Prognostic Significance of HER2 and HER4 Coexpression in Childhood Medulloblastoma

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ABSTRACT

Recent in vitro studies of the epidermal growth factor receptor (EGFR) family have revealed complex signaling interactions involving the production of ligand-mediated heterodimers synergistic for the transformation of cells in vitro. In a series of 70 patients with childhood medulloblastoma, we have used immunohistochemistry and Western blotting analysis to investigate the expression patterns of all four EGFR family members (EGFR, HER2, HER3, and HER4) and heregulin-α, a ligand for the HER3 and HER4 receptors. The majority of cases expressed two or more receptor proteins; coexpression of the HER2 and HER4 receptors occurred in 54%. Expression of the ligand heregulin-α was detected in 31% of tumors. To investigate whether coexpression results in receptor heterodimerization, we have also performed immunoprecipitation analysis of protein extracts from primary tumors, and we demonstrate various patterns of receptor interaction including between HER2 and HER4.

In multivariate 25-year survival analysis with clinicopathological disease features, no individual receptor or heregulin-α achieved significance. In contrast, when considered together in the multivariate model, coexpression of HER2 and HER4 demonstrated independent prognostic significance (P = 0.006). These data suggest the hypothesis that HER2-HER4 receptor heterodimerization is of particular biological significance in this disease, and this report is the first to demonstrate potential clinical significance of EGFR family heterodimerization in human cancer. Finally, we have also analyzed expression of the AP-2 transcription factor implicated in the positive regulation of HER2 and HER3 gene transcription in malignant cells and reveal an association between AP-2 expression and not only HER2 and HER3, but also HER4 levels in medulloblastoma primary tumors.

INTRODUCTION

The RTK family includes the EGFR and the HER2 (1), HER3 (2, 3), and HER4 (4) receptors, also termed c-erbB-2, c-erbB-3, and c-erbB-4. Substantial evidence exists in the literature implicating the RTK family in the development and progression of the malignant phenotype. With regard to HER2, its activated rodent counterpart neu was first identified in transplacentally induced central nervous system tumors in rats (1). Subsequently, introduction of high levels of either wild-type HER2 or EGFR into NIH 3T3 fibroblasts was shown to be transforming. The former led to focus formation and anchorage-independent growth in vitro (5, 6); the latter supported partial transformation in the presence of its cognate ligand transforming growth factor α or EGF (7, 8). Receptor overexpression with or without gene amplification has also been reported in a variety of tumor cell lines for each member of the family (2, 4, 9–12), and for some family members (e.g., EGFR), it appears to show a direct relationship with their capacity to form tumors in nude mice. The treatment of such mice with neutralizing antibody suppresses malignant growth (9, 13–15). Similar results have been obtained in mice bearing tumors produced by the expression of HER2 alone or in synergy with EGF (16, 17).

Such studies have led to intensive investigation of RTK family expression and prognosis in human tumors, particularly in breast cancer (18–20). In this disease, up to 40% and 30% of cases demonstrate elevated levels of EGF and HER2 receptor expression, respectively; the latter is most frequently mediated by gene amplification (21). However, in general, their influence on clinical outcome remains unclear. In studies of individual receptor expression and survival, only elevated HER2 protein levels in node-positive disease has consistently been associated with shorter relapse-free and overall patient survival (22). The prognostic significance of HER2 receptor expression in other pathological subgroups and the overall influence of EGF expression on patient survival in breast cancer remains controversial (21, 23, 24). A relatively small number of studies have investigated the role of the more recently described HER3 in breast cancer. These have also shown elevated receptor levels to be a frequent occurrence, but the analysis of its relationship to established prognostic factors, in particular nodal status, has produced conflicting results (25–27). There are no reports in the literature regarding the relationship of HER4 to prognosis in human cancer.

The frequent problem of small study population size is likely to have contributed to the variable prognostic significance of RTK family receptors expression in malignant disease. However, the recent identification of common ligands for the RTK receptors, including the heregulins (28–30) and β-cellulin (31), with the capacity to mediate functional heterodimerization between family members, is also likely to prove of major importance in the analysis of these receptors (32, 33). Such heterodimerization has been reported in a number of tumor cell lines (4, 12, 34, 35), is synergistic for the transformation of cells in vitro (36–39), and may result in differing signal responses including cell proliferation or differentiation (31, 40). Hence, the prognostic importance of one family member may critically depend on the expression of another and their cognate ligand(s). This emphasizes the need to co-analyze all RTK receptors in studies of their influence on clinical outcome in malignancy.

We have previously reported the prognostic significance of HER2 protein expression in childhood medulloblastoma (41, 42). Medulloblastoma (also termed central cerebellar primitive neuroectodermal tumor) is one of the most common solid tumors of childhood and continues to present a difficult management problem. Most centers using the current conventional treatment of surgery and radiotherapy with or without chemotherapy report long-term survival rates of only 50–60%, with significant iatrogenic sequelae in those achieving cure (43–45). Therefore, future goals in the management of medulloblastoma include the identification of patients with a more favorable prognosis, who may be treated less intensely, and the development of new effective therapies for those patients who will not be cured by existing protocols. Such progress in therapy is likely to come from better knowledge of molecular abnormalities and their prognostic significance in medulloblastoma.
In this report, we have expanded our earlier studies of the HER2 receptor in medulloblastoma and describe the expression and heterodimerization patterns and the prognostic significance of all four EGFR family members and their ligand heregulin-α in this disease. To our knowledge, this is the first report of a relationship between a specific EGFR family heterodimer and poor clinical outcome in human cancer. We have also analyzed expression of the transcription factor AP-2 implicated in the positive regulation of HER2 and HER3 gene transcription in malignant cells (46, 47), and we show that high expression levels of AP-2 are significantly related to elevated receptor levels of HER2, HER3, and HER4.

MATERIALS AND METHODS

Study Population. The patient population used in the current study has been described in detail elsewhere (42). In short, 82 children under 15 years of age with medulloblastoma were notified to the Northern Region Young People’s Malignant Disease Registry between 1968 and 1996. Of these, five patients died in the perioperative period from treatment complications. Tumor material was not available from seven cases. The remaining 70 were included in the study. Age at diagnosis ranged from 1 month to 14 years; the male:female sex ratio was 2.04:1.

All patients underwent surgery (24 total and 46 partial or biopsy resection), and 63 received postoperative PFI and CSI radiotherapy. Twenty-eight patients received PFI equal to or greater than the conventional dose of 50 Gy, whereas 43 were treated with CSI equal to or greater than the conventional dose of 35 Gy. In addition to surgery and radiotherapy, 28 patients underwent adjuvant chemotherapy. In 22 cases treated prior to 1989, this consisted of “empirical” non-trial-based treatments, whereas those treated from the late 1980s onward received trial-based chemotherapy; 1 and 5 patients received the International Society of Paediatric Oncology II and III protocols, respectively (45). Two patients less than 1 year old received surgery and the recently described “baby brain” chemotherapy protocol without radiotherapy (48).

Full reviews of the surgical, radiology, and tumor registry notes were made to obtain details of disease stage. Chaging staging of the primary tumor was carried out for all cases with full surgical descriptions of the primary tumor (n = 58; Ref. 49). However, metastasis staging could only be undertaken in the 29 patients with detailed neuroaxial imaging. Reliability of the surgical notes for tumor staging was confirmed by comparing surgical and radiology reports in the 29 patients with detailed imaging. All staging data are summarized in Table 1.

Table 1  Chang tumor and metastasis staging

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>No. of patients (n = 58)</th>
<th>Metastasis stage</th>
<th>No. of patients (n = 29)</th>
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<tbody>
<tr>
<td>T1</td>
<td>16</td>
<td>M0</td>
<td>20</td>
</tr>
<tr>
<td>T2</td>
<td>20</td>
<td>M1</td>
<td>1</td>
</tr>
<tr>
<td>T3</td>
<td>25</td>
<td>M2</td>
<td>3</td>
</tr>
<tr>
<td>T3b</td>
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<td>5</td>
</tr>
<tr>
<td>T4</td>
<td>7</td>
<td>M4</td>
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</tbody>
</table>

EGFR FAMILY AND PROGNOSIS IN CHILDHOOD MEDULLOBLASTOMA

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Table 1  Chang tumor and metastasis staging

Summary of tumor stage for 58 patients with full surgical notes and metastasis stage for 29 patients with neuro-axial imaging.

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>No. of patients (n = 58)</th>
<th>Metastasis stage</th>
<th>No. of patients (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>16</td>
<td>M0</td>
<td>20</td>
</tr>
<tr>
<td>T2</td>
<td>20</td>
<td>M1</td>
<td>1</td>
</tr>
<tr>
<td>T3</td>
<td>25</td>
<td>M2</td>
<td>3</td>
</tr>
<tr>
<td>T3b</td>
<td>10</td>
<td>M3</td>
<td>5</td>
</tr>
<tr>
<td>T4</td>
<td>7</td>
<td>M4</td>
<td>0</td>
</tr>
</tbody>
</table>

Each antibody for its respective antigen was confirmed using Western blot analysis as described below.

Briefly, 5-μm sections were mounted on siliconized slides and rehydrated in serial alcohol solutions. At this point, all sections except those for NCL-CB111 analysis were subject to high-temperature antigen unmasking as recommended by the manufacturers. This was performed by microwaving twice, at full power, for 5 min in 10 mM trisodium citrate buffer, pH 6.0. Following washing in tap water, endogenous peroxide activity was blocked in all slides by incubation in peroxide/methanol solution followed by blockade of nonspecific binding sites using 10% normal serum in TBS. Horse, goat, and rabbit blocking sera were used for all sections to be analyzed with mouse monoclonal antibody, rabbit polyclonal antibody, and goat polyclonal antibody, respectively, governed by the species of origin of secondary antibody. Sections were incubated at room temperature for 1 h in a solution of their respective primary antibody made up to the appropriate dilution in a 10% solution of normal serum. After the sections were washed in TBS, the primary antibody was detected using an antiprimary biotinylated secondary antibody and then avidin-peroxidase complex following another TBS wash (Vector Laboratories, Peterborough, United Kingdom). Finally, visualization of the primary antibody and hence antigen distribution within sections was achieved using 0.5% diaminobenzidine substrate for 8 min. Sections were then counter-stained in hematoxylin.

Four staining controls were used. Negative controls included substitution of either the primary or secondary antibody with normal serum and an antigen absorption control in which primary antibody was first incubated with its respective antigen (supplied by the manufacturers). All controls were negative. Consecutive sections of formalin-fixed, paraffin-embedded tissue taken from normal placenta (EGFR), Paget’s disease of the breast (HER2), normal kidney (HER3), normal skeletal muscle (HER4), normal cerebral cortex (heregulin-α), and normal testes (AP-2) were used as positive controls to ensure consistency of staining between IHC runs.

Staining was scored blind on separate occasions by two observers (R. H. P. and R. J. G.). Tumor sections were assigned a score based on the estimated percentage of tumor cells demonstrating immunopositivity. Scoring ranged from 0 to 100% positive tumor cells at 10% intervals. Discrepancies in staining analysis of greater than 10% occurred in 10% of all sections. These were reexamined on a multihued microscope and a consensus was reached.

Immunoprecipitation and Western Blot Analysis. Fresh surgical medulloblastoma specimens were obtained from patients undergoing surgery for their primary tumors at Newcastle General Hospital. Immediately following resection, samples were treated in Arcton (ICI Chemicals, Middleabrough, United Kingdom) cooled in liquid nitrogen and then stored at −80°C until analysis. Using the same antibodies used in IHC, samples were then assessed by Western blotting for the expression of the four RTK1 proteins, heregulin-α, and the AP-2 transcription factor.

First, frozen medulloblastoma tissue was transferred into a precooled mortar and pestle and ground under liquid nitrogen into a fine powder. Ground samples were then transferred to a precooled tube on ice containing 500 μl of lysis buffer (50 mM HEPES, pH 7.5; 100 mM NaCl; 1 mM EDTA; 1% Triton X-100; 20 mM NaF; 1 mM sodium orthovanadate; 1 mM p-nitrophenyl phosphate; and 10 μg/ml each of trypsin inhibitor, aprotinin, leupeptin, and pepstatin) and gently mixed with pipetting for 10 min. The cell lysates were then cleared by microcentrifugation at 12,000 rpm for 5 min at 4°C. Following centrifugation, samples were heated at 100°C for 4 min with an equal volume of SDS-PAGE sample loading buffer (0.1 M Tris-HCl, pH 6.8; 40% glycerol; 0.004% bromphenol blue; 2% SDS; and 4% β-mercaptoethanol). Protein samples were separated on an 8% SDS-polyacrylamide gel for at 200 V for 45 min and transferred to a nitrocellulose membrane (Amersham Corp., Arlington, Little Chalfont, United Kingdom) by electrophoresis using a wet transfer cell (Bio-Rad, United Kingdom). Prestained markers (Novel Experimental Technology, San Diego, CA) were also separated in parallel on the gel in tracks adjacent to each sample. Separate filters were blocked for 1 h with blocking buffer solution (5% nonfat milk powder and 0.05% Tween in TBS) and then probed with the appropriate antibody in blocking buffer at the concentration recommended by the manufacturer. The signals were visualized with the appropriate alkaline phosphatase labeled secondary antibody (Santa Cruz Biotechnology) and substrate solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as instructed by the manufacturers (Sigma Chemical Co., Poole, United Kingdom). Tumor samples that were...
to investigate the hypothesis that receptor coexpression detected by IHC was associated with the formation of heterodimers, coimmunoprecipitation analysis was performed for two of the primary tumor samples. Case 1 expressed the HER2, HER3, and HER4 receptors but not the EGFR; case 2 expressed EGFR, HER2, and HER4 (confirmed by IHC and Western blotting). Cell lysates from tumor samples were prepared exactly as described above. Lysates were then mixed with 1 μg of NCL-HER2 monoclonal antibody for 90 min at 4°C with gentle mixing. The immune complexes were collected with protein A-Sepharose beads (Pharmacia, Sweden) and washed three times in lysis buffer. Samples were then heated in an equal volume of SDS-PAGE sample loading buffer and analyzed by Western blotting as above.

**Statistical Analysis.** Expression relationships between members of the RTK I family, heregulin-α, and AP-2 transcription factor and tumor stage were analyzed using χ² analysis and the Mann-Whitney test. Both univariate and multivariate survival analysis was performed using the Cox regression model (50) and Kaplan-Meier survival curves with the log rank test (51). This permitted the analysis of variables in both continuous and categorical forms.

To assess their independent prognostic significance, all factors, including clinicopathological disease features, were then analyzed using backward stepwise Cox regression (50). All analysis was performed using the Stata statistics program (Stata Statistical Software, State Co., Texas).

## RESULTS

### IHC

**EGFR.** Sufficient tumor material was available for analysis of EGFR expression in 65 of the 70 cases. EGFR immunostaining was detected in 17 cases (26%). Although all cases demonstrated membrane and cytoplasmic positivity, two distinct staining patterns were observed. In three cases, over 70% of tumor cells demonstrated positive immunostaining with intense membrane staining (Fig. 1A). In contrast, the remaining 14 cases demonstrated only moderate to weak staining in 30% or less of the tumor cells. IHC staining was heterogeneous throughout the sections, with no relationship to vascularity or other tumor features. Moderate immunostaining was also demonstrated by normal Purkinje and choroid plexus cells.

**HER2.** All 70 cases were analyzed by IHC for HER2 expression. Sixty cases were positive in IHC analysis, with moderate to intense mixed membrane and cytoplasmic staining (Fig. 1B). Unlike EGFR receptor expression, no distinct subgroups were observed regarding the percentage of tumor cells expressing the HER2 protein. Expression ranged from 0 to 90%. We have previously reported a significant difference in patient survival between those cases with tumors in which greater than 50% of cells expressed the HER2 receptor versus those in which less than 50% of cells expressed the HER2 receptor (41). In the current expanded series, 23 cases had greater than 50% section positivity.

**HER3.** Eighteen of 65 cases (28%) showed positive HER3 immunostaining. In all cases, this was confined to a granular cytoplasmic pattern and involved 40% or less of tumor cells (Fig. 1C). Although no specific staining distribution was observed among tumor cells, a notable pattern occurred among normal astrocytes. In particular, reactive astrocytes in areas of high tumor invasion demonstrated intense cytoplasmic immunostaining, whereas those in more distant relatively normal nervous tissue were weakly or negatively stained.

**HER4.** Sixty-five cases were analyzed for HER4 immunoreactivity. Forty-three were positive (66%). All cases showed mixed cytoplasmic and membrane stain with a heterogeneous distribution throughout the section (Fig. 1D). As with HER2 staining, section tumor cell positivity varied over a broad range (0–90%); 49% of cases had greater than 50% HER4-immunopositive tumor cells. Weak staining was also observed in normal astrocytes and neurons, although a relationship with tumor invasion (as noted in HER3 IHC) was not seen.

**heregulin-α.** Fifty-one sections were analyzed for heregulin-α immunoreactivity, of which 16 (31%) were positive. Six cases demonstrated moderate to intense cytoplasmic staining in 30–60% of tumor cells (Fig. 1E). In the remaining 10 cases, 10% or less of tumor cells demonstrated moderate to faint cytoplasmic activity. With regard to normal tissue, staining of astrocytes was observed and, as with HER3, was particularly prominent in reactive astrocytes.

**AP-2 Transcription Factor.** Fifty-seven of the 59 tumors analyzed demonstrated AP-2 immunonegativity. As expected, staining was predominantly nuclear, ranging from moderate to intense in the majority of cases (Fig. 1F). In addition, some tumor cells demonstrated a faint to moderate cytoplasmic stain. The percentage of tumor cells involved varied between 0 and 90% with no specific distribution of positive cells within sections.

### Analysis of RTK I, Heregulin-α, and AP-2 Coexpression

All four RTK I receptors were analyzed by IHC in 63 cases. These cases were used in the analysis of coexpression patterns. Two cases were positive and two were negative for all four receptors. HER2 was the only family member detected in 12 cases (19%), and HER4 alone was present in another 6 cases (10%). The remaining 41 cases expressed two or more RTK I proteins. Coexpression of the HER2 and HER4 receptors alone was the most frequent pattern observed (n = 13 of 63), followed by HER2 and HER4 together with either EGFR (n = 9 of 63) or HER3 (n = 10 of 63) receptors. Various other combinations accounted for the remaining nine cases analyzed. Coexpression of HER2, HER4, and heregulin-α was observed in 13 cases.

No significant relationship was observed between either the expression of one RTK I family member and another or the expression of any RTK I receptor and heregulin-α (Table 2). However, on Mann-Whitney analysis, HER2 (≥50% versus <50%), HER3 (positive versus negative) and HER4 (≥50% versus <50%) receptor expression all demonstrated a significant positive relationship with the percentage of tumor cells expressing the AP-2 transcription factor, with P = 0.034, P = 0.024, and P < 0.0001, respectively (Table 2 and Fig. 2). No such relationship was seen between AP-2 transcription factor and EGFR expression (Table 2).

### Immunoprecipitation and Western Blot Analysis

The results of Western blotting and immunoprecipitation analysis are shown in Fig. 3. Each of the antibodies used in IHC identified the correct sized protein in Western blot analysis (Fig. 3A). Heterodimerization between the HER2 receptor and EGFR, HER3, and HER4 receptors was analyzed in two cases using immunoprecipitation. In case 1, the HER2 and HER4 receptors coimmunoprecipitated with HER2, supporting the hypothesis of HER2-HER3 and HER2-HER4 receptor heterodimerization in this primary tumor (Fig. 3B). Similar results were obtained for case 2 (expressing EGFR, HER2, and HER4), in which potential heterodimerization between HER2-EGFR and HER2-HER4 was identified.

### Survival Analysis

**Univariate Analysis of RTK I and Heregulin-α Immunostaining.** Survival curves were constructed, and the log rank test was performed to analyze the impact of RTK I and heregulin-α immunostaining on patient outcome. Because of the relatively small number of patients demonstrating EGFR or HER3 receptor immunonegativity,
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Fig. 1. Results of immunohistochemical staining of EGF, HER2, HER3, and HER4 receptors, their ligand heregulin-α, and the transcription factor AP-2 in childhood medulloblastoma. A, one of three cases demonstrating intense EGFR membrane staining. X400. B, primary tumor with mixed cytoplasmic and membrane HER2 immunostaining. X400. C, characteristic granular cytoplasmic reactivity in a primary tumor with HER3 receptor reactivity. X600. D, mixed membrane and cytoplasmic HER4 immunoreactivity in secondary medulloblastoma tumor deposit taken from the patient with extraneural metastases. X400. E, cytoplasmic heregulin-α reactivity in primary tumor. X600. F, primary tumor demonstrating intense AP-2 transcription factor nuclear reactivity. X600.

survival analysis for these two family members was performed by comparing the two groups (receptor-positive and receptor-negative cases). Neither receptor demonstrated prognostic significance in log rank or Cox univariate analysis (Table 3). In log rank analysis, $\chi^2 = 0.176, P = 0.67$ for EGF and $\chi^2 = 2.29, P = 0.13$ for HER3 receptors.

In view of our previous report of a significantly worse prognosis in patients with $\geq 50\%$ HER2 expressing tumor cells (41), HER2 and HER4 staining was analyzed in two forms. Survival curves for each were produced comparing outcome in patients with $\geq 50\%$ against those with $<50\%$ positive tumor cells. In addition, univariate Cox analysis was performed separately for HER2 and HER4 in continuous
Table 2 Relationships between RTK I and AP-2 expression in medulloblastoma primary tumors

<table>
<thead>
<tr>
<th></th>
<th>EGFR</th>
<th>HER2</th>
<th>HER3</th>
<th>HER4</th>
<th>AP-2</th>
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<tr>
<td>EGFR</td>
<td>1</td>
<td>0.07</td>
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<td>1.65</td>
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<tr>
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<td>0.00</td>
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<tr>
<td>HER3</td>
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<td>1.0</td>
<td>0.024</td>
</tr>
<tr>
<td>HER4</td>
<td></td>
<td>1</td>
<td>1</td>
<td>0.7</td>
<td>0.001</td>
</tr>
<tr>
<td>AP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 2. Relationship between AP-2 transcription factor and HER2, HER3, and HER4 receptor immunostaining. HER2 and HER4 staining was analyzed as ≥50% or <50% immunopositive tumor cells per section. HER3 was analyzed as sections showing positive versus negative staining. Boxes enclose the 25th and 75th percentiles for each data set and include a horizontal line indicating the median value. Outlying data points are represented by the bars. Ps relate to the results of Mann-Whitney analysis comparing the difference between staining groups for each receptor.

Finally, in view of the coexpression by some tumors of HER2, HER4, and heregulin-α (a potential ligand for any HER2-HER4 heterodimer), the possibility of an autocrine loop exists in these cases. Therefore, log rank analysis was performed to assess whether patients with this coexpression pattern had a different prognosis from the rest of HER2 and HER4 coexpression on survival was highly significant in log rank analysis ($\chi^2 = 17.95$, $P = 0.0001$; Fig. 5).

form. HER2 expression proved to be significantly related to survival in both analyses. In categorical form, 46% of patients with <50% HER2-positive tumor cells were alive at 25 years versus only 17% of patients with ≥50% immunopositivity ($\chi^2 = 8.32$, $P = 0.0039$; Fig. 4). These results for HER2 are very similar to those in our initial study of 55 patients (41). In continuous form, a worse prognosis was also seen to be associated with increasing HER2 expression ($P = 0.005$; Table 3). In contrast, although there was a trend toward decreased survival in patients expressing high levels of HER4, no significant relationship with patient outcome was observed in categorical or continuous analysis ($P = 0.25$ and $P = 0.4$, respectively; Table 3).

Heregulin-α expression was also analyzed as both a categorical (positive versus negative) and a continuous variable. Neither reached significance; 25-year survival of patients with negative tumors (38%) was almost identical to that of cases with heregulin-α immunopositive disease (36%; Table 3).

Because of the frequently observed pattern of HER2 and HER4 coexpression and evidence of their potential heterodimerization from our immunoprecipitation analysis, log rank analysis of survival was performed to assess whether coexpression patterns would allow for further refining of prognostic subgroups. A clear and significant pattern was observed (Fig. 5). No patients who were positive for HER4 and had ≥50% HER2-positive tumor cells ($n = 15$) were alive after 5 years. In contrast, 66% of cases negative for HER4 with <50% HER2-positive tumor cells ($n = 16$) were alive at a 25-year follow-up. Patients either negative for HER4 with ≥50% HER2 expression or positive for HER4 with <50% HER2 immunoreactivity ($n = 32$) demonstrated intermediate survival of 42% at 25 years. This influence of HER2 and HER4 coexpression on survival was highly significant in log rank analysis ($\chi^2 = 17.95$, $P = 0.0001$; Fig. 5).

Finally, in view of the coexpression by some tumors of HER2, HER4, and heregulin-α (a potential ligand for any HER2-HER4 heterodimer), the possibility of an autocrine loop exists in these cases. Therefore, log rank analysis was performed to assess whether patients with this coexpression pattern had a different prognosis from the rest.
Table 3 Cox univariate regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>Relative risk</th>
<th>95% confidence interval for relative risk</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPI*</td>
<td>0.568</td>
<td>0.135</td>
<td>1.76</td>
<td>1.35—2.30</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PFI*</td>
<td>-0.997</td>
<td>0.340</td>
<td>0.37</td>
<td>0.19—0.72</td>
<td>0.003</td>
</tr>
<tr>
<td>HER2 receptor*</td>
<td>0.016</td>
<td>0.006</td>
<td>1.02</td>
<td>1.00—1.03</td>
<td>0.005</td>
</tr>
<tr>
<td>Tumor stage* (T1 &amp; 2 vs. T3 &amp; 4)</td>
<td>0.890</td>
<td>0.363</td>
<td>2.43</td>
<td>1.19—4.96</td>
<td>0.014</td>
</tr>
<tr>
<td>CS*</td>
<td>-0.716</td>
<td>0.306</td>
<td>0.49</td>
<td>0.27—0.89</td>
<td>0.019</td>
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<tr>
<td>Year of diagnosis*</td>
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<td>0.320</td>
<td>0.49</td>
<td>0.26—0.91</td>
<td>0.024</td>
</tr>
<tr>
<td>Surgery (total vs. partial)</td>
<td>-0.601</td>
<td>0.361</td>
<td>0.55</td>
<td>0.27—1.11</td>
<td>0.096</td>
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<tr>
<td>HER3 receptor (positive vs. negative)</td>
<td>0.567</td>
<td>0.341</td>
<td>1.76</td>
<td>1.76—1.78</td>
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<tr>
<td>Heregulin-α</td>
<td>0.274</td>
<td>0.197</td>
<td>1.38</td>
<td>0.89—1.93</td>
<td>0.163</td>
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<td>Sex</td>
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<td>0.70</td>
<td>0.37—1.35</td>
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<td>HER4 receptor</td>
<td>0.009</td>
<td>0.004</td>
<td>1.04</td>
<td>0.996—1.01</td>
<td>0.421</td>
</tr>
<tr>
<td>EGFR receptor (positive vs. negative)</td>
<td>0.190</td>
<td>0.382</td>
<td>1.21</td>
<td>0.57—2.56</td>
<td>0.619</td>
</tr>
<tr>
<td>Age</td>
<td>-0.004</td>
<td>0.036</td>
<td>1.00</td>
<td>0.93—1.07</td>
<td>0.908</td>
</tr>
</tbody>
</table>

* Significant factors.

DISCUSSION

Despite being one of the most common solid tumors of childhood, comparatively little is known regarding the important molecular lesions in medulloblastoma (54). The most common nonrandom chromosomal abnormalities in this disease include an iso chromosome of 17q, double min, and deletions of 17p distinct from the p53 locus (55–57). With regard to oncogene and tumor suppressor gene studies in medulloblastoma, \( \text{EGFR, MYC, and MYCN gene amplification has been detected in cell lines and a small minority of primary tumors (54), whereas p53 accumulation has been reported in clinical samples (58). However, the biological and prognostic significance of these features remains to be established.} \)

In the present study, we have expanded our initial observations of the HER2 receptor in medulloblastoma to include all four RTK I receptors and their ligand heregulin-α. The EGF, HER2, HER3, and HER4 expression (categorical form) and presence or absence of metastases was performed separately to assess whether their expression was significantly related to this well-established prognostic factor (45, 52, 53). For the 29 patients with metastasis stage data, expression of either HER2 or HER4 receptor in ≥50% of tumor cells was significantly related to the presence of metastases (\( \chi^2 = 5.21 \) and \( \chi^2 = 5.45 \), respectively, and \( P = 0.002 \) for both). Insufficient data were available to analyze the relationship between HER2 and HER4 coexpression and metastasis stage.
HER2 receptors were identified by IHC in 26, 86, 28, and 66% of cases, respectively, whereas expression of heregulin-α was detected in 31% of tumors analyzed. Each of the antibodies used in IHC identified the correct sized protein in Western blotting analysis of primary tumors (Fig. 3A). This confirms their specificity and value for assessing the expression of these proteins in routine clinical samples. In keeping with reports on other tumor types, the EGFR, HER2, and HER4 receptors demonstrated mixed membrane and cytoplasmic immunoreactivity, whereas the HER3 receptor antibody produced a predominantly granular cytoplasmic staining pattern (18, 59). Although the significance of EGFR family cytoplasmic immunoreactivity is unclear, the results of our Western blot analysis support the hypothesis that cytoplasmic staining represents specific receptor immunoreactivity. To our knowledge there are no reports in the literature assessing the expression of heregulin by IHC. In this study, the heregulin-α polyclonal antibody C-19 produced a clear granular cytoplasmic immunostain with negligible background staining (Fig. 1E). The specificity of this immunoreactivity was also confirmed by Western blot analysis, in which the C-19 antibody identified the Mr 44,000 heregulin protein (Fig. 3A).

With regard to receptor coexpression, various patterns were identified in the 63 cases in which all four RTK I receptors were performed. Only two cases were negative for all four proteins, whereas two or more receptors were detected in 65% of tumors analyzed. The most frequently coexpressed family members were HER2 and HER4, identified in 54% of tumors. The importance of receptor coexpression has been highlighted in recent years by the characterization of various RTK I ligands, including the heregulins (28–30). Further in vitro studies of ligand and RTK I receptor interaction in cultured cell lines have revealed a complex picture of receptor heterodimerization involving different receptor combinations, including EGFR and HER2 (34, 60) and HER2 and HER4 (61). Evidence is emerging to suggest that the heterodimeric state would be favored over homodimer formation and may diversify the signaling repertoire of these receptors (31, 40, 62). Clearly, this may have significant implications for the biological role of RTK I receptors in tumor development and prognosis. The results of the current study are consistent with this hypothesis. In particular, the observed coimmunoprecipitation from primary tumor extracts of EGFR, HER3, and HER4 with HER2 strongly support the theory that heterodimerization occurs between these receptors in childhood medulloblastoma.

The results of survival analysis in the current study also support the potential biological importance of receptor interactions in this disease. Survival of patients with tumors overexpressing both the HER2 and HER4 receptors was significantly worse in univariate and multivariate analysis. No patients with HER4 immunopositivity and ≥50% HER2 tumor cell expression were alive at a 5-year follow-up (Fig. 5). In multivariate analysis, coexpression also achieved prognostic significance (P = 0.006) independent of age, CSI dose, tumor stage, MPI, and other clinical disease features, including year of diagnosis (Table 4). Although inclusion of metastasis stage was not possible in the assessment of prognosis, both receptors were significantly related to the presence of metastases in χ² analysis (P = 0.02).

Expression of heregulin-α was observed in 16 of 51 cases analyzed. Univariate survival analysis revealed identical survival rates for tumors with and without ligand expression. In contrast, the survival of patients coexpressing HER2, HER4, and heregulin-α (22% at 25 years) was approximately half that of the rest of the population (42% at 25 years); however, this did not reach significance. Study population size did not allow the further analysis of this combination in multivariate survival analysis. Nevertheless, the expression of HER2, HER4, and heregulin-α in 13 cases supports the hypothesis that an autocrine loop involving these three proteins may be important in the development and progression of medulloblastoma.

The heregulin ligand family includes a number of alternatively spliced variants encoded by the same gene and subclassified as α or β isoforms (63). Studies in the literature have revealed the latter to be more commonly expressed in neuronal tissue (63). Once antibodies are available for this second class of heregulin ligands, it will be important to investigate their expression in medulloblastoma.

Evidence suggests that medulloblastoma develops from the primitive cells of the cerebellar external granular layer during intra-uterine development (64–66). Studies of rodent tissue have revealed HER2/neu expression by the central nervous system to be confined to a relatively short period of early embryonic life, with levels peaking at day 14 postconception and declining toward birth (67). Similar analysis of human tissue has produced conflicting results; some studies, using IHC and Northern blot analysis, report the absence of HER2 expression from the nervous system (68). In contrast, expression of heregulin and HER4 occurs in the developing brain and remains elevated into adult life (4, 30, 69). It is possible that deregulation of HER2 receptor expression by developing cerebellar tissue may allow abnormal continued expression in the presence of HER4 and heregulin, contributing to malignant transformation.

To investigate this further, we have assessed possible mechanisms of HER2 overexpression in medulloblastoma. In human tumors, HER2 overexpression is frequently mediated by gene amplification (19). However, protein overexpression in the absence of gene amplification is also well documented. In such cases, the AP-2 transcription factor may play a major role in the positive regulation of HER2 and HER3 expression (46, 47). Using a quantitative PCR-based technique, we have previously analyzed 20 cases from the current study population for amplification of the EGFR, HER2, HER3, and HER4 genes (data not shown). HER2 gene amplification alone (five copies) was detected in only one tumor, suggesting transcriptional or posttranscriptional regulation may be more important mechanisms mediating receptor overexpression. Hence, in the present study we also analyzed expression of the AP-2 transcription factor by IHC and Western blotting (Figs. 1F and 3A) to assess whether it may be implicated in receptor overexpression in this disease. Western blot analysis of medulloblastoma primary tumors with the c-18 anti-AP-2 polyclonal antibody identified the correct sized Mr 50,000 protein (Fig. 3A). Fifty-seven of 59 tumors analyzed were immunopositive for the AP-2 transcription factor. In addition, HER2 (>50% versus ≤50%) and HER3 (positive versus negative) receptor expression was significantly related to the presence of tumor cells expressing the AP-2 transcrip-
tion factor (P = 0.034 and P = 0.024, respectively; Fig. 2). Interestingly, AP-2 expression was also related to HER4 receptor expression (P < 0.0001). The transcriptional regulatory region of the HER4 gene has not yet been reported, and AP-2 has not previously been implicated in the control of HER4 transcription. AP-2 expression in the central nervous system is confined to only a limited number of sites (including the cerebellum); AP-2 may play a key role as a transcriptional activator of early neuroectodermal differentiation events (70, 71). We are currently undertaking further investigation of the potential role for AP-2-mediated gene transcriptional control, including the possibility of regulation of HER4 in medulloblastoma.

In summary, these data reveal the independent prognostic significance of HER2 and HER4 coexpression in medulloblastoma. In addition, our immunoprecipitation analysis supports the hypothesis that coexpression of these receptors results in heterodimerization. Further investigation of the biological implications of HER2 and HER4 in this disease and other malignancies (e.g., breast and ovarian cancer) will provide further insights into their impact on disease behavior and may provide a target for the development of novel anticancer therapies.

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