Expression of Amphiregulin and EGFRvIII Affect Outcome of Patients with Squamous Cell Carcinoma of the Head and Neck Receiving Cetuximab–Docetaxel Treatment

Ingeborg Tinhofer1, Konrad Klinghammer2, Wilko Weichert2, Maren Knödler2, Albrecht Stenzinger3, Thomas Gauler4, Volker Budach1, and Ulrich Keilholz2

Abstract

Purpose: Constitutive activation of epidermal growth factor receptor (EGFR) as a result of gene amplification, mutation, or overexpression of its ligands has been associated with response to EGFR targeting strategies. The role of these molecular mechanisms for the responsiveness of squamous cell carcinoma of the head and neck (SCCHN) to cetuximab-containing regimens remains unknown.

Experimental design: Tumor biopsies from 47 patients, enrolled in a single-arm phase II multicenter study for second-line treatment of recurrent or metastatic SCCHN with cetuximab and docetaxel, were analyzed by immunohistochemistry for expression of EGFR, its deletion variant III (EGFRvIII) and its ligand amphiregulin (AREG). The relation between expression levels and disease control rate (DCR) was evaluated by logistic regression. Association between expression levels, progression-free survival (PFS), and overall survival (OS) was determined by Kaplan–Meier analysis, log-rank test, and uni- and multivariate Cox regression analysis.

Results: High expression of EGFR, EGFRvIII, and AREG was detected in 73%, 17%, and 45% of SCCHN cases, respectively. Expression levels of EGFR had no impact on PFS or OS. High expression levels of EGFRvIII were significantly associated with reduced DCR and shortened PFS (HR: 3.3, \( P = 0.005 \)) but not with OS. Patients with high AREG expression in tumor cells had significantly shortened OS (HR: 2.2, \( P = 0.019 \)) and PFS (HR 2.2, \( P = 0.019 \)) compared with patients with low expression score. Multivariate Cox analysis revealed an independent association of AREG and EGFRvIII with PFS but only AREG was an independent prognosticator of OS.

Conclusions: High EGFRvIII and AREG expression levels identify SCCHN patients who are less likely to benefit from combination treatment with cetuximab and docetaxel. Clin Cancer Res; 17(15); 1–8. ©2011 AACR.

Introduction

Epidermal growth factor receptor (EGFR) plays an important role in tumor growth, invasion, and metastasis and is commonly overexpressed in a variety of epithelial malignancies. In squamous cell carcinoma of the head and neck (SCCHN), EGFR expression has been reported in up to 90% of tumors (1, 2), and high expression levels have not only been associated with a more aggressive phenotype but also with decreased responsiveness to radio- or chemotherapy (3, 4) and reduced relapse-free and overall survival (OS) (4–6). Because of its prevalence and crucial role in the pathogenesis, targeting EGFR has thus become a rational approach for treatment of SCCHN. Indeed, combinations of cetuximab with radiotherapy (7, 8) or platinum-containing chemotherapy regimens (9) have already shown significant improvement of treatment outcome. However, for these cetuximab-containing treatment modalities, a local 2-year control rate of 50% (7) and a best overall response rate of 36% (9), respectively, were shown suggesting that a considerably large group of patients will not benefit from cetuximab.

In the search for biomarkers allowing prospective identification of patients with significant benefit of EGFR targeting therapy, the predictive role of EGFR gene...
amplification, activating mutations, and protein expression levels as well as the expression of EGFR ligands has already been evaluated. These studies revealed that EGFR gene amplification (10) or expression of EGFR (7, 9) did not predict for the response of SCCHN patients to EGFR targeting. EGFR-activating mutations are rather rare in SCCHN (11, 12) and thus, contrary to lung cancer, less likely to contribute to treatment outcome. In contrast, EGFR deletion variant III (EGFRvIII), which has been shown to be expressed in about 45% of SCCHN cases, was shown to be significantly associated with reduced response to cetuximab treatment in SCCHN cell line models (13). Regarding the role of EGFR ligands, high expression levels of amphiregulin (AREG) and epiregulin have been associated with improved disease control on cetuximab monotherapy (14) or combined cetuximab–irinotecan treatment (15) in patients with metastatic colorectal cancer (mCRC). In the SCCHN model, the predictive value of EGFR ligands is less well established and rather conflicting data exist: overexpression of AREG was found to be associated with reduced response to cetuximab or gefitinib in 1 study of SCCHN cell lines (16), whereas 2 other studies again using SCCHN cell lines as a model identified high AREG mRNA and protein expression as predictors for high sensitivity to EGFR targeting (17, 18).

Considering the paucity of information on the role of EGFRvIII and AREG for the response to EGFR targeting in SCCHN patient cohorts, we retrospectively evaluated their role for cetuximab efficacy within a multicenter clinical phase II trial for treatment of recurrent/metastatic SCCHN patients with cetuximab in combination with docetaxel.

Materials and Methods

Patients and treatment

Eighty-four patients with histologically confirmed recurrent or initially metastatic SCCHN were enrolled in a phase II multicentric clinical trial for treatment with cetuximab and docetaxel. Further eligibility criteria were tumor relapse after platinum-containing chemoradiotherapy or after platinum-containing first-line chemotherapy, no intermittent can treatment (15) in patients with metastatic colorectal cancer (mCRC). In the SCCHN model, the predictive value of EGFR ligands is less well established and rather conflicting data exist: overexpression of AREG was found to be associated with reduced response to cetuximab or gefitinib in 1 study of SCCHN cell lines (16), whereas 2 other studies again using SCCHN cell lines as a model identified high AREG mRNA and protein expression as predictors for high sensitivity to EGFR targeting (17, 18).

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Immunohistochemistry

Expression analysis of EGFR, EGFRvIII, and AREG was conducted on archival formalin-fixed paraffin-embedded (FFPE) primary tumor biopsy material. For the evaluation of AREG expression, conventional 3-µm sections from the original tissue blocks were used. For the analysis of EGFR and EGFRvIII expression, tissue microarrays (TMA) were generated using a precision instrument (Beecher Instruments). Two tissue cylinders of 1.5-mm diameter were punched from each tumor-bearing donor block, transferred to a TMA recipient block, and serial 3-µm sections were cut. For cases which were not informative on the TMA or for which the biopsy material was too small in size for a TMA, conventional 3-µm sections from the original tissue blocks were used for staining.

EGFR expression was detected by the EGFR pharmDx Kit for Manual Use (Dako), according to the manufacturer’s instructions. Immunohistochemical analysis of EGFRvIII was done using mAb L8A4 specific for the junction of the fusion of exons 1 to 8 found in EGFRvIII (ref. 19; kindly provided by Dr. Bigner, Duke University Medical Center, Durham, North Carolina). For specificity control of mAb L8A4, immunostaining was carried out using 3-µm sections from the FFPE glioma cell line U87 stably transfected with an EGFRvIII expression vector or an empty vector (kindly provided by Dr. Furnari, University of California, San Diego). Analysis of AREG expression was conducted using a polyclonal goat anti-AREG antibody raised against the cleaved mature form of AREG (clone AF262; R&D Systems).

After heat-induced antigen retrieval, slides were incubated with the specific primary antibody (mAb L8A4, dilution 1:200; clone AF262, dilution 1:75) at 4°C overnight. The omission of the primary antibody served as negative control. Bound antibody was detected by a Super Sensitive IHC Detection System (BioGenex), according to the manufacturer’s protocol. For color development, a Fast Red system (Sigma, Deisenhofen, Germany) was used. The slides were coverslipped after counterstaining.

Evaluation of immunostaining

Immunostainings were independently analyzed by 2 pathologists (W.W. and A.S.) who were blinded to the clinical data. The membrane, cytoplasmic, and nuclear expression levels of EGFR, EGFRvIII, and AREG were scored for Manual Use (Dako), according to the manufacturer’s instructions. Immunohistochemical analysis of EGFRvIII was done using mAb L8A4 specific for the junction of the fusion of exons 1 to 8 found in EGFRvIII (ref. 19; kindly provided by Dr. Bigner, Duke University Medical Center, Durham, North Carolina). For specificity control of mAb L8A4, immunostaining was carried out using 3-µm sections from the FFPE glioma cell line U87 stably transfected with an EGFRvIII expression vector or an empty vector (kindly provided by Dr. Furnari, University of California, San Diego). Analysis of AREG expression was conducted using a polyclonal goat anti-AREG antibody raised against the cleaved mature form of AREG (clone AF262; R&D Systems).

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was determined by multiplying the intensity scores with the extent of positivity scores of stained cells which resulted in a minimum score of 0 and a maximum score of 12. For all 3 markers, the consistency of scoring between the 2 observers was high [kappa scores (ref. 20): EGFR, 0.79; EGFRvIII, 0.83; AREG, 0.86].

**Detection of EGFRvIII transcripts by reverse transcription PCR**

For independent validation of IHC results, we selected 4 EGFRvIII-positive and 4 negative FFPE samples as well as the FFPE glioma cell line U87 stably transfected with an EGFRvIII expression vector or an empty vector for detection of EGFRvIII transcripts using the protocol established by Yoshimoto and colleagues (21). Briefly, total RNA from 3 to 5 10-μm slices of FFPE tumor tissue was extracted using the RecoverAll Total Nucleic Acid isolation Kit (Applied Biosystems/Ambion), according to the manufacturer’s protocol, with the exception that the proteinase K treatment was prolonged to 4 hours to increase RNA yield. RNA was quantified using the NanoPhotometer from Implen. Synthesis of cDNA was carried out in a total volume of 25 μL with 500-ng random hexamers (Roche) and 2 μg of total RNA using the Omniscript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) according to the supplied protocol. cDNA (2 μL) was used for the PCR reaction. The quality of RNA was checked by PCR detection of the housekeeping gene porphobilinogen deaminase (PBGD; forward primer: 5'-GCC ACA ACC GGG TCG GGC A-3', reverse primer: 5'-CAC TTC CAC GCC CAA GGC CCC-3', product: 80 bp), and only samples positive for PBGD transcripts were used for EGFRvIII PCR. Conditions for the EGFRvIII PCR reaction and primers used therein were the same as described by Yoshimoto and colleagues (21).

**Statistical analysis**

The threshold for differentiating between low and high IHC scores was set at 7 or more for EGFRvIII and AREG immunostaining scores. From left to right: lane 1, 100-bp size marker; lane 2, the glioma cell line U87; lane 3, U87-EGFRvIII; lanes 4–7, tumor samples positive for EGFRvIII in IHC; lanes 8–11, IHC-negative tumor samples; lane 12, aqua destillata (a.d.).

Figure 1. Detection of AREG and EGFRvIII in SCCHN. A, representative tumor samples with low and high IHC score for AREG (left) and EGFRvIII (right) are presented. B, EGFRvIII (top) or PBGD transcripts (bottom) were amplified by conventional RT-PCR. Products were separated by gel electrophoresis on a 3.5% agarose gel and detected by SybrGreen staining. From left to right: lane 1, 100-bp size marker; lane 2, the glioma cell line U87; lane 3, U87-EGFRvIII; lanes 4–7, tumor samples positive for EGFRvIII in IHC; lanes 8–11, IHC-negative tumor samples; lane 12, aqua destillata (a.d.).

Expression and subcellular localization of EGFR, EGFRvIII, and AREG

The analysis of expression and subcellular localization of EGFR, EGFRvIII, and AREG revealed positive tumor samples in 94% (EGFR), 80% (EGFRvIII), and 81% of cases (AREG). Although EGFR was predominantly localized at the cellular membrane, EGFRvIII and AREG were mainly detected in the cytoplasm. AREG expression could be found both in the cytoplasm and in the nucleus of tumor cells. High expression levels of EGFR, EGFRvIII (IHC score ≥7), and AREG (IHC score ≥4) were detected in 73%, 17%, and 45% of SCCHN cases, respectively. No significant associations of EGFR, EGFRvIII, and AREG immunostaining scores with sex, age, tumor localization, initial tumor stage, smoking, and alcohol history were observed. Representative tumor samples with low and high IHC scores for AREG (left) and EGFRvIII (right) are presented in Figure 1A.

To validate the results from the IHC analysis of EGFRvIII using the L8A4 antibody, we analyzed 4 EGFRvIII-positive significantly different disease control rates (DCR) were determined by using the receiver operating characteristic curve analysis (see Supplementary Fig. S1), and the same cutoff values were subsequently applied to the analysis of PFS and OS. Comparison of PFS and OS between the patient subgroups with low or high expression score for EGFR, EGFRvIII, or AREG was done using the Kaplan–Meier analysis, and significance was determined using the log-rank test. The relation between EGFR, EGFRvIII, and AREG immunostaining score and survival was evaluated using the Cox regression model. The interference of EGFR, EGFRvIII, and AREG expression with the clinical parameters sex, age, tumor localization, initial tumor stage, smoking, and alcohol history as well as the DCR [partial response (PR) and stable disease (SD)] was assessed using logistic regression. Statistical analyses were carried out using the Statview software (version 5.0.1, SAS Institute Inc.). The level of significance was set at P < 0.05.

Results
samples with an IHC score of 6 or more, 4 negative FFPE samples with an IHC score of 0 and the U87 cell line transfected with a vector encoding for EGFRvIII (U87\textsuperscript{EGFRvIII}) or an empty vector (U87\textsuperscript{VC}) by reverse transcription PCR (RT-PCR). In 3 of 4 FFPE samples positive in the IHC analysis and U87EGFRvIII cells, a specific EGFRvIII PCR amplicon could be detected. Conversely, 3 of 4 EGFR-vIII-negative FFPE samples and U87VC cells were negative in the EGFRvIII RT-PCR (Fig. 1B). Thus, the overall concordance between the 2 analyses was 80% which is equal to the value reported by Yoshimoto and colleagues (21).

**EGFRvIII and AREG expression and outcome**

Because EGFRvIII and AREG expression levels have been shown to negatively and positively interfere with the efficacy of cetuximab in SCCHN cell line models (13) and mCRC patient cohorts (14, 15), respectively, we first evaluated whether their expression would also correlate with the response to cetuximab–docetaxel in our study. Response data after 3 months were available for 46 of 47 patients. The overall response rate was 12% and the DCR (PR + SD) 54%. Although EGFR expression did not affect the DCR, there was a significant association of EGFRvIII expression and treatment efficacy: in the patient group with low EGFRvIII IHC score, DCR was 65%, whereas patients with high EGFRvIII IHC score had a DCR of 13% (P = 0.02; Table 1). Contrary to the mCRC model, where high AREG expression levels were associated with an improved response to cetuximab or cetuximab–irinotecan treatment, we observed a trend to reduced disease control in patients with high AREG IHC score (DCR 65%, P = 0.09). As already reported recently (22), the A genotype of the EGFR single-nucleotide polymorphism R521K and the absence of any skin reaction in the early treatment phase were also by trend correlated with reduced response to cetuximab–docetaxel (Table 1). Therefore, these factors were also included in our multivariate model. Multivariate logistic regression analysis confirmed the independent significant association of EGFRvIII with response to treatment, whereas AREG expression levels, EGFR R521K, and skin toxicity remained only by trend associated with DCR (Table 1).

To further determine whether expression levels of EGFR, EGFRvIII, or AREG were significantly related to PFS and OS, patients were divided into low and high IHC score groups, again using the cutoffs of 7 or more for EGFR and EGFRvIII and 4 or more for AREG established for response to treatment. As depicted in Figure 2A, expression of EGFR had no impact on PFS and OS. In contrast, patients with tumors that showed high expression of EGFRvIII had significantly shorter PFS than patients with low expression (log-rank, P = 0.0028; HR: 3.3; mean PFS, 2.0 vs. 5.4 months; Fig. 2B), whereas OS was not significantly affected by EGFRvIII expression levels. Patients with tumors with high AREG expression had significantly shortened PFS (HR: 2.2, log-rank P = 0.016, mean PFS, 3.1 vs. 5.9 months) and OS (HR: 2.2, log-rank P = 0.0016, mean OS 3.5 vs 9.5 months) compared with patients with low AREG expression score (Fig. 3). Multivariate Cox models confirmed AREG, EGFR-vIII, and skin toxicity as independent predictors for PFS (Table 2) but only AREG expression levels were identified as an independent predictor for OS (Table 3).

**Discussion**

We show that the majority of recurrent or initially metastatic SCCHN tumors express EGFR, EGFRvIII,
AREG. The major fraction of AREG was detected in the cytoplasm and the nucleus of tumor cells. It has previously been shown that after ectodomain cleavage from its membrane-anchored precursor (23), soluble AREG binds to its receptor which results in the internalization of the receptor–ligand complex (24). Thus it seems likely that in our study mainly receptor-bound internalized AREG was detected. Comparable with the staining pattern of AREG, EGFRvIII was also mainly detected in the cytoplasm. This is also in line with previous results in glioblastoma where even under nonstimulated conditions, EGFRvIII was primarily found in the cytoplasm (25). In the latter study, constitutive tyrosine phosphorylation of EGFRvIII was identified as the molecular mechanism leading to the translocation of EGFRvIII from the membrane to the cytoplasm (25). These data, together with our results, are suggestive of the EGFR signaling pathway being constitutively activated in recurrent or initially metastatic SCCHN.

Our study showed for the first time that high AREG expression in SCCHN tumors is an independent prognosticator of poor outcome on cetuximab–docetaxel treatment. This is in clear contrast to the results from clinical studies of mCRC (14, 15) and NSCLC (26), in which the clinical activity of treatment with the EGFR targeting agents cetuximab, gefitinib, or erlotinib was positively associated with AREG expression. Technical differences in the AREG immunostaining methods as a possible reason for differences in the prognostic value of AREG expression levels are rather unlikely because the same antibody was used in the NSCLC (26) and our study. Furthermore, although AREG mRNA and not, as in our study, protein levels were determined in mCRC (14, 15), a good correlation between mRNA and protein expression levels, at least in NSCLC...
and SCCHN cell line models, has been reported (17). Our results, therefore, suggest that differences in the biological function of AREG depending on the type of epithelial tissue from which the tumor originates might be responsible for the observed differences in the predictive value of AREG expression.

The assessment of the biological functions of AREG in animal models revealed moderate basal expression of AREG in normal gastrointestinal mucosa, which is upregulated after infection with *Helicobacter pylori* (27) or nematode parasites (28). During the transformation process, tumor cells derived from the intestinal mucosa (e.g., CRC) might have not only preserved the expression of AREG as tissue-protective factor but might even have upregulated its expression to acquire a survival advantage. Indeed, high AREG expression has been reported in CRC where it correlated with poor outcome (29). It is tempting to speculate that AREG-expressing tumor cells may become dependent on the constitutive activity of the EGFR signaling pathway. Such a mechanism, termed *pathway addiction*, has indeed been proposed as a possible explanation for the positive association of AREG expression with the efficacy of cetuximab treatment in mCRC (15). In line with the peculiar role of EGFR signaling in gastrointestinal mucosa,

### Table 2. Cox regression model for the interaction between molecular/clinical parameters and PFS

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<th>Multivariate analysis</th>
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<td><strong>P</strong></td>
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<td><strong>HR (95% CI)</strong></td>
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<td>EGFRvIII</td>
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<td>37 1.0</td>
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<td>AREG</td>
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<td>EGFR R521K</td>
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### Table 3. Cox regression model for the interaction between molecular/clinical parameters and OS

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<tr>
<td><strong>N</strong></td>
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<td>13 1.9 (0.9–3.8)</td>
<td>1.7 (.8–3.8)</td>
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there is also the clinical observation that cetuximab given concurrently with or after an aggressive induction chemotherapy such as docetaxel, cisplatin, 5-fluorouracil (TPF; ref. 30 and results from the EORTC24061, orally presented by Dr. Vermorken, Athens, November 2010) can result in severe and life-threatening gastrointestinal toxicities including mucositis, enteritis, and diarrhea. Such dose-limiting toxicities have never been reported for the mucosa in the head and neck region nor have we observed such effects on oral mucosa in our own ongoing clinical trials.

Such a difference in the biological role of EGFR signaling in different epithelial mucosal types, however, cannot explain the discrepant results on the role of AREG as a predictor of gefitinib or erlotinib treatment in NSCLC. High AREG protein expression was significantly associated with prolonged PFS and OS in a cohort of 73 NSCLC patients treated with gefitinib or erlotinib (26); however, increased AREG levels in plasma (31), serum (32), or tumor tissue (33) did not interfere with treatment efficacy or were associated with progressive disease on EGFR targeting in 3 further studies, although in all 4 study populations, the proportion of adenocarcinoma/squamous cell carcinoma was comparable. Certainly, additional studies are needed to understand the molecular basis for the different role of AREG for the prediction of treatment efficacy in CRC, NSCLC, and SCCHN.

In our study, the expression of wild-type EGFR did not interfere with the outcome, which is in line with results from studies combining cetuximab with platinum-based chemotherapy (9) or radiotherapy (7). However, we identified the expression of its deletion variant, EGFRvIII, as an independent prognostic factor of the DCR and PFS on cetuximab-docetaxel. Our data corroborate results from SCCHN cell line models in which ectopic expression of EGFRvIII not only increased tumor cell proliferation in vitro and tumor growth in a xenograft model but also rendered EGFRvIII not only increased tumor cell proliferation in vitro but also conferred resistance to cisplatin and cetuximab treatment (13). Lower sensitivity of EGFRvIII-positive tumors to cisplatin treatment might also explain the higher frequency of EGFRvIII detection in our study compared with the study of Sok and colleagues (13), considering that only patients who relapsed after cisplatin-containing first-line treatment were eligible for our study.

Given that EGFRvIII also negatively interferes with radiosensitivity (34), all 3 major cornerstones of SCCHN treatment are potentially compromised by EGFRvIII expression, which makes the development of novel strategies for this unfavorable patient group an urgent clinical need. Because EGFRvIII is expressed exclusively by tumor cells, targeting of EGFRvIII may represent an attractive strategy to improve the clinical efficacy of chemoradiation as well as cetuximab-containing regimens without increasing the side effects of such multimodal treatment regimens. A specific antibody to EGFRvIII conjugated with cytotoxic compounds is currently being developed and has already proven efficacious in animal models of glioblastoma (35). Alternatively, SCCHN patients with EGFRvIII-positive tumors might benefit from next-generation tyrosine kinase inhibitors like HKI-272, which, when compared with gefitinib or erlotinib, showed a 100-fold higher potency in inhibiting the growth of EGFRvIII-transformed lung tumor cancer cells in vitro and in vivo (36).

In conclusion, high expression levels of both AREG and EGFRvIII were associated with reduced efficacy of cetuximab-docetaxel treatment in recurrent or initially metastatic SCCHN. Because of the small cohort size and the nonrandomized design of our clinical study, the predictive values of AREG and EGFRvIII remain undetermined. Prospective clinical evaluation of these biomarkers and their plasticity over time in larger patient cohorts will be necessary to establish whether or not expression of EGFRvIII and AREG can be used for tailoring treatment or for selecting patients for novel treatment strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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