

Signaling via ErbB2 and ErbB3 Associates with Resistance and Epidermal Growth Factor Receptor (EGFR) Amplification with Sensitivity to EGFR Inhibitor Gefitinib in Head and Neck Squamous Cell Carcinoma Cells

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Abstract Purpose: The epidermal growth factor receptor (EGFR) inhibitor gefitinib (Iressa) has shown antitumor activity in clinical trials against cancers, such as non – small cell lung cancer and head and neck squamous cell carcinoma (HNSCC). Research on non – small cell lung cancer has elucidated factors that may predict response to gefitinib. Less is known about molecular markers that may predict response to gefitinib in HNSCC patients.

Experimental Design: We analyzed possible associations of responsiveness to gefitinib with molecular markers of the EGFR/ErbB receptor family signaling pathway using 10 established HNSCC lines *in vitro*. IC₅₀ of gefitinib sensitivity was determined using clonogenic survival assays. ErbB signaling was assessed by Western and real-time reverse transcription-PCR analyses of EGFR, ErbB2, ErbB3, and ErbB4 expression levels as well as by phosphorylation analysis of pEGFR, pErbB2, pErbB3, pAkt, and pErk. *EGFR* sequences encoding kinase domain and *EGFR* gene copy numbers were determined by cDNA sequencing and real-time PCR, respectively. Finally, responsiveness to gefitinib was compared with responsiveness to the anti-EGFR antibody cetuximab (Erbix).

Results: Expression levels of pErbB2 ($P = 0.02$) and total ErbB3 protein ($P = 0.02$) associated with resistance to gefitinib. Combining gefitinib with pertuzumab (Omnitarg), an antibody targeting ErbB2 heterodimerization, provided additional growth-inhibitory effect over gefitinib alone on relatively gefitinib-resistant HNSCC cell lines. The same markers did not predict resistance to cetuximab. In contrast, a similar trend suggesting association between *EGFR* gene copy number and drug sensitivity was observed for both gefitinib ($P = 0.0498$) and cetuximab ($P = 0.053$). No activating EGFR mutations were identified.

Conclusions: *EGFR* amplification may predict sensitivity to gefitinib in HNSCC. However, other EGFR/ErbB receptor family members than EGFR may contribute to resistance to gefitinib. ErbB2 and ErbB3 may have potential as predictive markers and as therapeutic targets for combination therapy in treatment of HNSCC with gefitinib.

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein that includes a tyrosine kinase enzyme within its intracellular domain (1). EGFR belongs to the ErbB subfamily of receptor tyrosine kinases that consists of EGFR (also called ErbB1 or HER-1), ErbB2 (HER-2 or Neu), ErbB3 (HER-3), and ErbB4 (HER4) (2). ErbB3 differs

from the other ErbB receptors in possessing a functionally inefficient tyrosine kinase domain (3). ErbB2 is unique, as none of the known ErbB family ligands activates ErbB2 homodimers (4). Instead, ErbB2 seems to function primarily as a heterodimerization partner for other ErbB family members (5).

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Table 1. Characteristics of the SCC cell lines

Cell line	Gender	Primary tumor location	Tumor-node-metastasis*	Specimen site	Type of lesion	Grade	Area under the curve (Gy), x ± SD	Gefitinib IC ₅₀ (μmol/L), x ± SD	Cetuximab IC ₅₀ (nmol/L), x ± SD
UT-SCC-8	Male	Supraglottic larynx	T ₂ N ₀ M ₀	Larynx	Primary	1	1.9 ± 0.1	0.30 ± 0.02	1.0 ± 0.3
UT-SCC-9	Male	Glottic larynx	T ₂ N ₁ M ₀	Neck	Metastasis	1	1.4 ± 0.1	0.93 ± 0.25	1.3 ± 0.2
UT-SCC-11	Male	Glottic larynx	T ₁ N ₀ M ₀	Larynx	Recurrence	2	2.0 ± 0.2	0.64 ± 0.15	2.7 ± 0.7
UT-SCC-19a	Male	Glottic larynx	T ₄ N ₀ M ₀	Larynx	Primary	2	1.7 ± 0.1	0.18 ± 0.02	6.8 ± 1.5
UT-SCC-24a1	Male	Tongue	T ₂ N ₀ M ₀	Tongue	Primary	2	2.6 ± 0.3	0.60 ± 0.10	2.1 ± 0.2
UT-SCC-24a2	Male	Tongue	T ₂ N ₀ M ₀	Tongue	Primary	2	2.6 ± 0.3	0.15 ± 0.03	1.2 ± 0.5
UT-SCC-29	Male	Glottic larynx	T ₂ N ₀ M ₀	Larynx	Primary	1	1.8 ± 0.2	0.82 ± 0.33	6.7 ± 2.3
UT-SCC-34	Male	Supraglottic larynx	T ₄ N ₀ M ₀	Supraglottic larynx	Primary	1	2.1 ± 0.1	0.32 ± 0.12	6.2 ± 1.2
UT-SCC-38	Male	Glottic larynx	T ₂ N ₀ M ₀	Larynx	Primary	2	2.3 ± 0.3	8.40 ± 1.10	8.2 ± 1.6
UT-SCC-40	Male	Tongue	T ₃ N ₀ M ₀	Tongue	Primary	1	2.3 ± 0.2	0.40 ± 0.03	0.6 ± 0.2

NOTE: Grade 1, well differentiated; grade 2, moderately differentiated; grade 3, poorly differentiated. Intrinsic radiosensitivity is expressed as the area under the survival curve equivalent to the mean inactivation dose.

*Tumor-node-metastasis classification according to the International Union against Cancer (1997).

There are several endogenous ligands for the EGFR, including the well-characterized epidermal growth factor and transforming growth factor- α (6). Ligand binding leads to receptor dimerization (homodimerization between two identical receptors or heterodimerization between different receptors of the same family), which produces structural changes in the intracellular portion of the receptor that activate the tyrosine kinase domain. Activation of the kinase initiates the intracellular signaling cascade by phosphorylation of the receptor molecule itself (autophosphorylation) as well as of several downstream targets. Major signal transduction pathways activated by EGFR include the Ras-Raf-Erk and the phosphoinositide 3-kinase/Akt cascades, which convey the signal to cellular responses, such as proliferation and survival (7).

Aberrant EGFR signaling has been suggested to play a role in the growth of several tumor types (8). In head and neck squamous cell carcinoma (HNSCC), EGFR overexpression has been detected in the majority of tumors (9). High EGFR expression levels in HNSCC have also generally been associated with reduced survival, increased risk of recurrence, metastasis, and resistance to radiotherapy (9–12). To specifically target cancer types with EGFR overactivity, two predominant classes of EGFR inhibitors have been developed: monoclonal antibodies that target the extracellular domain of EGFR, such as cetuximab (Erbix), and small-molecule tyrosine kinase inhibitors (TKI) that target the receptor catalytic domain of EGFR, such as gefitinib (Iressa) and erlotinib (Tarceva) (13). Both antibodies and TKIs targeting EGFR have shown antitumor effect on HNSCC patients in clinical trials (14–16).

Data from clinical trials indicate that only a minority of HNSCC patients benefit from EGFR-targeted therapies. Recent studies on non-small cell lung cancer (NSCLC) showed that specific mutations in the kinase domain of EGFR gene may predict sensitivity to EGFR TKIs (17, 18). Identical somatic mutations have been reported in tumor tissues derived from HNSCC patients of Asian origin (19), but the predictive significance of the mutations in HNSCC has not been

documented. To date, little is known about predictive markers of responsiveness of HNSCC patients to EGFR-targeted drugs.

In this study, we determined the sensitivity of 10 established HNSCC cell lines to gefitinib and analyzed the associations of gefitinib responsiveness with molecular markers of the ErbB signaling system. The results indicate that analysis of signaling variables of ErbB receptors may provide predictive information and that simultaneous targeting of EGFR and other ErbB family members may suppress the growth of HNSCC cells.

Materials and Methods

Cell culture. Ten recently established HNSCC cell lines were used. The cell lines UT-SCC-24a1 and UT-SCC-24a2 were derived from different parts of the patient's tumor. The characteristics of the cell lines are shown in Table 1.

Before experiments, the cells were maintained in logarithmic growth in T25 culture flasks by passing weekly in DMEM containing 2 mmol/L L-glutamine, 1% nonessential amino acids, 100 units/mL streptomycin, 100 units/mL penicillin, and 10% fetal bovine serum. Cells in midlogarithmic growth (40–60% confluent) were used for experiments and fed with fresh medium on the day before plating.

ErbB inhibitor drugs. Gefitinib (kindly provided by AstraZeneca, MacClesfield, United Kingdom) was freshly diluted in DMSO and used at concentrations ranging from 0.05 to 20 μ mol/L. Pertuzumab (Omnitarg; kindly provided by Dr. Mark Sliwkowski, Genentech, San Francisco, CA) was used at concentrations 0, 2, and 20 μ g/mL. Cetuximab (kindly provided by Merck KGaA, Darmstadt, Germany) was used at concentrations ranging from 0.5 to 20 nmol/L.

Growth curves. The cells were harvested with trypsin-EDTA, washed, resuspended, and counted. Cell density was adjusted to 10,000 cells/mL, and 1 mL suspension was placed in each well of a 24-well plate. Gefitinib was added 4 days after seeding at the indicated concentrations. The medium containing gefitinib was replaced daily to ensure that nutrient depletion did not affect cell growth and that fresh and effective drug was present during each day of the experiment. The cell number of triplicate wells for each drug concentration was counted under a microscope using a hemocytometer every other day until a plateau growth phase was reached. The average of triplicate wells is presented.

Clonogenic survival assay. The 96-well plate clonogenic assay based on limiting dilutions was used. The assay has been described earlier in detail (20, 21). Shortly, the cells were harvested with trypsin-EDTA, counted, and suspended in Ham's F-12 medium containing 15% fetal bovine serum. With a stock solution containing 4,167 cells/mL and 120 μ L of this solution diluted in 50 mL growth medium, a concentration of 2 cells per well was achieved by applying 200 μ L of this suspension to each well. The number of cells plated per well was adjusted according to the plating efficiency of the cell line. The desired gefitinib or cetuximab concentrations were added to the stock cell suspensions immediately before plating into culture plates. The plates were incubated at 37°C for 4 weeks, after which the number of wells containing coherent, living colonies, consisting of ≥ 32 cells, were counted using an inverted phase-contrast microscope. The drug was present in the medium throughout the whole incubation period. Every set with one cell line consisted of two control plates (without gefitinib or cetuximab) and five duplicate plates with five different gefitinib (0.05-10 μ mol/L) or cetuximab (0.5-20 nmol/L) concentrations. Thus, the whole set included 12 plates per cell line and drug tested. Each analysis with one cell line was repeated at least four times.

Combination of gefitinib and pertuzumab was tested with two cell lines: UT-SCC-29 and UT-SCC-38. In these studies, three different sets of plates with at least four repeats were used. The first set was used as a control with no pertuzumab, whereas in the other two sets pertuzumab was added in concentrations of 2 or 20 μ g/mL. Each set included 12 plates: 2 control plates (without gefitinib) and 5 duplicate plates with 5 different gefitinib concentrations (0.2-10 μ mol/L). The whole study with one cell line included 36 plates.

The drug sensitivities were expressed as IC_{50} s, corresponding to 50% clonogenic inhibition of the surviving fraction obtained from the dose-response curves, after fitting the data to the linear quadratic model. The plating efficiency was calculated using the formula: $-\ln[(\text{number of negative wells}) / (\text{total number of wells})] / (\text{number of cells plated per well})$. Fraction survival data as a function of the gefitinib or cetuximab dose were found to be fit in the linear quadratic equation. A microcomputer program was used to fit data to $F = \exp[-(\alpha D + \beta D^2)]$ and to obtain the area under the curve by numerical integration. Area under the curve, equivalent to mean inactivation dose, was used to compare the drug sensitivity of individual cell lines (22).

Western blot analyses. For protein expression and phosphorylation analysis, cells were lysed and samples equivalent to 50 to 75 μ g total protein were separated in 8% to 10% SDS-PAGE gels followed by Western

blotting as described previously (23). ErbB protein expression was analyzed with anti-ErbB1 antibody (sc-03), anti-ErbB2 antibody (sc-284), anti-ErbB3 antibody (sc-285), and anti-ErbB4 antibody (sc-283; all from Santa Cruz Biotechnology). Mitogen-activated protein kinase expression was analyzed with an anti-p44/42 antibody (Cell Signaling Technology, Danvers, MA) and Akt expression with an anti-Akt antibody (sc-1618; Santa Cruz Biotechnology, Santa Cruz, CA). Expression of actin was analyzed as a control with anti-actin antibody (sc-1616; Santa Cruz Biotechnology). For phosphorylation analyses, anti-pEGFR antibody (Tyr¹⁰⁶⁸), anti-phospho-HER-2/ErbB2 antibody (Tyr¹²⁴⁸), anti-phospho-HER-3/ErbB3 receptor antibody (Tyr¹²⁸⁹), anti-phospho-p44/42 mitogen-activated protein kinase antibody (Thr²⁰²/Tyr²⁰⁴), and anti-pAkt antibody (Ser⁴⁷³; all from Cell Signaling Technology), were used. The intensity of protein expression was quantitated by densitometry using MCID Image Analyzer (Imaging Research, Cambridge, United Kingdom).

The effect of pertuzumab on ErbB3 phosphorylation was analyzed for HNSCC lines UT-SCC-29 and UT-SCC-38. Cells were starved without serum for 2 hours followed by addition of pertuzumab (0 or 20 μ g/mL) for 1 hour. Cells were stimulated with or without NRG-1 (50 ng/mL; R&D Systems, Minneapolis, MN) for 10 minutes and lysed. Phosphorylation of ErbB3 was analyzed by Western as described above.

Real-time reverse transcription-PCR. To quantitate ErbB receptor and β -actin mRNA levels, total RNA was isolated from HNSCC cell lines as described (24). cDNA was prepared and real-time quantitative reverse transcription-PCR (Taqman) analysis was done as described previously (25). Samples were analyzed in duplicate, and in each measurement, range of the CT (threshold cycle) values was $<5\%$ of the mean. Expression of each ErbB transcript is presented as the percentage of ErbB mRNA expression relative to the expression of internal control, β -actin mRNA, measured from the same sample.

Real-time PCR analysis of EGFR amplification. For EGFR amplification analysis, genomic DNA was extracted from all HNSCC lines as described previously (26). Genomic DNA was sonicated for 5 minutes with Bioruptor UCD-200 ultrasonic homogenizer (Cosmo Bio, Tokyo, Japan). Real-time PCR was done as described (25) using the following primers and probes specific for EGFR gene (7p12) and for a reference gene MET (7q31.2) localized in the opposite arm of the same chromosome: 5'-GCGAATTCCTTTGGAAAACC-3' (EGFR 5' primer) and 5'-AAGGCATAGGAATTTTCGTAGTACA-3' (EGFR 3' primer), ProbeLibrary probe 6 (EGFR probe; Roche, Basel, Switzerland), 5'-CCCCTCATGTGAATTTCTCC-3' (MET 5' primer) and 5'-TGTATGCTCCA-CAATCACTTCTG-3' (MET 3' primer), and ProbeLibrary probe 47

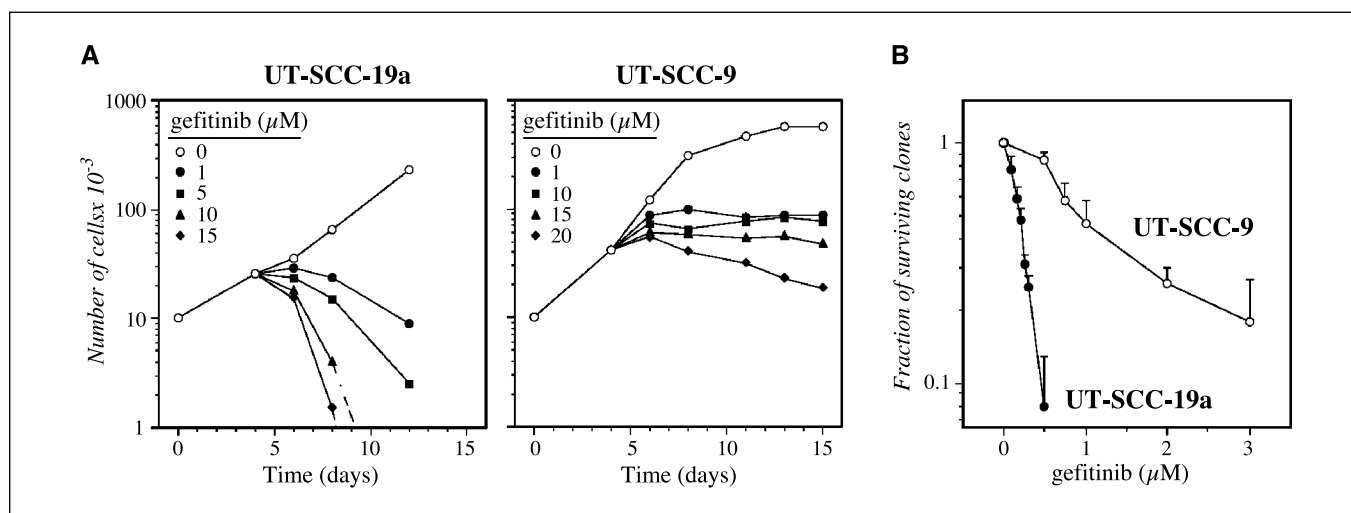


Fig. 1. Effect of gefitinib on growth of HNSCC cell lines. The effect of indicated concentrations of gefitinib on proliferation (A) and clonogenic survival (B) of UT-SCC-9 and UT-SCC-19a cell lines was measured. Proliferation was estimated by direct counting of cell numbers using hemocytometer at indicated time points. Clonogenic survival was evaluated by measuring the fraction of surviving cell colonies under microscope 4 weeks after plating approximately two viable cells per well (number of plated cells adjusted for cell line – specific plating efficiency as described in Materials and Methods).

(*MET* probe; Roche). Relative copy number of the *EGFR* gene was expressed as the ratio of PCR signals obtained for *EGFR* and *MET*. Samples were analyzed in triplicate.

Sequencing of cDNA encoding EGFR kinase domain. Total RNA was isolated from HNSCC lines and cDNA prepared as described (25). Sequence encoding the EGFR kinase domain was amplified by PCR as two overlapping fragments using the following primers: 5'-CCGGCCATGTGTGCCACCTGT-3', 5'-TCTTTGTGTCCCGGACATA-3', 5'-GTGGACAACCCACCGTGTGCC-3', and 5'-TAGGACTTGGCAAATGCATTCT-3'. Amplified DNA was separated with agarose gel electrophoresis and extracted using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). Sequencing was carried out with the same primers used for PCR amplification using BigDye Terminator version 3.1 Cycle Sequencing kit and ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

Statistical analysis. Associations between different molecular variables and sensitivity to gefitinib and cetuximab were analyzed using Spearman correlation coefficient. Statistical analysis was done with SAS System for Windows version 9.1.3.

Results

HNSCC lines are sensitive to gefitinib. To assay for responsiveness of the primary HNSCC lines to gefitinib, growth curves at different gefitinib concentrations were determined for six HNSCC lines. Cells were plated on 24-well plates at 10,000 per well and cultured in the presence of gefitinib concentrations ranging from 1 to 20 $\mu\text{mol/L}$ for up to 17 days, after which cells were counted. All six lines (UT-SCC-8, UT-SCC-9, UT-SCC-11, UT-SCC-19a, UT-SCC-24a2, and UT-SCC-34), representing HNSCC from supraglottic larynx, glottic larynx, and tongue (Table 1), responded to gefitinib, but with different sensitivity. Two sensitive cell lines (UT-SCC-8 and UT-SCC-19a) showed a clear growth inhibition already at 1.0 $\mu\text{mol/L}$, and practically all the cells died at 10 $\mu\text{mol/L}$ gefitinib in 10 days. However, although high gefitinib doses clearly slowed down the growth of two relatively resistant lines (UT-SCC-9 and UT-SCC-34), concentrations as high as 20 $\mu\text{mol/L}$ did not reduce the cell numbers below the initial plating density. Representative growth curves of relatively gefitinib-sensitive (UT-SCC-19a) and gefitinib-resistant (UT-SCC-9) HNSCC lines are shown (Fig. 1A). These data indicate that HNSCC lines differ in their sensitivity to gefitinib.

IC_{50} s for sensitivity of HNSCC lines to gefitinib. To determine the IC_{50} s of gefitinib sensitivity, all 10 HNSCC cell lines were assessed in clonogenic survival assays under different gefitinib concentrations. In these assays, cells were plated on 96 wells at predicted dilutions adjusted to plating efficiency and the cytotoxic effect (see Materials and Methods), and after 4 weeks in culture, colonies equivalent to or exceeding the size of 32 cells per well were scored as positive for growth. The IC_{50} s of gefitinib chemosensitivity varied between 0.15 and 8.40 $\mu\text{mol/L}$ (i.e., by 56-fold; Table 1), in line with a previous report on cultured HNSCC cells (27). The 10 HNSCC cell lines tested were established from tongue, glottic larynx, and supraglottic larynx cancers (Table 1). The most sensitive cell line and the most resistant cell line were both established from glottic larynx cancers, and there seemed to be no obvious association between gefitinib response and primary anatomic location of the tumor. Representative clonogenic survival assays of relatively gefitinib-sensitive (UT-SCC-19a) and gefitinib-resistant (UT-SCC-9) cell lines are shown (Fig. 1B).

ErbB expression in HNSCC lines. To identify molecular markers predicting gefitinib sensitivity, the expression of the

gefitinib target protein EGFR as well as other ErbB family members was analyzed by Western blotting. Most HNSCC lines expressed variable amounts of EGFR, ErbB2, and ErbB3, whereas no signal was detected for ErbB4 in any of the 10 cell lines (Fig. 2A). Nine of the 10 HNSCC lines used were also analyzed for ErbB mRNA expression by real-time quantitative reverse transcription-PCR. At mRNA level, the SCC cells expressed mostly EGFR followed by ErbB2 and ErbB3 mRNA expression (Fig. 2B). Consistent with the Western analysis,

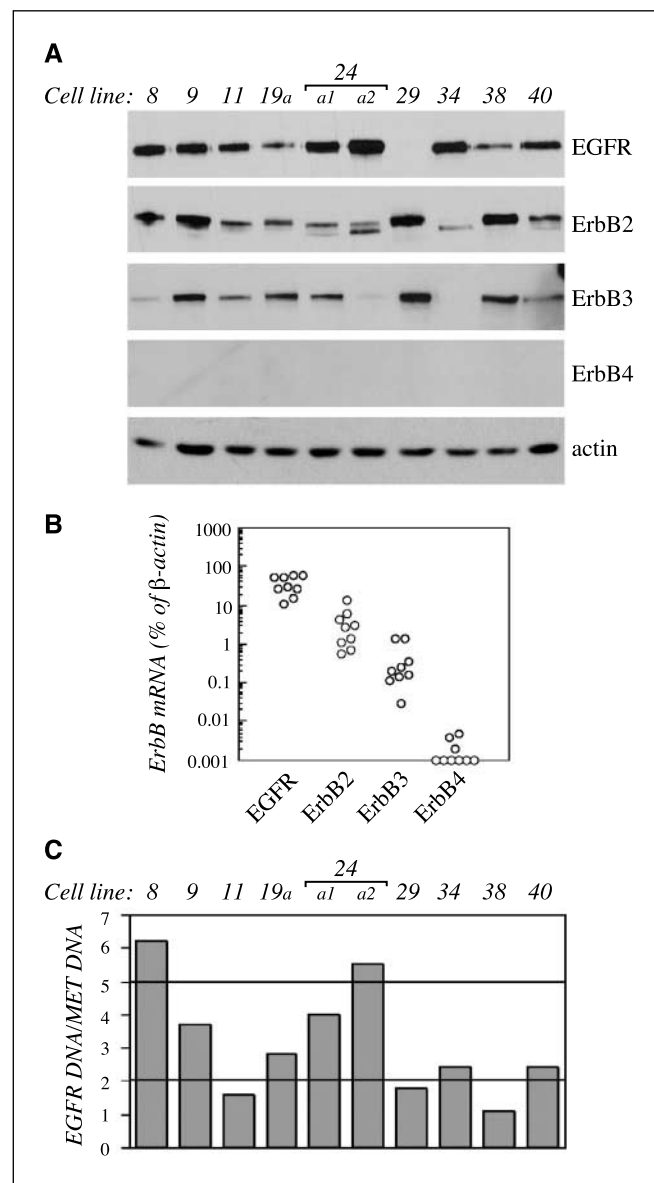


Fig. 2. Expression of EGFR, ErbB2, ErbB3, and ErbB4 in HNSCC cell lines. **A**, expression of ErbB and actin proteins was analyzed by Western blotting. **B**, expression of ErbB mRNAs was analyzed by real-time quantitative reverse transcription-PCR. For ErbB4, data on expression of JM-a isoform are shown. Similar low expression levels were detected for transcripts encoding the alternative JM-b isoform (data not shown). Expression of each ErbB transcript is presented as the percentage of ErbB mRNA expression relative to the mRNA expression of the internal control gene β -actin. **C**, genomic DNA extracted from the HNSCC lines was analyzed by real-time PCR with primers and probes specific for *EGFR* and *MET*, two genes located in opposite arms of the same chromosome 7. Relative *EGFR* gene copy number is expressed as the ratio of PCR signal obtained for *EGFR* and *MET*.

Table 2. Association of gefitinib IC₅₀ with molecular markers of ErbB signaling

	EGFR	ErbB2	ErbB3	EGFR copy no.	Cetuximab IC ₅₀
Correlation coefficient	-0.47	0.59	0.71	-0.63	0.36
<i>P</i>	0.17	0.07	0.02*	0.0498*	0.31
	pEGFR	pErbB2	pErbB3	pAkt	pErk
Correlation coefficient	-0.44	0.71	-0.08	-0.10	-0.12
<i>P</i>	0.20	0.02*	0.83	0.80	0.77

NOTE: Quantity of ErbB and pErbB signals was estimated by densitometric analysis of Western films. Associations were calculated using Spearman correlation coefficients.

**P* < 0.05, *n* = 10, except for pAkt and pErk (*n* = 9).

the signal for ErbB4 mRNA was small and close to background (Fig. 2B).

EGFR amplification and mutations in HNSCC lines. EGFR was expressed in relatively high quantities at both protein and RNA levels. To assess for *EGFR* gene amplification, the copy numbers of *EGFR* gene were determined by real-time PCR of genomic DNA isolated from the 10 HNSCC lines. Two (20%) of the lines showed relative *EGFR* copy numbers exceeding 5 (Fig. 2C), suggesting high-level amplification. In addition, 5 of the cell lines had relative *EGFR* copy numbers between 2 and 5, indicating *EGFR* gain or low-level amplification. Somatic mutations within the kinase domain of EGFR that putatively activate EGFR signaling have also been described in HNSCC patients of Asian origin (19). However, no similar mutations were identified in DNA encoding the EGFR kinase domain in any of the 10 HNSCC lines.

Association of ErbB expression and EGFR amplification with sensitivity to gefitinib. Relatively resistant SCC lines (UT-SCC-29 and UT-SCC-38) seemed to express relatively low levels of EGFR. In contrast, the expression of both ErbB2 and ErbB3 seemed to be consistently high in the three most resistant lines (UT-SCC-9, UT-SCC-29, and UT-SCC-38; Fig. 2A). For statistical analyses, the ErbB expression levels were quantitated by densitometry of the Western films. No statistically significant association was observed between gefitinib IC₅₀ measured with the clonogenic survival assays and EGFR expression levels (*P* = 0.17; Table 2). The association between high ErbB3 expression and high IC₅₀ for gefitinib, however, reached statistical significance (*P* = 0.02), and there was a tendency for similar association for ErbB2 (*P* = 0.07). These results indicate that high ErbB3, and possibly ErbB2, expression may predict resistance of HNSCC cells to gefitinib.

Two relatively sensitive lines UT-SCC-24a2 and UT-SCC-8 showed high-level *EGFR* amplification (Fig. 2C). Moreover, the most resistant UT-SCC-38 was the only line without any sign of *EGFR* gain (relative *EGFR* copy number, 1.1). A statistical significance was also reached (*P* = 0.0498) for an association between gefitinib sensitivity and *EGFR* relative copy number (Table 2). Taken together, these data suggest that *EGFR* gene amplification may predict sensitivity, whereas high protein levels of other ErbB family members are associated with resistance of HNSCC cells to gefitinib.

Predictive value of phosphorylation status of components in the ErbB signaling pathway. Molecular alterations relevant for

sensitivity to gefitinib could affect the activity of ErbB signaling pathway without changes in ErbB protein expression levels (17, 18). To address possible predictive value of ErbB phosphorylation status, the 10 HNSCC lines were subjected to Western analysis with phosphospecific anti-pEGFR, anti-pErbB2, and anti-pErbB3 antibodies (Fig. 3A). No association was observed between pEGFR (*P* = 0.20) or pErbB3 (*P* = 0.83) quantity and gefitinib IC₅₀ (Table 2). However, the association between pErbB2 levels and IC₅₀ was significant (*P* = 0.02). No predictive value was found with phospho-Western analysis of the major components of the ErbB signaling cascade, pAkt (*P* = 0.80) and the mitogen-activated protein kinases pErk1 and pErk2 (*P* = 0.77). These findings suggest that activation status of ErbB2, but not of EGFR, ErbB3, Akt, or Erk, may predict resistance to gefitinib.

Do markers that predict response to gefitinib also predict response to cetuximab? The findings suggested that pErbB2 and ErbB3 contents may predict resistance and *EGFR* amplification sensitivity to the EGFR TKI gefitinib. To address whether the observations could be extrapolated to another clinically relevant EGFR inhibitor, IC₅₀s were determined with clonogenic survival assays also for the anti-EGFR antibody cetuximab. The IC₅₀s for cetuximab varied between 0.6 and 8.2 nmol/L (i.e., by 14-fold; Table 1). In contrast to gefitinib, neither phosphorylated nor total ErbB2/ErbB3 protein levels predicted resistance to cetuximab (Table 3). However, there was a tendency of a similar association between cetuximab sensitivity and relative *EGFR* gene copy numbers (*P* = 0.053) as observed for gefitinib. Moreover, EGFR protein expression reached a significant level of association with sensitivity to cetuximab (*P* = 0.048). These findings suggest that different but partially overlapping markers may predict responsiveness to different EGFR inhibitors.

A combination of gefitinib and pertuzumab is superior to gefitinib alone in suppressing HNSCC cell growth. The observed associations of ErbB3 expression and ErbB2 phosphorylation with relative resistance to gefitinib implied that resistant cells may overcome EGFR suppression by activation of signaling via other EGFR/ErbB family members. To test this hypothesis, a humanized antibody targeting ErbB2 heterodimerization, pertuzumab, was combined with gefitinib and applied to two relatively resistant HNSCC lines (UT-SCC-29 and UT-SCC-38). Pertuzumab had a dose-dependent additive effect when combined with gefitinib in suppressing the clonogenic survival of both HNSCC lines (Fig. 4A and B). The activity of

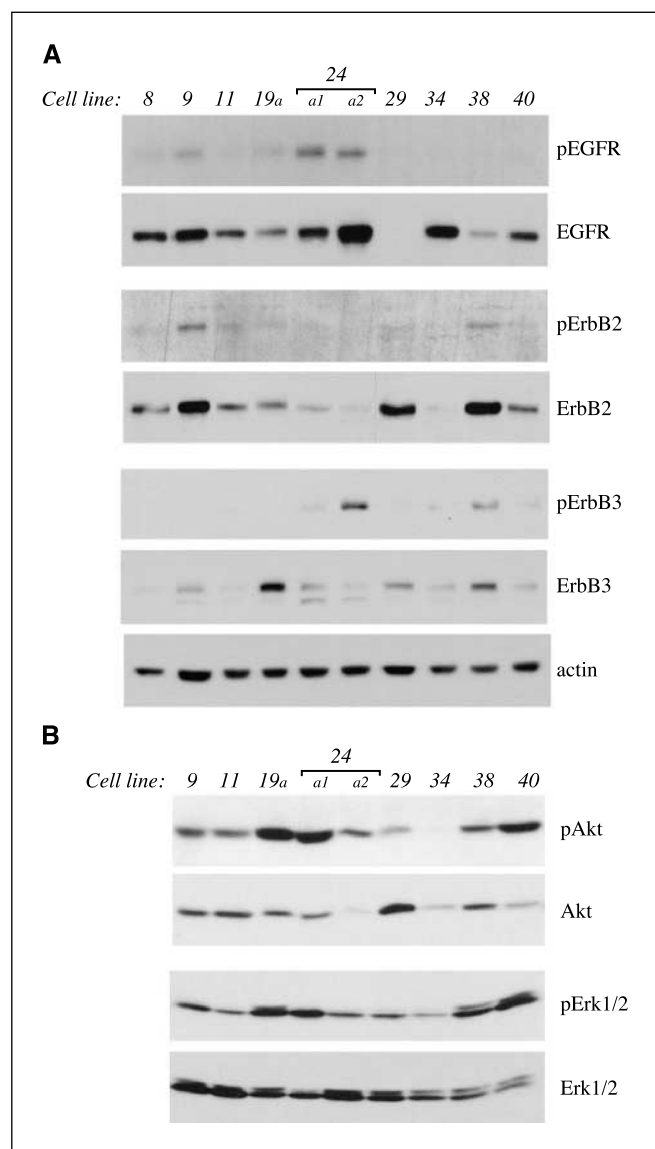


Fig. 3. Phosphorylation of EGFR, ErbB2, ErbB3, Akt, and Erk in HNSCC cell lines. Basal phosphorylation of pEGFR, pErbB2, and pErbB3 (A) or pAkt and pErk (B) was analyzed by Western blotting with phosphospecific antibodies. Membranes used for analysis of phosphorylated proteins were stripped and reblotted with the respective antibodies recognizing total EGFR, ErbB2, and ErbB3 (A) or total Akt and Erk1/2 (B). Expression of actin was analyzed from the same cell lines as control (A).

pertuzumab on the same two UT-SCC-29 and UT-SCC-38 lines was confirmed by showing that 1-hour treatment with 20 μ g/mL pertuzumab significantly reduced phosphorylation of ErbB3, a dimerization partner of the pertuzumab target protein ErbB2 (Fig. 4C and D). Taken together, these findings indicate that enhanced signaling via ErbB2 heterodimers, such as ErbB2/ErbB3, may contribute to resistance of HNSCC cells to gefitinib.

Discussion

Targeted therapeutics that specifically inhibit EGFR have shown clinical effect on patients with HNSCC (14, 15). However, there is a need for predictive markers that could be

used to identify patients that benefit from EGFR inhibitor therapy. Here, we analyzed the potential of molecules participating in signaling via EGFR as markers predicting responsiveness to gefitinib using 10 established HNSCC lines. Our findings indicate that pErbB2 and total ErbB3 may predict resistance to gefitinib *in vitro*. In accordance, combining gefitinib with pertuzumab, an antibody blocking signaling via ErbB2 heterodimers, provided an additive growth-inhibitory effect when compared with gefitinib alone. These data suggest a role for ErbB2/ErbB3 signaling in predicting and treating gefitinib-resistant HNSCC. In addition, our finding that EGFR amplification may predict sensitivity of HNSCC lines to gefitinib *in vitro* extends earlier clinical data showing an association between EGFR amplification and responsiveness to EGFR TKIs in NSCLC (28, 29).

Both Western and real-time reverse transcription-PCR analyses indicated that most HNSCC lines expressed variable amounts of EGFR, ErbB2, and ErbB3, whereas ErbB4 expression was low or nonexistent. No statistical association was observed between sensitivity to gefitinib and expression of the gefitinib target molecule EGFR, when EGFR was quantitated on either protein or mRNA level. Moreover, the quantity of activated EGFR analyzed by phosphotyrosine-specific Western blots did not correlate with responsiveness. Clinical data on predictive value of EGFR for gefitinib response in HNSCC are limited; however, in agreement with the *in vitro* data presented here, immunohistochemically determined expression of EGFR has been suggested not to predict response of HNSCC patients to gefitinib or erlotinib (15, 16).

Simply assessing the quantity of EGFR may not always give a reliable readout of the EGFR-stimulated signaling activity or dependence of the tumor on EGFR signaling. In NSCLC, EGFR amplification and activating mutations within the EGFR kinase domain have been suggested to associate with sensitivity to EGFR TKIs (17, 18, 28, 29). Thus, analyses of EGFR gene copy numbers and sequencing through the EGFR kinase domains were carried out for the 10 HNSCC lines. Consistent with the reports on EGFR amplification and NSCLC (28), high EGFR copy numbers were found to correlate with gefitinib sensitivity of the HNSCC lines. However, no activating mutations in the EGFR kinase domain were found in our sequencing analyses. EGFR mutations similar to those found in NSCLC have been observed previously in HNSCC patients of Asian origin (19) but not in an analysis of 82 HNSCC patients treated in U.S. hospitals (30), suggesting differences in prevalence between ethnic groups.

Oncogenic alterations in signaling via molecules along the EGFR signaling pathway could also alter the threshold at which the pathway is activated irrespective of changes in EGFR expression (30). Analysis of other ErbB family members indicated that the expression level of ErbB3 was significantly associated with resistance to gefitinib ($P = 0.02$), and there was a tendency for predictive significance also for total ErbB2 protein levels ($P = 0.07$). Furthermore, a statistically significant association was observed between the quantity of pErbB2, analyzed by phosphospecific Western blotting, and gefitinib resistance ($P = 0.02$). These findings suggest that receptor heterodimerization may contribute to signal transduction downstream of EGFR in HNSCC cells. Formation of ErbB heterodimers in HNSCC may also be clinically relevant as overexpression of multiple ErbB receptors in HNSCC has

Table 3. Association of cetuximab IC₅₀ with molecular markers of ErbB signaling

	EGFR	ErbB2	ErbB3	EGFR copy no.	Gefitinib IC ₅₀
Correlation coefficient	-0.64	0.01	0.27	-0.63	0.36
<i>P</i>	0.048*	0.99	0.45	0.053	0.31
	pEGFR	pErbB2	pErbB3	pAkt	pErk
Correlation coefficient	-0.47	0.44	-0.19	-0.03	-0.15
<i>P</i>	0.17	0.20	0.59	0.93	0.70

NOTE: Quantity of ErbB and pErbB signals was estimated by densitometric analysis of Western films. Associations were calculated using Spearman correlation coefficients.
**P* < 0.05, *n* = 10, except for pAkt and pErk (*n* = 9).

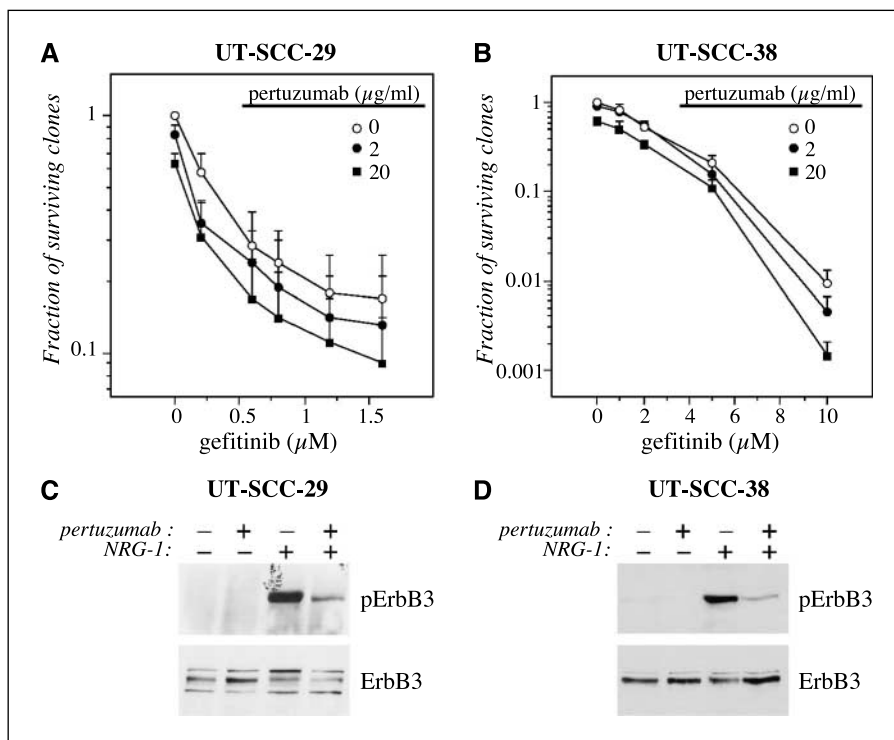
been reported to correlate with worse clinical outcome than overexpression of EGFR alone (31). Interestingly, gefitinib has been suggested to stimulate formation of inactive heterodimers of EGFR with ErbB2 and ErbB3 in a mechanism not requiring ligand stimulation (32, 33). A hypothesis that heterodimerization of EGFR with other ErbBs is associated with resistance to an EGFR inhibitor is in line with findings that higher concentrations of TKIs are needed to suppress EGFR phosphorylation in cancer cells that also express high levels of ErbB2 when compared with cells expressing EGFR alone (34). In contrast, studies of NSCLC have indicated a role for ErbB2 and ErbB3 as markers of sensitivity to gefitinib (35–37). These differences may be due to the inherent difficulties in extrapolating data from *in vitro* experiments to the clinic or to variation in cancer type or other molecular characteristics, such as frequency of *EGFR* amplification. As the 10 analyzed cell

lines originate from patients with variable genetic backgrounds, it is possible that there are also other predictive markers that were not identified in our analyses focusing on the ErbB signaling pathway.

In NSCLC, basal phosphorylation of Akt detected by immunohistochemistry has been suggested to predict responsiveness of patients to gefitinib (38). *EGFR* mutations that sensitize NSCLC cells to gefitinib have also been shown to activate the phosphoinositide 3-kinase/Akt signaling pathway (39). Furthermore, gefitinib sensitivity has been associated with the effect of the TKI to suppress phosphorylation of Erk (40). However, in our analyses of HNSCC lines, neither pAkt nor pErk correlated with responsiveness to gefitinib.

To address whether the markers identified here as predictors of gefitinib response could also be applied to assess responsiveness to other EGFR-targeting drugs, IC₅₀s of the 10 HNSCC

Fig. 4. Effect of pertuzumab in combination with gefitinib on HNSCC cell growth. The effect of the indicated concentrations of gefitinib in combination with 0, 2, or 20 μ g/mL pertuzumab on clonogenic survival of relatively gefitinib-resistant UT-SCC-29 (A) and UT-SCC-38 (B) cell lines was measured as described in Fig. 1. The effect of 1-hour treatment with 20 μ g/mL pertuzumab on ErbB3 phosphorylation was shown in the same two HNSCC lines UT-SCC-29 (C) and UT-SCC-38 (D) by Western blotting with a phosphospecific antibody. Membranes were stripped and reblotted with an anti-ErbB3 antibody.



lines were also determined for the anti-EGFR antibody cetuximab. Statistical analyses indicated that there indeed was a similar tendency for an association between high *EGFR* copy numbers and sensitivity to both gefitinib and cetuximab. In contrast, no similar association as found for gefitinib was observed between response to cetuximab and pErbB2/ErBB3 expression levels. These findings are in line with clinical observations, indicating that different molecular markers may predict responsiveness to gefitinib and cetuximab in patients with NSCLC (41).

One way to overcome resistance to targeted therapy is to use combination of monotherapeutic agents with different mechanisms of action (42). Our correlative data suggested that the expression of pErbB2 or ErbB3 may confer resistance to gefitinib. Heterodimerization between ErbB2 and ErbB3 was also shown in the HNSCC lines by coprecipitation experiments (data not shown). Therefore, the effect of combining gefitinib with pertuzumab, a monoclonal antibody that interferes with heterodimerization of ErbB2 with other ErbBs (43, 44), was tested. The combination of gefitinib and pertuzumab showed a dose-dependent additive effect on clonogenic survival of HNSCC cells when compared with treatment with gefitinib

alone. These data suggest that, in addition to EGFR, ErbB2 heterodimers, such as ErbB2/ErBB3, may promote HNSCC cell growth and predict resistance to gefitinib. Our findings are consistent with findings that down-regulation of ErbB2 suppresses EGFR-mediated transformation (45). In addition, it was recently reported that a combination of erlotinib and pertuzumab was superior to either drug alone in mouse xenograft models of human NSCLC (46).

Taken together, our data suggest that *EGFR* amplification may predict sensitivity and increased ErbB2/ErBB3 signaling resistance to EGFR TKI gefitinib in HNSCC. To date, most targeted therapies have focused on inhibition of EGFR in HNSCC. Data presented here indicate that combining EGFR inhibitors with drugs, such as pertuzumab, which target other members of the ErbB family, may provide additional benefit to targeted therapy of HNSCC.

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