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The Role of EphB4 and EphrinB2 in Head and Neck Squamous Cell Carcinoma

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the most common cancer arising in the upper aerodigestive tract. It is an epithelial tumor most commonly affecting the oral cavity, hypopharynx, and larynx. HNSCC is the fifth most common cancer worldwide with approximately 900,000 cases yearly worldwide. In the United States, there were approximately 36,000 cases in 2010 and 8,000 deaths. Men are at significantly greater risk with tobacco and alcohol consumption the most important etiologic risk factors.

The main treatment modality for HNSCC has traditionally been surgery and postoperative radiotherapy. However, over the past 30 years, no significant change has been made in treatment strategy with minimal improvement in survival. Overall survival at five years ranges from 70-85% for patients presenting with early-stage disease (stage I and II) to 30-40% for advanced-stage disease (stage III and IV).

2. Pathogenesis of head and neck cancer

Mutations in specific genes and alteration of their expression lead to neoplasia in the head and neck. The development of HNSCC is a multi-step process with sequential mutations in genes responsible for tumor surveillance. A microsatellite analysis of allelic alterations showed that with the accumulation of genetic mutations, one can follow the transformation of cells from simple squamous hyperplasia to severe dysplasia, and, ultimately, invasive squamous cell carcinoma. These changes include mutation of the p53 tumor suppressor, overexpression of epidermal growth factor receptor (EGFR), and inactivation of the cyclin dependent kinase inhibitor p16. Other changes such as Rb mutation, ras activation, cyclin D amplification, and myc overexpression are less frequent in HNSCC. There is also an alteration in those genes which control DNA repair, proliferation, immortalization, apoptosis, invasion, and angiogenesis in HNSCC.

The p53 gene is believed to be the most frequently mutated tumor suppressor in human cancer. p53 has been implicated in the early pathogenesis of HNSCC, as it controls cell growth through regulation of the cell-cycle and apoptosis. The p53 null keratinocytes possessing an activated ras oncogene proliferate at a higher rate than those expressing the tumor suppressor.

In a study by Kashiwazaki et al in HNSCC, 79% of cancers and 36% of dysplastic lesions were shown to have p53 mutations. Hyperplastic lesions were negative for p53 mutations in this study. A higher incidence of p53 mutations have been detected in invasive carcinomas (75%) than in non-invasive cancers (35%). p53 mutations were not detected in normal mucosal cells. This study also detected sequential mutations of different exons which suggested accumulation of alterations during neoplastic transformation. The incidence of p53 mutations correlated with the degree of dysplasia with significantly higher numbers found in smokers. In agreement with these studies, dysplastic lesions in non-smokers infrequently contained p53 mutations. These results indicate that p53 mutation and inactivation is an early event in head and neck tumorigenesis.

In addition to p53, alterations in the retinoblastoma (Rb) gene are involved in the pathogenesis of HNSCC. The Rb protein is also a tumor suppressor pathway. $p16^{INK4A}$, a major target of the Rb pathway is inhibited through a variety of pathways including loss of heterozygosity (LOH) of chromosome 9p21, where it is located. LOH of 9p21 is seen in 80% of malignant lesions.

In addition to the p53 and Rb genes, sphingosine kinase (SphK) has been implicated in HNSCC. SphK regulates levels of ceramide, sphingosine, and sphingosine-1-phosphate, influencing cells to enter proliferative states. SphK1, a SphK isozyme is upregulated in HNSCC with overexpression in recurrent and advanced stage tumors. Use of small molecular inhibitors or siRNA's targeting SphK1 sensitizes cells both in in vitro and in vivo studies leads to radiation induced cell death. As a cell cycle regulator overexpressed in HNSCC patients, SphK1 plays a significant role in the pathogenesis of HNSCC.

The Epidermal Growth Factor Receptor (EGFR) is of the most studied biomarkers in HNSCC. EFGR is a receptor tyrosine kinase that effects cell growth, angiogenesis, and invasion. The epidermal growth factor receptor gene encodes a transmembrane receptor for EGF and transforming growth factor (TGF)-α. Ligand binding to the extracellular domain induces receptor dimerization and activation of the cytoplasmic tyrosine kinase. Many epithelial cancers including that of the head and neck overexpress EGFR, its ligands, or both. EGFR has been detected in the basal layer of normal oropharyngeal mucosa. All cells from dysplastic head and neck lesions stain for EGFR as do the majority of carcinomas. Almost all cells in poorly differentiated head and neck tumors were positive for the receptor. Amplification of the EGFR gene has been demonstrated in cultured cells and tissues.

EGFR overexpression may result in constitutive activity of the kinase domain and consequently increase downstream signaling such as that of the mitogen activated protein kinase pathway. The tyrosine kinase activity of the receptor results in autophosphorylation and recruitment of a variety of intracellular signaling proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. This recruitment provides a means of assembling the complexes required for receptor signaling. Proteins such as Grb2 and Shc, which contain SH2 and SH3 domains, mediate interactions with signal transduction proteins linking EGFR with the ras/mitogen activated protein kinase (MAPK) pathway. Ras also interacts with many proteins such as raf and phosphatidylinositol 3-kinase (PI3-K) to simulate downstream effectors such as MEK and ERK. These MAPKs are translocated to the

nucleus where they activate a number of transcription factors which control cellular proliferation, migration, and differentiation.

With devastating effects on communication, swallowing, and most importantly, survival, new biomarkers and targeted therapies are needed to improve detection, treatment, and survival. Potential targeted therapies may be found in factors that regulate angiogenesis. Angiogenesis plays an important role in both tumor growth and metastasis. Tumors are unlikely to grow beyond 3mm without the growth of new vessels. Receptor tyrosine kinases (RTKs) have emerged as important molecules in the regulation of angiogenesis.

Abnormal RTK expression is characteristic of most human cancers. There are three families of receptor tyrosine kinases and their ligands important in vascular development, including the vascular endothelial growth factor receptor (VEGF) family, the angiopoietin family, and the ephrins and the Eph receptors. Of the three receptor tyrosine kinase families above, VEGF is the most extensively studied. VEGF has been shown to be overexppressed in tumor compared to normal cells. VEGF overexpression is associated with a 1.88 fold increased risk of death and is also shown to be associated with lymph node metastasis. This chapter focuses on the expression of EphB4 and EphrinB2 in HNSCC and possible therapeutic applications to reduce tumor burden and improve survival.

3. The Eph receptors and their ligands the ephrins

The Eph receptors (erythropoietin-producing human hepatocellcular carcinoma) form the largest family of RTKs. In this group of proteins, there are 15 members divided into EphA and EphB classes. The EphA subclass is tethered to the cell membrane by glycosyl phosphatidylinositol, and the EphB subclass has a transmembrane domain that is followed by a short cytoplasmic region.

Eph receptors have an extracellular domain composed of the ligand-binding globular domain, a cysteine rich region followed by a pair of fibronectin type III repeats. The cytoplasmic domain consists of a juxtamembrane region containing two conserved tyrosine residues, a protein tyrosine kinase domain, a sterile α-motif (SAM), and a PDZ-domain binding motif.

The ephrins (Eph family receptor interacting proteins) are the ligands for the Eph receptors, with 13 members, also divided into classes A and B. Class B Ephrins have a transmembrane domain and cytoplasmic region with five conserved tyrosine residues and a PDZ domain. EphrinB2 is the exclusive ligand for EphB4. EphB4 is normally expressed on venous endothelial cells while EphrinB2 on arterial endothelial cells. In contrast, the A class ligands have a glycosylphosphatidylinositol membrane anchor.

Eph receptors are activated by binding of clustered, membrane attached ephrins indicating that contact between cells expressing the receptors and cells expressing the ligands is required for Eph activation. A corollary of this is that soluble ligands would act as inhibitors of Eph activation. Ligand binding to the Eph receptor autophosphorylates the juxtamembrane tyrosine residues to acquire full activation. Specificity of the ligand to its receptor is mediated by the N-terminal domain of the receptor. The interactions between the Eph receptors and their ligands form a bi-directional signaling pathway with forward Eph receptor signaling and reverse ephrin signaling (Figure 1).

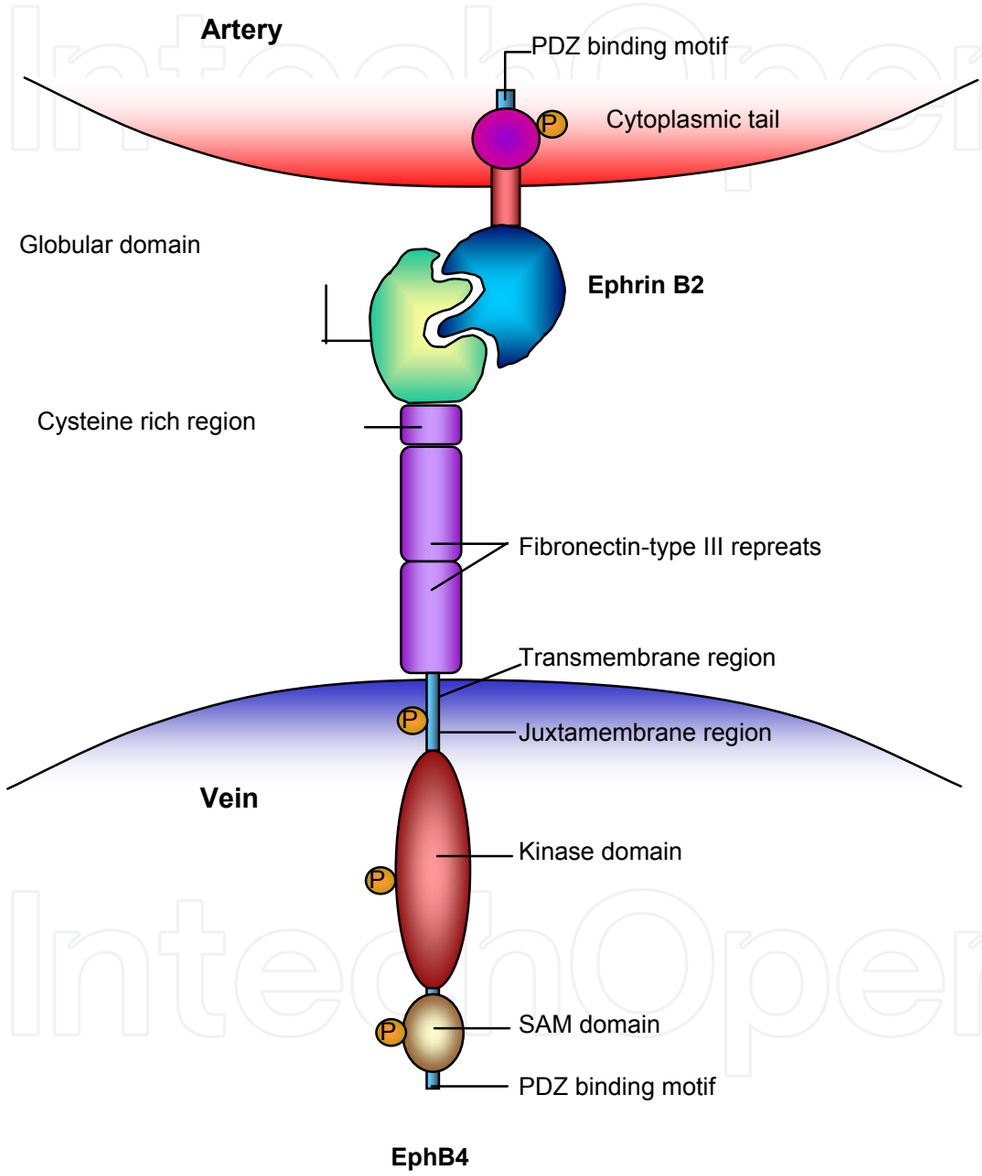


Fig. 1. Bidirectional signaling between EphB4 and EphrinB2.

When activated, EphB4 and EphrinB2 become phosphorylated, forming complexes with other proteins, and affect downstream signaling. Reverse signaling is initiated through recruitment of Src-family kinases followed by phosphorylation of ephrin B proteins. Evidence suggests that the Eph/ephrin interaction influences and is influenced in turn by other signaling pathways. The endothelial-specific receptor Tie-2 can directly phosphorylate ephrin cytoplasmic domains while EphrinB1 is phosphorylated by the PDGF receptor, and inhibits PDGF induced focus formation. Similarly, EphrinB2 inhibits VEGF signaling and the proliferation and migration of endothelial cells. The ephrins have also been shown to couple to GPCRs, such as the chemokine receptor CXCR4, via the PDZ linking proteins and a ternary complex involving the extracellular domains of EphrinB1, EphB2, and the 7-transmembrane GPCR subunit of the NMDA glutamate receptor has also been demonstrated.

The Eph receptor/ephrin system has been shown to play a role in several biologic processes. These processes include embryonic development, cell migration and aggregation, segmentation, pattern recognition, neural development, angiogenesis, vascular network development, and immune regulation. Recently, a role for these proteins has emerged in cancer.

Several studies have demonstrated that the Eph receptor/ephrin system plays a role in tumorigenesis. Dodolet et al and Wimmer-Kleikamp et al have shown involvement of the Eph receptor/ephrin system in angiogenesis, invasion, and tumor metastasis. There is also evidence that elevated expression of the Eph/Ephrin system correlates with increased invasiveness in tumors including malignant melanoma, ovarian carcinoma, breast cancer, kidney carcinoma, neuroblastoma, and prostate cancer. More specifically, elevated EphB4 expression has been shown in hematologic, breast, endometrial, prostate, bladder, ovarian, and colon cancers as well as malignant mesothelioma.

EphB4 activation has been shown to increase proliferation and survival of microvascular endothelial cells through increased phosphatidylinositol 3-kinase activity and phosphorylation of mitogen-activated protein kinase (MAPK) and protein kinase B (Akt). EphB4's involvement in cell migration and invasion is associated with EphB4 induction of MMP2 and MMP9, thus demonstrating a role for EphB4 in tumor metastasis.

4. Expression of EphB4 and EphrinB2 in HNSCC

As demonstrated in many other tumors, EphB4 is overexpressed in HNSCC. Through *in situ* hybridization, western blot analysis, and immunofluorescence of HNSCC tumor samples, EphB4 expression was found to be elevated in tumor tissue compared to normal adjacent tissue. In addition, EphB4 was overexpressed in metastatic lymph nodes (Figure 2). Furthermore, EphB4 overexpression correlated with advanced tumor stage (stage III or IV) and lymph node metastasis with stage III and IV tumors having 2.8 and 5.5-fold overexpression respectively compared to adjacent normal tissue.

Lymph nodes positive with tumor had 7.8-fold higher expression compared to normal adjacent tissue. In contrast, in patients with early-stage disease (stage I or II), EphB4 overexpression was 2.1-fold greater in tumor compared to adjacent normal tissue. Using

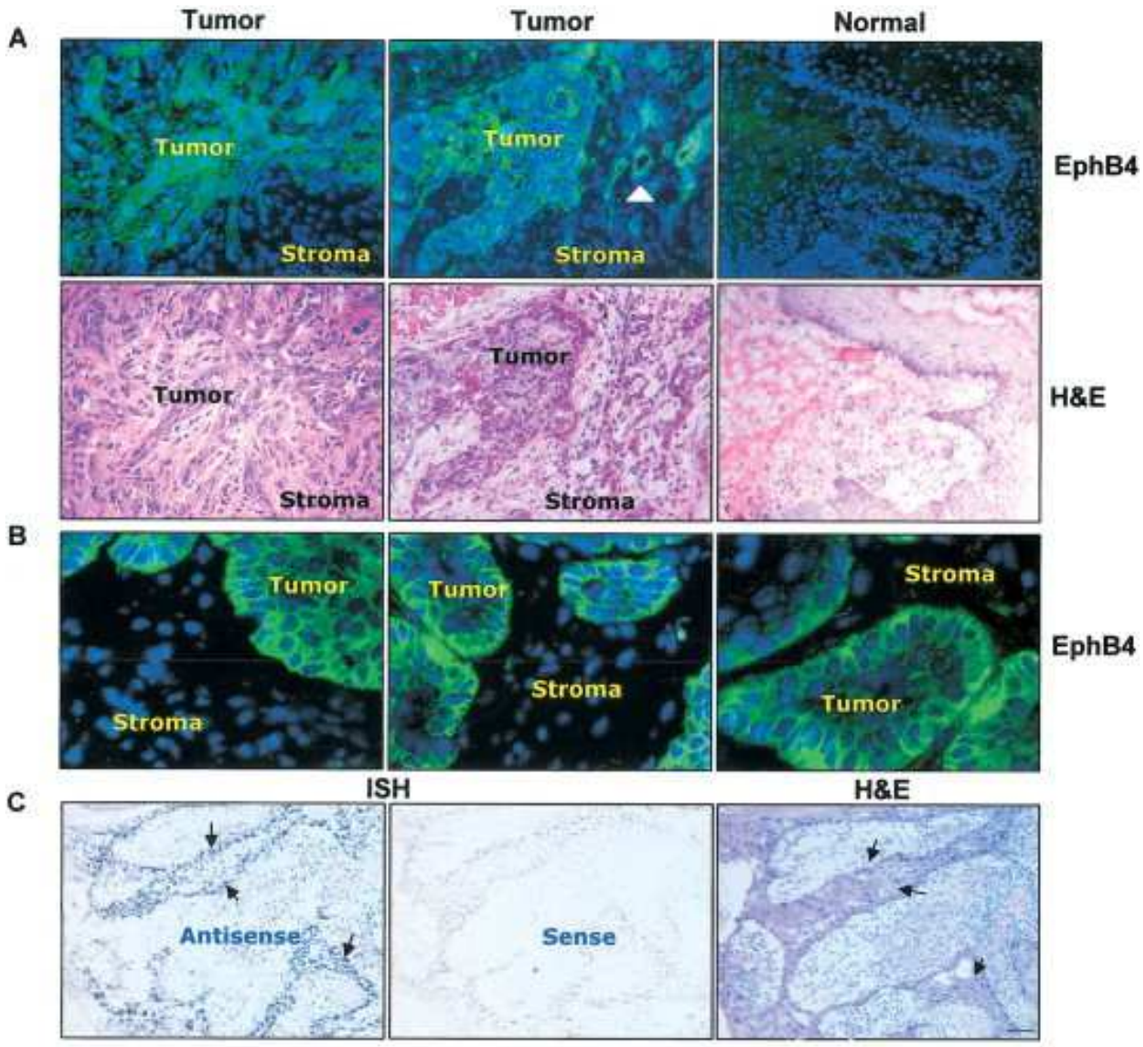


Fig. 2. EphB4 is expressed in HNSCC primary tissues and metastases. (*a*) Top panel: Immunofluorescence of representative fresh frozen sections of tumors (left and middle panels) or adjacent normal tissue (right panel) stained with EphB4-specific monoclonal antibody and visualized with FITC (green color). Sections were counter-stained with DAPI to identify cell nuclei. Bottom panel: Hematoxylin and Eosin (H&E) staining of the next serial section. Arrowhead in middle panel shows a vessel staining positive for EphB4. (*b*) Representative high power photomicrographs of tumor sections stained for EphB4 to document tumor cell membrane-specific expression. (*c*) *In situ* hybridization (ISH) of representative tumor sections with EphB4-specific antisense or sense probe. Arrows show positive signal for mRNA. H&E stain of the next section is shown in the right panel. Arrows indicate regions of the tumor.

quantitative PCR at the EphB4 gene locus, 30% of patients were found to have gene amplification of EphB4 with at least four copies of the gene locus. In a study of 42 patients with HNSCC, EphrinB2 expression was also analyzed with western blot analysis. EphrinB2 was found to be overexpressed in HNSCC tumor samples with an average overexpression of 2.2-fold greater when compared to normal adjacent tissue. Therefore, both EphB4 and EphrinB2 have been shown to be overexpressed in HNSCC.

5. HNSCC risk factors and EphB4 expression

The two main risk factors for HNSCC are alcohol and smoking. Studies have shown that tobacco use can lead to a 20-fold increased risk of HNSCC. Tobacco related substances can alter the genes and growth factors associated with HNSCC and can affect the genomic stability and extracellular environment in HNSCC. The expression of EphB4 in the oral mucosa of smokers without HNSCC was analyzed and results showed no expression of EphB4. However, in patients with HNSCC, EphB4 expression in tumor specimens in nonsmokers was compared to that of patients with a smoking history. There was a significantly increased expression of EphB4 in tumor samples from patients with a smoking history compared to nonsmokers, with a 3.8-fold overexpression of EphB4 in smokers compared to a 2.1 fold overexpression in nonsmokers. Therefore, tobacco-related substances may induce signaling changes that increase and activate EphB4 leading to changes in angiogenesis and tumor growth.

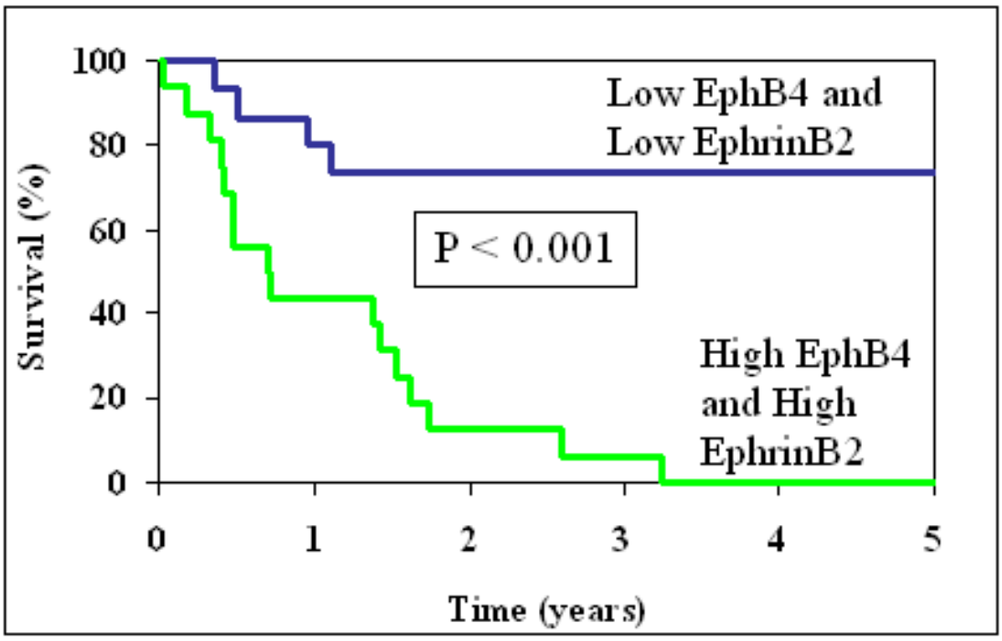


Fig. 3. Kaplan-Meier Curve for Overall Survival in Patients with Elevated Expression of EphB4 and EphrinB2.

In addition to smoking status, the effect of alcohol intake on EphB4 expression was also assessed in patients with HNSCC. Unlike with smoking status, EphB4 expression was not altered by a history of alcohol consumption. This is likely related to differing mechanisms of toxin induced carcinogenesis between alcohol and smoking.

6. EphB4 and EphrinB2 expression and survival

As increasing EphB4/EphrinB2 system expression is associated with advanced tumor stage and lymph node metastasis, the effect of EphB4 and EphrinB2 overexpression on survival was also studied. Patients who had high expression of EphB4 and EphrinB2 were compared to patients with low expression of EphB4 and EphrinB2. Those with high expression of EphB4 had a 5 year survival of 15% compared to 64% in patients with low EphB4 expression. Patients with elevated EphrinB2 expression had a 5 year survival of 9% compared to 79% in patients with low EphrinB2 expression.

In patients with elevated EphB4 and EphrinB2 expression, 5 year survival was 0% compared to 73% in patients with low EphB4 and low EphrinB2 expression (Figure 3). Therefore, elevated EphB4 and EphrinB2 expression is a significant predictor of poorer overall survival, even after adjusting for confounders including age, sex, race, stage, site of tumor, and mode of treatment. As all patients with high EphB4 and EphrinB2 expression died; this suggests a synergistic role between EphB4 and EphrinB2 in HNSCC.

7. Inhibition of EphB4 and tumor cell survival

EphB4 overexpression is associated with a worse overall survival in HNSCC; therefore, its inhibition in tumor cells is an important step to understanding possible therapeutic opportunities. Using small interfering RNA (siRNA) against the EphB4 sequence, which ablates EphB4 expression, results in a significant decrease in HNSCC tumor cells. In the presence of epidermal growth factor (EGF), which has been shown to induce EphB4 expression, inhibition with siRNA against EphB4 also led to a decrease in tumor cells (Figure 4). The population of cells exposed to the siRNA against EphB4 was found to accumulate in the sub-G0 phase, suggestive of apoptosis. EphB4 was shown to provide a survival advantage to cells by inhibition of apoptotic pathways. Inhibition of EphB4 in a murine HNSCC model showed a reduction in tumor growth. Its knockdown leads to an activation of capase-8 and subsequent cell death by apoptosis. Therefore, EphB4 expression in HNSCC provides a survival advantage to tumors cells and is an important potential biomarker whose inhibition may improve survival.

8. Therapeutic applications

Tumor biomarkers provide an opportunity with which one can improve early detection of tumor, monitoring, and treatment, and ultimately improve survival. Recently, several new biomarkers have emerged and are currently being studied for their effectiveness in HNSCC detection, prognosis, and treatment. One such molecule is cetuximab, a monoclonal antibody against the epidermal growth factor receptor. It is one of the most successful targeted therapies in HNSCC with a phase III clinical trial showing cetuximab in

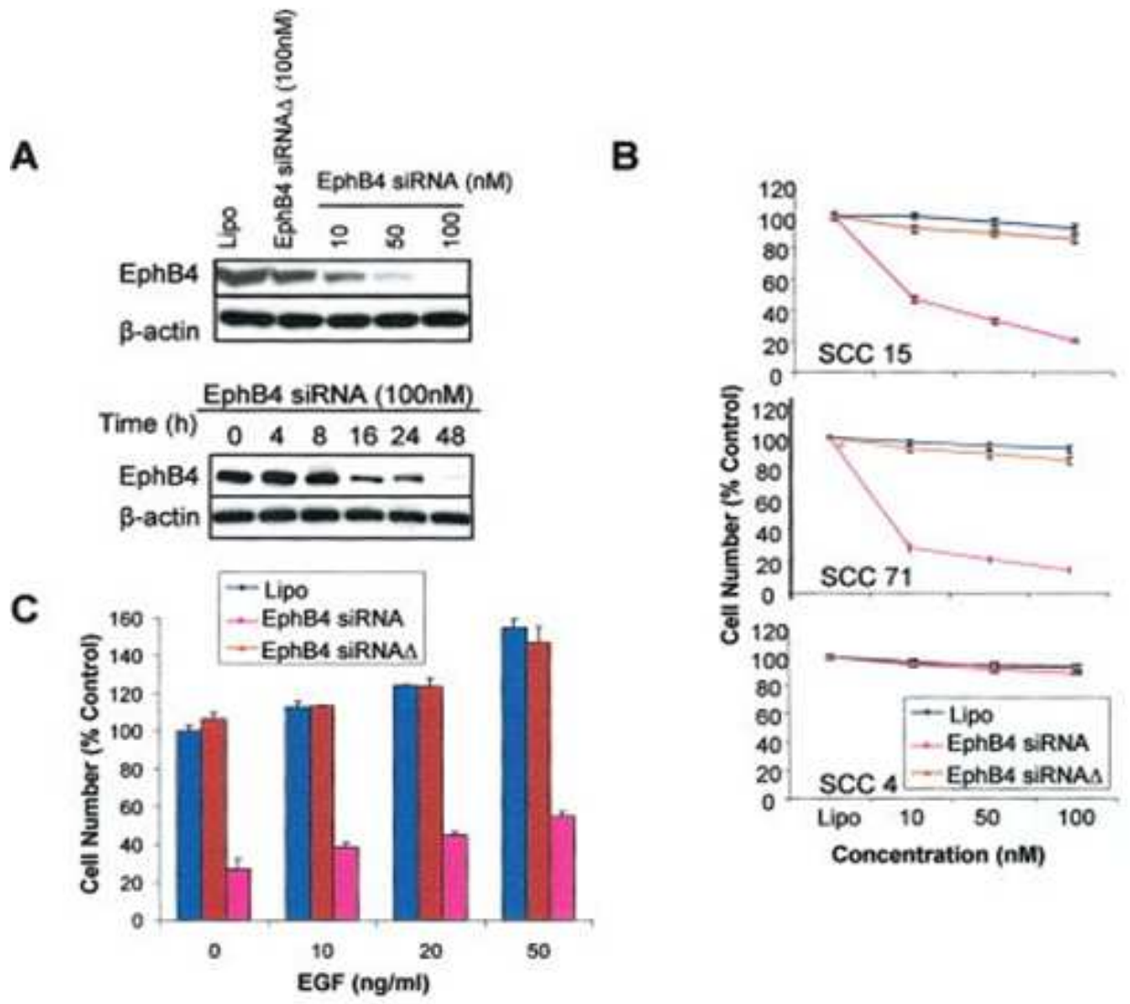


Fig. 4. Ablation of EphB4 in HNSCC cells lines results in reduction in cell numbers and inhibition of tumor cell migration/invasion. (*a*) Potent EphB4-specific siRNA chosen from their ability to block EphB4 was transfected at various concentrations into SCC-15 cells. A mutant siRNA (EphB4 siRNAΔ) with three base substitutions was used as negative control. Extracts of treated cells were analyzed by Western blotting to detect EphB4 and β-actin (upper panel). SCC-15 cells were transfected with 100 nM EphB4 siRNA and EphB4 expression analyzed at various time points (lower panel). (*b*) MTT cell number assays of EphB4-positive SCC cell lines (SCC-15 and -71) and an EphB4-negative cell line (SCC-4). Cell number was tested 48 hr following treatment with lipofectamine alone (Lipo), EphB4-specific siRNA (EphB4 siRNA) or mutant siRNA (EphB4 siRNAΔ). Data shown is mean ± SEM of triplicate samples. (*c*) MTT cell number assays of SCC-15 cells following treatment with increasing doses of EGF and lipofectamine alone (Lipo), EphB4-specific siRNA (EphB4 siRNA) or mutant siRNA (EphB4 siRNAΔ). Data shown is mean ± SEM of triplicate samples.

combination with radiotherapy provided an overall survival benefit of an additional 20 months compared to radiation alone. Downstream EGFR signaling activates the MAPK pathway as well as the PI3-K/Akt pathway. Signaling through the PI3-K/Akt pathway ultimately leads to inhibition of the tumor suppressor gene p53. EGFR has been shown to regulate EphB4 expression (Figure 5). EGFR signaling through the Akt pathway induces EphB4. Inhibition of EGFR through antibodies such as cetuximab may also downregulate EphB4 through Akt. Potentially, some of the survival benefit of cetuximab is achieved through EphB4 inhibition.

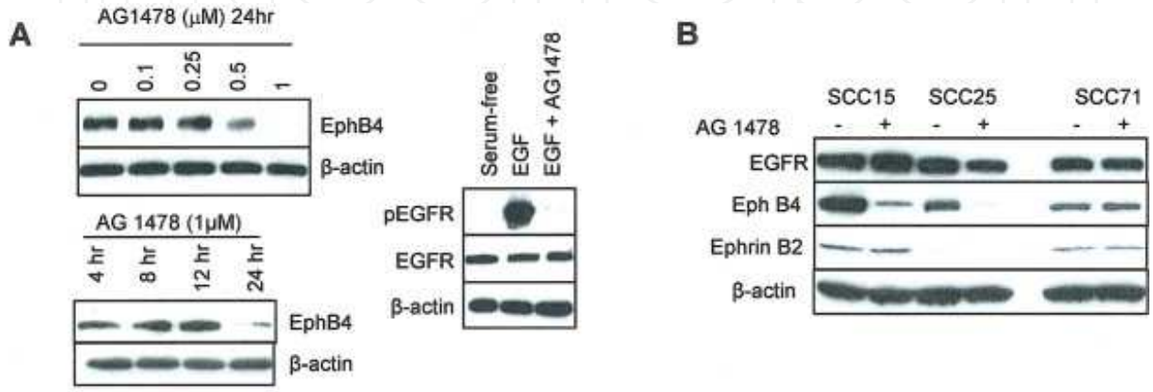


Fig. 5. Regulation of EphB4 expression by EGFR signaling pathway. (*a*) EGFR kinase inhibitor AG1478 was tested in SCC-15 for optimal dose (left upper panel) and time (left lower panel) for inhibition of EphB4 expression by Western blot of whole cell lysates. Equal loading of protein in each lane is shown by β-actin levels. Inhibition of EGFR activation by EGF in the presence of AG1478 (1 μM) is shown in right panel. (*b*) Western blot analysis of SCC-15, -25, and -71 cell lines for regulation of EphB4, EGFR and EphrinB2 in response to AG1478. Serial stripping and probing for various proteins was performed from the same blot.

Monoclonal antibodies to EphB4 have not been applied clinically as of yet, however, their development is crucial to improve survival in HNSCC. Xu et al have developed a humanized version of a mouse monoclonal antibody to EphB4 that binds the human EphB4 receptor. Krasnoperov et al have also developed two anti-ephB4 monoclonal antibodies targeting different EphB4 domains. These antibodies have yet to be tested in humans. Bardelle el al have demonstrated a non-benxodioxole inhibitor of EphB4 that may have applications in vivo but is also yet to be studied further.

In addition to the direct inhibition of the EphB4, several receptor tyrosine kinase inhibitors are being studied. These may also inhibit EphB4 function. Sunitinib, sorafenib, vandetanib, semaxanib, and foretinib are small molecule tyrosine kinase inhibitors currently being studied in phase II clinical trials. Machiels et al reviewed Sunitinib in a phase II clinical trial of 38 HNSCC patients in which it was given as a palliative treatment achieving a disease control rate of 50%. Due to several complications that occurred including bleeding, skin ulceration, and fistulas, they recommended further study of the drug to assess which patients would benefit. In recurrent/metastatic HNSCC and nasopharyngeal carcinoma,

Sorafenib's effect was studied and a response rate of 3.7% was achieved. As a multikinase inhibitor, its effect cannot be attributed only to its anti-angiogenic activity. As a single agent, Semaxanib was also studied in HNSCC, but was discontinued due to several adverse affects and its difficulty with administration. Given the potential improvement in survival with EphB4 inhibition in HNSCC, new therapies targeting EphB4 are essential and further investigation is necessary.

9. Conclusion

Head and neck squamous cell carcinoma is the most common cancer of the head and neck with devastating effects on communication, swallowing, quality of life, and, most importantly, survival. The Eph receptor family and its ligands, the ephrins, specifically EphB4 and EphrinB2, have an important role in many physiologic processes including cell aggregation and migration, angiogenesis, and vascular network development.

EphB4 and its sole ligand EphrinB2 are overexpressed in all HNSCC patients, with EphB4 overexpression correlating with advanced stage disease and lymph node metastasis. In vivo, EphB4 has also been demonstrated to provide a survival advantage to tumor cells, and, its inhibition has been shown to decrease the survival of the HNSCC tumor cells. Furthermore, EphB4/EphrinB2 overexpression is associated with a significantly poorer overall survival. Given that EphB4 and EphrinB2 are overexpressed in HNSCC and that this is associated with worse overall survival, EphB4 and EphrinB2 are potentially useful biomarkers that may provide another target for HNSCC treatment. While there are several investigators examining the therapeutic role of EphB4 inhibition in cancer, there is still a great deal of progress to be made to apply EphB4 and EphrinB2 inhibition in head and neck squamous cell carcinoma treatment.

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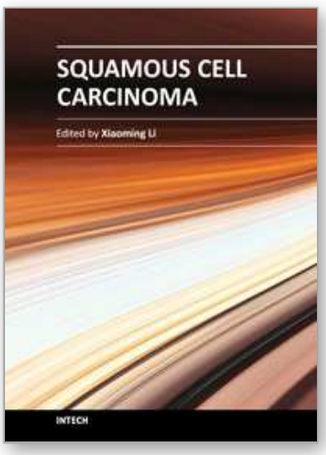
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This book points to some new areas for investigation on squamous cell carcinoma (SCC). Firstly, the features and management of some specific SCC is discussed to give the readers the general principles in dealing with these uncommon and sophisticated conditions. Some new concepts in adjuvant therapy including neoadjuvant therapy and gold nanoparticle-based photo dynamic therapy are introduced. Secondly, a detailed discussion of molecular aspects of tumor invasion and progression in SCC is provided with the emphasis on the roles of some important factors. The role of tumor microenvironment in head and neck SCC is specifically discussed. Thirdly, the roles of cancer stem cells (CSC) in cancer therapy of SCC are described. Molecular mechanisms involving therapeutic resistance and new therapeutic strategies targeting CSC are discussed in detail. Finally, other aspects concerning SCC are included, which involve the assessment, genetic manipulation and its possible clinical implications for the treatment of SCC.

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