Epidermal Growth Factor Receptor Expression and Gene Amplification in High-Grade Non-Brainstem Gliomas of Childhood

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INTRODUCTION
Brain tumors are the most common solid neoplasms of childhood and are second only to leukemias in overall incidence among children (1–3). Gliomas comprise ∼40% of all pediatric brain tumors (4). More than 20% of these lesions are histologically malignant tumors, such as AA and GBM, which infiltrate diffusely and often prove rapidly fatal.

Although the initiating factor for glioma development remains elusive, there is evidence that neoplastic glial cells may follow one of a number of pathways of genomic aberrations to acquire a common malignant phenotype (5–8). During the course of their evolution, these cells come to exhibit deregulation of a variety of GF receptor-mediated signaling pathways, which permits autocrine stimulation of glioma cell proliferation (9, 10) as well as paracrine stimulation of endothelial cell proliferation and neovascularization (11, 12).

Aberrant patterns of expression of the c-erbB1 proto-oncogene product EGFR, a 170-kDa transmembrane tyrosine kinase (11), have been associated with tumor progression and enhanced tumorigenicity in high-grade gliomas arising in adults (11–14). Amplification of the EGFR gene has been found in 40–50% of these tumors (15–18), often in association with rearrangement of the gene to produce a truncated, constitutively activated receptor (19). EGFR amplification has been observed to distinguish so-called “primary” glioblastomas (i.e., those arising de novo, usually in older patients) from “secondary” glioblastomas (i.e., those that seem to evolve in a more gradual fashion from lower-grade lesions, generally in younger patients; Refs. 5–8).

Although the EGFR gene is amplified in only a subset of adult malignant gliomas, the EGFR protein is expressed at high levels relative to nonneoplastic brain in up to 85% of adult AAs and 95% of adult GBMs (20–24), suggesting a potential role for this receptor in mediating tumor growth. Several groups have observed that EGFR gene amplification and/or protein expression are associated with outcome in newly diagnosed adult malignant gliomas (15, 20, 22, 23). However, because this factor correlates with tumor grade,
it is presently uncertain whether EGFR expression and gene amplification are of independent prognostic use. Although Hiesinger et al. (22) noted that EGFR immunoreactivity was associated with an adverse outcome in newly diagnosed GBMs, other studies have failed to detect an independent prognostic effect of EGFR expression (15, 20, 23, 24). Because prominent EGFR immunoreactivity is such a common feature in these tumors, therapeutic strategies targeting this receptor have been proposed for adult high-grade gliomas (25, 26).

To date, it has remained uncertain whether pediatric high-grade gliomas share this common pattern of EGFR immunoreactivity and whether a sizeable subset of these tumors exhibit EGFR gene amplification. Because childhood gliomas carry a more favorable prognosis than comparable lesions in adults (27–29), it has been suggested that these tumors may arise from distinct molecular pathways (27–31), a factor that has important implications for the design of rational therapeutic strategies for these lesions. To address this issue, we examined the expression of EGFR using immunohistochemistry and screened for amplification of the EGFR gene using competitive PCR in a series of 27 archival pediatric high-grade nonbrainstem gliomas, and questioned whether these factors were associated with patient outcome. Our results, which differ significantly from those observed in adult high-grade gliomas, indicate that although childhood high-grade gliomas seem to be similar histologically to their adult counterparts, they exhibit distinctive molecular features and, therefore, warrant independent examination in molecular diagnostic and therapeutic studies.

MATERIALS AND METHODS

Clinical Patient Data. Thirty-one children diagnosed with high-grade nonbrainstem 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 <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 been previously ascertained on each of the patients in a prior study by Campbell et al. (27).

The majority of patients were included in prospective multicenter trials that evaluated the efficacy of different chemotherapeutic regimens in conjunction with irradiation (28, 29). Patients older than 4 years of age were generally treated with at least 5000 cGy of irradiation 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 records; each patient alive at the time of the current study had undergone a magnetic resonance imaging scan within the previous year.

Tissue Specimens and Tumor Histopathology. The original patient slides were re-reviewed independently by two neuropathologists who had no knowledge of the patients’ outcome. The tumors were graded by criteria of the WHO (32), in which an AA (grade III tumor) exhibits focal or diffuse anaplasia with increased cellularity, pleomorphism, nuclear atypia, and mitotic activity, and a GBM (grade IV tumor) exhibits the above features in conjunction with prominent vascular proliferation and/or necrosis. Patients with pure AA or GBM were included in the study, as were patients with mixed gliomas that displayed a predominance of astrocytic features. In all cases, there was agreement between the two neuropathologists with regard to the final histopathological diagnosis.

Immunohistochemical Study of EGFR Expression. Appropriate blocks from the original tumor specimen were sectioned at a thickness of 4 μm. Adjacent sections were either: (a) stained with H&E to confirm that representative malignant glial tissue had been obtained; (b) subjected to immunohistochemical analysis of EGFR expression; or (c) processed for EGFR amplification analysis (see below). 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 microwave antigen enhancement (33) was performed by boiling the slides in 10 mM citrate buffer (pH 6.0). 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 (EGFR-overexpressing breast carcinoma) were then incubated for 1 h at room temperature with a mouse monoclonal antibody to the extracellular domain (residues 580–591) of human EGFR (Genosys Biotechnologies Inc., Cambridge, United Kingdom; 1:100) in blocking buffer. Negative controls were treated with blocking buffer alone. Specimens were then washed in PBS and incubated with biotinylated horse antimouse IgG antibody (Vector Laboratories; 1:350). Antibody binding was visualized with a peroxidase-conjugated Vector ABC Elite kit using the substrate 3,3′-diaminobenzidine (34) as per the supplier’s protocol. The sections were then counterstained with Shandon hematoxylin for 3 min and washed in distilled water. The slides were dehydrated through graded concentrations of ethanol, incubated in xylene for 5 min, mounted, and examined using an Olympus BH-2 microscope. Positive and negative controls were included with each batch of sections. Immunoreactivity was graded by two blinded observers (M. B. and R. L. H.) as either absent or present in single cells (0; 0–5%), present in a minority of cells (1; 5–50%), present in a majority of cells (2; 50–90%), or present in virtually all cells (3; >90%). The latter two groups were considered to have elevated immunoreactivity for EGFR, which exceeded levels observed in peritumoral brain, in which only scattered immunoreactive cells were observed.

Assessment of EGFR Amplification. Sectioned specimens were deparaffinized in xylene, and tumor tissue was scraped into a microcentrifuge tube containing 200 μl of a mixture consisting of 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.4 mg/ml proteinase K. The samples were digested at 55°C for 48 h, with fresh enzyme being added after 24 h. The specimens were then boiled for 10 min and diluted with 200 μl TE buffer [10 mM Tris-Cl and 1 mM EDTA (pH 8.0)]. The DNA solution was added to a Microcon-100
microcentrifugation. Sample concentrations were approximated by comparing their ethidium bromide fluorescence against DNA solutions of known concentration.

Competitive PCR amplifications were carried out in a final volume of 25 μl. Each reaction mixture contained 0.2 μg of template, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 μM dNTPs, 0.5 μM oligonucleotide primers for the target (EGFR) and reference genes, as described previously (35), and 0.625 units of AmpliTaq Gold (Perkin-Elmer Corp., Norwalk, CT). PCR profiles were as follows: 92°C for 2 min; cycles 1–5 at 97°C for 30 s, 53°C for 1 min, and 72°C for 1 min; cycles 6–30 at 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min; with final extension at 72°C for 10 min. Reaction products were electrophoresed through a 2.0% agarose gel, stained with ethidium bromide, and quantitatively analyzed using a Gel-Doc 100 photodocumentation system with Molecular Analyst software (Bio-Rad). All reaction series included both positive (samples previously determined to have gene amplification) and normal tissue controls. Normalized target/reference ratios of >2.0 were interpreted as evidence for gene amplification.

**Statistical Analysis.** The relationship between EGFR expression and patient outcome was examined using the Kaplan-Meier method with tumor progression and death as the two end points (36); patients who died perioperatively (n = 3) were excluded from this outcome analysis. Actuarial survival curves were generated for both the group with elevated EGFR immunoreactivity (n = 19) and the group without (n = 5). The association between EGFR expression and both PFS and OS was then analyzed using a rank sum test. Because, in previous analyses, tumor histology (27–29), basic fibroblast GF immunoreactivity (37), and proliferation index (as assessed by MIB-1 labeling of the Ki-67 nuclear antigen; Ref. 38) have been shown to be associated with outcome in these tumors, we also examined the relationship between these factors and EGFR expression using Fisher’s exact test.

**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 intraoperative complications and in one case from status epilepticus. Of the
remaining 24 patients, all but 2 (who were younger than 4 years of age at diagnosis) received radiotherapy and 18 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 100 months (range, 80–164 months): eight of these patients were progression-free throughout the follow-up period, and one patient with an initial episode of disease progression at 14 months after diagnosis is disease-free 66 months after reoperation and additional therapy.

Immunoreactivity of EGFR. Fig. 1 summarizes the EGFR staining distribution in our study cohort. EGFR-positive cells demonstrated a partly membranous, partly cytoplasmic staining pattern. In comparison with nonneoplastic brain, which exhibited low levels of EGFR immunoreactivity, EGFR expression was significantly increased in 22 of 27 high-grade gliomas (81%). Fifteen tumors (56%) had a positive immunoreaction in >90% of the cells. Fig. 2 shows a representative result of this high expression. Another seven tumors had immunopositivity in the majority of cells (50–90%). In contrast, only five tumors showed staining in <50% of cells, of which one exhibited staining in <5% of tumor cells.

EGFR Amplification. Only 2 of 27 tumors (7.4%) demonstrated an amplified EGFR gene copy number on competitive PCR (Fig. 3). Both tumors were among the 19 GBMs included in the study (Table 1) and, both exhibited elevated EGFR expression on immunohistochemical analysis, with immunoreactivity in >90% of cells.

Relationship between EGFR Immunostaining and Patient Outcome. No association was apparent between EGFR expression level and either PFS or OS (P > 0.3). A Kaplan-Meier approach showed nearly identical curves for PFS and OS in tumors that had elevated EGFR expression and those that did not (Fig. 4, A and B). Mean PFS was 12 months in the group with increased EGFR expression compared with 6 months in the group with low levels of expression. Mean OS was 19 months in patients with elevated EGFR labeling compared with 14 months in the group of patients without this finding. Similarly, no differences in survival were apparent when tumors with expression in >90% of cells were compared with those exhibiting lower levels of expression. Of the five tumors with the lowest levels of EGFR expression, four were among the 19 grade IV tumors (i.e., GBMs) and one was among the 8 grade III tumors (i.e., AAs and anaplastic mixed gliomas); thus, no significant association between tumor histology and EGFR expression status was apparent. Two of these tumors were located in the cerebral hemispheres, whereas three tumors were thalamic or cerebellar lesions. The patient with the lowest level of EGFR expression actually had among the shortest survivals, dying of progressive disease 4 months after diagnosis.

There was also no apparent association between EGFR immunoreactivity and either basic fibroblast GF overexpression or high MIB proliferation index (P > 0.3), which had been recorded on these tumors in prior studies (37, 38). Because of the low frequency of actual EGFR amplification, this factor could not be correlated with outcome in this cohort. However, one of the two patients with EGFR amplification was a long-term progression-free survivor.
DISCUSSION

The c-erbB1-encoded EGFR is a 170-kDa single-chain transmembrane glycoprotein consisting of an extracellular ligand binding region, an anchoring membrane spanning domain, and an intracellular catalytic domain that possesses tyrosine kinase activity (39). This polypeptide has been implicated as an important element mediating neuroglial cell growth and differentiation. The most extensively characterized ligands for EGFR are EGF and transforming GFα (13).

In addition to its pivotal role during normal body and organ function, the EGFR signaling pathway, when subverted, may be an important factor in the process of malignant progression in human gliomas (10–16). Numerous studies have demonstrated that EGFR amplification occurs in up to 50% of grade IV astrocytomas in adults versus <10% of grade III gliomas and virtually no lower-grade gliomas (11, 13, 17, 18). Many such tumors exhibit rearrangement of the EGFR gene, leading to constitutive activation of the gene product and, potentially, increased tumorigenicity (19, 40). Seven variants of the EGFR gene have been described for brain tumors, of which the type III deletion mutant EGFRvIII is the most common variety, resulting in a truncated form of EGFR lacking residues 6–274 (18, 41, 42). Increased expression of EGFR relative to nonneoplastic brain may also occur in gliomas in the absence of gene amplification, and this factor, likewise, seems to correlate with tumor grade (20, 23). In a series of 43 adult astrocytomas, Jaros et al. (20) observed that increased immunoreactivity for EGFR was not present in WHO grade I astrocytomas, but was apparent in 33% of grade II, 85% of grade III, and 95% of grade IV astrocytomas.

Because in vitro studies indicate that excessive activation of EGFR-mediated pathways may facilitate glioma cell proliferation, migration, and invasiveness, which can be inhibited by antibody-mediated or pharmacological blockade of EGFR-initiated signaling (43), it is conceivable that the extent of expression of EGFR might be an important factor influencing the biological behavior of human high-grade gliomas. Several studies have observed that the finding of EGFR amplification or increased EGFR immunoreactivity is an adverse prognostic factor among adults with glial neoplasms (15, 20, 22, 23). For example, Jaros et al. (20) observed that EGFR expression was significantly associated with poor prognosis in adults with malignant gliomas. Patients with tumors expressing EGFR showed a profoundly reduced survival compared with those with no EGFR expression; only 13% of the former survived a 100-week period in comparison with 60% of the latter. However, most studies have failed to demonstrate that this factor has independent prognostic use after taking into account the effect of tumor histology (15, 20, 23, 24). An exception is the study of Hiesiger et al. (22), which reported that expression of EGFR in human glioblastomas was associated with aggressive clinical behavior and resistance to treatment. In a series of 17 newly diagnosed adult glioblastomas, 4 of 7 patients with short-term survival had tumors with elevated EGFR expression, whereas none of the 10 patients who enjoyed long-term survival demonstrated EGFR expression.

In contrast to the extensive studies of EGFR gene am-
plification and EGFR protein expression in adult high-grade gliomas, comparatively little attention has been focused on evaluating the status of these factors in malignant gliomas of childhood. The goal of the current study was to address this issue using an institutional cohort of consecutively treated nonbrainstem high-grade gliomas. Because it has been observed that EGFR amplification is much more common in so-called “primary” glioblastomas that typically arise in older adults than in “secondary” glioblastomas that typically occur in younger adults (5–8), our initial expectation was that this feature might be particularly uncommon in pediatric high-grade gliomas. In accordance with this hypothesis, EGFR amplification was observed in only 2 of 27 malignant childhood gliomas, both of which were grade IV astrocytomas (i.e., GBM), a strikingly lower frequency than has been noted in comparable studies of adult high-grade gliomas. In contrast, prior studies of this cohort have observed that among grade IV astrocytomas, the frequency of p53 mutations, a hallmark of secondary glioblastomas, was actually somewhat greater (47%; Ref. 31) than that observed in prior studies of adult high-grade gliomas (44, 45).

In comparison with the low frequency of EGFR amplification detected in this series, elevated EGFR expression within these tumors relative to nonneoplastic brain was a common finding. The observation that the frequency of elevated EGFR immunoreactivity far exceeded that of EGFR gene amplification is consistent with prior results in adult high-grade gliomas (23), suggesting that the expression of this protein may be enhanced by mechanisms other than simple alteration in gene copy number. In the current study, 80% of the tumors examined demonstrated immunopositivity for EGFR in a majority of cells, a level of expression that far exceeded levels observed in the surrounding brain. For initial outcome analysis, we categorized our patients into two groups with one showing EGFR positivity in a minority of cells and the other with EGFR expression in >50% of cells (i.e., EGFR overexpression). This distinction was somewhat arbitrary in that all but one of the tumors demonstrated levels of expression that clearly exceeded those observed in nonneoplastic brain. However, regardless of how patients were subdivided on the basis of expression level, no clear association between this factor and outcome was apparent.

On the basis of these results, we conclude that EGFR gene amplification is much less common in pediatric high-grade gliomas than in histologically similar tumors in adults, suggesting that childhood malignant gliomas may follow a pathway of genetic alterations that more closely parallels that observed in secondary glioblastomas. Despite the infrequency of EGFR gene amplification, immunohistochemical evidence of elevated EGFR expression is a common finding in these tumors. The consistently high levels of expression of this glycoprotein suggests that this mitogenic receptor may play a role in mediating proliferation and malignant progression of pediatric high-grade gliomas. Moreover, in view of the high levels of immunoreactivity for EGFR in tumor versus nonneoplastic brain, this receptor may constitute a potential therapeutic target in pediatric high-grade gliomas.

REFERENCES