Cetuximab Resistance in Squamous Carcinomas of the Upper Aerodigestive Tract Is Driven by Receptor Tyrosine Kinase Plasticity: Potential for mAb Mixtures

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Abstract

Squamous cell carcinomas (SCC) arising in upper parts of the aerodigestive tract are among the leading causes of death worldwide. EGFR has been found to play an essential role in driving the malignancy of SCC of the upper aerodigestive tract (SCCUAT), but, despite this, clinical results using a range of different EGFR-targeted agents have been disappointing. Cetuximab is currently the only EGFR-targeted agent approved by the FDA for treatment of SCCUAT. However, intrinsic and acquired cetuximab resistance is a major problem for effective therapy. Thus, a better understanding of the mechanisms responsible for cetuximab resistance is valuable for development of the next generation of antibody therapeutics. In order to better understand the underlying mechanisms of cetuximab resistance in SCCUAT, we established from cetuximab-sensitive models cell lines with acquired resistance to cetuximab by continuous selective pressure in vitro and in vivo. Our results show that resistant clones maintain partial dependency on EGFR and that receptor tyrosine kinase plasticity mediated by HER3 and IGF1R plays an essential role. A multitarget mAb mixture against EGFR, HER3, and IGF1R was able to overcome cetuximab resistance in vitro. To our surprise, these findings could be extended to include SCCUAT cell lines with intrinsic resistance to cetuximab, suggesting that the triad consisting of EGFR, HER3, and IGF1R plays a key role in SCCUAT. Our results thus provide a rationale for simultaneous targeting of EGFR, HER3, and IGF1R in SCCUAT. Mol Cancer Ther; 15(7): 1614–26. ©2016 AACR.

Introduction

EGFR overexpression is a common characteristic of squamous cell carcinomas (SCC) arising in upper parts of the aerodigestive tract (UAT) including lips, oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, paranasal sinuses, nasal cavity, salivary glands, esophagus, and trachea (1–3). Gene amplification is a common cause of EGFR overexpression in SCCUAT and has been correlated with poor prognosis, indicating that these tumors have a dependency on EGFR (4–7). Hence, targeting of EGFR in SCCUAT is an attractive and pursued strategy.

Despite evidence demonstrating an important role of EGFR in SCCUAT, clinical results using a range of different EGFR-targeted agents have been disappointing (8, 9). Cetuximab is currently the only EGFR-targeted agent approved by FDA for treatment of SCC arising from the UAT. The overall response rate of cetuximab rapidly develop resistance to treatment (10, 11). Cetuximab combined with radiotherapy is approved for treatment of primary SCC of the oropharynx, hypopharynx, or larynx, and cetuximab combined with platinum is approved for treatment of recurrent or metastatic SCC of the oropharynx, hypopharynx, larynx, and oral cavity, based on positive clinical trials (12, 13). Cetuximab has been tested in combination with irinotecan, cisplatin, and concurrent radiotherapy in esophageal cancer in multiple phase II studies (14–17). The results are mixed regarding safety and question the efficacy of including cetuximab in treatment. Currently, two phase III studies are evaluating the efficacy of cetuximab in combination with paclitaxel, cisplatin, and radiotherapy in patients with locally advanced esophageal cancer (NCT00655876) and cetuximab in combination with radiotherapy and chemotherapy followed by surgery in patients with locally advanced esophageal cancer that can be removed by surgery (NCT01107639). Other anti-EGFR antibodies, such as panitumumab and zalutumumab, have been tested in open-label phase III trials in SCC of the head and neck, in combination with cisplatin and fluorouracil (NCT00460265) or together with best supportive care, respectively (NCT00382031; refs. 9, 18). These trials show no increase in overall survival when adding the anti-EGFR mAb in the treatment.

Efforts to establish a positive correlation between EGFR levels determined by IHC and/or EGFR gene amplification (FISH) with response to cetuximab in SCCUAT have so far been inconclusive (2, 19, 20). Generally, EGFR levels or gene amplification status fail to predict response to EGFR-targeted...
agents across indications, which is in contrast to, e.g., HER2 and MET, where gene amplification status correlates well with response to targeted agents (21, 22). The reason for this difference is currently unknown. A possible explanation could be that EGFR interacts with a larger repertoire of cell surface molecules and activates a larger network of downstream signaling pathways compared with HER2 and MET. Hence, resistance to EGFR-targeted agents occurs more readily relative to HER2- and MET-targeted agents.

Mechanistically, cetuximab blocks EGFR activity by stabilizing the closed conformation of the receptor, thereby preventing ligand binding and receptor dimerization (23–25). Secondary effector functions, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), mediated by the Fc region of the antibody are part of cetuximab’s antitumor activity in patients, but the relative contribution is unknown (26–28). It is likely that the above-described mechanism of cetuximab action is insufficient for efficient EGFR targeting due to the plasticity/promiscuity of the receptor. More effective EGFR targeting strategies are in clinical development, including mixtures of anti-EGFR antibodies that induce EGFR degradation and secondary effector enhanced antibodies (29–33). Whether or not these will turn out to be superior to cetuximab remains to be seen.

In order to enhance the understanding of the underlying mechanisms of cetuximab resistance in SCCUAT, we established two cell line models with acquired cetuximab resistance, by exposing cetuximab-sensitive cell lines to continuous selective pressure in vitro and in vivo. Results show that cetuximab-resistant clones sustain partial dependency on EGFR and that HER3 and IGFR1 play essential roles in mediating receptor tyrosine kinase (RTK) plasticity in the escape of cetuximab treatment. Acquired cetuximab resistance in vitro was defeated by a multitargeted mixture of five mAbs against EGFR, HER3, and IGFR1 (HEI). To our surprise, RTK plasticity mediated by HER3 and IGFR1 also existed in SCCUAT cell lines with intrinsic resistance to cetuximab, suggesting that the triad consisting of EGFR, HER3, and IGFR1 plays a substantial role in resistance in SCCUAT.

**Materials and Methods**

**Cell cultures**

The cell lines HN5 and HN6 were provided by the Department of Radiation Biology (Copenhagen University Hospital, Denmark) and maintained in 10% FBS DMEM (Invitrogen) with 1% penicillin and streptomycin. Detroit562, FaDu, SCC9, SCC15, SCC25, KYSE30, KYSE140, KYSE150, KYSE520, TE-11, COLO680N, A-253, and CGTH-W1 were obtained from the Danish Co-operation for Cell Cultures. Detroit562, FaDu, SCC9, SCC15, SCC25, KYSE30, KYSE140, KYSE150, KYSE520, TE-11, COLO680N, A-253, and CGTH-W1 were obtained from the American Type Culture Collection. Cell cultures were maintained in 10% FBS DMEM, 1% penicillin and streptomycin, and 50 μg/mL gentamicin and cultured according to the supplier’s recommendations. The cell lines were minced into small pieces, and tumor cells isolated as described previously (34). The established cell lines were maintained in 10% FBS DMEM, 1% penicillin and streptomycin, and 50 μg/mL gentamicin.

**Production of antibodies and antibody mixtures**

Eight- to 10-week-old BALB/c or C57BL/6 female mice were immunized with IGFR1-Fc. Spleens were recovered and macerated through a 74-μm cell strainer (Corning) generating single-cell suspensions. Antibodies specific for IGFR1 were cloned using the murine Symplex technology, as previously described by Koefoed and colleagues (30). The two anti-EGFR antibodies (1565, 1277) and the two anti-HER3 antibodies (5038, 5082) are described by Koefoed and colleagues. (30) and Jacobsen and colleagues (35). Antibody mixtures were generated freshly prior to the individual experiments and mixed in ratios of 1:1 or 1:1:1 (w/w).

**Viability assay**

A standard 4-day WST-1 viability assay was used to measure growth and growth inhibition following treatment with mAbs and mAb mixtures at a final concentration of 50 μg/mL and performed as described in Pedersen and colleagues (29). The number of viable cells was calculated as percentage of untreated control cells. Two-fold serial dilutions of mAbs or mAb mixtures were prepared immediately prior to performing the experiment.

**Immunoblot analyses**

Whole-cell lysis was performed in Pierce RIPA Buffer (Thermo Scientific) with Protease Inhibitor (Thermo Scientific) and Phosphatase Inhibitor (Calbiochem). Lysates were sonicated, and the amount of protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific). Proteins were separated by the NuPAGE SDS-PAGE Gel System (Life Technologies) with 3% to 8% Tris Acetate and 4% to 12% Bis-Tris precast gels as per the manufacturer’s instructions. Proteins

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were transferred to nitrocellulose membranes by iBlot (Life Technologies) and incubated with primary antibody overnight at 4°C. Immunocomplexes were visualized by addition of 800 CW fluorescently labeled secondary antibodies and imaged using the Odyssey System (LI-COR Bioscience) with Image Studio software version 2.1.

Locked nucleic acid–mediated EGFR knockdown
Cells were seeded in a 6-well plate and allowed to adhere before being transfected with 5 nmol/L EGFR-LNA (locked nucleic acid), 5′ATGGAGAGTGAGTC′3 (Exiqon), scramble-LNA, or HiPerfect being transfected with 5 nmol/L EGFR-LNA (locked nucleic acid), 5′ATGGAGAGTGAGTC′3 (Exiqon), scramble-LNA, or HiPerfect

Immunoprecipitation
Cells were lysed with 1X Cell Lysis Buffer (Cell Signaling Technology) with protease inhibitor and phosphatase inhibitor according to the manufacturer’s instruction (Thermo Scientific). Note that 500 μg to 1 mg of protein were incubated overnight with 2 μg of mAb5259 (HER3) with gentle rotation. Protein G magnetic beads (Cell Signaling Technology) were added for 30 minutes. The immunoprecipitates were pelleted down using DynaMag (Invitrogen) and washed three times with lysis buffer. The captured immunocomplexes were eluted, boiled in 1.5 X SDS sample buffer for 5 minutes, and subjected to immunoblot analysis as described above.

Cetuximab binding assay
Cells (1,000 cells/well) were seeded in 384-well Optical Assay Plates (Nunc). The following day, cells were fixed with 2% paraformaldehyde for 15 minutes at 37°C. Excess paraformaldehyde was removed by washing the plates. A 2-fold dilution of cetuximab (27 nmol/L) was added, and the plates were incubated at room temperature for 48 hours. Following washes with 1X PBS, cells were incubated with DyLight800-conjugated Fab Fragment Goat Anti-Human IgG (1:500; LI-COR Bioscience) for 30 minutes on ice. Next, DRAQ-5 (1:5,000) was added to the cells for nuclei staining followed by 15-minute incubation at room temperature. Binding of cetuximab to cell surface EGFR was visualized using the Odyssey System (LI-COR Bioscience).

Flow cytometry
Cells were harvested by applying Cell Dissociation Reagent (Life Technologies) and dissolved in normal growth media. A cell suspension containing 5 × 10^5 cells in 250 μL 1X PBS containing 2% FBS was incubated with 0.25 μg of either FITC-conjugated control IgG (Abcam) or FITC-conjugated anti-EGFR antibody (Abcam) for 15 minutes at 5°C. The cells were then washed twice and analyzed using a BD FACSVerse flow cytometer (BD Bioscience).

EGFR copy number
Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen). Quantitative PCR was carried out using qBiomer SYBR Green FAST Mastermix, qBiomer EGFR Copy Number PCR assay, and qBiomer Multicopy Reference PCR assay (Qiagen) with 2 ng DNA as template. Analysis was performed in triplicates in on a QuantStudio 6 Flex real time instrument (Life Technologies). EGFR primers and Multicopy Reference primers were supplied by SA Biosciences. EGFR gene copy number was calculated using the ΔΔ Ct method. The cell line U87-MG which contains a diploid copy number of chromosome 7 was used as reference genome for EGFR (36).

Quantitative real-time PCR
Total RNA was extracted using an RNasy Plus Mini kit (Qiagen). cDNA was synthesized using 500 ng RNA, Superscript III reverse transcriptase (Invitrogen), and oligo d/random hexamers (TAG Copenhagen). Quantitative PCR was carried out using Fast SYBR Green Master mix (Applied Biosystems). Primers were supplied by TAG Copenhagen (ERBB1 Fw 5′ to 3′: AGGCACGAGTAACAAGCTCAC, ERBB1 Rev 5′ to 3′: ATGAGGACATTACACGGAC, GAPDH Fw 5′ to 3′: GGAGCGGAGATCCCTCCAAAAT, GAPDH Rev 5′ to 3′: GGCTGTTGTCAAGTCCACAAACT, GAPDH Rev 5′ to 3′: GGCTGTTGTCAAGTCCACAACT, GAPDH Rev 5′ to 3′: GGCTGTTGTCAAGTCCACAACT, GAPDH Rev 5′ to 3′: GGCTGTTGTCAAGTCCACAACT). Analysis was done in triplicate, and GAPDH was used as internal control.

Statistical analysis
Data analysis was performed in GraphPad Prism version 5 for Windows. The difference between treatments was analyzed using unpaired Student t test, and P values less than 0.05 were considered statistically significant.

Results
EGFR status in cell lines originating from SCCUAT
According to published data in cBioPortal, cancer cell lines and tissues originating from SCCUAT have high levels of EGFR expression compared with other human tissues (Fig. 1A), and focal amplification of the EGFR gene is frequently observed (Fig. 1B). A representative panel of cell lines originating from SCCUAT were selected and characterized with regards to EGFR status and sensitivity to cetuximab (Fig. 1E). Overall, a good correlation between EGFR gene copy number, EGFR mRNA level, and EGFR protein levels was found (Fig. 1C and D). Four EGFR tyrosine phosphorylation sites, associated with activation of distinct downstream signaling pathways, were investigated: Tyr1068 and Tyr1173, primarily inducing RAS/RAF/ERK activation; Tyr992, which signals through PLC-γ, and Tyr1045, which binds CBL regulating EGFR ubiquitinylation. EGFR phosphorylation levels were found to correlate with the total EGFR levels, and a similar
pattern of phosphorylation was observed for the different tyrosine residues (Fig. 1C). Cetuximab sensitivity of the cell lines was determined by a standard viability assay, and the results are shown in Fig. 1E. HN5 and FaDu were the most sensitive and HN6 the least sensitive. No clear correlation was evident between response to cetuximab and EGFR amplification status, expression levels, or basal receptor activity (Fig. 1F; Supplementary Table S1). HN5 and KYSE520 were found to be EGFR amplified and cetuximab-sensitive, whereas HN6 was found to be EGFR amplified and resistant to cetuximab. In contrast, FaDu was found to be cetuximab-sensitive but not EGFR amplified. In this cell line, the effect of cetuximab could be explained by a secondary effect on alternative RTKs involved in driving growth and proliferation of the cell line and whose activity is regulated by EGFR.

Establishment and characterization of cetuximab-resistant HN5 cell lines in vitro

HN5 was selected for establishment of cetuximab-resistant cell lines in vitro due to its high sensitivity to EGFR inhibition. HN5 cells were cultured in the presence of increasing concentrations of cetuximab until a cell population emerged that was resistant to 100 μg/mL of cetuximab. From this heterogeneous pool of cells, individual clones were established by limited dilution. Four cetuximab-resistant clones, designated HN5 CR2, HN5 CR6, HN5 CR13, and HN5 CR14, were selected for further characterization (Fig. 2A). To exclude that the acquired resistance was due to lack of cetuximab binding, a cell-based binding assay was performed. The EC50 of cetuximab was unaltered in the resistant clones, whereas the maximum level of cetuximab binding was found to be lower (Supplementary Fig. S1). Immunoblot analysis and flow cytometry confirmed that the resistant clones had significantly lower levels of EGFR compared with the parental cell line (Fig. 2B; Supplementary Fig. S2).

The function of EGFR in resistant clones was investigated by measuring the levels of pEGFR and downstream signaling upon EGF stimulation. Stimulation of the resistant clones with EGF led to phosphorylation of EGFR as well as downstream signaling molecules. Higher levels of ERK1/2 phosphorylation were seen in resistant clones in the presence and absence of EGF compared with parental cells. The higher levels of pEGFR (Ser1046/1047) in parental cells suggest a stronger feedback inhibition in parental cells than in the resistant clones (Fig. 2B; Supplementary Fig. S3; refs. 37, 38). Collectively, these data demonstrate that, although EGFR levels are lower, the receptor remains functional and active in the investigated cetuximab-resistant cell clones.

It has previously been shown that antibody pairs targeting nonoverlapping epitopes on EGFR can induce internalization and near-complete degradation of the receptor (29). We therefore tested whether a mixture of two anti-EGFR antibodies targeting nonoverlapping epitopes induced EGFR degradation in resistant clones and whether this would overcome the cetuximab resistance. The mAb mixture was found to inhibit growth of the resistant clones (Fig. 2C) and to cause rapid EGFR degradation (Fig. 2D; Supplementary Fig. S4). In contrast, cetuximab did not result in EGFR degradation to any significant extent. Similar results were obtained using LNA-mediated knockdown of EGFR, which led to inhibition of growth and proliferation of the resistant clones (Supplementary Fig. S5). Collectively, these results indicate that the cetuximab-resistant HN5 clones remain partially dependent on EGFR.

Immunoblot analyses investigating EGFR phosphorylation and downstream signaling after 48 hours of treatment with anti-EGFR 2 mixture did not show any significant difference in EGFR phosphorylation between parental HN5 cells and resistant clones. However, both cetuximab-resistant cell lines had higher pAKT(Ser473) levels (Fig. 2D). Interestingly, in both parental and resistant clones, total levels of HER3 and pHER3 increased upon treatment with EGFR 2 mixture. Furthermore, IGF1R remained phosphorylated (Tyr1135) in the cetuximab-resistant clones despite treatment. These data suggest that alternative RTKs, such as IGF1R and HER3, are able to compensate to maintain downstream signaling when EGFR activity is inhibited.

Establishment of cetuximab-resistant FaDu cell lines in vivo

In order to extend and confirm the in vitro results, we wanted to establish and characterize cell lines derived from tumors resistant to cetuximab in vivo. Since HN5 grows poorly as xenografts, an alternative cetuximab-sensitive cell line, FaDu, was used for selection of tumors with acquired cetuximab resistance. FaDu cells were inoculated subcutaneously into the flanks of mice. Cetuximab treatment was initiated when tumors reached 200 mm³ and continued until tumor regrowth occurred. The cetuximab-resistant cell lines FaDu 1420, FaDu 1441, and FaDu 1446 (Fig. 3B) were derived from three different tumors (Fig. 3A). In contrast to the in vitro–generated HN5 cetuximab-resistant clones, in vivo–derived cetuximab-resistant FaDu clones were resistant to treatment with the anti-EGFR 2-mixture (Fig. 3C). Furthermore, the levels of EGFR in the cetuximab-resistant FaDu clones were similar to those in the parental FaDu cell line (Supplementary Fig. S6). Collectively, these results suggest that cetuximab-resistant FaDu clones derived from tumors no longer have a primary dependency on EGFR activity.

Triple blockade of EGFR, HER3, and IGF1R effectively inhibits proliferation of cell lines with acquired resistance to cetuximab

To investigate potential compensatory effects of HER3 and IGF1R in HN5 cells with acquired cetuximab resistance, cells were treated with increasing concentrations of single targeting mAb and antibody mixtures targeting two or more receptors simultaneously. Monotargeting of HER3 or IGF1R had no effect on viability of HN5, HN5 CR2, or HN5 CR14 cells (Fig. 4A; Supplementary Fig. S7), whereas mixtures of antibodies against EGFR and HER3 or EGFR and IGF1R led to significant inhibition of cell viability. Simultaneous targeting of all three receptors with a mixture of five antibodies against EGFR (2 mAbs), HER3 (2 mAbs), and IGF1R (1 mAb; HEI) had an even greater effect on cell viability. Similar results were obtained using FaDu 1441 cells (Fig. 4A).

To investigate the molecular mechanisms behind the enhanced activity of the dual and triple mixtures, HN5 and FaDu cells were treated with the different mAbs and mixtures for 48 hours, and the receptor levels were determined by Western blot analyses (Fig. 4B). Monotargeting clearly caused degradation of specific RTKs. The EGFR 2 mixture was found to increase HER3 and pHER3(Tyr1289) levels, thereby demonstrating upregulation of HER3 activity as a consequence of EGFR inhibition.
Figure 2.
Partial EGFR dependency and upregulated activity of HER3 and IGF1R in cetuximab-resistant HN5 cell lines. A, HN5 and cetuximab-resistant cell lines (HN5 CR2, HN5 CR6, HN5 CR13, and HN5 CR14) treated with cetuximab for 96 hours using WST-1 in a viability assay. Data points are represented as mean ± SEM, n = 6. B, immunoblot analyses of EGFR, phosphorylated EGFR, and downstream signaling molecules. Cells were untreated or stimulated with 1 nmol/L EGF for 15 minutes before harvesting. β-Actin was used as a loading control. C, HN5, HN5 CR2, and HN5 CR14 treated with cetuximab or EGFR 2 mixture for 96 hours using WST-1 in a viability assay. Data points are shown as mean ± SEM, n = 3. D, immunoblot analyses of the indicated proteins. β-Actin was used as a loading control. HN5, HN5 CR2, and HN5 CR14 treated with cetuximab or EGFR 2 mixture for 48 hours before harvesting.
Furthermore, anti-EGFR treatment reduced the activity of pIGF1R(Tyr1135) in HN5 and HN5 CR2 cells, indicating some level of cross-talk between EGFR and IGF1R.

In general, the RTK phosphorylation levels correlated well with total receptor levels, and the observed decrease in receptor phosphorylations by triple blockade of EGFR, HER3,
HER3 and IGF1R plasticity as a mechanism of acquired resistance to cetuximab in vitro. A, HN5, HN5 CR2, FaDu, and FaDu 1441 treated with the indicated antibody mixtures for 96 hours. The level of growth inhibition was measured using WST-1 in a viability assay. Data points are represented as mean ± SEM, n = 3. B, immunoblot analyses of the impact of the antibody mixtures targeting EGFR, HER3, and IGF1R on receptor level and receptor phosphorylation level. Cells were treated with 20 μg/mL of the indicated antibody mixtures for 48 hours before harvesting. β-Actin was used as a loading control. One milligram of whole-cell lysate was subjected to immunoprecipitation analysis with an anti-HER3 antibody followed by immunoblotting for either HER3 or pHER3(Tyr1289). C, immunoblot analysis of EGFR and downstream signaling pathway activation upon antibody treatment at different time points. β-Actin was used as a loading control.
and IGF1R is most likely caused by receptor elimination (Fig. 4B). However, in FaDu and FaDu 1441 cell lines, we observed high levels of total IGF1R, but no or very low levels of pIGF1R(Tyr1135), indicating that the activity of IGF1R in this cell line is kinase- and/or autophosphorylation independent. Still, targeting IGF1R in these cell lines led to effective receptor degradation and growth inhibition, suggesting that the anti-IGF1R antibody inhibits kinase-dependent as well as kinase-independent activity of IGF1R.

To further explore potential differences in the compensatory mechanisms induced by cetuximab and HEI (anti-HER3, EGFR, and IGF1R mAb mixture) treatments, HN5 CR2 and FaDu 1441 cells were treated for different periods of time (up to 24 hours) and the levels of EGFR phosphorylation and phosphorylation of downstream signaling molecules were investigated (Fig. 4C). No major effect on EGFR phosphorylation was evident upon cetuximab treatment, and although an initial inhibition of pERK (Thr202/Tyr204) was observed, the effect was transient and back to baseline after just 4 hours. No effect of cetuximab on Akt phosphorylation was observed in these resistant cell lines.

In contrast with cetuximab, HEI treatment led to a decrease in pEGFR(Tyr1068) levels in both cell lines correlating with the observed decrease in total receptor levels. A rapid decrease in pAkt(Ser473), pAkt(Thr308), and pERK(Thr202/Tyr204) was likewise seen in HEI-treated cells, indicating that the HEI mixture, by simultaneously targeting EGFR, HER3, and IGF1R, prevents compensatory signaling and thus growth of resistant cells (Fig. 4C). A small increase in pERK(Thr202/Tyr204) at the 24-hour time-point was observed in both cell lines, suggesting that additional compensatory mechanism may kick-in.

Triple blockade of EGFR, HER3, and IGF1R effectively inhibits proliferation of cell lines with intrinsic resistance to cetuximab

Next, we wanted to explore if the promising effect of the HEI mixture seen in cell lines with acquired resistance to cetuximab could be extended to include cell lines with intrinsic resistance to cetuximab. The activity of HEI was therefore explored in a panel of SCCUAT cell lines. In the majority of the cell lines, HEI had superior inhibitory activity compared with cetuximab (Fig. 5A; Supplementary Fig. S8). Even in cell lines with intrinsic resistance to cetuximab (SCC25, Detroit 562, A-253, HN6, and Colo680N), the HEI mixture had significant inhibitory activity. Full deconvolution of the activity of the HEI mixture was obtained in the cell lines SCC25 and KYSE140 to determine the contribution of the different components (Fig. 5B). In both cell lines, HEI was found to be superior to individual mAbs and the dual RTK targeting mixtures (Fig. 5B).

The level of phosphorylation of EGFR, HER3, IGF1R, and downstream signaling molecules was also investigated in a cell line with intrinsic resistance following treatment with cetuximab or HEI (Fig. 5C and D). Similar to the results found using the two cell lines with acquired cetuximab resistance (HN5 CR2 and FaDu 1441), HEI decreased EGFR, Akt, and ERK1/2 phosphorylations in SCC25 (Fig. 5D). However, in contrast with the acquired resistant cell lines, a strong compensatory increase in pEGFR (Tyr1068) was observed in the SCC25 cell line following 48 hours of treatment with mAbs or mixtures containing anti-EGFR antibodies (Fig. 5C). Such strong compensatory increase in EGFR phosphorylation is surprising and most likely mediated by increased ligand production or cross-talk with alternative RTKs.

Discussion

Despite evidence pointing to a role of EGFR as an oncogenic driver in SCCUAT, clinical responses to cetuximab treatment are infrequent and when observed often transient (2, 10, 12). Neither EGFR levels nor EGFR gene amplification status is predictive of a therapeutic response (19, 20). Hence, acquired and intrinsic resistance is a major barrier for the clinical utility of cetuximab. In this study, we characterized a panel of cancer cell lines originating from SCCUAT with regard to EGFR status and response to cetuximab. Although several cell lines were sensitive to cetuximab, we found no clear correlation between cetuximab sensitivity and EGFR status. To investigate mechanisms driving cetuximab resistance in SCCUAT, we established cetuximab-resistant clones of two of the most sensitive cell lines, HN5 and FaDu, by continuous cetuximab pressure in vitro and in vivo, respectively. It has previously been demonstrated that acquired resistance to cetuximab can occur as a result of altered functional status of EGFR but that resistant cells remain fully or partially EGFR dependent (39, 40).

We therefore investigated the status of EGFR in the resistant cells. Decreases in EGFR receptor levels were observed in the HN5-resistant cell lines, which is most likely due to selection of clones with lower levels of EGFR gene amplification. No marked differences in EGFR levels were found in cetuximab-resistant FaDu clones. Recently, a number of mutations in the extracellular EGFR have been identified leading to loss of cetuximab binding (41). Cetuximab resistance in the cell lines investigated here was not caused by such mutations as cetuximab maintained full binding in all clones.

We and others have previously shown that a mixture of two antibodies targeting nonoverlapping epitopes on EGFR is more effective at inhibiting EGFR activity compared with cetuximab (29, 42). Treatment with an anti-EGFR 2 mixture leads to a significant reduction in the viability of cetuximab-resistant HN5 clones confirming that these clones maintain a partial dependency on EGFR. On the contrary, the cetuximab-resistant FaDu clones were unresponsive to the EGFR 2 mixture. The observed difference in response to the EGFR 2 mixture between the resistant clones may be explained by differences in their primary dependency on EGFR. The HN5 cell line has a high level of EGFR amplification, and EGFR is the primary driver of growth and proliferation. Our results suggest that resistant clones from this cell line are not easily released from their EGFR dependency and will more likely escape the effect of cetuximab by altering the level or activity of EGFR or by cross-talk with other receptors. In contrast, the FaDu cell line has no EGFR amplification and relies on multiple RTKs to sustain growth and proliferation. Hence, the EGFR dependency in this cell line is plastic and more readily lost in response to a selective pressure by cetuximab.

Time profiles of ERK1/2 and Akt phosphorylation revealed an incomplete inhibition of the Akt signaling pathway in all resistant cell lines when targeting EGFR. In addition, a strong rephosphorylation of ERK1/2 was observed after 24 hours of cetuximab treatment. Collectively, these results indicate that signal rewiring has occurred and that compensatory RTKs have been mobilized to preserve activity of downstream signaling networks.

The plasticity of the RTK family members, and thus their ability to provide compensatory signaling when the driving RTK is blocked, is a well described mechanism of acquired resistance to monotherapied targeted therapy (43–46). HER3 and IGF1R...
Figure 5.
Intrinsic cetuximab resistance in SCCUAT. A, SCCUAT cell lines treated with 50 μg/mL cetuximab or HEI for 96 hours. Growth inhibition was measured using WST-1 in a viability assay. Data points are represented as mean ± SEM, n = 3; ** P < 0.01; *** P < 0.001 when comparing efficacy at the highest antibody concentration (50 μg/mL).

B, deconvolution of HEI in SCC25 and KYSE140 cell lines. Cells were treated with increasing concentrations of the indicated antibodies for 96 hours. The level of growth inhibition was measured using WST-1 in a viability assay. ** P < 0.01 when comparing efficacy at the highest antibody concentration (50 μg/mL).

C, immunoblot analyses of the indicated proteins upon 48-hour treatment with the indicated antibody mixtures in SCC25. β-Actin was used as a loading control.

D, immunoblot analysis of EGFR and downstream signaling pathway activation upon antibody treatment at different time points. β-Actin was used as a loading control.

Multitarget mAb Mixture Overcomes Cetuximab Resistance
are among the key players due to their powerful activation of AKT signaling. HER3 is transcriptionally regulated by PI3K/AKT signaling, and inhibition of AKT activity has been shown to induce HER3 transcription in a FOXO-dependent manner (47–49). Multiple studies have confirmed a role of HER-3 in acquired resistance to EGFR inhibition (35, 45, 50). Increased IGF1R activity is another well-described mechanism of resistance to EGFR inhibition. In squamous epidermoid carcinomas with amplified EGFR, acquired resistance to the EGFR inhibitor gefitinib was explained by reduced expression of IGFBP-3 and IGFBP-4, both of which negatively modulate IGF1R by sequestering the IGF ligands (51). In another study, blockade of EGFR activity was shown to potentiate IGF1R signaling by enhancing the ability of IGF1R to couple to IRS1 (52). In models of lung cancer, increased IGF1R activation was shown to mediate chromatin modifications that confer a reversible state of "drug tolerance" to gefitinib (53). Recently, IGF2 was shown to limit the response to cetuximab in models of colorectal cancer and synergistic activity of cetuximab, and a small molecule inhibitor of IGF1R was demonstrated (54).

We therefore investigated potential synergistic activity of simultaneous targeting of EGFR, HER3, and IGF1R with the multitarget mAb mixture HEI in cell lines with acquired cetuximab resistance. Triple blockade of EGFR, HER3, and IGF1R resulted in synergistic inhibition of cell proliferation in all acquired cetuximab-resistant cell lines tested. The superior inhibitory effect of HEI could be explained by a rapid and sustained shutdown of both AKT and ERK1/2 signaling. These findings highlight the importance of inhibiting both AKT and ERK1/2 signaling in cancer cells in order to effectively inhibit their proliferation and to prevent rapid resistance development caused primarily by relief of PI3K-AKT pathway feedback inhibition of HER3 and IGF1R (Fig. 6).

Next, we investigated if the synergistic inhibitory effect of targeting the EGFR, HER3, and IGF1R triad in acquired cetuximab-resistant cell lines could be translated to SCCUAT cell lines with intrinsic cetuximab resistance. Surprisingly, HEI caused substantial and synergistic growth inhibition in most cell lines with intrinsic cetuximab resistance. Similar to what was observed in the cell lines with acquired cetuximab resistance, HEI caused rapid shutdown of both AKT and ERK1/2 activities. The broad inhibitory effect of HEI seen in this panel of SCCUAT cell lines suggests a general cooperative role of EGFR, HER3, and IGF1R in driving oncogenic signaling in this indication. This cooperation allows rapid compensation by IGF1R and HER3 following EGFR inactivation. The ease by which this occurs raises a challenge for EGFR mono-targeted therapy in SCCUAT patients.

In summary, our study confirms that EGFR monotargeting is likely to be ineffective in patients with SCCUAT and underscores the role of RTK plasticity in intrinsic and acquired cetuximab resistance. A triad consisting of EGFR, HER3, and IGF1R appears to act as codrivers of oncogenic signaling and suggests that simultaneous targeting of these three receptors with a multitargeting mAb mixture may be an effective strategy in this indication.
Disclosure of Potential Conflicts of Interest

I.D. Horak has ownership interest (including patents) in, Symphogen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: I. Kjær, I.D. Horak, M. Kragh, M.W. Pedersen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.W. Pedersen
Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): I. Kjær, T. Lindsted, C. Frolich, M.W. Pedersen

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Cetuximab Resistance in Squamous Carcinomas of the Upper Aerodigestive Tract Is Driven by Receptor Tyrosine Kinase Plasticity: Potential for mAb Mixtures

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