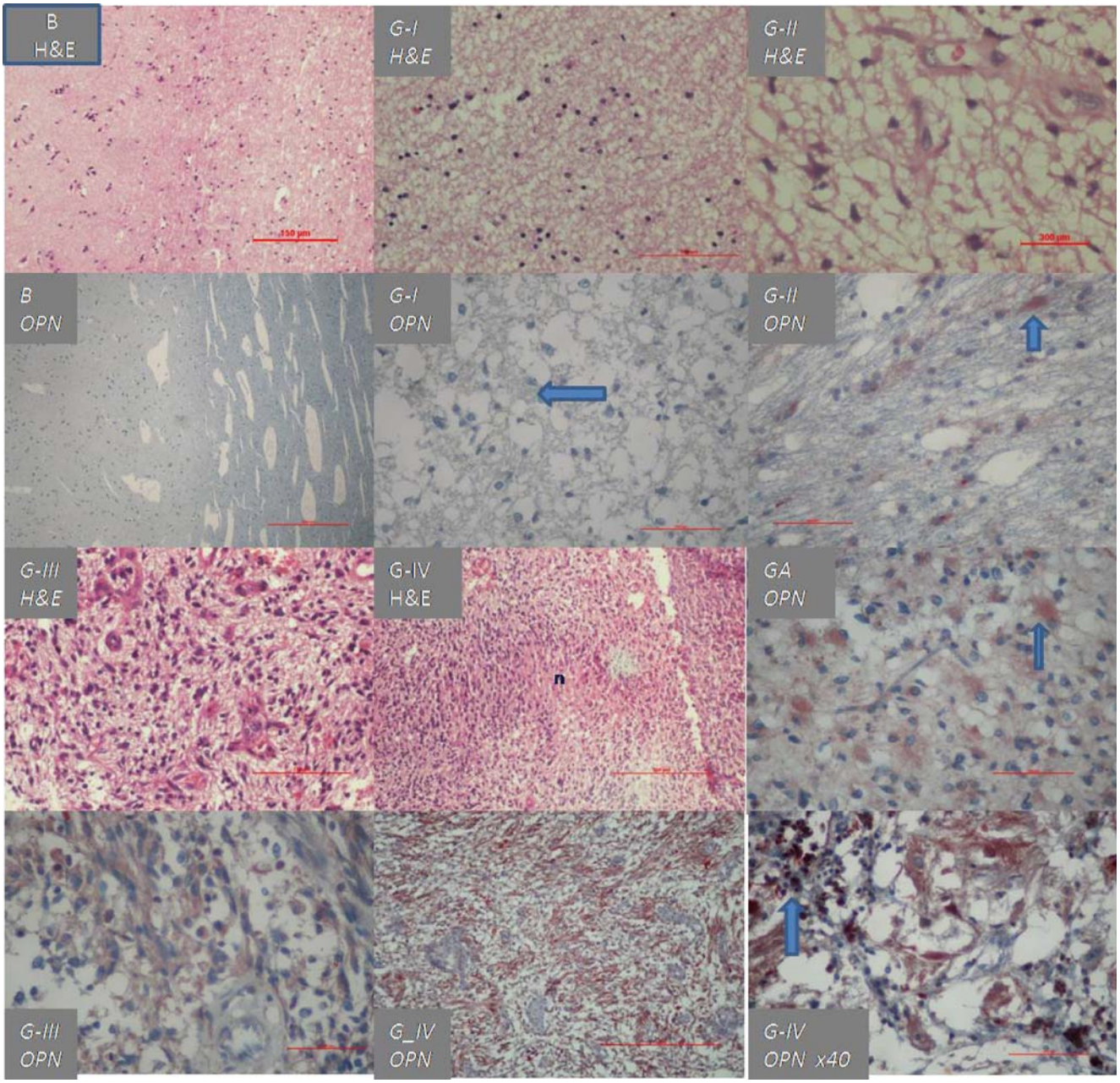

Fig. 1 Normal brain (*B*),grade I (*G-I*),gradeII (*G-II*),Grade III (*G-III*) and grade IV(G-*IV*) (*n:necrotic area*) astrocytoma's morphology with Hematoxylen & Eosin stain are shown. In *B -OPN ,* the normal brain tissue is OPN negative. Progressive increase in OPN expression in different grades of astrocytoma. Low expression of OPN is noted in grade I (G-I OPN) (*arrow: negative staining*) and II (*G-II OPN*) (*arrow:positive cytoplasmic staining*). The increasing degree of staining intensity is noted from grade III (G-III OPN) to grade IV (*G-IV OPN*) astrocytoma. Tumors were classified and graded according to WHO criteria. In Gemistocytic Astrocytoma (*GA OPN*) the Gemistocytic cells have positive OPN stain (arrow). In G-IV x40 the arrow shows *false positive inflammatory cells*

Discussion

The ability of glioma cells to infiltrate brain structures is the most important prognostic feature of the malignant gliomas. Glioma cell invasion is a complex and multistep mechanism involving a large array of molecules and cell-cell and cell-ECM interactions [13]. These processes allow individual tumor cells to migrate and invade the healthy surrounding brain. Proteases, ECM components, adhesion cell molecules, and related signaling pathways have been shown to play an important role in glioma cell migration and invasion. Alterations in the expression of both components of the ECM and cell surface receptors during tumorigenesis can thereby profoundly affect cell function [13].

OPN is a secreted, cell attachment protein and cytokine that is highly conserved among mammals. It is expressed in normal

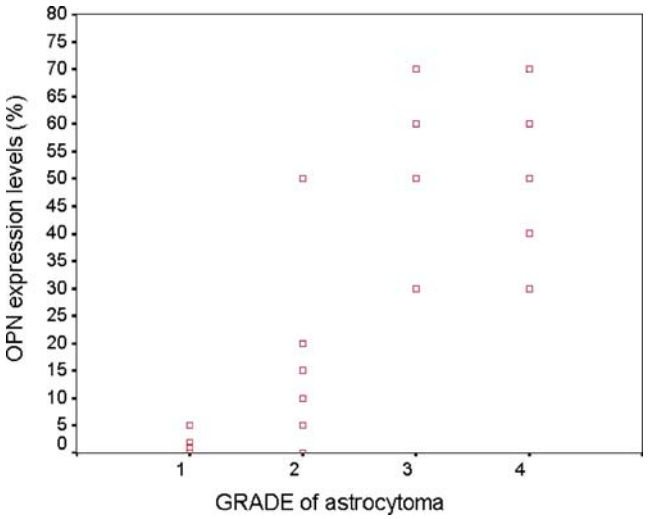

Fig. 2 The figure presents the data in Table 1 in a vertical scatter plot figure

Table 1 Comparison of OPN expression levels according to grade I–IV astrocytomas*

Kruskall Wallis test was used comparison of OPN level

	Grade 1 *N*=7	Grade 2 *N*=8	Grade 3 *N*=7	Grade 4 *N*=7	P value
OPN level Median IQR (25–75%)	1 (0–2)	7.5 (0–18.75)	60 (50–60)	50 (40–70)	*P*<0.005

Table 2 Post hoc analysis results

Two compared tumor grade group	P value
Grade 1–2	0.188
Grade 1–3	0.002
Grade 1–4	0.002
Grade 2–3	0.002
Grade 2–4	0.004
Grade 3–4	0.844

Mann Whitney U test was used comparison of OPN levels of two grade group
**P* value<0.008 is statistically significant in this analysis

Table 3 Comparison of OPN expression levels according to low (Grade I–II) or high grade (Grade III–IV) astrocytomas*

	Low grade *N*=15	High grade *N*=14	P value
OPN level Median IQR (25–75%)	2 (0–10)	55 (47.5–62.5)	*P*<0.005

Mann Whitney U test was used comparison of OPN level according to low vs high grade

mineralized bone, the mammary gland, smooth muscle, kidney, placenta, and several neoplastic tissues [1, 2]. In the last decade, several studies have defined an important role for OPN in carcinogenesis and metastasis [6–9].

Although it has not been extensively studied as a mediator of glioma pathology, OPN is known to be over expressed in human glial tumors. Saitoh et al showed in a cell line study that OPN mRNA and proteins are expressed in human glioma cells and that the extent of OPN expression may correlate with the grade of malignancy [14]. Another study performed by Tucker et al using brain tumor cell lines demonstrated that OPN was preferentially expressed in high grade and metastatic brain tumors compared to low grade brain tumors [8]. A study performed by Jang et al revealed that OPN was the most highly up-regulated gene in gliomas induced by N-ethyl-N-Nitrosourea (ENU) in rats [15]. These authors did not detect immunopositivity for OPN in normal brain tissue. They concluded that OPN overexpression occurred within a specific subset of intratumoral glial fibrillary acidic protein-positive cells and became evident at the stage of tumor progression. The study by Colin et al highlighted the crucial role of brain invasion in human GBM and identified specific molecules involved in this process. These authors reported that GBM differed from pleocytic astrocytoma by the expression of five genes (fibronectin, OPN, fibromodulin, chitinase-3-like-1 (YKL-40), and keratoepithelin) involved in invasion and angiogenesis [16]. However, Ding et al reported that OPN is expressed at similar levels in both normal adult brain tissue and grade III malignant astrocytic tumors [17].

Said et al investigated the hypoxia-related expression of OPN, CA9, erythropoietin, VEGF, and HIF-1 alpha both in vitro in human glioma cell lines as well as in vivo in human samples of GBM. They found that OPN mRNA and protein levels were higher in GBM than low grade astrocytoma, but they did not perform immunohistochemical analysis [18].

The current study confirmed the findings of Said et al in human gliomas as well as those from other previous studies in cell lines and animals by demonstrating the correlation between OPN expression and human astrocytoma tumor grade. Strong OPN immunoreactivity was present in high grade astrocytomas in this study, whereas slight staining was observed in low grade astrocytomas.

According to our results, OPN proteins are expressed in human astrocytomas and the extent of OPN expression correlates with the astrocytoma grade. It may also be considered that OPN is more highly involved with tumor progression than tumor initiation. In order to design future therapeutics and find potential targets for astrocytoma, the contribution of proteins and pathways that cause tumor development must remain an area of research.

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