The Significance of EphB4 and EphrinB2 Expression and Survival in Head and Neck Squamous Cell Carcinoma

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**Objectives:** To examine the expression of EphB4 and EphrinB2 in tumor tissue and surrounding normal tissue in patients with head and neck squamous cell carcinoma (HNSCC) and to evaluate its association with overall patient survival.

**Design:** Retrospective study.

**Setting:** University of Southern California–University Hospital, the Los Angeles County and University of Southern California Medical Center, and the Department of Otolaryngology–Head and Neck Surgery, University of Southern California, Los Angeles.

**Patients:** Fifty patients, 8 with early-stage (stages I and II) and 42 with advanced-stage (stages III and IV) HNSCC, were enrolled into this study. Staging was based on the system of the American Joint Committee on Cancer.

**Main Outcome Measures:** EphB4 and EphrinB2 expression was evaluated by Western blot analysis. Overall survival in patients was then compared with EphB4 and EphrinB2 expression.

**Results:** EphB4 and EphrinB2 expression was detected in all normal and tumor samples in patients with HNSCC, with the magnitude of EphB4 overexpression greater than that of EphrinB2 expression compared with normal tissue. There was a statistically significant decrease in overall survival among patients with elevated EphB4 and EphrinB2 expression ($P < .001$).

**Conclusions:** EphB4 and EphrinB2 overexpression is associated with poor overall survival in patients with HNSCC. Our results are the first to demonstrate an association between decreased survival and elevated expression of the receptor tyrosine kinase EphB4 and its ligand EphrinB2, suggesting that EphB4 and EphrinB2 may be used as biomarkers to predict prognosis and as targets in future HNSCC therapies.

Arch Otolaryngol Head Neck Surg. 2008;134(9):985-991

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**H**ead and neck squamous cell carcinoma (HNSCC) is an epithelial cancer that arises in the mucosa of the upper aerodigestive tract. It commonly affects the oral cavity, oropharynx, hypopharynx, and larynx. Head and neck squamous cell carcinoma is the sixth most common cancer worldwide, comprising about 4% of all malignant neoplasms, with 900,000 cases worldwide each year. In the United States, it was estimated that in 2008 there would be 48,000 cases and 11,000 deaths from the disease. Men are significantly more affected than women, with increased incidence and mortality among African American men. Important risk factors associated with the development of HNSCC are tobacco and alcohol use; therefore, abstinence from smoking and drinking is the best mode of prevention.

The major treatment modality for HNSCC has traditionally been surgical resection and postoperative radiotherapy. Approximately 30% to 40% of patients are initially seen with early-stage disease (stages I and II), with cure rates ranging from 60% to 80% depending on the stage. For patients with more advanced disease (stages III and IV), cure rates are below 30%. Five-year survival rates range from 70% to 85% for early-stage disease and from 30% to 40% for advanced-stage disease. Because of the poor prognosis of advanced-stage disease, prevention and early diagnosis are high priorities.
More recently, treatment modalities for locoregionally advanced disease have included new chemotherapeutic regimens. Cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR), is one of the therapies being examined. Many head and neck cancers demonstrated increased EGFR expression and subsequent poor prognosis, rendering EGFR a prime target for therapy. Novel therapies aimed at molecular markers with increased expression in HNSCC are essential to improve survival and prognosis in patients who have this disease.

Several potential targets in cancer therapy that may reduce tumor burden and improve survival may be found in factors that control angiogenesis. There are 3 families of ligands and their receptor tyrosine kinases (RTKs) implicated in vascular development. These include the vascular endothelial growth factor (VEGF) family, the angiopoietin family, and the ephrins and Eph receptors.

The Eph receptors form the largest family of RTKs, of which EphB4 is a member (EPHB4) (NM_004444; Entrez Gene 2050). There are 15 members in the Eph receptor family, divided into EphA and EphB classes. EphB4 is a component of the class B Eph receptors, which are transmembrane proteins. Ephrins are the ligands of the Eph receptors. There are 13 members in the ephrin family, which is also divided into classes A and B. EphrinB2 (EFNB2) (NM_004093; Entrez Gene 1948) is the exclusive ligand of EphB4, and (like EphB4) it is a component of the class B transmembrane proteins. EphB4 is normally expressed on venous endothelial cells, while EphrinB2 is expressed on arterial endothelial cells. The ephrins and Eph receptors have been shown to have an important role during embryonic development in cell aggregation and migration, segmentation, pattern recognition, neural development, angiogenesis, and vascular network development.

Previously, our group has studied the expression of EphB4 and EphrinB2 in HNSCC. We have shown that EphB4 is expressed in all primary and metastatic tumors and that levels of EphB4 expression correlate with advanced stage and with lymph node metastasis. EphB4 expression was also found to be associated with smoking status. In addition, we demonstrated that EphB4 provided a survival advantage to tumor cells and that its inhibition decreased survival of tumor cells in vivo. In this study, we show that EphrinB2 expression is increased in tumor samples compared with corresponding normal tissue. We also demonstrate that elevated expression of EphB4 and EphrinB2 correlates with poor overall survival in patients with HNSCC. The role that EphB4 and EphrinB2 have in angiogenesis, the elevated expression of EphB4 in advanced cases, and the lower overall survival among patients with elevated EphB4 and EphrinB2 expression support the importance of targeting EphB4 and EphrinB2 in HNSCC therapy.

PATIENT SELECTION

Fifty patients with HNSCC underwent surgical resection at the University of Southern California–University Hospital and the Los Angeles County and University of Southern California Medical Center and were retrospectively entered into the study. The study was approved by the appropriate institutional review board. Demographic data were collected from the patient hospital records. The staff members who collected the demographic data had no knowledge of the status of EphB4 or EphrinB2 expression in the patient tumors.

Perioperative data included the TNM stage of the head and neck cancer (stages I-IV according to the staging system of the American Joint Committee on Cancer), the site of the primary tumor, and the pathologic grade of the neoplasm on light microscopic examination. Tumor and normal adjacent tissues were collected in all 50 cases. The tumor tissues were not laser dissected; therefore, to control for the amount of histologically normal tissue within the tumor sample, we used tumor tissue sections that showed more than 70% of tumors cells by hematoxylin-eosin staining. Each tissue specimen was given a unique identification number and was sent to the laboratory for analysis. The laboratory was blinded to the status of the tissue specimen.

REAGENTS

Antibodies to EphB4 (C-16) and EphrinB2 (P20) were purchased (Santa Cruz Biotech, Santa Cruz, California). Additional monoclonal antibodies to the extracellular domain of EphB4 (25D, 94D, and 265D) were generated in house.

WESTERN BLOT

Cell lysates were prepared as previously described. Typically, 10-µg proteins from whole cell lysates were fractionated on a 4% to 20% Tris glycine polyacrylamide gel, electrotransferred to polyvinyl difluoride membrane, and probed with a primary antibody overnight. The blot was stripped with Western blot stripping buffer (Restore; Pierce Biotechnology Inc, Rockford, Illinois) and reprobed with β-actin to confirm equivalent loading and transfer of protein. Signal was detected using a substrate (Super Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology Inc). Western blots were digitized, and the relevant protein bands of EphB4 and EphrinB2 were normalized with β-actin and quantified (Fluro-S Multi-Imager System; Bio-Rad Laboratories, Hercules, California). Western blots of tissue from primary tumor and uninvolved tissue were performed to determine the relative levels of EphB4 and EphrinB2 expression in these sites.

STATISTICAL ANALYSIS

Overall survival was measured in years from the date of surgery to the date of death or last follow-up. EphB4 and EphrinB2 expression was not normally distributed. Patients were divided into high- and low-expressing EphB4 and EphrinB2 groups based on digitized quantification of Western blots using an imaging system (Fluro-S Multi-Imager System). Because EphB4 and EphrinB2 expression was not normally distributed, we used the median level of EphB4 and EphrinB2 expression to group patients into low- and high-expression categories. Survival curves were generated using the Kaplan-Meier method and were compared by the log-rank statistic using statistical software (SPSS; SPSS Inc, Chicago, Illinois).
PATIENT CHARACTERISTICS

From October 22, 2002, to May 20, 2005, 50 patients with HNSCC were seen at the University of Southern California–University Hospital and the Los Angeles County and University of Southern California Medical Center for surgical resection of their tumor. The clinicopathologic features of the patients are listed in Table 1.

EXPRESSION OF EPHB4 AND ITS LIGAND EPHRINB2

Our group has previously shown the expression of EphB4 in HNSCC.16,17 Patients with advanced cases of HNSCC have increased expression of EphB4 by Western blot analysis.17 In the present study, we also reviewed the expression of EphrinB2 by Western blot analysis among 43 patients in the HNSCC study group. Although the intensity of expression was not as high as that of EphB4, there was a trend toward increased expression of EphrinB2 in tumor samples vs normal tissue ($P < .001$) (Table 2). EphB4 expression and EphrinB2 expression were moderately correlated (Spearman rank correlation coefficient $r = 0.54$, $P < .001$).

SURVIVAL

We analyzed the prognosis of 50 patients who underwent curative resection of their tumor. Among the patients, there were 29 deaths, with no operative mortality. The mean follow-up period for all patients was 50 months (range, 1-132 months). For 29 patients who died, the mean follow-up period was 29 months (range, 1-98 months). Of 21 living patients, the mean follow-up period was 29 months (range, 1-132 months). Survival for all patients is shown in Figure 1, with overall survival for all patients at 1, 3, and 5 years of 64%, 42%, and 31%, respectively. The distribution of EphB4 and EphrinB2 levels in surviving and nonsurviving patients is plotted in Figure 2, which shows that surviving patients seemed to have lower expression of EphB4 and EphrinB2 than did nonsurviving patients.

Overall survival rates of patients with HNSCC were compared with levels of EphB4 or EphrinB2 using the Kaplan-Meier method (Figure 3). The median level of expression for EphB4 was 2.5-fold greater in tumor samples compared with adjacent normal tissue. This level was used to stratify patients into groups of low and high EphB4 expression. There were 25 patients in the low-expressing EphB4 group and 25 patients in the high-expressing EphB4 group. Patients with low EphB4 expression had survival at 1, 3, and 5 years of 68%, 64%, and 64%, respectively. For patients with high EphB4 expression, survival at 1, 3, and 5 years was 60%, 22%, and 15%, respectively. EphB4 overexpression was a significant predictor of overall survival ($P = .01$) (Figure 3A).

Because of the significant difference in survival among patients with elevated EphB4 expression, we also studied EphrinB2 expression in 43 patients. There were 19 patients in the low EphrinB2 group and 24 patients in the high EphrinB2 group. The median level of expres-
sion for EphrinB2 was 2.2-fold greater in tumor samples compared with adjacent normal tissue. This level was used to stratify patients into groups of low and high EphrinB2 expression. For patients with low EphrinB2 expression, survival at 1, 3, and 5 years was 84%, 79%, and 79%, respectively. For patients with high EphrinB2 expression, survival at 1, 3, and 5 years was 46%, 19%, and 9%, respectively. Like EphB4, EphrinB2 overexpression was a significant predictor of overall survival in HNSCC (P < .001) (Figure 3B).

In addition to comparing overall survival with EphB4 expression and EphrinB2 expression individually, we compared survival in patients with both elevated EphB4 expression and elevated EphrinB2 expression. There were 15 patients in the low EphB4 and EphrinB2 group and 16 patients in the high EphB4 and EphrinB2 group. All 16 patients in the high EphB4 and EphrinB2 group died, and their survival at 1, 3, and 5 years was 44%, 6%, and 0%, respectively. For patients with both low EphB4 expression and low EphrinB2 expression, survival at 1, 3,
Angiogenesis is an important factor in tumor growth and progression. For this reason, it is essential to find targets that inhibit this process. The RTK EphB4 and its ligand EphrinB2 have well-established roles in blood vessel formation and vessel maturation. EphB4, a transmembrane protein, is predominantly expressed on arterial endothelial cells, whereas its receptor, EphB4, is largely confined to veins. Ephrins (EphrinB2) and Ephs (EphB4) are membrane bound; therefore, binding and activation of EphrinB2 and EphB4 require cell-to-cell interaction rather than long-range communication. They mediate bidirectional signaling cascades, forward signaling from the EphB4 receptor, and reverse signaling from the EphrinB2 ligand. When activated, EphB4 and EphrinB2 become phosphorylated, form complexes with various proteins, and affect downstream signaling. Confirming their role in angiogenesis, EphB4 and EphrinB2 knockout mice developed defective vessel remodeling, organization, and sprouting.

Elevated expression and activity of Eph receptors have also been correlated with the growth of solid tumors. Along with that, high expression of ephrins may be associated with increased potential of tumor growth, tumorigenicity, and metastasis. Upregulation of EphB4 and EphrinB2 has been shown to enhance proliferation, migration, and metastatic potential of the tumor cells. In addition, our laboratory has previously shown that EphB4 promotes tumor cell longevity and that elevated EphB4 expression is associated with advanced HNSCC.

In this study, we evaluated the prognosis associated with EphB4 and EphrinB2 expression in HNSCC. We confirmed previously reported results of elevated EphB4 expression in tumor vs normal tissue, and we demonstrated elevated EphrinB2 expression in tumor samples. The intensity of EphB4 overexpression is greater than that of EphrinB2 overexpression. However, EphrinB2 is also significantly overexpressed in HNSCC compared with adjacent normal tissue. We also showed that increased expression of EphB4 or EphrinB2 is associated with decreased overall survival in patients with HNSCC. In addition, we demonstrated that, among patients in whom both EphB4 and EphrinB2 were elevated, overall survival was even worse than that among patients with increased expression of EphB4 or EphrinB2 alone. Furthermore, all patients with elevated EphB4 and EphrinB2 expression died. The poor prognosis of patients with elevated EphB4 and EphrinB2 levels suggests that the coexpression of EphB4 and EphrinB2 has a synergistic role in HNSCC that contributes to worse overall survival.

There was a significant relationship between EphB4 and EphrinB2 overexpression and poor overall survival. However, 9 patients with low EphB4 expression died. Four of 9 patients with low EphB4 expression also had low EphrinB2 expression. These patients had advanced-stage HNSCC, with 3 patients initially seen with positive lymph nodes and 4 patients having recurrent tumor at the time of surgery. In patients with recurrent tumor, the adjacent normal tissue also expressed elevated levels of EphB4 and EphrinB2. In these 9 patients, once EphB4 and EphrinB2 expression was normalized with expression in mucosa adjacent to the tumor, the relative expression of EphB4 and EphrinB2 was low compared with that in other patients with low EphB4 and EphrinB2 expression in their corresponding normal tissue. The deaths that occurred in the patients with low EphB4 and EphrinB2 expression in their tumor compared with their adjacent normal mucosa may reflect the “field effect” of the tumor on local expression of these proteins in adjacent normal mucosa. In addition, these patients were initially seen with advanced HNSCC, which (along with other causes) likely contributed to their death. Although in some patients EphB4 and EphrinB2 expression was low when normalized with adjacent normal tissue, there was significant association between EphB4 and EphrinB2 overexpression and decreased overall survival in patients with HNSCC.

When we compared EphB4 and EphrinB2 expression, EphB4 and EphrinB2 were moderately correlated. In addition, their expression was not significantly associated with sex, race/ethnicity, site of primary tumor, and radiotherapy and chemotherapy before surgery.

### Table 3. Multivariate Analysis of Demographic and Tumor Biologic Factors for Survival Associated With EphB4 and EphrinB2 Expression

<table>
<thead>
<tr>
<th>Model</th>
<th>Hazard Ratio (95% Confidence Interval)</th>
<th>P Value</th>
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<tr>
<td>EphB4 Expression</td>
<td></td>
<td></td>
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<tr>
<td>Unadjusted</td>
<td>2.64 (1.20-5.83)</td>
<td>.02</td>
</tr>
<tr>
<td>Adjusted</td>
<td>2.95 (1.13-7.74)</td>
<td>.03</td>
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<tr>
<td>EphrinB2 Expression</td>
<td></td>
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<tr>
<td>Unadjusted</td>
<td>3.45 (1.56-7.92)</td>
<td>.002</td>
</tr>
<tr>
<td>Adjusted</td>
<td>4.17 (1.55-11.04)</td>
<td>.004</td>
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*a Cox proportional hazards model (PHREG procedure in SAS version 9.1; SAS Institute Inc, Cary, North Carolina). b Adjusted for age, sex, race/ethnicity, clinical stage, site of primary tumor, and radiotherapy and chemotherapy before surgery.
mor stage, tumor size, or prior chemotherapy or radiotherapy. However, EphrinB2 expression was weakly correlated with age (Spearman rank correlation \( r = 0.25 \)), although this correlation was not statistically significant (\( P = 0.10 \)). This association could be an important finding and may suggest poor prognosis in patients with high EphrinB2 levels and with older age. Although there was no significant correlation between these variables and EphB4 or EphrinB2 expression, multivariate analysis was performed with adjustment for the effect of these variables on EphB4 and EphrinB2 expression and overall survival, which showed that elevated EphB4 or EphrinB2 expression is independently associated with decreased overall survival in HNSCC. For this study, we did not have information about the smoking status in the entire sample. Further adjustment for smoking in a subsample did not affect the risk estimates; therefore, it is unlikely that smoking could explain the observed associations. Therefore, a multivariate analysis adjusting for demographic and tumor biologic factors confirmed that elevated EphB4 expression and EphrinB2 expression are independent predictors of overall survival in HNSCC.

Patients with high EphB4 expression were also likely to overexpress EphrinB2. This relationship, along with the low overall survival in patients with overexpression of both EphB4 and EphrinB2, supports that the bidirectional signaling taking place between EphB4 and EphrinB2 has a significant effect on overall patient survival. Given that EphB4 is overexpressed in HNSCC, increases tumor cell lifespan, and is associated with poor overall survival along with EphrinB2, EphB4 and EphrinB2 are ideal targets for therapy in HNSCC and potential biomarkers for predicting prognosis.

Previously, our laboratory has shown an important relationship between EGFR and HNSCC.16 Epidermal growth factor receptor is also overexpressed in HNSCC and was found to activate EphB4 and to increase its expression. Activated EGFR was shown to induce EphB4 via activation of Akt in HNSCC.16 Furthermore, patients with HNSCC who had high EGFR expression had reduced survival.25 These data suggest that poor survival in patients with elevated EGFR may be related to induction and activation of EphB4.

The prognostic significance of EphB4 has been studied in several other tumors. EphB4 has been correlated with a more invasive and metastatic phenotype in prostate cancer.14 In ovarian cancer and breast cancer, EphB4 has been shown to increase tumor cell life, and in ovarian cancer it has been associated with poor patient survival.13,26 Others have studied the expression of EphB4 and EphrinB2 in uterine endometrial cancer.27 As we have seen in HNSCC, Alam et al27 reported elevated expression of EphB4 and EphrinB2 in uterine endometrial cancer and demonstrated that this overexpression is associated with reduced survival.

Receptor tyrosine kinases have recently become important targets in malignant neoplasms. There are a multitude of pathways that lead to RTK dysregulation, which ultimately promotes the development of cancer and tumor growth. These pathways range from mutations in the RTK genes to aberrant overexpression of the RTK or its ligand.28 Although there are many important factors involved in the development of HNSCC, we propose that the RTK EphB4 and its ligand EphrinB2 have an important role in the progression and prognosis of HNSCC.

Head and neck squamous cell carcinoma is a debilitating disease that diminishes quality of life and results in a poor outcome in most patients. The importance of investigation of the underlying molecular mechanisms responsible for the development and progression of HNSCC cannot be overlooked. The expression of EphB4 has been previously shown to be elevated in HNSCC.16,17 In this study, we show that EphrinB2 is also overexpressed. In addition, we demonstrate that elevated EphB4 and EphrinB2 expression predicts poor overall outcome in patients with HNSCC. These data suggest that EphB4 and EphrinB2 are prime targets for clinical therapy and may be used as biomarkers in patients with HNSCC.

Submitted for Publication: May 18, 2007; final revision received September 29, 2007; accepted November 13, 2007.

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Author Contributions: Drs Yavrouian and Masood had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Sinha, Rice, Gill, and Masood. Acquisition of data: Yavrouian and Masood. Analysis and interpretation of data: Salam and Masood. Drafting of the manuscript: Yavrouian, Gill, and Masood. Critical revision of the manuscript for important intellectual content: Sinha, Rice, Salam, and Masood. Statistical analysis: Yavrouian and Salam. Administrative, technical, and material support: Masood. Study supervision: Sinha, Rice, and Masood.

Financial Disclosure: None reported.

Funding/Support: This study was supported by funds from the Department of Otolaryngology–Head and Neck Surgery, University of Southern California.

Additional Contributions: Susan McDonald collected patient data, and Donna Hoffman, MA, provided editorial assistance.

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