Prognostic Significance of HER2 and HER4 Coexpression in Childhood Medulloblastoma[1]

Richard J. Gilbertson,[2] Robert H. Perry, Peter J. Kelly, Andrew D. J. Pearson, and John Lunec

Cancer Research Unit, The Medical School, Framlington Place, University of Newcastle upon Tyne [R. J. G., J. L.]; Department of Neuropathology, Newcastle General Hospital, Westgate Road [R. H. P.]; Department of Medical Statistics, Medical School, Framlington Place, University of Newcastle upon Tyne [P. J. K.]; North of England Children's Cancer Research Unit, Sir James Spence Building, Royal Victoria Infirmary, Queen Victoria Road [A. D. J. P.], Newcastle upon Tyne NE2 4HH, United Kingdom

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 I[3] receptor family includes the EGFR and the HER2 (1), HER3 (2, 3), and HER4 (4) receptors, also termed c-*erb*B-2, c-*erb*B-3, and c-*erb*B-4. Substantial evidence exists in the literature implicating the RTK I 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 antireceptor 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 EGFR (16, 17).

Such studies have led to intensive investigation of RTK I 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 EGFR 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 I receptor expression in malignant disease. However, the recent identification of common ligands for the RTK I 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 I 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. Chang 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.

IHC. IHC was performed on formalin-fixed, paraffin-embedded sections using the avidin-biotin-peroxidase complex technique as described previously (41). The most representative block for each case was selected following review of standard H&E sections. Consecutive sections were then taken from this block and used in all subsequent IHC. The following antibodies were used in the IHC analysis (dilution shown in parentheses): mouse monocolonal NCL-EGFR (1:20), NCL-CB11 (1:40), and NCL-c-erbB-3 (1:40), for detection of EGF, HER2, and HER3 receptors, respectively (NovoCastra Laboratories, Newcastle upon Tyne, United Kingdom). The HER4 receptor and heregulin-α ligand were detected using the rabbit polyclonal antiserum C-18: sc-283 (1:200) and the goat polyclonal antiserum C-19:sc-1791 (1:75), respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Finally, expression of the AP-2 transcription factor was analyzed using the rabbit polyclonal antiserum C-18:sc-184 at a dilution of 1:150 (Santa Cruz Biotechnology). Specificity of

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

Briefly, 5-μm sections were mounted on silanized slides and rehydrated in serial alcohol solutions. At this point, all sections except those for NCL-CB11 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 then 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 (Vecta 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 cases were reexamined on a multiheaded 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, Middlesbrough, 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 EGTA; 1% Triton X-100; 20 mM NaF; 1 mM sodium orthovanadate; 1 mM p-nitrophenyl phosphate; and 10 μg/ml each of trypsin inhibitor, apoprotinin, 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., Amersham, 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

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.

Tumor stage	No. of patients ($n = 58$)	Metastasis stage	No. of patients ($n = 29$)
T1	16	M0	20
T2	20	M1	1
T3a	5	M2	3
T3b	10	M3	5
T4	7	M4	0

negative on IHC were also analyzed using Western blots as negative controls (Fig. 3A).

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-CB11 anti-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 χ^2 analysis and the Mann-Whitney test. Both univariate and multivariate survival analysis was performed. Initially, univariate 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 EGF 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 heavy 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 reactivity. 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 immunopositivity. 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 ($\geq$50% versus <50%), HER3 (positive versus negative) and HER4 ($\geq$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). All samples judged negative by IHC were negative on Western blotting analysis (Fig. 3A). Heterodimerization between the HER2 receptor and EGF, HER3, and HER4 receptors was analyzed in two cases using immunoprecipitation. In case 1, the HER3 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 EGF or HER3 receptor immunopositivity,
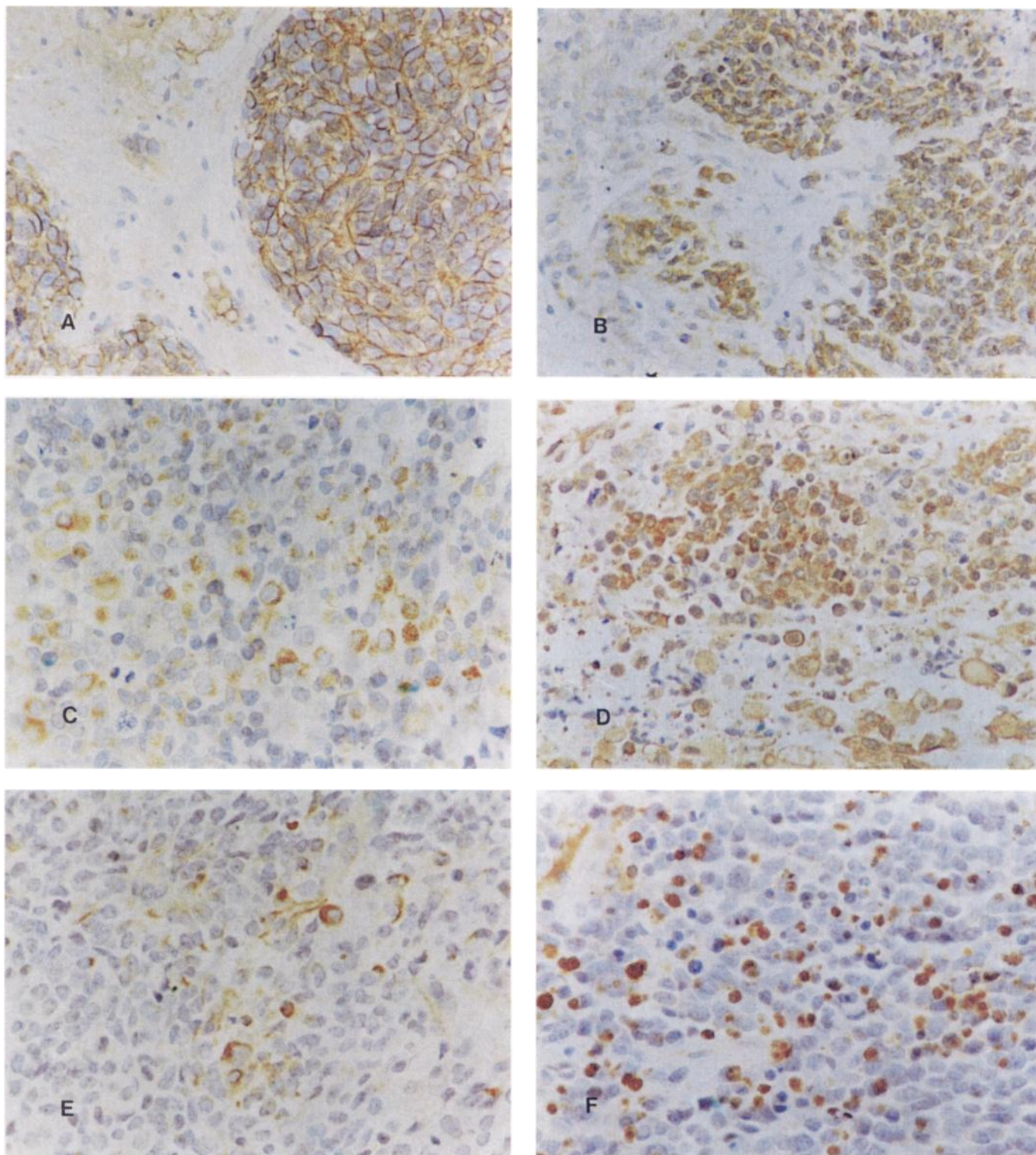
Fig. 1. Results of immunohistochemical staining of EGF, HER2, HER3, and HER4 receptors, their ligand heregulin-α, and the transcription factor AP-2 in childhood medulloblastoma. *A*, one of three cases demonstrating intense EGFR membrane staining. ×400. *B*, primary tumor with mixed cytoplasmic and membrane HER2 immunostaining. ×400. *C*, characteristic granular cytoplasmic reactivity in a primary tumor with HER3 receptor reactivity. ×600. *D*, mixed membrane and cytoplasmic HER4 immunoreactivity in secondary medulloblastoma tumor deposit taken from the patient with extraneural metastases. ×400. *E*, cytoplasmic heregulin-α reactivity in primary tumor. ×600. *F*, primary tumor demonstrating intense AP-2 transcription factor nuclear reactivity. ×600.

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 ≥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 ≥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 1 and AP-2 expression in medulloblastoma primary tumors*

Relationships between RTK 1 receptors were assessed by χ^2 analysis (given as statistic and *P* value). Relationship between AP-2 and RTK 1 family members was assessed using the Mann-Whitney test.

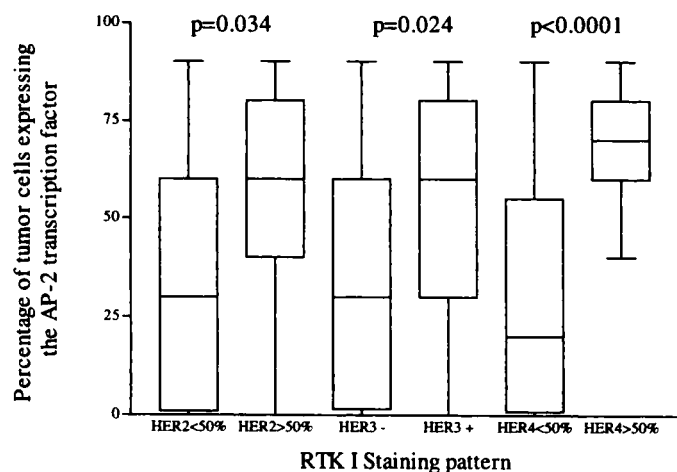|	EGFR	HER2	HER3	HER4	AP-2
EGFR	1	0.07, *P* = 0.8	0.02, *P* = 0.9	1.65, *P* = 0.2	*P* = 0.15
HER2		1	2.47, *P* = 0.1	0.00, *P* = 1.0	*P* = 0.034
HER3			1	1.25, *P* = 0.7	*P* = 0.024
HER4				1	*P* < 0.0001
AP-2					

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*. *P*s relate to the results of Mann-Whitney analysis comparing the difference between staining groups for each receptor.

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 (χ^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 (χ^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
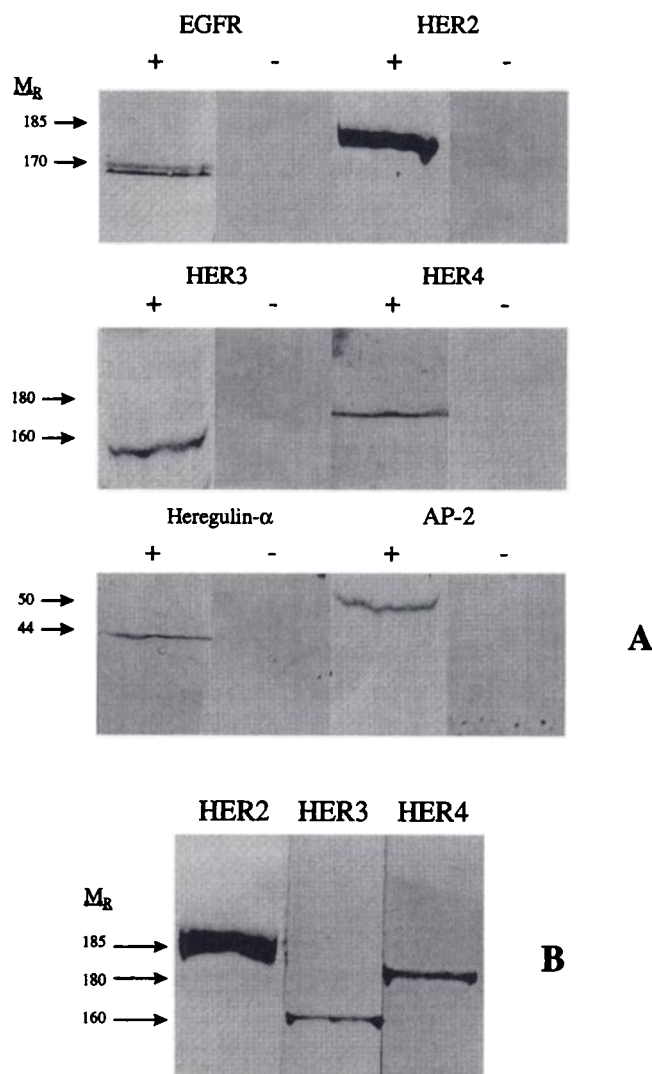

Fig. 3. *A*, Western blot analysis of medulloblastoma primary tumor: detection of EGFR (M_r 170,000), HER2 (M_r 185,000), HER3 (M_r 160,000), HER4 (M_r 180,000), heregulin-α (M_r 44,000), and AP-2 (M_r 50,000) proteins using antibodies NCL-EGFR, NCL-CB11, NCL-c-erbB-3, C-18:sc-283, C-19:sc-1791, and C-18:sc-184 respectively. + and −, analysis of tumors expressing and not expressing the antigen, respectively, by IHC. *B*, coimmunoprecipitation analysis of primary medulloblastoma sample expressing HER2, HER3, and HER4. Both HER3 and HER4 coimmunoprecipitated with HER2 using the mouse monoclonal antibody NCL-CB11.

Table 3 *Cox univariate regression analysis*

Cox univariate survival analysis for clinicopathological disease features in the study population.

| Variable	Coefficient	SE	Relative risk	95% confidence interval for relative risk	P
MPI[a]	0.568	0.135	1.76	1.35–2.30	<0.0001
PFI[a]	−0.997	0.340	0.37	0.19–0.72	0.003
HER2 receptor[a]	0.016	0.006	1.02	1.00–1.03	0.005
Tumor stage[a] (T1 & 2 vs. T3 & 4)	0.890	0.363	2.43	1.19–4.96	0.014
CSI[a]	−0.716	0.306	0.49	0.27–0.89	0.019
Year of diagnosis[a]	−0.722	0.320	0.49	0.26–0.91	0.024
Surgery (total vs. partial)	−0.601	0.361	0.55	0.27–1.11	0.096
HER3 receptor (positive vs. negative)	0.567	0.341	1.76	1.74–1.78	0.097
Heregulin-α	0.274	0.197	1.38	0.89–1.93	0.165
Chemotherapy	0.372	0.309	1.45	0.79–2.65	0.223
Sex	−0.352	0.333	0.70	0.37–1.35	0.291
HER4 receptor	0.004	0.004	1.00	0.996–1.01	0.421
EGF receptor (positive vs. negative)	0.190	0.382	1.21	0.57–2.56	0.619
Age	−0.004	0.036	1.00	0.93–1.07	0.908

[a] Significant factors.
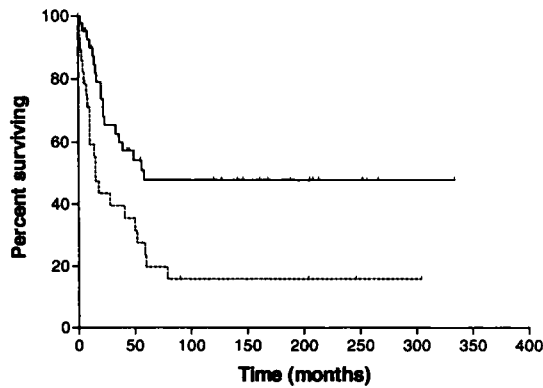
Fig. 4. Kaplan-Meier survival curves comparing 25-year survival for patients with ≥50% (- - - -; n = 23) and <50% (——; n = 47) HER2-positive tumor cells per section. Log rank test: χ^2=8.32, P = 0.0039.

of the study population. Although a trend toward decreased survival was observed in cases coexpressing all three proteins compared to all other patients (42% *versus* 22% 25-year survival), this did not reach significance (χ^2 = 0.77, P = 0.38).

Univariate Analysis of Clinical Disease Features. These have been extensively analyzed elsewhere (42). Results of univariate analysis of these factors are summarized in Table 3. MPI, included in this analysis, is a mitotic count score, which we have described previously as the most significant independent prognostic factor in the patient population used in the current study (42).

Multivariate Survival Analysis of Tumor RTK I and Heregulin-α Expression with Clinicopathological Disease Features. Initially, Cox backward stepwise regression was performed for all of the clinicopathological disease features shown in Table 3, with EGFR (positive *versus* negative), HER2 (categorical and continuous, separately), HER3 (positive *versus* negative), HER4 (categorical and continuous, separately) and heregulin-α (continuous form) expression. None of the RTK I receptors individually or heregulin-α expression reached significance. Only tumor MPI (P < 0.0001), CSI dose (P = 0.006), tumor stage (P = 0.025), and age at diagnosis (P = 0.048) achieved significance. Repeat Cox analysis was then performed using the same variables except for "HER2 and HER4 coexpression" (positive for both *versus* negative for both), as a single variable rather than HER2 and HER4 separate variables. In this second analysis, coexpression of HER2 and HER4 proved to be of independent prognostic significance (P = 0.006), along with MPI, CSI, age, and tumor stage (Table 4).

Although the lack of data regarding metastasis stage did not permit its inclusion in survival analysis, χ^2 analysis of HER2 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 (χ^2 = 5.21 and 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.

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, *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
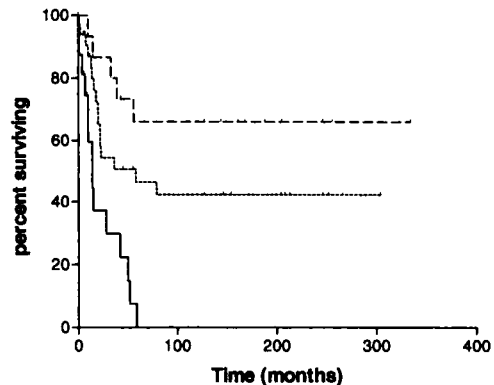

Fig. 5. Kaplan-Meier survival curves comparing 25-year survival for the three groups: ——, ≥50% HER2-positive tumor cells per section and HER4-positive (n = 15); - - - - -, ≥50% HER2-positive tumor cells per section and HER4 negative or <50% HER2-positive tumor cells per section and HER4 positive (n = 32); - - - - -, <50% HER2-positive tumor cells per section and HER4 negative. Log rank test: χ^2 = 17.95, P = 0.0001.

Table 4 *Cox multivariate analysis*

Table of independent significant prognostic factors in Cox multivariate regression analysis of the study population.

Variable	Coefficient	SE	Relative risk	95% confidence interval for relative risk	P
MPI	0.693	0.195	2.00	1.36–2.93	<0.0001
CSI	−1.792	0.543	0.17	0.06–0.48	0.001
HER2 & HER4 coexpression	1.181	0.432	3.25	1.40–7.60	0.006
Age	0.149	0.054	1.16	1.04–1.29	0.006
Tumor stage (T1 & 2 vs. T3 & 4)	0.966	0.392	2.63	1.22–5.66	0.014

HER4 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 EGF, 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 M_r 44,000 heregulin protein (Fig. 3A).

With regard to receptor coexpression, various patterns were identified in the 63 cases in which IHC for all four RTK I receptors was 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 EGF 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 χ^2 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 M_r 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 percentage 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 possible 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.

ACKNOWLEDGMENTS

REFERENCES

1. Schechter, A. L., Stern, D. F., Vaidyanathan, L., Decker, S. J., Drebin, J. A., Greene, M. I., and Weinberg, R. A. The *neu*-oncogene: an erb-B related gene encoding a 185,000 M_R tumour antigen. Nature (Lond.), *312:* 513–516, 1984.
2. Kraus, M. H., Issing, W., Toru, M., Popescu, N. C., and Aaronson, S. A. Isolation and characterisation of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA, *86:* 9193–9197, 1989.
3. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J., and Shoyab, M. Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc. Natl. Acad. Sci. USA, *87:* 4905–4909, 1990.
4. Plowman, G. D., Culcouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. Ligand specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA, *90:* 1746–1750, 1993.
5. Hudziak, R. M., Schlessinger, J., and Ullrich, A. Increased expression of the putative growth factor receptor p185^{HER2} causes transformation and tumorigenesis of NIH/3T3 cells. Proc. Natl. Acad. Sci. USA, *84:* 7159–7163, 1987.
6. DiFiore, P. P., Pierce, J., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. c-*erbB*-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science (Washington DC), *237:* 178–182, 1987.
7. DiFiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J., and Aaronson, S. A. Overexpression of the human EGF receptor confers an EGF dependent transformed phenotype to NIH 3T3 cells. Cell, *51:* 1063–1070, 1987.
8. Marco, E. D., Pierce, J. H., Fleming, T. P., Kraus, M. H., Molloy, C. J., Aaronson, S. A., and DiFiore, P. P. Autocrine interaction between TGF α and the EGF receptor: quantitative requirements for induction of the malignant phenotype. Oncogene, *4:* 831–838, 1989.
9. Filmus, J., Trent, J. M., Pollack, M. N., and Buick, R. N. Epidermal growth factor receptor gene-amplified MDA-A468 breast cancer cell line and its nonamplified variants. Mol. Cell. Biol., *7:* 251–257, 1987.
10. Gullick, W. J., Berger, M. S., Bennett, P. L. P., Rothbard, J. B., and Waterfield, M. D. Expression of the c-erbB-2 protein in normal and transformed cells. Int. J. Cancer, *40:* 246–254, 1987.
11. Carlin, C. R., Simon, D., Mattison, J., and Knowles, B. B. Expression and biosynthetic variation of the epidermal growth factor receptor in human hepatocellular carcinoma derived cell lines. Mol. Cell. Biol., *8:* 25–34, 1988.
12. Kraus, M. H., Fedi, P., Starks, V., Muraro, R., and Aaronson, S. A. Demonstration of ligand-dependent signalling by the erbB3 tyrosine kinase and its constitutive activation in human breast cancer. Proc. Natl. Acad. Sci. USA, *90:* 2900–2904, 1993.
13. Santon, J. B., Cronin, M. T., MacLeod, C. L., Mendelson, J., Masui, H., and Gill, G. N. Effects of epidermal growth factor receptor concentration on tumorigenicity of A431 cells in nude mice. Cancer Res., *46:* 4701–4705, 1986.
14. Aboud-Pirak, E., Hurwitz, E., Pirak, M. E., Bellot, F., Schlessinger, J., and Sela, M.

15. Efficacy of antibodies to epidermal growth factor receptor against Kb carcinoma cells *in vitro* and in nude mice. J. Natl. Cancer Inst., *80:* 1605–1611, 1988.
15. Muller, B. M., Romerdahl, C. A., Trent, J. M., and Reisfeld, R. A. Suppression of spontaneous melanoma metastasis in scid mice with an antibody to the epidermal growth factor receptor. Cancer Res., *51:* 2193–2198, 1991.
16. Stankovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y., and Sela, M. Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. Proc. Natl. Acad. Sci. USA, *88:* 8691–8695, 1991.
17. Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. The *neu* oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. Oncogene, *9:* 2109–2123, 1994.
18. Poller, D. N., Spendlove, I., Baker, C., Church, R., Ellis, I. O., Plowman, G. D., and Mayer, R. J. Production and characterisation of a polyclonal antibody to the c-erbB3 protein: examination of c-erbB-3 protein expression in adenocarcinomas. J. Pathol., *168:* 275–280, 1992.
19. Singleton, T. P., and Strickler, J. G. Clinical and pathological significance of the c-*erbB*-2 (*HER2/neu*) oncogene. Pathol. Annu., *27:* 165–190, 1992.
20. Khazaie, K., Schirrmacher, V., and Lichtner, R. B., EGF receptor in neoplasia and metastasis. Cancer Metastasis Rev., *12:* 255–274, 1993.
21. Rajkumar, T., and Gullick, W. J. The type 1 growth factor receptors in human breast cancer. Breast Cancer Res. Treat., *29:* 3–9, 1994.
22. Gullick, W. J., Love, S. B., Wright, C. B., Barnes, D. B., Gusterson, B., Harris, A. L., and Altman, D. G. c-erbB2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br. J. Cancer, *63:* 434–438, 1990.
23. Tandon, A. K., Clark, G. M., Chamness, G. C., Ullrich, A., and McGuire, W. L. HER2/neu oncogene protein and prognosis in breast cancer. J. Clin. Oncol., *7:* 1120–1128, 1989.
24. Klijn, J. G. M., Berns, P. M. J. J., Schmitz, P. I. M., and Foekens, J. A. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr. Rev., *13:* 3–17, 1992.
25. Lemoine, N. R., Barnes, D. M., Hollywood, D. P., Hughes, C. M., Smith, P., Dublin, E., Prigent, S. A., Gullick, W. J., and Hurst, H. C. Expression of the erbB3 gene product in breast cancer. Br. J. Cancer, *63:* 439–443, 1992.
26. Gasparini, G., Gullick, W. J., Maluta, S., Palma, P. D., Caffo, O., Leonardi, E., Borrachi, P., Pozza, F., Lemoine, N. R., and Bevilacqua, P. c-erbB-3 and c-erbB-2 gene expression in node negative breast cancer. Eur. J. Cancer, *30A:* 16–22, 1994.
27. Travis, A., Pinder, S. E., Robertson, J. F. R., Bell, J. A., Wencyk, P., Gullick, W. J., Nicholson, R. I., Poller, D. N., Blamey, R. W., Elston, C. W., and Ellis, O. I. C-erbB-3 in human breast carcinoma: expression and relation to prognosis and established prognostic indicators. Br. J. Cancer, *74:* 229–223, 1996.
28. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, O., Ogden, S. G., Ben-Levy, R., and Yarden, Y. Isolation of the neu/HER2 stimulatory ligand: a 44 kD glycoprotein that induces differentiation of mammary tumor cells. Cell, *69:* 205–216, 1992.
29. Holmes, W. E., Silkowski, M. W., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W. J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. Identification of heregulin, a specific activator of p180^{erbB2}. Science (Washington DC), *256:* 1205–1210, 1992.
30. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, D. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature (Lond.), *362:* 312–318, 1993.
31. Riese, D. J., II, Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D., and Stern, D. F. Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neurgulin-β. Oncogene, *12:* 345–353, 1996.
32. Carraway, K. L., III, and Cantley, L. C. A Neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerisation in growth signalling. Cell, *78:* 5–8, 1994.
33. Earp, H. S., Dawson, T. L., Li, X., and Yu, H. Heterodimerisation and functional interaction between EGF receptor family members: a new signalling paradigm with implications for breast cancer research. Br. Cancer Res. Treat., *35:* 115–132, 1995.
34. Goldman, R., Ben-Levy, R., Peles, E., and Yarden, Y. Heterodimerisation of the erbB-1 and erbB-2 receptors in breast-carcinoma cells: a mechanism for receptor transregulation. Biochemistry, *29:* 11024–11028, 1990.
35. Chan, S. D. H., Antoniucci, D. M., Fok, K. S., Alajoki, M. L., Harkins, R. N., Thompson, S. A., and Wada, H. G. Heregulin activation of extracellular acidification in mammary carcinomacells is associated with expression of HER2 and HER3. J. Biol. Chem., *270:* 22608–22609, 1995.
36. Kokai, Y., Myers, J. N., Wada, T., Brown, V. I., LeVea, C. M., Davis, J. G., Dobashi, K., and Greene, M. I. Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. Cell, *58:* 287–292, 1989.
37. Dougall, W. C., Qian, X., and Greene, M. I. Interaction of the Neu/p185 EGF receptor tyrosine kinases: implications for cellular transformation. J. Cell. Biochem., *53:* 61–73, 1993.
38. Alimandi, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., DiFiore, P. P., and Kraus, M. H. Cooperative signalling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene, *10:* 1813–1821, 1995.
39. Zhang, K., Sun, J., Liu, N., Wen, D., Chang, D., Thomason, A., and Yoshinaga, S. K. Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. J. Biol. Chem., *271:* 3884–3890, 1996.

40. Wallasch, C., Wieβ, F. U., Niederfellner, G., Jallal, B., Issing, W., and Ullrich, A. Heregulin-dependent regulation of HER2/neu oncogenic signalling by heterodimerisation with HER3. EMBO, 14: 4267–4275, 1993.

41. Gilbertson, R. J., Jaros, E., Perry, R. H., Kelly, P., and Pearson, A. D. J. Prognostic significance of the c-erb B2 oncogene product in childhood medulloblastoma. Br. J. Cancer, 71: 473–477, 1995.

42. Gilbertson, R. J., Jaros, E., Perry, R. H., Kelly, P., Lunec, J., and Pearson, A. D. J. Mitotic percentage index: a new prognostic factor for childhood medulloblastoma. Eur. J. Cancer, 33: 609–615, 1997.

43. Tait, D. M., Thornton-Jones, H., Bloom, H. J. G., Lemerle, J., and Morris-Jones, P. Adjuvant chemotherapy for medulloblastoma: the first multicentre control trial of the International Society of Paediatric Oncology (SIOP I). Eur. J. Cancer, 26: 464–469, 1990.

44. Evans, A. E., Jenkin, D., Sposto, R., Ortega, J. A., Wilson, C. B., Wara, W., Ertel, I. J., Kramer, S., Chang, C. H., and Leikin, S. L. The treatment of medulloblastoma: results of a prospective randomised trial of radiation therapy with and without CCNU vincristine and prednisone. J. Neurosurg., 72: 572–582, 1990.

45. Bailey, C. C., Gnewkow, A., Welleck, S., Jones, M., Round, C., Brown, J., Phillips, A., and Neidhardt, M. K. Prospective randomised trial of chemotherapy given before radiotherapy in childhood medulloblastoma. International Society of Paediatric Oncology (SIOP) and the German Society of Paediatric Oncology (GPO): SIOP II. Med. Pediatr. Oncol., 25: 166–178, 1995.

46. Skinner, A., and Hurst, H. C. Transcriptional regulation of the c-erbB-3 gene in human breast carcinoma cell lines. Oncogene, 8: 3393–3401, 1993.

47. Boshner, J. M., Williams, T., and Hurst, H. C. The developmentally regulated transcription factor AP-2 is involved in c-erbB-2 overexpression in human mammary cancer. Proc. Natl. Acad. Sci. USA, 92: 744–747, 1995.

48. Lashford, L. S., Campbell, R. H. A., Gattamaneni, H. R., Robinson, K., Walker, D., and Bailey, C. An intensive multiagent chemotherapy regimen for brain tumours occurring in very young children. Arch. Dis. Child., 74: 215–223, 1996.

49. Chang, C. H., Housepain, E. M., and Herbert, C. An operative staging system and a megavoltage and a megavoltage therapeutic technique for cerebellar medulloblastoma. Radiology, 93: 1352–1359, 1969.

50. Cox, D. R. Regression models and life tables. J. R. Stat. Soc., B34: 187–220, 1972.

51. Peto, R., and Pike, M. C. Conservatism in the approximation E (O-E)2/E in the log rank tests for survival data and tumour incidence data. Biometrics, 29: 579–584, 1973.

52. Jenkin, D., Goddard, K., Armstrong, D., Becker, L., Berry, M. P., Chan, H., Doherty, M., Greenberg, M., Hendrick, B., Hoffmanh, H., Senley, M., Wietzman, S., and Ziperskey, A. Posterior fossa medulloblastoma in childhood. Treatment results and a proposal of a new staging system. Int. J. Radiat. Oncol. Biol. Phys., 19: 265–274, 1990.

53. Zerbini, C., Gelber, R. D., Wienberg, D., Sallan, S. E., Barnes, P., Kupsky, W., Scott, M., and Tarbell, N. J. Prognostic factors in medulloblastoma including DNA ploidy. J. Clin. Oncology, 11: 616–622, 1990.

54. Batra, S. K., Rasheed, B. K. A., Bigner, S. H., and Bigner, D. D. Biology of disease: oncogenes and antioncogenes in human central nervous system tumours. Lab. Invest., 71: 621–637, 1995.

55. Bigner, S. H., Mark, J., Friedman, H. S., Biegel, J. A., and Bigner, D. D. Structural chromosomal abnormalities in human medulloblastoma. Cancer Genet. Cytogenet., 30: 91–101, 1988.

56. Fujii, Y., Hongo, T., and Hayashi, Y. Chromosome analysis of brain tumours in childhood. Genes Chromosomes & Cancer, 11: 202–215, 1995.

57. McDonald, J. D., Daneshvar, L., Willert, J. R., Matsumura, K., Waldman, F., and Cogen, P. H. Physical mapping of chromosome 17p13.3 in the region of a putative tumour suppressor gene important in medulloblastoma. Genomics, 23: 229–232, 1994.

58. Jaros, E., Lunec, J., Perry, R. H., Kelly, P. J., and Pearson, A. D. J. p53 overexpression identifies a group of central primitive neuroectodermal tumours with poor prognosis. Br. J. Cancer, 68: 801–807, 1993.

59. Haugen, D. R. F., Akslen, L. A., Varhaug, J. E., and Lillehaug, J. R. Expression of c-erbB-3 and c-erbB-4 proteins in papillary thyroid carcinomas. Cancer Res., 56: 1184–1188, 1996.

60. Wada, T., Qian, X., and Greene, M. I. Intermolecular association of the $p185^{neu}$ protein and EGF receptor modulates EGF receptor function. Cell, 61: 1339–1347, 1990.

61. Plowman, G. D., Green, J. M., Culouscou, J-M., Carlton, G. W., Rothwell, V. M., and Buckley, S. Heregulin induces tyrosine phosphorylation of $HER4/p180^{erbB4}$. Nature (Lond.), 366: 473–475, 1993.

62. Murali, R., Brennan, P. J., Kieber-Emmons, T., and Greene, M. I. Structural analysis of $p185^{c-neu}$ and epidermal growth factor receptor tyrosine kinases: oligomerisation of kinase domains. Proc. Natl. Acad. Sci. USA, 93: 6252–6257, 1996.

63. Ben-Baruch, N., and Yarden, Y. Neu differentiation factors: a family of alternatively spliced neuronal and mesenchymal factors. Proc. Soc. Exp. Biol. Med., 206: 221–227, 1994.

64. Rubenstein, L. J. Embryonal central neuroepithelial tumours and their differentiating potential. J Neurosurg., 62: 795–805, 1985.

65. Kozmik, Z., Sure, U., Ruedi, D., Busslinger, M., and Aguzzi, A. Deregulated expression of PAX5 in medulloblastoma. Proc. Natl. Acad. Sci. USA, 92: 5709–5713, 1995.

66. Yokota, N., Aruga, J., Takai, S., Yamamda, K., Hamazaki, M., Iwase, T., Sugimura, H., and Mikoshiba, K. Predominant expression of human zic in cerebellar granule cell lineage and medulloblastoma. Cancer Res., 56: 377–383, 1996.

67. Kokai, Y., Cohen, J. A., Drebin, J. A., and Greene, M. I. Stage and tissue specific expression of the neu oncogene in rat development. Proc. Natl. Acad. Sci. USA, 84: 8498–8501, 1987.

68. Press, M. F., Cordon-Cardo, C., and Slamon, D. J. Expression of the HER2/neu proto-oncogene in normal adult and fetal tissues. Oncogene, 5: 953–962, 1990.

69. Pinkas-Karmarski, R., Eilam, R., Spiegler, O., Lavi, S., Liu, N., Chang, D., Wen, D., Schwartz, M., and Yarden, Y. Brain neurons and glial cells express Neu differentiation factor/heregulin: a survival factor for astrocytes. Proc. Natl. Acad. Sci. USA, 91: 9387–9391, 1994.

70. Mitcell, P. J., Timmons, P. M., Herbert, J. M., Rigby, P. W., and Tjian, R. Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Genes Dev., 5: 105–119, 1991.

71. Philipp, J., Mitcell, P. J., Malipiero, U., and Fontana, A. Cell-type specific regulation of expression of transcription factor AP-2 in neuroectodermal cells. Dev. Biol., 165: 602–614, 1994.