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Expression of the EPHB4 receptor tyrosine kinase in head and neck and renal malignancies—implications for solid tumors and potential for therapeutic inhibition

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Abstract

Solid malignancies are often characterized by overexpression of various receptor tyrosine kinases (RTKs) against which many targeted therapies are currently in use and in active development. EPHB4 has recently emerged as a frequently overexpressed RTK in many types of cancer. Here, we demonstrate expression patterns of EPHB4 in two solid malignancies, squamous cell carcinoma of the head and neck (HNSCC) and renal cell carcinoma (RCC), by immunohistochemical analysis. We demonstrate the first association between EPHB4 expression and progression of HNSCC from normal tissue to dysplasia and to cancer. Interestingly, most RCC subtypes exhibited expression patterns that were opposite from that found in HNSCC, possibly owing to their unique biology and high degree of organ and tumor vasculature. Taken together, these results suggest a possible role for EPHB4 as a therapeutic target in these malignancies.

Keywords

EphB4; receptor tyrosine kinase; head and neck cancer; renal cell carcinoma

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Introduction

Solid tumors are frequently characterized by overexpression of receptor tyrosine kinases, including EGFR and its related family members, MET, KIT, RON, RET, ROS, and VEGFR (Blume-Jensen and Hunter, 2001; Wagh et al., 2008; Seiwert et al., 2009; Blumenschein et al., 2012; Roskoski, 2013). Expression of EPH receptors, another group of receptor tyrosine kinases, has also been documented in cancers of virtually every major organ system. The EPH family of RTKs is made up of 14 mammalian ephrin receptors and 8 mammalian ligands, called ephrins. These are functionally and structurally divided into A- and B-classes based on sequence similarity and ligand-binding affinity. EPHB receptors interact with ephrin-B ligands to effect downstream signaling, which has established roles in vasculogenesis, brain and eye development, and tumorigenesis (Kullander and Klein, 2002).

In particular, EPHB4 has emerged as a frequently overexpressed RTK in cancer. While the normal role of EPHB4 is typically associated with early development, its expression is typically low in adult tissues. We have previously shown that EPHB4 is overexpressed 4.3-fold more strongly in lung adenocarcinoma, 2.7-fold more strongly in lung squamous cell carcinoma, and 2.4-fold more strongly in small cell lung carcinoma relative to paired normal tissues (Ferguson et al., 2013) and is also overexpressed in pleural mesothelioma (Liu et al., 2013) and esophageal cancer (Hasina et al., 2013). Many other cancers, including those of the breast (Kumar et al., 2006), thyroid gland (Sharma et al., 2014), large bowel (Kumar et al., 2009; Stephenson et al., 2001), ovaries (Alam et al., 2008; Kumar et al., 2007; Spannuth et al., 2010), cervix (Alam et al., 2009), endometrium (Alam et al., 2007), prostate (Xia et al., 2005; Lee et al., 2005), bladder (Xia et al., 2006), and skin (Yang et al., 2006), have demonstrated increased expression of EPHB4. Despite this pattern, the prognostic value of EPHB4 in cancer remains somewhat unclear. Though many studies have found that stronger EPHB4 expression is correlated with disease progression and shorter survival (Lee et al., 2005; Brantley-Sieders et al., 2011; Guijarro-Muñoz et al., 2013), some have shown the opposite relationship (Battle et al., 2005; Ferguson et al., 2013).

Here, we present expression patterns of EPHB4 in solid tumors of the head and neck as well as renal malignancies.

Methods

Tissue procurement

Human HNSCC and renal cancer patient tissues were obtained from the University of Chicago Tumor Tissue Bank with informed consent and in accordance with IRB protocol.

Immunohistochemistry

Anti-EPHB4 antibody (#131) was generously provided by the Gill Laboratory, University of Southern California. Paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions to distilled water, and washed in Tris-buffered saline (TBS). Antigen retrieval was carried out by heating sections in citrate buffer (pH 6) for 15m in a microwave. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in methanol for 5m. Non-specific binding sites were blocked using Protein Block

(Dako, Carpinteria CA) for 20m. Sections were incubated for 1h at room temperature with anti-EPHB4 primary antibody (anti-mouse) at a concentration of 40 μ g/mL, then incubated for 30m with goat anti-mouse IgG conjugated to a HRP-labeled polymer (Bio SB, Santa Barbara CA). Slides were then developed for 5m with 3-3'-diaminobenzidine chromogen, counterstained with hematoxylin, and coverslipped. Negative controls were performed by substituting primary antibody with non-immune mouse immunoglobulins. Colon cancer and brain tissue sections served as positive controls for EPHB4 staining (Stephenson et al., 2001; Hafner et al., 2004). Tissue expression for each sample was quantified by manual pathology scoring on a 0/1+/2+/3+ scale.

Statistical analyses

Comparisons of three or more groups were made using one-way ANOVA. All statistical calculations were performed using Prism software (GraphPad, La Jolla CA).

Results

HNSCC and renal tumor tissues were analyzed for EPHB4 expression by IHC. The majority of normal tissues demonstrated EPHB4 expression at a low level with absent expression in 15% of specimens. However, during the progression from normal tissue to dysplasia to frank squamous cell carcinoma, there was a significant increase in the proportion of tissues strongly expressing EPHB4 and a concordant decrease in tissues with weak or absent EPHB4 expression ($p < 0.0001$, $n=15$ patients) in a paired-sample expression analysis (Figures 1 and 2). 70% of HNSCC specimens demonstrated moderate or strong (2+ or 3+) EPHB4 expression compared to 10% of normal specimens and 37% of dysplastic specimens.

One out of five renal cancer subtypes investigated, type II papillary RCC, as well as oncocytoma demonstrated strong EPHB4 expression in a majority of samples, while the remaining four RCC subtypes (clear cell, chromophobe, sarcomatoid, type I papillary) and angiomyolipoma exhibited weaker expression in tumor tissues (Figure 3). A statistical analysis using the Kruskal-Wallis one-way ANOVA test involving nonparametric samples demonstrated that the overall P value for variance in expression across tumor subtypes (with normal tissue samples excluded) was less than 0.0001; however, the only subtype that differed statistically significantly from normal renal tissue expression was clear cell RCC ($p < 0.001$), in which expression was less strong than normal. Type I and II papillary RCC samples also differed significantly from each other ($p < 0.01$).

Discussion

We have been investigating mechanisms of upper aerodigestive tumorigenesis, and our group has previously demonstrated increased EPHB4 expression patterns in cancers of the lung, pleural mesothelium, and esophagus, as well as in breast, colorectal, prostate, bladder, and ovarian cancers. A summary of studies demonstrating pro-tumorigenic roles of EPHB4 expression patterns across various cancer types is shown in Table 1. We now present these data for head and neck tumors as well as renal cell carcinoma as an independent entity to explore EPHB4 expression outside of the aforementioned group of systems.

The expression trend described here for HNSCC is in close agreement with reports by Masood et al. (Masood et al., 2006), which found that tumor expression of EPHB4 in HNSCC increased with disease stage (in primary tumor tissues and nodal metastases), by Sinha et al. (Sinha et al., 2006), which reported that EPHB4 expression correlated both with stage of HNSCC as well as with progression from normal status to cancer, and by Yavrouian et al. (Yavrouian et al., 2008), which demonstrated that increased EPHB4 expression in HNSCC is correlated with shorter survival. However, no previous studies have investigated the expression of EPHB4 in pre-HNSCC dysplastic tissue; to our knowledge, these data are the first to address the expression pattern of a kinase in paired head and neck tissues with progression from normal status through dysplasia to cancer and therefore shed significant light on the role of EPHB4 in HNSCC progression.

Currently, chemotherapy, radiation, and surgery are frequently combined in the management of HNSCC. Incorporation of targeted small-molecule and antibody-based therapies against RTKs has increasingly come into use, including those aimed at EGFR, MET, and VEGFR (Cohen et al., 2003; Price and Cohen, 2012; Choong and Cohen, 2006; Seiwert et al., 2009). Major challenges, however, remain the high rate of recurrence among patients treated for locoregionally advanced disease and the high frequency of presentation at advanced stages. Additionally, the development of resistance to kinase inhibition is a significant barrier to successful treatment with adequate progression-free disease (Cooper and Cohen, 2009). Identification of additional therapeutic targets in HNSCC remains an important challenge for improvement in treatment efficacy.

The expression pattern presented here suggests that EPHB4 may be both a good biomarker and promising target in HNSCC. EPH signaling in cancer commonly involves downstream activity of Src, STATs, and the PI3K/Akt axis, especially in concert with EGFR signaling (Kumar et al., 2006; Li et al., 2012; Ferguson et al., 2013). The interplay between EGFR and EPHB4 signaling is not well understood but may represent an important factor in HNSCC biology. It would also be interesting to correlate expression patterns of EPHB4 with the presence of mutations and copy number variations in solid tumors.

It is interesting to note that the predominant expression pattern in renal cancer specimens was discordant from that found in HNSCC. EPHB4 has previously been shown to be more strongly expressed in the endothelium of renal, bladder, and prostate cancers than in corresponding normal tissue (Ozgür et al., 2011; Xia et al., 2006), and other EPH family members, including EPHA2 and EPHB2, have been found to be strongly expressed in urothelial malignancies (Chetcuti et al., 2011; Abraham et al., 2006). EPHA2 overexpression has been successfully exploited for efficient delivery of paclitaxel in a mouse model of renal cancer (Wang et al., 2013). However, mRNA expression of EPHB4 was noted to be 2.2-fold lower in renal cancers than paired normal renal tissues (Hafner et al., 2004), which correlates with the protein expression data presented here. Because EPHB4 is commonly expressed within venous vasculature, it may be the case that its biology in renal malignancies, which are highly vascular, exhibits unique behavior relative to its other solid tumor counterparts. Furthermore, oncocytoma (a benign epithelial renal tumor arising from collecting ducts) and papillary type II RCC with oncocytic cytoplasm (M. Tretiakova, personal communication) exhibited the strongest expression of EPHB4. This may suggest

that these and other renal neoplasms frequently associated with oncocytic changes, such as rhabdoid tumors as well as variants of chromophobe and clear cell subtypes, could be targeted more effectively with EPHB4 inhibition. More studies are needed to fully clarify the role of EPHB4 in renal cancer.

A phase I trial investigating the effects of EPHB4 inhibition in solid tumors is currently underway. These data support the hypothesis that EPHB4 inhibition may be a promising strategy for the treatment of solid malignancies.

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Normal

Dysplasia

HNSCC

Figure 1. EPHB4 expression in HNSCC

Examples of EPHB4 expression in normal, dysplastic, and HNSCC are displayed.

Figure 2. Pattern of EPHB4 expression in HNSCC

A: Distribution of EPHB4 expression by IHC scoring across HNSCC tissues along its disease progression. B: Quantification of EPHB4 expression across HNSCC progression in a paired-sample analysis. Overall P value was calculated by one-way ANOVA.

Figure 3. EPHB4 expression across renal cancer subtypes

Low designation includes tissues demonstrating 0 or 1+ staining by manual IHC scoring; high designation includes tissues demonstrating 2+ or 3+ staining. Sample numbers are shown below subtype indications. CCRCC, clear cell RCC; Papillary I/II, papillary type I and II RCC; AML, renal angiomyolipoma. The overall P value shown was calculated using the Kruskal-Wallis one-way ANOVA test involving nonparametric samples.

Table 1

Summary of prior studies demonstrating pro-tumorigenic roles of EPHB4 expression patterns across solid malignancies.

Site	References
Head and neck cancer	(Masood et al., 2006; Sinha et al., 2003; Sinha et al., 2006; Yavrouian et al., 2008)
Thyroid cancer	(Sharma et al., 2014)
Esophageal cancer	(Hasina et al., 2013)
Lung cancer	(Ferguson et al., 2013)
Pleural mesothelioma	(Liu et al., 2013)
Breast cancer	(Kumar et al., 2006; Wu et al., 2004)
Colon cancer	(Kumar et al., 2009; Stephenson et al., 2001)
Bladder cancer	(Xia et al., 2006)
Kidney cancer	(Ozgür et al., 2011)
Prostate cancer	(Xia et al., 2005; Lee et al., 2005)
Uterine cancer	(Alam et al., 2009; Alam et al., 2007; Berclaz et al., 2003)
Ovarian cancer	(Kumar et al., 2007; Spannuth et al., 2010; Alam et al., 2008; Wu et al., 2006)
Leukemia	(Steube et al., 1999)
Skin cancer	(Yang et al., 2006)