

Critical Role for the Receptor Tyrosine Kinase EPHB4 in Esophageal Cancers

Rifat Hasina¹, Nathan Mollberg¹, Ichiro Kawada¹, Karun Mutreja¹, Geetanjali Kanade¹, Soheil Yala¹, Mosmi Surat¹, Ren Liu⁴, Xiuqing Li⁴, Yue Zhou⁴, Benjamin D. Ferguson¹, Vidya Nallasura¹, Kenneth S. Cohen¹, Elizabeth Hyjek², Jeffery Mueller², Rajani Kanteti¹, Essam El Hashani¹, Dorothy Kane², Yutaka Shimada⁵, Mark W. Lingen^{1,2}, Aliya N. Husain², Mitchell C. Posner³, Irving Waxman³, Victoria M. Villafior¹, Mark K. Ferguson³, Lyuba Varticovski⁶, Everett E. Vokes¹, Parkash Gill⁴, and Ravi Salgia¹

Abstract

Esophageal cancer incidence is increasing and has few treatment options. In studying receptor tyrosine kinases associated with esophageal cancers, we have identified EPHB4 to be robustly overexpressed in cell lines and primary tumor tissues. In total, 94 squamous cell carcinoma, 82 adenocarcinoma, 25 dysplasia, 13 Barrett esophagus, and 25 adjacent or unrelated normal esophageal tissues were evaluated by immunohistochemistry. EPHB4 expression was significantly higher in all the different histologic categories than in adjacent normal tissues. In 13 esophageal cancer cell lines, 3 of the 9 SCC cell lines and 2 of the 4 adenocarcinomas expressed very high levels of EPHB4. An increased gene copy number ranging from 4 to 20 copies was identified in a subset of the overexpressing patient samples and cell lines. We have developed a novel 4-nitroquinoline 1-oxide (4-NQO)-induced mouse model of esophageal cancer that recapitulates the EPHB4 expression in humans. A specific small-molecule inhibitor of EPHB4 decreased cell viability in a time- and dose-dependent manner in 3 of the 4 cell lines tested. The small-molecule inhibitor and an EPHB4 siRNA also decreased cell migration (12%–40% closure in treated vs. 60%–80% in untreated), with decreased phosphorylation of various tyrosyl-containing proteins, EphB4, and its downstream target p125FAK. Finally, in a xenograft tumor model, an EPHB4 inhibitor abrogated tumor growth by approximately 60% compared with untreated control. EphB4 is robustly expressed and potentially serves as a novel biomarker for targeted therapy in esophageal cancers. *Cancer Res*; 73(1); 184–94. ©2012 AACR.

Introduction

Despite substantial improvements in screening, diagnosis, and treatment of esophageal cancer, the prognosis of this disease remains bleak, underscoring the need for new targets in its treatment. Esophageal cancer is the ninth most frequent cancer in the world and fifth most frequent in developed countries (1). In the United States, its incidence has increased in recent years, largely because of a dramatic increase in the incidence of adenocarcinoma of the distal esophagus and

gastroesophageal junction. A profound epidemiologic shift in esophageal cancers has occurred as a result of a 350% increase in adenocarcinoma between 1974 and 1994. This epidemiologic shift reflects a change in etiology, with adenocarcinoma mirroring an increase in Barrett metaplasia, a preneoplastic condition induced by chronic gastroesophageal reflux. At present, Barrett esophagus is recognized as the single most important risk factor for esophageal adenocarcinoma.

We addressed the need for novel targeted biologic agents for the treatment of esophageal carcinoma by exploring the Eph/ephrin receptor tyrosine kinase pathway. The cellular responses to Eph receptor stimulation by their ephrin ligands are important in mediating a wide range of biologic activities, including angiogenesis, cell segregation, cell attachment, shape, and motility. Also Eph/ephrin signaling has been identified to play a role in many human cancers, such as lung, breast, and prostate cancers, as well as melanoma and leukemia (2). Besides their proposed role in cancer, the function of Eph receptors and their ephrin ligands has already become well established in nervous system development in vertebrates and tissue patterning. Several Eph/ephrin molecules are expressed in vascular systems, but EPHB4 and its ligand EPHRINB2 have been found to be the most significant. EPHRINB2 is predominantly expressed in arterial endothelial cells (EC), whereas EPHB4 is mostly venous-specific. Targeted inactivation of

Authors' Affiliations: ¹Section of Hematology/Oncology, Department of Medicine, Departments of ²Pathology and ³Surgery, The University of Chicago, Chicago, Illinois; ⁴Division of Hematology and Pathology, Department of Medicine, University of Southern California, Los Angeles, California; ⁵Department of Surgery and Science, University of Toyama, Toyama, Japan; and ⁶Laboratory of Receptor Biology and Gene Expression, National Cancer Institute, NIH, Bethesda, Maryland

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Corresponding Author: Ravi Salgia, Chest Oncology and Thoracic Oncology Research Program, The University of Chicago, Department of Medicine, Section of Hematology/Oncology, 5841 S Maryland Avenue, MC2115, Chicago, IL 60637. Phone: 773-702-4399; Fax: 773-702-3002; E-mail: rsalgia@medicine.bsd.uchicago.edu

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EPHB4 and EPHRINB2 have showed that both are essential for angiogenic remodeling and embryonic survival (3, 4). There are several signal transduction pathways and corresponding molecules that have been implicated in angiogenesis and tumorigenesis with regard to the Eph/ephrin axis (5–9); the Jak/Stat pathway, involved in cell growth and viability, is a novel signaling pathway of EphA4 (10). The Akt/PI3K (phosphoinositide 3-kinase) is another signaling cascade involved in regulating cell proliferation and viability (reviewed in ref. 11). A study by Steinle and colleagues (12) showed that EPHB4 receptor signaling mediates endothelial cell migration and proliferation via the PI3K pathway (proliferation increased by 38% and migration by 63% upon EPHB4 activation with EPHRINB2). Signal transduction molecules influencing cell motility include paxillin, focal adhesion kinase (FAK), CRK, Rho, and P130Cas (13–15).

Despite the progress made in understanding Eph/ephrin function in early developmental processes, as well as their role in cancer due to their complex nature, much remains to be understood about the mechanisms and signaling processes of Eph receptors.

In this study, we show that EPHB4 is overexpressed and has increased gene copy numbers in esophageal cancer. We also report that EPHB4 is involved in enhanced motility and migration of cancer cells. These findings show that EPHB4 contributes to tumor biology. In addition, we report the development of a carcinogen-induced esophageal cancer mouse model that recapitulates EPHB4 expression in human disease, which will serve as a useful model in which to study EPHB4 biology and its effectiveness as a novel biomarker and a molecular target for esophageal cancer. Ultimately, EPHB4 represents a novel target for esophageal cancer.

Materials and Methods

Cell lines

Esophageal cancer cell lines (FLO, Kyse-110, Kyse-140, Kyse-220, Kyse-410, Kyse-850, SKGT-4, TE-1, and TE-12) were cultured in Dulbecco's Modified Eagles' Medium (DMEM) or DMEM + Hank F12 media supplemented with 10% FBS and penicillin in a 37°C, 5% CO₂ environment. The FLO cells were a kind gift from Dr. David Beers of University of Michigan (Ann Arbor, MI) and the Kyse cells were acquired through a generous gift from Dr. Yutaka Shimada of Toyama University (Toyama, Japan). The cell lines used in this study were not authenticated by any tests in our laboratory.

Tissue acquisition

Samples for this study were acquired from paraffin-embedded, formalin-fixed tissues archived at The University of Chicago (Chicago, IL) Human Tissue Resource Center available through Institutional Review Board-approved protocols. Experienced pathologists (J. Mueller and M.W. Lingen) analyzed samples using conventional light microscopy and determined the histologic subtype as well as tumor grade.

Barrett tissue microarray

A Barrett esophagus tissue microarray (TMA) was created with samples selected from the archive: carcinoma ($n = 3$),

high-grade dysplasia ($n = 15$), low-grade dysplasia ($n = 10$), nondysplastic Barrett esophagus ($n = 13$), and normal control ($n = 4$; colon, placenta, liver, and kidney). Additional TMAs were purchased from US Biomax Inc. Catalog #BC02011 [multiple esophageal squamous cell carcinoma (SCC) tissue array, 69 cases/72 cores—69 SCC \times 1 + 3 normal \times 1]. Catalog #BS02051 [esophageal adenocarcinoma (AC) tissue array, 33 cases/63 cores—28 AC \times 2 + 1 mucinous AC \times 2 + 1 smooth muscle \times 2 + 3 normal squamous epithelium \times 1]. Catalog #ES208 [high-density esophageal cancer tissue array, with normal tissue, grade, and TNM, 69 cases/208 cores—24 SCC \times 3 + 2 ADSCC \times 3 + 23 AC \times 3 + 12 mets \times 3 + 9 normal \times 3 + 1 melanoma \times 1]. Catalog #ES8011 (esophageal AS tissue array, with normal tissue, TNM, and grade info, 40 cases/80 cores—35 AC \times 2 + 5 normal \times 2).

Immunohistochemistry

Immunohistochemistry for human-EPHB4 was conducted on tissue sections and all TMAs using a monoclonal mouse anti-EPHB4 antibody (clone EPHB4-131, gift of Dr. Parkash Gill, University of Southern California, Los Angeles, CA). The immunohistochemistry (IHC) was conducted on a Dako Autostainer (Dako) using their Advance HRP detection system. Briefly, following deparaffinization and quenching of endogenous peroxidase, sections were retrieved in a DAKO Target Retrieval Solution, transferred to the Dako Autostainer, and incubated with the anti-EPHB4 antibody at 1:6 dilution for 30 minutes followed by Advance HRP detection system. Peroxidase reaction was developed using 3,3'-diaminobenzidine provided in the kit and slides counterstained with hematoxylin.

IHC for ephB4 on mouse esophagus (mice described later in *in vivo* methods) was conducted similarly on the Dako Autostainer using polyclonal goat anti-ephb4 antibody (R&D # BAF446).

Tumor sections were evaluated by a pathologist who, blinded to the identity of the tissues, visually observed individual sections under the microscope and estimated total staining intensity and extent of staining. The final IHC score was obtained by a semiquantitative method that accounts for staining intensity and percentage of cells stained. This estimation resulted in an IHC score of 0, 1, 2, or 3 denoting negative, weak, moderate, and strong expression, respectively. This score was correlated with available clinical information in further analyses.

Statistical analyses

Within the Barrett TMA, EPHB4 expression in tumor samples was compared with normal tissue from the same patient. An averaged IHC expression score was calculated for all tumor and normal tissues that had multiple samples for a patient within the TMA. A sample was considered to have low expression if averaged expression score ranged between 0 and 1.0; medium if between 1.1 and 2.0; and high if between 2.1 and 3.0. Matched samples were compared using a Wilcoxon signed rank test (SPSS version 17.0). Expression was also correlated with grade. Grades in each of the US Biomax TMAs were defined on an ordinal scale of 1 to 4. They were coded as well

differentiated (1), moderately differentiated (2), poorly differentiated (3), and undifferentiated (4). The data were analyzed using a bivariate correlation coefficient. A Spearman correlation coefficient was used and missing data were excluded pairwise. The correlation coefficient was calculated separately for SCCs and ACs. To compare tumor and normal IHC data from US Biomax TMAs, a Mann–Whitney test was conducted on the 4 TMAs and EPHB4 expression in tumors correlated to that of normal samples in the corresponding TMA. There was no normal protein expression data available for BC2011 and BC2051. We acknowledge that the immunohistochemistry data was acquired by semiquantitative analysis by a single pathologist to have no associated errors, and we have not done repeat experiments to show that they are reproducible.

Immunoblot and phospho-protein immunoblot

Cells were lysed using radioimmunoprecipitation (RIPA) buffer supplemented with sodium orthovanadate, protease, and phosphatase inhibitors. About 150 µg of total protein was subjected to SDS-PAGE on 7.5% gel, transferred to polyvinylidene difluoride (PVDF) membranes, and probed with appropriate primary antibodies. After blocking with 5% bovine serum albumin, bands were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies exposed to 1:1 horseradish peroxidase ECL solutions (Bio-Rad Laboratories), and images were taken using chemiluminescent imaging system ChemiDoc XRS (Bio-Rad). The non-small cell lung cancer (NSCLC) cell line H1993 was used as positive control in some blots. β-Actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as loading controls.

Genomic DNA

The gDNA was collected according to procedures described previously (16) from a subset of the paraffin-embedded tissues used for EPHB4 IHC. The integrity of gDNA was evaluated by agarose gel electrophoresis followed by PCR with β-globin.

Real-time quantitative PCR

Using a short target sequence within *EPHB4*, patient gDNA was used in real-time quantitative PCR (RT-qPCR) to determine absolute gene copy number. Standard curves were generated using standard DNA to ensure a suitable range of sample detection; copy numbers were normalized to LINE-1 as a paired internal control. Samples and controls were amplified in triplicate. We interpreted these data descriptively by examining the relative frequencies of amplification status and noting remarkable findings. *EPHB4* was considered amplified if its average detected gene copy number was greater than 4 copies. DNA extracted from H1993 served as positive control.

Mutation analysis

The gDNA was used in PCR to amplify DNA fragments encoding each *EPHB4* exon. Successfully amplified PCR products were treated with ExoSAP-IT (USB Corporation) and sequenced at the University of Chicago Cancer Research Center DNA Sequencing Core Facility. Sequences were analyzed for mutations by direct comparison to the wild-type

gDNA sequence using Mutation Surveyor (Softgenetics). Each positive genetic variation was repeated for confirmation, mapped to the full-length *EPHB4* protein, and analyzed for amino acid changes.

Viability assay

Briefly, cells seeded in 96-well plates were incubated overnight and fed fresh media supplemented with the small-molecule inhibitor (SMI) of EPHB4, AZ12489875-002 (Astra-Zeneca) at concentrations of 1 and 10 µmol/L or dimethyl sulfoxide (DMSO) as vehicle control. The media were replaced with fresh drug every 48 hours. Cell viability was assessed at 0, 48, 72, and 96 hours of drug treatment using MTT reagent. Plates were read at 570 nm using Synergy HT Multi-Mode Microplate Reader.

Wound-healing assay

Cells were plated at 70% to 90% confluency in 35-mm dishes. Twenty-four hours after plating, a fine scratch was made in the center of the well, cells were washed in PBS, and media replaced with the appropriate dose of drug. Cells were photographed from 0 hour of scratch up to 20 hours. The wound closure was measured in images of 0 and 15 or 20 hours using tools in ImageJ program (17).

siRNA

Cells were transfected with *EPHB4* siRNA duplex (5'-GGU-GAAUGUAAGACGCUGUU-3', IDT) and control siRNA duplex (5'-AGUAAAUAUCAAGACGCUGUU-3', IDT) oligonucleotides at a final concentration of 20 µmol/L using Oligofectamine (Invitrogen) in serum-free OptiMem medium (Invitrogen). Mock-treated cells received transfection reagent only. After 48 hours, cells were collected for viability, wound-healing, and immunoblot assays.

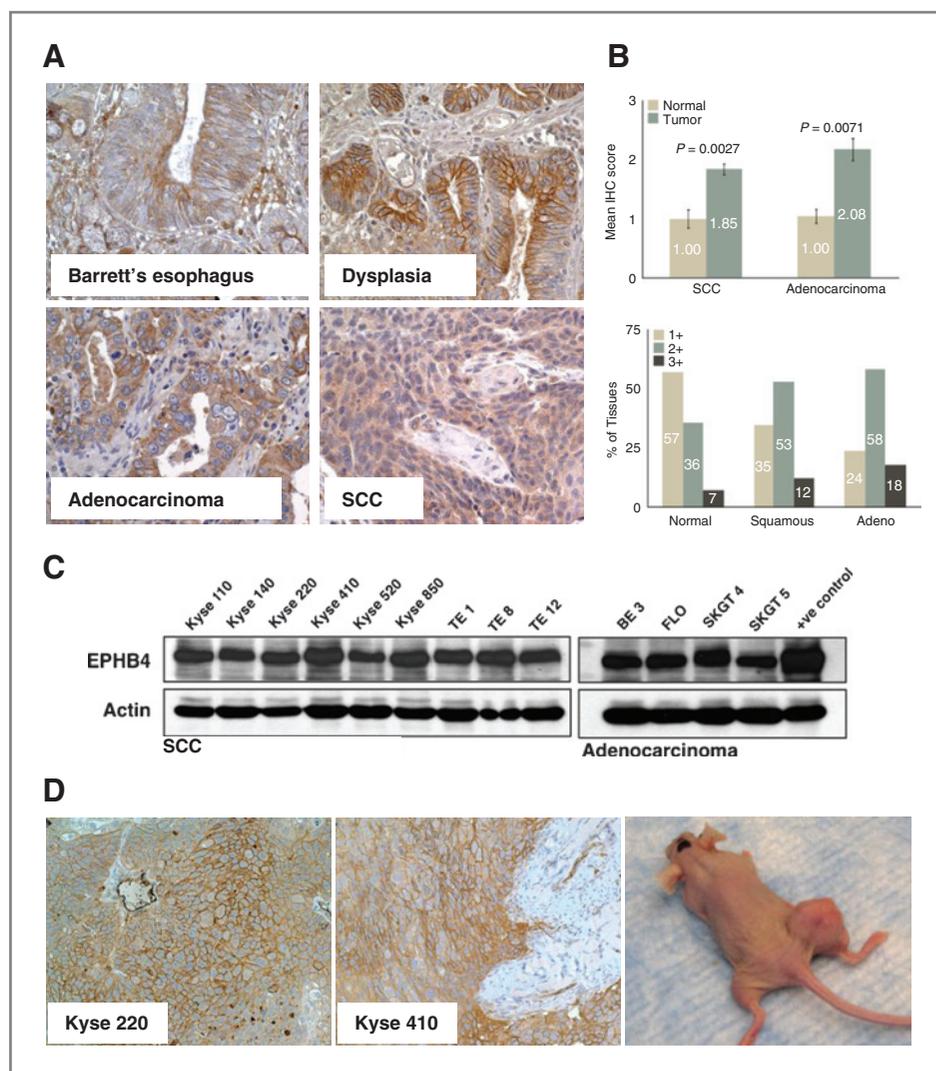
Human papillomavirus status

A representative sample of 10 SCC and adjacent normal tissue gDNA was used in a PCR-based method using the PGM09/11 primer set to determine human papillomavirus (HPV) status as described (18).

In vivo xenograft tumor growth

Five million FLO cell were mixed 1:1 with Matrigel (BD Biosciences) and injected subcutaneously in bilateral flanks of 6- to 8-week-old male Balb/C *nu/nu* mice. Tumor growth measured thrice weekly was calculated to estimate volume ($0.52 \times a \times b^2$, where a and b are the largest and smallest lengths of the tumor). Once tumors were approximately 100 mm³ (day 0), animals were divided into treatment and control groups ($n = 10$) such that the mean tumor volume of each group was comparable and the SE between groups was minimal. Animals were treated with PBS or 50 mg/kg sEPHB4-HSA. Mice were sacrificed after 37 days of treatment. All procedures were approved by Institutional Animal Care and Use Committee and conducted in accordance with the Animal Welfare Act. Data were analyzed comparing the mean tumor volume of the 2 groups on day 37, and the P value was calculated with 2-tailed Student t test.

Figure 1. A, Barrett TMA, archival tissue, and US Biomax TMAs containing 25 dysplasia, 82 adenocarcinoma, 94 SCCs, and 25 normal tissue samples were probed against EPHB4. A, representative samples expressing high (3+) EPHB4 levels in each histology. B, top graph, comparative EPHB4 scores in US Biomax TMAs; bottom graph, comparative EPHB4 scores in archival samples. C, immunoblots showing consistent expression of EPHB4 in both esophageal SCCs and adenocarcinoma cell lines. D, tissue sections of Kyse-220 and Kyse-410 cells xenografted in nude mice (Kyse-410 mouse shown) also show high expression of EPHB4 in these cells.



***In vivo* model of esophageal carcinoma**

Male CBA mice, 6 to 8 weeks, Jackson Laboratory, were used for this study. Mice were administered 4-nitroquinoline 1-oxide (4-NQO) in their drinking water on a continuous basis at 50 mg/mL for 8 weeks following protocols detailed previously (19). Control mice were given vehicle only. Immediately following sacrifice, whole esophagi were paraffin-embedded and sections evaluated for histologic changes and ephb4 expression. Dysplasia was characterized as a lesion that showed one or more histopathologic alterations, and esophageal cancer was characterized by invasion into the underlying connective tissue, stroma, according to previously described criteria (19).

Results

EPHB4 protein is overexpressed in esophageal cancer patient tissue samples

The EPHB4 protein is expressed in blood vessels, normal tissue, and cancer cells and is detectable in cell membranes and cytoplasm. The samples in this study contained squa-

mous cell carcinoma (94), adenocarcinoma (82), dysplasia (25), Barrett esophagus (13), and adjacent or unrelated normal esophageal tissues (25), all of which were evaluated for EPHB4 protein expression by immunohistochemistry using a specific antibody against EPHB4 (Fig. 1A). The data were analyzed in batches depending on the availability of background information and are depicted in Fig. 1B and shown as a table in Supplementary Table S1. Among esophageal cancer specimens in the University of Chicago Barrett TMA, expression of EPHB4 was found to be greater in tumor tissue than in matched normal tissue in 19 of 25 samples. The degree of difference was statistically significant (sum of ranks = 190; $P = 0.01$). These findings were reproduced in the US Biomax TMAs (ES208 and ES8011) where tumor tissue was found to exhibit higher protein expression than matched normal ($P = 0.011$ and 0.001 , respectively). IHC score was also correlated with grade within US Biomax TMA samples, where grade data were available. In the US Biomax TMAs (BC2011, BC2051, ES208, and ES8011), all 94 squamous cell carcinoma individuals

had both grade data and protein expression data. The correlation coefficient for grade and protein expression was $r = 0.63$ ($P < 0.01$). For adenocarcinoma, there were 79 individuals who had both grade and protein expression data. The correlation coefficient for this sample was $r = -0.03$ ($P = 0.077$; Fig. 1B).

EPHB4 protein expression in cell lines

We analyzed protein expression in 13 esophageal cancer cell lines by immunoblotting with a specific EPHB4 antibody; all cells showed robust EPHB4 expression at 120 kDa. In the 9 SCCs tested, Kyse-410, Kyse-850, and TE-8 had the highest expression. In the 4 adenocarcinomas, BE3, FLO, SKGT-4, and SKGT-5, all had significant baseline expression with SKGT-4 being the highest. H1993, an NSCLC cell line known to overexpress EPHB4 was the positive control and β -actin the loading control (Fig. 1C). Kyse-220 and Kyse-410 were xenografted in nude mice and tumors evaluated for EPHB4; a strong membrane-associated expression was evident in both (Fig. 1D).

Variability of EPHB4 gene copy number in esophageal cancer patient samples

Gene copy number was determined using gDNA from 15 archival SCCs and 8 archival adenocarcinoma patient samples, commercially available normal human DNA and 3 individual normal esophageal tissue samples as internal reference controls. In SCCs, 9 of 15 (60%) patients had a gene copy number ranging from 4 and 20; similarly, in adenocarcinomas, 5 of 8 (62%) had gene copy number ranging from 4 to 20 (Fig. 2A and B). In addition, 33% of the SCC and 25% of the adenocarcinoma cell lines had copy numbers 4 or more (Fig. 2C). Control copy numbers were 2 in each case. In an effort to determine gene copy number changes in EPHRINB2, we also carried out RT-PCR on a limited number of randomly selected samples, which did not show any significant increase in gene copy numbers.

SNP analysis in esophageal cancer cell lines

We sequenced all 17 exons of *EPHB4* in 13 esophageal cancer cell lines, revealing 2 SNPs, T483C and A584G, in the FN III and

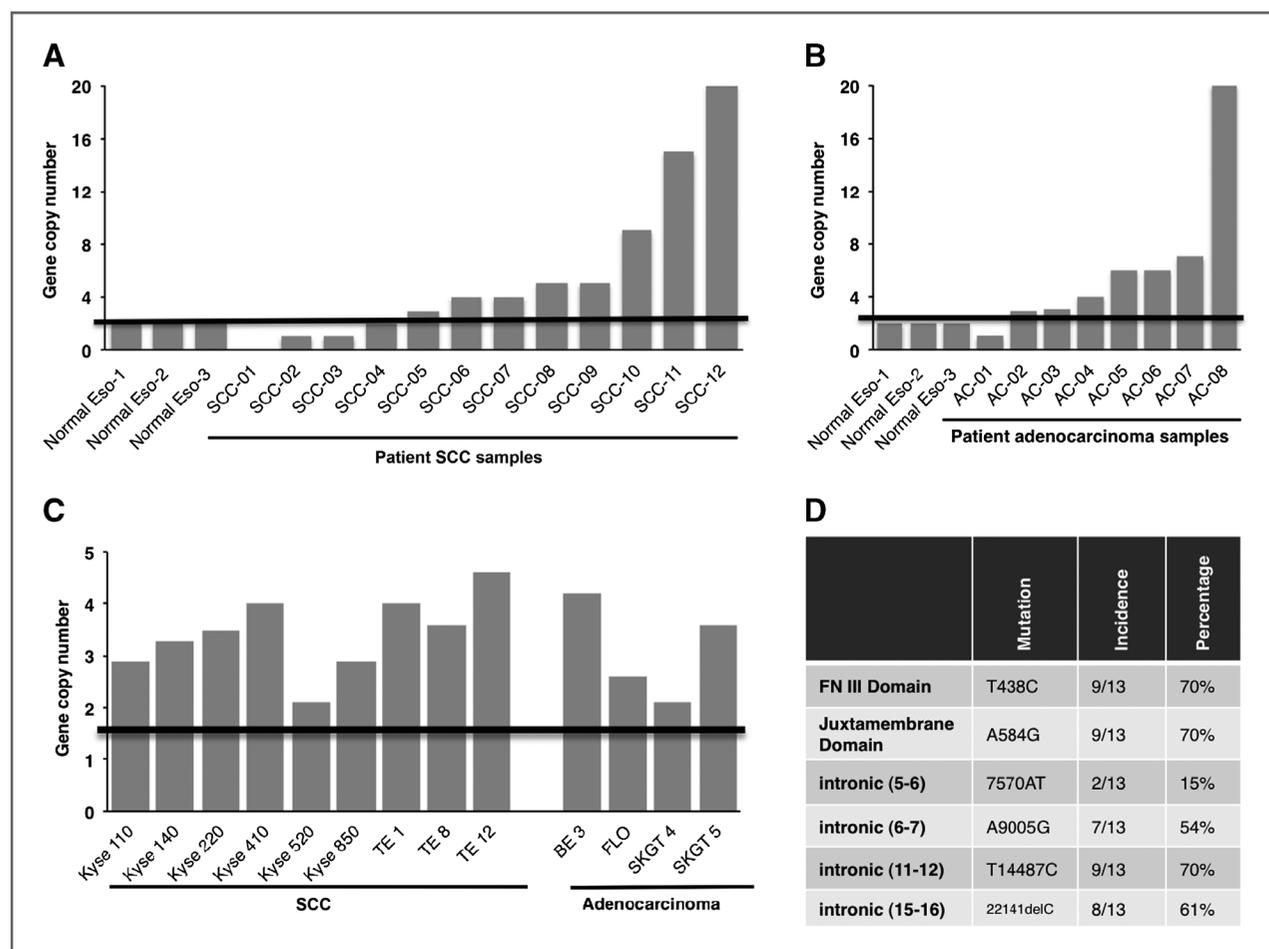


Figure 2. Distribution of *EPHB4* gene copy number by RT-PCR analysis in SCCs (A) and adenocarcinomas (B) in selected archival patient samples. C, gene copy number in cell lines. The horizontal bars represent a normal gene copy number of 2. D, the mutational status of *EPHB4* in esophageal cancer evaluated by PCR and sequence analysis. The figure shows 2 synonymous single-nucleotide polymorphisms (SNP) discovered in 70% of the cell lines analyzed as well as 4 intronic SNPs identified. Evaluation of patient samples did not reveal any new SNPs.

juxtamembrane domains respectively in 70% of the cells (Fig. 2D). Both these SNPs are synonymous and reported in the HapMap database. In addition, 4 intronic mutations A7570A/T, A9995G, T14487C, and 2241delC were detected in approximately 15% of cells. No significant nonsynonymous SNPs were detected in any exons examined.

Pharmacologic inhibition of EPHB4 by a small molecule inhibits cell proliferation

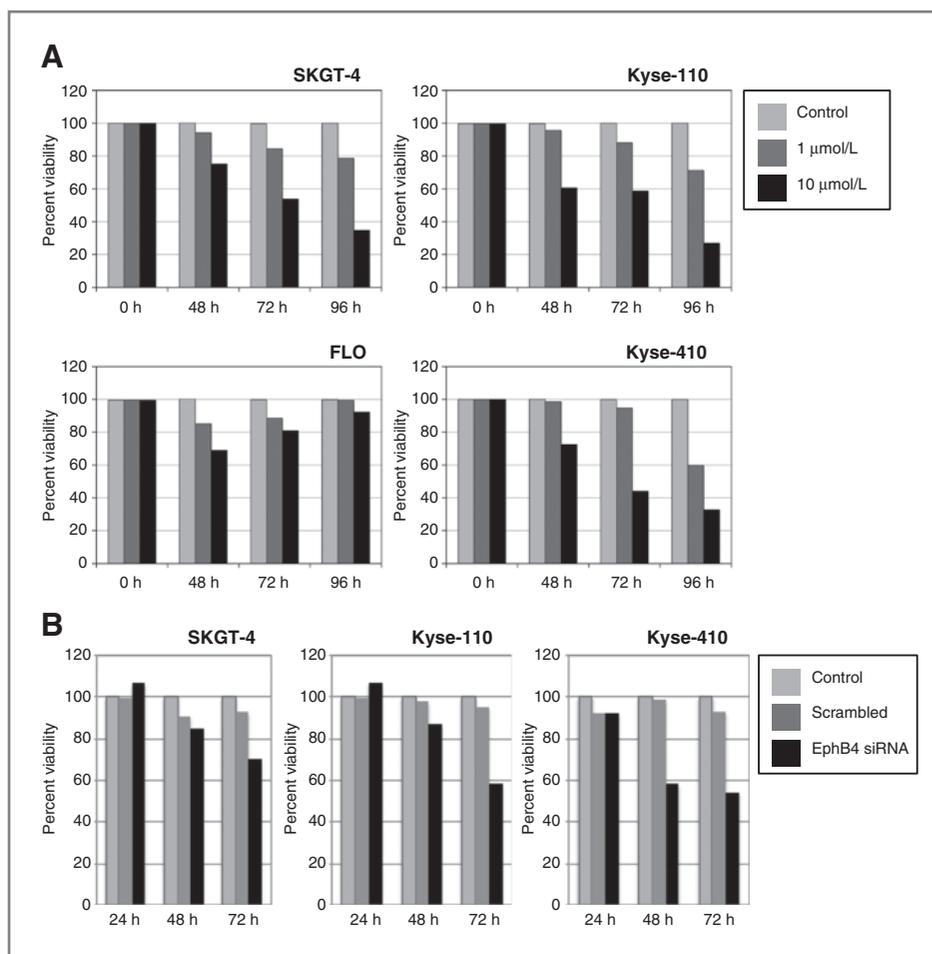
We used the EPHB4-SMI AZ12489875-002 (20–23) on 2 SCCs (Kyse-110 and Kyse-410) and 2 adenocarcinoma (SKGT-4 and FLO) cell lines. Cells were treated with 0 and 1 $\mu\text{mol/L}$ inhibitor for 0, 48, 72, and 96 hours. The data were plotted as a percentage of viable cells in treated wells compared with corresponding wells at 0 time point. Kyse-110, Kyse-410, and SKGT-4 growth was abrogated by EPHB4 inhibitor, whereas FLO cells did not show significant growth inhibition (Fig. 3A). For Kyse-110, Kyse-410, and SKGT-4, cell proliferation was inhibited in a dose-dependent manner as well as with increasing duration of treatment in a range of 40% to 65% inhibition. FLO cell proliferation was not inhibited by the highest dose of 10 $\mu\text{mol/L}$ inhibitor for 96 hours. These data indicate that 3 of the 4 cell lines tested were sensitive to the EPHB4-SMI, whereas one was resistant.

The same experiment was repeated with SKGT-4, Kyse-110, and Kyse-410 cells transfected with siRNA targeted against EPHB4 or a scrambled siRNA and untransfected controls. The FLO cell line is a combination of adherent and suspension cells and presented a technical challenge. The experimental design of siRNA treatment followed by viability or wound-healing assays rendered the acquired data unreliable due to uneven loss of suspension cells at each repeat instance. We lacked confidence in the consistency of the FLO data and decided not to use it. The gene-specific silencing yielded similar inhibition of cell proliferation. These data suggest that *EPHB4* is a target of the SMI (Fig. 3B).

Pharmacologic inhibition of EPHB4 inhibits cell migration

Figure 4A shows a representative snapshot of wounds at the beginning and end of 15-hour periods with or without EPHB4-SMI treatments. In the SCC cell lines Kyse-110 and Kyse-410, the cells behaved differently from each other. In the absence of EPHB4-SMI, the Kyse-410 cells closed the wound by 70% in 15 hours when compared with the size of the wound at 0 hour. When treated with the drug at 1 $\mu\text{mol/L}$, migration was strongly inhibited, with only about 28% of the wound closing (Fig. 4A and B). The Kyse-110 cells migrated more slowly in

Figure 3. A, inhibition of cell growth by a specific inhibitor of EPHB4. Two SCC and 2 adenocarcinoma cell lines were treated with 0, 1, and 10 $\mu\text{mol/L}$ of EPHB4-SMI AZ12489875-002 and viability measured at 0, 48, 72, and 96 hours. The graphs show SKGT-4, FLO, Kyse-110, and Kyse-410 cell growth inhibition. B, inhibition of cell growth in 3 cell lines in which *EPHB4* was silenced with siRNA.



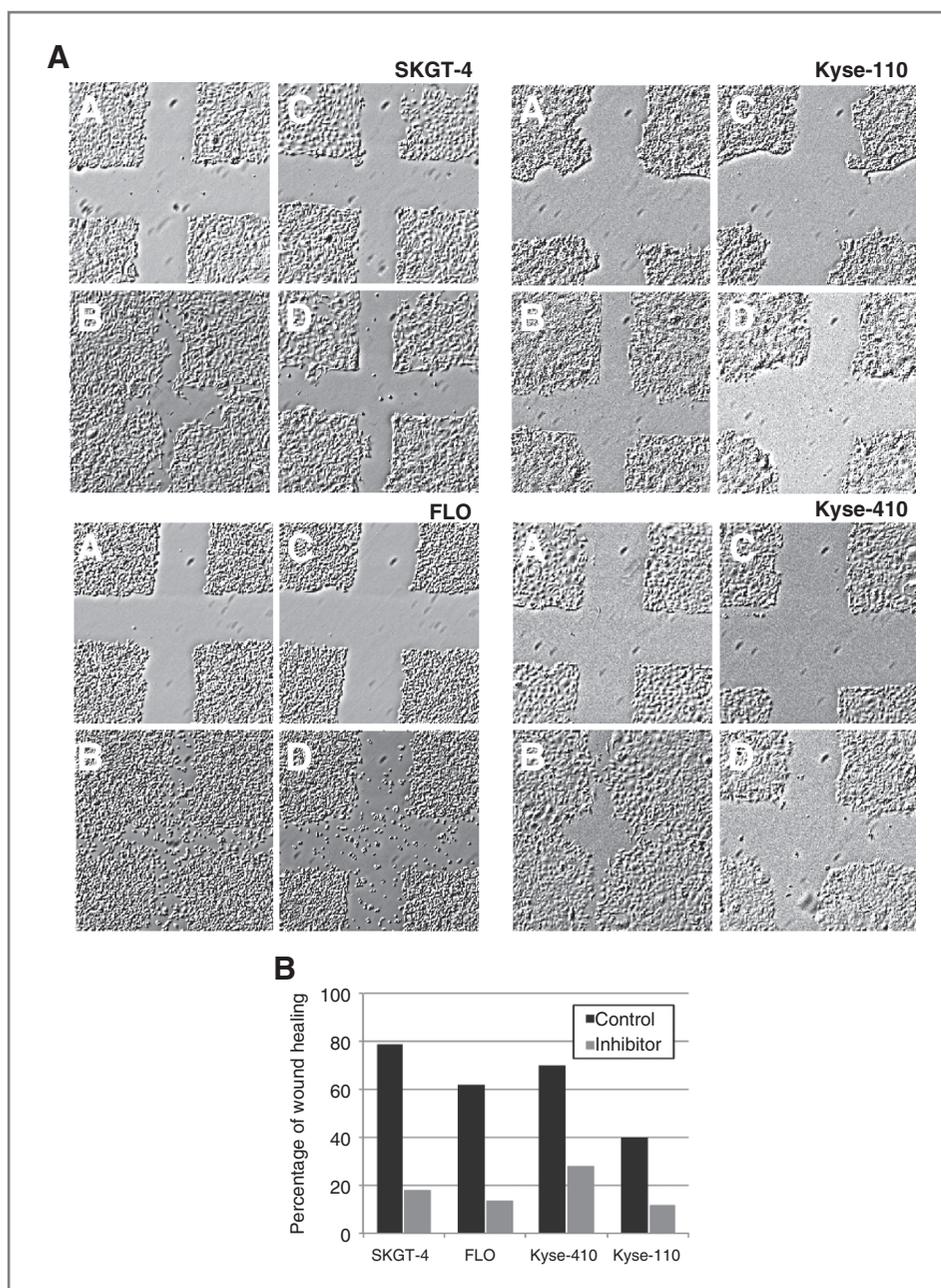


Figure 4. A, wound-healing assay to determine effect of EPHB4 inhibition on cell migration. Confluent SKGT-4, FLO, Kyse-110, and Kyse-410 cells were scratched to initiate a wound and treated with no or 1 $\mu\text{mol/L}$ inhibitor. The 4 panels in each cell group indicate: no treatment at 0 hour (A); no treatment at 15 hours (B); 1 $\mu\text{mol/L}$ EPHB4 inhibitor at 0 hour (C); 1 $\mu\text{mol/L}$ EPHB4 inhibitor at 15 hours (D). The graph in B plots the relative wound closure measured against untreated controls.

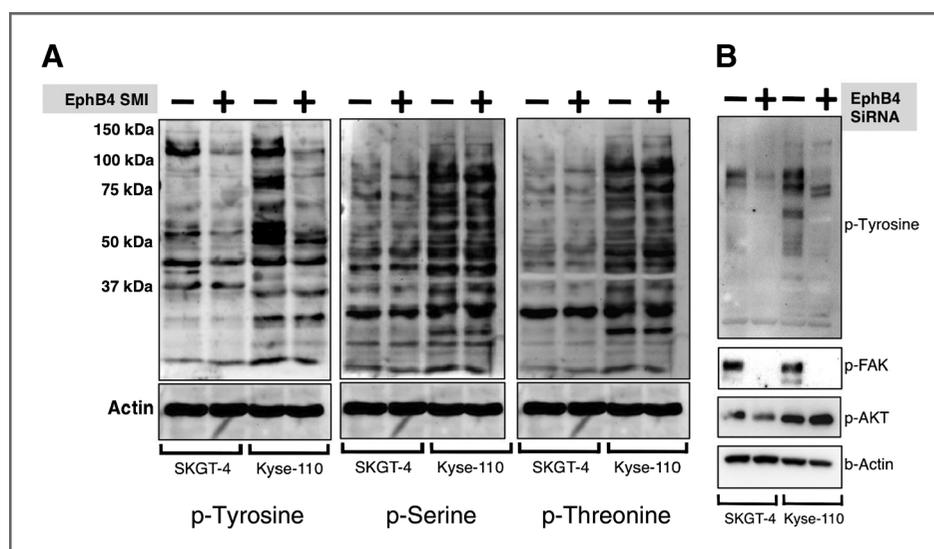
15 hours, only closing the wound 40% in the controls, whereas drug treatment almost completely inhibited migration with 12% of the wound healing. Both adenocarcinoma cells lines, SKGT-4 and FLO, showed 62% to 79% wound closure in the absence of the SMI, whereas 1 $\mu\text{mol/L}$ drug inhibited migration resulting in only 14% to 18% wound closure (Fig. 4A and B).

The same experiment was repeated with SKGT-4, Kyse-110, and Kyse-410 cells *EPHB4* silenced with siRNA, a scrambled siRNA, and untransfected controls. The gene-specific silencing yielded similar inhibition of wound closure, confirming again that the SMI used is specific to EPHB4 (data not shown).

EPHB4-SMI represses the serine, threonine, and tyrosine phosphorylation of esophageal cells

SKGT-4 and Kyse-110 cells were treated in culture with 1 $\mu\text{mol/L}$ EPHB4-SMI. Whole-cell lysates for untreated control and treated cells were used in an immunoblot against phospho-tyrosine, phospho-serine, and phospho-threonine to determine the effect of inhibition of EPHB4 on protein phosphorylation. A dramatic decrease in phosphorylation was evident as shown in Fig. 5A. Inhibition of EPHB4 induced decreased phosphorylation of p130, p125, p68, p66, and p39 kDa proteins in both SKGT-4 and Kyse-110 cells. In a phospho-protein immunoblot analysis for the *EPHB4* siRNA-silenced

Figure 5. A, immunoassay showing the effect of EPHB4 inhibition on protein phosphorylation. Whole-cell lysates of SKGT-4 and Kyse-110 cells treated with 10 μ mol/L EPHB4-SMI for 24 hours were immunoassayed with antibodies against phospho-tyrosine, phospho-serine, and phospho-threonine. B, SKGT-4 and Kyse-110 were used again to specifically inhibit *EPHB4* by siRNA, and lysates were immunoassayed with antibodies against phospho-FAK and phospho-AKT. In both, β -actin was used as loading control.



cell lines (SKGT-4 and Kyse-110), gene-specific silencing led to similar downregulation of phosphorylation. We also analyzed the effect of *EPHB4* siRNA on phospho-FAK and phospho-AKT proteins, which show that phospho-FAK was completely inhibited (Fig. 5B).

In vivo xenograft tumor growth is inhibited by blocking EPHB4

The effect of EPHB4 inhibition *in vivo* was determined using sEPHB4-HSA, which is the extracellular domain of EPHB4 fused with human serum albumin, and achieves its activity by blocking the biologic effects of EPHRINB2 binding to EPHB4 (24). FLO cells xenografted in nude mice were treated with

either the drug or PBS. Tumor volume plotted as a mean of 10 tumors for each group shows that, compared with PBS only, treatment with sEPHB4-HSA significantly blocked tumor progression ($P = 0.005$; Fig. 6). Thus, despite *in vitro* resistance of FLO cells to EPHB4, these cells require functional EPHB4 *in vivo*.

Chemical induction of esophageal carcinogenesis in a mouse model and *ephb4* expression

The mouse 4-NQO model development of oral and esophageal carcinogenesis has previously been described in detail by Tang and colleagues and us (19, 25, 26). Here, we report esophageal SCCs as a result of carcinogenic induction by 4-NQO ingestion and the expression of *ephb4*. In our model, 38% of mice developed invasive SCCs, 29% carcinoma *in situ*, 24% dysplasia, and only 9% remained histologically unchanged (Fig. 7A and B). Ten age-matched normal mice with no 4-NQO induction were used as normal controls; all 10 normal esophagi showed a score of 0 for *ephb4* staining. Of the 21 4-NQO-treated mouse esophagus, only 10 were used for IHC due to limitations of staining resources. Eight samples constituted invasive or early invasive cancer and had an average score of 1.8, whereas the remaining 2 were dysplasias with an average score of 3 (Fig. 7C).

Discussion

In determining the role of EPHB4 in esophageal cancer, we profiled its protein expression pattern and compared with adjacent normal tissue. We found that there was consistently higher expression of EPHB4 in both squamous cancer and adenocarcinoma than in adjacent normal tissue. IHC data showed a significantly greater protein expression in tumor tissues than in normal tissues. In addition, a significant correlation was established between EPHB4 expression and higher grade of SCCs, suggesting that overexpression of EPHB4 in SCCs plays a role in clinically aggressive tumors. We also detected a robust expression of EPHB4 in all of the 13 esophageal cancer cell lines. We further found that in more than 40%

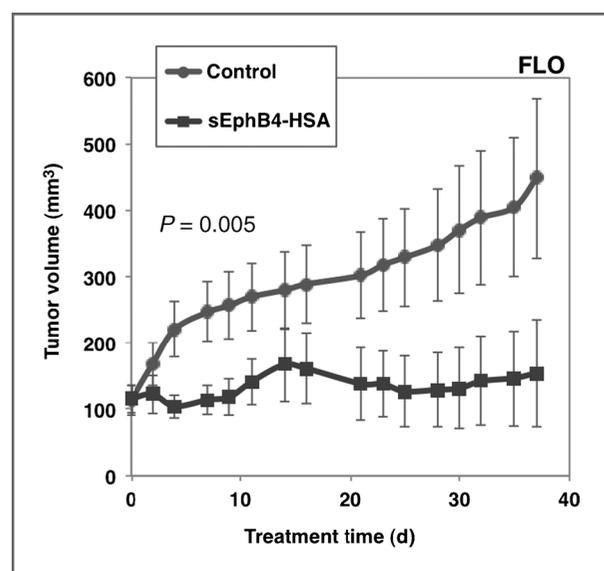


Figure 6. FLO cells injected subcutaneously in bilateral flanks of nude mice were treated with either sEPHB4-HSA (50 mg/kg) or PBS 3 times a week. Plotted tumor volume reflects the average of 10 tumors per group. P value was calculated with 2-tailed Student t test.

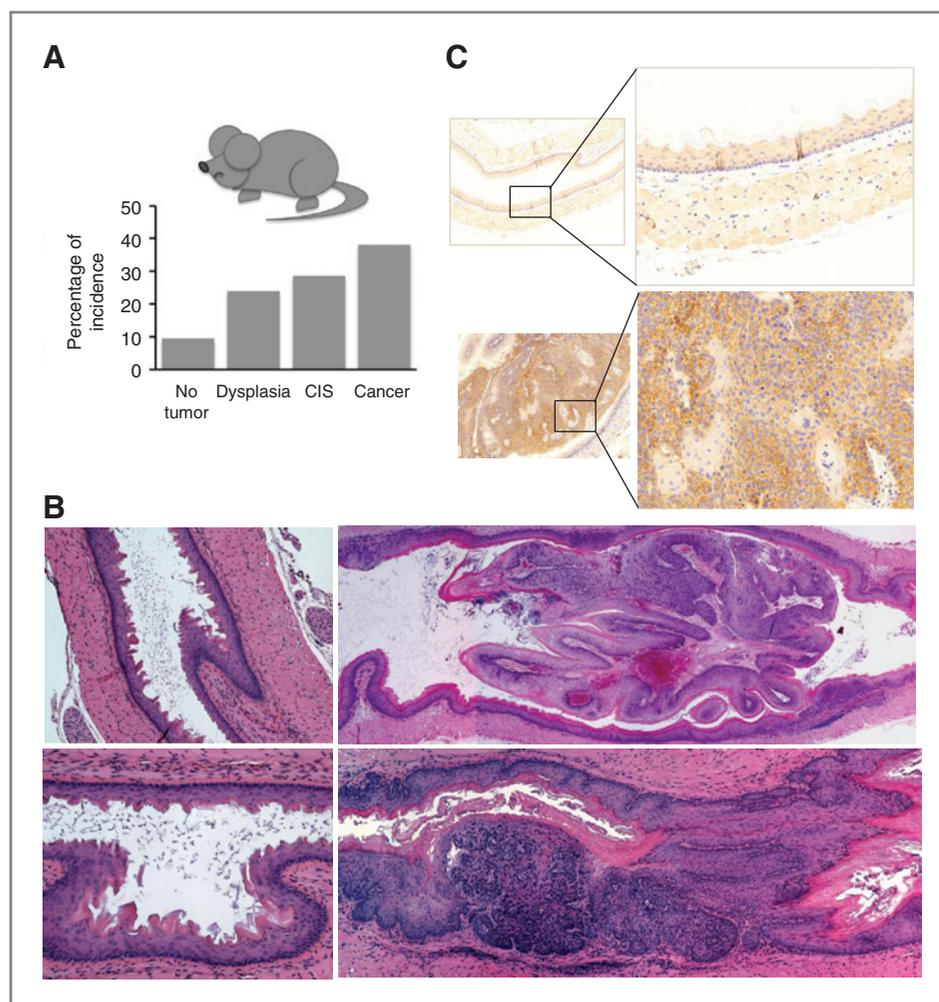


Figure 7. A, progressive chemical carcinogenesis. CBA mice develop esophageal SCCs by drinking 4-NQO. The incidence of carcinogenic changes after 8 weeks of 4-NQO is 38% SCCs, 29% carcinoma *in situ*, 24% dysplasia, and 9% no carcinogenic change. B, H&E staining of normal and 4-NQO-treated mouse esophagus. Left, longitudinal sections of 2 normal esophageal lumen and columnar epithelial lining; right, formation of SCCs in the esophageal lumen of 2 representative mice after 4-NQO treatment. C, top, 4-NQO untreated mouse esophagus expressing very low EPHB4 with some high background; bottom, 4-NQO-treated mouse esophagus bearing SCCs expressing high EPHB4.

of patients with SCCs, the *EPHB4* gene copy number ranged between 4 and 20 and more than 60% of patients had copy numbers in this range in adenocarcinomas. The gene copy number was similarly increased in cultured esophageal cancer cells. Increased gene copy number or copy number variations of oncogenes have been linked to increased protein expression with elevated risk to disease predisposition. This has also been implicated in disease progression and response to therapy (27, 28). Although activating mutations are a potential method by which receptor tyrosine kinases (RTK) are constitutively activated in cancer, we did not detect any significant mutations in *EPHB4* in cancer cells. Specific inhibition of *EPHB4* by an SMI showed that in 3 of 4 esophageal cancer cells, the drug impaired cell growth. Moreover, this compound also blocked migration in wound-healing assay. Both of these phenomena were clearly reproduced by silencing *EPHB4* with siRNA. These findings underscore that functional overexpression and *EPHB4* activity are regulated by other oncogenic drivers and that *EPHB4* represents an important pathway to target in the treatment of esophageal cancers.

The expression of *EPHB4* has been also shown to be higher in tumor tissues from lung, breast, prostate, bladder, ovarian, head and neck, and colon cancers among others (29–33).

Esophageal cancers are a heterogeneous group of disorders reflecting both adenocarcinoma and SCCs. In particular, as the lesion progresses from preneoplasia (Barrett esophagus or dysplasia) to cancer, *EPHB4* expression increases. Interestingly, the gene copy number was also increased in several tumor specimens. This could either represent polysomy or clustered amplification. It would be important to conduct further studies using a FISH analysis and compare with copy number of other genes, such as *MET* and *Her2/neu* that can be abnormal in adenocarcinomas.

EPHB4 has been reported to possess tumor suppressor properties (34). However, as shown here, an SMI against *EPHB4* or depletion with siRNA leads to a decrease in cell growth and viability. It would be important to study the effects of *EPHB4* in combination with cytotoxic chemotherapy or radiation therapy, as well as to conduct *in vivo* studies to validate these findings.

In generating a mouse model to study esophageal carcinoma, we were successful in showing that the 4-NQO model of oral carcinogenic induction resulted in high incidence of SCC development. In addition, the progressive induction of carcinogenic changes makes this an ideal model for prevention studies. For the first time, we were able to use this mouse model

to show that ephb4 is overexpressed in SCC as compared with normal esophagus. Moreover, in xenograft tumor model of FLO cells, we were able to see a large reduction in tumor volume with an EPHB4 inhibitor *in vivo*. The 4-NQO mouse model will significantly enhance not only the study of molecular markers such as EPHB4 but also help to define progression, invasion, angiogenesis, and possibly metastasis related biologic changes in this histology. This model will be also useful in future studies using inhibitors or EPHB4.

One of the features of esophageal SCC is the division of its incidence into low- and high-risk areas, based on geographical location. Some of the low risk areas include North America, along with countries in Western Asia and Northern and Southern Europe, where the incidence rates range from 1.5 to 6.0 of 100,000. High-risk areas include South Africa, China, Iran, and countries in Eastern Africa, where the incidence rates range from 10 to 25 of 100,000 (35, 36). In addition to the known risk factors such as alcohol and tobacco, gastroesophageal reflux disease, nutritional deficiencies, numerous studies have detected HPV DNA in esophageal cancer specimens, implicating HPV infection as a further risk factor in the development of esophageal cancer (37, 38). Interestingly, the highest frequencies of HPV infection are seen in the high-risk geographical locations. HPV infection has been implicated in other SCCs such as head and neck cancer (21) and non-melanoma skin cancer (39–41), and its involvement in cervical cancer is well established (42). We tested 10 representative esophageal SCC samples and their adjacent normal tissues of which 6 were African American and 4 Caucasian. None of the samples tested positive for HPV. In the past, it has been proposed that esophageal SCC can have HPV positivity. However, we did not identify this in our population.

Our study shows that EPHB4 is important not only in cell growth but also in cell motility and migration in several cell lines. This is most likely related to the cytoskeletal function, especially formation of focal adhesion complexes. With inhibition of EPHB4, there was decreased phosphorylation of p125FAK. FAK is a focal adhesion protein that is important both structurally and functionally. It is predicted that the EPHB4 molecule may be tightly linked to other proteins such as p130cas and paxillin. Previously, we have identified paxillin to be overexpressed or mutated in NSCLCs. It would be important to study this molecule in the context of EPHB4 in the future.

With increased motility and migration, tumors also gain more invasive properties that could lead to metastasis. Local proliferation and growth of tumor, invasion, and metastasis also leads to angiogenesis in the tumor bed. Because EPHB4 and its ligand, EPHRINB2, also participate in angiogenesis (3–5), it would be important to study the properties of vascular proliferation and neoangiogenesis in esophageal tumors as related to EPHB4 and EPHRINB2 *in vitro* and *in vivo*. Our results that one of the cell lines that was resistant to EPHB4 *in vitro* responded to the inhibition of EPHB4 pathway *in vivo* supports this hypothesis.

In summary, we have identified EPHB4 to be a key participant in esophageal preneoplastic and neoplastic lesions. It would be useful to explore EPHB4 inhibition strategy to clinical fruition in esophageal cancer.

Disclosure of Potential Conflicts of Interest

P. Gill has ownership interest (including patents) in Vasgene therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: R. Hasina, S. Yala, B.D. Ferguson, L. Varticovski, E.E. Vokes, P. Gill, R. Salgia

Development of methodology: R. Hasina, I. Kawada, K. Mutreja, G. Kanade, S. Yala, B.D. Ferguson, V. Nallasura, E. Hyjek, R. Kanteti, D. Kane, R. Salgia

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Hasina, N. Mollberg, I. Kawada, K. Mutreja, Y. Zhou, R. Kanteti, Y. Shimada, M.W. Lingen, A.N. Husain, M.C. Posner, I. Waxman, V.M. Villalflor, M.K. Ferguson, P. Gill, R. Salgia

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Hasina, N. Mollberg, I. Kawada, K. Mutreja, S. Yala, M. Surati, R. Liu, X. Li, Y. Zhou, V. Nallasura, J. Mueller, M.W. Lingen, A.N. Husain, L. Varticovski, P. Gill, R. Salgia

Writing, review, and/or revision of the manuscript: R. Hasina, N. Mollberg, R. Liu, B.D. Ferguson, K.S. Cohen, J. Mueller, Y. Shimada, M.W. Lingen, A.N. Husain, M.C. Posner, I. Waxman, V.M. Villalflor, M.K. Ferguson, E.E. Vokes, P. Gill, R. Salgia

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Hasina, I. Kawada, S. Yala, R. Liu, X. Li, R. Kanteti, E. El Hashani

Study supervision: R. Hasina, P. Gill, R. Salgia

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Correction: Critical Role for the Receptor Tyrosine Kinase EPHB4 in Esophageal Cancers

In this article (Cancer Res 2013 Jan 1;73:184–94), which was published in the January 1, 2013, issue of *Cancer Research* (1), references 20–23 were listed incorrectly. The online version has been corrected and no longer matches the print version. The authors regret this error. The correct references are as follows:

20. Bardelle C, Cross D, Davenport S, Kettle JG, Ko EJ, Leach AG, et al. Inhibitors of the tyrosine kinase EphB4. Part 1: Structure-based design and optimization of a series of 2,4-bis-anilinopyrimidines. *Bioorg Med Chem Lett* 2008;18:2776–80.
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1. Hasina R, Mollberg N, Kawada I, Mutreja K, Kanade G, Yala S, et al. Critical role for the receptor tyrosine kinase EPHB4 in esophageal cancers. *Cancer Res* 2013;73:184–94.

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Critical Role for the Receptor Tyrosine Kinase EPHB4 in Esophageal Cancers

Rifat Hasina, Nathan Mollberg, Ichiro Kawada, et al.

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