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Epidermal Growth Factor Receptor-stimulated Activation of Phospholipase Cγ-1 Promotes Invasion of Head and Neck Squamous Cell Carcinoma[1]

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ABSTRACT

Lymph node metastasis and local invasion of head and neck squamous cell carcinoma (HNSCC) is associated with a poor prognosis. However, little is known about the factors governing tumor cell invasion in HNSCC. Phospholipase Cγ-1 (PLCγ-1) contributes to tumor cell invasion in experimental systems when activated by the epidermal growth factor receptor (EGFR). We hypothesized that EGFR overexpression in HNSCC mediates invasion via PLCγ-1. On EGFR ligand stimulation, phosphorylation of PLCγ-1 increased in all of the HNSCC cell lines tested (4 of 4). In the presence of EGFR-specific tyrosine kinase inhibitor (PD153035) or an anti-EGFR antibody (C225), PLCγ-1 activation was abrogated indicating that PLCγ-1 was downstream of EGFR. Blocking cellular PLC with an inhibitor (U73122) reduced inositol phosphate turnover in all of the HNSCC cell lines examined, and treatment with the PLC inhibitor or antisense oligonucleotides targeting PLCγ-1 significantly reduced *in vitro* invasiveness of HNSCC cell lines through Matrigel. To determine the clinical relevance of these findings, we compared levels of PLCγ-1 in tumor and paired normal tissue from 33 patients with HNSCC. PLCγ-1 levels were significantly higher ($P < 0.0001$) in the tumors compared with the normal mucosa of HNSCC patients. Levels of activated PLCγ-1 were analyzed in 20 patients. Tumors expressed higher levels of phosphorylated PLCγ-1 compared with normal adjacent mucosa ($P = 0.05$). Thus, PLCγ-1 may mediate invasion and metastasis downstream of EGFR in HNSCC.

INTRODUCTION

Nearly 50% of patients with HNSCC[3] present with cervical lymph node metastases (1). Invasion of the tumor into the neck viscera is a primary cause of morbidity and mortality in this cancer. The mechanisms that govern tumor cell invasion in HNSCC are incompletely understood. Elucidation of the molecular events that mediate invasion is required to improve therapeutic approaches and, hence, survival.

More than 95% of HNSCC tumors express elevated levels of the EGFR (2, 3). We have shown previously that increased EGFR expression in HNSCC tumors correlates with reduced survival and an increased incidence of lymph node metastasis (4). An established oncogene, EGFR mediates cellular motility, proliferation, and prevents apoptosis in HNSCC cells via activation of a number of downstream signaling pathways. Activation of these pathways by EGFR is necessary for tumor progression (5). However, to rationally disrupt these events, an increased understanding of EGFR signaling is required to elucidate its biological role in cancers including HNSCC.

Several signaling pathways downstream of EGFR have been reported in HNSCC including the MAPK, phosphatidylinositol 3′-kinase, and STATs (6). Several lines of evidence suggest a redundancy among EGFR signaling pathways (7), whereas others have reported modulation of a specific phenotype when one pathway is specifically targeted. In HNSCC cells, EGFR-stimulated MAPK activation induced proliferation but not invasion (8). The phosphatidylinositol 3′-kinase pathway has been implicated in mediating anti-apoptotic functions and conferring radiation-resistance in HNSCC cells (9). We have shown previously that EGFR-mediated STAT3 activation is required for cell growth and survival *in vitro* (10). Additional evidence suggests that constitutively activated STAT3 in HNSCC tumors results in uncontrolled cell growth by an antiapoptotic mechanism (11). Phosphoinositide bisphosphate turnover downstream from PLCγ activation has not been studied previously in HNSCC.

The present study was undertaken to test the hypothesis that EGFR stimulation of PLCγ-1 mediates cell invasion in HNSCC. We examined PLCγ-1 expression and activation in a series of HNSCC cell lines and patient tissues. Blockade of EGFR abrogated PLCγ-1 levels suggesting that PLCγ-1 activation in HNSCC cells was primarily because of EGFR stimulation. Abrogation of PLCγ-1 decreased HNSCC cell invasion *in vitro* without affecting cell proliferation. *In vivo*, PLCγ-1 was expressed and phosphorylated at higher levels in tumor tissue compared with normal adjacent mucosa. These results indicate that EGFR-mediated PLCγ-1 activation modulates invasion of HNSCC cells and may contribute to tumorigenesis.

MATERIALS AND METHODS

Cell Lines and Tissues. Cell lines derived from HNSCC were maintained in DMEM with 10% FBS (Life Technologies, Inc., Grand Island, NY). The OSC-19 cell line was cultured in Eagle's MEM containing 10% FBS and nonessential amino acids (0.1 mM). Cell lines OSC-19 (12) and PCI-15b were derived from metastatic lymph nodes (13). UM-22a was derived from a SCC of the buccal mucosal (14). UPCI:SCC32, derived from the retromolar trigone, is a generous gift from Dr. Susanne M. Gollin (University of Pittsburgh, Pittsburgh, PA). A431 is a well-characterized vulvar SCC known to overexpress EGFR and was used as a positive control.

Patient tissues used in the study were obtained from patients undergoing surgery at the University of Pittsburgh Medical Center. Primary HNSCC and normal adjacent mucosa (~5 cm away from tumor site) were harvested under the auspices of an Institutional Review Board-approved protocol. Signed informed consent was obtained from each subject.

Reagents. For *in vitro* cell stimulation, recombinant human EGF (Sigma Chemical Co., St. Louis, MO) was used. U73122 (BioMol, Plymouth Meeting, PA) was used to block PLC activity. An inactive analogue of U73122, U73343 (BioMol), was used as a negative control. Specific EGFR tyrosine kinase inhibitor PD153035 was obtained from Calbiochem-Novabiochem Corporation (San Diego, CA). The EGFR blocking antibody C225 was obtained from Imclone Systems Incorporated (New York, NY). Antibodies used included mouse monoclonal anti-PLCγ -1 (Upstate Biotechnology, Lake Placid, NY), antiphospho-PLCγ-1 (Cell Signaling Technologies, Beverly, MA), and β-actin (Calbiochem-Novabiochem Corporation). Antisense and scrambled PLCγ-1 oligonucleotides were obtained from MWG Biotech (High Point, NC). The PLCγ-1 antisense oligonucleotide (5′AGGGGACGCGGCGCCCGCCAT3′) is a 21-mer fragment directed against the translation initiation site of human

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription; PLCγ, phospholipase Cγ; FBS, fetal bovine serum; SCC, squamous cell carcinoma; EGF, epidermal growth factor; IP, inositol phosphate; MMP, matrix metalloproteinase.

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PLCγ-1 (derived from Ref. 15). A control scrambled oligonucleotide sequence of the antisense sequence to PLCγ-1 (5′ATCCGCGGTGGCCGCCGCCAG3′) was also used in the study.

Effect of PLC Inhibition on Cellular Proliferation. The cytotoxic effects of the PLC inhibitor U73122 and antisense PLCγ-1 oligonucleotides were determined by incubating HNSCC cells in increasing concentrations of reagents for 2–4 days. Cells were plated in growth medium (MEM + 10% FCS + 1% nonessential amino acids) in triplicate wells at a density of 2×10^4 cells/well in 24-well plates. After 24 h, the medium was replaced with growth medium containing either the PLC inhibitor U73122 or vehicle control (chloroform). For studies with oligonucleotides, medium was replaced with serum-free medium containing the respective oligonucleotides. At a predetermined end point cells were trypsinized and counted on a hemocytometer.

Immunoblotting. Briefly, 80–90% confluent cells were serum starved for 48 h. Cells were then subjected to either 3 μM U73122 or U73343 for 25 min, 200 nM PD153035 or 7 μg/ml C225 for 2 h followed by stimulation with 10 ng/ml of recombinant human EGF for 5 min. After treatment, cells were harvested in lysis buffer [10 mM Tris HCl (pH 7.6), 50 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM NaV_3O_4, 1% Triton X-100 and 1× protease inhibitor mixture tablet that included a broad spectrum potent inhibitor of protein tyrosine phosphatases (Roche, Germany)]. Lysates were sonicated for 3 s and centrifuged at 4°C, 14,000 rpm for 5 min. The supernatant was collected for protein quantitation using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) and BSA of known concentration as the standard. Fifty μg of protein was size-fractionated through an 8% SDS-PAGE gel and immunoblotted with anti-pPLCγ-1 antibody (Cell Signaling Technologies) followed by anti-PLCγ-1 antibody (Upstate Biotechnology).

Frozen HNSCC tumor and normal tissue samples were finely minced and suspended in 1 ml of lysis buffer (1% NP40, 0.1 M phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin and aprotinin). The cell debrie was pelleted and the supernatant aliquoted, protein concentration estimated, and the proteins were size-fractionated on an 8% SDS-PAGE gel as mentioned before. Paired samples for tumor and normal tissue were always run next to each other on the same gel. Twenty μg of A431 protein extract was loaded on every gel as a positive control and normalization standard. Samples were electrophoresed along with a Bio-Rad molecular weight marker (Bio-Rad). The proteins were transferred to a nitrocellulose membrane in a semidry transfer apparatus (Bio-Rad). Western blots were probed with anti-PLCγ-1, anti-pPLCγ-1, or anti-β-actin antibody (Oncogene Research Products), and the signals were obtained on autoradiograms after enhanced chemiluminescent detection (Amersham ECL kit). Signals were quantified on a Molecular Dynamics Personal Densitometer SI and ImageQuant software (Image Products International, Chantilly, VA). Values obtained from PLCγ-1 and pPLCγ-1 quantification were normalized to β-actin levels as well as A431 levels. The ratio of the tumor:normal PLCγ-1 and phospho-PLCγ-1 levels were calculated.

***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, Bedford, MA). Cells were plated at a density of 2×10^4 cells/well in the chamber or insert. Both the insert and the holding well were subjected to the same medium composition with the exception of serum. The insert contained no serum, whereas the lower well contained 10% FBS that served as a chemoattractant. Chambers were treated with reagents depending on the experiment. Reagents used for invasion assays were EGF (10 ng/ml), PLCγ-1 antisense oligonucleotides (5′AGGGGACGCGGCGCCCGCCAT3′), or scrambled control oligonucleotides (5′ATCCGCGGTGGCCGCCGCCAG3′; 12.5 μM), U73122 (3 μM), U73343 (3 μM), or PD153035 (200 nM). After 48 h of treatment at 37°C in a 5% CO_2 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 with Hema 3 (Fisher Scientifics) according to the manufacturer's instructions. Invading cells in 4 representative fields were counted using light microscopy at ×400 magnification. Mean ± SE was calculated from two independent experiments.

IP Turnover Assay. The accumulation of IP was measured in cells as described previously (16). Briefly, cells were incubated with 0.5 μCi/ml myo [1,2-^{3}H (N)]-inositol for 24 h followed by incubation with 10 mM $LiCl_2$ for 10 min and then subjected to U73122 (3 μM) treatment for 25 min and/or recombinant human EGF (10 ng/ml) for 5 min. Cell lysates were run on AG1-X8 100–200 mesh anion-exchange resin-containing columns. IP was eluted using ammonium formate/formic acid (200 mM/100 mM). Levels of IP were obtained from radioactive counts in the ammonium formate/formic acid fraction.

Statistical Analysis. The signed rank test was applied to test the differences between PLCγ-1 levels in tumor compared with matched adjacent normal mucosa of 33 subjects. To analyze the differences between phosphorylated PLCγ-1 levels in tumor and normal tissue from 20 subjects, the exact sign rank test was used. Cell counts from invasion experiments were analyzed by a one-way ANOVA. Pairwise differences were tested with the Sidak procedure.

RESULTS

EGFR Stimulation Increases PLCγ-1 Activation in HNSCC Cells. Expression and activation of PLCγ-1 has not been studied previously in HNSCC cells. Four HNSCC cell lines were screened for expression of PLCγ-1. All four of the cell lines were found to express basal levels of PLCγ-1 (Fig. 1*A*). Phosphorylation of PLCγ-1 by EGFR tyrosine kinase results in activation of PLCγ-1. The ability of EGFR ligand, EGF, to induce PLCγ-1 phosphorylation was tested. HNSCC cells were serum starved for 48 h to minimize the effects of growth factors in the serum and signaling from secreted autocrine ligands. Cells were then stimulated with recombinant EGF (10 ng/ml for 5 min). Total cellular protein was harvested and fractionated on a SDS-PAGE gel. Western blotting was performed with anti-phospho-PLCγ-1 followed by anti-PLCγ-1 antibody to demonstrate equal loading. In all four of the cell lines tested, the EGFR ligand increased phosphorylation of PLCγ-1 compared with corresponding unstimulated cells (Fig. 1*B*). These findings suggest that EGFR ligand stimulation induces phosphorylation and, hence, activation of PLCγ-1 in HNSCC cells.

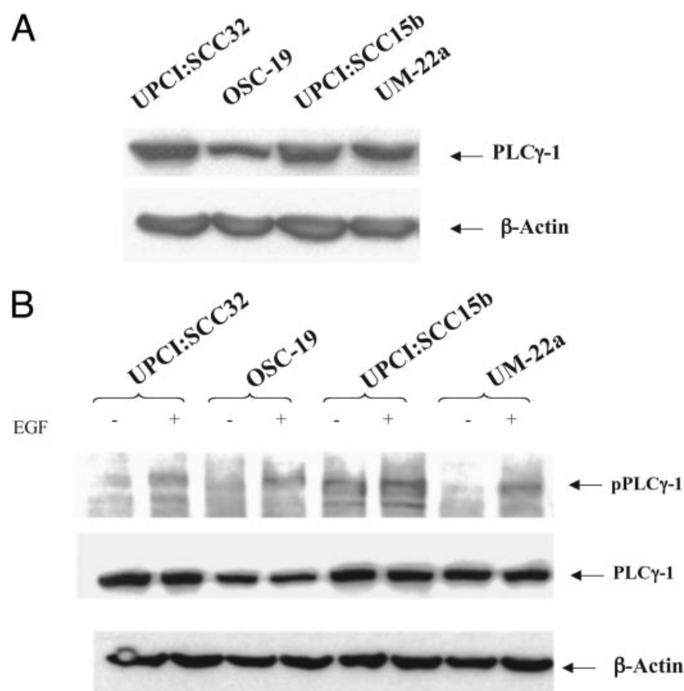

Fig. 1. EGFR stimulation activates PLCγ in HNSCC cells. *A*, PLCγ-1 levels in HNSCC cell lines. Four representative HNSCC cell lines were analyzed for PLCγ-1 expression by Western blotting. The same membrane was probed for β-actin to demonstrate equal loading. *B*, four representative HNSCC cell lines were analyzed for PLCγ-1 activation. Cell lysates from representative HNSCC cell lines (*UPCI:SCC32, OSC-19, PCI-15b,* and *UM-22a*) were treated with recombinant EGF (10 ng/ml for 5 min) followed by immunoblotting with anti-pPLCγ-1 antibody. The same blot was probed with anti-PLCγ-1 and β-actin antibodies to show equal loading of protein in all lanes. EGFR ligand stimulation results in increased activation of pPLCγ-1(*top panel*), whereas total levels of PLCγ-1 (*bottom panel*) remain unchanged.

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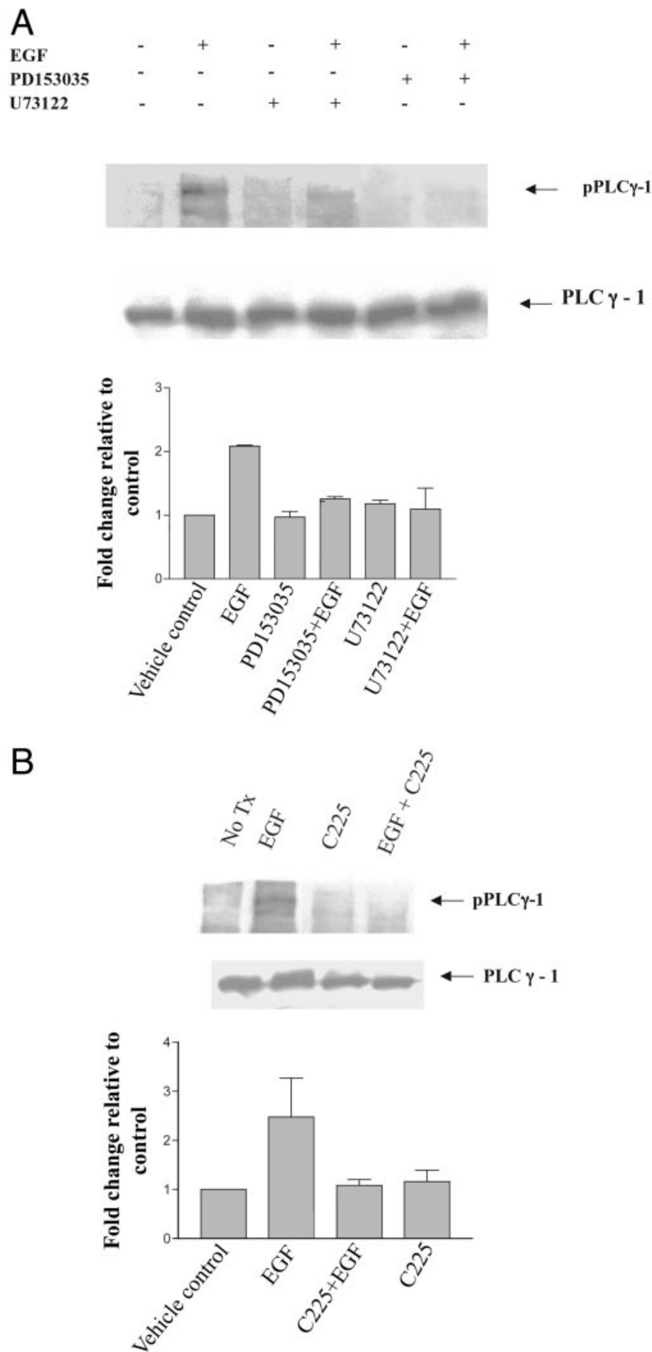

Fig. 2. EGFR mediates activation of PLCγ-1 in HNSCC cells. *A,* a representative HNSCC cell line (*OSC 19*) was pretreated for 2 h with an EGFR-specific inhibitor PD153035 (200 nM) or a PLC inhibitor U73122 (3 μM for 25 min) and/or treated with EGF (10 ng/ml for 5 min). Cell lysates were size fractionated on a SDS-PAGE gel and immunoblotted with anti-phospho-PLCγ-1 antibody. The same blot was probed for PLCγ-1 to show equal loading of protein in all lanes. Densitometric analysis was carried out, and gels were normalized to values of corresponding PLCγ-1 levels. *Bars,* ±SE from two separate experiments. *B,* a representative HNSCC cell line (*OSC 19*) was pretreated for 2 h with anti-EGFR antibody C225 (7 μg/ml) and/or treated with EGF (10 ng/ml for 5 min). Cell lysates were size fractionated on a SDS-PAGE gel and immunoblotted with anti-phospho-PLCγ-1 antibody. The same blot was probed for PLCγ-1 to show equal loading of protein in all lanes. Densitometric analysis was carried out and gels were normalized to values of corresponding PLCγ-1 levels. *Bars,* ±SE from two separate experiments.

Blockade of EGFR and PLC Abrogates the Activation of PLCγ-1. PLCγ-1 can be stimulated after activation of several growth factor receptors including EGFR. To determine whether activation of PLCγ-1 was mediated via an EGFR signaling pathway, HNSCC cells were treated with an EGFR-specific tyrosine kinase inhibitor (PD153035) at a dose reported previously to block EGFR phosphorylation in HNSCC cells (17). A representative HNSCC cell line (OSC-19) was pretreated for 2 h with either EGFR inhibitor PD153035 (200 nM) or anti-EGFR antibody C225 (7 μg/ml), or for 25 min with PLC inhibitor U73122 or the inactive analogue U73343 (3 μM). Pretreatment was followed by EGF stimulation (10 ng/ml for 5 min). Protein extracts were fractionated on a SDS-PAGE gel. Immunoblots were probed with anti-phospho-PLCγ-1 antibody and PLCγ-1 antibody to demonstrate equal loading. Activation of PLCγ-1 was reduced by EGFR or PLC inhibition by more than 90% (Fig. 2, *A* and *B*). These results indicate that EGFR activation stimulates PLCγ-1 phosphorylation in HNSCC cells.

Inhibition of PLCγ-1 Signaling Reduces Cell Invasion *in Vitro*. To determine whether PLC blockade abrogated HNSCC cell invasion *in vitro*, six HNSCC cell lines were plated in duplicate in transwell chambers at 2×10^4 cells/well with either EGF or the PLC inhibitor in the upper and lower chambers. After 24 h the cells on the lower side of the chamber were fixed and stained. The number of cells that invaded the Matrigel coated transwell chamber was counted using a light field inverted microscope. An average of four fields of cells counted under 400× magnification was determined. We found that

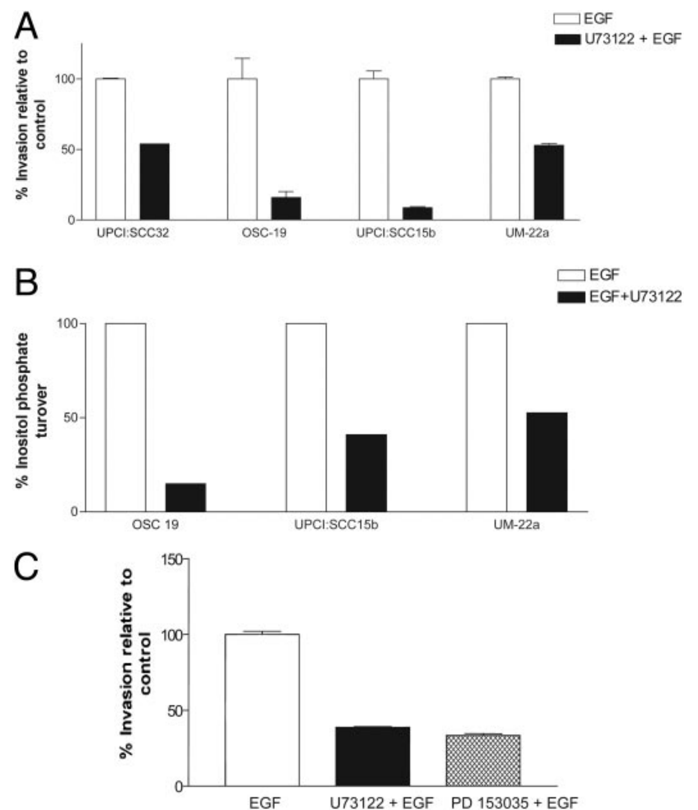

Fig. 3. PLC inhibition decreases invasion of representative HNSCC cell lines *in vitro*. *A,* the percent invasion of HNSCC cells (*UPCI:SCC32, OSC-19, PCI-15b,* and *UM-22a*) through Matrigel-coated transwell chambers was calculated relative to the control *Bars,* ±SE of duplicate samples. There was a reduction in the number of invading cells in the presence of the PLC inhibitor U73122 as compared with the untreated control. *B,* IP turnover in representative HNSCC cell lines (*OSC-19, PCI-15b,* and *UM-22a*). PLC activity was measured as the production of IPs by anion-exchange chromatography. The effect of U73122 (3 μM for 25 min) on IP turnover in EGF-treated (10 ng/ml for 5 min) cells was calculated and normalized to the control (EGF-treated). *C,* effect of PLC or EGFR inhibition on cell invasion *in vitro*. A representative HNSCC cell line (*OSC-19*) was plated in transwell invasion chambers in the presence of either an EGFR inhibitor PD153035 (200 nM) or PLC inhibitor U73122 (3 μM). The percentage invasion was determined by normalizing the number of cells that migrated in the control group. The experiment was repeated three times with similar results.

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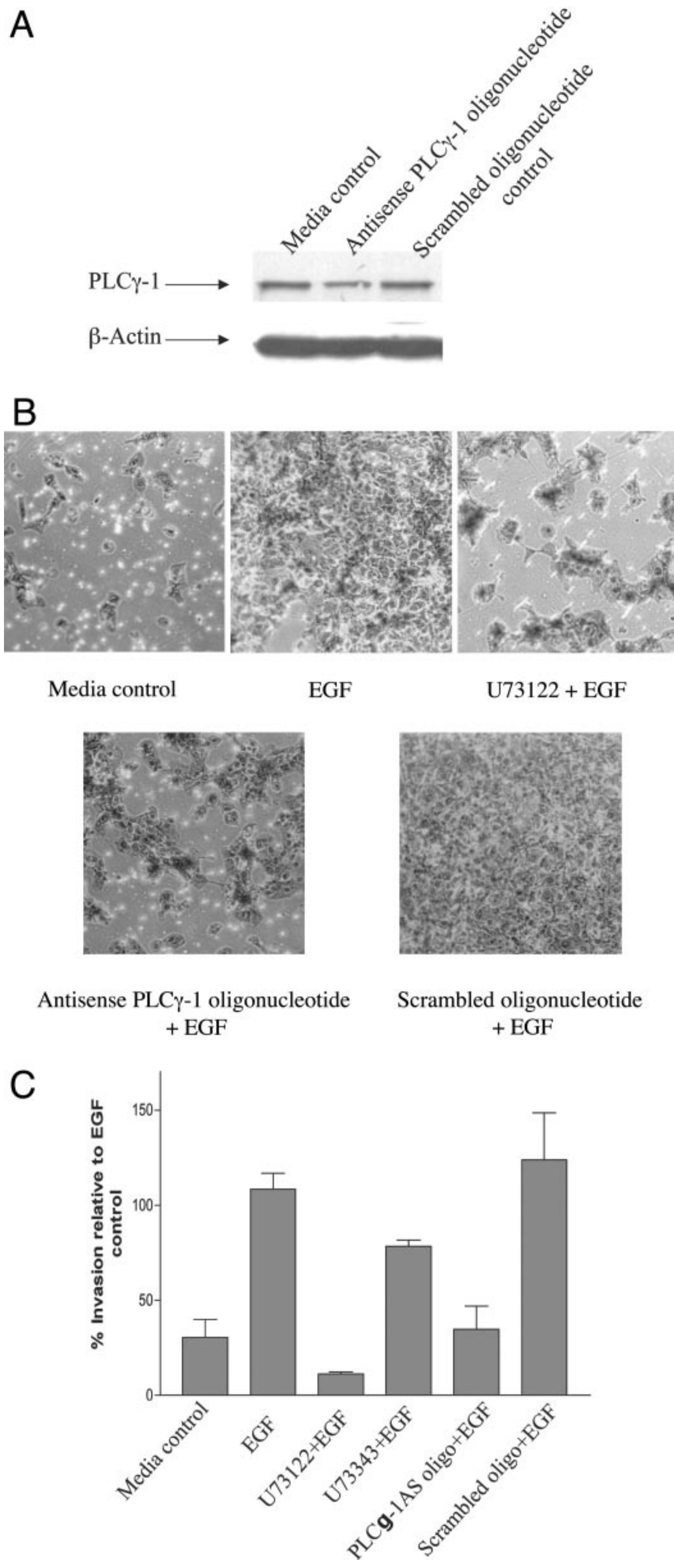

Fig. 4. The effect of PLCγ-1 inhibition on invasion *in vitro*. *A*, PLCγ-1 expression is decreased by anti-PLCγ-1 antisense oligonucleotides. Cells were treated with 12.5 μM of either antisense PLCγ-1 or scrambled control in the absence of serum for 72 h. Cell lysates were subjected to Western blotting, and the blots were probed with anti-β-actin antibody to demonstrate equal loading. Results from densitometric analysis were plotted as fold change of pPLCγ-1 compared with untreated control. Ratios of pPLCγ-1:β-actin were plotted to account for any variation in loading. *B*, a representative HNSCC cell line (*OSC-19*) was plated in transwell invasion chambers in the absence or presence of EGF (10 ng/ml). Duplicate wells were treated in the presence of EGF (10 ng/ml) with either 12.5 μM of PLCγ-1 antisense oligonucleotides, scrambled control oligonucleotides or 3 μM of PLC inhibitor U73122, or medium alone or EGF alone. After 48 h, noninvading cells inside the wells were wiped off the insert with a cotton-tipped applicator. Invading cells were fixed, stained, and either counted or photographed using ×400 magnification with an inverted microscope. The experiment was repeated three times with similar results. *C, in vitro* invasion in presence of PLC and PLCγ-1 inhibition was quantified by counting four fields under ×400 magnification, and the results were plotted as percentage invasion relative to EGF treated control. *Bars,* ±SE of duplicate wells for each treatment from two independent experiments.

the percentage of cells invading the chamber was reduced in the presence on 3 μM of U73122 in all of the cell lines tested (Fig. 3*A*).

Activated PLCγ-1 hydrolyzes phosphatidylinositol biphosphate to inositol triphosphate and diacylglecerol. The amount of the inositol triphosphate produced indicates the activity of PLC enzyme within cells and is a measure of PLCγ-1 activation. IP turnover in HNSCC cell lines treated with a PLC inhibitor was measured to determine whether reduced invasion correlated with lowered PLC activity. Cells were quiesed for 24 h and then treated with U73122 (3 μM for 25 min) and EGF (10 ng/ml for 5 min). We found that PLC inhibition by U73122 resulted in a decrease in IP turn over all of the HNSCC cell lines tested (Fig. 3*B*).

To determine whether invasion was mediated by EGFR signaling *in vitro*, HNSCC cells were plated in Matrigel-coated transwell chambers in the presence of either PLC or EGFR-specific inhibitors (U73122 or PD153035, respectively). After 48 h, the cells that invaded the transwell chamber were fixed, stained, and counted. We found that inhibition of either EGFR or PLC significantly reduced the percentage of cells invading the Matrigel as compared with untreated control wells by >50% (Fig. 3*C*).

Because U73122 inhibits all of the cellular PLCs, we designed PLCγ-1 antisense oligonucleotides to test the consequence of specific abrogation of PLCγ-1. The antisense oligonucleotide was directed against the ATG start site. HNSCC cells (OSC-19) were plated in invasion chambers at a high density and treated with EGF (10 ng/ml), EGF + U73122 (3 μM), EGF + antisense oligonucleotides against PLCγ-1 (12.5 μM), or EGF + control scrambled oligonucleotides (12.5 μM). Cells were treated for 48 h, fixed, stained, and counted. Immunoblotting demonstrated that the PLCγ-1 antisense oligonucleotides decreased expression of PLCγ-1 protein by 50% (Fig. 4*A*). Both the PLC inhibitor as well as PLCγ-1 antisense oligonucleotides abrogated *in vitro* invasion of a representative HNSCC cell line (Fig. 4*B*). On quantifying the number of invading cells it was found that the number of invading cells in wells with inhibitors was reduced significantly compared with the corresponding controls ($P = 0.05$ when tested with the Sikak procedure; Fig. 4*C*).

PLC Inhibition Does Not Abrogate HNSCC Proliferation. We have reported previously that EGFR inhibition abrogates growth of HNSCC cells (17). To determine the effect of PLCγ-1 inhibition on cell proliferation, a representative HNSCC cell line (OSC-19) was treated with 12.5 μM of PLCγ-1 antisense oligonucleotides, scrambled control oligonucleotides, or PLC inhibitor U73122. The cell number in each well was determined at several time points by cell counting using vital dye exclusion. There was no significant difference in cell viability after PLCγ-1 blockade (Fig. 5). The lack of effect of PLCγ-1 inhibition on cell proliferation was corroborated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as well as flow cytometric analysis (data not shown).

Increased Expression and Activation of PLCγ-1 in Tumors Compared with Corresponding Normal Mucosa from HNSCC Patients. Our *in vitro* studies suggest that PLCγ-1 plays an important role in invasion of HNSCC cell lines. To explore the biological significance of this finding, we compared protein expression levels of PLCγ-1 in 33 HNSCC tumors and paired normal mucosa harvested several centimeters away from the tumor (see Table 1 for patient

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characteristics). Using immunoblotting of tissue lysates, we found a 1.8-fold increase in PLCγ-1 expression levels in HNSCC tumors compared with levels in paired adjacent normal mucosa ($P = 0.0001$; Fig. 6). In 30 of 33 (91%) cases, PLCγ-1 expression levels in the tumor were greater than those in the corresponding normal mucosa. We also examined levels of phosphorylated PLCγ-1 in a subset of the paired tissues where adequate material was available for analysis (20 subjects). In these samples, activated PLCγ-1 levels were 1.65-fold higher in HNSCC tumors compared with levels in paired normal mucosa ($P = 0.05$). These *in vitro* and *in vivo* results indicate that EGFR-mediated PLCγ-1 activation may play an important role in invasion of HNSCC cells.

Table 1 *Clinicopathologic characteristics of 33 head and neck cancer patients evaluated*

Gender	
Male	25 (76%)
Female	8 (24%)
Age	Mean, 61 years; Median, 60 years; range, 47–82 years
Tumor site	
Oral cavity	10 (30.3%)
Oropharynx	3 (9.09%)
Larynx	19 (57.57%)
Hypopharynx	1 (3.03%)
T stage	
1–2	14 (45%)
3–4	19 (55%)
N stage	
0	21 (63.6%)
1	3 (9%)
2	69 (27.3%)

DISCUSSION

In this study we present evidence to support the hypothesis that EGFR mediates invasion of HNSCC cells via activation of PLCγ-1 and that PLCγ-1 may be involved in tumor progression. Upon ligand stimulation, EGFR activated PLCγ-1 in a series of HNSCC cell lines and mediated cellular invasion *in vitro*. Targeting PLCγ-1 reduced invasion of HNSCC cells through Matrigel. Furthermore, abrogation of PLCγ-1 did not modulate proliferation or survival. These findings implicate a specific invasive function of EGFR-mediated signaling via PLCγ-1 activation. To determine the clinical relevance of our *in vitro* findings, we examined PLCγ-1 and phosphorylated PLCγ-1 levels in HNSCC tumors compared with levels in paired normal adjacent mucosa. This is the first study, to our knowledge, of activated PLCγ-1 in *de novo* human cancers. Expression levels of both PLCγ-1 and phosphorylated PLCγ-1 were elevated nearly 2-fold in the tumors. These results suggest that PLCγ-1 plays an important role in EGFR-mediated invasion of HNSCC.

EGFR elicits multiple biological effects in cells overexpressing the receptor including proliferation, prevention of apoptosis, invasion, and transformation (18). Despite the multiplicity of downstream EGFR signaling pathways, several lines of evidence support a redundancy in EGFR signaling. For example, ligand-stimulated EGFR mutants unable to bind SH2 domain proteins use alternate mechanisms including tyrosine-phosphorylated proteins (Shc and Gab1) to activate downstream effectors (19). Thus, an alternate pathway resulting in phenotypic effects expected on EGFR ligand stimulation can overcome the specific inhibition of a downstream effector. However, other studies have reported specificity associated with signaling pathways activated by EGFR. We have shown previously that activation of EGFR stimulates STAT activation pathways that regulate proliferative and antiapoptotic signals (20). Down-modulation of STAT3 using antisense oligonucleotides or dominant-negative approaches resulted in abrogation of growth and apoptosis in HNSCC cells (21). In HNSCC cells, EGFR has been shown to modulate proliferation via MAPK where inhibition of MAPK activation by EGFR did not affect invasion *in vitro* (8). To elucidate the role of EGFR-mediated signaling via PLCγ-1 in both mitogenic and motogenic responses, we specifically blocked PLCγ-1 using several approaches in HNSCC cell lines and determined the effects on proliferation. Our results suggest that PLCγ-1 inhibition decreases invasion but not proliferation of HNSCC cells *in vitro*. Similar findings have been reported in prostate carcinoma cells where inhibition of PLCγ-1 *in vitro* did not decrease cell growth, whereas *in vivo* PLC blockade reduced invasion but not tumor volumes (22, 23). Thus, activated EGFR may specifically mediate cancer cell invasion by activation of PLCγ-1.

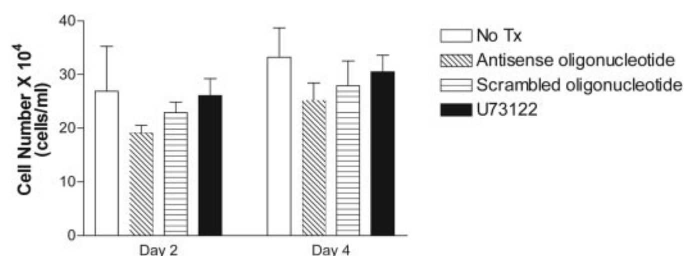

Fig. 5. Effect of PLCγ-1 blockade on cell proliferation. HNSCC cells (*OSC-19*) were treated with either antisense, or scrambled oligonucleotides (12.5 μM) or U73122 (3 μM) over a 4-day period to determine the effect of PLCγ-1/PLC blockade on cellular proliferation. Cells were treated in triplicate with U73122 (3 μM) or with 12.5 μM of either PLCγ-1 antisense oligonucleotides or scrambled control oligonucleotides in serum-free medium. At the 2-day and 4-day time points, cells were trypsinized with 0.5% trypsin-EDTA and counted using a hemocytometer in presence of 0.1% trypan blue dye. All treatments were carried out in triplicate, and the experiment was repeated twice with similar results.

This is the first report to demonstrate a correlation between EGFR-mediated PLCγ-1 signaling in HNSCC and its effects on cellular behavior. Little is known about the biology of invasion in HNSCC cells. Given the high rate of invasion and its associated morbidity and mortality, elucidation of the molecular mechanisms that govern tumor cell invasion is critical for the design of improved therapeutic strategies. To invade, cells secrete proteases that remodel the extracellular matrix and/or activate the tumor cells enabling the cells to migrate although the basement membrane. The MMP family of proteolytic enzymes has been implicated in tumor cell invasion. Several studies have reported up-regulation of MMP-2 and -9 in HNSCC tissues (24–26). Increased expression levels of MMP-9 were found to correlate with overexpression of EGFR in HNSCC tumors (27). Incubation of HNSCC cells with EGFR ligands increased MMP-9 gene expression and *in vitro* invasion in HNSCC cells (28). The mechanisms that govern EGFR regulation of MMPs are incompletely understood. Another protease linked to increased cell motility, the urokinase-type plasminogen activator, has also been shown to play a role in EGFR-mediated invasion of HNSCC cells (29). However, these proteases appear to function downstream of EGFR activation and are not directly modulated by cytoplasmic EGFR signaling pathways. We found decreased invasion of HNSCC cells after treatment with an EGFR-specific tyrosine kinase inhibitor. Abrogation of HNSCC invasion after EGFR or a PLCγ-1 inhibition suggests that PLCγ-1 is activated immediately downstream of EGFR leading to HNSCC cellular invasion.

To examine the clinical relevance of our *in vitro* data, we measured the levels of PLCγ-1 and phosphorylated PLCγ-1 in HNSCC tumors and paired normal mucosa specimens. Our results suggest that HNSCC tumors express significantly higher levels of PLCγ-1 in tumors compared with adjacent normal epithelial mucosa. Mammary carcinoma cells were reported to express increased levels of EGFR

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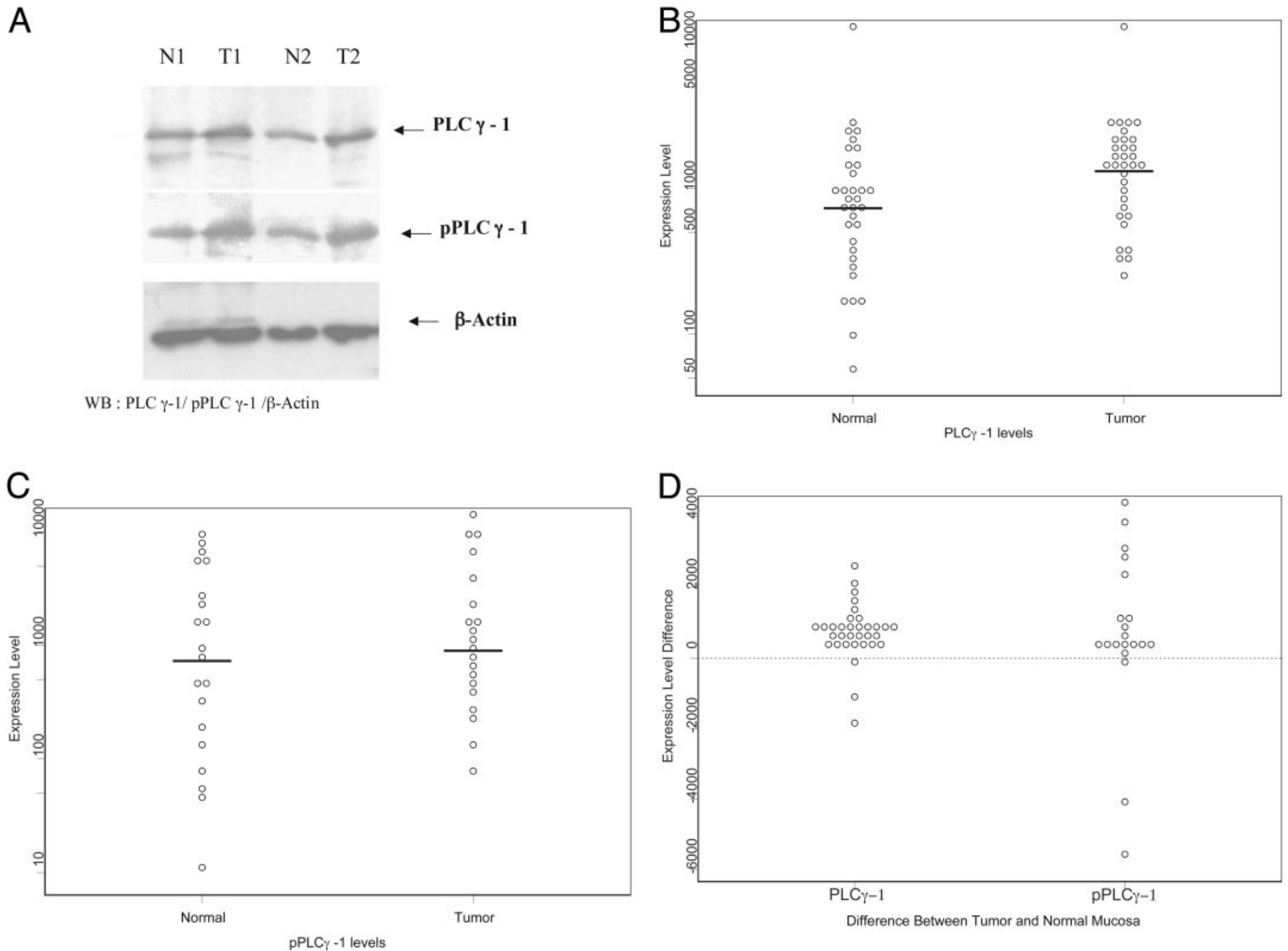

Fig. 6. PLCγ-1 levels are increased in HNSCC tumors compared with paired normal tissues. *A,* immunoblot showing PLCγ-1 levels (*top panel*), phospho-PLCγ-1 levels (*middle panel*), and β-actin levels (*loading control*) in tumor (*T*) and adjacent normal mucosa (*N*) from 2 representative HNSCC patients. Densitometric analysis was carried out, and gels were normalized to values of corresponding β-actin levels. Cumulative results showing levels of (*B*) PLCγ-1 and (*C*) activated PLCγ-1 in tumors and normal tissue. The levels of PLCγ-1 and phospho-PLCγ-1 in tumor tissues were higher (1.8- and 1.6-fold, respectively) than that in the normal adjacent mucosa ($P < 0.0001$ and $P = 0.05$, respectively).

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and erbB-2, as well as PLCγ-1 when compared with levels in nonmalignant breast tissue (30). Others have reported *in vivo* overexpression of PLCγ-1 protein in meningiomas (31) and colorectal carcinomas (32–34). However for the first time, we examined activation status by probing for phosphorylated PLCγ-1 and found it to be increased in the tumor tissues. Up-regulation of PLCγ-1 downstream of EGFR may contribute to the invasive phenotype of HNSCC tumors. Therapies that specifically target PLCγ-1 may reduce local and regional tumor invasion and, hence, improve survival.

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