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EGF-R and PDGF-R, But Not bcl-2, Overexpression Predict Overall Survival in Patients With Low-Grade Astrocytomas

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Background and Objectives: Therapy of malignant glioma tumors is based on histology and clinical factors. However, comparable lesions may correspond with important prognostic differences. Our purpose was to analyze retrospectively the prognostic input of platelet-derived growth factor receptor (PDGF-R), epidermal growth factor (EGF-R), and bcl-2 expression in 103 malignant gliomas from uniformly treated patients.
Methods: The expression of the antigens was analyzed by immunohistochemistry (IHC). Prognostic evaluation was performed with the multivariate proportional hazards model. The follow-up period lasted 19 (5–122) months for survivors.
Results: We observed that almost 50% of gliomas showed high expression of PDGF-R, while a lower expression of EGF-R and bcl-2 was found. No association between the main prognostic factors in malignant glioma (sex, age, histological grade, and Karnofsky score) and the labeling index (LI) of these antigens was observed. We found that only PDGF-R and EGF-R overexpression were associated with a shorter survival in patients with World Health Organization (WHO) II astrocytomas, being both associations independent of known prognostic factors, as shown by Cox model. Besides, we confirmed other authors' results that high histological grade and low performance score were associated with worse prognosis.
Conclusions: PDGF-R and EGF-R expression could be relevant in determining the prognosis of low-grade astrocytomas (LGAs) and in providing a more objective mechanism for their classification.
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KEY WORDS: tumor marker; malignant glioma; invasion; EGF-R; PDGF-R; bcl-2

INTRODUCTION

Malignant gliomas are the most common tumors of the central nervous system, accounting for more than 60% of all primary brain tumors. A cardinal property of these tumors is the propensity to invade the brain, making complete surgery almost impossible. Besides these tumors are largely resistant to radiation and to chemotherapy. These reasons can explain the high rate of recurrence, frequently to more anaplastic entities, and the short survival these patients have.

Malignant gliomas are classified by the World Health Organization (WHO) into grades II–IV based on the degree of malignancy, as determined by histopathological criteria: low-grade astrocytoma (LGA, grade II), anaplastic astrocytoma (AA, grade III), and glioma multi-

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forme (GBM, grade IV). GBM is one the most aggressive human cancers, showing a median (Md) survival of less than 1 year since the past two decades [1].

The clinical factors useful for assessing individual glioma prognosis are patient age, tumor grade, tumor type, the volume of tumor rejected, condition (reflected in the Karnofsky performance score), the use of radiotherapy, and some neuroradiological data such as the absence of necrosis in imaging studies [2]. However, these tumor parameters are insufficient to predict the evolution of each individual patient. So it is a high priority to find tumoral markers able to differentiate subtypes of malignant gliomas allowing the selection of patients with bad prognosis to apply an earlier and more aggressive therapy.

It is widely known that tumor formation is a complex, multistep process involving the accumulation of genetic lesions in genes that normally regulate the pathways of cell proliferation, differentiation, and death required for normal development. Tumors often overexpress different growth factors and their respective receptors suggesting the establishment of autocrine stimulatory loops, leading to the activation of the cell cycle machinery and uncontrollable cell proliferation [3]. In particular, the epidermal growth factor (EGF-R) and the platelet-derived growth factor receptor (PDGF-R) are glycoproteins with tyrosine kinase activity that have been found overexpressed in a high percentage of malignant gliomas [4–7].

The protooncogene bcl-2 is a member of the superfamily of proteins that are involved in the regulation of apoptosis. Bcl-2, localized preferentially in the mitochondrial membranes [8], blocks apoptosis by inhibiting the release of cytochrome-*c* [9] and by negatively regulating molecules able to induce apoptosis such as Apaf-1. Furthermore, bcl-2 seems to enhance migration and invasion capability of human glioma cells [10] and its expression has been reported to be altered in human gliomas [11].

Recent clinical and molecular studies indicate that two different genetic pathways leading to the development of GBM. Kleihues and Cavenee [2] and Louis and Gusella [12], among others, have outlined the following model for malignant progression of gliomas: loss of chromosomes 17p (carrying p53 gene), loss of heterocygosis of 19q and 10q, and PDGF-R alteration in secondary GBM, while amplification and overexpression of EGF-R and Mdm2 together with p16 inactivation have been involved in primary GBM. Primary gliomas occur more frequently in older people and are more aggressive [13,14]. Both the histological examination and the known prognostic factors are unable to differentiate subtypes of malignant gliomas.

The aim of this study was to analyze the expression pattern of EGF-R, PDGF-R, and bcl-2 in 103 malignant gliomas tumors receiving uniform treatment and to correlate it with clinical and anatomo-pathological parameters related to established prognostic factors, including survival rate.

MATERIALS AND METHODS

Patients and Tumors

One hundred three paraffin embedded brain tumors corresponding to 1995–2000 period were obtained from the ''Hospital Italiano de Buenos Aires.'' All tissue specimens, obtained from untreated patients by surgical resection, were classified morphologically and graded according to the current WHO system.

Table I shows some features of the studied population. All patients presented a performance score (Karnofsky) higher than 80. Patients were only included if a gross total or subtotal tumor resection was possible. This was confirmed by magnetic resonance imaging within the first 48 hr after surgery.

Patients were treated postoperative according to the histological grade: GBM and AA patients always received postsurgery radiotherapy and chemotherapy, while LGA patients received radiotherapy/chemotherapy when studies indicated residual tumor immediately after surgery or during recurrence.

The follow-up period lasted a Md time of 19 months with a range of 5–122 months for the survivors. The numbers of survivors, at the end of the study, were 11, 6, and 10 for LGA, AA, and GBM, respectively. All patients

TABLE I. Characteristics of the Studied Population

Histologic type (WHO grade)	n (103)	Age[a] (year)	Sex		Survival time[a] (month)
			M (n = 65)	F (n = 38)	
Low-grade astrocytoma (LGA) (II)	25	37 (18–72)	16	9	42 (15–122)
Anaplastic astrocytoma (AA) (III)	24	55 (25–72)	16	8	23.5 (9–96)
Glioblastoma multiforme (GBM) (IV)	54	59 (18–76)	33	21	14 (5–31)

[a]Expressed as Md (range).

who died had clear evidence of uncontrolled tumor growth at the time of death.

Immunohistochemistry (IHC)

Tumor specimens were fixed immediately after removal in 10% formalin and processed to paraffin blocks.

Representative serial sections (5-μm thick) were placed on positively charged slides and microwaved in citrate buffer (pH = 6) to recover antigenicity. Sections were incubated with commercial monoclonal primary antibodies: EGF-R (M3563, Dako Corporation, Glostrup, Denmark; diluted 1/50), PDGF-R (sc-338, Santa Cruz, Biotechnology, Santa Cruz, CA; 1/100), bcl-2 (M 0887, Dako Corporation; 1/40), and Ki-67 (A0047, Dako Corporation; 1/50) followed by biotinylated anti-mouse or anti-rabbit antibody (Gibco BRL, Gaithersburg, MD). After washing, sections were treated with Vectastain ABC kit Universal (Vector Laboratories, Burlingame, CA) and then incubated with the chromogen 3, 3′-diaminobencidine (7%) plus 3% H_2O_2 in PBS. Finally, they were counterstained with Harris hematoxylin. Negative controls, missing out the first antibody, were performed to discriminate background staining.

The expression of the different antigens was analyzed by three independent observers and was scored according to the number of cells with positive bright brownish staining. Differences in the intensity of staining were not considered. The labeling index (LI) for each antibody was calculated as the percentage of labeled cells out of the total number of tumor cells counted. For statistical analysis, scores were later condensed to a score of ''negative'' or ''positive.'' For EGF-R, Ki-67, and bcl-2, a value of 10% was required before a case was accepted as positive. For PDGF-R2α, the threshold of positivity was raised to 25%, due to the high staining observed.

Statistical Analysis

The relationships between various parameters were evaluated statistically using χ^2 Wilcoxon test and Pearson correlation coefficients. A difference of $P < 0.05$ was considered to be significant.

We used linear regression to summarize the joint effects of histologic type, age, sex, and Karnofsky performance status on antigen positivity. As all the selected patients had similar grade of resection after surgery and received similar type of therapy, these variables were not included in the study.

The Kaplan–Meier method was used to estimate survival, defined as the time between tumor diagnosis and the patient's death or last official contact. In univariate survival analyses, two-sided log-rank tests for equality of survivor functions were used to assess the prognostic significance of different parameters on PDGF-R, EGF-R, bcl-2, and Ki-67 positivity. Multivariate analysis was performed using the stepwise Cox proportional hazards model to evaluate the predictive power of each variable independently of the others. We employed a model starting with the Cox model containing all four variables and successively eliminated the least statistically significant variable until only statistically variables were left ($P < 0.05$). All variables were entered in the multivariate analysis as categorical ones.

SPSSPC+ (version 10) for Windows software was used for the aforementioned analyses.

RESULTS

Malignant Glioma Immunostaining for PDGF-R, EGF-R, and bcl-2

The expression of PDGF-Rα, EGF-R, bcl-2 was analyzed in 103 malignant gliomas of different histological grade. PDGF-R and EGF-R stained preferentially the plasmatic membrane, but some cytoplasmatic staining was also observed. On the other hand, bcl-2 staining was restricted to the cytoplasm.

Possible relationships between antigen immunostaining and clinicopathological features relevant in prognosis as sex, age, histological grade, and Karnofsky status are shown in Table II. As can be observed, no significant association between PDGF-R, EGF-R, and bcl-2 LI and these different prognostic factors was found.

Almost 50% of the studied malignant gliomas overexpressed PDGF-R, while only 22% of tumors overexpressed EGF-R, without statistically significant differences among tumor grades. On the other hand, we found a low percentage of LGA showing high bcl-2 staining (8%), while about 20% of the gliomas of high grade did.

Besides, as a measure of cycling cells, Ki-67 LI was also included. Ki-67 showed a specific nuclear staining, either granular or diffuse with nucleolar accentuation. The adjacent ''peritumoral'' nervous tissue was always negative for this antigen. Increasing Ki-67 LI was associated with malignant progression, as statistically significant differences in Ki-67 LI among LGA, AA, and GBM were observed (Table II) (Pearson coefficient 0.35, $P < 0.001$). This association remained in a multivariate analysis (data not shown).

No correlation was observed among the overexpression of the growth factors receptors and bcl-2. On the other hand, we found a positive correlation between PDGF-R overexpression and Ki-67 staining (Pearson correlation, $P < 0.01$). Besides, a positive correlation between histological grade of gliomas and age and Ki-67 expression (Pearson $P < 0.001$ in both cases) as well as an inverse correlation between histological malignancy and Karnofsky ($P < 0.005$) were found.

TABLE II. Relationship Between the Expression of the Different Studied Molecules and Some Clinicopathological Features Relevant in the Prognosis of Malignant Gliomas

Parameter	PGDF-R+/total (%)	EGF-R+/total (%)	bcl-2/total (%)	Ki-67+/total (%)
Sex				
M	29/63 (46.0)	14/63 (22.2)	9/63 (14.3)	37/57 (64.9)
F	21/38 (55.3)	8/37 (21.6)	8/38 (21.1)	29/40 (72.5)
Age				
<35	8/19 (42.1)	2/20 (10.0)	0/20 (0)	9/19 (47.4)
36–35	18/35 (51.4)	10/34 (29.4)	9/35 (25.7)	24/35 (68.6)
>56	24/47 (51.1)	10/46 (21.7)	8/46 (17.4)	33/43 (76.7)
Histological grade				
LGA	12/25 (48.0)	7/25 (28.0)	2/25 (8.0)	10/23 (43.5)
AA	10/23 (43.5)	3/22 (13.6)	4/24 (16.7)	11/18 (61.1)
GBM	28/53 (52.8)	12/53 (22.6)	11/52 (21.2)	41/50 (82.0)**
Karnofsky				
<80	22/42 (52.4)	9/40 (22.5)	6/42 (14.3)	24/37 (64.9)
>80	18/41 (43.9)	13/59 (22.0)	11/59 (18.6)	41/59 (69.5)

**$P < 0.01$, χ^2 test.

About 25% of malignant gliomas that overexpressed PDGF-R presented EGF-R overexpression simultaneously, while bcl-2 alteration occurred together with PDGF-R overexpression in nine cases and with EGF-R in six tumors.

Uni- and Multivariate Analysis of Survival

The multivariate analysis of the known clinico-pathological parameters useful in predicting glioma outcome confirmed that histological grade, age, and performance status are independent prognostic factors in our series of 103 malignant gliomas (Table III).

The univariate analysis showed significant correlation between PDGF-R overexpression and overall survival (OS) in malignant gliomas stratified by histological grade (log-rank test = 3.88, $P < 0.05$). When Cox proportional hazards analysis was performed for each histological grade, PDGF-R demonstrated to be an independent prognostic factor only in patients with LGA (Fig. 1; Table IV). The Md survival time for LGA patients with PDGF-R overexpression was 39.5 (21–75) months versus 57.0 (15–122) months for those patients whose gliomas were negative for PDGF-R.

Besides, only in patients suffering from LGA, overexpression of EGF-R was also significantly associated with worse OS (log-rank test = 8.59, $P < 0.01$, Fig. 2). Multivariate Cox analysis showed that EGF-R overexpression was a statistically significant prognostic factor in LGA patients after adjustment for the effects of histologic grade, age, and on-study performance score (Table V). The survival time for LGA patients with EGF-R overexpression [Md 25 (21–71) months] was lower than that of patients negative for EGF-R [64.5 (15–122) months].

TABLE III. Multivariate Study Showing the Effect of Known Clinico-Pathological Parameters in the Survival of the Studied Malignant Glioma Patients

Independent variable	Beta coefficient	*P*-value	Relative risk	95% CI
Histological grade	0.36	0.02	1.43	1.05–1.96
Age	0.55	0.01	1.73	1.14–2.62
Karnofsky	−0.49	0.03	0.61	0.40–0.94
Sex	−0.28	0.25	0.75	0.46–1.22

CI, confidence interval.

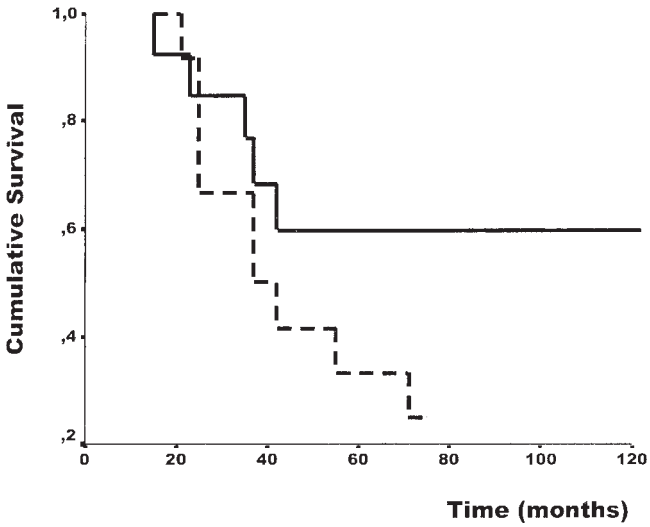

Fig. 1. Kaplan–Meier curves of overall survival categorized by PDGF-R labeling index in LGA patients (—-, PDGF-R overexpression and - - - -, normal PDGF-R expression).

TABLE IV. Cox Survival Model for PDGF-R Overexpression in LGA Patients

Independent variable	Beta coefficient	*P*-value	Relative risk	95% CI
PDGF-R	1.62	0.03	5.03	1.23–20.63
Age	0.68	0.14	1.96	0.81–4.76
Sex	−1.20	0.10	0.30	0.07–1.26
Karnofsky	−0.11	0.92	0.79	0.11–7.60
Ki-67	−0.24	0.72	0.79	0.21–2.95

CI, confidence interval.

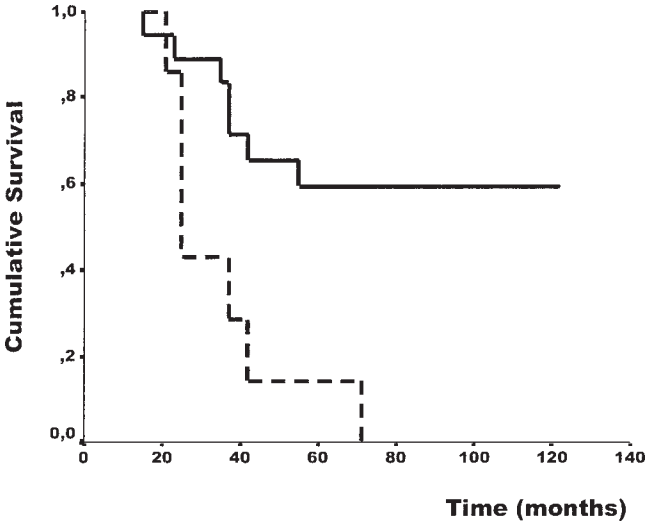

Fig. 2. Kaplan–Meier curves of overall survival categorized by EGF-R labeling index in LGA patients (—-, EGF-R overexpression and - - - -, normal EGF-R immunostaining).

The survival curves for malignant glioma patients categorized by bcl-2 LI did not show statistical differences (data not shown).

Besides, patients whose tumor presented a high number of cycling cells, as measured by Ki-67 expression, showed a worse survival (log-rank test 4.31, $P < 0.05$). However, the Cox analysis showed that the statistical significance is lost when the variable histological grade was included (data not shown).

DISCUSSION

The molecular changes within each grade of malignant gliomas, including activating mutations, amplification, and overexpression of various growth factor receptors, such as PDGF-R and EGF-R, and signaling intermediates have been fairly well defined [2,12,13].

PDGF, a major mitogen for connective tissue cells and glia is recognized by two different receptors: PDGF-R α and β. Our study indicates that almost 50% of the studied malignant gliomas overexpressed PDGF-R α, approximately equal in all grades of glioma, suggesting that such overexpression is important since the initial stages of glioma formation. A similar level of expression of this molecule was found by other authors [4,15]. Moreover, gliomas often overexpress PDGF ligands also, suggesting the establishment of autocrine stimulatory loops [4]. Although we did not analyze the expression of the PDGF ligands, the fact that the number of cycling cells, measured by Ki-67 expression, was directly associated to PDGF-R expression suggests the functionality of PDGF-R. On the other hand, loss of p53 has been closely correlated with PDGF-R α receptor overexpression, both associated with secondary GBM (2). In our population, almost 70% of gliomas that overexpressed PDGF-R α also expressed mutated p53 (our unpublishing data), suggesting that the alteration of both genes may have a potent oncogenic effect.

Several lines of evidence support a main role for EGF-R/EGF system in the gliomatogenesis, such as the frequent co-expression of EGF-R and its respective ligands EGF and TGFα [3,16], the EGF-R gene amplification and/or rearrangement generating a constitutively autophosphorylated receptor [17,18], as well as the fact that the introduction of mutated EGF-R into brain tumor cells dramatically enhances their tumorigenicity in vivo [19]. Several works report that about 40–60% of GBM present amplified and/or overexpressed EGF-R [2,20–22]. We found that only 22% of GBM overexpressed EGF-R. A possible explanation for this rather low expression is that

TABLE V. Cox Survival Model for EGF-R Overexpression in LGA Patients

Independent variable	Beta coefficient	*P*-value	Relative risk	95% CI
EGF-R	1.75	0.02	5.75	1.34–24.76
Age	0.28	0.95	1.03	0.48–2.19
Sex	−0.55	0.42	0.58	0.16–2.17
Karnofsky	−0.61	0.53	0.54	0.08–3.63
Ki-67	−0.54	0.49	0.58	0.12–2.75

CI, confidence interval.

these GBM could be mainly secondary GBM, as it has been described that only 10% of secondary GBM show this alteration [6,14]. However, results from other authors [23] suggest that perhaps the molecular distinction between primary and secondary GBM is not so clear-cut.

Overexpression of EGF-R has been associated with primary GBM [2], thus, from a theoretical point of view, only very few cases of LGA must be positive for this antigen. However, we found that 28% of LGA overexpressed EGF-R and that most of them had aggressive behavior. Related to our finding, other authors have also found immunopositivity for EGF-R in LGA [22,24]. Besides, von Bossanyi et al. [25] found that several LGA showed small foci of EGF-R expression, and suggested an early stimulation of malignant transformation. As the bulk of works are performed on GBM patients, further studies on EGF-R expression in malignant glioma series with a larger number of LGA cases must be undergone.

Individual prediction of clinical outcome is an elusive goal in glioma tumors. Some clinical factors as complete resection, age lower than 45, and high Karnosfky score favor longer survival [2]. Our results confirmed that histological grade, age, and performance status are independent prognostic factors in our population, assuring that this series of gliomas constitute a representative sample of this pathology. Our study demonstrates that LGA patients with PDGF-R or EGF-R overexpression had a significantly worse outcome, being the risk of short survival time about fivefold higher than that of patients with no overexpression of these growth factors receptors. On the other hand, the overexpression of PDGF-R or EGF-R seems not to have a prognostic value either in AA or GBM patients of this series. The multivariate analysis demonstrated that the overexpression of either PDGF-R or EGF-R behaved as an independent marker of poor survival in LGA. Interestingly, as the Md time of survival for these groups was similar to that observed with the more aggressive WHO III gliomas, we consider that these studies could help to select an LGA subgroup of patients with worse prognosis that could receive a more aggressive therapy.

Few studies have investigated the role of PDGF-R as prognostic factor. In contrast with our results, Ribom et al. found an association between high expression of PDGF-R α in WHO II gliomas and long survival [26]. However, these authors have analyzed together tumors of different histological origin. On the other hand, several works studied the value of EGF-R alterations as a prognosis factor, though there is no uniform consensus in the results. While some studies have reported that its amplification/overexpression is associated with poor prognosis [27,28], other investigators have not confirmed these findings [22,29]. Smith et al. [7] identified EGF-R amplification as an independent predictor of prolonged survival in patients with GBM older than 60 years of age and Simmons et al. [30] found that EGF-R overexpression was negatively associated with survival only in the cases expressing p53 wt. The results of a meta-analysis of the data about the value of EGF-R gene amplification in GBM patients suggest that the available studies are insufficient for determining its prognostic value [31].

Apoptosis is the necessary mechanism complementary to proliferation that ensures homeostasis of all tissues. Bcl-2 is one of the best-known antiapoptotic molecules [32,33]. Interestingly, overexpression of bcl-2 is also known to cause resistance against adjuvant treatment in malignant tumors [11] and to enhance migration and invasion of tumoral cells [10]. We studied bcl-2 expression in the same series of malignant gliomas. We observed a progressive increase in bcl-2 expression according to the histological grade though no statistical difference was found. There are a scarce number of papers addressing the expression of this antiapoptotic molecule in gliomas and no uniform conclusion can be drawn. In this sense, Carroll et al. [34] found similar bcl-2 expression in all glioma WHO grades that did not correlate with the number of apoptotic cells, while others [11,35] reported higher expression in gliomas of lower grade than in GBM. Recently, the prognostic value of bcl-2 immunoexpression for the various human cancers has come under intensive examination. We were unable to find any association between the level of bcl-2 immunopositivity and survival. These data agree with other authors' results that have not found a clear relationship between bcl-2 immunoreactivity and clinical outcome [36]. On the contrary, Fels et al. [11] showed that bcl-2 expression was associated with a significantly shorter survival time in patients with a diagnosis of AA, but not in GBM population. Finally, Schiffer et al. [37] discussed a possible dual role for bcl-2 in brain tumors, as a protective factor in lower grade tumors and as a negative factor by inhibiting therapy-induced apoptosis in high-grade neoplasms.

CONCLUSIONS

Our results demonstrate that overexpression of PDGF-R and EGF-R could be used to identify LGA (WHO II) patients with a potentially more aggressive disease. It is clear that histologically comparable lesions may exhibit diverse patterns of gene expression and genomic alterations, which may correspond with important prognostic distinctions. So, our findings together with others could improve existing strategies for the stratification and management of these patients.

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