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ONCOLOGY: RESEARCH ARTICLE

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

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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,

Abbreviations: Cen7, centromeric region of chromosome 7; CEP17, centromeric probe for chromosome 17; CLIA, Clinical Laboratory Improvement Amendments; CNS, central nervous system; DDISH, dual-color, dual-hapten in situ hybridization; DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymomas; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ hybridization; HER2, 3, 4, human epidermal growth factor receptor 2, 3, 4; INFORM, INdividualized therapy FOr Relapsed Malignancies in childhood; ISH, in situ hybridization; MB, medulloblastomas; NB, neuroblastomas; NSCLC, non-small cell lung cancer; PNET, primitive neuroectodermal tumors; RLGA, recurrent or refractory low-grade astrocytoma; RMS, rhabdomyosarcoma; WHO, World Health Organization.

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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

1 | 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 therelevance 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.[2,15–21] 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,[32,33] 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

TABLE 1 Summary of patients for each tumor type

Tumor type	Patients (*n*)	Normalized NanoString data available
DIPG	24[a]	17
EP	48	44
HGG	48	39
MB/PNET	45 (40 MB/5 PNET)	41 (38 MB/3 PNET)
RLGA	40	24
RMS	26[b]	22
Embryonal	12	10
Alveolar	6	3
Other/not specified	10	9
NB	66[c]	54
Total	297	241

Abbreviations: DIPG, diffuse intrinsic pontine glioma; EP, ependymoma; HGG, high-grade gliomas; MB/PNET, medulloblastoma/primitive neuroectodermal tumors; NB, neuroblastoma; RLGA, recurrent low-grade astrocytoma; RMS, rhabdomyosarcoma.
[a]Sixty-nine tissues (42 untreated and 27 treated); three patients provided two measurements (untreated and treated).
[b]Thirty tissues (16 untreated and 14 treated); four patients provided two measurements (untreated and treated).
[c]Includes four archived tissue samples and 20 autopsy samples from patients with DIPG.

initiatives are characterizing pediatric tumor samples to identify druggable targets and provide individualized molecular information to guide treatment decisions (NCT02613962; NCT03155620; DRKS-ID: DRKS00007623).[34]

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),[35] 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 were 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),[36] 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 ≥40% of the cells (high polysomy); (d) presence of a gene cluster (four to 10 copies) in ≥10% of the cells (gene amplification).

HER2 amplifications were detected using DDISH (INdividualized 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 ≥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™ 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*-score = (1 × % cells weak staining [intensity: 1+]) + (2 × % cells moderate staining [2+]) + (3 × % 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™ 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; 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; 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						
	DIPG	EP	HGG	MB/PNET	RLGA	RMS	NB
Patients, *n* (%)	24	48	48	45	40	26[b]	66[a]
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.

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

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

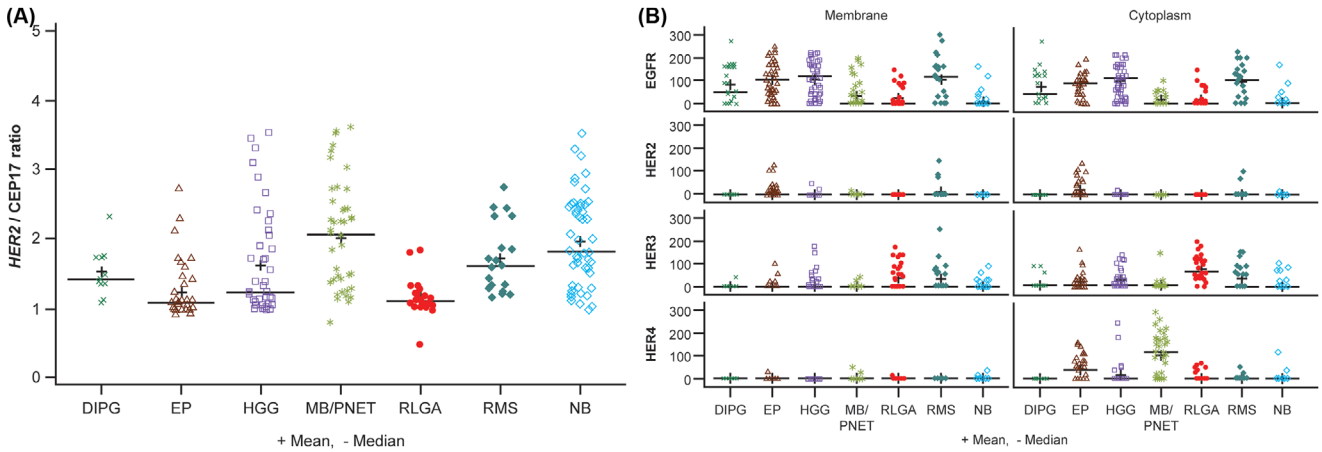

FIGURE 1 Investigation of ErbB family member gene amplification and protein expression for the seven investigated tumor types. (A) Distribution of *HER2*/CEP17 ratio. Amplification of *HER2* in pediatric tumor samples ($n = 297$) was determined using DDISH. *HER2* DDISH status was defined as positive 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),[36] 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).[37]

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,

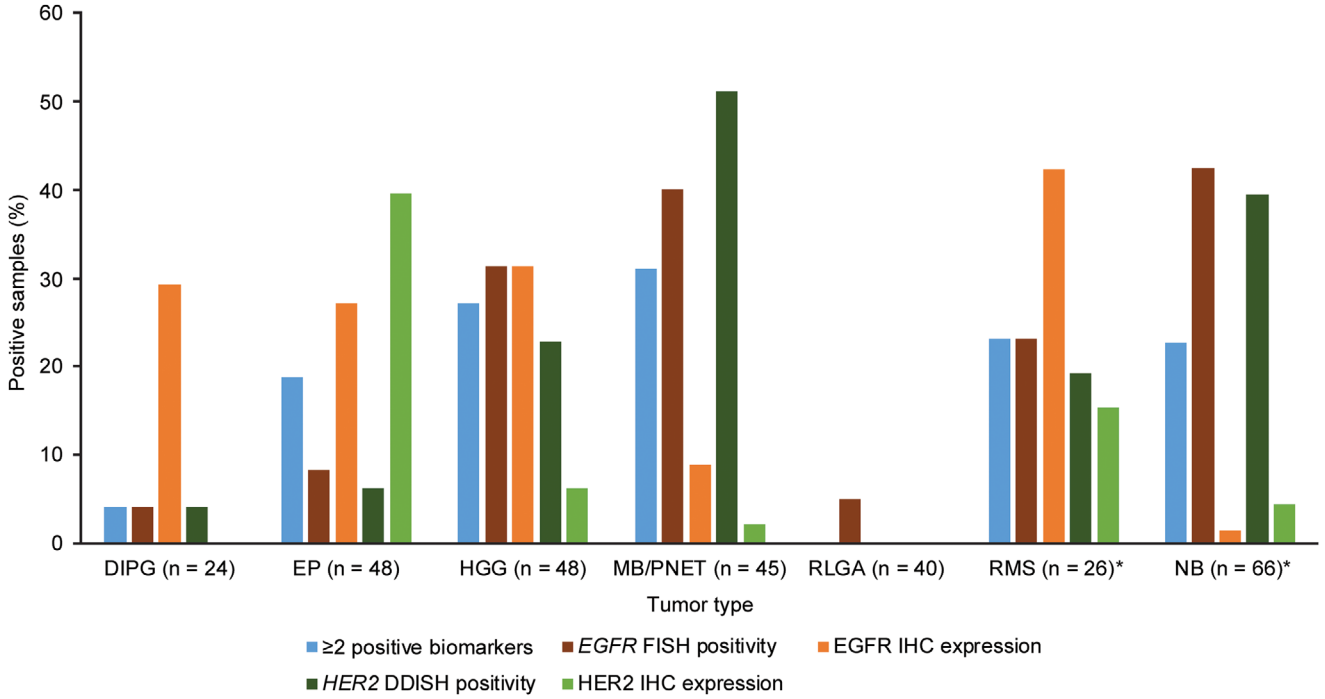

FIGURE 2 Prevalence of the investigated molecular markers and percentage of patients exhibiting positivity for greater than or equal to two markers. Prevalence of investigated molecular markers was determined based on the following criteria: *EGFR* FISH positivity: EGFR/Cen7 ≥2.0; ≥10% of cells with ≥15 copies; ≥40% of cells with greater than or equal to four copies; or gene cluster in ≥10% of cells; *HER2* DDISH positivity: HER2/CEP17 ≥2.0; EGFR IHC overexpression: *H*-score >150 (membrane staining); and HER2 IHC overexpression: *H*-score >0 (membrane staining). *Forty-two untreated and 24 treated; three patients provided two measurements; only the first (untreated) measurement was used. †Sixteen untreated and 10 treated; four patients provided two measurements; only the first (untreated) measurement was used. Cen7, centromeric region of chromosome 7; DDISH, dual-hapten in situ hybridization; DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymoma; *H*-score, Hirsch-score; HER2, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization; HGG, high-grade gliomas; IHC, immunohistochemistry; MB/PNET, medulloblastoma/primitive neuroectodermal tumor; NB, neuroblastomas; RLGA, recurrent low-grade astrocytoma; RMS, rhabdomyosarcoma

with 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 DIPG samples, although the one patient with two positive biomarkers showed *EGFR* FISH and *HER2* DDISH positivity, neither of which were observed in other samples. *EGFR* FISH and *HER2* DDISH positivity were 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

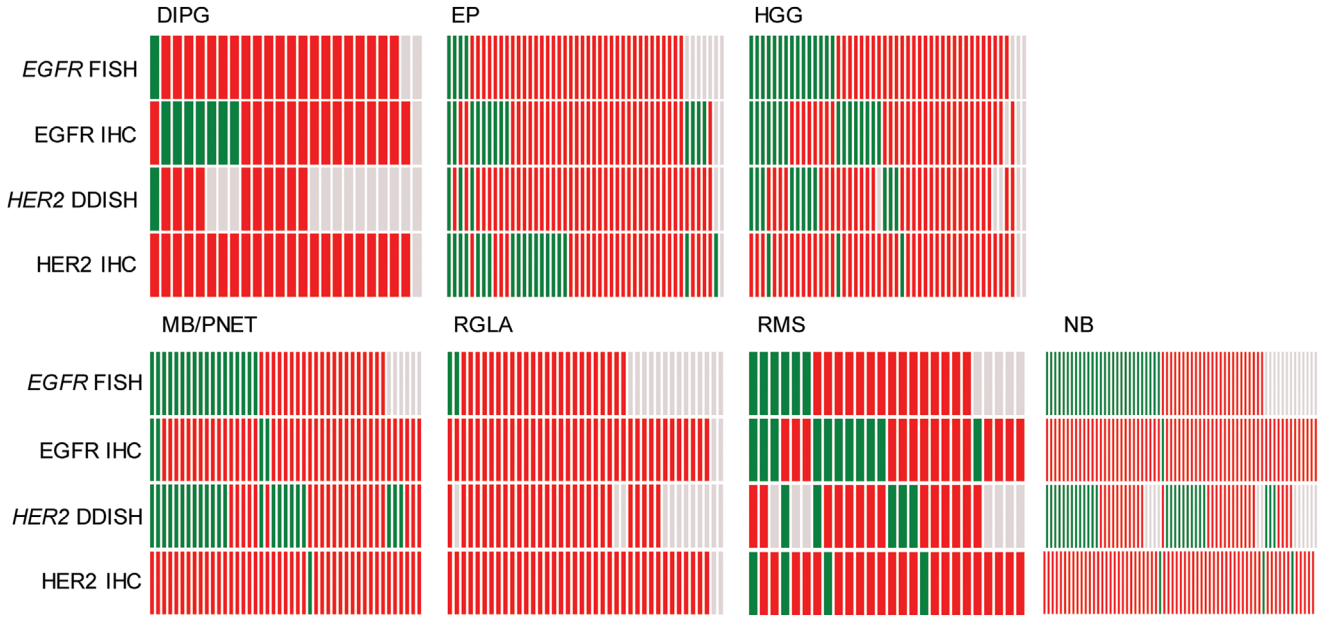

FIGURE 3 Heatmap showing the co-occurrence of the selected markers among individual patients with each of the seven investigated tumor types. Green shading indicates positive; red shading, negative; grey shading, missing. DDISH, dual-hapten in situ hybridization; DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymoma; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; HGG, high-grade gliomas; IHC, immunohistochemistry; MB/PNET, medulloblastoma/primitive neuroectodermal tumor; NB, neuroblastomas; RLGA, recurrent low-grade astrocytoma; RMS, rhabdomyosarcoma

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.[41] 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.[27,29] The changes in the 2016 CNS WHO also altered the classification of several tumor types. While our samples

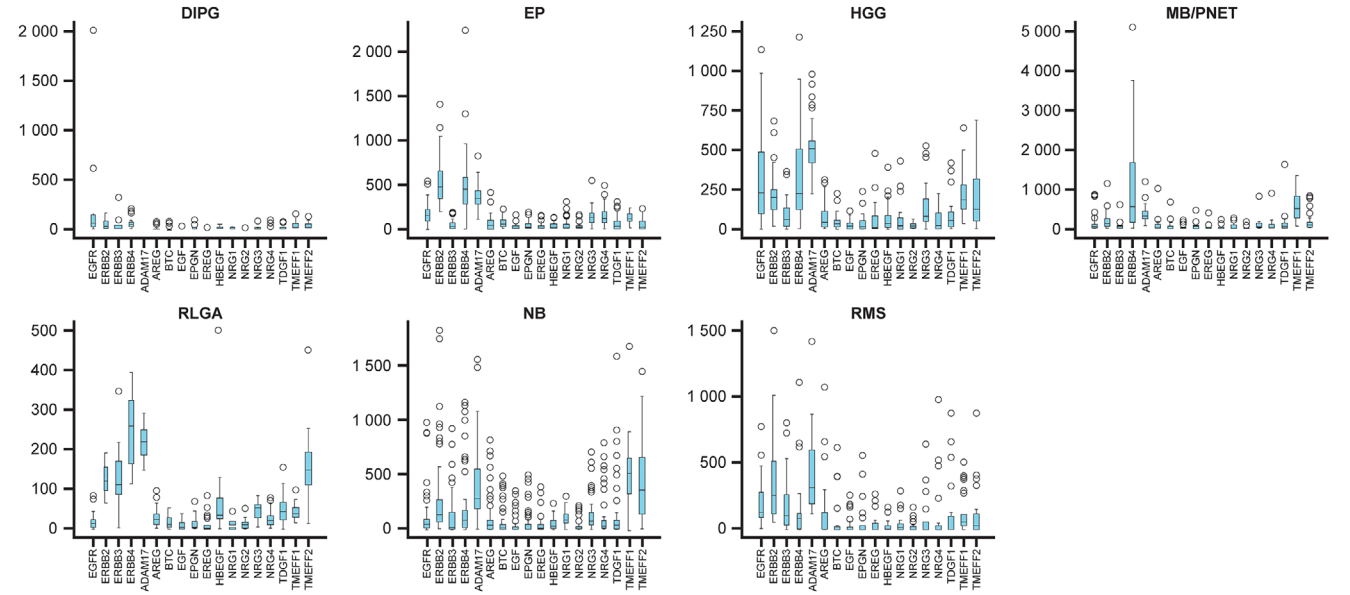

FIGURE 4 Distribution of normalized mRNA expression in each tumor type. Normalized mRNA expression counts using NanoString are plotted in box plots for the ErbB receptors and their ligands. Each box represents the 25th–75th percentiles of the distribution, and the central line indicates the median. The 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,[42–44] 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.[43,45] 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 with those overexpressing only EGFR[46,47]; NSCLC tumors overexpressing both EGFR and HER2 are also more responsive to EGFR tyrosine kinase inhibitors.[46] 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.[48–51] 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.[52,53]

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.[50] 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.[54,55]

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,[18]

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[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.[56,57] Preliminary evidence indicates that such patients could potentially benefit from EGFR targeted agent/temozolomide-based combination regimens.[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.[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 RLGA 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.[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.[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.

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CONFLICT OF INTEREST

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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 data 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.

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SUPPORTING INFORMATION

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

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