

# IGFBP-2 EXPRESSION, ANGIOGENESIS AND PSEUDOPALISADES IN GLIOBLASTOMA

## Abstract

Glioblastoma is the most frequent, the most malignant and the best vascularised primary brain tumor. Substantial evidence suggests that IGFBP-2 (insulin-like growth factor binding protein) may play a significant role in the development and progression of various types of cancer, including the anaplastic progression of astroglial tumors. The correlation of IGFBP-2 and VEGF over-expression in diffuse gliomas and strong IGFBP-2 immunostaining and co-expression with VEGF in the cells of pseudopalisading necrosis suggests the induction of IGFBP-2 expression by hypoxia and/or possible involvement of its protein product in angiogenesis. The aim of this study was to analyze the expression of IGFBP-2 and the relationship between the IGFBP-2 expression, extent of bizarre angiogenesis and the presence of pseudopalisades. The prognostic impact of these variables was estimated, as well. Our results revealed that most glioblastoma (75%) express IGFBP2 and that IGFBP2 expression was associated with the presence of pseudopalisading necrosis as well as extensive bizarre angiogenesis. IGFBP-2 immunoreactivity in more than 5% of tumor cells and the presence of pseudopalisading necrosis were prognostically significant in univariate analysis ( $p=0.034$ ) while in multivariate analysis only a patient's age and the presence of pseudopalisades remained statistically significant ( $p=0.004$ ). The results of our study showed the association of IGFBP-2 expression in glioblastoma with pseudopalisades and bizarre angiogenesis thus confirming on morphological grounds presumed induction of this gene under hypoxic conditions and its possible role in angiogenesis.

## Keywords

• IGFBP-2 • Angiogenesis • Pseudopalisades • Glioblastoma • Glioma

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## 1. Introduction

Glioblastoma is the most common primary brain tumor and one of the most malignant human neoplasms. It accounts for 12-15% of all intracranial neoplasms and 60-75% of glial tumors [1]. Median survival time is only 12-14 months [2,3].

Characteristic histological features of this tumor include cellular proliferation and pleomorphism, mitotic activity, necrosis and microvascular proliferation. Microvascular proliferation results in bizarre vascular formations such as glomeruloid bodies, vascular garlands and vascular clusters [4]. Necrosis, particularly pseudopalisading type, and bizarre angiogenesis are morphological hallmarks of glioblastoma that distinguish this glioma from other infiltrative astrocytomas of lower grade [5].

IGFBP-2 (*insulin-like growth factor binding protein*) is one of the seven peptides with

a high affinity for insulin-like growth factors (IGF) [6,7]. By sequestering IGFs away from the type I IGF receptor, IGFBPs may inhibit mitogenesis, differentiation, survival, and other IGF-stimulated events [6,8,9]. Substantial evidence suggests that IGFBP-2 over-expression might be related to development and progression of various types of cancer [9-16]. Serum IGFBP-2 level is elevated in several malignant diseases such as prostatic [17-19], ovarian [20-22], and gastro-intestinal cancers [23,24]. IGFBP-2 expression in atypical and neoplastic prostatic and epithelium, but not in normal or hyperplastic type, suggests that it could be an independent marker of neoplastic transformation [9,25,26]. The strong correlation between over-expression of IGFBP-2 and histologic grade implies that IGFBP-2 may play a significant role in glioma progression [27-33].

IGFBP-2 possibly controls the cellular functions that are crucial in determining the phenotypes of advanced stages of cancer, such as cell proliferation, angiogenesis and invasion [32,34,35]. Reactivation of IGFBP-2 in glioblastoma is accompanied by the over-expression of VEGF and vascular proliferation suggesting the possible role of IGFBP-2 in tumor angiogenesis. Strong IGFBP-2 immunostaining and co expression with VEGF in the cells of pseudopalisades suggests the induction of this gene by hypoxia [16,33,36,37].

In order to further explore the presumed proangiogenic and hypoxia-associated role of IGFBP-2 the aim of this study was to determine the relationship between the IGFBP-2 expression, extent of bizarre vascular formations and the presence of pseudopalisades. Additionally, the prognostic significance of these variables on overall patient survival was also estimated.

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## 2. Experimental Procedures

Fifty-six patients, 30 male and 26 female, who underwent surgery of primary glioblastoma between January 2003 and December 2007 at the Department of Neurosurgery Clinical Hospital Center Sestre Milosrdnice were included in this retrospective study. Median age at surgery was 62.5 years (range 47-77 years) and median survival time was 162 days. All tumor specimens were reviewed and confirmed as primary glioblastomas according to the criteria of World Health Organization (WHO) Classification of Brain Tumors [1]. Recurrent tumors or tumors with a history of previous low-grade astrocytoma were excluded from this study. No chemotherapy or radiotherapy was administered to the patients before the surgery.

Surgical specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. For each sample, all sections were taken from the same paraffin block in order to evaluate IGFBP-2 expression, neovascularisation and the presence of pseudopalisades in the identical tumor areas. Immunohistochemistry was performed on 4- $\mu$ m formalin-fixed paraffin-embedded tissue sections using the LSAB method and Dako TechMate TM automatic immunostainer according to the manufacturer's instructions. Primary antibodies used were goat polyclonal antihuman insulin-like growth factor-binding protein 2 (IGFBP-2, Santa Cruz Biotechnology, Santa Cruz, California, 1:75) and mouse monoclonal antihuman endoglin (CD105) (DAKO, Glostrup, Denmark, 1:25). Human placenta served as a positive control, while the negative control was obtained by substituting the primary antibody with normal goat IgG. Endoglin (CD105) was used as immunohistochemical marker for activated endothelial cells which participate in the neovascularisation in order to facilitate the recognition of the angiogenesis pattern. All immunopositive neoplastic cells were included in this study. IGFBP-2 expression was estimated by using a semiquantitative four-tiered scale: score 0 = no IGFBP-2 positive cells; score 1 = up to 5% of IGFBP-2 positive tumor cells; score 2 = >5-25% of IGFBP-2 positive tumor cells; score 3 = > 25% of IGFBP-2 positive tumor cells [28].

Bizarre vascular formations (glomeruloid bodies, vascular clusters and vascular garlands) and the three-tiered scoring system of bizarre angiogenesis were defined according to Birner *et al.* [4]. The scoring system was used as follows: 0 points: no bizarre vascular formations (glomeruloid bodies or vascular clusters or vascular garlands) evident; 1 point: only single bizarre vascular formation in the whole tumor specimen detectable; 2 points: easily detectable multiple bizarre vascular formations. Scoring points of glomeruloid bodies, vascular clusters and vascular garlands in the individual tumor were added giving the final bizarre angiogenesis score (maximum score of 6). Pseudopalisades were defined as an irregular focus of tumor necrosis surrounded by the palisading tumor cell nuclei [1].

Data were analyzed by Statistica 6.0 software (StatSoft Inc., Tulsa, OK, USA). Spearman correlation was used to assess the correlation between categorical variables. Survival probabilities were computed according to the Kaplan-Meier method. Survival data were obtained from the National Cancer Register ending with September 5, 2009 (census date). Overall survival was defined from the day of the initial surgery until death of the patient. Survival until the end of the observation was considered as censored observation. For all tests, a 2-tailed p-value of  $\leq 0.05$  was considered as significant.

## 3. Results

IGFBP-2 immunoreactive cells (Figure 1) were found in 75% of (42/56) analyzed tumors. IGFBP-2 expression in up to 5% of tumor cells was observed in 28.6% (16/56) tumors, >5 to 25% tumor cells in 35.7% (20/56) and >25% of tumor cells in 10.8% (6/56) cases. IGFBP-2 expression was limited to the tumor cells only. IGFBP-2 immunoreactivity was found diffusely throughout the neoplastic cells but was enhanced in the tumor cells forming pseudopalisades.

Vascular garlands, glomeruloid bodies and vascular clusters were observed in 14 (25.0%), 34 (60.7%) and 43 (76.8%) cases, respectively. Only 2 cases had no bizarre vascular formations, and only 2 cases had the maximum bizarre angiogenesis score of 6. At least one bizarre vascular formation was observed in 54 (96.4%) cases. Pseudopalisades were observed in 31/56 (55.4%) cases. IGFBP-2 expression was positively associated with vascular clusters, bizarre angiogenesis score and pseudopalisades (Table 1).

Patient age was negatively associated with glomeruloid bodies and bizarre angiogenesis score (Table 2). The age of the patient was negatively associated with the survival and this association was statistically significant (Spearman Rank correlation,  $r = -0.26$ ,  $p = 0.044$ ). No associations between the

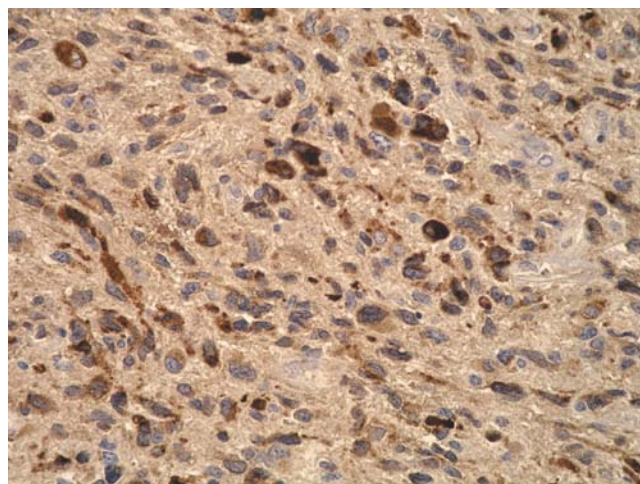


Figure 1. Variable cytoplasmic IGFBP-2 immunoreactivity is present in majority of glioblastoma cells. Magnification 400x."

bizarre vascular formations and survival were observed.

When IGFBP-2 expression was classified in 4 categories it showed no statistically significant correlation with survival ( $\chi^2=5.218$ ,  $p=0.157$ , Kaplan-Meier). However, if IGFBP-2 expression was classified in only two categories, one with less or equal 5% immunoreactive cells and the other with >5% immunoreactive cells, the patients with more IGFBP-2 immunoreactive cells survived longer and this was statistically significant ( $Z=-2.120$ ,  $p=0.034$ , Kaplan-Meier) (Figure 2).

Pseudopalisades were also a statistically significant predictor of longer survival in univariate analysis. Median survival of patients with pseudopalisades was 6.6 months while median survival of patients without pseudopalisades was 4.6 months ( $Z=-2.036$ ;  $p=0.0418$ , Kaplan Meier) (Figure 3).

When pseudopalisades were analyzed in a model including patient age (most significant predictor of survival) previously determined statistical significance of pseudopalisades as an independent predictor of survival was lost ( $p=0.092$ ), although the model was a statistically significant predictor of survival ( $\chi^2=10.554$ ;  $df=2$ ;  $p=0.005$ ). As there was no statistically significant difference for age between groups with and without pseudopalisades this was most probably not the cause of the loss of statistical significance for pseudopalisades in a two variant model.

Adding more factors in a multivariate model predicting survival (bizarre angiogenesis score and IGFBP-2 expression), the multivariate model showed a statistically significant influence on survival ( $p=0.043$ ) with significant independent influence of age ( $p=0.004$ ) and pseudopalisades ( $p=0.034$ ) in a way that younger individuals (aged 47-55 years) having pseudopalisades have longer survival.

#### 4. Discussion

Glioblastoma is the most frequent, the most malignant and the best vascularised primary brain tumor. Pseudopalisades, bizarre angiogenesis and IGFBP-2 over-expression are morphological and molecular features distinguishing glioblastoma from better

Table 1. IGFBP-2 correlation with bizarre angiogenesis and pseudopalisades (N=56).

|                            | Spearman - R | t (N-2) | p            |
|----------------------------|--------------|---------|--------------|
| vascular garlands          | 0,0039       | 0,029   | 0,976        |
| glomeruloid bodies         | 0,105        | 0,778   | 0,439        |
| vascular clusters          | 0,272        | 2,082   | <b>0,042</b> |
| bizarre angiogenesis score | 0,281        | 2,155   | <b>0,035</b> |
| pseudopalisades            | 0,346        | 2,712   | <b>0,008</b> |

Table 2. Patient's age correlation with bizarre angiogenesis (N=56).

|                            | Spearman - R | t (N-2) | p            |
|----------------------------|--------------|---------|--------------|
| vascular garlands          | -0,030       | -0,224  | 0,823        |
| glomeruloid bodies         | -0,359       | -2,832  | <b>0,006</b> |
| vascular clusters          | -0,127       | -0,948  | 0,347        |
| bizarre angiogenesis score | -0,375       | -2,976  | <b>0,004</b> |

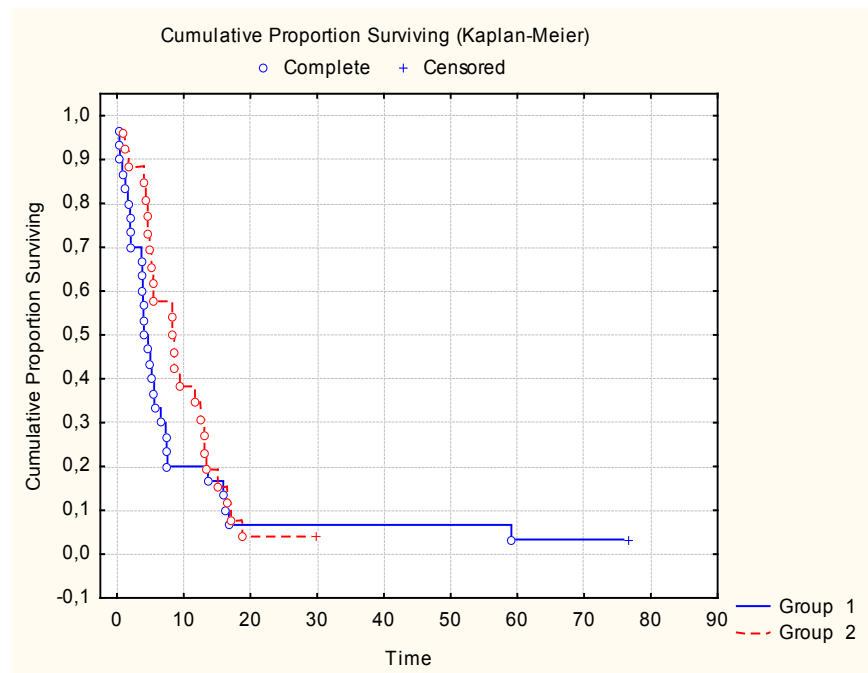


Figure 2. Kaplan-Meier survival curves in patients stratified by IGFBP-2 expression. Group 1 are patients with no immunoreactive tumor cells (stratum 0) and patients with less or equal 5% of immunoreactive tumor cells (stratum 1). Group 2 are patients with >5% of immunoreactive tumor cells (stratum 2 and 3). Censored data are presented as „+“.

differentiated astroglial tumors. IGFBP-2 over-expression directly correlates with the tumor grade of diffuse astroglial tumors

and is one of the most frequent molecular events in glioblastomas [16,27,31]. IGFBP-2 immunoreactivity in our study was found in

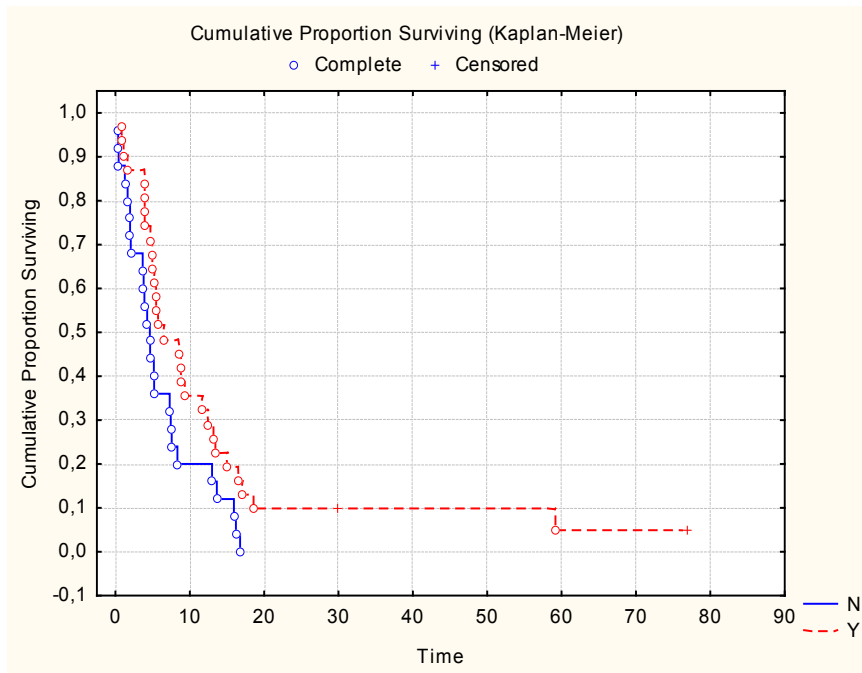


Figure 3. Kaplan-Meier survival curves in patients with (Y) and without (N) pseudopalisades in analyzed tumor samples. Censored data are presented as „+“.

75% of glioblastomas confirming the previously published results indicating IGFBP-2 positivity in 69% to 100% glioblastomas [28,29,31,38].

IGFBP-2 likely exerts its oncogenic potential through the control of cellular functions that are crucial in determining the phenotypes of advanced stages of cancer, such as cell proliferation, angiogenesis and invasion [32]. Hsieh *et al.* demonstrated IGFBP-2 over-expression within the stem-cell compartment of glioblastomas and its significance for clonal expansion and proliferation of stem-cells [39]. They suggest that IGFBP-2 may contribute significantly to glioblastoma pathogenesis by promoting stem-cell proliferation and survival. Moore *et al.* indicated that IGFBP-2 behaves as an oncogenic driver in glioma progression [40].

Association of immunohistochemically determined IGFBP-2 expression with morphological correlates of neovascularisation (bizarre angiogenesis score) and hypoxia (pseudopalisades) in our study might be explained with their presumed identical hypoxic pathogenesis. The correlation of IGFBP-2 and VEGF over-expression in diffuse gliomas and strong IGFBP-2 immunostaining

and co-expression with VEGF in the cells of pseudopalisades suggest the induction of this gene by hypoxia through the HIF-1 $\alpha$  (hypoxia-inducible factor) activated transcription and/or possible involvement of its protein product in angiogenesis [16,33,36,37]. Evidence that IGFBP-2 expression can be induced under anoxic or hypoxic conditions in glioblastoma cell lines and mouse embryonic stem cells and hypoxic injury to the rat brain supports this hypothesis [33,36]. Hypoxic conditions associated with necrosis probably induce angiogenic response and this could explain the intimate relationship between necrosis and glomeruloid bodies in glial neoplasms [41]. However, it remains unclear whether IGFBP-2 expression is intricately involved in the process of angiogenesis or whether it is merely a consequence of the hypoxic environment [28].

According to some authors vascular patterns significantly influence clinical outcome in primary glioblastomas [4] but not in ependymomas [42]. In our study no association was found between the extent of bizarre angiogenesis and survival. This disagreement with the previously published results [4] might

be a consequence of the poor interobserver agreement already noticed in the vascular pattern recognition [43].

Although the prognosis for patients with glioblastoma is poor, survival is variable and 3-5% are known to survive for more than 3 years [44]. Established good prognostic factors include young age, high Karnofsky Performance Status (KPS), high mini-mental status examination score, O6-methylguanine methyltransferase promoter methylation, and resection of > 98% of the tumor [45]. Few studies investigated the prognostic significance of IGFBP-2 expression with the contradictory results. McDonald *et al.* found no difference in the median survival of IGFBP-2 positive and negative glioblastomas (182 days with IGFBP-2 expression compared to 186 with no IGFBP-2 expression) [28] although long-term survival of more than 3 years was observed in patients with IGFBP-2 negative glioblastoma [28]. In the report of Santosh *et al.* univariate analysis showed statistically significant association between the IGFBP-2 labeling index and worse clinical outcome [33]. However, the patients in our study with > 5% IGFBP-2 immunoreactivity cells survived longer and this was statistically significant. Positive association of IGFBP-2 and survival in analyzed tumors might be the consequence of enhanced IGFBP-2 expression in tumor cells forming pseudopalisades which in multivariate analysis had statistically significant and independent influence on survival.

Multivariate analysis in our study showed that patient age and the presence of pseudopalisades had statistically significant and independent influence on survival in a way that younger individuals having pseudopalisades had longer survival. Because necrosis and apoptosis can lead to cell removal, one might suggest that they are both desirable in cancer treatment. However, in the setting of glioblastoma multiforme, clinical studies indicate that as the degree of necrosis advances, the patient's prognosis worsens. It has been shown that the presence of palisading necrosis in glioblastoma is an indicator of poor prognosis [46]. Although the magnitude of the survival difference between patients with and without tumor necrosis was small, absence of necrosis, after adjustment for age and extent

of resection, was still a statistically significant predictor of longer survival in multivariate analysis [47]. Pierallini A *et al.* evaluated the prognostic significance of the extent of the necrotic area as seen on contrast-enhanced CT and/or MRI [48]. Their survival data suggested that only patients with a small area of necrosis (less than 35% of the tumor) had a significantly longer survival time. When necrosis involved more than 35% of the mass, patients had a

shorter survival time, without any further correlation with the extent of necrosis [48].

The results of our study showed the association of IGFBP-2 expression in glioblastoma with pseudopalisades and bizarre angiogenesis thus confirming presumed induction of this gene under hypoxic conditions and its possible role in angiogenesis. Positive prognostic influence of IGFBP-2 expression and pseudopalisades on overall survival is surprising and has to be further

evaluated because our sample was small and there is a possibility that observed differences may be accidental.

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