

# UBE4B Levels are Correlated With Clinical Outcomes in Neuroblastoma Patients and With Altered Neuroblastoma Cell Proliferation and Sensitivity to Epidermal Growth Factor Receptor Inhibitors

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**BACKGROUND:** The *UBE4B* gene, which is located on chromosome 1p36, encodes a ubiquitin ligase that interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a protein involved in epidermal growth factor receptor (EGFR) trafficking, suggesting a link between EGFR trafficking and neuroblastoma pathogenesis. The authors analyzed the roles of UBE4B in the outcomes of patients with neuroblastoma and in neuroblastoma tumor cell proliferation, EGFR trafficking, and response to EGFR inhibition. **METHODS:** The association between *UBE4B* expression and the survival of patients with neuroblastoma was examined using available microarray data sets. UBE4B and EGFR protein levels were measured in patient tumor samples, EGFR degradation rates were measured in neuroblastoma cell lines, and the effects of UBE4B on neuroblastoma tumor cell growth were analyzed. The effects of the EGFR inhibitor cetuximab were examined in neuroblastoma cells that expressed 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 tumor samples. EGFR degradation rates correlated with cellular UBE4B levels. Enhanced expression of catalytically active UBE4B resulted in reduced sensitivity to EGFR inhibition. **CONCLUSIONS:** The current study demonstrates associations between *UBE4B* expression and the outcomes of patients with neuroblastoma and between UBE4B and EGFR expression in neuroblastoma tumor samples. Moreover, levels of UBE4B influence neuroblastoma tumor cell proliferation, EGFR degradation, and response to EGFR inhibition. These results suggest UBE4B-mediated growth factor receptor trafficking may contribute to the poor prognosis of patients who have neuroblastoma tumors with 1p36 deletions and that UBE4B expression may be a marker that can predict responses of neuroblastoma tumors to treatment. *Cancer* 2013;119:915-23. © 2012 American Cancer Society.

**KEYWORDS:** neuroblastoma, UBE4B, Hrs, epidermal growth factor receptor, EGFR, cetuximab.

## INTRODUCTION

Overall survival rates for children with high-risk neuroblastoma remain approximately 30% with current treatment regimens.<sup>1,2</sup> Cases of high-risk neuroblastoma are associated with frequent relapses and tumors that are resistant to treatment,<sup>1,2</sup> and children with refractory or recurrent neuroblastoma have poor responses to salvage therapy and very poor survival rates.<sup>3,4</sup> 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 growth factor receptor (GFR) expression and function are common features of neuroblastoma tumors. Ubiquitin-mediated sorting of activated GFRs for lysosomal degradation can regulate the amplitude and kinetics of GFR signaling,<sup>5,6</sup> 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.<sup>7</sup> Appropriate trafficking of GFRs is critical for control of unrestrained signaling that is characteristic of transformed cells.<sup>5,6,8,9</sup>

Deletions in the short arm of chromosome 1 (chromosome 1p36) are identified in approximately one-third of neuroblastoma tumors and are associated with high-risk tumor features and a poor prognosis.<sup>10-12</sup> The *UBE4B* gene is located

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in the 1p36 region and encodes an E3/E4 ubiquitin ligase.<sup>13,14</sup> Recently, Krona and colleagues identified a mutation in the *UBE4B* gene in the tumor from a patient with neuroblastoma with a fatal outcome.<sup>15</sup> 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<sup>15,16</sup>, and *UBE4B* was therefore suggested to be a candidate tumor suppressor gene.<sup>15</sup>

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.<sup>14</sup> 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 the outcomes of patients with neuroblastoma and with 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 epidermal growth factor receptor (EGFR) expression, and in the responses of neuroblastoma tumor cells to EGFR inhibition. The results of these studies suggest that UBE4B-mediated GFR trafficking may contribute to the poor prognosis of patients who have neuroblastoma tumors with 1p36 deletions and that UBE4B expression may be a marker that can predict responses of neuroblastoma tumors to treatment.

## MATERIALS AND METHODS

### Cell Culture

The characteristics of the 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 described previously<sup>17-20</sup> and were generously provided by Susan Cohn (The University of Chicago Children's Hospital, Chicago, Ill) 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<sub>2</sub> in RPMI-1640 (Invitrogen, Carlsbad, Calif) supplemented with 10% heat-inactivated fetal bovine serum (USB, Minneapolis,

Minn), 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 (COG) Neuroblastoma Biology Committee and the Biopathology Center in Columbus, Ohio, as previously described.<sup>21</sup>

We obtained microarray analysis results of neuroblastoma patient tumor samples from the National Cancer Institute (NCI) Oncogenomics Data Center Section (available at: <http://pob.abcc.ncicrf.gov/cgi-bin/JK>; accessed January 2012) 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 have 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 results from the "Neuroblastoma Prognosis Database-Seeger Lab" dataset.

### Cell Proliferation Assay

SK-N-AS neuroblastoma tumor cells were infected with lentivirus constructs expressing green fluorescent protein (GFP) alone, wild-type UBE4B, or a mutant UBE4B isoform with absent ubiquitin ligase activity (UBE4B<sub>P1140A</sub>),<sup>13</sup> as previously described.<sup>22</sup> Four thousand SK-N-AS neuroblastoma cells were plated in each well of 96-well plates in 100 µL of culture media with serum or with serum-free media supplemented with 50 ng/mL epidermal growth factor (EGF). At baseline and after 24, 48, and 72 hours of incubation at 37°C, 10 µL of WST-1 reagent (Roche, Indianapolis, Ind) was added to each well in each plate, and absorbance at 450 nm was determined.

To evaluate cell proliferation in response to cetuximab, SK-N-AS cells were plated as described above. After assessing baseline proliferation on day 1, existing media was discarded for all other plates, and 100 µL of media supplemented with cetuximab (400 nM, 1 µM, or 4 µM; provided by The University of Texas M. D. Anderson Cancer Center pharmacy) was added to each well. Proliferation was assessed at 24, 48, and 72 hours as described above and calculated at each time point as the percent change in absorbance compared to baseline absorbance. Analyses of variance analysis (ANOVA) 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 1 hour. Cells were washed 3 times with cold medium A and were either kept on ice (0 minutes) or incubated with warm medium A for either 30 or 60 minutes. Following incubations, cells were rinsed with cold phosphate-buffered saline (PBS) and scraped into 1.5 mL PBS. Samples were centrifuged at  $\times 2500g$ , and cell pellets were resuspended in 30  $\mu$ L lysis buffer (1  $\mu$ L each of 10 mM leupeptin, 1  $\mu$ g/ $\mu$ L pepstatin, 0.3 mM aprotinin, and 1.74  $\mu$ g/ $\mu$ L phenylmethanesulfonylfluoride [PMSF] per 100  $\mu$ L of M-PER [Pierce, Rockford, Ill]). The samples were incubated for 1 hour at 4°C and then centrifuged at  $\times 15,000g$ .

Supernatant was collected, and 50  $\mu$ g of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as previously described.<sup>21,23</sup> Membranes were incubated in blocking buffer (5% nonfat dry milk in PBS) and then with a rabbit polyclonal anti-EGFR antibody (ABR, Golden, Colo; 1:1000 dilution). Proteins were visualized with ECL, and bands were quantified using ImageJ (version 1.42; National Institutes of Health, Bethesda, Md). ANOVA 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 Mikrodis-membrator S (Sartorius, San Diego, Calif), then incubated for 10 minutes in protein lysis buffer containing a phosphatase inhibitor (Sigma Chemical Company) and protease inhibitor cocktail (Roche). Lysates were centrifuged at  $\times 15,000g$ , and supernatants were collected. 30  $\mu$ g of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes as described above.

The membranes were blocked in 5% milk in TBS-T (100 mM Tris; 1.5 M NaCl; 0.1% Tween-20, pH 7.9) and probed with antibodies directed against UBE4B, EGFR, and actin. Proteins were visualized and quantified as described 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

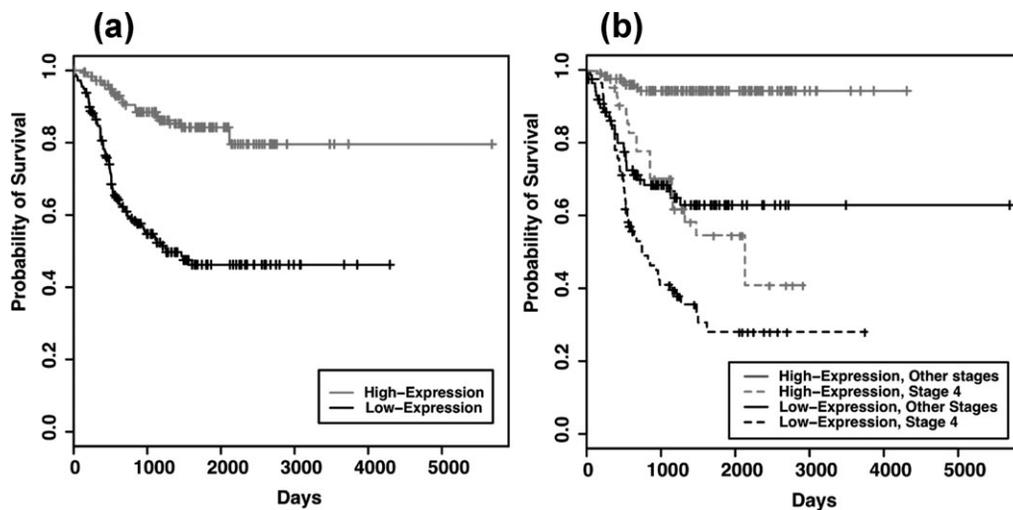
*UBE4B* gene expression groups by median-centered log<sub>2</sub> ratios as detailed on the NCI Oncogenomics database website. The high-expression group contained 177 patients, and the low-expression group contained 176 patients. Kaplan-Meier survival curves were plotted using the open-source statistical packages in R (R Foundation for Statistical Computing, Vienna, Austria; available at: <http://www.r-project.org>; [accessed January 2012]). We compared survival curves between the *UBE4B* gene expression groups using log-rank tests 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. Fisher's 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-Seegeer Lab dataset, the median-centered log<sub>2</sub> ratios for each of 7 probes for *UBE4B* were standardized to have a mean of zero and unit length by location and scale transformation. The standardized log<sub>2</sub> ratios were averaged across all 7 probes. Based on the average of log<sub>2</sub> ratios for each patient, 50 and 52 patients were in the 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,<sup>16</sup> 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 outcomes of patients with neuroblastoma ( $P < .00001$ ) (Fig. 1a) and with worse outcomes in patients with either stage 4 disease ( $P = .007$ ) or with stage 1, 2, 3, and 4S disease ( $P < .00001$ ) (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 ( $P < .00001$ ) (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 patients with neuroblastoma.

To evaluate the prognostic relevance of *UBE4B* expression in patients with neuroblastoma tumors without *N-myc* gene amplification, we separately analyzed the



**Figure 1.** Outcomes of patients with neuroblastoma based on *UBE4B* gene expression. The NCI Oncogenomics gene expression databases were evaluated for outcomes of patients with neuroblastoma, and Kaplan-Meier survival curves were generated. (a) Estimated overall survival for patients who have tumors with high *UBE4B* gene expression (gray;  $n = 177$ ) and low *UBE4B* gene expression (black;  $n = 176$ ; log-rank test;  $P = 3.02e-13$ ). (b) Estimated overall survival for patients with stage 4 neuroblastoma who have high (dashed gray;  $n = 40$ ) and low (dashed black;  $n = 87$ ) *UBE4B* gene expression (log-rank test;  $P = .007$ ) and for patients with tumors of all other stages with high (solid gray;  $n = 137$ ) and low (solid black;  $n = 89$ ) *UBE4B* gene expression (log-rank test;  $P = 7.59e-09$ ).

outcomes of the 308 patients with neuroblastoma without *N-myc* gene amplification in these databases. Low *UBE4B* expression was significantly associated with lower survival rates in these patients ( $P < .00001$ ) (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 patients who have neuroblastoma 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.<sup>24</sup> In these 102 patients, low *UBE4B* expression was also significantly associated with lower survival rates ( $P < .05$ ) (Fig. 2c), confirming the association of *UBE4B* gene expression with overall survival in patients who have neuroblastoma 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 *UBE4B*<sub>P1140A</sub>, a mutant isoform with absent ubiquitin ligase activity,<sup>13</sup> were grown in media supplemented with either 10% FBS or 50 ng/mL EGF. Wild-type *UBE4B* expression, but not expression

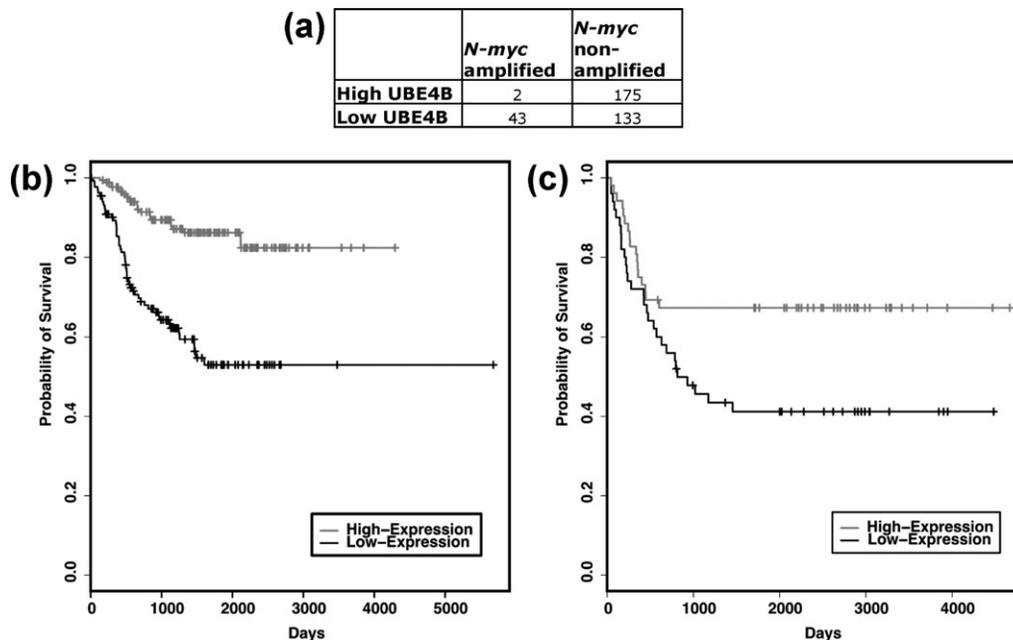
of the *UBE4B*<sub>P1140A</sub> mutant, resulted in reduced neuroblastoma tumor cell proliferation under both conditions ( $P < .05$ ) (Fig. 3), suggesting that *UBE4B* expression and, more specifically, *UBE4B* ubiquitin ligase function can reduce neuroblastoma tumor cell proliferation.

#### Association of *UBE4B* and EGFR Protein Levels

Aberrant GFR expression and function have been shown to be important in the pathogenesis of neuroblastoma.<sup>25-30</sup> We have observed that the *UBE4B* protein interacts with Hrs, a key regulator of EGFR endosomal trafficking and degradation,<sup>31-34</sup> and that the Hrs-*UBE4B* interaction is critical for efficient GFR trafficking and degradation,<sup>14</sup> 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 blot analysis. *UBE4B* protein expression in these patient samples (Spearman's correlation [ $\rho$ ] =  $-0.4545$ ;  $P < .05$ ) (Fig. 4), suggesting a possible role for *UBE4B* in the regulation of EGFR levels in neuroblastoma tumors.

#### *UBE4B*-Mediated EGFR Degradation in Neuroblastoma Cells

To explore the mechanisms by which *UBE4B* regulates EGFR expression in neuroblastoma tumor cells, we investigated the expression levels of *UBE4B* and the



**Figure 2.** Outcomes of patients with neuroblastoma who have *N-myc*-nonamplified tumors compared with *UBE4B* expression. The NCI Oncogenomics gene expression databases were evaluated for the outcomes of patients with neuroblastoma, and Kaplan-Meier survival curves were generated. (a) Numbers of patients with high or low *UBE4B* gene expression compared to the presence or absence of *N-myc* gene amplification (Fisher exact test;  $P=2.87\text{e-}14$ ). (b) Estimated overall survival for patients with *N-myc*-nonamplified neuroblastoma tumors and high *UBE4B* gene expression (gray;  $n=175$ ) or low *UBE4B* gene expression (black;  $n=133$ ; log-rank test,  $P=9.0\text{e-}9$ ). (c) Estimated overall survival for patients with neuroblastoma from the Neuroblastoma Prognosis Database-Seeger Lab dataset with tumors having high *UBE4B* gene expression (gray;  $n=52$ ) or low *UBE4B* gene expression (black;  $n=50$ ; log-rank test;  $P=.021$ ).

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 blot analysis (Fig. 5a). 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.

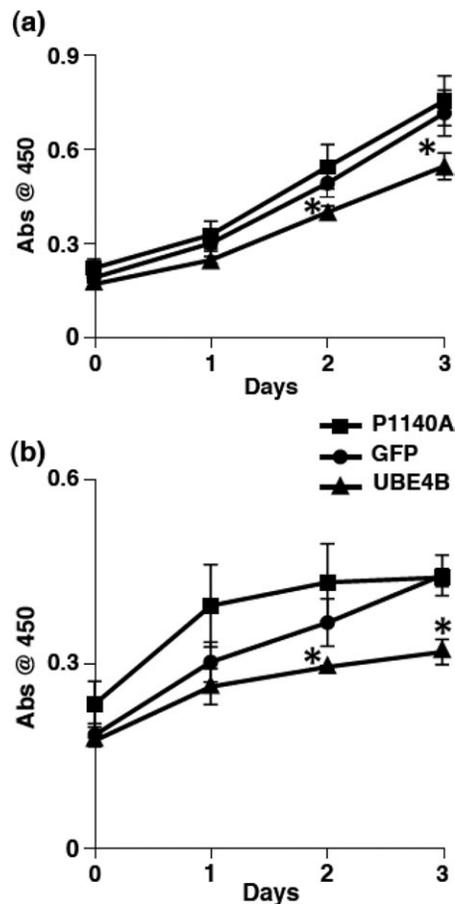
#### Association of UBE4B With Neuroblastoma Tumor Cell Response to EGFR Inhibition

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 4  $\mu\text{M}$

cetuximab resulted in significant inhibition of neuroblastoma tumor cell proliferation (Fig. 6a) ( $P < .01$ ). 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 UBE4B<sub>P1140A</sub> mutant, or GFP were grown as described above and treated with 4  $\mu\text{M}$  cetuximab. Overexpression of UBE4B, but not of 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 response of neuroblastoma tumor cells to EGFR inhibition.

#### DISCUSSION

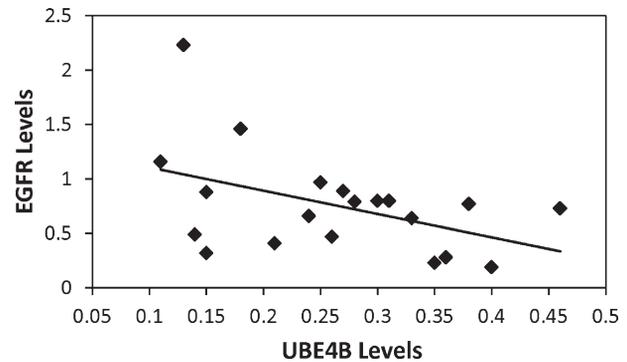
Children with high-risk neuroblastoma have extremely poor outcomes, and additional understanding of the pathways involved in neuroblastoma pathogenesis will assist in the development of improved therapies. The *UBE4B* gene is located in chromosome 1p36, a commonly deleted chromosomal region in neuroblastoma tumors, and UBE4B interacts with proteins involved in the trafficking of GFRs that ultimately results in their lysosomal



**Figure 3.** UBE4B effects on the growth of neuroblastoma cells. SK-N-AS neuroblastoma cells expressing either green fluorescent protein (GFP) (circles), wild-type UBE4B (triangles), or a mutant UBE4B isoform with absent ubiquitin ligase activity (UBE4B<sub>P1140A</sub>) (squares) were grown in media containing (a) 10% fetal bovine serum or (b) 50 ng/mL epidermal growth factor. Cell growth was assessed using the WST-1-based spectrophotometric assay. Abs @ 450 indicates absorbance at 450 nm; \* $P < .05$ .

degradation, suggesting a role for UBE4B-mediated GFR trafficking in neuroblastoma pathogenesis. Our results demonstrate an association between *UBE4B* gene expression and neuroblastoma patient outcomes and of UBE4B function with neuroblastoma tumor cell proliferation and response to EGFR inhibitors.

A number of genes located within the 1p36 region have been proposed as tumor suppressors in neuroblastoma and other tumors, including chromodomain helicase DNA binding protein 5 (*CHD5*),<sup>35</sup> calmodulin binding transcription activator 1 (*CAMTA1*),<sup>36,37</sup> microRNA-34a,<sup>38,39</sup> castor zinc finger 1 (*CASZ1*),<sup>40</sup> kinesin family member 1B (*KIF1B*),<sup>41,42</sup> and the hairy and enhancer of split-1 (*HES*) gene family.<sup>43</sup> Studies are ongoing to deter-

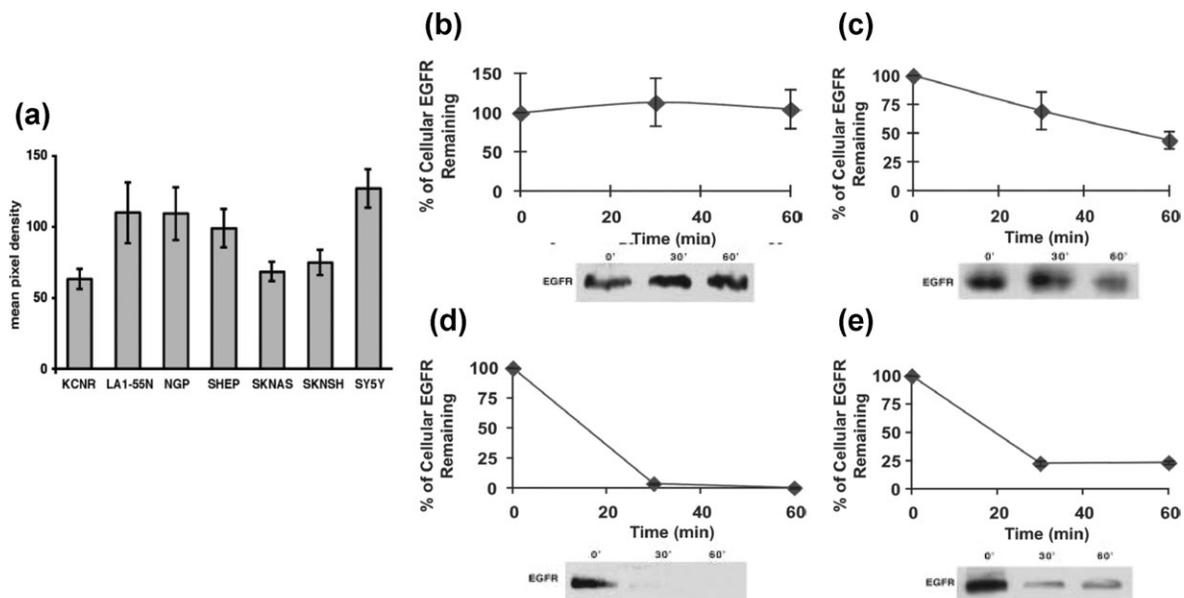


**Figure 4.** UBE4B and EGFR expression levels in neuroblastoma tumors. Human neuroblastoma tumor samples were obtained from the Children's Oncology Group (COG). Tumor sample lysates were separated on SDS-PAGE gels. Immunoblots for UBE4B and EGFR were performed, and relative protein levels were determined. Spearman's correlation analysis was performed with UBE4B and EGFR protein levels as shown ( $\rho = -0.4545$ ;  $P < .05$ ).

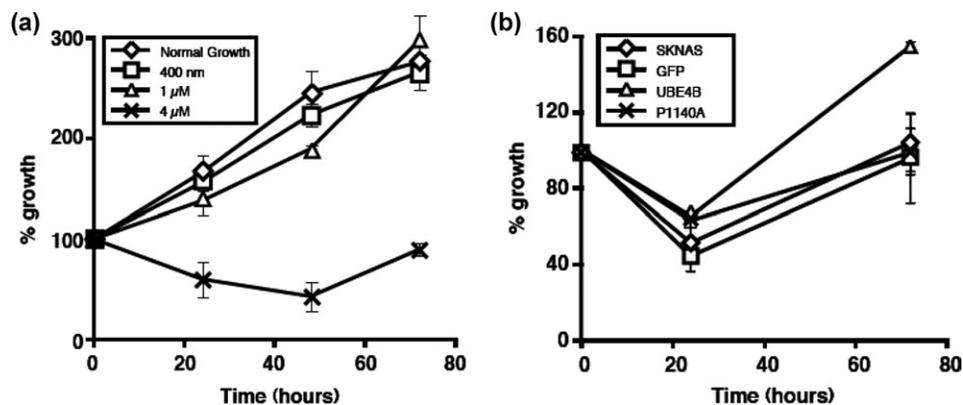
mine the role of UBE4B expression and function in other tumors with 1p36 aberrations. Alterations of the chromosome 1p36 region are found in a wide range of tumors, including brain tumors, breast cancers, and leukemias, and are often associated with aggressive tumor features and a poor prognosis.<sup>44</sup> In medulloblastoma tumor cells, UBE4B has been shown to induce degradation of p53,<sup>45</sup> 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 cancer treatment.<sup>46</sup> EGFR expression has been documented in neuroblastoma tumors and cell lines, and inhibition of EGFR signaling has been shown to reduce the growth of neuroblastoma tumor cells in vitro and in vivo.<sup>21,30,47</sup> Cetuximab has been shown to be effective against a variety of tumor cell lines, with 50% inhibitory concentration (IC<sub>50</sub>) values between 10 nM and 10  $\mu$ M.<sup>48</sup> Therefore, our identified effective dose of 4  $\mu$ M 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.<sup>47,49</sup> Additional studies have identified EGFR ubiquitination and degradation as a mechanism for colon cancer cell resistance to cetuximab.<sup>50</sup> The etiology of this EGFR-associated resistance and the



**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. (b) SK-N-SH, (c) SK-N-AS, (d) SH-SY5Y, and (e) LA1-55N neuroblastoma tumor cells were then starved and stimulated with epidermal growth factor (EGF) for 0, 30, or 60 minutes. Cell lysates were examined by immunoblot analysis for EGFR levels, and levels were plotted over time.



**Figure 6.** UBE4B effects on the growth of neuroblastoma cells and the 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 green fluorescent protein (GFP) (control), UBE4B, or a mutant UBE4B isoform with absent ubiquitin ligase activity (UBE4B<sub>P1140A</sub>) were grown in the presence of 4 μM cetuximab with growth assessed as described above.

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 resist-

ance. 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 cell 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 in which 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 for patients who have 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.

## FUNDING SOURCES

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## CONFLICT OF INTEREST DISCLOSURES

The authors have no conflicts of interest to disclose.

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