Cross-Talk between Estrogen Receptor and Epidermal Growth Factor Receptor in Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: This study aimed to characterize estrogen receptor expression and signaling in head and neck squamous cell carcinoma (HNSCC) cell lines and patient tissues, and to evaluate estrogen receptor and epidermal growth factor (EGF) receptor (EGFR) cross-activation in HNSCC.

Experimental Design: Estrogen receptor expression and signaling in HNSCC cell lines were assessed by immunoblotting. In vitro proliferation and invasion were evaluated in HNSCC cell lines in response to estrogen receptor and EGFR ligands or inhibitors. Estrogen receptor and EGFR protein expression in patient tissues was assessed by immunohistochemical staining.

Results: Phospho–mitogen-activated protein kinase (P-MAPK) levels were significantly increased following combined estrogen and EGF treatment. Treatment of HNSCC cells with estrogen and EGF significantly increased cell invasion compared with either treatment alone, whereas inhibiting these two pathways resulted in reduced invasion compared with inhibiting either pathway alone. EGFR (P = 0.008) and nuclear estrogen receptor α (ERα_nuc; P < 0.001) levels were significantly increased in HNSCC tumors (n = 56) compared with adjacent mucosa (n = 30), whereas nuclear estrogen receptor β (ERβ_nuc) levels did not differ (P = 0.67). Patients with high ERα_nuc and EGFR tumor levels had significantly reduced progression-free survival compared with patients with low tumor ERα_nuc and EGFR levels (hazards ratio, 4.09; P = 0.01; Cox proportional hazards). In contrast, high ERβ_nuc tumor levels were not associated with reduced progression-free survival alone or when combined with EGFR.

Conclusions: ERα and ERβ were expressed in HNSCC, and stimulation with estrogen receptor ligands resulted in both cytoplasmic signal transduction and transcriptional activation. Estrogen receptor and EGFR cross-talk was observed. Collectively, these studies indicate that estrogen receptor and EGFR together may contribute to HNSCC development and disease progression. (Clin Cancer Res 2009;15(21):6529–40)

Epidermal growth factor receptor (EGFR) is overexpressed in 40% to 90% of head and neck squamous cell carcinoma (HNSCC), and EGFR overexpression is associated with reduced HNSCC patient survival (1, 2). The EGFR-targeted chimeric monoclonal antibody cetuximab (C225, ImClone) has been Food and Drug Administration–approved for the treatment of HNSCC. Although EGFR is overexpressed in many HNSCC, clinical response to cetuximab and other EGFR-targeted therapies has been modest in clinical trials (3–5). In addition, response to EGFR-targeted treatment has not positively correlated with tumor EGFR levels in several studies (5–7). These data suggest that signaling pathways working in parallel or in concert with EGFR may modulate tumor response to EGFR-targeted therapies.

The mechanisms of acquired or de novo resistance to EGFR targeting in EGFR-expressing tumors are incompletely understood. Estrogen receptor signaling independent of EGFR and/or in concert with EGFR has been reported for cancers of the lung and esophagus (8–11), and combined EGFR and estrogen receptor targeting in lung cancer has been previously reported by our group to be a more effective antitumor therapy than targeting either alone (9).

EGFR overexpression is common in HNSCC (12–14), and the role of EGFR signaling in HNSCC growth and invasion has been well established (15, 16). In contrast, reports of estrogen receptor α (ERα) and estrogen receptor β (ERβ) expression in HNSCC are conflicting, and reports characterizing estrogen receptor function in HNSCC are scarce. In addition, reported

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survival. In addition, we have shown estrogen receptor and epidermal growth factor receptor (EGFR) cross-activation in these cells. Inhibiting these two pathways resulted in reduced invasion compared with inhibiting either pathway alone in vitro. Primary human HNSCC tumor tissue expressed significantly higher levels of ERα and EGFR compared with normal mucosa, and patients with high ERα and EGFR tumor expression levels had significantly reduced progression-free survival compared with patients with lower ERα and EGFR tumor levels. These results suggest that both the estrogen receptor and EGFR pathways together contribute to HNSCC and provide a rationale for targeting these pathways in combination as a new treatment strategy for head and neck cancer.

activities of estrogen receptor in breast cancers where estrogen receptor activities have been most characterized suggest that results differ according to experimental system or that estrogen receptor may play a complex role in cancer, as both tumorigenic and antitumor properties have been associated with specific estrogen receptor subtypes (17–19).

We sought to evaluate the expression of estrogen receptor subtypes, to determine whether estrogen receptor activation was associated with cell proliferation and/or invasion, and to examine the functional interaction between EGFR and estrogen receptors in HNSCC cell lines. To evaluate the putative role of estrogen receptor in HNSCC, we characterized estrogen receptor subtype expression in patient HNSCC tumors and paired adjacent mucosal tissues. We hypothesized that the estrogen receptor and EGFR pathways interact in HNSCC and we would achieve greater cell proliferation and/or invasion with combined treatment with estrogen and epidermal growth factor (EGF), an EGFR ligand agonist. We further hypothesized that inhibition of HNSCC invasion and/or proliferation would be greater with combined inhibition of estrogen receptor and EGFR than with targeting either pathway alone. In order to test these hypotheses, we biochemically evaluated estrogen receptor and EGFR signaling in several HNSCC cell lines in vitro and assessed estrogen receptor, EGFR, and their combined expression and EGFR signaling in several HNSCC cell lines.

**Cell lines and culture conditions.** HNSCC cell lines PCI-15B, PCI-37A, 1483, UM-22B, Detroit-562, and UPCI SSC-103 were maintained in DMEM with 10% fetal bovine serum at 37°C with 5% CO2. MCF7 breast cancer cells were purchased from the American Type Culture Collection and maintained in beta mercaptoethanol (BME) with 10% fetal bovine serum. HNSCC cell lines were of human origin and derived from an oropharyngeal tumor (1483), metastatic cervical lymph node (UM-22B and PCI-15B), metastatic pleural effusion (Detroit-562), or epiglottis (PCI-37A and UPCI SCC-103) as described previously (20–23). UM-22B, Detroit-562, and UPCI SCC-103 were derived from female patients, whereas PCI-15B, PCI-37A, and 1483 were derived from male patients.

**Protein extraction and Western analysis.** Whole cell extracts from cultured HNSCC cells were prepared as described previously (9). Equal amounts of protein (25 μg) from each sample were analyzed by immunoblotting for ERα, ERβ, EGFR, and β-actin. Proteins were fractionated using 10% SDS-Tricine gels and transferred to nitrocellulose membranes. Membranes were blocked by incubation in 1× TBS-tween/5% milk for 1 h at room temperature, followed by incubation overnight at 4°C with the following primary antibodies: anti-ERα antibody, HC-20 (1:1,000; Santa Cruz Biotechnology); anti-ERβ antibody, H-150 (1:1,000; Santa Cruz Biotechnology); anti-EGFR antibody, 1005 (1:500; Santa Cruz Biotechnology); or anti-actin antibody (1:10,000; Millipore Corporation). Blots were washed in 1× TBS-tween and incubated with horseradish peroxidase–conjugated antimouse or antirabbit IgG (1:2,000; Amersham). Immune complexes were detected using SuperSignal West Pico Chemiluminescent substrate (Pierce Biotechnology) and exposure to autoradiography film. Densitometry was done using Molecular Dynamics ImageQuant software version 5.2.

For induction of phospho–p44/42 mitogen-activated protein kinase (P-MAPK), HNSCC cells were grown to 75% confluency. Cells were serum-deprived for 48 h in phenol red–free media. Estrogen and/or EGF was added for 5 min. Inhibitors and NAs were added for 2 h prior to ligand stimulation. Whole cell protein extracts were prepared, gel-fractionated, transferred, and blocked as described above. Membranes were probed with anti–P-MAPK antibody (1:1,000; Cell Signaling Technology) or anti–total-p44/p42 MAPK (T-MAPK) antibody (1:1,000). Secondary was horseradish peroxidase–conjugated antirabbit IgG (1:2,000). Washes, detection, and quantification were done as described above. The quantified results represent the mean ± SE of two samples per experimental treatment for three independent experiments.

**Cell proliferation assay.** Cells were plated at 3.5 × 103 cells per well in complete media on 96-well plates and allowed to attach overnight. Cells were serum-deprived in phenol red–free medium for 48 h. Treatments were added for 72 h with media replenishment every 24 h. Samples were analyzed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) as described previously (9). The quantified results represent the mean ± SE of two independent experiments, each with six samples per experimental treatment.

**Transient transfection and luciferase assay.** Cells were plated in complete media at 1 × 104 cells/well in 6-well plates. The next day, the medium was changed to one containing 10% charcoal-stripped serum and no phenol red to deprive the cells of estrogen. Cells were transfected the following day, harvested, and analyzed for luciferase activity as previously described (24). Values were corrected for protein concentration and are presented as the mean ± SE of three independent experiments, each with two samples per experimental treatment.

**ELISA assays.** PCI-37A cells were grown to 75% confluency. The cells were then serum-deprived in phenol red–free medium for 48 h and treated with 1 mmol/L estrogen for 10 min. Supernatants were collected and cells were centrifuged at 1,200 × g for 10 min. The resulting supernatants were concentrated to 300 μL using Amicon ultrafilter devices and tested for levels of TGFα, HB-EGF, and amphiregulin by ELISA following the manufacturers’ instructions. Results are expressed as the fold-increase with estrogen treatment compared with controls. Results represent the average ± SE from five samples per experimental treatment assay in duplicate.

**Materials and Methods.**

**Reagents.** Estrogen was purchased from Sigma-Aldrich. Recombinant human EGF and transforming growth factor α (TGFα) neutralizing antibody (NA) were purchased from Oncogene Research Products. M225 was obtained from Imclone Systems, Inc. Marimistat was obtained from British Biotech. TGFα QuantiKine ELISA kit, human HB-EGF and amphiregulin DuoSet ELISA kits, and amphiregulin NA were from R&D Systems. HB-EGF NA was from Calbiochem. Gefitinib was purchased from Tocris. Fulvestrant was purchased from Tocris.
**Invasion assay.** For the stimulation experiments shown in Fig. 3A, PCI-37A cells that had been serum- and phenol red–deprived for 24 h were plated at a density of $7.5 \times 10^3$ cells/well in a 24-well BD Biocoat Matrigel growth-factor reduced invasion chamber (BD Biosciences). Estrogen (1 nmol/L) and/or EGF (0.5 ng/mL) was added to the media plus 10% charcoal-stripped serum in the lower chamber as indicated in the figures and incubated for 24 h. For the inhibition experiments shown in Fig. 4B, PCI-37A cells grown in complete media were plated 7.5 × 10^3 cells/well in a 24-well BD Biocoat Matigel invasion chamber. Gefitinib (10 μmol/L) and/or fulvestrant (5 μmol/L) was added to the media in both the upper and lower chambers and incubated for 24 h. The lower chamber also contained 10% fetal bovine serum. For both experiments, noninvading cells were removed, and invading cells were fixed and stained with the Diff-Quik staining kit (VWR International). Invasion is expressed as the mean number of cells invading through the Matrigel matrix. Control treatment was set to 100 and all results are expressed relative to control. Results are the mean ± SE of three independent experiments with two membranes per experimental treatment and four independent regions counted per membrane at ×40 magnification.

**Study subjects and tissue samples.** Patients who were to undergo surgical resection with curative intent for the treatment of HNSCC with pathologically confirmed cancer of the upper aerodigestive tract (oral cavity, oropharynx, hypopharynx, or larynx) gave written informed consent and donated tumor tissues and adjacent mucosa for study. Tumor specimens from 56 HNSCC patients, 30 with paired adjacent histologically normal mucosa, were incorporated into a tissue microarray (TMA). Tissues were collected under the auspices of a tissue bank protocol approved by the University of Pittsburgh Institutional Review Board. Subject smoking histories and body mass index data were obtained for 55 and 44 subjects, respectively, through administered questionnaire or clinical chart review. A summary of subjects with tumor specimens incorporated into TMAs is provided in Table 1.

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<td>5 (31.3)</td>
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<tr>
<td>Regression‡</td>
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<td>8</td>
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<td>Median (range), in mo</td>
<td>17.9 (1.2-81)</td>
<td>32 (2.7-78.3)</td>
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</table>

Abbreviations: CT, chemotherapy; RT, radiotherapy.  
*Fisher’s exact test.  
†Pack-year smoking data were available for 33 male and 16 female subjects.  
‡Rank sum test.  
§Log rank test.  
∥Progression defined as new HNSCC, recurrent HNSCC, metastases or death.
**TMA construction.** Cores were sampled from paraffin-embedded tissue blocks from surgical specimens by a head and neck cancer pathologist (RS). TMAs were constructed from 0.6-mm triplicate tissue cores extracted from HNSCC tumors or adjacent histologically normal tissues arrayed on two recipient paraffin blocks. The newly constructed array was then warmed to 37°C for 10 min to allow annealing of donor cores to the paraffin wax of the recipient block.

**Immunohistochemical staining and quantification.** TMA sections were deparaffinized with successive ethanol and xylene treatments, rehydrated, and stained for ERα, ERβ, or EGFR. For ERα staining, sections underwent heat-induced antigen retrieval in citrate buffer. Following three washes with 3% hydrogen peroxide and one wash with TBS (25 mmol/L Tris, 0.15 mol/L NaCl, pH 7.5), slides were blocked with Dako Protein Block Serum (X0909, Dako) for 5 min then incubated for 30 min with anti-ERα antibody (HC-20, SC-543, Santa Cruz) diluted 1:200 in antibody diluent (S0809, Dako). Signal amplification was done using the Dako EnVision kit (K1392, Dako). Immunoreactive cells were visualized following incubation with diaminobenzidine chromogenic substrate (K3468, Dako) at room temperature for 10 min. For ERβ staining, heat-induced antigen retrieval was done with DivaDecloaker antigen retrieval buffer (DV2004, Biocare). Slides were treated with 3% hydrogen peroxidase for 10 min to block endogenous peroxidases and then treated with protein block for 10 min (BS966L, Biocare). Slides were incubated for 30 min with anti-ERβ antibody (H-150, SC-8974, Santa Cruz; 1:100). Signal amplification was done using the Dako Cytomation Envision Dual Link System Peroxidase (K4063, Dako); immunoreactive cells were visualized following incubation with Dako DAB chromogen substrate (K3468, Dako) at room temperature for 5 min.
Fig. 2. A, stimulation of phospho-p44/p42 MAPK by EGF and estrogen. PCI-37A HNSCC cells were serum-deprived for 48 h followed by treatment with 1 nmol/L estrogen (E2), 1 ng/mL EGF, or a combination for 5 min. Cell extracts were prepared and 25 μg of each sample were analyzed by Western blotting using an anti-phospho-p44/p42 MAPK antibody (P-MAPK) or an anti-p44/p42 MAPK antibody (T-MAPK). Quantitation was done by densitometry and ImageQuant analysis. Quantitation was set to 100 and fold increases were calculated versus no treatment. Representative blot and quantitative data of three independent experiments each of which had two samples per experimental treatment. *, P < 0.05; **, P < 0.01; ***, P < 0.001, one-way ANOVA.

B, estrogen-induced phospho-p44/p42 MAPK depends on EGFR activation. PCI-37A cells were pretreated for 2 h with the EGFR blocking antibody (M225, 6 μg/mL) or 20 μmol/L marimastat or not treated at all followed by stimulation with 1 nmol/L estrogen for 5 min. Each sample (25 μg) was analyzed by Western analysis as in A. Representative Western blot is shown along with quantitation. Graph represents the average results of three independent experiments each of which had two samples per experimental treatment. **, P < 0.01, one-way ANOVA.

C, EGFR ligands mediate estrogen-induced EGFR activation. PCI-37A cells were pretreated with TGFα NA (7 μg/mL), amphiregulin NA (15 μg/mL), HB-EGF NA antibody (5 ng/mL) for 2 h before treatment with 1 nmol/L estrogen (E2) for 5 min. Each sample (25 μg) was analyzed by Western analysis using anti-phospho-p44/p42 MAPK antibody and anti-p44/p42 MAPK antibody. Quantitation was done using densitometry and ImageQuant analysis. No treatment was set to 100. Graph represents average results ± SE of three independent experiments each of which had two samples per experimental treatment. ***, P < 0.001, ANOVA.

D, cell culture supernatant was removed from PCI-37A cells that were either stimulated with estrogen for 5 min or not treated at all and analyzed for release of TGF-α, amphiregulin (AR), or HB-EGF in the supernatant by ELISA. *, P < 0.05.
EGFR staining was done without antigen retrieval using the anti-EGFR antibody (M3563, Dako; 1:500). Signal amplification was carried out using an antibody-conjugated proprietary micropolymer peroxidase (ImmPRESS, Vector). Immunoreactive cells were visualized as described for ERβ. All sections were counterstained with hematoxylin for 2.5 min. Staining intensity for each core was scored as 0 (none), 1+ (weak), 2+ (moderate), or 3+ (strong). The percentage of immunoreactive cells was recorded and rounded to the nearest 5th percentile. For ERα and ERβ, nuclear (nuc) and cytoplasmic (cyto) staining was evaluated independently. Tumor cores with >10% of cells staining +1 or greater were defined as positive. A composite score (immunohistochemical score) was defined as positive. A composite score (immunohistochemical score) was used to divide tumors into high and low categories for each protein evaluated.

Results

**Estrogen receptors were expressed in HNSCC cell lines.** We first examined protein expression of EGFR and estrogen receptors in a panel of six HNSCC cell lines derived from both male and female patients (Fig. 1). MCF7 breast cancer cells were used as a positive control for ERα and ERβ. All cell lines examined expressed full-length ERα (66 KDa) and ERβ (59 KDa) protein, although ERα was expressed at relatively lower levels compared with MCF7 cells (Fig. 1A). There was no difference in ERα or ERβ expression between cell lines derived from males (PCI-15B, PCI-37A, and 1483) and those from females (UM-22B, Detroit-562, and UPCI SCC-103). Although the highest ERα expression was consistently observed in the UM-22B cell line, the lowest ERα expression was also observed in a female-derived cell line, UPCI SCC-103. EGFR expression was variable in the cell lines examined. PCI-15B, 1483, and UM-22B expressed high levels of EGFR whereas PCI-37A, Detroit-562, and UPCI SCC-103 expressed relatively low levels of EGFR. No relationship was observed between EGFR expression and ERα or ERβ expression. β-Actin protein expression showed no differences between these cell lines. Reproducibility of protein expression levels was confirmed in at least two separate experiments for each cell line.

**Estrogen receptors are functional in HNSCC cell lines.** If estrogen influences HNSCC development, a stimulatory effect attributable to estrogen on the growth of HNSCC cells would be expected. In order to determine if estrogen could induce tumor growth in HNSCC cells, cell proliferation was assessed by MTS assay in four HNSCC cell lines. Figure 1B shows the effect of EGFR and estrogen on cell growth. EGF alone significantly stimulated cell growth by 1.4- to 1.8-fold in all cell lines examined. Estrogen stimulated cell proliferation to a lesser extent (1.1- to 1.5-fold) than EGF compared with vehicle control, but was statistically significant. One mechanism of ligand-dependent nuclear estrogen receptor action is through genomic responses whereby nuclear estrogen receptors are activated by estrogen binding at either estrogen-responsive elements (ERE) or activator protein 1 (AP-1) sites in estrogen-responsive genes. To verify a biologically functional role of estrogen receptors in HNSCC cells, we used a gene reporter assay with a single vitellogenin ERE upstream of a minimal thymidine kinase promoter and the firefly luciferase gene (pERE-TK-LUC) to determine if the endogenous estrogen receptors present in HNSCC cell lines, expressing different amounts of EGFR, could activate...
transcription in this manner. pRL-CMV was co-transfected to control for transfection efficiency. The results from three independent experiments are shown in Fig. 1C. Estrogen consistently increased estrogen receptor transcription with doses as low as 0.1 nmol/L estrogen, but the increase was not statistically significant in PCI-15B cells. No correlation was found between ERα or ERβ protein expression levels and extent of transcriptional response. An inverse correlation was observed between EGFR expression and transcriptional response. ERE-luciferase induction was highest in PCI-37A cells, which also had the lowest EGFR expression of the cell lines tested whereas PCI-15B cells had the highest EGFR expression and the lowest transcriptional activation. This suggests that if EGF signaling is low, estrogen signaling is more functional and vice versa.

In order to determine the relative contributions of ERα and ERβ signaling in HNSCC, we transiently transfected an AP-1 luciferase construct into HNSCC cells. Estrogen activates transcription from AP-1 sites when complexed to ERα and inhibits transcription when complexed to ERβ, allowing for assessment of the relative activities of ERα and ERβ (25). MCF7 cells, which express high amounts of ERα and low amounts of ERβ, showed a 3.5-fold increase in luciferase activity. In UM-22B and PCI-37A HNSCC cells, luciferase activity remained the same or slightly decreased upon estrogen treatment (Fig. 1D). These results suggest that the estrogen receptors present in HNSCC are functional and that ERβ is the predominant transcriptionally active estrogen receptor in UM-22B and PCI-37A cells.

Rapid stimulation of phospho-p44/p42 MAPK by estrogen and EGF. P-MAPK is a downstream signaling mediator of the EGFR pathway. We have previously shown that rapid activation of P-MAPK is a surrogate end point for EGFR activation (9). To determine if the estrogen receptors in head and neck cancer cell lines can transactivate EGFR downstream signaling pathways, PCI-37A cells (the cell line that responded best to estrogen in cell proliferation and transcription assays) were treated for 5 minutes with estrogen (1 nmol/L), EGF (0.5 ng/mL), or a combination of the two treatments, and were analyzed for P-MAPK expression (Fig. 2A). Submaximal concentrations of estrogen and EGF were used in order to observe a combined effect of the two ligands. Higher concentrations of EGF resulted in maximal stimulation of the P-MAPK pathway (data not shown). A 2.8-fold and 3.8-fold stimulation of P-MAPK was observed with estrogen and EGF treatment alone, respectively. The combination achieved an almost additive effect of 6-fold compared with control-treated cells (P < 0.001 for estrogen versus estrogen plus EGF, and EGF versus estrogen plus EGF). P-MAPK induction by estrogen is maximal at 5 to 10 minutes and then returns to basal levels (data not shown). To determine if estrogen-induced P-MAPK stimulation was dependent on EGFR, the cells were pretreated with an EGFR NA, M225, for 2 hours prior to ligand stimulation. Figure 2B shows that M225 almost completely abrogated P-MAPK induction by estrogen. It has previously been shown that transactivation of EGFR by other receptors involved extracellular release of EGFR ligands from the plasma membrane mediated by matrix metalloproteinases (MMP; ref. 26). Pretreatment of PCI-37A cells with the MMP inhibitor marimastat also completely inhibited the estrogen-induced P-MAPK (Fig. 2B), indicating that MMP activity was required for this signaling.

We next examined which EGFR ligands were involved in this response. Pretreatment of cells with TGFA, amphiregulin, and HB-EGF NAs followed by estrogen treatment diminished the P-MAPK response compared with estrogen treatment alone (Fig. 2C). TGFA NA resulted in complete inhibition of estrogen-induced P-MAPK whereas amphiregulin NA and HB-EGF NA resulted in only partial inhibition (amphiregulin NA versus amphiregulin NA plus estrogen, P > 0.05, not significant; HB-EGF NA versus HB-EGF NA

![Fig. 4.](https://example.com/fig4.png)
plus estrogen, $P < 0.005$). Additionally, ELISA assays for each of these three ligands showed a 3.34-, 2.21-, and 1.59-fold increase in secretion of TGFα, amphiregulin, and HB-EGF, respectively, in the supernatant upon estrogen stimulation compared with no estrogen treatment ($P < 0.05$; Fig. 2D). These results suggest that TGFα, followed by amphiregulin and HB-EGF are the primary ligands cleaved by estrogen stimulation and support a functional interaction between estrogen receptor and EGFR in head and neck cancer cells.

**Combination estrogen and EGF can maximally induce cell invasion.** We and others have previously shown that EGF can mediate invasion in HNSCC (27–29). To examine the effect of estrogen alone and in combination with EGF on cell invasion, PCI-37A cells were grown on Matrigel invasion chambers and treated with either estrogen, EGF, or the combination, and the number of invading cells was determined after 24-hour treatments. Both estrogen and EGF alone significantly stimulated cell invasion by 3.8- and 4.2-fold, respectively ($P < 0.001$; Fig. 3A). The combination of estrogen and EGF further enhanced cell invasion significantly over single-agent treatment with a 5.7-fold increase in invading cells observed (estrogen versus estrogen plus EGF, $P < 0.01$; EGF versus estrogen plus EGF, $P < 0.01$; Fig. 3A).

We next examined the ability of the cells grown in complete media containing serum to respond to drugs that target either the estrogen receptor or EGFR in an invasion assay. We used media containing serum to respond to drugs that target either EGF, estrogen, or both at the same time. We and others have previously shown that EGF can mediate invasion in HNSCC (27–29). To examine the effect of estrogen alone and in combination with EGF on cell invasion, PCI-37A cells were grown on Matrigel invasion chambers and treated with either estrogen, EGF, or the combination, and the number of invading cells was determined after 24-hour treatments. Both estrogen and EGF alone significantly stimulated cell invasion by 3.8- and 4.2-fold, respectively ($P < 0.001$; Fig. 3A). The combination of estrogen and EGF further enhanced cell invasion significantly over single-agent treatment with a 5.7-fold increase in invading cells observed (estrogen versus estrogen plus EGF, $P < 0.01$; EGF versus estrogen plus EGF, $P < 0.01$; Fig. 3A). The combination of estrogen and EGF further enhanced cell invasion significantly over single-agent treatment with a 5.7-fold increase in invading cells observed (estrogen versus estrogen plus EGF, $P < 0.01$; EGF versus estrogen plus EGF, $P < 0.01$; Fig. 3A).

We next examined the ability of the cells grown in complete media containing serum to respond to drugs that target either the estrogen receptor or EGFR in an invasion assay. We used the pure antiestrogen, fulvestrant, and the EGFR tyrosine kinase inhibitor, gefitinib, to inhibit either estrogen receptor or EGFR, respectively. We then measured the number of invading cells after 24-hour treatments. Both estrogen and EGF alone significantly stimulated cell invasion by 3.8- and 4.2-fold, respectively ($P < 0.001$; Fig. 3A). The combination of estrogen and EGF further enhanced cell invasion significantly over single-agent treatment with a 5.7-fold increase in invading cells observed (estrogen versus estrogen plus EGF, $P < 0.01$; EGF versus estrogen plus EGF, $P < 0.01$; Fig. 3A).

Using agents that target both the estrogen receptor and EGFR signaling pathways together may have enhanced benefit compared with single-pathway targeting.

**EGFR and nuclear ERα protein expression was elevated in HNSCC tumors and high nuclear ERα and EGFR HNSCC tumor levels were associated with reduced PFS.** In order to determine whether increased expression of ERα and/or ERβ is associated with tumorigenesis, TMA-arrayed HNSCC tumors and adjacent mucosal tissues were evaluated for ERα and ERβ protein levels by immunohistochemical staining. EGFR has been previously reported by us and others to be overexpressed in HNSCC (13, 30). In order to evaluate EGFR in this cohort and assess correlations between estrogen receptors and EGFR, we also evaluated EGFR expression in these arrayed tissues. ERα and ERβ showed predominantly nuclear staining, whereas EGFR staining was distributed through the plasma membrane and cytoplasmic compartments (Fig. 4A). Of the HNSCC tumors evaluated, 52%, 95%, and 44% were positive for EGFR, nuclear ERα (ERαnuc), and nuclear ERβ (ERβnuc), respectively. Levels of EGFR and ERαnuc were found to be significantly higher in tumor than in paired adjacent mucosa whereas ERβnuc levels did not differ between tumors and adjacent tissues (Fig. 4B). Patient characteristics by tumor EGFR and ERαnuc status are provided in Supplemental Tables 1 and 2, respectively. Of note, ERαnuc and ERβnuc expression levels in tumors and adjacent mucosal tissues did not differ by patient sex ($P = 0.81$ and $P = 0.66$, respectively) by the rank sum test (data not shown). Cytoplasmic ERα (ERαcyto) and cytoplasmic ERβ (ERβcyto) were detected although staining was less robust than the nuclear compartment. Seventy percent and 60% of HNSCC tumors were positive for ERαcyto and ERβcyto, respectively. Both ERαnuc and ERβnuc levels were elevated in HNSCC tumors compared with adjacent mucosa ($P < 0.001$ and $P = 0.008$, respectively; data not shown). Neither ERα nor ERβ nuclear or cytoplasmic levels differed by patient sex or tumor anatomical site (data not shown). Only paired adjacent mucosal tissues confirmed by our pathologist (RS) to be histologically normal were included in each analysis. The number of HNSCC tumors evaluated with paired adjacent mucosal tissues confirmed to be histologically normal for each protein evaluated is indicated in Fig. 4B.

ERα, ERβ, and EGFR high versus low tumor levels were independently evaluated for association with PFS. Kaplan-Meier plots indicated that patients with high tumor levels of ERαnuc or EGFR tended to have shorter PFS than patients with low tumor levels (Fig. 5A). High versus low tumor ERβnuc levels were not associated with differential PFS (Fig. 5A). Neither ERαnuc nor ERβnuc levels were associated with differential PFS ($P = 0.20$ and $P = 0.99$, respectively; log rank test). PFS did not differ by subject sex ($P = 0.22$; Table 1). However, women with high tumor ERαnuc levels tended to have shorter PFS as assessed by Cox proportional hazards models than women with low ERαnuc (HR, 6.32; $P = 0.09$), a trend not observed for male cases (HR, 0.65; $P = 0.25$) or for female cases with high versus low EGFR tumor levels (HR, 1.06; $P = 0.94$). Our cohort had significantly more women than men never smokers at diagnosis, and women tended to smoke fewer pack-years than did men (Table 1). Body mass index did not differ for subjects with high versus low ERαnuc tumor levels ($P = 0.09$, rank sum test).

We confined analyses evaluating EGFR and estrogen receptor tumor levels together to the estrogen receptor nuclear component, the fraction associated with survival. Although ERαnuc levels were elevated in many tumors that expressed relatively low levels of EGFR, tumor ERαnuc, and EGFR protein levels were found to be positively correlated (Fig. 5B, left). Interestingly, patients with high ERαnuc and high EGFR tumor levels tended to have reduced PFS compared with patients with high tumor levels of either ERαnuc or EGFR or neither (Fig. 5C, left).

Recently, the presence of human papilloma virus in oropharyngeal HNSCC has been identified as an important prognostic indicator (31, 32). Human papilloma virus detected by quantitative PCR or in situ hybridization has been reported to be present in 40% to 60% of oropharyngeal HNSCC and present in only small proportions of tumors from other sites (32). We did a subset analysis excluding oropharyngeal tumors. Although the findings were not significant in these analyses with reduced power, the similarly increased hazards associated with high tumor EGFR or ERαnuc levels are likely not dependent on tumor human papilloma virus status.

EGFR expression in tumors is a proven prognostic factor for HNSCC. These findings indicate that the inclusion of ERαnuc tumor levels enhances the prognostic significance of EGFR tumor levels. In contrast, ERβnuc and EGFR tumor levels were not...
correlated (Fig. 5B), and PFS for patients with high ERβ<sub>nuc</sub> and high EGFR levels did not differ from patients whose tumors expressed high levels of either ERβ<sub>nuc</sub> or EGFR (Fig. 5C, right). Patients with EGFR<sup>H</sup>, ER-α<sub>nuc</sub><sup>H</sup> tumors were estimated to have significantly decreased PFS compared with patients with EGFR<sup>L</sup>, ER-α<sub>nuc</sub><sup>L</sup> (HR, 4.09; P = 0.01, univariate Cox proportional hazards) even after adjusting for age, sex, and clinical disease stage (HR, 4.19; P = 0.03, Cox proportional hazards). The HR for patients with EGFR<sup>H</sup>, ERβ<sub>nuc</sub><sup>H</sup> versus EGFR<sup>L</sup>, ERβ<sub>nuc</sub><sup>L</sup> was estimated to be 2.27 (P = 0.11; Fig. 5C, right) using univariate Cox proportional hazards models and 2.41 (P = 0.12) after adjusting for age, sex, and clinical disease stage.

Fig. 5. A, Kaplan-Meier curves for HNSCC patients by high versus low ERα<sub>nuc</sub>, ERβ<sub>nuc</sub>, or EGFR tumor expression level as evaluated by immunohistochemical staining. Univariate Cox proportional hazards estimates and associated P values are provided. B, tumor levels of EGFR and ERα<sub>nuc</sub> (left) and EGFR and ERβ<sub>nuc</sub> (right) by immunohistochemical staining were assessed for correlations. EGFR and ERα<sub>nuc</sub> levels in HNSCC tumors were correlated; EGFR and ERβ<sub>nuc</sub> were not correlated. Spearman’s correlation coefficient (ρ) and associated P value are provided for each comparison. C, Kaplan-Meier plots by combined EGFR and estrogen receptor tumor status. Estimated HRs from univariate Cox proportional hazards models for patients with tumor high in both markers compared with low in both markers and associated P values are provided.
Our study was not adequately powered to determine whether patient PFS differed significantly for patients with both high EGFR and high ERα_nuc compared with patients with tumors expressing high levels of only one of these markers. However, the approximate 2-fold increase in the HR when ERα_nuc and EGFR status are both considered compared with either protein alone suggests that combining ERα_nuc and EGFR tumor status likely improved predicted survival discrimination over ERα_nuc or EGFR tumor status alone.

**Discussion**

We have shown that estrogen receptors are expressed in HNSCC cell lines and tumors. We report here that the addition of exogenous estrogen stimulated HNSCC proliferation and invasion in vitro, indicating that estrogen receptor activation contributes to HNSCC cell growth and invasion. Estrogen receptor has well-described genomic transcriptional and cytoplasmic signal transduction activities (33). Our findings are consistent with estrogen receptor having both of these properties: we found that estrogen increased transcription from ERE and induced activation of MAPK in HNSCC cell lines. Estrogen receptor expression and function in HNSCC cell lines did not differ by sex of the patient from whom the cell lines were derived. In addition, estrogen receptor expression was detected in the majority of HNSCC tumors, and expression levels did not differ by patient sex. Taken together, these data indicate that estrogen receptor likely contributes to HNSCC growth and invasion in both men and women.

The literature regarding estrogen receptor function in HNSCC is mixed. Our data support studies reporting a positive role for estrogen receptors in HNSCC growth and invasion. These reports include the findings that estrogen treatment potentiated the growth of laryngeal xenograft tumors in nude mice, and estrogen was found to stimulate oral squamous cell carcinoma invasion in vitro (34, 35). Also, consistent with our data were the reported findings that the inhibition of estrogen receptor activity by tamoxifen reduced HNSCC cell growth in vitro (36) and that tamoxifen treatment induced apoptosis and inhibited invasion of oral squamous cell carcinoma in vitro (35). However, the majority of the HNSCC cell lines found to be inhibited by tamoxifen in the cell growth study were reported to not express estrogen receptor (36).

Reports of the frequency of estrogen receptor–positive HNSCC vary widely. Expression of either estrogen receptor subtype has been reported to be present in only 2.7% of HNSCC tumors by estrogen receptor receptor assay and in 50.7% of HNSCC tumors by immunohistochemistry (37, 38). There have been several reports that HNSCC tumors and cell lines do not express estrogen receptor or the frequency of estrogen receptor expression was <10% of tumors or HNSCC cell lines evaluated (37, 39, 40). In contrast, estrogen receptor expression has been described in patient tumors with expression of the ERα subtype predominating over the ERβ subtype in an immunohistochemical study with PCR confirmation of estrogen receptor subtype expression of 67 oral cavity and laryngeal/hypopharyngeal cancers (38). In a separate study of 15 primary HNSCC tumors, ERβ expression was observed in all HNSCC tumors whereas ERα expression was observed in only 2 of the 15 HNSCC tumors (35). Our data are consistent with more recent findings that estrogen receptors are expressed in the majority of HNSCC tumors and cell lines. In fact, all HNSCC cell lines that we evaluated expressed estrogen receptors. An earlier report suggested that estrogen receptors were more frequently expressed in tumors of larynx than other head and neck cancers (41); however, we found both ERα and ERβ were expressed in the majority of HNSCC tumors with no difference in expression level by anatomical tumor site. Importantly, we found high ERα_nuc levels were associated with reduced PFS but there was no association of ERβ levels in either the nuclear or cytoplasmic compartment with PFS. Interestingly, we noted a trend in women for reduced PFS with high ERα_nuc levels, which was not observed in men. Female cases tended to smoke less than male cases, suggesting the possibility that estrogen receptor may be especially important for HNSCC etiologies less related to tobacco exposure. Although the patient tumor data suggest a more prominent role for ERα_nuc in HNSCC for women, a larger cohort will be required to definitively assess the relationships among estrogen receptor expression, gender, and smoking.

Although we saw no sex-based estrogen receptor expression level differences in HNSCC cell lines or tumors, it is possible that estrogen receptor activity may play a role in the sex differences in tobacco-related susceptibility to HNSCC, as has been proposed for lung cancer. Tobacco use is an identified risk factor for HNSCC. Although men were found to smoke more than women, hazards associated with smoking were reported to be higher for women than men in a large prospective cohort study of 476,211 participants (42). Smoking-related risk of oral cancers has also been reported to be higher for women than for men in at least one independent study (43). Estrogen receptor–mediated events may be responsible for at least some of the increased tobacco-related HNSCC risk for women compared with men because women have higher circulating levels of estrogen. Estrogen receptor–mediated events may also contribute to HNSCC in men. Tissue levels of estrogen in men may be high enough to show biological effects because testosterone can be converted to estrogen through the action of aromatase, which has been shown to be expressed in head and neck tissue samples (44).

Our finding is the first evaluation of estrogen receptor levels and HNSCC prognosis. To date, there are no reports evaluating estrogen receptor expression and HNSCC disease progression. Several studies have evaluated the relationship between estrogen receptor levels and disease prognosis in upper aerodigestive cancers including lung cancers (45–48). Of these, elevated ERα_nuc level by immunohistochemistry was associated with poorer overall survival in a study of 132 non–small cell lung carcinomas (45). In this same study, loss of ERβ_nuc expression associated with poorer survival, and ERα_nuc–positive–ERβ_nuc–negative patients had significantly reduced survival compared with ERα_nuc–negative–ERβ_nuc–patients (45). Two of these lung cancer studies reported that elevated nuclear ERα levels were associated with better survival in men only (47, 48). These data and our data suggest that estrogen receptor subtype and subcellular localization may differ for HNSCC and lung cancers. However, subtype and localization are important determinants of estrogen receptor involvement in upper aerodigestive cancers including HNSCC. The ERα and ERβ antibodies used in our study are the same antibodies that we and others have used previously to detect estrogen receptor expression (24, 45, 46).

It is important to note that our in vitro studies characterize estrogen receptor function whereas our evaluation of estrogen...
receptor protein expression in tumors does not, and assessment of estrogen receptor levels in patient tumors included subcellular localization whereas in vitro studies evaluated whole cell lysates. At least one study has reported that estrogen receptor expression level and estrogen receptor activity were not correlated (49), and in this study of lung adenocarcinoma–derived cells, estrogen receptor expression levels did not differ by patient sex but estrogen receptor activity was higher in cells derived from females (49). Therefore, although we found that high ERα levels by immunohistochemistry were associated with reduced PFS, our data do not necessarily indicate that ERα activity is elevated in tumors with high ERα protein. In addition, the in vitro analysis indicates that several possible signaling mechanisms occur in HNSCC, including both nuclear and cytoplasmic estrogen signaling. However, the predominant mechanism involved in the survival analysis seems to be the nuclear estrogen signaling.

We were especially interested in the evaluation of estrogen receptor and EGFR cross-activation. We report here evidence that estrogen receptor and EGFR cross-talk is present in HNSCC. The rapid activation of EGFR by nonnuclear estrogen receptor was dependent upon MMPs and was present in HNSCC cell lines derived from both males and females. We found that combined estrogen receptor and EGFR inhibition in vitro reduced HNSCC invasion but not proliferation compared with single targeting. Although estrogen receptor and EGFR ligand activation promoted both invasion and proliferation when administered separately, we did not observe enhanced inhibition of proliferation with dual inhibition. Our data suggest that there is likely redundancy in the pathways leading to proliferation for EGFR and estrogen receptor, whereas as at least some independent contributions to invasion are provided by EGFR- and estrogen receptor–mediated signaling events. Interestingly, in vitro we found that the highest estrogen-induced transcriptional activity was observed in cells with low EGFR expression whereas low transcriptional activity was observed in cells with high EGFR expression. This is in agreement with a previously reported reciprocal control mechanism for estrogen-EGF signaling reported for lung cancer and breast cancer cells (9, 50). Our evaluation of estrogen and estrogen receptor expression in HNSCC tumors indicated that in a subset of tumors, coordinated elevated expression of ERγ and ERα was associated with poor prognosis. EGF and estrogen independently activate signaling pathways known to be involved in tumorigenesis, and these data combined with our in vitro data suggest that EGFR and estrogen receptor cross-talk promote tumor invasion, which may contribute to poor patient prognosis. The cross-signaling between the estrogen receptor–EGFR pathways in HNSCC provides a rationale to combine anti-estrogen therapy with EGFR inhibition for head and neck cancer treatment. These results suggest that increased estrogen and EGF signaling contribute to the invasive properties of HNSCC and that combined inhibition of these two pathways augment the inhibition of invasion compared with blockade of each pathway separately. These data provide a rationale for further investigation of the mechanism of combined estrogen receptor and EGFR targeting in HNSCC with expression of these proteins.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Cross-Talk between Estrogen Receptor and Epidermal Growth Factor Receptor in Head and Neck Squamous Cell Carcinoma

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