Detection of protein targets associated with survival in malignant pediatric brain tumors

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Abstract

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Background: Brain tumors are the leading cause of pediatric cancer death. Specific protein detection by immunohistochemistry may predict patient survival and identify opportunities for targeted therapy. The purpose of this study was to evaluate the prevalence of selected protein markers to prioritize targets for further therapeutic study.

Procedure: We studied a single institution cohort of pediatric patients who had pathologic diagnosis of malignant brain tumors at Seattle Children's Hospital between the years 2000-2011. EGFR, ERBB2, pS6, KIT, pERK, and PDGFRA were evaluated in tissue microarrays (TMA) by immunohistochemical staining. Clinical data were obtained from the electronic medical record. Survival was analyzed using the Kaplan-Meier method, log-rank test and multivariable Cox regression.

Results: One hundred thirty eight patients met inclusion criteria. Sixty-one patients with samples obtained at diagnosis were included in TMA studies including medulloblastoma (n=28), ependymoma (n=17), high grade glioma (=10) and supratentorial primitive neuroectodermal tumor (n=6). Thirteen of these patients had paired tumor samples analyzed from the time of relapse. The majority (89%) of tumors obtained at diagnosis and 100% of tumors at relapse were positive for at least one of the six proteins studied. Five-year survival was 20% for subjects with EGFR positive tumors, compared to 81% for EGFR negative tumors ($p<0.001$); 62% and 100% for PDGFRA positive and negative tumors ($p=0.04$); and 56% and 89% for pS6 positive and negative tumors ($p=0.02$), but not associated with ERBB2, pERK or KIT ($p>0.05$). The associations between positive status and poor survival persisted after adjustment for tumor histology, age and extent of resection.

Conclusions: Potential therapeutic targets were detected by immunohistochemistry in the majority of newly diagnosed malignant pediatric brain tumors. EGFR, PDGFRA and pS6 positivity at diagnosis were each found to be associated with poor survival.

INTRODUCTION:

Brain tumors are the most common solid tumor and leading cause of cancer death in the pediatric population.[1] Contemporary therapy for malignant tumors including maximal surgical resection, radiation, and chemotherapy is curative for many children.[2-5] The maximal benefit from increasing the intensity of cytotoxic therapy has likely been reached.[5-9] Current therapy adversely affects pediatric growth and development and results in considerable long-term endocrine, functional and neurocognitive toxicity.[10, 11] Pediatric brain tumor case-fatality is approximately 30%, and no new cytotoxic agent studied in the past decade has improved long-term survival.[12-19] The next generation of clinical trials in pediatric brain tumors will aim to selectively incorporate novel targeted therapeutics.

In recent decades, there has been an explosion in novel therapeutic drug development. Over 100 anti-neoplastic drugs have been FDA approved, and many of these new therapeutics target specific proteins and cellular pathways.[20, 21] The presence of protein as measured by immunohistochemical testing may be used to predict tumor response to these targeted agents. For example, EGFR expression in pulmonary adenocarcinoma predicts response to the EGFR inhibitor erlotinib,[22] and ERBB2 expression in breast cancer predicts response to trastuzumab or lapatinib.[23]

Using a single-institution cohort of clinically well-characterized pediatric brain tumors, investigated a focused panel of proteins chosen based on the availability of targeted therapeutic agents for which pediatric safety testing had already been completed.[24-31] To this end, EGFR, ERBB2, pS6, KIT, pERK, and PDGFRA were chosen for study. Pediatric brain tumors have previously been described to express some of these proteins, although the reported prevalence of expression is quite variable,[32] and the prognostic significance of protein expression has not been established. Proteins associated with poor survival represent targets which warrant prioritization for further therapeutic development in the pediatric population.

METHODS:

Study subjects and participation:

This study included subjects less than 22 years of age at the time of brain tumor diagnosis, who underwent neurosurgical resection of brain tumor leading to diagnosis of medulloblastoma, ependymoma,

high-grade glioma (HGG) or supratentorial primitive neuroectodermal tumor (sPNET) at Seattle Children's Hospital between January 1, 2000 and April 1, 2011. Due to limited tissue availability, subjects with primary spinal tumors or pineal region tumors were not included in this study, nor were subjects with any other histological diagnosis. Routine clinical care of pediatric patients diagnosed with brain tumor includes follow-up at minimum three-month intervals for the first two years from diagnosis, then at six-month interval until 5 years from diagnosis, then yearly until adulthood (age >21). Follow-up for vital status was assessed from the electronic medical records and Washington State Tumor Registry, which includes cross-referencing of national death index. Samples included in the tissue microarray were obtained from subjects whose legal guardians had given written informed consent to have tumor banked and clinical information collected for the general purpose of research. This specific study received further approval from the Seattle Children's Institutional Review Board to obtain clinical information for all patients diagnosed during the study time period, including subjects with and without research tissue banked.

Tumor biology studies:

Archived formalin-fixed paraffin-embedded tissue was used to create tissue microarrays (TMA) for each of four tumor types: Tumors from initial diagnosis included 28 medulloblastoma, 17 ependymoma, 10 high-grade glioma, and 6 supratentorial primitive neuroectodermal tumor (sPNET). In addition, 13 paired tumors obtained at the time of relapse included 2 medulloblastoma, 8 ependymoma, 1 high-grade glioma and 2 sPNET. Each tumor was sampled in duplicate or triplicate. Microarrays were constructed using Arraymold Kit B (IHC World, Woodstock, MD) with 1.5 mm diameter tissue cores.

Four-µm-thick paraffin sections of each TMA were stained using a Ventana Benchmark Stainer (Tucson, AZ). Sections were incubated with primary antibodies in the following concentrations: EGFR, 1:100 (Invitrogen, Carslbad, CA); ERBB2, 1:1000 (Dako, Carpinteria, CA); KIT, 1: 2000 (Dako, Carpinteria, CA); pERK, 1:50 (Cell Signaling Technology, Danvers, MA); PDGFRA, 1:100 (Santa Cruz Biotechnology Inc., Dallas, TX); and pS6, 1:150 (Cell Signaling Technology, Danvers, MA) diluted in phosphate-buffered solution (PBS). Slides were incubated with biotinylated secondary antibodies, followed by incubation with the streptavidin and biotinylated peroxidase complex. Sections were counterstained with hematoxylin and mounted.

Data Analysis:

The following clinical variables were collected from the electronic medical records: date of diagnosis, age at diagnosis, gender, histologic diagnosis, extent of resection, survival status, and either date of death or date of last clinical follow-up. Data regarding race and ethnicity were not obtained due to limited availability in the medical record. The date of diagnostic surgery was considered to be the date of diagnosis for all subjects. Age was analyzed as a binary variable with a cutoff value of 3 years since a radiation-sparing treatment strategy was employed for children under the age of 3 during the study period. It is the routine clinical practice of the Seattle Children's Brain Tumor Clinic to see patients for MRI and clinic visit at a minimum frequency of every three months until two years from date of diagnosis, then every six months until five years from diagnosis, then yearly until ten years from diagnosis.

Two pathologists (BC and ER) independently interpreted immunohistochemical results and consensus was reached regarding any discrepancies. Six protein markers (EGFR, ERBB2, KIT, PDGFRA, pERK and pS6) were initially scored between 0 and 4+ and then further rated as binary variables (positive/negative). Example of scoring is shown in figure 1A.

The scoring system used for antibodies was as follows: 0, absent staining in tumor cells; 1+, <10% of cells; 2+, 10-50% of cells; 3+, 50-90% of cells; and 4+, >90% of cells. For pERK, only the proportion of cells with nuclear staining was reported. For ERBB2, the 0-3+ breast cancer scoring system was used,[33] modified to include consideration of cytoplasmic as well as membranous staining. Cutoff points for each antibody were determined prior to survival analysis based on visual appearance of each antibody leading to pathologist confidence in tumor staining over background, and prevalence of expression levels between 0 and 4+ across all tumors tested. A score of 1+ or greater was considered positive for EGFR, ERBB2, KIT and PDGFRA. A score of 2+ or greater was considered positive for pERK and pS6. In cases where duplicate tumor samples had differential results, the highest area of positivity was used.

Survival analysis was conducted using the Kaplan-Meier method. Patients who were alive were censored at the time of last clinical follow-up as documented in the medical record. The log-rank test was used to evaluate the significance of associations between survival and each clinical and biological variable described above. Stepwise Cox regression was used to conduct multivariable analysis. Hazard of death

associated with each biological marker was presented with adjustment for tumor histology, age and extent of resection. STATA software, version 11.0, was used to perform all analysis.

RESULTS:

One hundred thirty-eight patients were diagnosed with sPNET (n=12), medulloblastoma (n=63), ependymoma (n=31), or high-grade glioma (n=32) within the study timeframe. Demographic factors for all patients, and according to whether tissue was included in biology studies are detailed in Table 1. The median age at diagnosis was 8.6 years (range 0-21.7) and 63% of patients were male. Eighty-two patients were living and 56 patients were deceased at the time of last follow-up. Median time between diagnosis and death was 18 months for deceased patients. Median follow-up was 49 months for the entire cohort, 70 months for patients who remained alive at the time of last follow-up. There were 10 patients who were lost to follow-up within two years from diagnosis, six of these with documentation of transfer of care to other centers, and four who were lost to follow-up without documentation of reason.

Biology studies were performed on a subset of 61 patients. Tumor tissue obtained from initial diagnostic sample was evaluated for all 61 patients, and paired tumor tissue from relapse was available for 13 of these patients. Compared to the 77 patients who did not have tumor obtained for banking and biology studies, patients with tumors included in the biology studies tended to be younger, were more likely to have had gross total tumor resection, and had higher survival (72% 5 year survival for patients with samples included in biology studies versus 49% 5 year survival without tumor biology samples included).

Fifty-four (89%) of 61 patients with biology testing by TMA had at least one positive marker. The percent of positive tumors was 16% for EGFR, 25% for ERBB2, 31% for KIT, 73% for PDGFRA, 43% for pERK and 54% for pS6. Each marker was positive in a subset of tumors of each histologic type, with the exception of ERBB2, which was negative in all high-grade glioma tumors; and EGFR, which was negative in all ependymoma tumors. Details of the prevalence of each marker within histologic subtype are provided in supplementary table 1. There were only seven primary tumors that did not express any of the study markers, all of which were medulloblastoma.

Five-year survival for all 138 patients was 59% (95% CI 50-67%), 70% for medulloblastoma (95% CI 56-80%), 79% for ependymoma (95% CI 59-90%), 42% for sPNET (95% CI 15-67%), and 27%

for HGG (95% CI 13-43%). In addition to tumor histology, survival was associated with extent of resection, EGFR, PDGFRA and pS6 (log rank $p<0.05$, figure 2). EGFR positivity was the variable associated with the greatest difference in survival, with 5-year survival 81% for patients with EGFR negative tumors (95% CI 67-90%) versus 20% for patients with EGFR positive tumors (95% CI 1-55%, $p<0.001$). Five-year survival was not associated with gender (59% for both males and females, $p=0.75$), age at diagnosis (65% if < 3 years vs. 57% if > 3 years, $p=0.93$), year of diagnosis (64% for prior to 2/1/2005 vs. 54% for after 2/1/2005, $p=0.18$), ERBB2 positivity (75% vs. 68% for ERBB2 negative, $p=0.91$), KIT positivity (62% versus 76% for KIT negative, $p=0.31$), or pERK positivity (77% versus 67% for pERK negative, $p=0.28$).

Multivariable analysis was conducted to evaluate the independence of clinical and biological variables. The Hazard of death for each immunohistochemical marker is shown in table II. EGFR, PDGFRA and pS6 positivity remained independently associated with survival when adjusted for tumor type, age and extent of resection (Table II).

Considering exclusively the three markers associated with decreased survival (EGFR, PDGFRA and pS6), we evaluated the association between co-expression of multiple markers and survival. Survival was associated with the number of markers expressed, with 5-year survival of 100% if all three markers were negative (n=10 tumors), 85%, 68% and 13% if one (n=23), two (n=20) or three (n=8) markers were positive ($p<0.001$). One patient with all three markers negative at diagnosis experienced late (>5 years) relapse and death. Tumor sample obtained at the time of relapse was found to be positive for PDGFRA in this case. All eight patients with three markers positive at diagnosis were deceased at the time of this analysis ($p<0.001$). In multivariable analysis adjusting for tumor type, patient age and extent of resection, the number of positive markers remained independently associated with survival. The chance of death was 5.3 times greater (95% CI 2.1-13.4) for each unit increase in number of positive markers between 0 and 3 ($p<0.001$).

In addition to tumors obtained from initial diagnosis, 13 paired samples obtained at the time of relapse were evaluated. An example of marked change in EGFR expression at diagnosis and relapse is shown in figure 1B. All relapsed tumors expressed at least one marker. While most marker expression was the same at primary diagnosis and relapse, all 13 tumors had at least one change in immunoreactivity.

Eleven of 13 expressed a marker at relapse that was negative in the primary tumor sample obtained at diagnosis, whereas seven of 13 were positive for a marker in the primary tumor that was negative at relapse. Details of each marker positivity at initial diagnosis and relapse for the 13 paired tumors are presented in supplementary table II.

DISCUSSION:

In this study, 89% of malignant pediatric brain tumors expressed at least one of only six protein targets studied, with 84% of all patients at diagnosis and 100% of patients who ultimately died of disease expressing at least one of three proteins (EGFR, PDGFRA or pS6) associated with poor survival. The targets in this study were selected primarily based on practical factors, specifically the availability of FDA approved inhibitors which have already undergone initial safety testing in the pediatric cancer population, examples of which are detailed in Table III.[24-28, 30, 31, 34] No conclusions regarding the effect of pathway inhibition or the efficacy of currently available targeted agents can be made from this study since patients studied here were not treated with targeted agents.

We found a proportion of positive tumors for most markers across the four tumor types studied. While EGFR was rarely positive (in only 3 medulloblastoma, 3 high-grade glioma, 4 sPNET), it was associated with decreased survival across tumor types. Multivariable analysis suggests that EGFR is not merely a marker of histologic type (high-grade glioma), but independently associated with decreased survival.

While EGFR positivity was rare, PDGFRA was positive in most (44/60) tumors. In the case of PDGFRA, the rare negative tumors (11 medulloblastoma and 5 ependymoma) had an excellent survival of 100% at five years, an association also unlikely to be due to chance ($p<0.05$).

One methodological point that should be emphasized regarding this study in comparison to others[22, 23, 32] is the cutoff points for marker positivity selected *a priori* were intentionally low. We hypothesized that low-level expression at diagnosis could represent a biologically important subset of tumor population represented at higher level at the time of relapse such as the tumor shown in figure 1b. Our evaluation of the few paired relapse samples suggests that examination of additional tissue obtained at the time of relapse may be essential to evaluation of target expression and appropriate therapeutic selection.

Limitations of this study include the small sample size, particularly limiting the power to further explore multivariable analyses and the ability to precisely estimate the magnitude of effect of each marker. The use of TMA rather than whole slides may have decreased the sensitivity of detection of markers in cases of heterogeneous expression. We attempted to address this by sampling multiple areas for each tumor. The convenience cohort (the subset of patients who donated tissue for research) used in the biology studies may be a potential source of bias. The higher survival in the group of patients with tissue included in the tissue microarray can be at least partially explained by the higher proportion of patients with tumors on the TMA who had gross total resection.

Pediatric brain tumors represent a group of rare diseases. Large genome-based unbiased approaches have facilitated the categorization of malignant pediatric tumors by biologic subtypes.[36, 37] This presents a practical challenge for clinical trial development, as rare tumors are made even rarer if specific biologic subsets are to be studied. New therapeutic agents may be better evaluated by defining patient subgroups according to pathway activation. It will be important to develop companion diagnostic studies along with novel agents. Due to practicality, efficiency and widespread availability, immunohistochemistry remains an important and useful adjunct in clinical practice even in the era of genomic medicine.[38]

We have demonstrated that EGFR, PDGFRA, and pS6 are positive by immunohistochemical assessment in a subset of malignant pediatric brain tumors, and positivity is associated with poor survival, with co-expression predicting a dismal outcome. Further studies are warranted to define the functional interactions between these pathways in pediatric brain tumors, evaluate whether these pathways correlate with subgroups as defined by gene expression, and evaluate the effect of pathway inhibitors in appropriately selected populations.

TABLES AND FIGURES:

Table I: The clinical characteristics of all patients, and according to whether tumor was included on tissue microarray for immunohistochemical analysis. sPNET: supratentorial primitive neuroectodermal tumor, TMA: tissue microarray, GTR: gross total resection, including near-total resection, STR: subtotal resection, including biopsy only.

Table II: Survival according to marker positivity, adjusted for tumor type, patient age and extent of resection.

Table III: Examples of targeted therapeutic agents with pediatric safety experience. All drugs listed are FDA approved for at least one indication in the adults. Imatinib and Everolimus are also FDA approved for a specific pediatric indication.

Table SI: Positivity of each marker by tumor type. sPNET: supratentorial primitive neuroectodermal tumor. Few missing data points were due to technical constraints of specific TMA slides (5 patients without data for ERBB2, 1 patient for each PDGFRA and pERK).

Table SII: Comparison of positivity between initial diagnosis and relapse. 13 patients had tumor samples evaluated at initial diagnosis and relapse. All 13 pairs were evaluable for KIT, PDGFRA, pERK and pS6. 12 pairs were evaluable for EGFR, and 11 pairs evaluable for ERBB2.

Figure 1: Representative images of tumor samples on the tissue microarray a) showing a range of pS6 expression as reported using the 1-4+ scoring system based on proportion of positive tumor cells, and b) differential EGFR expression between initial diagnostic biopsy sample (left panel) and a later sample obtained from the same patient at the time of relapse (right panel).

Figure 2: Kaplan Meier survival curves according to clinical factors (a-c) and marker positivity (d-f). a) 5 year survival was 79% for 31 ependymoma, 70% for 63 medulloblastoma, 42% for 12 sPNET, and 27% for

32 high-grade glioma ($p<0.001$). b) 5 year survival was 57% for 112 children who were greater than 3 years old at diagnosis versus 65% for 26 children less than 3 years old at diagnosis ($p=0.92$). c) 5 year survival was 69% for 92 children with surgical gross total resection versus 38% for 46 children without gross total resection ($p<0.001$). d) 5 year survival was 20% for 10 EGFR negative tumors versus 81% for 51 EGFR positive tumors ($p<0.001$). e) 5 year survival was 100% for 16 PDGFRA negative tumors versus 62% for 44 PDGFRA positive tumors ($p=0.04$). f) 5 year survival was 89% for 28 pS6 negative tumors versus 56% for 33 pS6 positive tumors ($p=0.02$).

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