Mutant Epidermal Growth Factor Receptor (EGFRvIII) Contributes to Head and Neck Cancer Growth and Resistance to EGFR Targeting

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) is overexpressed in head and neck squamous cell carcinoma (HNSCC) where expression levels correlate with decreased survival. Therapies that block EGFR have shown limited efficacy in clinical trials and primarily when combined with standard therapy. The most common form of mutant EGFR (EGFRvIII) has been described in several cancers, chiefly glioblastoma. The present study was undertaken to determine the incidence of EGFRvIII expression in HNSCC and the biological consequences of EGFRvIII on tumor growth in response to EGFR targeting.

Experimental Design: Thirty-three HNSCC tumors were evaluated by immunostaining and reverse transcription-PCR for EGFRvIII expression. A representative HNSCC cell line was stably transfected with an EGFRvIII expression construct. EGFRvIII-expressing cells and vector-transfected controls were compared for growth rates in vitro and in vivo as well as chemotherapy-induced apoptosis and the consequences of EGFR inhibition using the chimeric monoclonal antibody C225/cetuximab/Erbitux.

Results: EGFRvIII expression was detected in 42% of HNSCC tumors where EGFRvIII was always found in conjunction with wild-type EGFR. HNSCC cells expressing EGFRvIII showed increased proliferation in vitro and increased tumor volumes in vivo compared with vector-transfected controls. Furthermore, EGFRvIII-transfected HNSCC cells showed decreased apoptosis in response to cisplatin and decreased growth inhibition following treatment with C225 compared with vector-transfected control cells.

Conclusions: EGFRvIII is expressed in HNSCC where it contributes to enhanced growth and resistance to targeting wild-type EGFR. The antitumor efficacy of EGFR targeting strategies may be enhanced by the addition of EGFRvIII-specific blockade.

Analysis of genetic and epigenetic alterations in human tumors has revealed potential molecular targets for cancer therapy. The epidermal growth factor receptor (EGFR) is a well-characterized proto-oncogene that is present in multiple cancers where it has been shown to promote tumor progression. EGFR is ubiquitously distributed on normal epithelial tissues and is overexpressed in several cancers, such as those of the breast, prostate, lung, and glioma (3). We reported previously that >95% of HNSCCs express elevated EGFR levels compared with levels in normal mucosa from patients without cancer (4). Further investigation showed that elevated EGFR expression levels in HNSCC serve as an independent indicator of poor prognosis and decreased overall survival (5, 6).

Strategies that target EGFR are actively under investigation for the treatment of HNSCC. EGFR-targeted therapies include monoclonal antibodies (mAb) that block the extracellular ligand-binding domain and tyrosine kinase inhibitors (TKI) that prevent activation of the cytoplasmic tyrosine kinase of EGFR. EGFR-specific TKIs and mAbs have shown great promise in cancer cell lines and animal models (7, 8). When combined with high-dose radiation in patients with locoregionally advanced HNSCC, the addition of C225 (EGFR-specific mAb) showed a statistically significant prolongation in overall...
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The presence of naturally occurring mutations of the EGFR gene in tumors may account for the limited clinical response to EGFR-targeted therapies. Various mutations of the EGFR gene have been described. However, the presence of mutant EGFR (EGFRvIII) and/or EGFRwt has not been systematically evaluated in HNSCC tumors before treatment with EGFR-targeted therapy. Recently, somatic mutations in the tyrosine kinase domain of the EGFR gene have been described in non–small cell lung carcinomas that are associated with increased sensitivity to EGFR-specific TKIs (13, 14). However, in HNSCC, the incidence of these mutations is low and varies according to ethnic origin (1% of Caucasians versus 7% of Asians with HNSCC; refs. 15, 16). A commonly described EGFR mutation is a truncation mutation, EGFR variant III (EGFRvIII). In gliomas, where it has been most extensively studied, EGFRvIII expression correlates with increased tumorigenicity in mouse models (17) and poor prognosis in the clinical setting (18). Moreover, the expression of EGFRvIII is unique to cancer. EGFRvIII has not been observed in normal tissue, but it has been detected in other malignancies, such as non–small cell lung carcinoma, breast cancer, and ovarian carcinoma (19–22). EGFRvIII harbors an in-frame deletion mutation of exons 2 to 7 spanning the extracellular ligand-binding domain. This deletion produces a truncated 150-kDa protein that is weakly constitutively phosphorylated in a ligand-independent manner (23–25). Ligand-independent activation of EGFRvIII may explain the relative inability of blocking mAbs to down-regulate this receptor.

The present study was undertaken to test the hypothesis that EGFRvIII is expressed in HNSCC and contributes to the tumor phenotype. We examined 33 HNSCC tumors for EGFRvIII and EGFRwt overexpression using reverse transcription-PCR (RT-PCR) and immunohistochemistry. We identified EGFRvIII expression in 42% of HNSCC tumors by immunostaining with an EGFRvIII-specific antibody and RT-PCR using primers specific for this mutant receptor. In addition, the expression of EGFRvIII was only detected in the presence of EGFRwt. Because tumors that express EGFRvIII do not retain EGFRvIII expression when grown in long-term tissue cultures, we stably transfected a HNSCC cell line with an EGFRvIII vector. In vitro and in vivo studies using EGFRvIII-expressing HNSCC cells showed a decreased response to the well-characterized EGFR mAb C225/cetuximab/Erbitux when compared with the parental cells, which overexpress only the EGFRwt. Taken together, these data suggest that EGFRvIII is expressed in HNSCC, where this naturally occurring EGFR mutation may contribute to the limited clinical response to EGFR-targeted therapy.

Materials and Methods

Cell lines, tumors, and reagents. C225/cetuximab/Erbitux was kindly provided by ImClone Systems, Inc. (New York, NY). NR6 (Swiss 3T3 murine fibroblasts) cells expressing EGFRwt (NR6W) was a generous gift from Dr. Alan Wells (University of Pittsburgh School of Medicine; ref. 26). NR6 cells expressing human EGFRvIII (NR6M) were generated as described previously (23). All cells were maintained in DMEM (Mediatech, Inc., Herndon, VA) with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and incubated at 37°C in the presence of 5% to 10% CO2.

Paired primary HNSCC tumor samples were obtained from 33 HNSCC patients undergoing surgical excision with curative intent at the University of Pittsburgh Medical Center under the auspices of an institutional review board–approved protocol (Table 1). Signed informed consent was obtained from each subject.

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Tumor grade</th>
<th>N stage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>65</td>
<td>Male</td>
<td>Well-differentiated</td>
<td>0</td>
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<tr>
<td>Oropharynx</td>
<td>6</td>
<td>Female</td>
<td>Moderately differentiated</td>
<td>13</td>
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<tr>
<td>Larynx</td>
<td>7</td>
<td>Male</td>
<td>Poorly differentiated</td>
<td>15</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>6</td>
<td>Female</td>
<td>1-2</td>
<td>1</td>
</tr>
<tr>
<td>T stage**</td>
<td>3-4</td>
<td>Male</td>
<td>3-4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Three of the patients presented with recurrences.

**One patient presented with an unknown primary so therefore could not be staged.

Table 1. Clinicopathologic characteristics of 33 HNSCC patients evaluated for EGFRvIII

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(5'-ATGCCAGCTCGGGGACG-3' and 5'-ATCCGTACACTTTTGGCCG-3') designed to flank the deletion of exons 2 to 7. The primers were diluted to a final concentration of 0.6 µmol/L and incorporated into the reaction mixture. The remaining PCR reagents were diluted according to the manufacturer’s protocol. Reverse transcription was done at 40°C for 30 minutes followed by enzyme inactivation and hot-start PCR at 95°C for 15 minutes. Denaturation, annealing, and extension were done at 94°C, 55°C, and 72°C, respectively, for 1 minute each for a total of 35 cycles. The reaction was completed with an extension period at 72°C for 10 minutes. PCR products were visualized on a 1% agarose gel containing ethidium bromide. For confirmatory cDNA sequencing, the agarose-fractionated amplicon corresponding to the EGFR mutant band was excised from agarose and purified using the Qiagen Gel Extraction kit. The DNA product was sequenced by capillary gel electrophoresis using Big Dye Terminator chemistry version 3.1 kit and the ABI Prism Kinetic Analyzer model 3100 by the DNA Core Facility at the University of Pittsburgh School of Medicine.

Immunohistochemical analysis was done on paraffin-embedded HNSCC tumor tissue with mAb L8A4 (28) specific for the junction of the fusion of exons 1 to 8 found in EGFRvIII and a polyclonal rabbit antiseraum raised against the protein product of EGFR exons 2 to 7 (EGFR-1, specific for EGFRwt and unreactive for EGFRvIII; refs. 29, 30). Positive controls for antibody activity were verified by analysis of formalin-fixed cytospins of NR6M (EGFRvIII-expressing cells) and NR6W (EGFRwt-expressing cells). The slides were coded and the intensity and extent of EGFRwt and EGFRvIII expression were independently assessed by a board-certified pathologist and an immunohistochemist. The intensity of staining was graded on a scale of 0 to 4, with 4 being highest possible intensity. In addition, the extent of tumor staining ranged from a factor of 1 (0.25% of tumor positive), 2 (25-49% of tumor positive), 3 (50-74% of tumor positive), to 4 (75% or more of tumor positive).

In vitro growth of EGFRvIII-expressing cells and sensitivity to cisplatin or EGFR inhibition. To determine the growth kinetics of EGFRvIII-expressing HNSCC cells, transfected UM-22B cells (6 × 10⁴) were seeded onto 35-mm plates. Each cell population was then harvested in duplicate, transferred to a hemocytometer, and counted daily over a period of 6 days. This experiment was done twice. To determine if the sensitivity of HNSCC cells to EGFR inhibition was affected by EGFRvIII expression, vector-transfected and EGFRvIII-expressing HNSCC cells were treated with an EGFR-specific antibody C225 and the viability of each cell population was assessed. HNSCC cells were plated in 24-well plates at a density of 20,000/mL in DMEM containing 10% charcoal-stripped fetal bovine serum. After 24 hours, cells were treated with 200 µmol/L C225. Following a 72-hour treatment with C225, a tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for metabolically active cells was carried out according to the manufacturer’s protocol. For the in vitro apoptosis studies, HNSCC cells transfected with EGFRvIII cDNA or vector control were treated with 20 µmol/L cisplatin in DMEM containing 10% charcoal-stripped fetal bovine serum. Following treatment with cisplatin, the cells were detached by trypsinization, counted, and pelleted at 1,000 rpm for 5 minutes. Cell pellets were washed once with PBS (pH 7.4) and resuspended in 100 µL Annexin V binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂] as described previously (31). Cells (5 × 10⁵) were incubated with 5 µL Annexin V-Cy3 (BioVision Research Products, Mountain View, CA) at room temperature and in the absence of light for 15 minutes. The stained cell suspension was placed onto a hemocytometer and analyzed under fluorescence microscopy. The ratio (percentage) of apoptotic to total cells (apoptotic + nonapoptotic cells) was calculated for each high-power field. For each treatment, 5 to 10 high-power fields of view were quantitated on each section.

In vivo growth of HNSCC cells expressing EGFRvIII and sensitivity to C225. HNSCC cell line UM-22B-expressing EGFRvIII (vIII-1) or empty vector-transfected parental cells (PLV-1) were cultured in DMEM containing 10% fetal bovine serum and G418 (1.0 mg/mL). Cells were trypsinized and washed thrice with HBSS (Life Technologies, Carlsbad, CA). Cell number and viability of the cells were determined using trypan blue dye exclusion using a hemocytometer. A suspension of 2 × 10⁶ HNSCC cells in 100 µL HBSS was injected s.c. on the right and left flanks of nu/nu athymic nude mice (n = 10; Harlan Sprague-Dawley, Indianapolis, IN). The left flank was injected with vector-transfected cells and the right flank was injected with EGFRvIII-expressing cells. Tumor volumes were measured over 24 days in two dimensions with Vernier calipers. Tumor volumes were calculated using the formula: (length × width²) × 0.5. After 24 days, the mice were sacrificed by cervical dislocation under anesthesia. The tumors were surgically excised and divided into three sections; the first section was fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis with L8A4 or vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology, Santa Cruz, CA), the second section was snap frozen in a dry ice-ethanol bath for gene expression analysis by RT-PCR, and the third section was subjected to cellular lysis for Western blot analysis (below). To investigate the sensitivity to C225 in vivo, xenograft-inoculated animals (n = 4) were treated with i.p. injections of 100 mg/kg C225 thrice weekly for 3 weeks and the tumors were measured biweekly with a Vernier caliper in two dimensions. This experiment was done twice; in the second experiment, the number of vector control cells inoculated was doubled to 4 × 10⁶ cells to ensure equal tumor volumes at the start of treatment. Animal use and care was in strict compliance with institutional guidelines established by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Western analysis of HNSCC xenografts. Tumors were harvested and cell lysates were prepared and transferred to an Eppendorf tube and centrifuged for 30 minutes at 14,000 rpm. The supernatant was transferred to a clean tube, and protein quantitation was done on the supernatant using Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Proteins (40 µg) were loaded on a 10% SDS-polyacrylamide gel and electrophoresed along with 1 µL prestained broad-range protein marker (Cell Signaling Technology, Beverly, MA). After electrophoresis, proteins were transferred to nitrocellulose filters (Protran, Schleicher & Schuell, Inc., Floharn Park, NJ) in a semidry transfer apparatus (Bio-Rad Laboratories). The filters were blocked in 1× PBS with 0.2% Tween 20 and 0.5% nonfat milk for 1 hour in room temperature. The filters were incubated with primary antibody and subsequently washed with Blotto solution [50 mmol/L Tris (pH 7.4), 0.9% NaCl, 0.5% Tween 20] thrice for 15 minutes. The filters were then incubated with secondary antibody for 1 hour and washed with Blotto solution thrice for 15 minutes. The filters were quickly rinsed with rinsing solution, and the blot was developed with Luminol Reagent (Santa Cruz Biotechnology) by autoradiography. Antibodies used for blotting included VEGF and β-actin (Oncogene Research Products, Boston, MA) to show equal loading.

Statistics. Fisher’s exact test was used to show correlation between EGFRvIII expression and EGFRwt in HNSCC tumor tissues. Wilcoxon signed rank test was used for the analysis of growth rates in HNSCC xenografts, comparing EGFRvIII and vector controls. Wilcoxon-Mann-Whitney test was used for the analysis of VEGF expression in HNSCC xenografts, comparing EGFRvIII and vector controls. All tests were exact and two-tailed. Statistical analysis was done on the StatExact v.6.1 software (Cytel Software Corp., Cambridge, MA).

Results

EGFRvIII is expressed in HNSCC tumors. Cumulative evidence suggests that EGFR signaling contributes to the pathogenesis of HNSCC. However, the role of EGFRvIII has not been examined previously in HNSCC tissues or cell line models. We first sought to determine if EGFRvIII, a mutation correlated with increased tumorigenicity in glioblastoma, is present in HNSCC tumors. Thirty-three HNSCC tumors were stained...
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Using a EGFRwt-specific antibody (anti-exons 2-7) and an EGFRvIII-specific antibody (L8A4) to determine prevalence of the EGFRvIII and EGFRwt expression. Both antibodies were mutually exclusive, as they have been shown not to cross-react with their competing target proteins (29, 30). This was confirmed by immunocytochemical analysis on the control cells, NR6W and NR6M (data not shown). Patient demographic information used in this study and their corresponding clinicopathologic characteristics are summarized in Table 1. EGFRvIII expression was shown in 14 of 33 (42%) of HNSCC tumors (Table 2). Intriguingly, EGFRvIII was shown exclusively in tumors that also stained positive for EGFRwt expression. Although 12 (36%) of the samples showed EGFRwt without EGFRvIII expression, all of the tumors expressing the mutant receptor also showed coexpression of the wild-type receptor.

<table>
<thead>
<tr>
<th>Characteristics of EGFRvIII and EGFRwt expression in 33 HNSCC patients</th>
<th>No. (%) patients (N = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFRvIII (+)</td>
<td>14 (42)</td>
</tr>
<tr>
<td>EGFRwt (+)</td>
<td>26 (79)</td>
</tr>
<tr>
<td>EGFRvIII (+) / EGFRwt (+)</td>
<td>14 (42)</td>
</tr>
<tr>
<td>EGFRvIII (+) / EGFRwt (-)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>EGFRvIII (-) / EGFRwt (+)</td>
<td>12 (36)</td>
</tr>
<tr>
<td>EGFRvIII (-) / EGFRwt (-)</td>
<td>7 (21)</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of EGFRvIII and EGFRwt expression in 33 HNSCC patients

NOTE: Thirty-three HNSCC tumor samples were sectioned and stained for EGFRvIII and EGFRwt expression. Of these tumor samples, 26 (79%) stained positive for EGFRwt expression and 14 (42%) stained positive for EGFRvIII expression. Although 12 (36%) of the samples showed EGFRwt without EGFRvIII expression, all of the tumors expressing the mutant receptor also showed coexpression of the wild-type receptor.

To determine the subcellular localization of EGFRvIII, formalin-fixed tissues stained with specific antibodies to either EGFRvIII (L8A4) or EGFRwt (EGFR-1) were examined. Figure 1A shows serial sections from a representative HNSCC tumor sample. Figure 1A (I) was stained with L8A4 and Fig. 1A (II) was stained with EGFR-1 (anti-exons 2-7, the ligand-binding segment of EGFR known to be absent in the EGFRvIII gene). Staining for both receptors was specific to the HNSCC tumor nests and was not reactive to the adjacent normal tissue. At ×66 magnification, EGFRvIII staining was homogeneous diffusely with mildly higher intensity in the peripheral regions of the tumor nests (Fig. 1A, I), whereas EGFRwt staining showed areas of focal intensity in addition to homogeneous distribution (Fig. 1A, II). At ×132 magnification (Fig. 1A, III), EGFRvIII expression showed diffuse cytoplasmatic staining in addition to strong membranous staining. Negative primary reagent controls were provided by murine IgG at the same concentration as L8A4 and purified normal rabbit IgG at the same concentration as anti-exons 2-7 (Fig. 1A, IV). There was no evidence of EGFRvIII expression in the adjacent normal mucosa or in the submucosa.

To confirm the protein expression data, RT-PCR was done using primers that flank exons 2 to 7 designed to synthesize two discrete amplicons distinguishing the expression of EGFRvIII from the larger EGFRwt transcript. Figure 1B shows mRNA expression of the mutant receptor in one of three representative tumor samples where coexpression with EGFRwt is evident, consistent with the protein expression data. Moreover, DNA sequencing of the PCR products confirmed the identity of the 5' regions of EGFRvIII and EGFRwt (data not shown). These results showed that EGFRvIII is present in nearly half of HNSCC tumors.

EGFRvIII contributes to increased HNSCC cell proliferation and tumor growth. A universal feature among cells undergoing malignant transformation is the increase in cellular growth rates. Overexpression of EGFR has been associated with increased tumor growth and metastasis (32, 33). Therefore, a constitutively active form of this receptor, EGFRvIII, could contribute to increased growth potential. Human tumors that express EGFRvIII do not retain expression of the mutant

Fig. 1. EGFRvIII is expressed in HNSCC. A, immunohistochemical staining of HNSCC samples for EGFRvIII and EGFRwt. Serial sections from a representative HNSCC tumor sample. I, a section that has been stained with L8A4, an EGFRvIII-specific antibody raised against the novel glycine epitope created by the junction of exons 1 and 8. II, same section stained with a EGFRwt-specific antibody raised against exons 2 to 7. This antibody has been shown not to cross-react with the mutant receptor on NR6M cytospins. III, a section stained with L8A4 that shows diffuse cytoplasmatic staining as well as cell membrane staining. IV, stained with an IgG murine isotype negative control. B, EGFRvIII mRNA levels were examined in 30 HNSCC tumor tissues by RT-PCR. Representative ethidium bromide–stained gel showing EGFR and EGFRvIII PCR products from three HNSCC tumors (lanes 71–73), A431 human vulvar SCC line expressing EGFRwt as a negative control for EGFRvIII (lane A), EGFRvIII-transfected NR6M cell line as a positive control (lane D), and a negative control for the RT-PCR without template reaction (lane W). Left, sequence of the primers used and their locations flanking the 801-bp deletion of the EGFR mRNA.
receptor when grown in vitro. To investigate the contribution of EGFRvIII in HNSCC proliferation, we established an in vitro model by generating a stable cell line that constitutionally expresses this mutant receptor. Twenty-four clones of a total of $2 \times 10^8$ transfected cells (HNSCC cell line, UM-22B) were selected, all of which initially scored positive for EGFRvIII expression by RT-PCR (data not shown). Four of the most stable clones constitutively expressing EGFRvIII were selected for further study. RT-PCR analysis of two representative clones confirmed stable expression of EGFRvIII as well as the absence of EGFRvIII expression in the vector-transfected control cells (Fig. 2A). When these paired cells were grown in culture, the population doubling time was shortened in EGFRvIII-expressing cells and was immediately evident by day 3. By day 5, the number of EGFRvIII-expressing HNSCC cells was greater than doubled when compared with the vector-transfected control cell population (Fig. 2B).

To investigate the mitogenic effect of EGFRvIII expression in vivo, athymic nude mice were inoculated with HNSCC cells expressing EGFRvIII on the right flank and vector-transfected control cells on the left flank over a period of 24 days (Fig. 2C). The tumor volumes were measured in 10 athymic nude mice. By day 24, the median tumor volume from HNSCC xenografts expressing EGFRvIII measured 87-fold greater in size in comparison with the contralateral HNSCC xenografts derived from vector-transfected control cells ($P < 0.004$ for days 5, 9, 16, 20, and 24 and $P < 0.032$ for day 12). The median tumor size difference between VIII-1 and PLV-1 was calculated from each individual mice and was increased to 751 mm$^3$ by day 24 (Fig. 2D). The tumors were harvested at the conclusion of the experiment and stained for expression of EGFRvIII by immunohistochemistry. Figure 2E depicts a representative slide showing the expression of the EGFRvIII protein in vivo. This was further validated at the gene expression level by RT-PCR, showing the coexpression of EGFRwt (also seen in the control xenografts) in addition to EGFRvIII mRNA expression (Fig. 2F).

HNSCC tumors expressing EGFRvIII appeared more erythematous by gross observation, characteristic in appearance for tumor angiogenesis (data not shown). Previous studies in astrocytoma cells expressing EGFRvIII have shown increases in VEGF secretion through the activation of the Ras oncogene (34). To investigate the possible role of VEGF expression in EGFRvIII-mediated tumorigenesis in head and neck cancer, xenografts derived from EGFRvIII-transfected HNSCC cells were stained for VEGF (Fig. 3A). Quantitation of VEGF expression by densitometry revealed that the level of VEGF was nearly doubled in HNSCC xenografts expressing the EGFRvIII (Fig. 3B; $P > 0.04$). This observation was confirmed by Western blot analysis showing increased VEGF expression in EGFRvIII-transfected xenografts from three representative nude mice (Fig. 3C). These results suggest that the expression of EGFRvIII in HNSCC contributes to increased growth in vitro and in vivo and is associated with elevated expression of VEGF.

**EGFRvIII abrogates antitumor responses to chemotherapy and EGFR targeting.** Chemotherapy (generally in combination with radiation therapy) is the standard approach for the treatment of patients with recurrent and/or metastatic HNSCC and, more recently, for patients with advanced locoregional disease. The benefits of cisplatin were shown ~20 years ago (35) and this remains a common agent either alone or in combination with other chemotherapy drugs. EGFRvIII has been reported to confer resistance to cisplatin in glioma cells (36). To investigate the role of EGFRvIII in response to cisplatin, HNSCC cells expressing EGFRvIII- or vector-transfected controls were treated with cisplatin followed by staining for apoptotic cells with Annexin 5-Cy3 fluorophore. Cisplatin treatment induced apoptosis in 43% of the vector-transfected controls. However, the percentage of cells undergoing apoptosis in HNSCC cells expressing EGFRvIII was reduced by 2-fold (19.6%; $P < 0.001$; Fig. 4). EGFRvIII-expressing cells are, therefore, more resistant to apoptosis when treated with a potent cytotoxic agent, such as cisplatin.

Several strategies designed to block EGFR activity are currently under clinical investigation or have been approved by the Food and Drug Administration for use in selected cancer patients. These include receptor-blocking α-EGFR antibodies, TKIs, EGFR ligand-conjugated toxins, and EGFR antisense gene therapy (37). Among these agents, the monoclonal α-EGFR antibodies (C225/cetuximab/Erbilux) and the small-molecule TKIs (ZD1839/gefitinib/Erlysaa or OSI774/erlotinib/Tarceva) are the most extensively studied. Clinical trials of these agents administered as monotherapy showed limited antitumor effects (38). We hypothesized, therefore, that the aberrant expression of EGFRvIII may contribute to the limited response of HNSCC to EGFR targeting. To investigate the contribution of EGFRvIII to EGFR-blocking strategies, HNSCC cells expressing EGFRvIII- or vector-transfected controls were treated with the chimeric human-murine mAb to EGFR (C225) followed by a tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] viability assay to monitor cell death. The percentage of killing from C225 treatment was reduced in EGFRvIII cells (33%) compared with untransfected parental cells (55%) or vector-transfected controls (54%; Fig. 5A). In contrast to the relative resistance to treatment with an α-EGFR antibody, EGFRvIII-expressing cells and vector-transfected controls were equally susceptible to EGFR TKI treatment (data not shown). This is consistent with the fact that both EGFRvIII and EGFRwt contain an intact tyrosine kinase domain.

The effects of C225 on EGFRvIII-expressing HNSCC tumor growth were also evaluated in vivo. Athymic mice were inoculated s.c. with HNSCC cells expressing EGFRvIII- and vector-transfected control cells and subsequently treated with C225 (100 mg/kg, three injections weekly). The tumor volumes were measured in four athymic nude mice over a period of 3 weeks. By day 22, the mean tumor volume from HNSCC xenografts expressing EGFRvIII nearly doubled in size when compared with the vector control tumors (235 versus 128 mm$^3$, respectively; $P < 0.04$; data not shown). Due to the faster growth rates observed in the EGFRvIII-expressing xenografts, this experiment was done twice. In the second experiment, athymic nude mice were inoculated with twice the number of vector control cells ($4 \times 10^8$ versus $2 \times 10^8$ cells) to ensure equal tumor volumes at the start of the experiment. By day 24, the mean tumor volume from the EGFRvIII-expressing xenografts more than tripled in size when compared with the vector control tumors (54 versus 171 mm$^3$, respectively; $P < 0.008$). The median tumor size difference between VIII-1 and PLV-1 was also calculated from each individual mice and was increased to 117 mm$^3$ by day 24 (Fig. 5B). Taking together, these results suggest that the limited efficacy of chemotherapy and specific agents that inhibit the ligand-binding activity of EGFRwt in a
Fig. 2. EGFRvIII contributes to increased HNSCC growth in vitro and in vivo. A, RT-PCR of HNSCC cell line UM-22B showing expression of EGFRvIII following transfection in representative stable lines (clones 1 and 2), vector-transfected control UM-22B cells (PLV-1), untransfected UM-22B as a negative control, and EGFRvIII-transfected NR6M cell line as a positive control. B, HNSCC cells expressing EGFRvIII (vIII-1) show increased growth rates compared with the vector-transfected cells (PLV-1). The growth curve of the transfected cell lines in vitro was obtained by cell counting using vital dye exclusion at several time points for 6 days. Representative of two independent experiments. C, HNSCC xenografts expressing EGFRvIII grow more rapidly than tumors derived from vector-transfected control cells. Athymic nude mice (n = 10) were inoculated s.c. with vector-transfected cells (PLV-1) on the left flank and EGFRvIII-expressing cells (vIII-1) on the right flank. Three representative mice were photographed to show the asymmetrical tumor volumes on the hind limbs. D, 10 athymic nude mice were serially measured over 24 days and plotted to evaluate the tumor growth rate. Top left, vIII-1, EGFRvIII-transfected xenograft; top right, PLV-1, the vector-transfected xenograft; bottom, vIII-1 minus PLV-1, median flank difference of tumor volumes between vIII-1 and PLV-1 xenografts within each individual mouse. E, formalin-fixed xenograft tumors derived from EGFRvIII-transfected cells (left) or vector-transfected control cells (right) were examined by immunohistochemistry using the L8A4 antibody for the expression of EGFRvIII. F, RT-PCR of xenograft tumors showing EGFRvIII expression. RNA was extracted from EGFRvIII (M) or vector-transfected control (C) xenografts and RT-PCR products were fractionated on a 1% ethidium bromide–stained agarose gel using primer sets in Fig. 1B.
subset of HNSCC patients may be due to the contribution of an aberrant, ligand-independent EGFRvIII expression.

**Discussion**

The EGFR has long been associated with increased tumorigenesis in cancer models where expression levels in human tumors correlate with poor prognosis in the clinical setting. As a growth-promoting molecule, overexpression of EGFR leads to up-regulation of downstream signaling pathways leading to increased cell proliferation, invasion, and metastasis (39). EGFR is up-regulated in >90% of HNSCC tumors where overexpression of this receptor as well as its autocrine ligand, transforming growth factor-α, are independent indicators of poor prognosis (4).

EGFR-driven carcinogenesis has been investigated in several tumor systems. Genomic analysis of EGFR amplification in glioblastomas led to the discovery of tumor-specific EGFR mutations (25, 40). The most common mutant found is EGFRvIII, a ligand-independent, constitutively active receptor that was subsequently shown to correlate with poor prognosis in glioblastoma patients (41). A novel truncation mutation of EGFR was detected in malignant oral keratinocytes as well as oral SCC (42, 43). The presence of EGFRvIII has not been reported in HNSCC.

In this study, we show EGFRvIII expression in 42% of HNSCC by immunohistochemistry. EGFRvIII was also detected at the mRNA level by RT-PCR. Sequencing of the RT-PCR product confirmed the characteristic mRNA deletion of exons 2 to 7 corresponding to the EGFRvIII. This frequency of EGFRvIII expression by immunohistochemistry is similar to that seen in other tumors types, including glioblastomas, anaplastic astrocytomas, non–small cell lung carcinoma, breast cancers, and ovarian carcinomas (19–22, 44). More recent mutational analysis of EGFR in lung cancers has shown that EGFRvIII is uncommon. In this study, EGFRvIII mutation was present in 5% (3 of 56) of lung SCC but was not present in lung adenocarcinomas (0 of 123; ref. 45). To our knowledge, this study is the first report of EGFRvIII expression in HNSCC.

We find the extensive and homogeneous expression of EGFRvIII in head and neck carcinoma similar to that found in glioblastomas. Demonstration of EGFRvIII in HNSCC is not entirely unexpected given its presence in other cancers of ectodermal cell origin. In contrast to glioblastomas, where 40% of the tumors overexpress EGFR at the genomic level by gene amplification (25), increased expression of EGFR in HNSCC is accompanied by gene amplification in ~10% of cases (46). HNSCC cells expressing elevated levels of EGFR have been
shown to proliferate, invade, and metastasize to a greater extent than those expressing lower EGFR levels. We hypothesize that EGFR overexpression leads to rapid cell proliferation resulting in late-stage mutations, such as EGFRvIII. In support of this hypothesis, we found that EGFRvIII expression in HNSCC is only evident in the presence of EGFRwt overexpression. In glioblastomas, EGFR mutations are generally not detected in tumors without EGFRwt amplification (47). It is also possible that, given our relatively small cohort of 33 HNSCC patients, analysis of additional cases may reveal a tumor uniquely expressing EGFRvIII in the absence of EGFRwt.

Functional characterization studies of EGFRvIII have shown that several downstream modulators confer increased tumorigenicity to EGFRvIII-expressing cells. In vitro studies suggest that EGFRvIII-expressing cells may be more chemotherapy and radiotherapy resistant as well as display a more aggressive phenotype (36, 48–50). In contrast to EGFRwt, EGFRvIII does not activate the Ras-Raf-MEK pathway and instead seems to preferentially activate the phosphatidylinositol 3-kinase pathway (51). It has recently been shown that astrocytic cells expressing EGFRvIII may be radioresistant in part due to increased phosphatidylinositol 3-kinase signaling (52). Other studies have shown that EGFRvIII induces metalloproteinases and extracellular matrix components known to be involved in tumor invasion (50). Nagane et al. showed down-regulation of Bcl-XL as well as decreased apoptosis in response to cisplatin treatment (36, 53). Moreover, Holland et al. showed that all of the tumors generated in their in vivo gene transfer model for gliomagenesis required EGFRvIII expression (54).

Because of the relatively high frequency of EGFR overexpression and its correlation with decreased survival in HNSCC, this receptor has been investigated as a target for therapeutic intervention in this malignancy. EGFR-targeted therapies, such as TKIs, EGFR-specific mAbs, and ligand-linked toxins, have successfully reduced HNSCC tumor burden in vivo (55). However, clinical trials evaluating these reagents as monotherapy have achieved only modest results (56). This may be because patients are not routinely selected for treatment based on EGFR overexpression and/or activation status. Consequently, those patients who do not show increased EGFR expression or activation may not be amenable to EGFR-targeted therapies. Alternatively, the modest clinical response may be due to the presence of EGFRvIII.

Our study shows that the expression of EGFRvIII abrogates antitumor responses to EGFR-specific mAb, C225. C225 binds to the extracellular ligand-binding domain of EGFRwt with an affinity equal to its ligand and blocks its activation (57). Therefore, it is not surprising that EGFRvIII expression could circumvent the effects of C225, given the fact that this truncated mutant receptor is ligand independent and constitutively active.

Fig. 5. EGFRvIII contributes to “resistance” to EGFR targeting. A, HNSCC vector-transfected or EGFRvIII-expressing cells were treated with 200 nmol/L of the EGFR-specific antibody C225 for 72 hours before a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Columns, percent killing of cells by C225. B, EGFRvIII-expressing HNSCC cells are partially resistant the EGFR inhibitor C225 in vivo. Athymic nude mice (n = 4) were inoculated with EGFRvIII-expressing cells (top left, vii-1) and vector-transfected cells (top right, PLV-1) and subsequently treated with i.p. injections of C225 (100 mg/kg, three injections weekly). The tumors were measured twice weekly over 24 days and plotted to evaluate the tumor growth rate. Bottom, vii-1 minus PLV-1, median flank difference of tumor volumes between vii-1 and PLV-1 xenografts within each individual mice. To ensure equal tumor volumes at the start of the experiment, the mice were inoculated with 4 \times 10^5 vector-transfected cells on the left flank and 2 \times 10^5 EGFRvIII-expressing cells on the right flank.
Although there is no conclusive evidence of C225 binding of EGFRvIII, the development of EGFR-targeted C225 immunoliposomes that bind and internalize in tumor cells, which overexpress EGFRvIII, have been described \textit{in vitro} (58). However, in a follow-up study by the same authors, specific binding of C225 to EGFRvIII \textit{in vivo} could not be determined because the EGFRvIII-expressing xenografts also expressed EGFRwt (59). The Food and Drug Administration has recently granted priority review for the approval of C225 as mono-therapy in HNSCC patients with recurrent and/or metastatic disease where prior platinum-based chemotherapy has failed or in combination with radiation for locally or regionally advanced disease (Iدارة press, released March 1, 2006).

EGFRvIII is an attractive candidate target for therapeutic intervention because, unlike EGFRwt, EGFRvIII is not found in normal tissue. The in-frame recombination of exons 1 and 8 produces a novel glycine epitope for EGFRvIII-specific antibodies. Antibody-linked chemotherapies would target tumor tissue with theoretically little or no toxicity to normal tissue. Several data support this hypothesis. An EGFRvIII peptide vaccine increased survival time in mice with established i.c. tumors (60). Systemic administration of an EGFRvIII-specific mAb (806) to nude mice bearing i.c. glioblastoma xenografts led to tumor shrinkage and increased survival (61).

In conclusion, EGFRvIII is expressed in more than one third of HNSCC tumors. This mutant receptor is a tumor-specific, constitutively active, ligand-independent EGFR mutant that may contribute to the initiation, promotion, or progression to the malignant phenotype of HNSCC. Expression of EGFRvIII, at least in part, abrogates antitumor responses to chemotherapy and EGFR targeting \textit{in vitro} and \textit{in vivo}. Functional studies in HNSCC models are required to further elucidate the role of EGFRvIII in HNSCC pathogenesis and its potential as a therapeutic target.

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