Critical role for the receptor tyrosine kinase EPHB4 in esophageal cancers

Running title: EPHB4 biomarker in esophageal cancers

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Abstract:

Esophageal cancer incidence is rising and has few treatment options. In studying receptor tyrosine kinases associated with esophageal cancers, we have identified EPHB4 to be robustly over expressed in cell lines and primary tumor tissues. In total, 94 squamous cell carcinoma (SCC), 82 adenocarcinoma, 25 dysplasia, 13 Barrett's esophagus and 25 adjacent or unrelated normal esophageal tissues were evaluated by IHC. EPHB4 expression was significantly higher in all the different histologic categories compared to adjacent normal tissues. In 13 esophageal cancer cell lines, three of the nine SCC cell lines and one of the four adenocarcinomas expressed very high levels of EPHB4. An increased gene copy number ranging from 4-20 copies was identified in a subset of the overexpressing patient samples and cell lines. We have developed a novel 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 three of the four 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 to untreated control. EphB4 is robustly expressed and potentially serves as a novel biomarker for targeted therapy in esophageal cancers.
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 epidemiological shift in esophageal cancers has occurred as a result of a 350% increase in adenocarcinoma between 1974 and 1994. This epidemiological shift reflects a change in etiology, with adenocarcinoma mirroring an increase in Barrett’s metaplasia, a preneoplastic condition induced by chronic gastroesophageal reflux. At present, Barrett’s 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 biological activities, including angiogenesis, cell segregation, and 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 (ECs), whereas EPHB4 is mostly venous-specific. Targeted inactivation of EPHB4 and EPHRINB2 have demonstrated 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 regards 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 (phosphatidylinositol 3-kinase) is another signaling cascade involved in regulating cell proliferation and viability (reviewed in (11)). A study by Steinle et al. (12) demonstrated 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 includes 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 demonstrate that EPHB4 is over expressed 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 demonstrate 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 DMEM or DMEM+Hank’s 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, USA and the Kyse cells were acquired through a generous gift from Dr. Yutaka Shimada of Toyama University, 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 Human Tissue Resource Center (HTRC) available through IRB-approved protocols. Experienced pathologists (JM and MWL) analyzed samples using conventional light microscopy and determined the histological subtype as well as tumor grade.

Barrett’s TMA

A Barrett’s Esophagus Tissue Microarray (TMA) was created with samples selected from the archive: carcinoma (n=3), high-grade dysplasia HGD (n=15), low-grade dysplasia LGD (n=10), non-dysplastic Barrett’s esophagus (n=13) and normal control (n=4; colon, placenta, liver, and kidney). Additional TMAs were purchased from US Biomax Inc. (Rockville, MD). Catalog #BC02011 (Multiple Esophageal Squamous Cell Carcinoma (SCC) tissue array, 69 cases/72 cores - 69 SCC x1 + 3 normal x1). Catalog #BS02051 (Esophageal Adenocarcinoma (AC) tissue...
array, 33 cases/63 cores - 28 AC x2 + 1 mucinous AC x2 + 1 smooth muscle x2 + 3 normal squamous epithelium x1). Catalog #ES208 (High Density Esophageal Cancer tissue array, with normal tissue, grade and TNM, 69 cases/208 cores - 24 SCC x3 + 2 ADSCC x3 + 23 AC x3 + 12 mets x3 + 9 normal x3 + 1 melanoma x1). Catalog #ES8011 (Esophageal Adenocarcinoma tissue array, with normal tissue, TNM and grade info, 40 cases/80 cores - 35 AC x2 + 5 normal x2).

**Immunohistochemistry (IHC):** IHC for human-EPHB4 was performed 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 IHC was performed on a Dako Autostainer (Dako, Carpinteria, CA) 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 min followed by Advance™ HRP detection system. Peroxidase reaction was developed using DAB provided in the kit and slides counterstained with Hematoxylin.

IHC for ephB4 on mouse esophagus (mice described later in in vivo methods) was performed 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, visibly observed individual sections under the microscope and estimated total staining intensity and extent of staining. The final IHC score was obtained by a semi-quantitative method that accounts for staining intensity and percent 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’s 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, Chicago, IL). Expression was also correlated with grade. Grades in each of the US Biomax TMAs were defined on an ordinal scale of 1-4. They were coded as well differentiated (1), moderately differentiated (2), poorly differentiated (3), and undifferentiated (4). The data was 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 squamous cell carcinomas and adenocarcinomas. To compare tumor and normal IHC data from US Biomax TMAs, a Mann Whitney test was performed 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 semi-quantitative 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 RIPA buffer supplemented with sodium orthovanadate, protease and phosphatase inhibitors. 150 µg of total protein were subjected to SDS-PAGE on 7.5% gel, transferred to PVDF membranes, and probed with appropriate primary antibodies. After blocking with 5% BSA, bands were visualized using HRP-conjugated secondary antibodies exposed to 1:1 horseradish peroxidase ECL solutions (Bio-Rad Laboratories, Hercules, CA), 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 GAPDH served as loading controls.

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

Real-time quantitative PCR (RTQ-PCR): Using a short target sequence within EPHB4, patient gDNA was used in RTQ-PCR 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 this 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:** gDNA was used in PCR to amplify DNA fragments encoding each *EPHB4* exon. Successfully amplified PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH) 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; State College, PA). 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 (AstraZeneca, Wilmington, DE) at concentrations of 1 uM and 10 uM or DMSO as vehicle control. The media was replaced with fresh drug every 48H. Cell viability was assessed at 0, 48, 72 and 96H 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 70-90% confluency in 35mm dishes. 24H 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 zero hour of scratch up to 20H. The wound closure was measured in images of zero hour and 15H or 20H using tools in ImageJ program (17).

**siRNA:** Cells were transfected with *EPHB4* siRNA duplex (5’-GGUGAAUGUAAGACGCUGUU-3’, IDT) and control siRNA duplex (5’-
AGUUAAUAUCAAGACGCUGUU-3’, IDT) oligonucleotides at a final concentration of 20 uM using Oligofectamine (Invitrogen) in serum free OptiMem medium (Invitrogen). Mock-treated cells received transfection reagent only. After 48H, cells were collected for viability, wound healing and immunoblot assays.

**HPV status:** A representative sample of ten SCC and adjacent normal tissue gDNA was used in a PCR based method utilizing the PGMY09/11 primer set to determine 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 6-8-week-old male Balb/C nu/nu mice. Tumor growth measured thrice weekly was calculated to estimate volume (0.52 × a × b², where a and b are the largest and smallest lengths of the tumor). Once tumors were approximately 100 cubic mm (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 standard error 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 (IACUC) and performed in accordance with the Animal Welfare Act. Data was analyzed comparing the mean tumor volume of the two groups on day 37, and the P value was calculated with student T-test, 2 tailed.

**in vivo model of esophageal carcinoma:** Male CBA mice, 6-8 weeks, Jackson Laboratory (Bar Harbor, ME) 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 histological changes and ephb4 expression. Dysplasia was characterized as a lesion that demonstrated 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 over expressed 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 squamous cell carcinoma (94), adenocarcinoma (82), dysplasia (25), Barrett’s 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 (Figure 1A). The data were analyzed in batches depending on the availability of background information and are depicted in Figure 1B and shown as a table in supplemental Table 1. Among esophageal cancer specimens in the University of Chicago Barrett’s TMA, expression of EPHB4 was found to be greater in tumor tissue compared with 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 was 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 $r=-0.03$ ($p=0.077$) (Figure 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 nine SCCs tested, Kyse-410, Kyse-850 and TE-8 had the highest expression. In the four adenocarcinomas, BE3, FLO, SKGT-4 and SKGT-5, all had significant baseline expression with SKGT-4 being the highest. H1993, a NSCLC cell line known to over express EPHB4 was the positive control and β-actin the loading control (Figure 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 (Figure 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 three 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 (Figure 2A, 2B). In addition, 33% of the SCC and 25% of the adenocarcinoma cell lines had copy numbers of $\geq 4$ (Figure 2C). Control copy numbers were 2 in each case. In an effort to determine gene copy number changes in EPHRINB2, we also
conducted real-time PCR on a limited number of randomly selected samples, which did not show any significant rise 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 two SNPs, T483C and A584G, in the FN III and juxtamembrane domains respectively in 70% of the cells ((Figure 2D). Both these SNPs are synonymous and reported in the HapMap database. In addition, four intronic mutations A7570A/T, A9995G, T14487C and 2241delC were detected in approximately 15% of cells. No significant non-synonymous 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 two SCC (Kyse-110, Kyse-410) and two adenocarcinoma (SKGT-4 and FLO) cell lines. Cells were treated with 0 and 1 uM inhibitor for 0, 48, 72 and 96H. The data were plotted as a percentage of viable cells in treated wells compared to 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 (Figure 3A). For Kyse-110 and 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 40-65% inhibition. FLO cell proliferation was not inhibited by the highest dose of 10 uM inhibitor for 96H. This data indicates that three of the four cell lines tested were sensitive to the EPHB4-SMI, while 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. This data suggests that \textit{EPHB4} is a target of the SMI (Figure 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 15H when compared with the size of the wound at 0 hour. When treated with the drug at 1 \textmu M, migration was strongly inhibited, with only about 28% of the wound closing (Figure 4A and B). The Kyse-110 cells migrated more slowly in 15H, only closing the wound 40% in the controls, while drug treatment almost completely inhibited migration with 12% of the wound healing. Both adenocarcinoma cells lines, SKGT-4 and FLO, showed 62-79% wound closure in the absence of the SMI, whereas 1 \textmu M drug inhibited migration resulting in only 14-18% wound closure (Figure 4A and 4B).

The same experiment was repeated with SKGT-4, Kyse-110 and Kyse-410 cells \textit{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 uM EPHB4-SMI. Whole cell lysates for untreated control and treated cells were used in an immunoblot against phosphotyrosine, 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 Figure 5A. Inhibition of EPHB4 induced decreased phosphorylation of p130, p125, p68, p66, p39 kDa proteins in both SKGT-4 and Kyse-110 cells. In a phosphoprotein immunoblot analysis for the EPHB4 siRNA silenced cell lines (SKGT-4 and Kyse-110), gene specific silencing led to similar down regulation 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 (Figure 5B).

**in vivo xenograft tumor growth is inhibited by blocking EPHB4:** The effect of EPHB4 inhibition in vivo was determined utilizing 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 ten tumors for each group shows that, compared to PBS only, treatment with sEPHB4-HSA significantly blocked tumor progression (p=0.005) (Figure 6). Thus, in spite of 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 et al. and us (19, 25, 26). Here, we report esophageal SCC as a result of carcinogenic induction by 4-NQO ingestion and the expression of ephb4. In our model, 38% of mice developed invasive SCC, 29% carcinoma in situ, 24% dysplasia and only 9% remained any histologically unchanged (Figure 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 while the remaining two were dysplasias with an average score of 3 (Figure 7C).

Discussion:
In determining the role of EPHB4 in esophageal cancer, we profiled its protein expression pattern and compared to adjacent normal tissue. We found that there was consistently higher expression of EPHB4 in both squamous cancer and adenocarcinoma compared to adjacent normal tissue. IHC data showed a significantly greater protein expression in tumor tissues compared to normal tissues. In addition, a significant correlation was established between EPHB4 expression and higher grade of SCC, suggesting that overexpression of EPHB4 in SCC 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% of SCC patients, the EPHB4 gene copy number ranged between 4 and 20 and more than 60% of patients
had copy numbers in this range in adenocarcinoma. 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 RTKs are constitutively activated in cancer, we did not detect any significant mutations in EPHB4 in cancer cells. Specific inhibition of EPHB4 by a small molecule inhibitor showed that in three out of four 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 \textit{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 SCC. In particular, as the lesion progresses from preneoplasia (Barrett’s 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 perform 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, a small molecule inhibitor 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 squamous cell cancer 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 utilize this mouse model to demonstrate that ephb4 is overexpressed in squamous cell carcinoma as compared to 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 will also help to define progression, invasion, angiogenesis and possibly metastasis related biological changes in this histology. This model will be also useful in future studies using inhibitors or EPHB4.

One of the features of esophageal squamous cell carcinoma 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 6.0/100,000. High risk areas include South Africa, China, Iran and countries in Eastern Africa, where the incidence rates range from 10 to 25/100,000 (35, 36). In addition to the known risk factors such as alcohol and tobacco, GERD, nutritional...
deficiencies etc., numerous studies have detected human papillomavirus (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 squamous cell carcinomas 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 ten representative esophageal SCC samples and their adjacent normal tissues of which six were African American and four Caucasian. None of the samples tested positive for HPV. In the past, it has been proposed that esophageal squamous cell cancer can have HPV positivity. However, we did not identify this in our population.

Our study shows that, not only is EPHB4 important 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 over expressed or mutated in NSCLC. 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. Since 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 which was resistant to EPHB4 \textit{in vitro} responded to the inhibition of EPHB4 pathway \textit{in vivo}, supports this hypothesis.

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


Figure Legends

Figure 1A. Barrett’s TMA, archival tissue and US Biomax TMAs containing 25 dysplasia, 82 adenocarcinoma, 94 SCC and 25 normal tissue samples were probed against EPHB4. Panel A shows representative samples expressing high (3+) EPHB4 levels in each histology. 1B-top graph: comparative EPHB4 scores in US Biomax TMAs; bottom graph: comparative EPHB4 scores in archival samples. 1C. Immunoblots demonstrating consistent expression of EPHB4 in both esophageal SCC and adenocarcinoma cell lines. 1D. 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.

Figure 2. Distribution of EPHB4 gene copy number by real-time PCR analysis in SCC (2A) and adenocarcinoma (2B) in selected archival patient samples. 2C shows gene copy number in cell lines. The horizontal bars represent a normal gene copy number of two. 2D shows the mutational status of EPHB4 in esophageal cancer evaluated by PCR and sequence analysis. The figure shows two synonymous SNPs discovered in 70% of the cell lines analyzed as well as four intronic SNPs identified. Evaluation of patient samples did not reveal any new SNPs.

Figure 3A. Inhibition of cell growth by a specific inhibitor of EPHB4. Two SCC and two adenocarcinoma cell lines were treated with 0, 1 and 10 uM of EPHB4-SMI AZ12489875-002 and viability measured at 0, 48, 72 and 96H. The graphs demonstrate SKGT-4, FLO, Kyse-110, Kyse-110 cell growth inhibition. 3B shows inhibition of cell growth in three cell lines in which EPHB4 was silenced with siRNA.
Figure 4. 4A shows 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 uM inhibitor. The four panels in each cell group indicate: A: No treatment at 0 Hour; B: No treatment at 15 Hour; C: 1 uM EPHB4 inhibitor at 0 Hour; D: 1 uM EPHB4 inhibitor at 15 Hour. The graph in 4B plots the relative wound closure measured against untreated controls.

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

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

Figure 7. 7A. Progressive chemical carcinogenesis. CBA mice develop esophageal SCC by drinking 4-NQO. The incidence of carcinogenic changes after 8 weeks of 4-NQO is 38% SCC, 29% carcinoma in situ, 24% dysplasia and 9% no carcinogenic change. 7B. H&E staining of normal and 4-NQO treated mouse esophagus. The left panels represent longitudinal sections of two normal esophageal lumen and columnar epithelial lining and right panels represent
formation of SCC in the esophageal lumen of two representative mice after 4-NQO treatment.  

7C. Top panel: 4-NQO untreated mouse esophagus expressing very low EPHB4 with some high background. Bottom panel: 4-NQO treated mouse esophagus bearing SCC expressing high EPHB4.
Figure 1

A

Barrett's esophagus
Dysplasia
Adenocarcinoma
SCC

B

Normal
Tumor

1.00
1.00
1.00

0.0017
0.0011

Squamous Cell Carcinoma
Adenocarcinoma

C

EPHB4
Actin

Squamous cell carcinoma
Adenocarcinoma

D

Kyse 220
Kyse 410
Figure 2

A. Gene Copy Number for Squamous Cell Carcinoma Samples

B. Gene Copy Number for Adenocarcinoma Samples

C. Gene Copy Number Distribution

D. Table of Mutations

<table>
<thead>
<tr>
<th>Domain</th>
<th>Mutation</th>
<th>Incidence</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN III Domain</td>
<td>T438C</td>
<td>9/13</td>
<td>70%</td>
</tr>
<tr>
<td>Juxtamembrane Domain</td>
<td>A584G</td>
<td>9/13</td>
<td>70%</td>
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<tr>
<td>Intronic (5-6)</td>
<td>7570AT</td>
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<td>15%</td>
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<tr>
<td>Intronic (6-7)</td>
<td>A9005G</td>
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<td>54%</td>
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<tr>
<td>Intronic (11-12)</td>
<td>T14487C</td>
<td>9/13</td>
<td>70%</td>
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<tr>
<td>Intronic (15-16)</td>
<td>22141delC</td>
<td>8/13</td>
<td>61%</td>
</tr>
</tbody>
</table>
Figure 3

A

SKGT-4

Kyse-110

Control
1 μM
10 μM

Percent Viability

0H 48H 72H 96H

FLO

Kyse-410

Percent Viability

0H 48H 72H 96H

B

SKGT-4

Kyse-110

Kyse-410

Control
Scrambled
EphB4 siRNA

Percent Viability

24H 48H 72H
Figure 4

A

SKGT-4

Kyse-110

FLO

Kyse-410

B

Percentage of Wound Healing

Control
Inhibitor

SKGT-4
FLO
Kyse-410
Kyse-110
Figure 5
Figure 7

A

<table>
<thead>
<tr>
<th>Percentage of Incidence</th>
</tr>
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<tbody>
<tr>
<td>No tumor</td>
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<tr>
<td>Dysplasia</td>
</tr>
<tr>
<td>CIS</td>
</tr>
<tr>
<td>Cancer</td>
</tr>
</tbody>
</table>

B

C

Research.
Critical role for the receptor tyrosine kinase EPHB4 in esophageal cancers

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

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