Combined Inhibition of PLCγ-1 and c-Src Abrogates Epidermal Growth Factor Receptor–Mediated Head and Neck Squamous Cell Carcinoma Invasion

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Abstract

Purpose: Mortality from head and neck squamous cell carcinoma (HNSCC) is usually associated with locoregional invasion of the tumor into vital organs, including the airway. Understanding the signaling mechanisms that abrogate HNSCC invasion may reveal novel therapeutic targets for intervention. The purpose of this study was to investigate the efficacy of combined inhibition of c-Src and PLCγ-1 in the abrogation of HNSCC invasion.

Experimental Design: PLCγ-1 and c-Src inhibition was achieved by a combination of small molecule inhibitors and dominant negative approaches. The effect of inhibition of PLCγ-1 and c-Src on invasion of HNSCC cells was assessed in an in vitro Matrigel-coated transwell invasion assay. In addition, the immunoprecipitation reactions and in silico database mining was used to examine the interactions between PLCγ-1 and c-Src.

Results: Here, we show that inhibition of PLCγ-1 or c-Src with the PLC inhibitor U73122 or the Src family inhibitor AZD0530 or using dominant-negative constructs attenuated epidermal growth factor (EGF)–stimulated HNSCC invasion. Furthermore, EGF stimulation increased the association between PLCγ-1 and c-Src in HNSCC cells. Combined inhibition of PLCγ-1 and c-Src resulted in further attenuation of HNSCC cell invasion in vitro.

Conclusions: These cumulative results suggest that PLCγ-1 and c-Src activation contribute to HNSCC invasion downstream of EGF receptor and that targeting these pathways may be a novel strategy to prevent tumor invasion in HNSCC.

Head and neck squamous cell carcinoma (HNSCC) continues to be the sixth most common cancer in the world and a major cause of cancer morbidity and mortality. The treatment of HNSCC has primarily relied on standard approaches, including surgery, radiation, and chemotherapy or a combination of these methods, with little improvement in outcome. Most HNSCC tumors rapidly invade the normal surrounding stroma and metastasize to regional lymph nodes. Understanding the molecular mechanisms responsible for the highly invasive nature of HNSCC tumors is vital to develop more effective strategies to manage this disease.

Epidermal growth factor (EGF) receptor (EGFR) is essential not only for cell survival but also plays an important role in cell motility and invasion. Several signaling pathways leading to altered cellular phenotypes are triggered downstream of EGF in HNSCC cells. We previously showed that EGFR activates PLCγ-1, resulting in an increase in HNSCC cell invasion (1). Others have reported that PLCγ-1 inhibition decreases the invasion of prostate and breast carcinoma cells (2). Thus PLCγ-1 plays a crucial role in invasion of HNSCC and other tumor types. In addition to PLCγ-1, c-Src has also been implicated in cancer invasion (3–5). A nonreceptor tyrosine kinase, c-Src, is a proto-oncogene that is overexpressed in several cancers including HNSCC, breast, prostate, and colon carcinoma (6–9). We have previously reported that c-Src directly associates with EGFR in HNSCC cells upon EGF stimulation (10).

The cooperative role of PLCγ-1 and c-Src in mediating tumor cell invasion downstream of EGF has not been previously investigated. The present study was undertaken to test the hypothesis that PLCγ-1 and c-Src interact with each other upon EGF stimulation, and activation of these proteins contribute to promoting HNSCC tumor cell invasion. We examined PLCγ-1 expression levels in HNSCC cells derived from primary and metastatic HNSCC tumors. Inhibition of PLCγ-1 or c-Src activation, using either pharmacologic inhibitors or dominant-negative mutants, abrogated EGF-stimulated HNSCC cell invasion. Additional investigation showed that EGF stimulation increased the association between PLCγ-1 and c-Src in HNSCC cells and that combined inhibition of PLCγ-1 and c-Src resulted in further attenuation of HNSCC cell invasion in vitro.
in further abrogation of HNSCC cell invasion. These cumulative results suggest that PLCγ-1 and c-Src interact with each other on EGF stimulation and that activation of PLCγ-1 and c-Src via EGFR contributes to HNSCC invasion. These results suggest that combined inhibition of PLCγ-1 and c-Src represents a potential strategy to prevent tumor invasion and metastasis in HNSCC.

**Materials and Methods**

**Cell lines.** HNSCC cell lines PCI-15A, PCI-37A, and UM-22A were derived from primary HNSCC tumor tissue, and PCI-15B, PCI-37B, and UM-22B were derived from matched metastatic lymph nodes, respectively, as described previously (11). Cells were maintained in DMEM with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc.) at 37°C with 5% CO2. Previously described human HNSCC cell line OSC-19, derived from a metastatic lymph node, was cultured in improved Eagle’s MEM containing 10% heat-inactivated FBS and nonessential amino acids (0.1 mmol/L; ref. 11).

**Reagents.** For in vitro cell stimulation, recombinant human EGF (Sigma Chemical Co.) was used. U73122 (BioMol) was used to block PLC activity. An inactive analogue of U73122, U73343 (BioMol), was used as a negative control. Antibodies used included mouse monoclonal anti–PLCγ-1 (Upstate Biotechnology), anti–phosphorylated PLCγ-1 (Cell Signaling Technologies), anti–phosphorylated focal adhesion kinase (FAK; Cell Signaling Technologies), tubulin (Abcam, Inc.), and β-actin (Calbiochem-Novabiochem Corporation). Antibodies against the activation loop of Src (PY418) and total c-Src were purchased from Biosource International and Santa Cruz Biotechnology, respectively. The c-Src inhibitor AZD0530 was supplied by AstraZeneca Pharmaceuticals.

**Transfection of HNSCC cells with dominant-negative PLCγ-1.** Previously described HNSCC cell line PCI-37A engineered to express dominant-negative Src (K296R/528F) DNA was used in these studies (12). An expression vector coding for a dominant-negative PLCγ-1 fragment (PLCz), as previously described, was stably transfected into a representative HNSCC cell line (OSC-19; ref. 13). Colonies obtained after selection were characterized by immunoblotting for levels of activated PLCγ-1 with or without EGF stimulation. Clones where EGF stimulation failed to activate PLCγ-1 were used in this study.

**Immunoblotting.** HNSCC cells were plated at 4 × 10^4 cells per 100-mm dish. Twenty-four hours after plating, cells were serum starved for 72 h. During serum starvation the media was changed every 24 hours. For the experiments with inhibitors, cells were treated with 3 μmol/L of either U73122 or U73343 for 25 min or 1 μmol/L AZD0530 for 4 h followed by stimulation with 10 ng/ml of recombinant human EGF or 10% FBS for 5 min. After EGF stimulation, cells were washed thrice with cold PBS and lysed as previously described (14). Forty-microgram protein was size-fractionated through an 8% SDS-PAGE gel and immunoblotted for phosphorylated and total PLCγ-1, c-Src, FAK, and β-actin.

**Immunoprecipitation.** For immunoprecipitation, 200 μg of protein was precipitated with anti–c-Src antibody (Cell Signaling Technologies) or anti–PLCγ-1 antibody (Upstate Biotechnology) or antinouse IgG as a control and protein agaroce G beads (Invitrogen). The immunoprecipitated proteins then were resolved on an 8% SDS-PAGE gel and immunoblotted for anti–PLCγ-1 antibody (Cell Signaling Technologies) or PY418 antibody. To show equal loading of protein among various lanes, immunoblots were stripped in Restore Western Blot Stripping Buffer (Pierce), blocked, and probed with anti–PLCγ-1 antibody (Cell Signaling Technologies) or anti–c-Src antibody (Santa Cruz Biotechnology).

**In vitro invasion of HNSCC cells.** Cell invasiveness was evaluated in vitro using Matrigel-coated semipermeable-modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocoat). HNSCC cells (2.5 × 10^4) were plated in serum-free medium in the insert. The lower chamber contained DMEM + 10% FBS that served as a chemotacticant. Cells were treated in the presence or absence of EGF (10 ng/ml) and/or U73122 (3 μmol/L), U73343 (3 μmol/L), or AZD0530 (1 μmol/L). To control for effect of inhibitors or growth factors on cell growth, the cells were also plated in parallel in a 96-well plate under identical conditions. After 48 h of treatment at 37°C in a 5% CO2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained using Hema 3 (Fisher Scientifics) according to the manufacturer’s instructions. Invading cells in four representative fields were counted using light microscopy at 200× magnification. Mean ± SE was calculated from three independent experiments. Cells plated on the 96-well plate were assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for metabolically active cells. Under the conditions used in the assay, there was no significant difference in the cell number between various treatment conditions.

**Database mining to examine interactions between EGF, PLCγ-1, and c-Src.** The Human Protein Reference database was used to identify publications documenting physical pair-wise interaction between PLCγ-1, EGFR, and c-Src. In addition, a total of 27 features from eight different data sources were collected to identify indirect evidences, further supporting their interactions. Three types of “similarity” features were derived from the Gene Ontology database (15), subcellular localization, functional categorization, and pathway membership. Domain-domain interactions were derived based on the hypergeometric distribution (16). Finally, we compared if there are homologous proteins in yeast and the known yeast protein-protein interactions derived from the corresponding interactions between the human proteins (17).

In addition to qualitatively comparing the entries in the databases for the three proteins, we also investigated the similarities quantitatively, using a classification framework (18). Each of the indirect data provides
partial information about interacting pairs and, together, contributes to the likelihood that two proteins interact. We developed a classification strategy to integrate the evidences from different data sources (18). By transforming the multiple data sources into a feature vector for every pair of proteins, we learned from HPRD-derived training data those features that distinguish interacting from noninteracting proteins. In a binary classification computational framework, we applied the Random Forest method to differentiate the two classes of proteins (17). Based on the score assigned to an interaction by the Random Forest, we estimated the confidence in an interaction based on the multiple evidences.

Statistical analysis. Statistical analysis was done using the exact Wilcoxon test to determine the statistical significance of the differences between groups using STATXact Version 6.0 (Cytel, Inc.).

Results

PLCγ-1 expression levels are elevated in the metastatic tumor-derived HNSCC cell lines. We previously reported that PLCγ-1 mediates cellular migration and invasion downstream of EGRF where increased expression of PLCγ-1 was observed in HNSCC tumors compared with levels in normal adjacent mucosa (1). To determine the role of PLCγ-1 in tumor progression from primary tumor to metastatic tumor, four HNSCC cell lines derived from the primary tumor and the paired metastatic lymph node from the same patient were analyzed via immunoblotting for total and phosphorylated PLCγ-1 levels. The primary and metastatic HNSCC cell lines were analyzed by immunoblotting for PLCγ-1. In all three paired cell lines, a 1.4-fold to 2.4-fold increase in PLCγ-1 expression levels were detected in the metastatic tumor-derived HNSCC cell line compared with paired primary tumor-derived cell line (Fig. 1).

HNSCC cell lines derived from metastatic lymph nodes are more invasive than those derived from primary tumors. Molecular alterations in the primary tumor contribute to the metastatic spread of HNSCC tumors (19). Previous reports have shown that up-regulation of molecules involved in motility, including integrins, are associated with HNSCC invasion (20). Because expression and activation of PLCγ-1 was increased in cells derived from metastatic lymph nodes, we examined the invasive capacity of the paired primary and metastatic lymph node-derived HNSCC cells in vitro. Matrigel invasion assays were done in three paired HNSCC cell lines over 48 h. HNSCC cells derived from the primary tumor were less invasive than cells derived from the lymph node metastasis, suggesting that increased PLCγ-1 expression may correlate with an increase in the invasive capacity of HNSCC cells (Fig. 2).

Inhibition of PLCγ-1 partially abrogates HNSCC cell invasion in vitro. PLCγ-1 is phosphorylated by EGFR on ligand stimulation (1). Further phosphorylation of PLCγ-1 results in an increase in inositol phosphate turnover, indicating increased PLCγ-1 activity (1). Thus, phosphorylation of PLCγ-1 results in activation of the enzyme triggering downstream effects, including increase in inositol phosphate and diacyl glycerol levels. To examine the effect of decreased PLCγ-1 on HNSCC invasion in vitro, HNSCC cells were transfected with dominant-negative PLCγ-1 or with the empty vector. Transfected clones were characterized by testing for PLCγ-1 activation in the presence of EGF stimulation. EGFR stimulation increased activation of PLCγ-1 in vector control–transfected HNSCC cells, but not in dominant-negative PLCγ-1 expressing cells (Fig. 3A). HNSCC cells expressing dominant-negative PLCγ-1 were next examined for invasion in vitro in response to EGF or serum stimulation. Cells expressing dominant-negative PLCγ-1 did invade in response to EGF and serum stimulation but to a lesser extent when compared with the vector control cells (Fig. 3B). Thus, although PLCγ-1 plays a role in EGFR-mediated HNSCC cell invasion, other molecules downstream of EGFR may also contribute to HNSCC invasion.

Inhibition of c-Src activation on EGF stimulation abrogates HNSCC cell invasion in vitro. We have previously shown that c-Src may be involved in EGFR-mediated invasion in HNSCC (12). To examine the efficacy of combined inhibition of both PLCγ-1 and c-Src in abrogating HNSCC invasion, we first determined strategies to inhibit EGFR-mediated c-Src activation in HNSCC cells. We have previously shown that c-Src directly associates with EGFR upon EGF stimulation in HNSCC cells (12). To determine the effect of c-Src inhibition on EGFR-mediated invasion, we tested the efficacy of a small molecule inhibitor and a dominant-negative c-Src approach. Cells were pretreated for 4 h with the Src inhibitor AZD0530 and were then stimulated with recombinant EGF (10 ng/mL) or 10% FBS containing medium for 5 min. Protein extracts were fractioned on an SDS-PAGE gel. Immunoblotting was done with anti–phosphorylated c-Src antibody (PY418) followed by anti-c-Src antibody to show equal loading. HNSCC cells treated with the Src inhibitor AZD0530 did not activate c-Src on EGF stimulation compared with the vehicle control–treated cells (Fig. 4A). This indicates that AZD0530 effectively inhibits tyrosine kinase receptor–mediated c-Src activation in HNSCC cells. Recently, c-Src was reported to mediate its invasive effects in breast cancer cells through activation of FAK (21). To determine if the increase in phosphorylated c-Src on EGF stimulation correlates with any meaningful change in c-Src kinase activity, we examined the effect of c-Src inhibition on activation of FAK. Our data show that in 37A cells EGF stimulation results in increase in phosphorylation of FAK (Fig. 4B). Pretreatment of the cells with Src inhibitor AZD0530 resulted in a reduction in EGF-mediated phosphorylation of FAK. However, in addition to c-Src, AZD0530 also inhibits...
control cells were plated at a density of 2 × 10^4 cells per well in Matrigel-coated transwell chambers at 2.5 × 10^5 cells per well in the presence of EGF (10 ng/mL) or 10% FBS containing medium and were treated with the PLC inhibitor U73122 (3 μmol/L) or the Src inhibitor AZD0530 (1 μmol/L) or a combination of both inhibitors for 48 hours. As shown in Fig. 5A, HNSCC cell invasion upon EGFR stimulation was attenuated by combination treatment with PLC and Src inhibitors compared with the controls. In addition to small molecule inhibitors, we tested combined inhibition of PLCγ-1 and Src in HNSCC cells expressing the dominant-negative constructs.

Dominant-negative c-Src or PLCγ-1 expressing cells and vector control cells were plated in Matrigel-coated transwell chambers at 2.5 × 10^5 cells per well in the presence of EGF (10 ng/mL) or 10% FBS containing medium and treated with either the PLC inhibitor or the Src inhibitor. Dominant-negative c-Src expressing cells and vector control cells were treated with PLC inhibitor U73122 (3 μmol/L) and dominant-negative PLCγ-1 expressing cells and vector control cells were treated with c-Src inhibitor AZD0530 (1 μmol/L) for 48 h. We found that dominant-negative c-Src or PLCγ-1 expressing cells showed reduced invasion compared with vector-transfected control cells (Fig. 5B and C) in presence of small molecule inhibitors compared with the controls. These cumulative results suggest that dual inhibition of c-Src and PLCγ-1 induces effectively attenuates EGFR-mediated HNSCC cell invasion.

Inhibition of PLCγ-1 and c-Src Abrogates HNSCC Invasion

Database mining results. The human protein reference database lists publications documenting physical pair-wise interactions (24–27). All three proteins PLCγ-1, EGFR, and c-Src are known to interact with each other physically. In

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**Fig. 3.** Inhibition of PLCγ-1 results in reduced EGFR-mediated invasion in HNSCC. A, HNSCC cell line OSC-19 cells expressing dominant-negative PLCγ-1 (PLCγ-1 clones 4 and 7) and vector control transfected cells were serum starved for 24 h before EGF stimulation. Protein extracts were fractionated on a SDS-PAGE gel. Immunoblotting was done with anti-phosphorylated PLCγ-1 followed by anti-PLCγ-1 antibody to show equal loading. EGFR failed to phosphorylate PLCγ-1 in dominant-negative PLCγ-1 cells compared with the vector control cells. B, dominant-negative PLCγ-1 expressing OSC-19 cells (PLCγ-4) or vector-transfected control cells were plated at a density of 2 × 10^5 cells per well in Matrigel-coated transwell chambers in the presence of EGF (10 ng/mL) or 10% FBS containing medium. After 48 h, cells on the reverse side of the upper chamber were fixed, stained, and counted. Columns, mean of at least two independent experiments; bars, SE. EGFR-mediated HNSCC cell invasion to a lesser degree in cells expressing dominant-negative PLCγ-1 compared with vector control cells indicating that PLCγ-1 plays a role in EGFR-mediated invasion of HNSCC.

Bcr-Abl (22). To verify the specific effects of c-Src inhibition on EGFR-mediated invasion, we used a dominant-negative c-Src expressing HNSCC cell line. HNSCC cells expressing dominant-negative c-Src and control vector were assessed for c-Src activation on EGFR stimulation. Cells were serum starved for 72 hours to reduce the effects of autocrine ligands. Cells were then stimulated with EGF (10 ng/mL) for 10 min. Cell lysates were analyzed via immunoblotting for c-Src activation. EGFR stimulation increased activation of c-Src in control vector–transfected HNSCC cells, but not in dominant-negative c-Src expressing cells (Fig. 4C). Thus, overexpression of dominant-negative c-Src effectively abrogates EGFR-mediated c-Src activation in HNSCC cells.

Previous reports show the HNSCC cells fail to invade in the presence of a small molecule Src inhibitor, dasatinib (23). We examined the effects of src inhibitor AZD0530 on HNSCC invasion and found that cells treated with the inhibitor have fewer invading cells despite stimulation of the EGFR signaling axis (data not shown). The reduced invasion of HNSCC cells as a result of c-Src inhibition emphasizes the role of c-Src signaling in HNSCC invasion. The invasive capacity of HNSCC cells (37A) stably transfected with a dominant-negative c-Src construct was analyzed. As shown in Fig. 4D, vector control cells responded to EGFR stimulation by invading the Matrigel-coated transwell chamber. In contrast, dominant-negative c-Src expressing HNSCC cells failed to invade under the same conditions. These cumulative results suggest that c-Src plays a role in EGFR-mediated HNSCC cell invasion.

Combined inhibition of c-Src and PLCγ-1 induces further abrogation of HNSCC cell invasion upon EGFR stimulation. Our data suggest that several pathways contribute to the metastatic phenotype of HNSCC cells. Combined inhibition of multiple molecules involved in invasion may be effective in curtailing tumor dissemination. Our data show that specific inhibition of PLCγ-1 or c-Src abrogates EGF-stimulated HNSCC cell invasion in vitro. To examine the effects of combined PLCγ-1 and c-Src blockade on HNSCC invasion, we used both small molecule inhibitors and dominant-negative approaches. HNSCC cells were plated in Matrigel-coated transwell chambers at 2.5 × 10^5 cells per well in the presence of EGF (10 ng/mL) or 10% FBS containing medium and were treated with the PLC inhibitor U73122 (3 μmol/L) or the Src inhibitor AZD0530 (1 μmol/L) or a combination of both inhibitors for 48 hours. As shown in Fig. 5A, HNSCC cell invasion upon EGFR stimulation was attenuated by combination treatment with PLC and Src inhibitors compared with the controls. In addition to small molecule inhibitors, we tested combined inhibition of PLCγ-1 and Src in HNSCC cells expressing the dominant-negative constructs.

Dominant-negative c-Src or PLCγ-1 expressing cells and vector control cells were plated in Matrigel-coated transwell chambers at 2.5 × 10^5 cells per well in the presence of EGF (10 ng/mL) or 10% FBS containing medium and treated with either the PLC inhibitor or the Src inhibitor. Dominant-negative c-Src expressing cells and vector control cells were treated with PLC inhibitor U73122 (3 μmol/L) and dominant-negative PLCγ-1 expressing cells and vector control cells were treated with c-Src inhibitor AZD0530 (1 μmol/L) for 48 h. We found that dominant-negative c-Src or PLCγ-1 expressing cells showed reduced invasion compared with vector-transfected control cells (Fig. 5B and C) in presence of small molecule inhibitors compared with the controls. These cumulative results suggest that dual inhibition of c-Src and PLCγ-1 induces effectively attenuates EGFR-mediated HNSCC cell invasion.

PLCγ-1 and c-Src associate upon EGFR stimulation in HNSCC cells. Analyses of the protein domains in PLCγ-1 and c-Src indicate a potential for interaction between the two proteins via the SH2 and SH3 domains. To determine if PLCγ-1 and c-Src interaction is induced by EGFR stimulation, we carried out immunoprecipitation and immunoblotting of PLCγ-1 and c-Src in the presence or absence of EGF. Cells expressing dominant-negative c-Src or PLCγ-1 and control vector–transfected cells were stimulated with EGF or 10% serum after 72-hour serum starvation. Cell lysates were subject to immunoprecipitation with PLCγ-1 followed by immunoblotting for c-Src and PLCγ-1. EGFR stimulation increased the association of PLCγ-1 and c-Src in HNSCC cells expressing the dominant-negative constructs.

Database mining results. The human protein reference database lists publications documenting physical pair-wise interactions (24–27). All three proteins PLCγ-1, EGFR, and c-Src are known to interact with each other physically. In
addition to the direct evidence for physical interaction, we also investigated other biological databases that could provide indirect evidence corroborating the physical and functional linkage between these proteins. Each of these indirect data provides partial information about interacting pairs, and all features together contribute to the likelihood that two proteins interact. We analyzed the features both qualitatively and using a computational framework, in which interacting proteins are distinguished from noninteracting proteins by solving a binary classification task (18). We found that the scores for all three sets of interactions are very high, typical of interactions of high confidence. The highest score was found in the case of the EGFR/c-Src interaction, second highest in the case of the PLCγ-1/c-Src, and third highest in the case of the EGFR/PLCγ-1 interaction. In terms of individual features, gene ontology functional similarity was found for the PLCγ-1/c-Src pair, which share signal transducer and protein binding categories, and the EGFR/c-Src pair, which share protein kinase and transferase activities.

Discussion

Due to the close proximity of vital structures in the head and neck region, tumor invasion increases morbidity and mortality in HNSCC patients. Therapies that prevent HNSCC tumor invasion and metastasis may facilitate the management of this disease by containing of the tumor at the site of origin. Aberrant expression and signaling of several proteins has been implicated in tumor progression to metastatic phenotypes. Up-regulation of EGFR has been associated with increased...
proliferation, survival, and invasion of HNSCC tumors. EGFR mediates its effects on HNSCC invasion via activation of downstream signal transduction molecules. In this study, we present evidence to support the hypothesis that EGFR mediates invasion of HNSCC via activation of PLC\(\gamma\)-1 and c-Src.

We previously reported that PLC\(\gamma\)-1 levels are higher in HNSCC tumor tissue compared with normal adjacent mucosa and PLC\(\gamma\)-1 blockade reduces EGFR ligand–mediated cell invasion in vitro (1). In the present study, we showed that PLC\(\gamma\)-1 expression and activation levels are elevated in the metastatic tumor–derived HNSCC cell lines compared with levels in paired primary tumor–derived cell lines. PLC\(\gamma\)-1 is activated by receptor tyrosine kinases by recruitment of the lipase to the autophosphorylated receptor and subsequent tyrosine phosphorylation (28–30). Activation of PLC\(\gamma\)-1 results in the production of the second messengers, such as diacylglycerol and inositol 1,4,5-trisphosphate and subsequent activation of protein kinase C isoforms, plays an important role in diverse cellular responses, including cytoskeletal rearrangement (31, 32). Inositol 1,4,5-trisphosphate binding to intracellular receptors induces an increase in cytosolic calcium levels and increased intracellular calcium levels in tumor cells are associated with increased cell motility (33). Thus, elevated PLC\(\gamma\)-1 expression and activation may cause increased cell motility in HNSCC cells. Our results, comparing the invasive-ness between metastatic and primary tumor-derived HNSCC cells, suggest that elevated PLC\(\gamma\)-1 expression and activation may correlate with cell invasion and that PLC\(\gamma\)-1 activity is required for EGFR-mediated cell motility in HNSCC. Furthermore, we also showed that inhibition of PLC\(\gamma\)-1 abrogates EGFR-mediated HNSCC cell invasion in PLC inhibitor–treated and dominant-negative PLC\(\gamma\)-1 expressing HNSCC cells. These

**Fig. 5.** Combined inhibition of PLC\(\gamma\)-1 and c-Src results in attenuation of EGFR-mediated invasion in HNSCC cells. A, pharmacologic inhibitors of PLC\(\gamma\)-1 and c-Src are active against EGFR-mediated invasion in HNSCC. HNSCC cells PCI-37B were plated at a density of 2.5 \(\times\) 10^5 cells per well in Matrigel-coated transwell chambers in the presence of EGF (10 ng/mL) or 10% FBS containing medium and treated with AZD0530 (1 \(\mu\)mol/L), PLC inhibitor (U73122; 3 \(\mu\)mol/L), control inactive compound (U73343; 3 \(\mu\)mol/L), and AZD0530 (1 \(\mu\)mol/L) plus U73122 or U73343 (3 \(\mu\)mol/L) for 48 h. Combined inhibition of PLC and c-Src in HNSCC cells results in abrogation of EGFR-mediated invasion compared with single agent–treated cells \((P < 0.05)\). B, PLC inhibition in HNSCC cells expressing dominant-negative c-Src results in minimal EGFR-mediated invasion. Dominant-negative c-Src expressing HNSCC cells or vector-transfected control cells were plated at a density of 2.5 \(\times\) 10^5 cells per well in the presence of EGF (10 ng/mL) or 10% FBS containing medium and treated with U73122 (3 \(\mu\)mol/L). Treatment of dominant-negative c-Src expressing cells with PLC inhibitor U73122 attenuated EGFR-mediated invasion in HNSCC cells compared with vector control cells \((*, P < 0.05)\). C, c-Src inhibition in HNSCC cells expressing dominant-negative PLC\(\gamma\)-1 results in minimal EGFR-mediated invasion. Dominant-negative PLC\(\gamma\)-1 expressing HNSCC cells were plated and treated with AZD0530 (1 \(\mu\)mol/L). After 48 h of treatment, the cells on the lower side of the chamber were counted at 200 \(\times\) magnification. Columns, mean of at least two independent experiments; bars, SE. HNSCC cells expressing dominant-negative PLC\(\gamma\)-1 when treated with the Src inhibitor AZD0530 have significantly fewer invading cells of EGFR stimulation compared with vector-transfected control under the same conditions \((**, P < 0.05)\).
cumulative results suggest that PLCγ-1 plays an important role in EGFR-mediated invasion of HNSCC cells.

In addition to PLCγ-1, it has been reported that c-Src expression and activation are elevated in a various of human tumors, including breast, colon, prostate, and head and neck (6, 9, 34, 35). Increased Src activity has been reported to correlate with the loss of epithelial differentiation and acquisition of a fibroblastic-like phenotype, which is involved in the metastatic potential of carcinoma cells (4). We previously showed that c-Src directly associates with EGFR upon EGF stimulation and c-Src activation contributes to HNSCC cell invasion (12). Although c-Src expression levels were not elevated in the metastatic tumor–derived HNSCC cell lines compared with levels in paired primary tumor–derived cell lines (data not shown), inhibition of c-Src activation on EGFR stimulation by pharmacologic (AZD0530) and dominant-negative methods abrogates HNSCC cell invasion. AZD0530 is a highly selective inhibitor of nonreceptor tyrosine kinases, including c-Src, c-Yes, Lck, and Abl (22). We show that AZD0530 inhibited c-Src activation mediated by EGFR stimulation in HNSCC cells and significantly suppressed the invasive nature of HNSCC cells in vitro. The reduced invasion of HNSCC cells, as a result of c-Src inhibition by AZD0530, emphasizes the important role of c-Src signaling in HNSCC cell invasion. Others have reported that AZD0530 is a potent inhibitor of cell migration, and combined treatment with EGFR inhibitor showed markedly additive effects toward inhibition of cell motility and invasion in breast carcinoma cells (36). Recently, it has been shown that an EGFR tyrosine kinase inhibitor suppresses c-Src and p21-activated kinase 1 activation and invasiveness of HNSCC and breast cancer cells on EGF stimulation (37). Taken together, these data indicate that c-Src activation mediated by EGFR likely contributes to the invasiveness and metastatic potential of HNSCC cells.

Because inhibition of PLCγ-1 or c-Src abrogates EGFR-stimulated HNSCC cell invasion in vitro, we hypothesized that combined inhibition of both PLCγ-1 and c-Src would further abrogate HNSCC cell invasion. In the present study, we showed that HNSCC cell invasion upon EGF stimulation was almost completely blocked by combined inhibition of both PLCγ-1 and c-Src by pharmacologic and dominant-negative methods. To our knowledge, this is the first report to show the combined inhibition of both PLCγ-1 and c-Src abrogates EGFR-mediated tumor cell invasion. Further testing in in vivo preclinical models will be required to fully explore the translational significance of this strategy. To elucidate the mechanism of the combined effect, we examined the interaction of these molecules in HNSCC cells stimulated with EGF. Our results indicate that the interaction between PLCγ-1 and c-Src was significantly increased in HNSCC cells stimulated with EGF and that this effect could be blocked in dominant-negative PLCγ-1 or c-Src–transfected HNSCC cells. c-Src contains SH2 and SH3 domains which mediate intramolecular protein-protein interactions (38, 39) and

Fig. 6. PLCγ-1 and c-Src interact with each other upon EGFR stimulation in HNSCC cells. HNSCC cells expressing dominant-negative c-Src (A) or PLCγ-1 (B), and vector-transfected control cells were stimulated with EGF (10 ng/mL) or 10% FBS containing medium after serum starvation for 72 h. PLCγ-1 was immunoprecipitated from cell lysates followed by immunoblotting for c-Src and PLCγ-1. Densitometric analysis was done, and expression levels relative to PLCγ-1 of IgG control lysates show that there is no nonspecific binding of antimouse antibody to PLCγ-1 or c-Src.

Cancer Therapy: Preclinical
The SH2 domain of c-Src has been shown to directly interact with activated EGFR in vitro (26). In contrast, others have reported that c-Src interacts with EGFR indirectly through intermediary proteins, such as the mucin-like transmembrane glycoprotein via subsequent SH2-dependent binding of c-Src (45). Similarly, the SH2 domains of PLC-γ also mediate the association with a receptor tyrosine kinase, such as EGFR (46, 47). The SH2 domains of PLC-γ-1 bind to phosphotyrosine-containing peptides and mediate the recruitment of SH2 domain–containing targets proteins to activated EGFR. PLC-γ-1 can interact with the activated EGFR by a mechanism that involves the N-SH2 domain as a primary association event and the C-SH2 domain as a secondary event necessary for a maximal level of association (48). Furthermore, PLC-γ-1 can associate with several signaling molecules, including kinases of the Src family (49, 50).

The binding of EGFR to its receptor induces dimerization of receptor subunits and stimulation of the tyrosine kinase activity of EGFR and results in autophosphorylation of EGFR on specific tyrosine residues. These phosphorylated tyrosine residues in EGFR initiate cellular signaling by acting as high-affinity binding sites for the SH2 domains of various effector proteins, such as PLC-γ-1 and c-Src. PLC-γ-1 and c-Src bind directly or indirectly to phosphorylated tyrosine residues on EGFR. The close proximity of PLC-γ-1 and c-Src in the receptor complex may promote reciprocal SH2 domain interactions (26, 45–47, 51, 52). Further exploration of in silico databases revealed that PLC-γ-1 and c-Src can directly interact with each other and also share numerous indirect features that make them likely to interact with each other, corroborating our findings in HNSCC cells. We previously showed that EGFR and PLC-γ-1 are overexpressed in HNSCC (1, 53). Overexpression of SH2 domain–containing proteins may lead to signal amplification through enhanced recruitment of the enzyme to activated receptors, suggesting that the resulting synergistic signaling will occur to a much greater extent in HNSCC cells and may increase EGFR-mediated HNSCC cell invasion.

In conclusion, we report here that EGFR activation increases the interaction of both PLC-γ-1 and c-Src in HNSCC cells and combined inhibition of these molecules can block EGFR-mediated HNSCC cell invasion.

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

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