Comprehensive analysis of the ErbB receptor family in pediatric nervous system tumors and rhabdomyosarcoma

Pascale Varlet1 | Eric Bouffet2 | Michela Casanova3 | Felice Giangaspero4,5 | Manila Antonelli4 | Darren Hargrave6 | Ruth Ladenstein7 | Andy Pearson8,9 | Cynthia Hawkins2 | Fatima Barbara König10 | Josef Rüschoff10 | Christian Schmauch10 | Claudia Bühnemann11 | Pilar Garin-Chesa12 | Norbert Schweifer12 | Martina Uttenreuther-Fischer11 | Neil Gibson11 | Carina Ittrich11 | Nicole Krämer12 | Flavio Solca13 | Britta Stolze11 | Birgit Geoerger14

1 GHU Psychiatrie et Neurosciences, site Sainte-Anne, service de Neuropathologie, Paris, France
2 The Hospital for Sick Children, Toronto, Ontario, Canada
3 Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy
4 Università Roma Sapienza, Rome, Italy
5 IRCCS Neumedi, Pozzilli, Italy
6 Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK
7 Department of Paediatrics, St. Anna Children’s Cancer Research Institute, Medical University, Vienna, Austria
8 Paediatric Drug Development, Children and Young People’s Unit, Royal Marsden Hospital, London, UK
9 Division of Clinical Studies, Institute of Cancer Research, London, UK
10 Targos Molecular Pathology GmbH, Kassel, Germany
11 Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
12 Staburo GmbH, Munich, Germany, on behalf of Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
13 Boehringer Ingelheim RCV GmbH & Co. KG, Vienna, Austria
14 Gustave Roussy Cancer Center, Department of Pediatric and Adolescent Oncology, INSERM U1015, Université Paris Saclay, Villejuif, France

Correspondence
Pascale Varlet, GHU Psychiatrie et Neurosciences, site Sainte-Anne, service de Neuropathologie, 1 Rue Cabanis, 75674 Paris, Cedex 14, France.
Email: p.varlet@ch-sainte-anne.fr

Abstract

Background: There is a paucity of knowledge regarding pediatric biomarkers, including the relevance of ErbB pathway aberrations in pediatric tumors. We investigated the occurrence of ErbB receptor aberrations across different pediatric malignancies,
to identify patterns of ErbB dysregulation and define biomarkers suitable for patient enrichment in clinical studies.

**Procedure:** Tissue samples from 297 patients with nervous system tumors and rhabdomyosarcoma were analyzed for immunohistochemical expression or gene amplification of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2). Exploratory analyses of HER3/HER4 expression, and mRNA expression of ErbB receptors/ligands (NanoString) were performed. Assay validation followed general procedures, with additional validation to address Clinical Laboratory Improvement Amendments (CLIA) requirements.

**Results:** In most tumor types, samples with high ErbB receptor expression were found with heterogeneous distribution. We considered increased/aberrant ErbB pathway activation when greater than or equal to two EGFR/HER2 markers were simultaneously upregulated. ErbB pathway dysregulation was identified in ~20%–30% of samples for most tumor types (medulloblastoma/primitive neuroectodermal tumors 31.1%, high-grade glioma 27.1%, neuroblastoma 22.7%, rhabdomyosarcoma 23.1%, ependymoma 18.8%), 4.2% of diffuse intrinsic pontine gliomas, and no recurrent or refractory low-grade astrocytomas. In medulloblastoma/primitive neuroectodermal tumors and neuroblastoma, this was attributed mainly to high EGFR polysomy/HER2 amplification, whereas EGFR gene amplification was observed in some high-grade glioma samples. EGFR/HER2 overexpression was most prevalent in ependymoma.

**Conclusions:** Overexpression and/or amplification of EGFR/HER2 were identified as potential enrichment biomarkers for clinical trials of ErbB-targeted drugs.

**KEYWORDS**
biomarkers, CNS cancers, drug targets, molecular oncology, pediatric cancers, protein tyrosine kinases

---

**INTRODUCTION**

Recent progress has been made in developing new mechanism-based therapies in pediatric malignancies.\(^1\) Signaling through the ErbB receptor family—comprising epidermal growth factor receptor (EGFR; ErbB1), human epidermal growth factor receptor 2 (HER2; ErbB2), ErbB3 (HER3), and ErbB4 (HER4)—mediates important cell functions, and is involved in the pathogenesis and progression of some cancers.\(^2\)–\(^4\) Accordingly, ErbB receptors have been studied intensely as therapeutic targets, and many ErbB inhibitors are registered to treat adult malignancies with EGFR mutations or HER2 amplifications.\(^3,4\) However, there remains a paucity of knowledge regarding the relevance of ErbB pathway aberrations across pediatric malignancies.

Nervous system tumors, including astrocytomas, high-grade gliomas (HGG), ependymomas (EP), medulloblastomas (MB), and neuroblastomas (NB), together with rhabdomyosarcoma (RMS), a mesenchymal tumor, represent a large proportion of pediatric malignancies.\(^5,6\) The molecular characteristics of these tumors frequently differ between adult and pediatric patients.\(^7\)–\(^10\) For many pediatric tumors, there is some limited evidence of ErbB pathway dysregulation.\(^10\)–\(^15\) In several tumor types, including NB, EP, and MB, levels of ErbB receptor expression vary by histological subgroup and/or risk category.\(^2\)–\(^5\),\(^10\)–\(^15\) For example, HER2 expression is common in NB and may be related to favorable prognosis.\(^17\),\(^18\) In contrast, HER2 expression predicts poor outcomes in MB.\(^22\) High HER4 expression has been observed in NB patients with metastatic disease.\(^18\),\(^20\) and a subgroup of MB patients.\(^23\) While EGFR overexpression is related to poor outcomes in patients with intracranial EP.\(^16\) EGFR expression (in association with fibrillin-2) is a marker for the embryonal subtype of RMS, and is related to favorable outcomes.\(^24\)–\(^26\)

Several phase I/II studies evaluating EGFR- and/or HER2-targeted agents have been conducted in pediatric malignancies.\(^21\)–\(^27\)–\(^30\) However, clinical activity in molecularly unselected populations is limited.\(^21\)–\(^27\),\(^29\),\(^31\) indicating that agents that target individual ErbB receptors may be insufficient to inhibit tumor growth. Afatinib is an ErbB family blocker that irreversibly inhibits signaling through all homo- and heterodimers of ErbB family members.\(^22\)\(^,\)\(^23\) and, as such, could be efficacious in tumors with multiple ErbB pathway aberrations including activating mutations, gene amplifications, and/or overexpression of ErbB receptors. However, to date, biomarkers for clinical trials of ErbB family inhibitors have not been reported in pediatric cancers, although several ongoing molecular profiling...
initiatives are characterizing pediatric tumor samples to identify druggable targets and provide individualized molecular information to guide treatment decisions (NCT02613962; NCT03155620; DRKS-ID: DRKS00007623). In this study, we assessed the prevalence/distribution of ErbB receptor aberrations across different pediatric malignancies, adopting a histology-agnostic approach. Additionally, we established hypothetical assay cutoffs/criteria to define ErbB pathway activation for patient enrichment in a phase I/II pediatric trial of afatinib (NCT02372006; 1200.120).

2 METHODS

2.1 Patients and tumor tissue samples

In total, tissue samples from 297 pediatric patients were analyzed, as formalin-fixed, paraffin-embedded (FFPE), 4–5 μm thick tissue sections. Tumor types (Table 1) included EP, HGG, MB/primitive neuroectodermal tumors (MB/PNET), recurrent/refractory low-grade astrocytoma (RLGA), diffuse intrinsic pontine glioma (DIPG), based on the 2007 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (2007 CNS WHO), as well as NB and RMS. The criteria for determination of ErbB receptor dysregulation were based on combined analysis of EP, HGG, MB/PNET, RLGA, NB, and RMS samples (DIPG was not included due to small sample size [n = 4]; however, exploratory analysis was subsequently performed on DIPG tissue samples from an additional 20 patients).

All tissue samples were derived from patients who were screened for, but not participating in, 1200.120, and full ethics approval was obtained in advance. The only clinical data collected were age and gender, and, in some patients, information on previous treatments (Table S1).

2.2 Biomarker evaluation

The following molecular markers were investigated: amplification of EGFR by fluorescence in situ hybridization (FISH); amplification of HER2 using dual-color, dual-hapten in situ hybridization (DDISH); and protein expression of EGFR and HER2 (membrane and cytoplasmic) determined by immunohistochemistry (IHC). HER3 and HER4 expression by IHC, and RNA expression of ErbB receptors and ligands via NanoString, were assessed in an exploratory fashion.

2.3 Measurement of receptor expression/amplifications of EGFR and HER2-4

Analyses were performed by Targos (College of American Pathologists-accredited central laboratory). For each tumor type, control tissue sections with varying IHC staining intensities and different EGFR FISH categories were reviewed by internal and external advisors to establish provisional cutoffs. Representative sections analyzed by IHC and DDISH are presented in Figure S1 (clear EGFR FISH images were not available).

2.4 FISH/DDISH

EGFR amplifications were detected using FISH (Vysis® EGFR FISH Kit, Abbott Molecular). EGFR FISH positivity was defined according to the International Union against Cancer Criteria for stratification of non-small cell lung cancer (NSCLC), based on meeting greater than or equal to one of the following criteria: (a) EGFR gene to centromeric region of chromosome 7 (Cen7) ratio ≥2 (gene amplification); (b) ≥15 copies of the EGFR signal in ≥10% of the cells (gene amplification); (c)
greater than or equal to four copies of the EGFR signal in \( \geq 40\% \) of the cells (high polysomy); (d) presence of a gene cluster (four to 10 copies) in \( \geq 10\% \) of the cells (gene amplification).

HER2 amplifications were detected using DDISH (INIndividualized therapy FOR Relapsed Malignancies in childhood [INFORM] HER2 Dual Probe Cocktail, Ventana). Positivity was based on the HER2 in situ hybridization (ISH) algorithm in the VENTANA INFORM HER2 Dual ISH Interpretation Guide.\(^{37}\) HER2 DDISH status was defined as positive if the HER2/centromeric probe for chromosome 17 (CEP17) ratio was \( \geq 2 \).

### 2.5 | IHC

EGFR and HER2 expression levels were determined using IHC assays previously validated in adult, but not pediatric, cancers (DAKO EGFR pharmDx\textsuperscript{TM} Kit; Dako HercepTest).\(^{38}\) Exploratory analysis of HER3/HER4 expression used IHC assays that are analytically validated for use in pediatric cancers.

Distribution of ErbB receptor expression was assessed based on staining intensity distribution, using the “magnification rule,”\(^{38}\) and calculation of a final Hirsch (H)-score, based on the following equation:\(^{39}\)

\[
H\text{-score} = (1 \times \% \text{ cells weak staining [intensity: 1+]}) + (2 \times \% \text{ cells moderate staining [2+]}) + (3 \times \% \text{ cells strong staining [3+]})
\]

### 2.6 | NanoString assays

A customized NanoString assay was designed to analyze multiplexed gene expression in EGFR and HER2-4 and their cognate ligands. The NanoString nCounter\textsuperscript{TM} system (NanoString Technologies) uses target-specific color-coded barcodes to label oligonucleotides that can hybridize directly to target mRNA molecules, allowing sensitive profiling of specific mRNAs in a complex mixture, as previously described.\(^{40}\)

CodeSets for 31 probes targeting 21 pathway-related genes and eight reference genes were custom designed and manufactured in collaboration with NanoString Technologies (Table S2). RNA expression of the following 18 genes was investigated: EGFR, HER2, HER3, HER4, ADAM17, AREG, BTC, EGF, EPGN, EREG, HBEGF, NRG1, NRG2, NRG3, NRG4, TDGF1, TMEFF1, and TMEFF2. RNA from representative FFPE tissue samples was isolated using the RNeasy FFPE Kit (73504; Qiagen) and quantified using NanoDrop (Supporting Methods). Data collection was performed using the nCounter Digital Analyzer, and results were analyzed using nCounter software (NanoString Technologies).

### 2.7 | Assay validation

IHC and ISH validation was conducted using adult glioblastoma samples \( (n = 7) \) and pediatric neuroectodermal tumor samples \( (n = 31; \text{Supporting Methods}) \). General procedures included proof-of-principle staining on a small sample cohort; verification of specificity; precision (inter- and intra-assay repeatability); robustness (cut slide stability and matrix effect where applicable); and establishment of a suitable scoring algorithm (EGFR/HER IHC and ISH).

Additional analytical validation for EGFR/HER2 IHC and ISH was performed to qualify the assays for use for recruitment to the 1200.120 expansion cohort/phase II part. Validation followed general procedures as described above, and US Food and Drug Administration/Clinical Laboratory Improvement Amendments (CLIA) requirements for off-label use of approved assays were addressed by inclusion of patient sample-derived data from the 1200.120 study \( (n = 171; \text{Supporting Methods}) \).

Validation procedures for HER3/HER4 IHC and NanoString analyses are provided in the Supporting Methods.

### 3 | RESULTS

#### 3.1 | EGFR FISH and HER2 DDISH

The frequency of EGFR and HER2 amplification varied across tumor types. In general, EGFR FISH positivity was driven mainly by high polysomy rather than specific EGFR gene amplification (Table 2). High polysomy was most prevalent in MB/PNET and NB (37.8% and 30.3% of samples), whereas EGFR gene amplification was observed predominantly in HGG and NB (16.7% and 12.1%).

On average, the HER2/CEP17 ratio was highest in MB/PNET and NB tumor samples, although HER2 DDISH positivity was observed in samples from all tumor types except RLGA (Figure 1A).

#### 3.2 | ErbB family receptor expression

The distribution of ErbB receptor expression based on IHC varied across and within each tumor type (Figure 1B and Figure S1). EP, HGG, RMS, and DIPG were the main entities expressing ErbB receptors in the membrane, with mean EGFR H-scores across all samples of 105.8, 107.7, 101.2, and 79.1, respectively, versus 37.2 for MB/PNET, 20.6 for RLGA, and 7.2 for NB. Mean H-scores for membrane-bound HER2 in EP, HGG, and RMS were 16.3, 1.7, and 10.8, respectively, versus 0–0.4 for the other four tumor types. Some HGG samples expressed HER3 (mean H-score 16.2) but, overall, HER3 expression in the membrane was observed mainly in RLGA and RMS (mean H-scores 38.1 and 28.3, respectively). Across all tumor types, the relative expression of ErbB receptors was similar between membrane and cytoplasm, except for HER4, which was expressed almost exclusively in the cytoplasm and mainly in a subpopulation of MB/PNET. Subsequent analyses focused on membrane EGFR and HER2 expression, which were sometimes co-expressed, particularly in EP and RMS samples (Figure S2).

For NB and RMS, patterns of EGFR and HER2 staining were generally consistent between initial diagnostic and post-treatment samples, with higher expression observed in RMS compared with NB samples from both untreated and treated patients. In RMS, EGFR, and HER2, H-scores tended to be higher in initial diagnostic samples versus post-treatment samples. However, for patients who provided samples at two
TABLE 2  Prevalence of EGFR FISH types in the seven investigated tumor types

| Tumor type | Patients, n (%) | DIPG | EP | HGG | MB/PNET | RLGA | RMS | NB |
|------------|----------------|------|----|-----|---------|------|-----|-----|
|            |                |      |    |     |         |      |     |     |
| Disomy     |                |      |    |     |         |      |     |     |
| 3 (12.5)   | 15 (31.3)      | 10 (20.8) | 2 (4.4) | 12 (30.0) | 0 (0) | 2 (3.0) |
| Low trisomy|                |      |    |     |         |      |     |     |
| 3 (12.5)   | 14 (29.2)      | 8 (16.7) | 7 (15.6) | 8 (20.0) | 3 (11.5) | 7 (10.6) |
| High trisomy|               |      |    |     |         |      |     |     |
| 10 (41.7)  | 0 (0)          | 0 (0) | 0 (0) | 0 (0) | 4 (15.4) | 6 (9.1) |
| Low polysomy|               |      |    |     |         |      |     |     |
| 5 (20.8)   | 8 (16.7)       | 12 (25.0) | 12 (26.7) | 4 (10.0) | 8 (30.8) | 10 (15.2) |
| High polysomy|              |      |    |     |         |      |     |     |
| 1 (4.2)    | 3 (6.3)        | 7 (14.6) | 17 (37.8) | 1 (2.5) | 4 (15.4) | 20 (30.3) |
| EGFR amplification | | 0 (0) | 1 (2.1) | 8 (16.7) | 1 (2.2) | 1 (2.5) | 2 (7.7) | 8 (12.1) |
| Missing    | 2 (8.3)        | 7 (14.6) | 3 (6.3) | 6 (13.3) | 14 (35.0) | 5 (19.2) | 13 (19.7) |

Note: Alterations shown in bold, bold text were defined as FISH positive.

Abbreviations: DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymoma; FISH, fluorescence in situ hybridization; HGG, high-grade gliomas; MB/PNET, medulloblastoma/primitive neuroectodermal tumor; NB, neuroblastoma; RLGA, recurrent low-grade astrocytoma; RMS, rhabdomyosarcoma.

*Sixteen untreated and 10 treated; four patients provided two measurements; only the first (untreated) measurement was used.

**Forty-two untreated and 24 treated; three patients provided two measurements; only the first (untreated) measurement was used.

FIGURE 1  Investigation of ErbB family member gene amplification and protein expression for the seven investigated tumor types. (A) Distribution of HER2/CEP17 ratio. Amplification of HER2 in pediatric tumor samples (n = 297) was determined using DDISH. HER2 DDISH status was defined as positive if the HER2/CEP17 ratio was ≥2. (B) H-score distributions, representing expression of EGFR, HER2, HER3, and HER4. EGFR, HER2, HER3, and HER4 expression was determined by IHC. Expression is presented as H-score for each of the seven tumor types. CEP17, centromeric probe for chromosome 17; DDISH, dual-hapten in situ hybridization; DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymoma; HGG, high-grade gliomas; H-score, Hirsch-score; HER, human epidermal growth factor; HER2, HER receptor 2; HER3, HER receptor 3; HER4, HER receptor 4; IHC, immunohistochemistry; MB/PNET, medulloblastoma/primitive neuroectodermal tumor; NB, neuroblastomas; RLGA, recurrent low-grade astrocytoma; RMS, rhabdomyosarcoma.

different time points (NB n = 3, RMS n = 4), H-scores were generally similar between pre- and post-treatment samples from the same patient (data not shown).

3.3 Selection of criteria for ErbB receptor positivity status

EGFR and HER2 amplifications based on FISH/DDISH and receptor overexpression based on IHC were considered for further exploration as potential biomarkers of ErbB dysregulation. The predefined definition for EGFR amplification was based on the widely adopted University of Colorado system (EGFR/Cen7 ratio ≥2.0, ≥10% of cells with ≥15 copies, ≥40% of cells with greater than or equal to four copies, or gene cluster in ≥10% of cells), as a higher gene copy number than normal suggests aberration. The definition for HER2 amplification (gene copy number gain) was per the diagnostic test label in adult gastric and breast cancer (HER2/CEP17 ratio ≥2.0).

The criteria selected for EGFR/HER2 overexpression were based on the distribution of H-scores observed across the four initial tumor types (EP, HGG, MB/PNET, RLGA), according to membranous staining. H-score thresholds of EGFR > 150 and HER2 > 0 were proposed,
Given the following rationale. High EGFR expression may relate to real aberration, as most samples expressed low levels, and a high threshold ($H$-score $> 150$) was chosen to ensure true overexpression in tumor areas displaying a staining intensity above $1+$. For HER2, expression is required as a heterodimer partner to EGFR; therefore, a cutoff of $> 0$, that is, any membrane staining, was chosen to demonstrate the potential for active signaling. To define overall positivity status, we proposed that patients should have greater than or equal to two positive molecular markers indicative of ErbB dysregulation.

### 3.4 Prevalence of ErbB dysregulation based on selected criteria

Some samples from six of the seven tumor types investigated exhibited positivity for greater than or equal to two markers (Figures 2 and 3), the exception being RLGA, in which only $EGFR$ FISH positivity was observed (2/40 samples). Across the other six tumor types, the proportion of samples with greater than or equal to two positive biomarkers was: EP 18.8%, NB 22.7%, RMS 23.1%, HGG 27.1%, MB/PNET 31.1%, and DIPG 4.2%.

ErbB dysregulation in MB/PNET and NB was driven mainly by positivity in $EGFR$ FISH, based on polysomy, and $HER2$ DDISH, which occurred together in some samples. In EP, $EGFR$, and HER2, IHC positivity was most prevalent, co-occurring frequently in the absence of genetic alterations. $EGFR$ IHC positivity was also prevalent in HGG, with some samples showing specific focal $EGFR$ amplification; $EGFR$ overexpression based on IHC was also prevalent. A similar proportion of HGG samples showed $EGFR$ FISH positivity and $EGFR$ overexpression, and some samples were positive for both (Figure 3); additionally, several samples showed both $EGFR$ FISH and $HER2$ DDISH positivity, with or without $EGFR$ overexpression. Otherwise, no clear correlation between $EGFR$ FISH/$HER2$ DDISH positivity and $EGFR$/$HER2$ overexpression was identified. In RMS, the main drivers of overall positivity varied between individual tumor samples, while no RLGA samples were positive for greater than or equal to one marker.

There was no consistent trend in the proportion of tumor samples with greater than or equal to two positive markers between untreated and treated NB and RMS patients (Table S3). In NB, a slightly higher...
proportion of treated patient samples had greater than or equal to two positive markers versus untreated patients, driven by a higher prevalence of HER2 expression and HER2 DDISH positivity. Conversely, in RMS, the prevalence of greater than or equal to two positive markers was higher in untreated patients, reflecting higher EGFR and HER2 IHC positivity. No differences in positivity status for individual markers were observed between pre- and posttreatment samples from the same patient, except for one NB patient with a negative EGFR FISH sample pretreatment, followed by a positive result posttreatment. Overall prevalence of ErbB markers was not compared for pre-/post-treatment samples from these patients due to small sample sizes.

3.5 mRNA expression pattern of ErbB pathway-related genes

NanoString analysis was performed for 241 patients (Table 1). Similar to gene amplifications/protein-based biomarkers, mRNA expression across the ErbB network was observed, and patterns varied across the seven tumor types (Figure 4). HGG samples differed from the other tumor types by displaying higher EGFR expression, and high expression of TMEFF 1 and 2; however, the values should not be directly compared because of separate normalization within each tumor type. Analysis of co-occurrence at the individual patient level indicated that in NB and RMS, there was a distinguishable set of patients with concomitant high expression of all four ErbB receptors and some cognate ligands (Figure S3). High expression of some genes was also observed in EP, HGG, MB/PNET, and RLGA samples, but no individual patients were identified with overall high ERBB gene expression. In general, lower ERBB gene expression was observed in RLGA samples than most other tumor types, except for high HER4 expression in some samples. Expression of all ERBB and ERBB-related genes was also very low or absent in DIPG samples. Otherwise, there was no apparent association between mRNA expression levels assessed by NanoString and gene amplifications or protein-based markers of ErbB family members.

4 DISCUSSION

To the best of our knowledge, this biomarker prevalence study is the first to comprehensively address the importance of the ErbB receptor repertoire in pediatric malignancies. We developed a series of comparative tests under standardized laboratory conditions, to be further validated for their clinical application. ErbB dysregulation was identified in most tumor types assessed. Of note, the 2016 update to the 2007 CNS WHO classification system now incorporates molecular as well as histological parameters to define CNS tumor entities. In this regard, it is conceivable that many molecular variables, including individual ErbB receptor aberrations, may vary between and within histopathological categories of CNS tumors and could potentially correlate with sensitivity of some tumors to ErbB family inhibition. Hence, it is important to develop molecular biomarkers for patient enrichment in further clinical studies of ErbB family inhibitors in pediatric populations, given the limited activity observed to date with agents such as lapatinib and erlotinib. The changes in the 2016 CNS WHO also altered the classification of several tumor types. While our samples...
FIGURE 4  Distribution of normalized mRNA expression in each tumor type. Normalized mRNA expression counts using NanoString are plotted in box plots for the ErbB receptors and their ligands. Each box represents the 25th–75th percentiles of the distribution, and the central line indicates the median. The upper whisker is the highest observation still within 1.5× the IQR of the 75th percentile. The lower whisker is the lowest observation still within 1.5× IQR of the 25th percentile. The dots show individual data points outside the whiskers. DIPG, diffuse intrinsic pontine glioma; EP, ependymoma; HGG, high-grade gliomas; IQR, interquartile range; MB/PNET, medulloblastoma/primitive neuroectodermal tumor; NB, neuroblastoma; RLGA, recurrent low-grade astrocytoma; RMS, rhabdomyosarcoma

were analyzed historically using the 2007 classification, we recognize that the groupings would differ using the 2016 version. For example, MB/PNET tumors may now be better classified as embryonal tumors and DIPG tumors would now be classified as diffuse midline gliomas.

ErbB pathway dysregulation was considered when greater than or equal to two EGFR/HER2 markers—EGFR or HER2 amplification, or EGFR or HER2 protein overexpression—were simultaneously upregulated, suggesting overall “pathway activation.” The ErbB receptor network signals through mandatory homo- or heterodimerization of ErbB family members required for ligand-induced activation of the single intracellular tyrosine kinase domain; this contributes to the complexity of the signaling network and offers multiple options for aberrant signaling. It has been proposed that HER2 is the preferred heterodimerization partner for other ErbB family members, which could be explained by the fact that HER2 lacks a bona fide ligand and that its extracellular domain is permanently positioned in an “active-like” conformation. Indeed, heterodimerization of EGFR and HER2 leads to more potent activation of the EGFR tyrosine kinase than EGFR homodimerization does, and NSCLC and breast tumors that overexpress both EGFR and HER2 are more aggressive compared to those overexpressing only EGFR; NSCLC tumors overexpressing both EGFR and HER2 are also more responsive to EGFR tyrosine kinase inhibitors. The rationale for considering increased/aberrant ErbB pathway activation is also supported by presence of a positive feedback loop resulting in the increased activation and/or expression of some ErbB receptors and/or ligands as target genes. Additionally, overexpression of HER2 has been demonstrated to inhibit downregulation of EGFR and HER2, and to enhance EGFR recycling, further strengthening the concept that HER2 overexpression increases the overall level of activated EGFR.

Given these signaling mechanisms, we selected and qualified EGFR or HER2 amplification by FISH, and EGFR or HER2 overexpression by IHC as tests that are indicative of ErbB pathway activation. These tests have obtained CLIA approval for adult indications, enabling their use as potential enrichment biomarkers for clinical trials of ErbB-targeted drugs. In addition, we assessed HER3 and HER4 expression and NanoString assays of ERBB-related gene expression as potential exploratory biomarkers for ErbB dysregulation. The rationale for measuring ERBB-related genes is based on the observation that high ERBB gene expression possibly reflects ErbB network activation. Moreover, data on ErbB receptor expression are expected to be more robust compared to activation hallmarks such as phospho-EGFR and phospho-HER2, which are deemed too labile and have not been evaluated clinically.

Similar to individual ErbB receptors, the prevalence of overall positivity for ErbB pathway dysregulation varied across tumor types. Positivity was identified in approximately 20%–30% of samples overall, the exceptions being DIPG (4% positivity) and RLGA (0% positivity). ErbB dysregulation in MB/PNET and NB in the form of EGFR FISH/HER2 DDISH positivity was driven by high polysomy that, given the locations of EGFR and HER2 (7p11.2 and 17q12, respectively), suggests the occurrence of multiple chromosomal alterations in these tumor types; however, this did not translate into high EGFR/HER2 protein expression. This lack of a correlation between EGFR FISH status and EGFR expression is in line with previous findings in NB; however, it should be acknowledged that an earlier study found no evidence of EGFR gene clusters or increased gene copy numbers.
whereas we identified specific EGFR gene amplifications in 12% of NB samples, using the same criteria.

In contrast with MB/PNET and NB, EGFR and HER2 were overexpressed in EP mainly in the absence of genetic alterations, likely due to epigenetic/differentiating factors. These differences, together with the lack of a clear correlation between EGFR FISH/HER2 DDISH positivity and EGFR/HER2 overexpression in most tumor types, reflect earlier findings\textsuperscript{17,25} and highlight the complexity of ErbB signaling in the development and progression of pediatric malignancies. Notably, some HGG samples were positive for both EGFR FISH and EGFR protein overexpression and thus could be particularly sensitive to ErbB receptor-targeted drugs. Interestingly, recent studies have identified a distinct, epigenetically homogenous, molecular class of pediatric thalamic gliomas that show a high prevalence of EGFR amplification and EGFR mutations.\textsuperscript{56,57} Preliminary evidence indicates that such patients could potentially benefit from EGFR targeted agent/temozolomide-based combination regimens.\textsuperscript{56} ErbB dysregulation was also prevalent in RMS, but the main drivers of overall positivity varied between individual tumor samples. Although not investigated due to small sample sizes, variation in ErbB marker expression across RMS samples may at least partly reflect different histological subtypes, as expressions of EGFR and HER2 are considered as markers for embryonal and alveolar RMS, respectively.\textsuperscript{24–26} Unfortunately, several RMS tumors were not subtyped, which is a limitation of the study. Finally, the prevalence of ErbB receptor aberrations was very low for RLG and DIPG in this study. Other studies indicate that EGFR amplification is rare in patients with DIPG, but some cases demonstrate high levels of EGFR expression.\textsuperscript{58} Accordingly, a recent phase 1/2 assessed the combination of erlotinib with bevacizumab/irinotecan in nine children with DIPG. The combination was tolerable and was associated with median overall survival of 13.8 months.\textsuperscript{59}

Analysis of the distribution and prevalence of markers of ErbB dysregulation in individual tumor types using a histology-agnostic approach allowed us to propose criteria that could be applied across all of the investigated tumor types, regardless of histopathology. Further, we chose a conservative approach in considering only strong markers of positivity for EGFR and HER2, and no other receptors, which may underestimate the prevalence of ErbB dysregulation in some tumor types. The results provide only an indication of the prevalence of ErbB dysregulation. Nevertheless, they suggest that it is possible to define a set of potential enrichment biomarkers and cutoffs that could be used across the investigated tumor types to identify patients with tumors showing increased ErbB pathway activation.

Finally, the assays used were analytically validated, both previously and in this study, with additional validation performed to qualify EGFR and HER2 IHC/FISH/DDISH for patient selection in the absence of clinically validated biomarkers. Evaluation of the clinical utility of the selected criteria and their potential as enrichment biomarkers for pan-ErbB-targeted therapy in pediatric cancers is one of the objectives in the phase I/II clinical trial of afatinib in pediatric cancers (NCT02372006; 1200,120). This trial consists of a dose-finding part and an expansion part, and the biomarker-defined criteria are part of the inclusion criteria for the expansion part.

Based on our findings, overexpression and/or amplification of EGFR/HER2 were identified as potential enrichment biomarkers for clinical trials of ErbB-targeted drugs.

ACKNOWLEDGMENTS

Medical writing assistance, supported financially by Boehringer Ingelheim, was provided by Fiona Scott of GeoMed, an Ashfield company, part of UDG Healthcare plc, during the preparation of this article. All research at Great Ormond Street Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child Health is made possible by the NIHR Great Ormond Street Hospital Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the UK Department of Health. This study was sponsored by Boehringer Ingelheim.

CONFLICT OF INTEREST

Pascale Varlet, Fatima Barbara König, Josef Rüschoff, and Christian Schmauch have received research grants/funds from Boehringer Ingelheim. Eric Bouffet has received research grants from Roche and Bristol Myers Squibb. Claudia Bühnemann, Pilar Garin-Chesa, Norbert Schweifer, Neil Gibson, Carina Ittrich, Flavio Solca, and Britta Stolze are employees of Boehringer Ingelheim. Martina Uttenreuther-Fischer was an employee of Boehringer Ingelheim during the preparation of this manuscript. Nicole Krämer is an external statistician at Staburo Gmbh on behalf of Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany. Birgit Geoerger has received consulting fees from Boehringer Ingelheim. No potential conflicts of interest were disclosed by the remaining authors.

AUTHOR CONTRIBUTIONS

Pascale Varlet, Andy Pearson, Claudia Bühnemann, Pilar Garin-Chesa, Norbert Schweifer, Martina Uttenreuther-Fischer, Neil Gibson, Carina Ittrich, Flavio Solca, and Birgit Geoerger contributed to the conception and design. Pascale Varlet, Eric Bouffet, Felice Giangaspero, Manila Antonelli, Darren Hargrave, Ruth Ladenstein, Andy Pearson, Cynthia Hawkins, Fatima Barbara König, Josef Rüschoff, Christian Schmauch, Claudia Bühnemann, Norbert Schweifer, Martina Uttenreuther-Fischer, Neil Gibson, Britta Stolze, and Birgit Geoerger contributed to the collection and assembly of data. Pascale Varlet, Eric Bouffet, Michela Casanova, Andy Pearson, Claudia Bühnemann, Pilar Garin-Chesa, Norbert Schweifer, Martina Uttenreuther-Fischer, Neil Gibson, Carina Ittrich, Nicole Krämer, Flavio Solca, Britta Stolze, and Birgit Geoerger contributed to the computing and analysis and interpretation. Pascale Varlet, Eric Bouffet, Michela Casanova, Felice Giangaspero, Manila Antonelli, Darren Hargrave, Ruth Ladenstein, Andy Pearson, Cynthia Hawkins, Fatima Barbara König, Josef Rüschoff, Christian Schmauch, Claudia Bühnemann, Pilar Garin-Chesa, Norbert Schweifer, Martina Uttenreuther-Fischer, Nicole Krämer, Britta Stolze, and Birgit Geoerger contributed to the drafting of the manuscript. Pascale Varlet, Eric Bouffet, Andy Pearson, Neil Gibson, Carina Ittrich, Nicole Krämer, Flavio Solca, and Birgit Geoerger contributed to the manuscript writing. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work.
which includes ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the study are available from the corresponding author upon reasonable request.

REFERENCES

1. Pearson AD, Herold R, Rousseau R, et al. Implementation of mechanism of action biology-driven early drug development for children with cancer. *Eur J Cancer*. 2016;62:124-131.

2. Bodey B, Kaiser HE, Siegel SE. Epidermal growth factor receptor (EGFR) expression in childhood brain tumors. *In Vivo*. 2005;19(5):931-941.

3. Lv Q, Meng Z, Yu Y, et al. Molecular mechanisms and translational therapies for human epidermal receptor 2 positive breast cancer. *Int J Mol Sci*. 2016;17(12):2095.

4. Jacobi N, Seebeck R, Hofmann E, Eger A. ErbB family signalling: a paradigm for oncogene addiction and personalized oncology. *Cancers (Basel)*. 2017;9(4):33.

5. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7-30.

6. Ward E, DeSantis C, Robbins A, Kohler B, Jemal A. Childhood and adolescent cancer statistics, 2014. *CA Cancer J Clin*. 2014;64(2):83-103.

7. Gilheeney SW, Kieran MW. Differences in molecular genetics between pediatric and adult malignant astrocytomas: age matters. *Future Oncol*. 2012;8(5):549-558.

8. Jones C, Karajannis MA, Jones DTW, et al. Pediatric high-grade glioma: biologically and clinically in need of new thinking. *Neuro Oncol*. 2017;19(2):153-161.

9. Egas-Bejar D, Huh WW. Rhabdomyosarcoma in adolescent and young adult patients: current perspectives. *Adolesc Health Med Ther*. 2014;5:115-125.

10. de Bont JM, Packer RJ, Michiels EM, den Boer ML, Pieters R. Biological background of pediatric medulloblastoma and ependymoma: a review from a translational research perspective. *Neuro Oncol*. 2008;10(6):1040-1060.

11. Bax DA, Gaspar N, Little SE, et al. EGFRVIII deletion mutations in pediatric high-grade glioma and response to targeted therapy in pediatric glioma cell lines. *Clin Cancer Res*. 2009;15(18):5753-5761.

12. Chmielecki J, Bailey M, He J, et al. Genomic profiling of a large set of diverse pediatric cancers identifies known and novel mutations across tumor spectrums. *Cancer Res*. 2017;77(2):509-519.

13. Gajjar A, Bowers DC, Karajannis MA, Leary S, Witt H, Gottardo NG. Pediatric brain tumors: invaive genomic information is transforming the diagnostic and clinical landscape. *J Clin Oncol*. 2015;33(27):2986-2998.

14. Gilbertson RJ, Perry RH, Kelly PJ, Pearson AD, Luncs J. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. *Cancer Res*. 1997;57(15):3272-3280.

15. Gilbertson RJ, Bentley L, Hernan R, et al. ERBB receptor signaling promotes ependymoma cell proliferation and represents a potential novel therapeutic target for this disease. *Clin Cancer Res*. 2002;8(10):3054-3064.

16. Mendryzky F, Korshunov A, Benner A, et al. Identification of gains on 1q and epidermal growth factor receptor overexpression as independent prognostic markers in intracranial ependymoma. *Cancer Res*. 2006;66(17 Pt 1):2070-2079.

17. Izycka-Swieszewska E, Wozniak A, Kot J, et al. Prognostic significance of HER2 expression in neuroblastic tumors. *Mod Pathol*. 2010;23(9):1261-1268.

18. Izycka-Swieszewska E, Wozniak A, Drozynska E, et al. Expression and significance of HER family receptors in neuroblastic tumors. *Clin Exp Metastasis*. 2011;28(3):271-282.

19. Richards KN, Zweidler-McKay PA, Van Roy N, et al. Signaling of ERBB receptor tyrosine kinases promotes neuroblastoma growth in vitro and in vivo. *Cancer*. 2010;116(13):3233-3243.

20. Hua Y, Gorski K, Yang Y, Wang W, Zhang N, Hughes DP. Slow down to stay alive: HER4 protects against cellular stress and confers chemoresistance in neuroblastoma. *Cancer*. 2012;118(20):5140-5154.

21. Georger B, Hargrave D, Thomas F, et al. Innovative therapies for children with cancer pediatric phase I study of erlotinib in brainstem glioma and relapsing/refractory brain tumors. *Neuro Oncol*. 2011;13(1):109-118.

22. Gilbertson RJ. ERBB2 in pediatric cancer: innocent until proven guilty. *Oncologist*. 2005;10(7):508-517.

23. Forget A, Martignetti L, Puget S, et al. Aberrant ErbB4-SRC signaling as a hallmark of group 4 medulloblastoma revealed by integrative phosphoproteomic profiling. *Cancer Cell*. 2018;34(3):379-395.

24. Grass B, Wachtel M, Behnke S, Leuschner I, Niggli FK, Schafer BW. Immunohistochemical detection of EGFR, fibrillin-2, P-cadherin and AP2beta as biomarkers for rhabdomyosarcoma diagnostics. *Histopathology*. 2009;54(7):873-879.

25. Ganti R, Skapek SX, Zhang J, et al. Expression and genomic status of EGFR and ErbB-2 in alveolar and embryonal rhabdomyosarcoma. *Mod Pathol*. 2006;19(9):1213-1220.

26. Wachtel M, Runge T, Leuschner I, et al. Subtype and prognostic classification of rhabdomyosarcoma by immunohistochemistry. *J Clin Oncol*. 2006;24(5):816-822.

27. Jakacki RI, Hamilton M, Gilbertson RJ, et al. Pediatric phase I and pharmacokinetic study of erlotinib followed by the combination of erlotinib and temozolomide: a Children's Oncology Group Phase I Consortium study. *J Clin Oncol*. 2008;26(30):4921-4927.

28. Daw NC, Furman WL, Stewart CF, et al. Phase I and pharmacokinetic study of gefitinib in children with refractory solid tumors: a Children's Oncology Group study. *J Clin Oncol*. 2005;23(25):6172-6180.

29. Fouladi M, Stewart CF, Blaney SM, et al. Phase I trial of lapatinib in children with refractory CNS malignancies: a Pediatric Brain Tumor Consortium study. *J Clin Oncol*. 2010;28(27):4221-4227.

30. DeWire M, Fouladi M, Turner DC, et al. An open-label, two-stage, phase II study of bevacizumab and lapatinib in children with recurrent or refractory ependymoma: a collaborative ependymoma research network study (CERN). *J Neurooncol*. 2015;123(1):85-91.

31. Thiessen B, Stewart C, Tsao M, et al. A phase II/II trial of GW572016 (lapatinib) in recurrent glioblastoma multiforme: clinical outcomes, pharmacokinetics and molecular correlation. *Cancer Chemother Pharmacol*. 2010;65(2):353-361.

32. Solca F, Dahl G, Zoepfel A, et al. Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker. *J Pharmacol Exp Ther*. 2012;343(2):342-350.

33. Modjahahi H, Cho BC, Michel MC, Solca F. A comprehensive review of the preclinical efficacy profile of the ErbB family blocker afatinib in cancer. *Naunyn Schmiedebers Arch Pharmacol*. 2014;387(6):505-521.

34. Worst BC, van Tilburg CM, Balasubramanian GP, et al. Next-generation personalised medicine for high-risk paediatric cancer patients - the INFORM pilot study. *Eur J Cancer*. 2016;65:91-101.

35. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol*. 2007;114(2):97-109.

36. Varella-Garcia M, Stratification of non-small cell lung cancer patients for therapy with epidermal growth factor receptor inhibitors: the EGFR fluorescence in situ hybridization assay. *Diagn Pathol*. 2006;1:19.

37. VENTANA INFORM HER2 dual IHC interpretation guide. US Food & Drug Administration. Accessed January 25, 2017. http://www.accessdata.fda.gov/cdrh_docs/pdf10/p100027c.pdf
38. Ruschoff J, Kerr KM, Grote HJ, et al. Reproducibility of immunohistochemical scoring for epidermal growth factor receptor expression in non-small cell lung cancer: round robin test. Arch Pathol Lab Med. 2013;137(9):1255-1261.
39. Hirsch FR, Varella-Garcia M. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol. 2003;21(20):3798-3807.
40. Geiss GK, Bumgarner RE, Birditt B, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26(3):317-325.
41. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. Acta Neuropathol. 2016;131(6):803-820.
42. Tai W, Mahato R, Cheng K. The role of HER2 in cancer therapy and targeted drug delivery. J Control Release. 2010;146(3):264-275.
43. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997;16(7):1647-1655.
44. Roskoski Jr. The ErbB/HER family of protein-tyrosine kinases: structures and small molecule inhibitors. Pharmacol Res. 2014;79:34-74.
45. Garrett TP, McKern NM, Lou M, et al. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell. 2003;11(2):495-505.
46. Hirsch FR, Varella-Garcia M, Cappuzzo F. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. Oncogene. 2009;28(Suppl1):S32-S37.
47. Jeon M, You D, Bae SY, et al. Dimerization of EGFR and HER2 induces breast cancer cell motility through STAT1-dependent ACTA2 induction. Oncotarget. 2017;8(31):50570-50581.
48. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006;7(7):505-516.
49. Shvartsman SY, Hagan MP, Yacoub A, Dent P, Wiley HS, Lauffenburger DA. Autocrine loops with positive feedback enable context-dependent cell signaling. Am J Physiol Cell Physiol. 2002;282(3):C545-C559.
50. Avraham R, Yarden Y. Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nat Rev Mol Cell Biol. 2011;12(2):104-117.
51. Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J. Analysis of the transcriptional program induced by Raf in epithelial cells. Genes Dev. 2001;15(8):981-994.
52. Hendriks BS, Opresko LK, Wiley HS, Lauffenburger D. Coregulation of epidermal growth factor receptor/human epidermal growth factor receptor 2 (HER2) levels and locations: quantitative analysis of HER2 overexpression effects. Cancer Res. 2003;63(5):1130-1137.
53. Worthylake R, Opresko LK, Wiley HS. ErbB-2 amplification inhibits down-regulation and induces constitutive activation of both ErbB-2 and epidermal growth factor receptors. J Biol Chem. 1999;274(13):8865-8874.
54. Hutchinson RA, Adams RA, McArt DG, Salto-Tellez M, Jasani B, Hamilton PW. Epidermal growth factor receptor immunohistochemistry: new opportunities in metastatic colorectal cancer. J Transl Med. 2015;13:217.
55. O’Hurley G, Sjostedt E, Rahman A, et al. Garbage in, garbage out: a critical evaluation of strategies used for validation of immunohistochemical biomarkers. Mol Oncol. 2014;8(4):783-798.
56. Mondal G, Lee JC, Ravindranathan A, et al. Pediatric bithalamic gliomas have a distinct epigenetic signature and frequent EGFR exon 20 insertions resulting in potential sensitivity to targeted kinase inhibition. Acta Neuropathol. 2020;139(6):1071-1088.
57. Sievers P, Sill M, Schrimpf D, et al. A subset of pediatric-type thalamic gliomas share a distinct DNA methylation profile, H3K27me3 loss and frequent alteration of EGFR. Neuro Oncol. 2021;23(1):34-43.
58. Zarghooni M, Bartels U, Lee E, et al. Whole-genome profiling of pediatric diffuse intrinsic pontine gliomas highlights platelet-derived growth factor receptor alpha and poly (ADP-ribose) polymerase as potential therapeutic targets. J Clin Oncol. 2010;28(8):1337-1344.
59. El-Khouly FE, Veldhuijzen van Zanten SEM, Jansen MHA, et al. A phase I/II study of bevacizumab, irinotecan and erlotinib in children with progressive diffuse intrinsic pontine glioma. J Neurooncol. 2021;153(2):263-271.

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Varlet P, Bouffet E, Casanova M, et al. Comprehensive analysis of the ErbB receptor family in pediatric nervous system tumors and rhabdomyosarcoma. Pediatr Blood Cancer. 2021:e29316. https://doi.org/10.1002/pbc.29316