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UBE4B Levels Are Correlated with Clinical Outcomes in Neuroblastoma Patients and with Altered Neuroblastoma Cell Proliferation and Sensitivity to EGFR Inhibitors

Peter E. Zage, MD, PhD[1,2,3,o], **Natalie Sirisaengtaksin**[4,o], **Yin Liu, PhD**[4,5], **Monica Gireud**[4], **Brandon S. Brown**[4], **Shana Palla**[6], **Kristen N. Richards**[7], **Dennis P.M. Hughes, MD, PhD**[7], and **Andrew J. Bean, PhD**[4,7]

[1] Department of Pediatrics, Section of Hematology-Oncology, Baylor College of Medicine, Houston, TX

[2] Texas Children's Cancer and Hematology Centers, Baylor College of Medicine, Houston, TX

[3] Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX

[4] Department of Neurobiology and Anatomy, The University of Texas Health Science Center at Houston, Houston, TX

[5] Department of Biomedical Engineering, University of Texas at Austin, Austin, TX

[6] Division of Quantitative Sciences, The University of Texas M.D. Anderson Cancer Center, Houston, TX

[7] Division of Pediatrics, The University of Texas M.D. Anderson Cancer Center, Houston, TX

Abstract

Background—The *UBE4B* gene, located on chromosome 1p36, encodes a ubiquitin ligase that interacts with Hrs, a protein involved in EGFR trafficking, suggesting a link between EGFR trafficking and neuroblastoma pathogenesis. We have analyzed the roles of UBE4B in the outcomes of neuroblastoma patients and in neuroblastoma tumor cell proliferation, EGFR trafficking, and response to EGFR inhibition.

Methods—We examined the association of *UBE4B* expression with neuroblastoma patient survival using available microarray datasets. We measured UBE4B and EGFR protein levels in patient tumor samples and EGFR degradation rates in neuroblastoma cell lines and analyzed the effects of UBE4B on neuroblastoma tumor cell growth. The effects of the EGFR inhibitor cetuximab were examined in neuroblastoma cells expressing wild-type and mutant UBE4B.

Results—Low *UBE4B* gene expression is associated with poor outcomes in patients with neuroblastoma. UBE4B overexpression reduced neuroblastoma tumor cell proliferation, and UBE4B expression was inversely related to EGFR expression in patient tumor samples. EGFR degradation rates correlated with cellular UBE4B levels. Enhanced expression of catalytically active UBE4B resulted in reduced sensitivity to EGFR inhibition.

Conclusions—We have demonstrated associations between *UBE4B* expression and neuroblastoma patient outcomes and between UBE4B and EGFR expression in neuroblastoma tumor samples. Moreover, levels of UBE4B influenced neuroblastoma tumor cell proliferation, EGFR

Correspondence to: Peter Zage, MD, PhD, Department of Pediatrics, Section of Hematology-Oncology, Baylor College of Medicine, 1102 Bates, Suite 1220, Houston, TX 77030, Tel: 832-824-4615, Fax: 832-825-4039, zage@bcm.tmc.edu.
[o]These authors contributed equally to this manuscript

degradation, and response to EGFR inhibition. These results suggest UBE4B-mediated GFR trafficking may contribute to the poor prognosis of neuroblastoma tumors with 1p36 deletions, and that UBE4B expression may be a marker that can predict responses of neuroblastoma tumors to treatment.

Keywords

neuroblastoma; UBE4B; Hrs; EGFR; cetuximab

Introduction

Overall survival rates for children with high-risk neuroblastoma remain approximately 30% with current treatment regimens[1,2]. Cases of high-risk neuroblastoma are associated with frequent relapses and tumors that are resistant to treatment[1,2], and children with refractory or recurrent neuroblastoma have poor responses to salvage therapy and very poor survival rates[3,4]. A better understanding of the mechanisms of neuroblastoma tumorigenesis will provide improved treatment options for these children.

Growth factors and their receptors play a critical role in the survival, growth, and differentiation of normal and malignant cells, and aberrant GFR expression and function are common features of neuroblastoma tumors. Ubiquitin-mediated sorting of activated growth factor receptors (GFRs) for lysosomal degradation can regulate the amplitude and kinetics of GFR signaling[5,6], and defects in the pathways for intracellular GFR sorting and degradation leading to altered GFR expression and activity have been identified in many cancer types[7]. Appropriate trafficking of GFRs is critical for control of unrestrained signaling that is characteristic of transformed cells[5,6,8,9].

Deletion of the chromosome 1p36 region is found in approximately one-third of neuroblastoma tumors and is associated with high-risk tumor features and a poor prognosis[10-12]. The *UBE4B* gene is located in the 1p36 region and encodes an E3/E4 ubiquitin ligase[13,14]. Recently, Martinsson and colleagues identified a mutation in the *UBE4B* gene in the tumor of a patient with neuroblastoma with a fatal outcome[15]. The expression of UBE4B was shown to be markedly diminished in a cohort of high-stage/poor-outcome tumors compared to low-stage/favorable-outcome tumors[15,16], and *UBE4B* was therefore suggested to be a candidate tumor suppressor gene[15].

We have observed that UBE4B interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a key regulator of the endosomal machinery for GFR trafficking, and that the UBE4B-Hrs interaction is critical for appropriate GFR trafficking and degradation[14]. Therefore, loss of UBE4B expression and function may be associated with aberrant GFR expression in neuroblastoma tumors. However, the roles of UBE4B in GFR trafficking in neuroblastoma tumor cells and of UBE4B-mediated GFR trafficking in the outcomes of neuroblastoma patients are unknown.

We hypothesized that UBE4B would be associated with neuroblastoma patient outcomes and neuroblastoma tumorigenesis. To explore the roles of UBE4B expression and function in the pathogenesis of neuroblastoma, we evaluated the association of *UBE4B* gene expression with neuroblastoma patient outcomes, and we investigated the roles of UBE4B in neuroblastoma tumor cell growth, in the regulation of EGFR expression, and in the responses of neuroblastoma tumor cells to EGFR inhibition. The results of these studies suggest UBE4B-mediated GFR trafficking may contribute to the poor prognosis of neuroblastoma tumors with 1p36 deletions and that UBE4B expression may be a marker that can predict responses of neuroblastoma tumors to treatment.

Methods

Cell culture

The characteristics of neuroblastoma cell lines SMS-KCNR, LA1-55N, NGP, SH-EP, SK-N-AS, SK-N-SH, and SH-SY5Y used in this study have been previously described[17-20] and were generously provided by Susan Cohn (The University of Chicago Children's Hospital, Chicago, IL) and John Maris (Children's Hospital of Philadelphia, Philadelphia, PA). Cell lines used in these studies were authenticated by DNA profiling. Neuroblastoma cell lines were grown at 37°C in 5% CO_2 in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (USB, Minneapolis, MN), L-glutamine, sodium pyruvate, non-essential amino acids, and penicillin/streptomycin (Sigma Chemical Company, St. Louis, MO).

Neuroblastoma Patient Tumor Samples and Data

The patient tumor samples employed in these studies were provided by the Children's Oncology Group Neuroblastoma Biology Committee and the Biopathology Center in Columbus, OH, as previously described[21].

We obtained microarray analysis results of neuroblastoma patient tumor samples from the National Cancer Institute (NCI) Oncogenomics Data Center Section (http://pob.abcc.ncicrf.gov/cgi-bin/JK) from the databases ";Neuroblastoma Prognosis Database," "Neuroblastoma Prognosis Database - Oberthuer Lab," and "Exon Array Neuroblastoma Database." These databases include patients with all tumor stages and included information regarding *N-myc* gene amplification status, and all patient data from these databases was included in our analysis. Additional studies were performed as detailed below using data from the "Neuroblastoma Prognosis Database - Seeger's Lab" dataset.

Cell proliferation assay

SK-N-AS neuroblastoma tumor cells were infected with lentivirus constructs expressing GFP alone, wild-type UBE4B, or UBE4BP1140A, a mutant isoform with absent ubiquitin ligase activity[13], as previously described[22]. 4,000 SK-N-AS neuroblastoma cells were plated in 96-well plates in 100 μL of culture media with serum or serum-free media supplemented with 50 ng/mL EGF. At baseline and after 24, 48, and 72 hours of incubation at 37°C, 10 μL of WST-1 reagent (Roche, Indianapolis, IN) was added to each well in each plate and absorbance at 450 nm was determined.

To evaluate proliferation in response to cetuximab, SK-N-AS cells were plated as above. After assessing baseline proliferation on day 1, existing media was discarded for all other plates, and 100μL of media supplemented with cetuximab (400nM, 1μM, or 4μM; provided by the M.D. Anderson Cancer Center pharmacy) was added to each well. Proliferation was assessed at 24, 48, and 72 hours as above and calculated at each time point as the percent change in absorbance compared to baseline absorbance. ANOVA analysis and post hoc Tukey tests were performed to establish significance.

EGFR degradation assay

For each experiment, cells at 80% confluency were incubated for 2 hours in DMEM supplemented with 1% BSA (Medium A). Media was then aspirated and cold medium A with EGF (50 ng/mL) was added. Plates were maintained at 4°C for one hour. Cells were washed three times with cold medium A and either kept on ice (0 min) or incubated with warm medium A for either 30 or 60 minutes. Following incubations, cells were rinsed with cold PBS and scraped into 1.5 mL PBS. Samples were centrifuged at 2,500g and cell pellets were resuspended in 30μL of lysis buffer (1μL each of 10mM leupeptin, 1μg/μL pepstatin, 0.3mM aprotinin,

and 1.74μg/μL PMSF per 100 μL of M-PER (Pierce, Rockford, IL)). Samples were incubated for 1 hour at 4°C and then centrifuged at 15,000g.

Supernatant was collected and 50 μg of protein from each sample was separated by SDS-PAGE and transferred to nitrocellulose membranes as previously described[21,23]. Membranes were incubated in blocking buffer (5% nonfat dry milk in PBS) and then with a rabbit polyclonal anti-EGFR antibody (ABR, Golden, CO; 1:1000 dilution). Proteins were visualized with ECL and bands were quantified using ImageJ (NIH, ver 1.42). ANOVA analysis and post hoc Tukey tests were performed to establish significance.

Expression of UBE4B and EGFR in neuroblastoma tumor samples

Patient samples were homogenized using a Mikrodismembrator S (Sartorius, San Diego, CA), then incubated for 10 minutes in protein lysis buffer containing a phosphatase inhibitor (Sigma) and protease inhibitor cocktail (Roche). Lysates were centrifuged at 15,000g and supernatants collected. 30μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes as above.

Membranes were blocked in 5% milk in TBST (100mM tris, 1.5M NaCl, 0.1% Tween-20, pH 7.9) and probed with antibodies directed against UBE4B, EGFR, and actin. Proteins were visualized and quantified as above. Spearman's correlation analysis was performed on levels of UBE4B and EGFR.

Statistical analysis

Using gene expression results from the databases described above, patients were divided into high and low *UBE4B* gene expression groups by median-centered log2 ratios as detailed in the NCI Oncogenomics database website. The high-expression group contained 177 patients and the low-expression group contained 176 patients. Kaplan-Meier survival curves were plotted using the open-source statistical packages in R (http://www.r-project.org). We compared the survival curves by *UBE4B* gene expression groups using the log-rank test to examine the association between expression and patient survival outcomes in the whole cohort and in patients with stage 4 neuroblastoma and in those with stage 1, 2, 3, or 4S neuroblastoma. The Fisher-exact test was performed to determine whether the proportion of tumors with *N-myc* gene amplification was associated with *UBE4B* gene expression. For separate analyses of the Neuroblastoma Prognosis Database - Seeger's Lab dataset, the median-centered log2 ratios for each of seven probes for *UBE4B* were standardized to have a mean of zero and unit length by location and scale transformation. The standardized log2 ratios were averaged across all seven probes. Based on the average of log2 ratios for each patient, 50 and 52 patients were in low and high *UBE4B* expression groups, respectively.

Results

Association of *UBE4B* Gene Expression with Neuroblastoma Patient Outcomes

Due to the location of the *UBE4B* gene and its low expression in advanced neuroblastoma tumors[16], loss of UBE4B expression and function may be critical for neuroblastoma tumorigenesis. Therefore, we evaluated the association of *UBE4B* gene expression with neuroblastoma patient outcomes, using results from microarray analyses of 353 neuroblastoma tumors obtained from the NCI as detailed above. Low expression of *UBE4B* was significantly associated with poor neuroblastoma patient outcomes ($p<0.00001$, Fig. 1A) and with worse outcomes in patients with either stage 4 disease ($p=0.007$) or with stage 1, 2, 3, and 4S disease ($p<0.00001$, Fig. 1B). Additionally, amplification of the *N-myc* oncogene was only seen in 2 of 177 patients with high *UBE4B* gene expression, as opposed to 43 of 176 with low *UBE4B* expression ($p<0.00001$, Fig. 2A), indicating an association of *N-myc* gene amplification with

low *UBE4B* expression. These results demonstrate that *UBE4B* expression is associated with the outcomes of neuroblastoma patients.

In order to evaluate the prognostic relevance of *UBE4B* expression in patients without *N-myc* gene amplification, we separately analyzed the outcomes of the 308 patients without *N-myc* gene amplification in these databases. Low *UBE4B* expression was significantly associated with lower survival rates in these neuroblastoma patients ($p<0.00001$, Fig. 2B), suggesting that *UBE4B* expression is prognostically relevant in this subset of patients. For further confirmation of the prognostic value of *UBE4B* gene expression in neuroblastoma patients without *N-myc* amplification, we independently evaluated the association of *UBE4B* expression with patient survival in the Neuroblastoma Prognosis Database - Seeger Lab, a database with results from patients with neuroblastoma without *N-myc* gene amplification[24]. In these 102 patients, low *UBE4B* expression was also significantly associated with lower survival rates ($p<0.05$, Fig. 2C), confirming the association of *UBE4B* gene expression with overall survival in neuroblastoma patients without *N-myc* amplification.

Effects of UBE4B on Neuroblastoma Cell Proliferation

We then explored the mechanisms by which UBE4B might affect neuroblastoma patient outcomes. To investigate the effects of UBE4B on neuroblastoma tumor cell proliferation, SK-N-AS neuroblastoma tumor cells infected with lentivirus constructs expressing GFP alone, wild-type UBE4B, or UBE4BP1140A, a mutant isoform with absent ubiquitin ligase activity[13], were grown in DMEM supplemented with either 10% fetal bovine serum or 50ng/mL EGF. Wild-type UBE4B expression, but not expression of the UBE4BP1140A mutant, resulted in reduced neuroblastoma tumor cell proliferation under both conditions ($p<0.05$, Fig. 3), suggesting that UBE4B expression and, more specifically, UBE4B ubiquitin ligase function, can reduce neuroblastoma tumor cell proliferation.

Association of UBE4B and EGFR Protein Levels

Aberrant GFR expression and function have been shown to be important in the pathogenesis of neuroblastoma[25-30]. We have observed that the UBE4B protein interacts with Hrs, a key regulator of EGFR endosomal trafficking and degradation[31-34], and that the Hrs-UBE4B interaction is critical for efficient GFR trafficking and degradation[14], suggesting that UBE4B expression and function may be associated with EGFR expression in neuroblastoma tumor cells. Therefore, we evaluated a cohort of 20 neuroblastoma tumor samples from the Children's Oncology Group (COG) for expression of UBE4B and EGFR proteins by quantitative Western blotting. UBE4B protein expression was inversely correlated with EGFR protein expression in these patient samples (Spearman's correlation $Rho = -0.4545$; $p<0.05$, Fig. 4), suggesting a possible role for UBE4B in regulation of EGFR levels in neuroblastoma tumors.

UBE4B-mediated EGFR Degradation in Neuroblastoma Cells

In order to explore the mechanisms by which UBE4B regulates EGFR expression in neuroblastoma tumor cells, we investigated the expression levels of UBE4B and the degradation rates of EGFR in neuroblastoma cell lines. Total UBE4B protein levels were measured in a panel of neuroblastoma tumor cells at baseline by quantitative Western blotting (Fig. 5A). In order to measure EGFR degradation rates in neuroblastoma tumor cells, cells with high (LA1-55N, SH-SY5Y) or low (SK-N-SH, SK-N-AS) UBE4B levels were briefly starved in serum-free media and then stimulated with EGF. Total cellular EGFR levels were then measured over the subsequent 60 minutes. Neuroblastoma tumor cells with higher levels of UBE4B had faster rates of EGFR degradation compared to those with lower UBE4B levels (Fig. 5B-E), suggesting that UBE4B affects EGFR levels through regulation of EGFR degradation.

Association of UBE4B with Neuroblastoma Tumor Cell Response to EGFR Inhibition

The association of UBE4B levels with EGFR degradation and with total EGFR levels suggested that UBE4B levels may also be associated with the response of neuroblastoma tumor cells to EGFR inhibitors. To investigate this possibility, neuroblastoma tumor cells were treated with a range of concentrations of cetuximab, an FDA-approved inhibitory antibody to EGFR. Treatment with 4μM cetuximab resulted in significant inhibition of neuroblastoma tumor cell proliferation (Fig. 6A, $p<0.01$). To investigate the role of UBE4B in the cetuximab-mediated inhibition of cell proliferation, parental neuroblastoma tumor cells or cells infected with lentivirus vectors expressing wild-type UBE4B, the UBE4BP1140A mutant, or GFP were grown as above and treated with 4μM cetuximab. Overexpression of UBE4B, but not the ligase-deficient mutant, resulted in loss of the cetuximab-mediated inhibition of proliferation (Fig. 6B), suggesting a role for UBE4B expression and its ubiquitin ligase activity in the responses of neuroblastoma tumor cells to EGFR inhibition.

Discussion

Children with high-risk neuroblastoma have extremely poor outcomes, and additional understanding of the pathways involved in neuroblastoma pathogenesis will assist in the development of improved therapies in the future. The *UBE4B* gene is located in chromosome 1p36, a commonly deleted chromosomal region in neuroblastoma tumors, and UBE4B is involved in the trafficking of GFRs that ultimately results in their lysosomal degradation, suggesting a role for UBE4B-mediated GFR trafficking in neuroblastoma pathogenesis. Our results demonstrate an association of *UBE4B* gene expression with neuroblastoma patient outcomes and of UBE4B function with neuroblastoma tumor cell proliferation and response to EGFR inhibitors.

A large number of genes located within the 1p36 region have been proposed as tumor suppressors in neuroblastoma and other tumors, including *CHD5*[35], *CAMTA1*[36,37], miRNA-34a[38,39], *CASZ1*[40], *KIF1B*[41,42], and the *HES* gene family[43]. Studies are ongoing to determine the role of UBE4B expression and function in other tumors with 1p36 aberrations. Alterations of the chromosome 1p36 region are found in a wide range of tumors, including brain tumors, breast cancers, and leukemias, among others, and are often associated with aggressive tumor features and a poor prognosis[44]. In medulloblastoma tumor cells, UBE4B has been shown to induce degradation of p53[45], suggesting a potential role for UBE4B in tumorigenesis independent of its effect on EGFR trafficking, and studies are ongoing to investigate the role of UBE4B in the trafficking of other transmembrane proteins in neuroblastoma.

EGF-induced signaling has been shown to be important in the pathogenesis of a variety of malignancies, and EGFR inhibitors are currently in use for treatment of a variety of malignancies[46]. EGFR expression has been documented in neuroblastoma tumors and cell lines, and inhibition of EGFR signaling has been shown to reduce growth of neuroblastoma tumor cells *in vitro* and *in vivo*[21,30,47]. Cetuximab has been shown to be effective against a variety of tumor cell lines, with IC50 values between 10nM and 10μM[48]. Therefore, our identified effective dose of 4μM fits within the observed cancer cell sensitivity range.

Previous studies have demonstrated that EGFR expression is associated with chemotherapy resistance in neuroblastoma cells and that chemotherapy is synergistic with EGFR inhibitors[47,49]. Additional studies have identified EGFR ubiquitination and degradation as the mechanism for colon cancer cell resistance to cetuximab[50]. The etiology of this EGFR-associated resistance and the mechanisms responsible for this association of EGFR with resistance to chemotherapy in neuroblastoma tumor cells are unknown. We have demonstrated that overexpression of UBE4B resulted in reduced sensitivity to cetuximab (Fig. 6), implicating

UBE4B and the GFR trafficking pathway in the sensitivity of neuroblastoma tumor cells to treatment with EGFR inhibitors. These data also suggest that UBE4B-mediated GFR trafficking, through regulation of EGFR expression, may be responsible for the association of EGFR expression with treatment resistance. However, the mechanisms by which alterations in UBE4B expression and GFR trafficking might affect neuroblastoma tumor cell responses to therapy are unknown, and a better understanding of these mechanisms will likely lead to improved efficacy of current therapy and allow for identification of patient populations that might be more or less sensitive to GFR inhibitor treatment.

The results of these studies provide important information about the previously undescribed roles and mechanisms of GFR trafficking in neuroblastoma tumor cells and of the UBE4B protein in neuroblastoma tumor proliferation and response to treatment. Our results have identified a previously undiscovered link between GFR trafficking and neuroblastoma pathogenesis, and the correlation between UBE4B and EGFR levels we describe in neuroblastoma tumors may be relevant for multiple tumor types with known 1p36 alterations where EGFR inhibition is therapeutically effective. We have demonstrated associations between *UBE4B* expression and neuroblastoma patient outcomes, and we have shown that levels of UBE4B influence neuroblastoma tumor cell proliferation, EGFR trafficking, and response to EGFR inhibition. Our results suggest that inhibition of UBE4B-mediated GFR degradation may contribute to the poor prognosis of neuroblastoma tumors with 1p36 deletions, and that UBE4B expression may be a marker that can predict responses of neuroblastoma tumors to treatment. The roles of UBE4B levels as a prognostic marker for treatment response and of UBE4B-mediated EGFR degradation as a mechanism for resistance to EGFR inhibition are currently being explored. These results demonstrate a novel pathway with the potential for targeted drug development and a novel mechanism for tumor treatment sensitivity that may provide a prognostic marker to identify a patient population most likely to respond to inhibition of GFRs.

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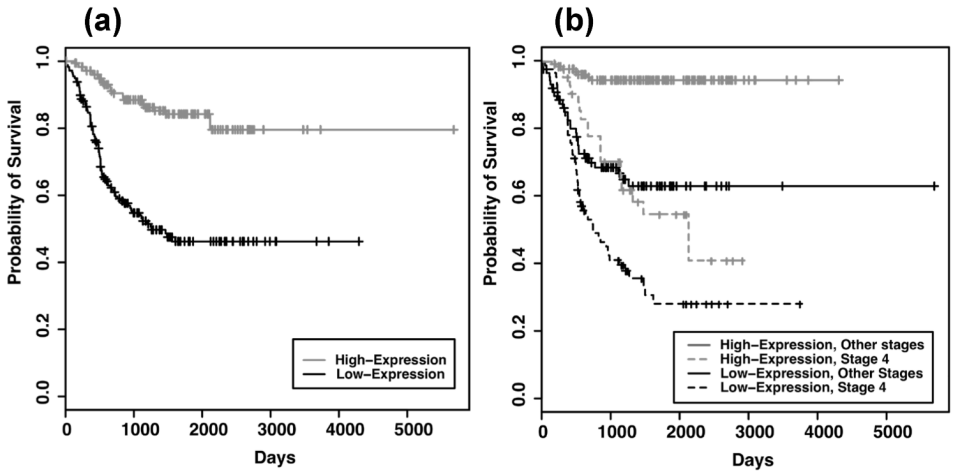

Figure 1. Outcomes of neuroblastoma patients based on UBE4B expression

The NCI Oncogenomics gene expression databases were evaluated for outcomes of neuroblastoma patients and Kaplan-Meier survival curves were generated. (A) Estimated overall survival for neuroblastoma patients with tumors having high *UBE4B* gene expression (grey, n=177) and low *UBE4B* gene expression (black, n=176) (log-rank test, p=3.02e-13) (B) Estimated overall survival for neuroblastoma patients with stage 4 tumors with high (grey, dashed; n=40) and low (black, dashed; n=87) *UBE4B* gene expression (log-rank test, p=0.007) and for patients with tumors of all other stages with high (grey, solid; n=137) and low (black, solid; n=89) *UBE4B* gene expression (log-rank test, p=7.59e−09).

(a)

	N-myc amplified	*N-myc* non-amplified
High UBE4B	2	175
Low UBE4B	43	133

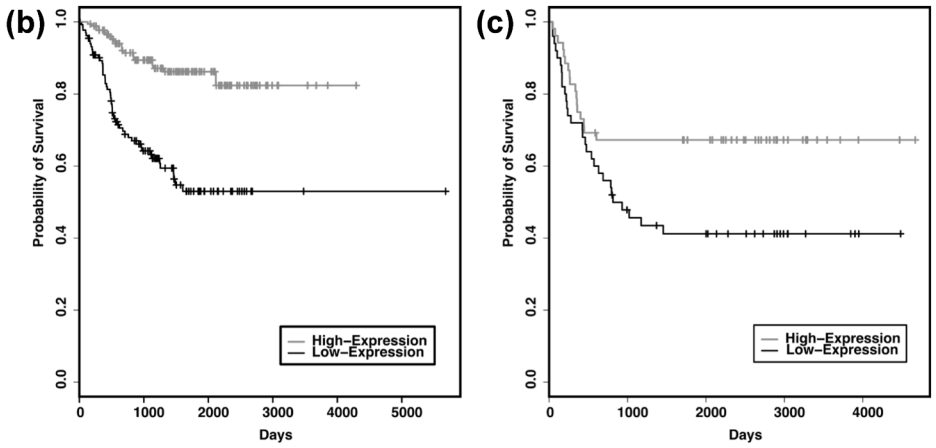

Figure 2. Outcomes of neuroblastoma patients with non-amplified N-myc based on UBE4B expression

The NCI Oncogenomics gene expression databases were evaluated for outcomes of neuroblastoma patients and Kaplan-Meier survival curves were generated. (A) Numbers of patients with high or low *UBE4B* gene expression compared to presence or absence of *N-myc* gene amplification (Fisher-exact test, p=2.87e-14). (B) Estimated overall survival for neuroblastoma patients with *N-myc* non-amplified tumors having high *UBE4B* gene expression (grey, n=175) and low *UBE4B* gene expression (black, n=133) (log-rank test, p=9.0e-9). (C) Estimated overall survival for neuroblastoma patients from the Neuroblastoma Prognosis Database-Seeger Lab dataset with tumors having high *UBE4B* gene expression (grey, n=52) and low *UBE4B* gene expression (black, n=50) (log-rank test, p=0.021).

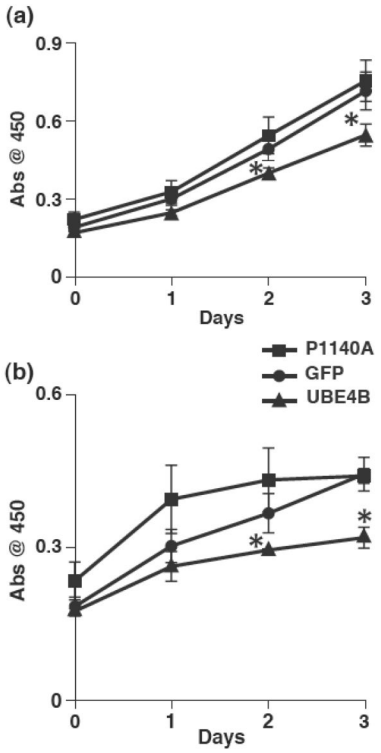

Figure 3. UBE4B effects on growth of neuroblastoma cells

SK-N-AS neuroblastoma cells expressing either GFP (circles), wild type UBE4B (triangles), or UBE4BP1140A (squares) were grown in media containing 10% FBS (A) or 50ng/ml EGF (B). Cell growth was assessed using the WST-1 based spectrophotometric assay. *p<0.05.

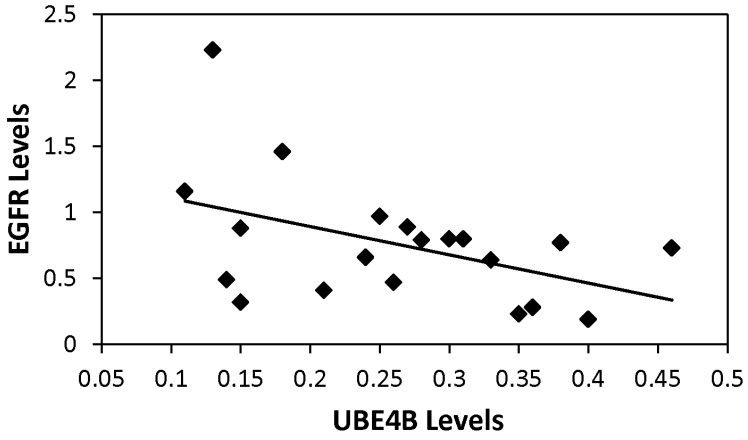

Figure 4. UBE4B and EGFR expression levels in tumors

Human neuroblastoma tumor samples were obtained from the Children's Oncology Group (COG). Tumor sample lysates were separated on SDS-PAGE gels. Immunoblots for UBE4B and EGFR were performed and relative protein levels were determined. A Spearman's correlation was performed with UBE4B and EGFR protein levels as shown (Rho = −0.4545; $p<0.05$).

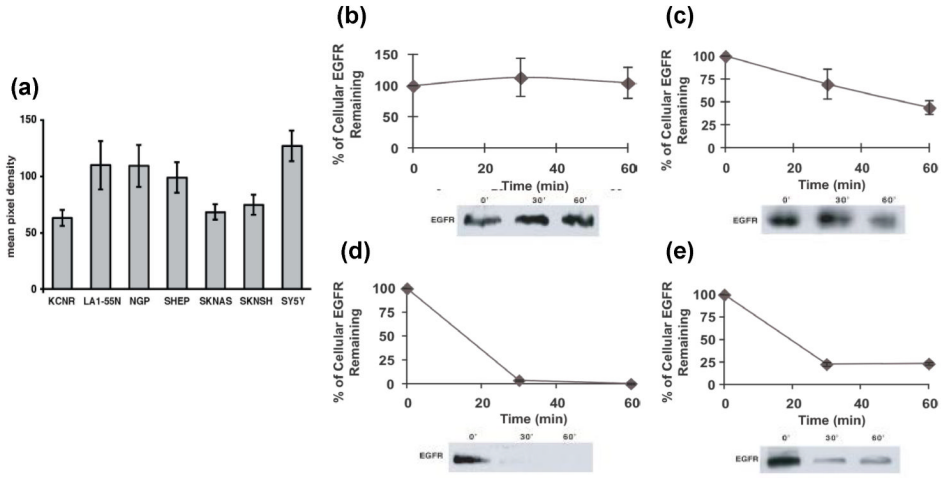

Figure 5. UBE4B expression and EGFR degradation rates in neuroblastoma cell lines

A. UBE4B protein levels were determined in neuroblastoma cell lines by immunoblot analysis of cell lysates. SK-N-SH (B), SK-N-AS (C), SH-SY5Y (D), and LA1-55N (E) neuroblastoma tumor cells were then starved and stimulated with EGF for 0, 30, or 60 minutes. Cell lysates were examined by immunoblotting for EGFR levels and levels were plotted over time.

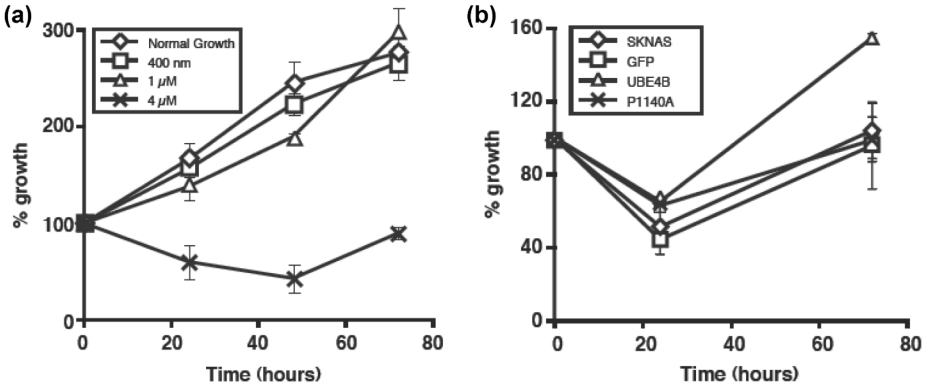

Figure 6. UBE4B effects on growth of neuroblastoma cells and response to EGFR inhibitors

A. SK-N-AS neuroblastoma tumor cells were treated with escalating concentrations of cetuximab. Cell growth was assessed at the indicated time points using the WST-1 based spectrophotometric assay. B. Parental SK-N-AS cells and SK-N-AS cells expressing either GFP (control), UBE4B, or UBE4BP1140A were grown in the presence of 4μM cetuximab (Erbitux) with growth assessed as above.