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Research Paper

Prognostic Value of CXCL12 Expression in 40 Low-Grade Oligodendrogliomas and Oligoastrocytomas

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KEY WORDS

CXCL12, TTP, PDGFR-β, PDGF-B, Nestin, MVD, oligoastrocytoma, oligodendroglioma

ABBREVIATIONS

CXCL12/SDF-1	stromal cell-derived factor
CXCR4	CXC chemokine receptor 4
ERK	extracellular signal-regulated kinase
KPS	Karnofsky performance score
MVD	microvessel density
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
RT	radiotherapy
ST	survival time
TNF	tumor necrosis factor
TTP	time to tumor progression

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ABSTRACT

Both clinical and biological features have been reported as prognostic factors in low-grade gliomas. Among these, histotype, tumor size, enhancement, age and genetic pattern. Microvessel density (MVD) has been correlated to clinical outcome in astrocytomas, but its impact in oligodendrogliomas and mixed tumors is not sure. The pro-angiogenic chemokine stromal cell-derived factor (SDF-1/CXCL12) and its receptor CXC chemokine receptor 4 (CXCR4) have been described in low-grade gliomas, with a correlation between CXCL12 expression and shorter time to progression (TTP). The intermediate filament Nestin is expressed in proliferating vessels. Platelet-derived growth factor B (PDGF-B) and its receptor PDGFR-β are also involved in angiogenesis and malignant progression in gliomas.

The aim of this study was to retrospectively investigate the MVD and immunohistochemical expression of CXCL12, CXCR4, PDGF-B, PDGFR-β and Nestin in 40 patients (11 oligodendrogliomas and 29 oligoastrocytomas).

In our study, oligodendroglioma histotype was associated with a trend to a more prolonged TTP than mixed tumors (p = 0.12). Age younger than 32, presurgery lack of enhancement at CT/MRI and total versus partial resection were not associated with longer TTP. Positivity for CXCL12 on tumor/endothelial cells was the only factor associated with a significantly shorter TTP (p = 0.011). Positivity for CXCL12 in tumor cells was predictive of a shorter survival time (p = 0.014).

Since CXCL12 is not only related to angiogenesis, but also exerts an anti-apoptotic effect that may contribute to tumor progression/endothelial escape from apoptotic mechanisms, expression of CXCL12 by these tumors might add prognostic information to available clinical and bio-molecular indexes.

INTRODUCTION

Within the heterogeneous group of low-grade gliomas, prognostic factors include histotype, tumor size and location, age, neurological signs, entity of surgical resection.[1] As a matter of fact, oligodendrogliomas are tumors with a longer median survival (12.1 years)[2] than low-grade astrocytomas (5 years in Pignatti),[1] with oligoastrocytomas displaying an intermediate behaviour.[2] Some works underscore the role of genetic alterations, with loss of heterozygosity at 1p and 19q playing a positive prognostic role in terms of survival/response to chemotherapy[3] and loss at 10q predicting a worse outcome.[4]

These prognostic elements add to previously identified ones, such as CT enhancement, calcification and radiation dose in oligodendroglioma/oligoastrocytoma.[5]

The study of clinical and biological prognostic factors in gliomas with oligodendroglial component could improve the management of these patients.

The vascular supply is known to be crucial in the development of tumors beyond a size of a few millimeters. In the present study we therefore investigated a pattern of factors related to angiogenesis and glioma growth, such as microvessel density (MVD) and immunohistochemical expression of CXCL12, CXCR4, PDGF-B, PDGFR-β and Nestin, and their relationship to clinical course in a group of low-grade oligodendrogliomas and oligoastrocytomas.

Histological studies have previously correlated MVD and various indexes related to vascularization[6] to the clinical outcome both in astrocytomas[7] and in oligodendrogliomas.[8]

Rempel has described the presence of the pro-angiogenic chemokine stromal cell-derived factor (SDF-1/CXCL12) and of its receptor CXC chemokine receptor 4 (CXCR4) in the context of human high grade glial tumors.[9] We have extended this finding to a group of 50 low-grade gliomas and we have found a correlation between CXCL12 expression

and shorter time to progression (TTP).[10] Experimental evidence suggests that CXCL12 may be expressed both by tumor cells and by endothelial cells, and that it may induce proliferation and inhibit apoptosis of glioma cells.[11-13]

Also platelet-derived growth factor (PDGF) is related to glioma growth and angiogenesis: platelet-derived growth factor receptor β (PDGFR-β) has been detected mainly in endothelial cells and PDGFR-α in tumor cells, suggesting the presence of autocrine and paracrine loops in glioma.[14,15]

Finally, we have assessed the expression of Nestin, that is a type VI intermediate filament protein originally expressed by neuroepithelial stem cells, but its presence has been documented also in newly formed vessels and it represents a marker of neovascularization.[16,17] Moreover, Nestin is also expressed by glioma cells, more so in high-grade than in low-grade tumors.[18,19]

This retrospective study was performed on 11 patients with oligodendroglioma and 29 with oligoastrocytoma operated on at a single Institution from 1992 to 1998. The impact of clinical variables (including age, presurgery enhancement at CT/MRI, KPS, extent of resection), of MVD and of the expression of CXCL12, CXCR4, PDGF-B, PDGFR-β and Nestin, was evaluated on TTP and on survival time (ST).

MATERIALS AND METHODS

Patients. 40 patients referred to Istituto Nazionale Neurologico "C. Besta" and deemed suitable for surgery in the years 1992–1998 were included in this retrospective study. Histological diagnosis, according to WHO, was grade II oligodendroglioma in 11 and grade II oligoastrocytoma in 29.

Age, gender, initial symptom, tumor location, imaging findings, extent of resection, initial treatment and performance status were recorded. Out of 40 patients, 24 were male, 16 female and age ranged from 14 to 68 years (median 32). Seizures (partial or generalized) were the onset symptom in 34 patients, headache was present at onset in 9, motor deficits in 1, cranial nerve dysfunction in 2, cognitive disturbances in 1. Tumor location was supratentorial in all patients. MRI was performed in 22 patients, CT in 3, both MRI and CT in 15 prior to surgery. As observed at MRI/CT, mass-effect was present in 14 patients and enhancement in 10. In 9 patients, surgery as judged by the operating neurosurgeon was partial, in 28 grossly total. After surgery, 7 underwent early radiotherapy (RT) and 4 early chemotherapy. Post-surgery Karnofsky performance score (KPS) was 40 in 1 patient, 60 in another, and 80 or more in all the remaining patients.

Immunohistochemistry. Surgical specimens were fixed in Carnoy's fixative, paraffin-embedded and cut at 3μm with stains for standard histological diagnosis. Sections were deparaffinized and rehydrated with standard procedures, placed in H_2O_2 3% for 15' to block endogenous peroxidase activity and then incubated with normal goat serum for 30'. For immunohistochemical detection of CXCL12 and PDGF-B, slides were pretreated in citrate buffer (Target Retrieval Solution, Dako, Carpineteria, CA) in a microwave at 350Watt 3 times for 3 minutes. The primary anti-CD34 (1:200, Dako) and anti-Nestin (1:200, R&D System, Abingdon, UK) antibodies were incubated for 1 hour, while anti-CXCL12 (1:10, R&D system), anti-PDGF-B (1:100, R&D system) and anti-PDGFR-β (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were incubated overnight. As secondary antibody, monoclonal anti-mouse envision® peroxidase conjugated (Dako) was used for CD34, CXCL12 and Nestin, while anti-rabbit envision® peroxidase conjugated (Dako) was used for PDGF-B and PDGFR-β.

As far as CXCR4 stain is concerned, we tested the anti-CXCR4 antibody at different dilutions, along with various antigen-retrieval methods. The best results were obtained by processing slides with 121°C hydrated autoclaving pretreatment and overnight primary antibody (1:1000, R&D system) incubations. Staining was performed by the avidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA).

Detection of immunostaining was performed using diaminobenzidine (DAB, Dako) as chromogene, followed by counterstaining with hematoxylin. Negative controls (i.e., sections in which the primary antibody was substituted by non immune serum) were also stained each run.

Staining was evaluated by two observers blinded to diagnosis and samples were scored according to the number of positive cells, regardless of the cell type expression (endothelial cells, tumor cells, or both). For statistical analysis, scores were condensed to a score of positive or negative and a value of 10% was considered to accept a sample as positive.

MVD quantification. To evaluate MVD, sections CD34-stained were used. Four fields at 250x were selected randomly in the context of the tumor and examined for number of microvessels (i.e., single-layered $CD34^+$ structures) by a single observer unaware of clinical and histological variables (M.G.). The average value of 4 fields was used for statistical analysis (indicated as MVD mean). Also the apparently most vascularized area of the tumor, as identified at low magnification, was assessed (one field at 250x) for MVD (indicated as "high MVD").

Statistical analysis. TTP and ST were analyzed by Kaplan-Meier survival curves and differences in this parameter between subgroups of patients by log-rank test. The variables considered for the analysis consisted of patient-related and tumor-related features, such as age, sex, initial symptoms, tumor location, presence of mass-effect or enhancement at MRI/CT, extent of resection, initial treatment (radiotherapy/chemotherapy), KPS and angiogenesis-related factors, such as MVD and immunohistochemical expression of CXCL12, CXCR4, PDGF-B, PDGFR-β and Nestin. The association between MVD and expression of the various angiogenesis-related factors was assessed by Fisher test. For the analyses, the age of 32, "MVD mean" value of 13 and "high MVD" value of 25 were selected as cut-off based on the median of the whole group.

The analyses were carried out using StatView 5.0.1 computer software (SAS Institute, Cary, NC) for Kaplan-Meier survival curves and Jandel SigmaStat statistical software, version 2.0, NL (Jandel SigmaStat, Jandel, San Rafael, CA) for Fisher test.

RESULTS

Clinical features. After a follow-up ranging from 20 to 153 months (median 84), 20 patients have progressed; of these, 8 have died due to disease progression. Three patients have been lost to follow-up.

Early RT was delivered in 7 patients. Of these, 4 have progressed and 2 have died.

Differences in histotype were associated with a trend to different TTP: pure oligodendroglioma patients had a more prolonged TTP than mixed tumors (p = 0.12 log-rank) (Fig. 1); of note, while median TTP in oligoastrocytomas was 44 months, median TTP was not reached in pure oligodendrogliomas, with an estimated percentage of progression-free patients of 64% after 122 months of follow up. We

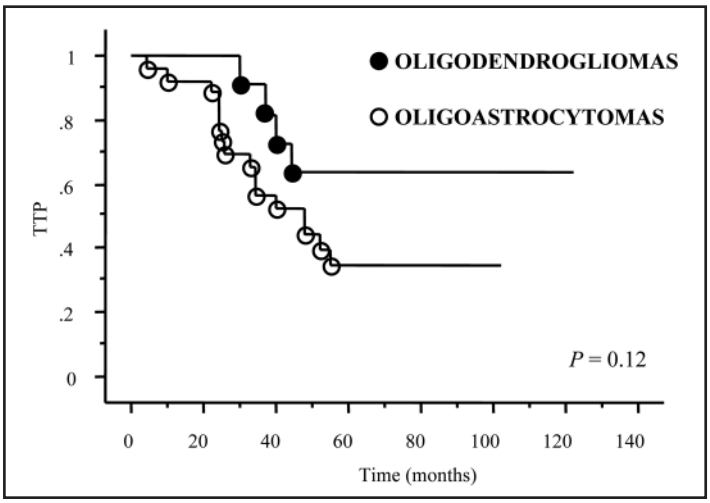

Figure 1. Kaplan-Meier curve shows time to progression (TTP) of patients with oligodendroglioma (11) and oligoastrocytoma (29) (p = 0.12 log-rank).

Table 1 **Evaluation of MVD mean and "high MDV" in CD34-stained sections of mixed and pure oligodendrogliomas**

	Oligodendrogliomas (11)	Oligoastrocytomas (29)
MVD mean[a]	13.8 ± 5.2	13.4 ± 5.7
high MVD[b]	24.6 ± 6.3	25.9 ± 11.8

MVD, microvessel density. [a]Average value of 4 fields at 250x selected randomly. [b]One field at 250x of the apparently most vascularized area of the tumor.

early RT versus the other patients, nor between patients with KPS of 100 and the other patients.

As far as ST is concerned, only presence of presurgery enhancement at CT/MRI (p = 0.067 log-rank) was of borderline significance as predictive of a shorter ST.

Immunohistochemistry and MVD. In immunohistochemical analysis, CXCL12 showed the highest number of positive samples (66%) in vessels, and in most cases endothelial and neoplastic cells immunoreactivity were associated (Table 2, Fig. 2A). Similar results were obtained with Nestin: 54% of samples were positive in endothelium and 39% in tumor cells (Fig. 2C). PDGFR-β was found in 44% of cases in endothelial cells and only in 18% of cases in tumor cells (Fig. 2B). PDGF-B immunoreactivity was observed in 37% of cases in vessels and in 7% in tumor cells (Fig. 2D). No differences were found in expression of investigated proteins between oligodendro-gliomas and oligoastrocytomas, with the exception of CXCL12 which was more frequently expressed by tumor cells in oligoastrocytomas than in oligodendrogliomas, although this difference did not reach statistical significance.

CXCR4 expression was detected in almost all tissue specimens, but with an ambiguous pattern; most cases showed nuclear staining in both neoplastic and apparently healthy surrounding tissue; CXCR4 nuclear immunoreactivity was seen mainly in tumor and endothelial cells, with only some cases showing positivity in the cytoplasm of tumor cells (not shown).

We didn't find any significant differences between oligodendrogliomas and oligoastrocytomas in MVD (Table 1), and when the impact of mean MVD on TTP was investigated, no significant relationship emerged between higher MVD and shorter TTP (not shown).

Positivity for CXCL12 either on tumor (p = 0.0058 log-rank) or on endothelial cells (p = 0.0065 log-rank) was associated with a significantly shorter TTP; positivity for CXCL12 regardless of the expressing cells was also associated with a shorter TTP (p = 0.011 log-rank) (Fig. 3); no significant correlation between immunoreactivity for PDGF-B, PDGFR-β, Nestin and TTP was found (not shown).

We addressed the issue whether CXCL12, Nestin, PDGFR-β and PDGF-B expression was more frequent in patients with a higher MVD: using a Fisher's exact test, no significant relationship emerged.

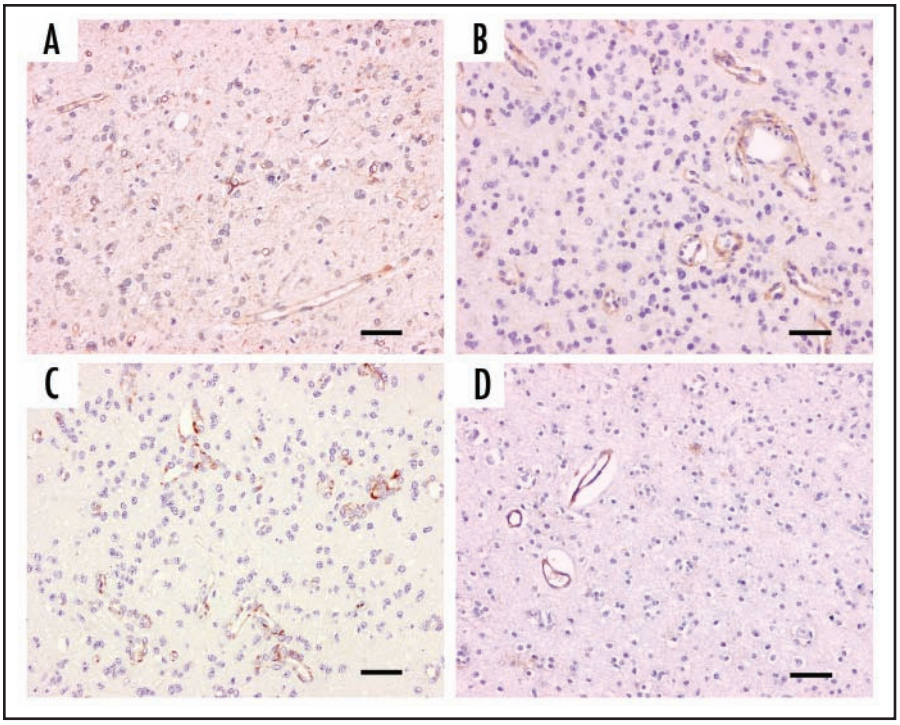

Figure 2. Immunohistochemistry on paraffin sections of human glioma: indirect immunoperoxidase staining with monoclonal antibodies which recognize (A) CXCL12 in tumor and endothelial cells on a oligoastrocytoma, (B) PDGFR-β in endothelial cells on a oligoastrocytoma, (C) Nestin in endothelial cells on a oligoastrocytoma, (D) PDGF-B in endothelial cells on a oligodendroglioma. The internal scale marker represents a length of 50 μm.

cannot calculate the impact of histotype on ST, because at the end of follow-up all patients with oligodendroglioma were alive.

Age younger than 32 was associated with a more prolonged TTP, although this did not reach statistical significance.

No significant differences were seen in TTP between patients having undergone partial versus grossly total tumor removal, or between patients with or without enhancement at preoperative CT/MRI. No differences in TTP were detected in patients undergoing

Table 2 **Expression of CXCL12, Nestin, PDGFR-β and PDGF-B in endothelial and tumor cells evaluated by immunohistochemistry**

ID#	CXCL12 endo	CXCL12 tum	Nestin endo	Nestin tum	PDGFRβ endo	PDGFRβ tum	PDGFB endo	PDGFB tum
1	+	+	-	-	-	-		
2	-	-	-	-	+	+	-	-
3	-	-	+	-	-	-	-	+
4	-	+	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	+	+	+	+	-	-		
7	+	+	-	+	-	-	-	-
8	-	-	-	-	+	+		
9	+	+	+	+	+	-	+	+
10	+	+	-	-	-	-		
11	+	-	+	-				
12	-	-	+	-	+	-	-	-
13	+	+	-	-				
14	+	+	-	-				
15	+	-	+	-	+	-	+	-
16	-	-	-	-	+	-	+	-
17	+	-	-	-	-	-	-	-
18	+	+	-	+	-	-	+	-
19	+	-	+	+	-	-	+	-
20	-	+						
21	+	+	+	+	-	-	+	-
22	-	-	-	-	-	-	-	-
23	+	-	+	-	+	-	-	-
24	+	+	+	+	+	-	-	-
25	+	-	-	+	-	-	+	-
26	+	-	+	+	-	-	+	-
27	+	+	+	+	+	+	-	-
28	+	-	+	+	+	+	+	-
29	+	+	+	+	+	+	-	-
30	+	-	-	+	-	+		
31	+	-	+	-	+	-	-	-
32	+	-	+	-	-	-		
33	+	+	-	-	-	-	-	-
34	-	-			+	-	-	-
35	-	-	+	+	-	-	+	-
36	-	-	+	+	+	-	-	-
37			-	-			-	-
38	+	-	-	-				
39	-	-	-	-	-	-	-	-
40	+	-	+	-	-	-	+	-

Abbreviation: endo, endothelial cells; tum, tumor cells

As far as ST is concerned, only positivity for CXCL12 expression on tumor cells (p = 0.014 log-rank) was associated with a shorter ST.

DISCUSSION

In the last years, many studies have highlighted as gliomas with oligodendroglial component are more responsive to chemotherapy and with a more favorable prognosis compared to pure astrocytomas.[20] Differences in clinical outcome of these patients are partly associated to genetic alterations, as loss of heterozygosity at 1p and 19q.[3]

In order to investigate the prognostic significance of clinical and biological features and their possible role in malignant progression, we have analyzed a cohort of 29 oligoastrocytomas and 11 oligodendrogliomas.

Among clinical/histological features, pure oligodendrogliomas had a longer TTP compared to mixed tumors, but not statistically significantly so (p = 0.12). Previous studies have shown a more favorable survival in oligodendrogliomas compared to mixed oligoastrocytomas: patients with oligoastrocytomas have 5- and 10-year survival rates of 63% and 33%, respectively, whereas patients with oligodendrogliomas have 5- and 10-year survival rates of 73% and 49%, respectively.[20] In our data, limited patient number and a less strong end-point could explain the lack of statistical significance for TTP; on the other hand, although a statistical analysis by survival curves could not be performed, no deaths occurred after a similar median follow-up (80 months in oligoastrocytoma and 98 months in oligodendroglioma) in the patients with pure oligodendroglioma, whereas all deaths occurred in the oligoastrocytoma subgroup, in agreement with available literature data concerning survival.

When the predictive value of age was considered, we failed to detect a shorter TTP in older patients. Grossly total removal and preoperative enhancement were also not significantly related to subsequent TTP in this cohort.

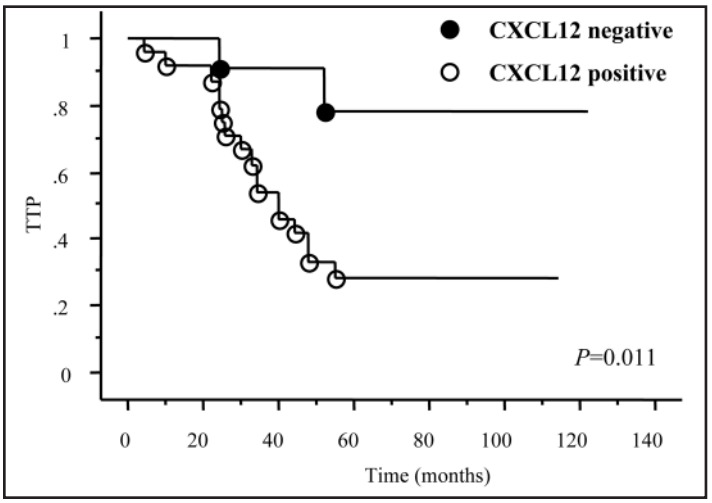

Figure 3. Kaplan-Meier curve shows time to progression (TTP) of patients with oligodendroglioma and oligoastrocytoma positive or negative for CXCL12 expression as assessed by immunohistochemistry (p = 0.011 log-rank).

The extent of MVD has also been assessed together with the expression of tumor growth/or angiogenesis-related factors such as CXCL12, CXCR4, PDGF-B, PDGFR-β and Nestin.

While it is known that similar MVD values in oligodendroglioma as compared with low-grade astrocytoma do not necessarily relate with a similar prognosis, it is true that they correlate with tumor grade within each histotype.[21] In our samples MVD was similar in oligodendrogliomas compared to oligoastrocytomas.

The lack of prognostic significance of MVD on TTP is in agreement with previous evidence by Vaquero and colleagues:[6] in their work, these authors lacked to detect differences in survival time in 36 low-grade oligodendroglioma patients according to age at surgery (younger or older than 40) or high- or low-MVD. In a subsequent study in 26 patients,[8] the same authors showed a predictive value of a "vascular endothelial surface index" (i.e., the CD34-immunostained area in μm^2 per 1000 tumor cells). However, this index is obtained via a rather complex and time-consuming histological observation which includes use of specific software, and it is not likely to be widely used with a high inter-observer agreement.

Whereas MVD per se does not seem to add prognostically relevant information to clinical-genetic data in oligodendroglial tumors, it has been reported to predict survival time in a large cohort of fibrillary astrocytoma patients;[7] this suggests that disease progression is more closely related to early angiogenic switch in astrocytic tumors.

We have investigated the expression of Nestin, an intermediate filament characteristic of proliferating vessels, detected also in glioma cells. In tissues undergoing neovascularization, in physiologic conditions such as during tumor growth, Nestin participates in formation of the cytoskeleton of the immature precursors of endothelial cells. Nestin expression is decreased when endothelial cells differentiate and their rate of growth diminishes.[16,17] In adult, glial cells express Nestin only when transformed, suggesting that CNS tumor cells can reexpress a gene normally active during CNS development.[18] Thus, Nestin detection in gliomas could be useful as an auxiliary indicator of dedifferentiation and progression.[19]

Dahlstrand and colleagues[18] have found a weak Nestin expression in oligodendrogliomas, mainly in endothelial cells. In agreement with these results, in our specimens Nestin expression was not as strong as in high grade glioma (Pollo, unpublished data) and it was localized predominantly, although not exclusively, in endothelial cells.

Also PDGFR-β was mainly confined to endothelial cells, in agreement to previous findings.[14,15] Guo and colleagues[22] suggest that an angiogenic switch could be promoted by overexpression of PDGF-B by tumor cells and PDGFR-β by endothelial cells, leading to vascular growth and malignant progression. Moreover, in a murine model PDGF-B in the presence of its receptor, PDGFR-β, was recently shown to be essential to maintain the features of high grade oligodendroglioma. Blocking the PDGFR-β, the major effect was the inhibition of oligodendroglioma cells proliferation.[23]

In our samples, we have detected vascular expression of PDGFR-β in 44% of cases, whereas PDGF-B immunoreactivity was very low and primarily localized in endothelial cells.

No correlation with unfavorable TTP was found in expression of Nestin, PDGFR-β and PDGF-B. We therefore conclude that Nestin and PDGF-B/PDGFR-β could have a marginal role in tumor progression in low grade oligodendrogliomas/oligoastrocytomas, but could be more important in a following phase of tumor development, related to angiogenesis and to a higher tumor proliferation rate.

Among the investigated clinico-histological features, only the expression of CXCL12 in tumor/ endothelial cells was predictive of a shorter TTP and it was a more powerful predictor than histotype. This stresses the relevance of this chemokine which has been shown to be present in high grade gliomas and glial tumor cell lines;[9,11,13] we have shown that high levels of CXCL12 in the post-surgical cavity of recently operated high-grade gliomas were associated to a short progression free survival.[12] The possible activity in vivo of elevated CXCL12 concentrations in the context of glial tumors is supported by our findings of expression of the receptor for this chemokine, CXCR4, on endothelial cells and primary tumor cultures derived from glioma patients.[12] Actually, a cross-talk between endothelium and tumor cells may lead to enhanced growth and vascularization. CXCR4 acts via extracellular signal-regulated kinase (ERK) and Akt activation as chemotactic factor, increasing proliferation and inhibiting apoptosis in tumor cells.[11,13,24] We observed that CXCL12 could protect from apoptosis also endothelial cells.[12] Furthermore, as suggested by Rempel,[9] CXCL12 and CXCR4 interactions could protect the tumor by suppressing immune response: high CXCL12 expression may induce macrophages and microglial cells to attack T cells via tumor necrosis factor (TNF)/TNF-R interactions.[25-27] Moreover, we[10] showed that CXCL12 was expressed also in a proportion of 53 low-grade glioma specimens, and that it was predictive of shorter TTP on the whole cohort of patients.

As far as CXCR4 immunoreactivity is concerned, its meaning was difficult to understand because of its inconstant and ambiguous pattern; as a matter of fact, widespread nuclear staining has been reported also by other authors.[28-31]

Regarding ST, we detected a borderline significant predictive value of presurgical contrast-enhancement and a highly significant predictive value for CXCL12 expression (with shorter ST), while the absence of deaths during follow-up precludes statistical analysis by survival curves, but is indeed highly suggestive for a better outcome in oligodendrogliomas. However, it should be stressed that survival time—although a stronger outcome parameter—may be influenced by differences in therapeutic attitudes at disease progression, which are common in these patients.

In conclusion, in our work we have analyzed a group of factors related to angiogenesis and tumor growth and their predictive value on TTP and ST in a cohort of low-grade oligodendrogliomas and oligoastrocytomas. The interest for these tumors is also related to

their less unfavorable clinical outcome compared to other glial tumors. Among the analyzed factors (histotype, clinical features, expression of Nestin, PDGFR-β and PDGF-B), CXCL12 expression was the unique with a predictive significance on TTP and ST. To our knowledge, this and our previous,[10] are the sole studies about CXCL12 in low grade oligodendrogliomas and oligoastrocytomas, while several studies have investigated its expression and its biological role in high grade gliomas. Further researches are required to assess the role of this chemokine in malignant progression of oligodendroglial tumors.

Expression of CXCL12 by these low-grade tumors might add prognostic information to available clinical[1] and molecular biology indexes,[3] thereby contributing to optimal management in patients with variable life expectancy.

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