ERBB1 Is Amplified and Overexpressed in High-grade Diffusely Infiltrative Pediatric Brain Stem Glioma[1]

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ABSTRACT

Purpose: **This study was conducted to investigate the incidence of *ERBB1* amplification and overexpression in samples of diffusely infiltrative (WHO grades II–IV) pediatric brain stem glioma (BSG) and determine the relationship of these abnormalities to expression and mutation of *TP53* and tumor grade.**

Experimental Design: **After central pathology review, the incidence of *ERBB1* amplification and overexpression was determined in 28 samples (18 surgical biopsy and 10 postmortem specimens) of BSG using quantitative PCR and immunohistochemistry, respectively. Mutation and expression of *TP53* were also determined in these same samples by direct sequence analysis of microdissected tumor material and immunohistochemistry, respectively. All experimental procedures were performed blind to tumor grade.**

Results: **Twelve, 9, and 7 tumors were classified as WHO grades II, III, and IV, respectively. A significant increase in ERBB1 expression was observed with increasing tumor grade ($P < 0.001$). Two grade IV tumors displayed intense membranous ERBB1 expression in 90% of tumor cells in association with high-level *ERBB1* gene amplification. One grade III tumor also contained low-level amplification of *ERBB1*. Six tumors demonstrated TP53 nuclear immunoreactivity, and six contained a mutation in *TP53*. No correlation was observed between abnormalities in *TP53* and either tumor grade or amplification and overexpression of *ERBB1*.**

Conclusions: **These data suggest that ERBB1 signaling is important for the development of childhood BSG and is worthy of study as a therapeutic target in this disease. Our data also indicate that the genetics of childhood BSG are complex and include both grade-dependent amplification and overexpression of *ERBB1* and grade-independent expression and mutation of *TP53*.**

INTRODUCTION

Over 90% of children with diffusely infiltrative BSG[3] succumb to their disease within 2 years of diagnosis (1). These tumors are not amenable to surgical management (2, 3), and there is no evidence that radiation therapy, including hyperfractionated and accelerated regimens (4–6), or chemotherapy (1, 7) improves patient survival. The failure of conventional treatment to reduce the mortality of children with BSG has intensified the search for novel therapeutic approaches for this disease.

The PBTC in the United States recently commenced a Phase I/II study of ZD1839 (Iressa), an inhibitor of the EGFR (ERBB1) tyrosine kinase, in children with high-grade supratentorial astrocytoma and nondisseminated diffuse intrinsic BSG. There is considerable evidence that inhibitors of the ERBB1 tyrosine kinase might have therapeutic efficacy against high-grade gliomas. *ERBB1* is amplified and overexpressed in up to one-half of adult high-grade gliomas (8–10) and overexpressed, usually in the absence of gene amplification, in ~30% of pediatric nonbrain stem high-grade gliomas (11–14). Furthermore, up-regulation of ERBB1 cell signaling promotes gliomagenesis in mice (15, 16) and an invasive (17, 18) and radioresistant phenotype in human glioma cells (19). Although these data indicate that ZD1839 might be a useful therapy for children with supratentorial high-grade glioma, there are no direct data to support its use in BSG. Therefore, in this study, the PBTC analyzed the incidence of *ERBB1* amplification and expression in 28 diffusely infiltrative (WHO grades II–IV) pediatric BSGs. Our data show that *ERBB1* is amplified and overexpressed in a significant proportion of high-grade pediatric BSG and support the rationale for treating children with this disease with inhibitors of the ERBB1 tyrosine kinase.

PATIENTS, MATERIALS, AND METHODS

Patients and Tumor Material. With Institutional Review Board approval, formalin-fixed tumor material was collected from 43 children (≤17 years) with a radiological or

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[3] The abbreviations used are: BSG, brain stem glioma; PBTC, Pediatric Brain Tumor Consortium; qPCR, quantitative PCR; EGFR, epidermal growth factor receptor; PA, pilocytic astrocytoma; IHC, immunohistochemical.

histological (biopsy performed at time of diagnosis or postmortem) diagnosis of intrinsic BSG. Tumor material was first subject to central histopathology review to confirm the diagnosis and grade of BSG and assess the quantity and quality of tumor material within sections. Tumors were classified according to WHO criteria (20) as PA (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), or glioblastoma multiforme (grade IV). Although PA can present clinically as intrinsic BSG, they appear biologically distinct from more aggressive diffusely infiltrative tumors (21). Therefore, only tumors classified as grade II or higher were included in the current study. After central pathology review, eight samples were judged to contain insufficient tumor material for analysis, six tumors were classified as PA, and one tumor was reclassified as a primitive neuroectodermal tumor. The remaining 28 tumors included 18 surgical biopsy samples (obtained at diagnosis) and 10 postmortem specimens. The postmortem delay was <3 h in all autopsy-derived samples, and none of these displayed significant autolysis. Sixteen patients were female, and 12 were male. The mean age at diagnosis was 6.9 years (range 2–14 years). All 28 samples of diffusely infiltrating BSG were then submitted for further analysis by investigators who were blinded to tumor grade.

IHC. Tumor expression levels of ERBB1 and TP53 protein were determined by IHC using the NCL-EGFR (Novocastra, Newcastle, United Kingdom) and DO-7 (DAKO, Carpinteria, CA) primary antibodies, respectively (22, 23). Sections used in IHC were adjacent to those used for histological review. The intensity of ERBB1 immunostaining (0, +, ++, +++; relative to positive and negative controls) and percentage of ERBB1 immunopositive tumor cells were recorded for all cases. TP53 expression was scored as described previously using a semiquantitative scale from 0 to 4 based on the proportion of immunopositive tumor cell nuclei (22).

qPCR Analysis. *ERBB1* amplification in tumors was detected using a qPCR-based assay described previously (24). Briefly, DNA was extracted from 10-μm-thick tumor sections by xylene dewaxing and digestion with Proteinase K. Serial dilutions of this template DNA were then simultaneously and independently amplified using *ERBB1* (5′ ACA GCC ATG CCC GCA TTA GCT CTA 3′; 5′ GGA ATG CAA CTT CCC AAA ATG TGC C 3′) and internal reference gene (*NACH*; 5′ TC GGC ATC TGC TTC TTC TGC A 3′; 5′ TCG AAC TTC TCC CAT GTC TCG 3′)-specific primers that have equal amplification efficiencies under the following reaction conditions: ×10 reaction buffer (2.5 μl; Advanced Biotechnologies); 2.5 mM each of dATP, dCTP, dTTP, and dGTP triphosphates (Pharmacia); Taq polymerase (0.75 units; Advanced Biotechnologies); 12 pM of sense and antisense primers; and 10 μl of template DNA amplified over 28 cycles of 95°C, 55°C, and 72°C for 1 min each. The amount of product generated from amplification of *ERBB1* relative to *NACH* (*ERBB1*:*NACH* ratio), within the linear range of the PCR, provides an estimate of *ERBB1* copy number. The quantity of PCR products generated from each reaction was determined by agarose gel electrophoresis and densitometry. To determine the sensitivity of the qPCR assay to detect an increase in *ERBB1* copy number, we calculated the *ERBB1*:*NACH* ratio in genomic DNA extracted from 18 samples of formalin-fixed *ERBB1* diploid control tissue (tonsil). The mean *ERBB1*:*NACH* ratio in these normal tissue samples was 1.22 (±0.49 SD). Tumor samples that displayed an *ERBB1*:*NACH* ratio ≥3 SD above this control mean (*i.e.*, *ERBB1*:*NACH* ratio ≥2.69) were judged to contain an amplified *ERBB1* locus. We have used previously this qPCR assay to reliably detect *ERBB2* and *MYCN* amplification in human breast and neuroblastoma tumor material, respectively (data not shown). A431 cells, in which *ERBB1* is amplified and overexpressed (25), served as a positive control for the qPCR assay in the current study.

Analysis for Mutations in TP53. Representative areas of fixed tumor were microdissected, and exons 5–8 were individually amplified by PCR as described (22). The PCR products were isolated and directly sequenced by dideoxy chain termination with the use of ^{35}S-labeled dATP, and the sequences read from autoradiograms of 6% polyacrylamide gels.

Statistical Analysis. The results of all molecular analyses were entered into the PBTC remote database. The PBTC central office then analyzed the relationships among clinicopathological variables. We used the Cochran-Armitage Trend Test to detect trend differences between bivariate and ordinal variables and the Cochran-Mantel-Haenszel Test for correlations between two ordinal variables.

RESULTS

ERBB1 is amplified and overexpressed in high-grade diffusely infiltrative pediatric BSG. Twelve, 9, and 7 tumors were classified as grade II–IV, respectively (Fig. 1). One oligodendroglioma was identified among the grade II tumors. No significant age or sex difference was observed among patients with different grades of tumor. A significant increase in ERBB1 expression was observed with increasing tumor grade ($P < 0.001$ for both intensity and percentage of cell ERBB1 expression; Fig. 1). ERBB1 expression was detected at low levels in only 2 of 12 grade II tumors (#4 and 12). In keeping with these expression data, no grade II tumor contained an amplified *ERBB1* gene (Fig. 1). In contrast, ERBB1 protein was detected in 7 of 9 grade III tumors, including 4 with moderate levels of protein expression in 10–30% of tumor cells (#14, 16, 18, and 21) and three with low-level expression in 30–90% of tumor cells (#13, 15, and 17). Tumor #15 also contained low-level *ERBB1* gene amplification (*ERBB1*:*NACH* = 2.8). ERBB1 protein was detected in all grade IV tumors; three of these displayed intense membranous ERBB1 immunoreactivity (#24–26). In two of these cases, ERBB1 was expressed in 90% of tumor cells in association with high-level *ERBB1* gene amplification (*ERBB1*:*NACH* > 5; Figs. 1 and 2).

Together, these data demonstrate that around one-half of diffusely infiltrative pediatric BSGs are high-grade (grade III or IV) tumors and that a significant proportion of these contain amplification and/or overexpression of *ERBB1*. Although the six grade I BSGs that were identified during central pathology review were excluded from the formal study, none of these cases contained detectable *ERBB1* amplification or expression. These data also support the hypothesis that *ERBB1* amplification and overexpression is restricted to high-grade BSG.

TP53 Is Frequently Mutated in Pediatric BSGs. Mutations in *TP53* are relatively uncommon in primary adult high-

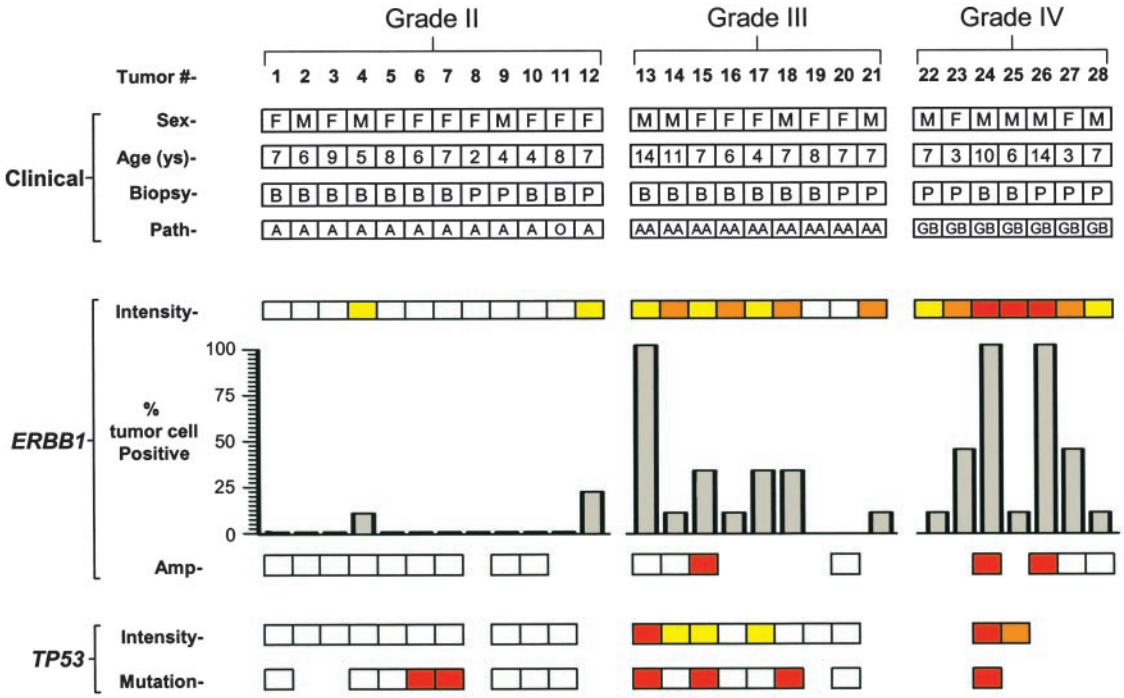

Fig. 1 BSG clinical and genetic data. Cases are numbered consecutively (1–28) through three groups separated by tumor grade (WHO II-IV). Clinical variables include: sex (*M*, male; *F*, female), age (years), method of tumor retrieval (*B*, surgical biopsy; *P*, postmortem), and pathology (*A*, diffuse astrocytoma; *O*, oligodendroglioma; *AA*, anaplastic astrocytoma; *GB*, glioblastoma). The results of ERBB1 IHC are shown by intensity of immunoreactivity (□, negative; , +; , ++; , +++) and graphically as the percentage of immunopositive tumor cells. The results of qPCR analysis of *ERBB1* amplification (Amp) are summarized as □, normal; , amplified. The results of TP53 IHC are summarized on a scale of 1–4 (see "Patients, Materials, and Methods" section; □, 0 or 1; , 2; , 3; , 4). The results of *TP53* mutational analysis (Mut) are summarized as □, normal; mutant. Tumors with insufficient material for analyses are shown as missing squares.

grade gliomas, but high-level expression and/or mutation of *TP53* affects over one-third of supratentorial and brain stem high-grade gliomas in children (11, 14, 22, 26). Six tumors in the current study demonstrated TP53 nuclear immunoreactivity (Fig. 1). A trend toward an increase in TP53 protein expression was observed among grade III and IV BSGs, although the small numbers of cases involved precluded formal statistical analysis. Mutations in *TP53* were detected in 6 of 16 analyzed tumors. These included two grade II tumors (#6, del exon 6; #7, Arg175His), three grade III tumors (#13, del exon 5; #15, Leu252Phe; #18, Tyr126Cys), and one grade IV tumor (#24, Arg248Trp). *ERBB1* amplification and *TP53* mutation were observed concurrently in two tumors (#15 and 24).

DISCUSSION

With an incidence of $\sim 7/10^6$ person years, BSGs account for 20% of all childhood brain tumors (27). This disease has been subclassified as either focal or diffusely infiltrative (21). The latter carries a particularly dismal prognosis, with patients rarely surviving beyond 2 years (1, 4–6, 28). Efforts to identify causative molecular abnormalities that might serve as therapeutic targets in BSG have been severely restricted by a lack of tumor material. Here, we report the largest molecular analysis of diffusely infiltrative BSG performed to date and show that *ERBB1* is amplified and overexpressed in a significant proportion of high-grade pediatric BSGs. These data suggest the hypothesis that ERBB1 signaling is important for the development of childhood BSG and is worthy of further study as a therapeutic target in this disease.

Genetic abnormalities in adult high-grade gliomas vary among a number of distinct tumor subgroups (29). Primary high-grade gliomas (primary glioblastoma) arise *de novo* in older patients and frequently contain an amplified and rearranged *ERBB1* locus, deletion of *INK4A/ARF*, loss of *PTEN*, and intact *TP53* (8–10, 30–34). In contrast, secondary glioblastomas that progress from lower grade tumors in younger patients rarely contain an amplified *ERBB1* locus but frequently contain mutations in *TP53* (30–32, 34, 35). Tumors with combinations of these abnormalities, including some containing concurrent mutation of *TP53* and amplification of *ERBB1*, have also been described (11, 34). Far less is known about genetic abnormalities in pediatric glioma. Some studies indicate that nonbrain stem pediatric high-grade gliomas are genetically similar to adult secondary glioblastoma. In this regard, although *ERBB1* amplification is rarely detected in pediatric high-grade gliomas ($n = 2$ of 119 cases in the literature), mutation of *TP53* occurs relatively frequently (11, 13, 14, 36). However, ERBB1 overexpression has been identified in a significant proportion of childhood high-grade gliomas (12, 13), and the precise pattern of genetic abnormalities within pediatric gliomas remains to be determined. Our study suggests that the genetics of pediatric BSG are complex and include grade-dependent amplification

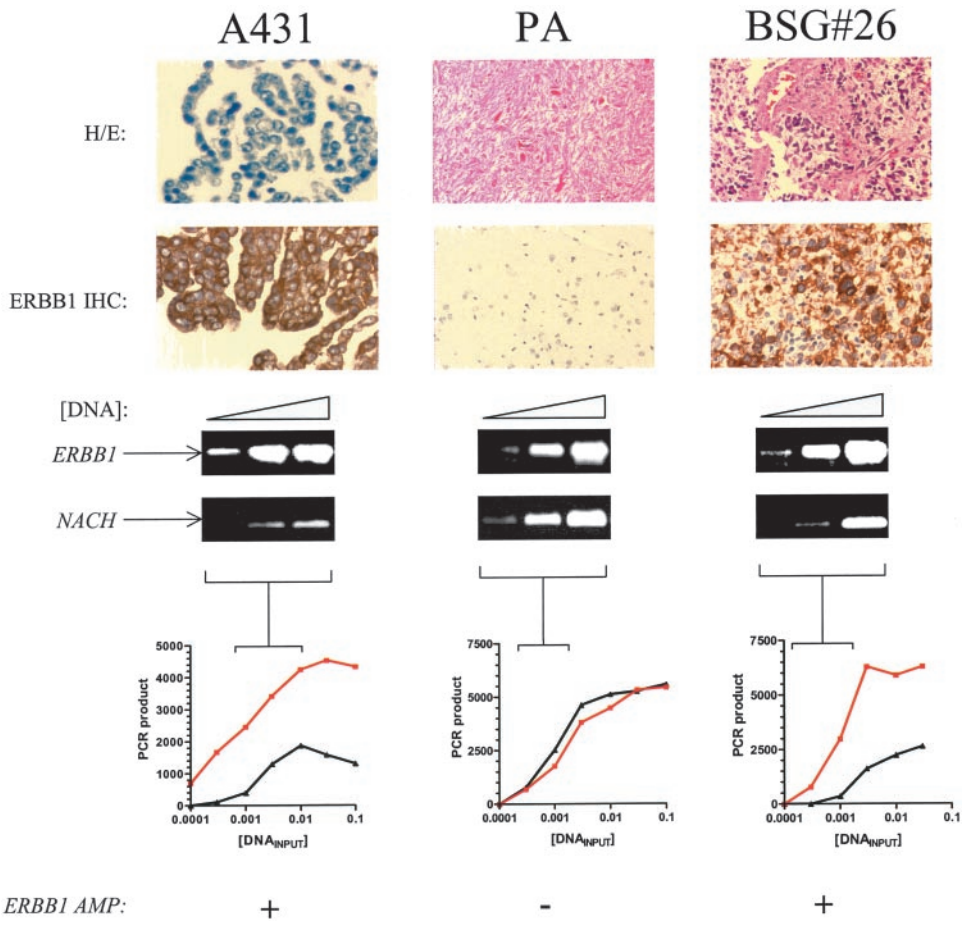

Fig. 2 *ERBB1* expression and amplification in A431 cells and two brain stem glioma samples. Top photomicrographs show H&E (hematoxylin only for A431 cells) staining of A431 human carcinoma cells, a brain stem PA, and grade IV tumor #26. Bottom photomicrographs show the results of ERBB1 immunostaining of these same samples. Agarose gels containing PCR products derived from amplification of *ERBB1* and the internal control gene (*NACH*) at three different concentrations of template DNA are shown immediately below the photomicrographs of the corresponding sample. Graphs display the results of densitometric analysis of PCR products derived from amplification of *ERBB1* (–) and *NACH* (–) over a range of template DNA concentrations. The linear range of PCR amplification from which *ERBB1* gene copy number was calculated is bracketed.

and overexpression of *ERBB1* and grade-independent expression and mutation of *TP53*. The *ERBB1* primers and antibody used in our study do not distinguish between the wild-type and truncated, constitutively active ERBB1 (EGFRvIII). EGFRvIII is present in 50% of adult glioblastomas that contain an amplified *ERBB1* locus (9, 10). Therefore, additional analyses are required to establish whether the EGFRvIII mutation also occurs in pediatric BSG.

Although the precise role of *ERBB1* in gliomagenesis remains to be established, evidence indicates that cell signaling via this receptor promotes an aggressive phenotype, *e.g.*, ERBB1 mediates glioma cell resistance to radiation and chemotherapies (19, 37–39) and increases the motility and invasive capacity of glioma cells (18, 40). Taken together with the results of the current study, these data suggest that inhibitors of the ERBB1 tyrosine kinase represent an attractive new therapeutic approach for childhood BSG.

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