Basic Fibroblast Growth Factor Expression as a Predictor of Prognosis in Pediatric High-Grade Gliomas

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ABSTRACT

Clinical and histopathological factors fail to adequately predict outcomes in children with high-grade gliomas, indicating a need to identify relevant biological markers of tumor behavior to guide therapeutic decision-making. Basic fibroblast growth factor (bFGF) is a mitogenic and angiogenic factor that has been observed to be overexpressed in a significant percentage of malignant gliomas, although the prognostic significance of this expression is unknown. To address this issue, the expression status of bFGF was examined immunohistochemically in a series of 27 archival pediatric malignant non-brainstem gliomas treated consecutively at our institution between 1975 and 1992. Tumors were categorized based on expression levels, and the association between expression status and outcome was examined. Sixteen cases showed high levels of expression of bFGF, and 11 showed low levels. There was no correlation between expression status and either tumor histology, patient age, or tumor location. However, there was a significant difference in outcome between patients with high levels of bFGF immunoreactivity and those with low expression. Median progression-free survival was >66 months in the low bFGF group as compared to 6 months in the high bFGF group (P = 0.006). Median overall survival was >66 months in the low bFGF group as compared to 18 months in the high bFGF group (P = 0.03). Tumor bFGF expression seems to be strongly associated with outcome in children with high-grade gliomas and, consequently, may serve as a biological correlate of patient prognosis in conjunction with other prognostic variables.

INTRODUCTION

Gliomas comprise a significant percentage of intrinsic neoplasms of the central nervous system during childhood (1, 2). More than 20% of these tumors are malignant lesions, such as anaplastic astrocytoma and glioblastoma multiforme (1), which generally have a poor prognosis. Although approximately 30% of patients experience prolonged disease control after neurosurgical intervention and adjuvant therapy, such as chemotherapy and irradiation, others with comparable histology and treatment exhibit rapid disease progression and death.

Numerous studies have identified clinical factors that are associated with outcome in children with these tumors, including extent of resection, tumor histology (anaplastic astrocytoma versus glioblastoma), tumor location, age, the use of postoperative chemotherapy in addition to radiotherapy, and the presence of seizures as a presenting symptom (3–7). However, even taking into account these factors, the prognosis for individual children with high-grade glioma remains largely unpredictable, which indicates the need for identifying relevant biological factors that better categorize these malignancies both to improve outcome predictions and to guide therapeutic decision making.

Because there is substantial evidence that malignant glial transformation may result from subversion of growth factor/receptor-mediated signaling pathways that control cell proliferation and differentiation (8) and that abnormal expression patterns of a variety of polypeptide growth factors are linked with autocrine- and paracrine-stimulated proliferation of glial neoplasms (9–17), it follows that patterns of growth factor and receptor expression might correlate with outcome in these tumors. bFGF3 (also referred to as FGF-2) is a particularly compelling potential marker. This polypeptide, which is normally not expressed by mature astrocytes, is a potent mitogen for both glial cells and endothelial cells (18–20). Its abnormal expression by neoplastic astrocytes stimulates the growth of astrocytoma cells in an autocrine fashion and endothelial cells in a paracrine fashion (11) and may contribute to the dense cellularity and characteristic hypervascularity of these tumors (14, 15, 19). Conversely, inhibition of the expression or receptor binding of bFGF has been shown to inhibit glioma growth in vitro (21, 22) and in vivo (23–25). To determine whether bFGF expression is a prognostic marker in malignant gliomas, we examined the relationship between bFGF expression and outcome in a well-characterized institutional cohort of children with high-grade gliomas (3).

Our observation of a strong association between bFGF expression and survival in these patients suggests that this factor may have utility in refining predictions of the biological behavior of malignant gliomas.

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3 The abbreviations used are: bFGF, basic fibroblast growth factor; PFS, progression-free survival; OS, overall survival.
PATIENTS AND METHODS

Clinical Patient Data. Thirty-one children diagnosed with high-grade non-brainstem gliomas between 1975 and 1992 were identified from a detailed review of the Tumor Registry of the Children’s Hospital of Pittsburgh; in 27 children, sufficient histopathological material was available for inclusion in the current study. All patients were less than 18 years of age at diagnosis and had undergone neuroimaging by computed tomography or magnetic resonance imaging both preoperatively and postoperatively. Detailed information on tumor location and extent of resection based on imaging and operative criteria had previously been ascertained on each of the patients in a prior study (3).

The majority of these patients were included in prospective multicenter protocols that evaluated the efficacy of different chemotherapeutic regimens in conjunction with irradiation (5, 7). Patients older than 4 years of age were generally treated with at least 5000 cGy to the tumor and a generous margin of the surrounding brain. Patients younger than 4 years were treated with similar regimens but with deferred radiotherapy or reduced radiation doses.

Patient outcome was assessed using hospital and outpatient charts. If a patient had not been evaluated within the previous year, a telephone interview was conducted with the family. Neuroimaging was obtained regularly on these children, and their films were reviewed for evidence of recurrent disease. Each patient alive at the time of the current study had undergone computed tomography or magnetic resonance imaging within the previous year.

Tissue Specimens and Tumor Histopathology. The original tumor specimens were re-reviewed independently by two neuropathologists who had no knowledge of the patients’ outcome. The tumors were graded by criteria of the WHO (26) in which an anaplastic astrocytoma exhibits focal or diffuse anaplasia with increased cellularity, pleomorphism, nuclear atypia, and mitotic activity, and a glioblastoma multiforme exhibits the above features in conjunction with prominent vascular proliferation and/or necrosis. Patients with pure anaplastic astrocytoma or glioblastoma were included in the study, as were patients with mixed gliomas that displayed a predominance of astrocytic features. Children with anaplastic oligodendroglioma or ganglioglioma and those with pleomorphic xanthoastrocytoma were excluded, because these tumors differ prognostically from other high-grade gliomas. In all cases, there was agreement between the two neuropathologists with regard to the final histopathological diagnosis.

Immunohistochemical Study of bFGF Expression. Slides from the original tumor specimen were reviewed, and appropriate blocks were sectioned at a thickness of 4 μm. Adjacent sections were stained with H&E to confirm that representative malignant glial tissue had been obtained and were subjected to immunohistochemical analysis of bFGF expression. Sections for immunohistochemistry were baked overnight at a temperature of 60°C, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol and distilled water. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol for 15 min. Specimens were then rehydrated in PBS and rinsed in 10 mM citrate buffer (pH 6.0). Microwave antigen enhancement (27) was performed by boiling the slides in 10 mM citrate buffer for 5 min; additional buffer was added, and the slides were boiled for another 5 min and subsequently cooled to room temperature. Sections were rinsed and washed in PBS, and nonspecific antibody binding was blocked by incubation with 10% normal horse serum (Vector Laboratories, Burlingame, CA) for 20 min in a humidified chamber.

Tumor slides and positive controls were then incubated for 1 h at room temperature with mouse monoclonal anti-bFGF antibody (Upstate Biotechnology, Lake Placid, NY; dilution, 1:500) in blocking buffer. Negative control samples were treated with blocking buffer alone. Specimens were then washed in PBS and incubated with biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA; dilution, 1:350). Antibody binding was visualized with a peroxidase-conjugated Vector ABC Elite kit using the substrate 3,3’-diaminobenzidine (28) per the supplier’s protocol. The sections were then counterstained with Shandon hematoxylin for 3 min and washed in distilled water. The slides were rehydrated through graded concentrations of ethanol, incubated in xylene for 5 min, mounted, and examined using an Olympus BH-2 microscope. Positive and negative control sections were included with each batch of glioma specimens to confirm the consistency of the analysis. Intensity of labeling was assessed independently by two observers (M. B. and R. L. H.). Immunoreactivity was recorded as either absent (0), present in a minority of cells (1), present in a majority of cells (2), or present in virtually all cells (3). The former two groups were considered to have “low expression,” which was comparable to that of normal brain, whereas the latter two groups were considered to have “overexpression.” In cases in which bFGF-positive cells were found to be clustered in discrete foci within the tumor sample, the region with maximal bFGF staining was used to determine expression level.

Statistical Analysis. After expression levels were recorded, the study cohort was subdivided into groups with high and low levels of bFGF immunoreactivity. Actuarial survival curves were generated using the Kaplan-Meier method with tumor progression and death as the two end points (29). Patients who died perioperatively (n = 3) were excluded from outcome analysis. The relationship between bFGF expression and both PFS and OS was examined in a series of univariate analyses performed using a rank sum test to assess the strength of association between individual parameters and outcome (30). Because a previous analysis of this (3, 31) and other (4–7) groups of children with high-grade gliomas has indicated that extent of resection, tumor location, age, tumor histopathology, and MIB-1 proliferation index were each significantly associated with outcome, we further examined the relationship between these factors and bFGF expression using Fisher’s exact test. Finally, multivariate regression analysis (32) was performed to identify those factors that were independently associated with outcome after adjusting for the other covariates.

RESULTS

Patient Characteristics. Relevant features of the study group are summarized in Table 1. Three patients died within several weeks of surgery, in two cases from sequela of intraoperative complications and in one case from intractable status epilepticus. Of the remaining 24 patients, all but 2 (who were younger than 4 years of age at diagnosis) had received radiotherapy, and 18 had received chemotherapy. The median OS for the group was 18
months, and for the 24 patients surviving the perioperative period, 22 months. The median PFSs were 7 and 11 months, respectively. Nine patients were alive (all without evidence of disease) at last follow-up with a median survival of 78 months (range, 55-142 months). The median PFSs were 7 and 11 months, respectively. For the 24 patients surviving the perioperative period, median PFS was >66 months when bFGF expression was low, whereas children with bFGF overexpression had a PFS of 6 months in the low bFGF group as compared to only 6 months in the group with bFGF overexpression.

**Immunoreactivity of bFGF.** Fig. 1 shows representative results of bFGF immunohistochemistry. BFGF immunoreactivity, which was apparent predominantly in a cytoplasmic distribution, varied widely in our patient population, ranging from no labeling in two patients to an immunoreactivity of more than 90% of tumor cells in three patients. A sizable group of patients (n = 11, 10 of whom survived the perioperative period) exhibited low bFGF immunoreactivity, and a slightly larger group (n = 16, 14 of whom survived the perioperative period) exhibited bFGF overexpression, with staining in most to virtually all cells. In some patients, bFGF-positive cells were found to be clustered in discrete tumor areas (Fig. 1C).

**Table 1 Clinical features, bFGF immunohistochemistry, and MIB-1 indices in a series of 27 high-grade gliomas in childhood**

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- Patient characteristics, treatment, and outcome are updated from the previous report of our clinical series by Campbell et al. (3).
- Tumor location: H, supratentorial hemispheric; C, cerebellum; T, thalamus/basal ganglia.
- Tumor histology: GBM, glioblastoma multiforme; AA, anaplastic astrocytoma; mixAA, anaplastic mixed glioma.
- Resection extent: ST, subtotal; B, biopsy; GT, gross total.
- XRT, radiotherapy, delivered in 180–200 cGy/day fractions.
- Chemo, chemotherapy: VCP, vincristine (VCR)/CCNU/prednisone; 8:1, “8 drugs in one day” regimen (vincristine, hydroxyurea, procarbazine, CCNU, cisplatin, cytosine arabinoside, methylprednisolone, and cyclophosphamide).
- Numbers under bFGF histo refer to bFGF staining distribution: 0, absent; 1, present in a minority of cells; 2, present in a majority of cells; 3, present in virtually all cells.
- Published in a previous study by Pollack et al. (31).
- F/U, follow-up [months to last contact (patient alive)].
- Etoposide/5-fluorouracil.
- Etoposide/ifosfamide/2-mercaptoethane sulfonic acid.

**Relationship between bFGF Expression and Patient Outcome.** Because there was an apparent grouping of our patients into two populations, with low and high immunoreactivity for bFGF, we performed a univariate comparison of outcome between these two groups. We also analyzed separately those patients in both groups with cerebral hemispheric tumors to examine whether statistical findings also applied when focusing exclusively on these lesions.
Fig. 1  bFGF immunolabeling in three patients, one with low bFGF staining (A), another with homogeneous bFGF overexpression (B), and a third with bFGF overexpression in discrete foci within the tumor specimen (C).
Fig. 2 The relationship between bFGF expression and PFS. Outcome was significantly better among patients with low levels of bFGF expression (solid line) than among patients with bFGF overexpression (dotted line; $P = 0.006$).

Fig. 3 The relationship between bFGF expression and OS. Patients with low bFGF staining exhibited a significantly better outcome (solid line) than those with overexpression (dotted line; $P = 0.03$).

of 10 months ($P = 0.001$). This association between bFGF expression and PFS was also apparent in multivariate analysis incorporating other potential prognostic factors, both in the overall study cohort ($P = 0.08$) and in the subgroup of children with hemispheric tumors ($P = 0.02$).

bFGF expression was also strongly associated with overall survival. Median OS was $>66$ months in the low bFGF group as compared to 18 months in the patients with bFGF overexpression ($P = 0.03$; Fig. 3). In the hemispheric subgroup, children with low bFGF expression exhibited an OS of $>66$ months, whereas those with overexpression had an OS of 25 months ($P = 0.006$).

**Relationship between bFGF Immunostaining and Other Prognostic Factors.** In previous studies, we (3, 31) and others (4–7) have shown that a number of factors are associated with outcome in children with high-grade gliomas. These include: (a) extent of resection; (b) tumor location (cerebral hemispheric versus midline and cerebellar); (c) patient age; (d) tumor histopathology; and (e) MIB-1 proliferation index. We, therefore, examined the relationship between bFGF immunolabeling and these prognostic factors.

A weak association between bFGF status and resection extent was apparent; only 2 of 7 patients who underwent gross total tumor resection showed bFGF overexpression as compared to 14 of 20 patients who underwent less extensive resections ($P = 0.06$). In contrast, there was no apparent association between tumor histopathology and bFGF expression; 12 of 19 patients with glioblastoma multiforme exhibited FGF overexpression versus 4 of 8 patients with anaplastic astrocytoma or anaplastic mixed glioma ($P = 0.27$). There was also no significant association between tumor location and bFGF labeling. Ten of 18 children with cerebral gliomas had bFGF overexpression, compared to 6 of 9 patients with tumors in the cerebellum...
or thalamus ($P = 0.28$). In addition, there was no correlation between bFGF labeling and patient age. Four of 7 patients younger than 4 years of age exhibited bFGF overexpression versus 12 of 20 older patients ($P = 0.34$).

In contrast, there was a strong association between bFGF expression and MIB-1 staining; 7 of 10 patients with low MIB-1 labeling (defined as less than 12% in this population of tumors; Ref. 31) also exhibited low bFGF immunostaining, whereas 13 of 17 children with a MIB-1 index of greater than 12% also had bFGF overexpression ($P = 0.02$, Fisher's exact test). Furthermore, we compared the outcome of the children who showed high levels of both bFGF and MIB-1 staining with the outcome of the patients who exhibited low bFGF levels and low MIB-1 indices. This demonstrated a dramatic association between the combination of bFGF and MIB-1 expression and both PFS ($P = 0.001$, rank sum test; Fig. 4) and OS ($P = 0.004$; Fig. 5). Median PFS was $>66$ months in the low bFGF/MIB-1 group compared to only 6 months in the high bFGF/MIB-1 group. Median OS was $>66$ months in the low-expression group compared to 14 months in the high-expression group. The association was even stronger in the subgroup of patients with cerebral hemispheric tumors ($P = 0.001$ and 0.0002, respectively). Patients with a combination of low bFGF and low MIB-1 expression exhibited a median PFS of $>66$ months compared to only 6 months in patients with both high bFGF and high MIB-1 indices. Median OS was $>66$ months in the low bFGF/MIB-1 group compared to <25 months in the high bFGF/MIB-1 group.

**DISCUSSION**

In general, the prognosis for children harboring malignant gliomas remains poor despite progressive refinements in neurosurgical, chemotherapeutic, and radiotherapeutic treatment, and the majority of patients die within 2–3 years of diagnosis (3–7).
However, there is a sizable subgroup of patients that enjoys long-term survival for several years, many of whom appear to be “cured” of their disease. The basis for the widely differing outcomes of these patients is as yet largely unknown. Several clinical factors have been noted to have an association with prognosis, particularly extent of resection and tumor histopathology (3-7), but even taking these into account, the prognosis for children with these tumors remains somewhat unpredictable. Accordingly, there is a strong need for identifying biologically relevant prognostic features to refine outcome predictions and to guide effective therapeutic decision-making.

In this context, several lines of evidence have suggested that bFGF overexpression may be associated with malignant progression of glial neoplasms. This polypeptide is one of a family of at least nine proteins that, although similar structurally, differ in terms of their expression patterns in different tissues, their contribution to various normal and abnormal processes, and their affinity for each of the four characterized tyrosine kinase FGF receptors (33-37). bFGF (38, 39), in particular, has been implicated as an important element mediating neuronal cell growth and differentiation, which may be subverted in malignant glioma cells (11, 14, 15, 17) as a result of deregulated expression. Because this polypeptide is a mitogen for endothelial cells as well as glioma cells, its overexpression may contribute significantly to the growth of malignant gliomas by favoring proliferation not only of glioma cells but also of the surrounding vasculature. Moreover, several studies have shown that inhibiting bFGF by antisense techniques (11, 19, 21, 22) or interference with bFGF-mediated signaling by antibody neutralization (23-25) blocks the proliferation of a percentage of malignant glioma cell lines. Accordingly, we examined the expression status of bFGF in a well-characterized study cohort of pediatric malignant glioma patients to determine whether there was an association between the expression of this factor and patient outcome.

This analysis showed that pediatric malignant gliomas could be categorized into distinct groups based on their levels of bFGF immunostaining. More importantly, both OS and PFS were significantly associated with the expression level of this factor on univariate analysis. However, despite the striking association between bFGF expression and outcome, this correlation was not absolute. Four patients with low bFGF expression exhibited a poor outcome, and two patients with high bFGF status enjoyed long-term survival, which undoubtedly reflects the contribution of other important prognostic variables to patient outcome. For instance, both long-term survivors with high bFGF expression had undergone gross total resection of cerebral hemispheric tumors, factors that have been associated with a favorable patient outcome (3). Conversely, three of the four patients with low bFGF immunoreactivity and poor outcome had cerebellar lesions, and all four had undergone only subtotal tumor resection or biopsy, features associated with an adverse prognosis (3).

In this context, it is likely that bFGF expression status will have the most prognostic utility when applied in combination with other relevant factors to discriminate patients at high and low risk for disease progression. From a biological standpoint, the combination of proliferation index (as assessed by MIB-1 labeling of the Ki-67 nuclear antigen; Refs. 31 and 40-42) and bFGF expression was particularly accurate in predicting survival. For example, none of the 11 patients who survived the perioperative period and had a combination of high bFGF expression, high MIB-1 labeling, and obvious residual disease enjoyed long-term survival.

A recognized limitation of this study was the small sample size, which precluded demonstration of an independent association between bFGF expression and outcome. Although the association between bFGF expression and PFS approached significance on multivariate analysis (P = 0.08), it was not possible to establish conclusively that bFGF expression was an independent predictor of outcome, particularly in view of the association between bFGF expression and resection extent, which itself is a strong prognostic factor in pediatric high-grade gliomas. In addition, the fact that only one antibody was used to screen for bFGF immunoreactivity leaves open the possibility that cross-reaction with another FGF family member may have contributed to the results observed. Although this is unlikely, given the fact that the antibody chosen for the analysis exhibits high specificity for bFGF versus other FGFs, further support for the association between bFGF expression and outcome would be provided by screening for bFGF immunoreactivity using a panel of antibodies targeted to different bFGF epitopes as well as by surveying the expression patterns of other related FGFs.

The above caveats notwithstanding, the present study indicates that immunohistochemical evidence of bFGF overexpression is a common finding in pediatric high-grade gliomas, which suggests that this mitogen may be important for the growth of childhood malignant gliomas, and, consequently, may also constitute a potential therapeutic target in these neoplasms. The observation of a strong association between bFGF expression and outcome in our cohort of childhood malignant gliomas provides a rationale for exploring this issue further in a larger study cohort. The finding that bFGF expression and proliferation index were more accurate predictors of patient outcome than was tumor histology suggests that the histological criteria conventionally used to subdivide high-grade gliomas may need to be evaluated in the context of these and other biological criteria to refine outcome predictions and to optimize treatment stratification.

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Basic fibroblast growth factor expression as a predictor of prognosis in pediatric high-grade gliomas.

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