ERBB Receptor Signaling Promotes Ependymoma Cell Proliferation and Represents a Potential Novel Therapeutic Target for This Disease

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Abstract

Purpose: This study was designed to investigate the biological and therapeutic significance of ERBB1, ERBB2, ERBB3, and ERBB4 in childhood ependymoma.

Experimental Design: The expression frequency and clinical significance of ERBB1–4 was analyzed in a large cohort of pediatric ependymoma (n = 121) using immunohistochemistry, Western blotting, and reverse transcription-PCR analysis. Histological markers of anaplasia (necrosis, microvascular proliferation, and Ki-67 proliferative index) were also determined. Functional assessment of ERBB-dependent cell signaling and proliferation, in addition to novel therapeutic inhibition of these processes, was conducted using short-term cultures of human ependymoma cells.

Results: Coexpression of ERBB2 and ERBB4 was identified in over 75% of tumors. High-level coexpression of these receptors was significantly related to tumor proliferative activity [P < 0.05; Ki-67 labeling index (LI)] and, in a combined survival analysis of clinical (degree of surgical resection) and molecular (ERBB2/ERBB4 expression status and Ki-67 LI) factors, enabled a greater resolution of patient prognosis than any individual variable alone. Ligand-dependent activation of ERBB receptor–signaling in cultured ependymoma cells resulted in AKT phosphorylation and cellular proliferation that was significantly blocked in a dose-dependent manner using WAY-177820, a novel inhibitor of ERBB2 tyrosine kinase activity.

Conclusions: This study suggests that ERBB receptor signaling results in aggressive disease behavior in ependymoma by promoting tumor cell proliferation. An analysis of ERBB2 and ERBB4 expression, in association with Ki-67 LI and the degree of surgical resection, may provide an accurate tool for assessing disease risk in children with this disease. In addition, these receptors may serve as a target for novel therapeutic approaches in ependymoma.

Introduction

Less than one-half of all children receiving contemporary treatment for ependymoma, the third most common intracranial tumor of childhood, will be disease free at 5 years (1–4). Clinical outcome is significantly better for patients whose tumors are totally resected (1–6). However, attempts to define optimum adjuvant treatment have been hindered by the relatively low incidence of the disease and the subsequent paucity of definitive clinical trials (1). A lack of consensus regarding the prognostic value of histological grading and poor understanding of disease biology has compounded this situation further (1, 5, 6). Substantial improvements in clinical management are unlikely to be achieved in the absence of reliable morphological and molecular predictors of disease behavior.

The WHO classification of nervous system tumors recognizes four major subgroups of ependymal tumor: subependymoma (WHO grade I), myxopapillary ependymoma (WHO grade II), ependymoma (WHO grade II), and anaplastic ependymoma (WHO grade III). The last of these is generally believed to demonstrate more aggressive clinical behavior. However, the diagnosis of anaplasia has proved subjective, and its prognostic significance remains controversial (1–7). The identification of molecular genetic abnormalities responsible for promoting aggressive disease behavior would provide a more accurate means of predicting disease risk in ependymoma. Such abnormalities may also prove useful as novel therapeutic targets. However, although non-random chromosomal alterations have been described in ependymoma (8–13), their clinical and molecular significance remains unclear.

In this study, we investigated the expression and clinical significance of the RTK family in childhood ependymoma.
The RTK I family includes the EGFR (also known as ERBB1), and the ERBB2, ERBB3, and ERBB4 receptors (14, 15). Affinity-based, hierarchical interaction between these receptors and their numerous ligands results in the formation of a network of transmembrane homo- and heterodimers. Signaling via this network has been implicated in the development of both normal and malignant tissues within the nervous system (16–19), including the pediatric brain tumor medulloblastoma (20–23). Here, we show that the ERBB2 and ERBB4 receptors are frequently coexpressed in childhood ependymoma and are associated with increased tumor-proliferative capacity and a poor clinical outcome. In addition, we demonstrate that activation of the ERBB signaling network promotes ependymoma cell proliferation in vitro and that this can be blocked using a novel inhibitor of ERBB2 tyrosine kinase activity.

Patients and Methods

Patients and Tumor Material. Two populations of children (≤18 years) with ependymoma were used in the study. Group A included 99 newly diagnosed consecutive patients with posterior fossa ependymoma (Table 1). Patients were treated in six (Bristol, n = 17; Edinburgh, n = 27; London, n = 20; Newcastle, n = 16; Oxford, n = 8; Southampton, n = 11) United Kingdom Children’s Cancer Study Group centers, between 1972 and 1998. A total of 89 cases were diagnosed after 1980. Formalin-fixed paraffin-embedded material obtained at primary surgery was available from each case. In addition, five of these cases had samples of relapsed tumor available that was obtained during surgical resection of progressive disease. Protocols for the fixation and histological processing of tumor samples were identical between study centers. The diagnosis of ependymoma was reviewed for each case by a single neuropathologist (D. W. E.). Subependymomas and other variants of classic ependymoma were excluded from the study.

The presence of tumor anaplasia was also assessed during this review. In an attempt to make this more objective, three diagnostic features of anaplasia were scored independently. These included the presence or absence of necrosis and the presence or absence of MVP. The third feature, the degree of tumor cell proliferation, was assessed by Ki-67 LI as described in “Immunohistochemistry.” The extent of primary surgical resection was determined using the surgeon’s operative notes and, when available, postoperative computed tomography and magnetic resonance brain imaging. Survival for all of the patients was recorded from the date of diagnosis to the time of death or last follow-up. Median survival for the whole population was 52 months (range, 2–312 months).

Group B included 15 posterior fossa, 4 supratentorial, and 1 spinal tumor(s), from St. Jude Children’s Research Hospital, Memphis, TN, and 2 posterior fossa cases from Tampere University Hospital, Tampere, Finland. Fresh frozen tumor material obtained at primary surgery was available from each case. All of the patients were diagnosed and treated between 1985 and 1998 (Table 1). Diagnosis was confirmed by Drs. Ashley Hill and Jesse Jenkins, Department of Pathology, St. Jude Children’s Research.

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A</th>
<th>Group B</th>
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<tbody>
<tr>
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<td>58</td>
<td>6 50.0</td>
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<td>Age (3–10 yr)</td>
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<td>6 27.3</td>
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<td>6 27.3</td>
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The second column of values for ErbB mRNA expression by tumors derived from Group B denotes the mean transcript expression value with the expression range shown in parentheses.
Research Hospital, and one of us (H. H.). In addition to fresh-frozen tumor material, total RNA extracted from normal fetal brain (14 weeks) and normal frontal cortex (75 years) was available for analysis.

**Ependymoma Cell Explants.** We analyzed cells explanted from two ependymomas treated at St. Jude Children’s Research Hospital. SJEp1 cells were derived from a recurrent posterior fossa ependymoma demonstrating classic histology excised from a 9-year-old female. SJEp2 cells were explanted from a primary ependymoma demonstrating anaplastic foci taken from a 2-year-old female. Tumor tissue was minced, plated on BD BioCoat Poly-Lysine Cellware (BD Biosciences, Bedford, MA) in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 4 mM glutamine (BioWhittaker, Walkersville, MD) and cultured in a 37°C humidified incubator gassed with 10% CO2 in air. Once confluent, cells were subcultured 1:4. Cells were evaluated by immunofluorescence for expression of GFAP (anti-GFAP; DAKO) and vimentin (anti-vimentin; Biomeda, Foster City, CA).

**Immunohistochemistry.** Expression of Ki-67 and the four ERBB receptors was determined by IHC in 5-μm sections of formalin-fixed paraffin-embedded tumor material derived from patients in Group A as described previously (20, 24). Primary antibodies included, anti-Ki67 antibody (DAKO, Carpinteria, CA), and NCL-EGFR, NCL-CB11 (against ERBB1 and ERBB2, respectively; Novocastra, Newcastle, United Kingdom) and c-17/ERBB3 and c-18/ERBB4 (against ERBB3 and ERBB4, respectively; Santa Cruz Biotechnology, Santa Cruz, CA). Sections used in IHC were adjacent to those used for histological review. Ki-67 LI was calculated using a method described elsewhere (24). ERBB receptor expression was scored as described previously using a scale from 0 to 100% (10% increments) based on the estimated percentage of immunopositive tumor cells (20).

**Extraction of Total Protein, RNA, and DNA.** Total protein, RNA, and high-molecular-weight DNA was extracted from Group B samples using the TriStar reagent as described by the manufacturer (Hybaid, Franklin, MA). Total protein was extracted from cultured ependymoma cells by washing cells in ice-cold PBS and scraping into ice-cold lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EGTA, 1% Triton X-100, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM p-nitrophenyl phosphate, and 10 μg/ml each: trypsin inhibitor, aprotinin, leupeptin, and pepstatin (Sigma Chemicals, St. Louis, MO)).

**Western Blotting.** Total protein lysate (50 μg/sample) was analyzed by Western blotting using standard techniques (20). Blots were probed with the same primary antibodies used in ERBB receptor IHC analysis. Protein extracted from the A431 (ERBB1-expressing), SKBR3 (ERBB2- and ERBB3-expressing), and MIIH-MED-1 (ERBB4-expressing; Ref. 22) cell lines were used as positive protein-expressing controls. Blots were reprobed with a monoclonal antibody for β-actin (Sigma Chemicals) to control for protein loading.

**TaqMan Analysis of ERBB Receptor mRNA Levels in Ependymoma Primary Tumors.** Sufficient total RNA was available from 16 cases derived from Group B and both normal brain samples. This was reverse transcribed using the Super-Script premplification system (Life Technologies, Inc., Rockville, MD), used in TaqMan quantitative RT-PCR analysis, to determine the expression level of each ERBB receptor mRNA including the JM-a, JM-b, CYT-1, and CYT-2 ERBB4 splice variants. The design and evaluation of ERBB mRNA quantitation by TaqMan analysis will be described in detail elsewhere.4 The oligonucleotide primers used were as follows (probes are shown in parentheses): ERBB1, 5’-caacctgtgccatacaaatc-3’; 5’-ggcatgggaacccgatctt-3’; 5’-ccaattactccatctatac-3’; 5’-ccacctgcgatctcagagcagaa-3’; 5’-aaatgccaaccagaaatctgtgctttt-3’; ERBB2, 5’-aggtggcccacccatactg-3’; 5’-aattgactcgaataggaaccagttgtataccgagat-3’; ERBB3, 5’-ccctgcca-ccagagcagaa-3’; 5’-tcaatggaacccgatctt-3’; ERBB4-CYT-1, 5’-ccatgggctctaccatctatac-3’; 5’-acaatgctgttctgctttt-3’; 5’-aattgactcgaataggaaccagttgtataccgagat-3’; 5’-ccatgggctctaccatctatac-3’; 5’-acaatgctgttctgctttt-3’. Overview of the procedure is as follows. PCR was performed using 300 nm primer, 200 nm (50 nm for CYT-2) probe, 12.5 μl of TaqMan universal PCR Master mix (PE Biosystems, Foster City, CA), and 0.5-μl template cDNA in 25-μl total volume. Thermal cycling (ABI PRISM 7700 Sequence Detector; PE Biosystems) started with initial steps of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C (63°C when ERBB4 CYT-2 was probed). Cx values (the cycle number in which the signal exceeds the fixed threshold value set above the background) were determined. cDNA quality was first confirmed in each case by RT-PCR analysis of β-actin internal control expression. Expression of each ERBB transcript was then determined and recorded as the percentage expression relative to the internal (β-actin) control RNA. All of the analyses were conducted in triplicate.

**Southern Blotting.** High-molecular-weight DNA (10 μg/sample), extracted from ependymoma tumor samples and normal peripheral WBCs (diploid control), was digested with EcoRI overnight at 37°C and was subject to Southern blotting analysis as described in detail previously (22). Membranes were probed with a [32P]dCTP-labeled 1.7-kb fragment of the human ERBB2 coding region for 16 h at 68°C. After washing in 2× SSC + 0.1% SDS and 0.5× SSC + 0.1% SDS, the membrane was analyzed by PhosphorImaging.

**[3H]Thymidine Incorporation Assay.** SJEp1 or SJEp2 cells at passage 2 and 3 were seeded into each well (3 × 103 cells/well) of a 96-well plate in DMEM supplemented with 10% FBS and were allowed to adhere over night. The medium was then replaced with serum-free DMEM, and incubation proceeded for 12 h. Ten μl of PBS, EGF, or NGFβ (both 10 ng/ml final concentration) were added and incubated with cells for 12 h, followed by a 4-h long pulse of [methyl-3H]thymidine (0.1 μCi/well). Cells were then harvested onto Unifilter GF/C 96-well microfilter plates (Packard BioScience, Meriden, CT), and incorporated radioactivity was measured by scintillation (TopCount NXT microplate counter; Packard BioScience). The

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4 T. T. Juntilla et al., manuscript in preparation.
ability of the novel ERBB2 tyrosine kinase inhibitor WAY-177820 (gift of Dr. S. Rabindran, Wyeth-Ayerst Pharmaceuticals, St. Davids, PA) to block EGF stimulated [methyl-\(^{3}H\)] thymidine incorporation was performed in an identical manner, except cells were incubated in the presence of the inhibitor (1.8–1800 nM) or of the vehicle for only 2 h before the addition of EGF. WAY-177820 is a potent and selective inhibitor of ERBB2 kinase activity in cell-free systems (IC\(_{50}\), 70 nM) and has been shown to preferentially inhibit cell proliferation in ERBB2-transfected 3T3 cells and ERBB2-overexpressing human breast cancer cell lines (SKBr-3 and BT474; IC\(_{50}\), 2–4 nM; Ref. 25). WAY-177820 was dissolved in DMSO and stored in aliquots at −20°C before use. Final DMSO concentration in all of the experiments was maintained at <0.1%.

WAY-177820 Inhibition of EGF-induced ERBB2, ERK1/2, and AKT Phosphorylation. SJEp1 or SJEp2 cells were grown to 80% confluence at passage 2 and 3 in DMEM supplemented with 10% FBS before incubation in serum-free DMEM for 14 h. Cells were then preincubated in 180 nM WAY-177820 or DMSO for only 2 h before the addition of EGF or an equal volume of PBS. After 10 min, cells were lysed, and total protein lysates were analyzed by Western blotting. Activation of ERBB2 receptor, ERK1/2, and AKT signaling in the presence or absence of the inhibitor was detected by phosphospecific Western blotting analysis. Western blotting was performed as described above using antibodies raised against Y1248 of ERBB2 (Upstate Biotechnology, Lake Placid, NY) Y204 of ERK1/2 (Santa Cruz Biotechnology) and Ser473 of AKT (New England Biolabs, MA). After probing with phosphospecific antibodies, all of the blots were stripped (62.5 mM Tris, 2% SDS, 100 mM \(\beta\)-mercaptoethanol) for 30 min at 50°C and reprobed with antibodies against ERBB2 (NCL-CB11; Novoceastra), ERK1 (Santa Cruz Biotechnology), and AKT (New England Biolabs).

Statistical Analysis. Relationships among clinicopathological variables were assessed using standard statistical techniques including Fisher’s (2 \(\times\) 2 comparisons) and Mann-Whitney tests. Univariate survival analysis was performed by construction of Kaplan-Meier survival curves and the log-rank test (26). Because we have previously demonstrated that a Ki-67 LI of \(\geq 25\%\) confers a significantly worse prognosis than lower scores (24), patients were divided into two groups based on this cutoff. Retrospective analyses may be biased by the inclusion of patients treated over broad time frames; therefore, survival analyses were restricted to patients diagnosed after 1980 (n = 89).

Results

The ERBB2 and ERBB4 Receptors Are Frequently Coexpressed in Childhood Ependymoma. The availability of tumor material allowed an assessment of ERBB1, ERBB2, ERBB3, and ERBB4 protein expression by IHC in 60 (61%), 59 (60%), 59 (60%), and 48 (48%) Group A cases, respectively. Total protein and RNA was available from 20 and 16 Group B cases, respectively. The results of expression analyses are summarized in Table 1.

ERBB4 immunoreactivity was observed in 100% of Group A samples examined with a median percentage tumor cell expression rate of 60% (Fig. 1). The majority of Group A cases also coexpressed ERBB2. This receptor was detected in 78% (46 of 59) of tumors with a median percentage tumor cell expression rate of 30% (Fig. 1). Similar high ERBB2 and ERBB4 coexpression levels were detected in Group B tumors. Nineteen of 20 and 14 of 20 samples demonstrated readily detectable levels of ERBB2 and ERBB4 by Western blotting, respectively (Fig. 2). In keeping with these data, all of the Group B tumors that were analyzed expressed the ERBB4-JM-a mRNA at levels higher than that detected in normal brain (Fig. 2). Tumor expression of the ERBB4-JM-b variant proved much less common and was observed at similar levels to that displayed by normal control tissue. All analyzed Group B cases also expressed the ERBB2 mRNA (Fig. 2). In the majority, this was detected at levels greater than that observed in normal brain. In addition, a significant positive relationship was observed between the expression levels of ERBB2 and ERBB4-JM-a transcripts in Group B samples (\(r^2 = 0.92; P < 0.0001;\) Fig. 3A). These data indicate that ERBB2 and ERBB4 are frequently co-overexpressed in childhood ependymoma and that this may be governed by a common mechanism(s).

In contrast, ERBB1 and ERBB3 protein and mRNA were absent or present at low levels in ependymoma (Figs. 1 and 2). Four Group B cases (Fig. 2, F9, F12–14) did demonstrate relatively high ERBB1 transcript levels. This did not appear to be translated into detectable protein (Fig. 2).

Elevated Expression of ERBB2 Is Not Mediated by Gene Amplification in Ependymoma. ERBB2 overexpression in human cancer commonly results from gene amplification, although overexpression from a nonamplified locus is also well described. High-level expression of ERBB4 in human tumors invariably occurs in the absence of gene amplification (14, 15, 22). The close correlation between ERBB2 and ERBB4 levels observed in Group B strongly suggests that mechanisms other than gene amplification are important in maintaining elevated ERBB2 expression in ependymoma. Southern blotting analysis of six samples from Group B, including two with high ERBB2 mRNA and protein expression (Fig. 3B, F13 and F14) confirmed this, demonstrating diploid ERBB2 copy number only.

High ERBB2 and ERBB4 Receptor Coexpression Levels Are Associated with Elevated Tumor Proliferative Activity in Childhood Ependymoma. We next sought to determine the significance of ERBB2 and ERBB4 coexpression in Group A cases of ependymoma. First, we analyzed the relationship between receptor expression and histological anaplasia. To increase the objectivity of our assessment of anaplasia, we analyzed independently the importance of three of its characteristic features (MVP, necrosis, and mitotic activity). MVP and necrosis were detected in 46 (46.4%) and 64 (64.6%) cases respectively (Table 1). The median and mean Ki-67 LI for Group A tumors was 16.8% (range, 0.1–71.3%) and 20.5 ± 15.4% (mean ± SD), respectively. Ki-67 LIs were significantly higher in tumors with elevated ERBB2 and ERBB4 coexpression (up to 50% tumor cell receptor immunoreactivity; \(P = 0.02;\) Fig. 4A). In contrast, neither the presence of necrosis nor MVP was related to the expression of any ERBB receptor or tumor Ki-67 LI. Therefore, elevated ERBB2 and ERBB4 receptor
expression may promote tumor cell proliferation but not necrosis or MVP in childhood ependymoma.

Evidence that tumor proliferation, but not necrosis or MVP, is more indicative of an anaplastic phenotype, was provided by analysis of paired primary and relapsed tumors from Group A. Although a significant increase in Ki-67 LI was observed with disease progression ($P < 0.01$, Fig. 4b), no significant difference in the presence of MVP or necrosis was seen between primary and relapse samples. Insufficient material was available to assess potential differences in ERBB receptor expression between primary and relapsed disease.

**ERBB2 and ERBB4 Coexpression and Ki-67 LI Improve the Accuracy of Clinical Risk Assessment in Childhood Ependymoma.** Having demonstrated that ERBB2 and ERBB4 coexpression levels and Ki-67 LI are significantly related in ependymoma, we next evaluated the importance of these variables for patient survival. First, we analyzed the prognostic significance of all clinicopathological variables including patient age ($\leq 3$ versus $>3$ years), sex, degree of surgical resection (total versus partial), adjuvant therapy, tumor necrosis, MVP, Ki-67 LI, and ERBB receptor expression (all receptors independently and in combination) for Group A patients. To mini-
mize the bias inherent in survival analyses of patients treated over broad time frames (Group A, 1972–1998), analysis was restricted to patients diagnosed after 1980 (n = 89).

In agreement with the existing literature, subtotal tumor resection (1–6) and high Ki-67 LI (24, 27–29) were significantly associated with a worse prognosis (Fig. 5, A and B). This latter finding is also in keeping with our observation that Ki-67 LI increased with disease progression (Fig. 4B). A young age at diagnosis (≤3 years) also resulted in a poorer survival, although this effect was marginal (Fig. 5C). ERBB2 and ERBB4 coexpression data were available for only 39 cases diagnosed after 1980. Although the impact of these receptors on patient survival did not reach statistical significance, a clear trend toward a worse outcome was seen in patients whose tumors coexpressed elevated levels of the ERBB2 and ERBB4 receptors (≥50% immunoreactivity for both receptors, Fig. 5D). The 10-year overall survival rate for patients with elevated ERBB2/ERBB4 coexpressing disease (20.6 ± 12.8%, mean ± SE) was less than one-half that of patients with lower levels of receptor expression (44.5 ± 10.1%, mean ± SE). Therefore, we determined whether a combination of Ki-67 LI and ERBB2 and ERBB4 coexpression levels might afford a more accurate assessment of disease risk. For this analysis, patients were divided into two groups. High-molecular-risk (Ki-67 LI ≥25% or high ERBB2 and ERBB4 coexpression; n = 41) or low-molecular-risk (neither Ki-67 LI ≥25% nor high ERBB2 and ERBB4 coexpression; n = 21). This risk assessment had a prognostic value that was superior to that of either variable alone and was almost equal to that associated with the degree of tumor resection (Fig. 5E). Finally, we recently demonstrated the value of combining clin-
ical and molecular risk markers, including ERBB2 expression and the degree of surgical resection, in disease-risk stratification of children with medulloblastoma (23). Therefore, we next analyzed whether a combination of molecular and clinical (degree of surgical resection) risk factors may also improve the accuracy of disease risk assessment in ependymoma. For this final analysis, patients were divided into three groups. High-risk (subtotal resection and high-molecular-risk, \( n \)) intermediate-risk (either total resection and high-molecular-risk or subtotal resection and low-molecular-risk, \( n = 31 \)) and low-risk (total resection and low-molecular-risk, \( n = 8 \)). This analysis provided the greatest degree of survival discrimination (\( P < 0.0001 \); Fig. 5). Of particular note, this stratification identified patients with an especially poor clinical outcome. Of 23 patients classified as “high-risk,” 19 have died with a median survival of only 2 years.

**RTK I Signaling Promotes Second Messenger Activation and Proliferation of Ependymoma Cells in Vitro That Can Be Blocked with an Inhibitor of ERBB2 Tyrosine Kinase Activity.** To further investigate the ability of ERBB receptor signaling to support ependymoma cell proliferation, we analyzed the impact of exogenous ligand treatment on the proliferation of SJEp1 and SJEp2 cells in vitro. These explants were established from histologically proven ependymoma, and both expressed the ependymoma-associated antigens GFAP and vimentin (Fig. 6A). Both SJEp1 and SJEp2 also expressed readily detectable levels of ERBB1, ERBB2, and ERBB3 (Fig. 6B). However, ERBB4 protein expression was not detected. Nonetheless, we sought to characterize ERBB signaling in these cells by activating ERBB2 in the context of an ERBB1/ERBB2 heterodimer using EGF or an ERBB3/ERBB2 heterodimer using NRG1β. Exogenous EGF induced a significant and approximately equal proliferative response in both cell types (Fig. 6C). In contrast, although NRG1β significantly stimulated the proliferation of SJEp2 cells, a more limited effect was observed in SJEp1 cells.

We next investigated the ability of the ERBB tyrosine kinase inhibitor WAY-177820 to abrogate ERBB receptor signaling and proliferation in SJEp1 and SJEp2 cells (Fig. 7). Because both explants responded significantly and equally to EGF, this ligand was used to stimulate cells in inhibitor assays. Treatment of EGF-stimulated SJEp1 and SJEp2 cells with WAY-177820, significantly inhibited their proliferation in a dose-dependent manner, with the maximum inhibition occurring at \( \approx 180 \text{ nM} \). In addition, whereas EGF treatment induced significant phosphorylation of Y1248ERBB2 and Ser473Akt in both cell types, this was markedly inhibited by exposure of cells to 180 nM of WAY-177820. These data support the hypothesis that RTK I signaling via ERBB2, stimulates the proliferation of ependymoma cells through pathways that include AKT. Furthermore, inhibitors of this pathway may represent a novel therapeutic mechanism for ependymoma.

**Discussion**

The management of pediatric ependymoma is unlikely to improve without a greater understanding of disease biology. Such knowledge may provide a more accurate means of determining disease risk. Although surgery has an established role in the management and clinical outcome of ependymoma (1–6), the value of adjuvant chemotherapeutically and radiotherapy is less clear (1). Therefore, a more accurate means of disease risk stratification...
would allow better evaluation of the efficacy of these conventional therapies. Furthermore, tumorigenic signal pathways may in turn serve as cellular targets for novel therapeutic approaches. The anti-ERBB2 monoclonal antibody Herceptin (30) and the BCR-ABL inhibitor STI571 (31) provide precedents for this approach.

In this comprehensive study of RTK I expression in childhood ependymoma, we have identified coexpression of ERBB2 and ERBB4 to be a frequent event in this disease, affecting over 70% of cases. Our quantitative RT-PCR analysis demonstrated that in the great majority of cases, this expression level is greater than that found in normal brain. Our study also provides evidence that this expression is significant for the biology of ependymoma. First, we show that tumors co-overexpressing ERBB2 and ERBB4 demonstrate significantly elevated proliferative activity. Therefore, signaling via ERBB2 homodimers or ERBB2/ERBB4 heterodimers may deregulate normal cell cycle and proliferative control in this disease. Indeed, our in vitro data indicate that signaling via ERBB2 is pro-proliferative in ependymoma. High tumor proliferative activity is one of a number of features associated with anaplasia (5). This histological diagnosis is based on a constellation of morphological features that include tumor necrosis and MVP in addition to proliferative activity. The individual survival significance of each of these factors is unknown. This may explain, at least in part, the controversy surrounding the prognostic value of anaplasia. Here, we demonstrate that, although elevated ERBB2 and ERBB4 coexpression is significantly related to Ki-67 LI, neither of these variables is associated with tumor necrosis or MVP. Furthermore, we show that, although Ki-67 LI increases with disease progression, no difference in necrosis or MVP can be detected between primary and relapsed tumor samples. Finally, our survival analyses indicate that, although Ki-67 LI significantly relates to patient outcome, no prognostic signifi-

**Fig. 5** Prognostic significance of clinicopathological factors in Group A patients. Kaplan-Meier survival curves and the associated log-rank statistic are shown for: A, degree of surgical resection; B, Ki-67 LI; C, age at diagnosis; D, ERBB2/ERBB4 coexpression status; E, molecular risk [High-risk: Ki-67 LI ≥25% or elevated ERBB2/ERBB4 coexpression; and Low-risk, neither Ki-67 LI ≥25% nor elevated ERBB2/ERBB4 coexpression]; F, combined molecular and clinical risk stratification [High-risk, molecular high-risk and subtotal tumor resection; intermediate-risk (Inter-risk), molecular high-risk and total tumor resection or molecular low-risk and subtotal tumor resection; Low-risk, molecular low-risk and total tumor resection].
cance is associated with tumor necrosis or MVP. Therefore, we propose that Ki-67 LI, but not necrosis or MVP, is a reliable marker of anaplastic, aggressive ependymoma. Furthermore, our data suggest that ERBB2 and ERBB4 receptor signaling may contribute to an aggressive tumor phenotype by mediating tumor proliferative activity.

The results of this study are intriguing in the light of our recent analyses of RTK I expression in medulloblastoma, a second posterior fossa tumor of childhood. In these studies, we also show ERBB2 and ERBB4 co-overexpression to be a frequent event in medulloblastoma (20, 22, 23), to significantly associate with increased proliferative potential (21), and to predict for poor clinical outcome (20, 23). ERBB2 and ERBB4 receptor cooperative signaling is crucial for normal hind brain development (16–19). Therefore, deregulation of these receptors may be important for tumorigenesis in a variety of cellular backgrounds within the developing posterior fossa.

In addition to proliferation, signaling via the ERBB4 receptor is associated with a diverse number of cellular responses that include AKT-mediated cell survival and chemotaxis (32,
33). The exact cell response elicited by ERBB4 signaling is determined in part by the expression of alternative receptor isoforms. These isoforms are generated by RNA splicing of the juxtamembrane (JM-a to d isoforms; Refs. 22, 34) and cytoplasmic (CYT-1 and -2 isoforms; Refs. 35, 36) regions of the ERBB4 primary transcript. Heterodimerization of the ERBB4 receptor with other RTK I family members may also modulate the signal response (14, 15, 37, 38). This is particularly true of ERBB2, which significantly enhances signal potency. Here, we have demonstrated that ERBB4 expression in ependymoma includes both JM-a/CYT-1 and JM-a/CYT-2 forms, whereas JM-b variants are much less common. The juxtamembrane region of ERBB4 appears to dictate the sensitivity of the JM isoforms to receptor ectodomain proteolytic cleavage (34). The JM-a variant is reported to be more sensitive to such processing. However, the overall effect that this exerts on receptor function remains to be determined. The CYT-1 sequence includes a phosphoinositide 3-kinase binding site (35, 36) that retains the capacity to induce cell proliferation and protection from apoptosis and to increase chemotaxis. CYT-2, which lacks phosphoinositide 3-kinase binding supports only ligand-induced proliferation (32). The identification of both CYT forms in ependymoma suggests that ERBB4 signaling in this disease may mediate a broad range of cellular responses, all of which may contribute to malignant behavior.

The results of this study highlight two important areas for future clinical evaluation. To our knowledge, this is the first report indicating that a combination of molecular and clinical markers provides an accurate means of predicting prognosis in ependymoma. We propose that this should be studied in a larger series of contemporarily treated patients. Limited patient numbers in the present study prevented us from accounting for patient age in our combined molecular and clinical survival analyses. Therefore, a larger prospective study would allow the importance of this and other clinical covariables to also be assessed. Finally, this study suggests that ERBB2 receptor-targeted therapies may represent a useful novel therapeutic approach for childhood ependymoma. The ependymoma cell explants described in this study expressed ERBB1, ERBB2, and ERBB3 but not the ERBB4 receptor. Therefore the culture and analysis of ERBB4-expressing ependymoma cells will be important for the future analysis of RTK I signaling biology in ependymoma.

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