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Original Article

High expression of EphB6 protein in tongue squamous cell carcinoma is associated with a poor outcome

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Abstract: EphB6 is a member in the receptor tyrosine kinase Eph family in that its kinase domain contains several alterations in conserved amino acids and is catalytically inactive. Although EphB6 is expressed both in a variety of embryonic and adult tissues, biological functions of this receptor are largely unknown. In this study, we examined the expression of EphB6 protein in 54 of tissue specimens of tongue squamous cell carcinoma by using a specific polyclonal anti-EphB6 antibody. The relationship between expression of EphB6 and clinical pathologic parameters was analyzed. The expression level of EphB6 in carcinoma cells from 34 out of 54 (63%) specimens was no alterative compared with normal squamous cells in same patient. The level of EphB6 protein staining was increased in carcinoma cells in 20 out of 54 (37%) specimens compared with normal squamous cells in same patient. The high-expression of EphB6 was significantly associated with age (P=0.021), tumor TNM stage (P=0.026) and lymph node metastasis (P=0.046). Patients with high expressed EphB6 protein had a high mortality (P=0.057). No significant relationship between expression of EphB6 and sex, tumor grade, HPV infection, relapse and smoke was found. We showed that patients with high expression of EphB6 had a significantly poor overall survival (OS) compared to patients with negative or weak expression (P=0.042). Our results indicated that EphB6 protein may be used as a new marker for prognosis for tongue squamous cell carcinoma.

Keywords: EphB6, receptor tyrosine kinase, tongue squamous cell carcinoma

Introduction

Squamous cell carcinoma of the head and neck comprising tumors in the oral cavity, nasal cavity, pharynx and larynx, is among the 10 most common malignancies in the world. Tongue squamous cell carcinoma is a lethal disease estimated to have 45,780 new cases in 2015 in the United States [1]. It accounts for more than 90% of all head and neck cancers. Despite increasing knowledge about the development and great advances in multimodal therapies against tongue squamous cell carcinoma over the past decades, the molecular mechanisms behind tongue squamous cell carcinoma and the 5-year overall survival for patients is still only around 50%. One important reason for this is that tumors often are diagnosed at a late stage and thus have spread to cervical lymph nodes. Another reason is that the limited therapeutic options available. The standard of care for tongue squamous cell carcinoma used to be surgery and radiation. Several biomarkers have been studied in the search for new diagnostic and prognostic factors for tongue squamous cell carcinoma but none has so far turned out to be suitable for routine use in the clinic [2-5].

Erythropoietin producing hepatocellular carcinoma (Eph) receptors make up the largest family of receptor tyrosine kinases in humans with 14 members identified to date. These proteins are divided into A and B groups based on their homology to one another as well as their affinity for their ligands ephrins. There are some documented cases of cross talk between receptor and ligand families. Eph receptors are involved in the development such as hindbrain patterning, axon guidance and angiogenesis [6-8]. The Eph receptors have also been implicated in several cancers. EphB6 is a kinase-dead receptor tyrosine kinase in that its kinase domain contains several alterations in conserved amino acids and is catalytically inactive. EphB6 is lost

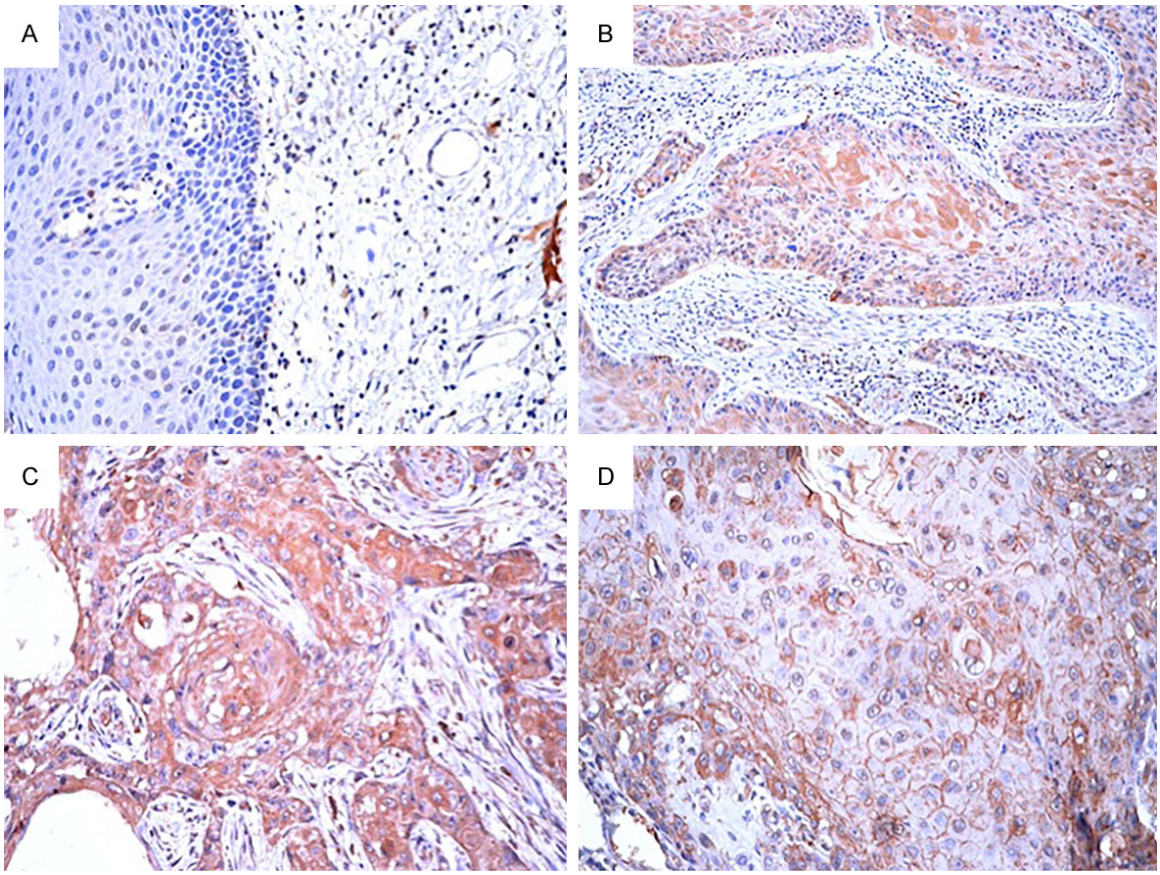

Figure 1. The expression of EphB6 protein was detected in tongue squamous cell carcinoma. A: Negative staining of EphB6 in normal tissue; B: Moderate expression of EphB6 in carcinoma; C: Strong positive expression of EphB6 in carcinoma; D: Cellular membrane positive expression of EphB6 in carcinoma.

in more aggressive breast cancer, melanomas, and neuroblastomas [9-13]. It has been previously shown that down-regulated of EphB6 is related to epigenetic silencing of this gene by promoter methylation [11, 14]. We previously investigated expression of EphB6 protein in colorectal cancers and colorectal adenomas. We found that decreased expression of EphB6 was found in colorectal cancers as compared with adenoma and normal tissues. EphB6 protein expression is associated with a shorter mean duration of survival in colorectal cancer [15]. Although the expression profile of EphB6 was documented in certain human cancers, no data can be found about expression level of EphB6 protein in Tongue squamous cell carcinoma, and the clinical relationship between the expression of EphB6 and clinical pathologic parameters. In this study, we examined the expression of EphB6 protein in 54 of tissue specimens of tongue squamous cell carcinoma by using a specific polyclonal anti-EphB6 antibody. The relationship between expression of EphB6 and clinical pathologic parameters was analyzed.

Materials and methods

Patients and clinicopathological variables

The study cohort consisted of 54 patients with tongue squamous cell carcinoma (age range 27-69 years, mean age 49 years), who underwent surgery from 2002 to 2011 in Institute and Hospital of Stomatology, Nanjing University Medical School, China. All hematoxylin and eosin stained slides were reviewed by two pathologists to verify the diagnosis, histological grade and stage.

54 patients with tongue squamous cell carcinoma were followed up until April 2011. The records of patients who were alive at follow-up or who did not die of disease were considered to be censored. None of the patients received preoperative chemotherapy or radiation therapy. This investigation was performed following

Table 1. Expression of EphB6 and its association with clinical pathologic parameters

		No difference	Up regulation	*P* value
		34	20	
Age (years)				
	<50	9	12	0.021
	≥50	25	8	
Sex				
	male	15	11	0.574
	female	19	9	
TNM stage				
	I+II	23	7	0.026
	III+VI	11	13	
Grade				
	I	12	7	1
	II	21	12	
	III	1	1	
Lymph node metastasis				
	yes	9	11	0.046
	no	25	9	
HPV infection				
	yes	12	8	0.772
	no	24	12	
Dead				
	yes	1	4	0.057
	no	33	16	
Relapse				
	yes	1	1	1
	no	33	19	
Smoke				
	yes	9	5	1
	no	25	15	

approval from the Ethics Committee of Institute and Hospital of Stomatology, Nanjing University Medical School, China. Informed consent was obtained from each patient.

Immunohistochemistry

Sections from surgical specimens were fixed in 10% formalin and embedded in paraffin and were used for immunohistochemical staining according to a standard method. Briefly, each 4-μm tissue section was deparaffinized and rehydrated. After rehydration through a graded ethanol series, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min for antigen retrieval, then cooled to 30°C and washed with phosphate-buffered saline (PBS, pH 7.3). After endogenous peroxidase had been quenched with aqueous 3% H_2O_2 for 10 min, the sections were washed with PBS, incubated at 4°C overnight with primary rabbit polyclonal anti-EphB6 antibody (Abgent, San Diego, CA, USA) at a dilution of 1:400 and then washed with PBS. The sections were incubated with secondary antibody (Dako REAL EnVision Detection System, Dako, UK) for 20 min at room temperature. This was followed by color development with 3, 3'-diaminobenzidine solution for 1 min and counterstaining with hematoxylin for 3 min. After counterstaining, the slides were washed with PBS, dehydrated, cleared in xylene, and mounted in neutral balsam. Primary antibody was replaced with antibody diluent for negative controls. The colon mucosa with known positivity was used as a positive external control. No reliable internal controls were available.

EphB6 staining was independently evaluated for immunoreactivity by two pathologists who were double-blinded to clinical data according to the scoring criteria. Immunoreactivity was determined according to the intensity of cytoplasmic staining. EphB6 expression was assessed by 4 staining intensities (0= no staining, 1= weak, 2= moderate, 3= strong). The level of EphB6 expression was assessed by comparing the score of the tumor cells to adjacent normal epithelial cells.

Statistical analysis

The Chi-square test (Fisher's exact test) was used to assess the associations between EphB6 protein expression and clinicopathological variables. *P*-values <0.05 (two-sided) were considered statistically significant. All analyses were performed by SPSS software (version 16.0, Chicago, IL, USA).

Results

EphB6 expression

The expression of EphB6 protein was determined in tongue squamous cell carcinoma by immunohistochemical staining. As shown in **Figure 1**, EphB6 staining was predominantly

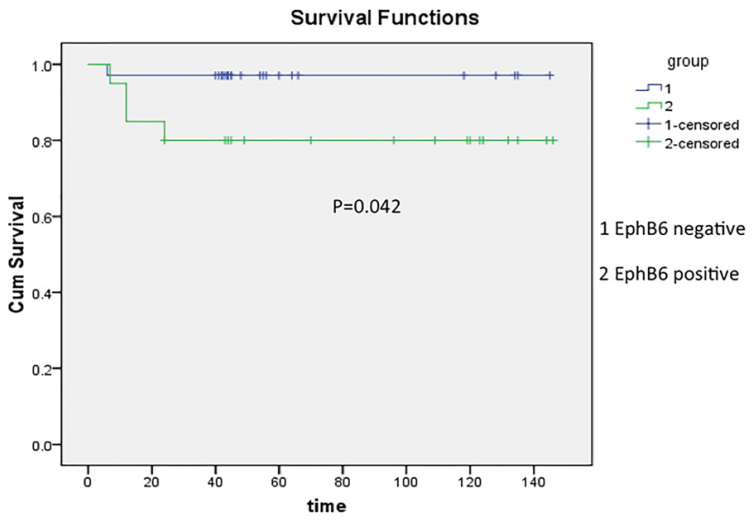

Figure 2. Patients with high expression of EphB6 had a significantly poor overall survival compared to patients with negative or weak expression.

localized in the cytoplasm and some in the cellular membrane. The expression level of EphB6 in carcinoma cells from 34 out of 54 (63%) specimens was no alterative compared with normal squamous cells in same patient (**Table 1**).

EphB6 expression in tongue squamous cell carcinoma and its correlation with clinicopathological features

The level of EphB6 protein staining was increased in carcinoma cells in 20 out of 54 (37%) specimens compared with normal squamous cells in same patient. The high-expression of EphB6 was significantly associated with age (P=0.021), tumor TNM stage (P=0.026) and lymph node metastasis (P=0.046). Patients with high expressed EphB6 protein had a high mortality (P=0.057). No significant relationship between expression of EphB6 and sex, tumor grade, HPV infection, relapse and smoke was found.

Association between EphB6 expression and overall survival in patients

Using the follow-up data of the 54 patients in conjunction with the results from the EphB6 IHC staining experiments, we showed that patients with high expression had a significantly poor overall survival (OS) compared to patients with negative or weak expression (P=0.042, **Figure 2**).

Discussion

The expression level of EphB6 was detected in several types of human cancers and the significance of EphB6 in progression, metastasis and prognosis was analyzed. Tang *et al.* detected the expression level of EphB6, Ephrin B2, and Ephrin B3 transcripts in human neuroblastomas [16]. They found that higher levels of EphB6, EphrinB2 and EphrinB3 expression were found in low-stage tumors than in advanced-stage tumors. Their data suggest that high level of EphB6 is associated with favorable neuroblastomaand is predictive of outcome of neuroblastom [17]. Fox *et al.* has demonstrated the loss of EphB6 protein in invasive breast carcinoma cell lines and absence of EphB6 transcript in a metastatic breast tumor specimen [10]. Yu *et al.* detected expression of EphB6 in normal lung tissues, NSCLC without metastasis and with metastasis and matched tumor-normal pairs of NSCLC patients [14]. They found that EphB6 mRNA and protein levels were significantly reduced in NSCLC tumors compared with matched normal lung tissues. Decreased EphB6 expression levels were associated with an increased risk for metastasis development in NSCLC patients. They demonstrated that loss of expression correlated with EphB6 hypermethylation.

The varied biological effects of the Eph receptors are mediated in part by the expression of these receptors and their intracellular binding proteins [9, 13, 18-20]. The ability of Eph molecules to form heterodimers within their own class has been suggested, although not exhaustively characterized. Fox *et al.* has clarified this phenomenon by showing that EphB6 can interact with EphA2 and EphB2 in mammalian cells [21]. They correlated relative expression of EphB6, EphB2 and EphA2 with non-invasive and invasive phenotypes of breast tumor cell lines. These data indicated that tumor invasiveness-suppression activity of EphB6 is mediated by its ability to sequester other kinase-sufficient and other oncogenic Eph receptors.

Till now, most studies show that EphB6 works as a tumor suppressor in cancers. However, in this study we found that the high-expression of EphB6 was significantly associated with advanced tumor TNM stage and lymph node metastasis. Patients with high expressed EphB6 protein had a high mortality. We postulate that the role of EphB6 in the carcinogenesis is variable between adenocarcinoma and squamous cell carcinoma. The loss of expression of EphB6 was frequently observed in adenocarcinoma including breast carcinoma, lung adenocarcinoma, and colorectal cancer. The expression and role of EphB6 were not intensively investigated in squamous cell cancers. The present study is the first time to describe expression level of EphB6 and its correlation with clinical pathological parameters in squamous cell carcinoma. Matsuoka *et al.* showed that EphB6 exerts biphasic function in adhesion and migration in response to different concentration of ligand, with a functional transition from promotion to inhibition [22]. In the present study, we did not detect the EphrinB2 and EphrinB3 ligands expression level in tongue squamous cell carcinoma and we should explore the relation between ligands and EphB6 receptor expression in the future.

In summary, we found that high-expression of EphB6 in tongue squamous cell carcinoma is associated with advanced tumor TNM stage, lymph node metastasis and poor outcome of patients.

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Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.

[2] Tsai CY, Lai YS and Chen MK. Prediction of lymph node metastasis in T1/T2 tongue squamous cell carcinoma. B-ENT 2014; 10: 179-184.

[3] Peng F, Zhang H, Du Y and Tan P. miR-23a promotes cisplatin chemoresistance and protects against cisplatin-induced apoptosis in tongue squamous cell carcinoma cells through Twist. Oncol Rep 2015; 33: 942-950.

[4] Cao J, Guo T, Dong Q, Zhang J and Li Y. miR-26b is downregulated in human tongue squamous cell carcinoma and regulates cell proliferation and metastasis through a COX-2-dependent mechanism. Oncol Rep 2015; 33: 974-980.

[5] Goineau A, Piot B, Malard O, Ferron C, Lisbona A, Cassagnau E, Delamazure AS, Campion L and Bardet E. Postoperative interstitial brachytherapy for resectable squamous cell carcinoma of the tongue. Brachytherapy 2015; 14: 71-76.

[6] Miao H, Burnett E, Kinch M, Simon E and Wang B. Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. Nat Cell Biol 2000; 2: 62-69.

[7] Naruse-Nakajima C, Asano M and Iwakura Y. Involvement of EphA2 in the formation of the tail notochord via interaction with ephrinA1. Mech Dev 2001; 102: 95-105.

[8] Duffy SL, Steiner KA, Tam PP and Boyd AW. Expression analysis of the Epha1 receptor tyrosine kinase and its high-affinity ligands Efna1 and Efna3 during early mouse development. Gene Expr Patterns 2006; 6: 719-723.

[9] Truitt L, Freywald T, DeCoteau J, Sharfe N and Freywald A. The EphB6 receptor cooperates with c-Cbl to regulate the behavior of breast cancer cells. Cancer Res 2010; 70: 1141-1153.

[10] Fox BP and Kandpal RP. EphB6 receptor significantly alters invasiveness and other phenotypic characteristics of human breast carcinoma cells. Oncogene 2009; 28: 1706-1713.

[11] Fox BP and Kandpal RP. Transcriptional silencing of EphB6 receptor tyrosine kinase in invasive breast carcinoma cells and detection of methylated promoter by methylation specific PCR. Biochem Biophys Res Commun 2006; 340: 268-276.

[12] Hafner C, Bataille F, Meyer S, Becker B, Roesch A, Landthaler M and Vogt T. Loss of EphB6 expression in metastatic melanoma. Int J Oncol 2003; 23: 1553-1559.

[13] Tang XX, Zhao H, Robinson ME, Cohen B, Cnaan A, London W, Cohn SL, Cheung NK, Brodeur GM, Evans AE and Ikegaki N. Implications of EPHB6, EFNB2, and EFNB3 expressions in human neuroblastoma. Proc Natl Acad Sci U S A 2000; 97: 10936-10941.

[14] Yu J, Bulk E, Ji P, Hascher A, Tang M, Metzger R, Marra A, Serve H, Berdel WE, Wiewroth R, Koschmieder S and Muller-Tidow C. The EPHB6 receptor tyrosine kinase is a metastasis suppressor that is frequently silenced by promoter DNA hypermethylation in non-small cell lung cancer. Clin Cancer Res 2010; 16: 2275-2283.

[15] Peng L, Tu P, Wang X, Shi S, Zhou X and Wang J. Loss of EphB6 protein expression in human colorectal cancer correlates with poor prognosis. J Mol Histol 2014; 45: 555-563.

[16] Tang XX, Evans AE, Zhao H, Cnaan A, London W, Cohn SL, Brodeur GM and Ikegaki N. High-level expression of EPHB6, EFNB2, and EFNB3 is associated with low tumor stage and high TrkA expression in human neuroblastomas. Clin Cancer Res 1999; 5: 1491-1496.

[17] Tang XX, Zhao H, Robinson ME, Cnaan A, London W, Cohn SL, Cheung NK, Brodeur GM, Evans AE and Ikegaki N. Prognostic significance of EPHB6, EFNB2, and EFNB3 expressions in neuroblastoma. Med Pediatr Oncol 2000; 35: 656-658.

[18] Yu J, Bulk E, Ji P, Hascher A, Koschmieder S, Berdel WE and Muller-Tidow C. The kinase defective EPHB6 receptor tyrosine kinase activates MAP kinase signaling in lung adenocarcinoma. Int J Oncol 2009; 35: 175-179.

[19] Freywald A, Sharfe N and Roifman CM. The kinase-null EphB6 receptor undergoes transphosphorylation in a complex with EphB1. J Biol Chem 2002; 277: 3823-3828.

[20] Munthe E, Rian E, Holien T, Rasmussen A, Levy FO and Aasheim H. Ephrin-B2 is a candidate ligand for the Eph receptor, EphB6. FEBS Lett 2000; 466: 169-174.

[21] Fox BP and Kandpal RP. A paradigm shift in EPH receptor interaction: biological relevance of EPHB6 interaction with EPHA2 and EPHB2 in breast carcinoma cell lines. Cancer Genomics Proteomics 2011; 8: 185-193.

[22] Matsuoka H, Obama H, Kelly ML, Matsui T and Nakamoto M. Biphasic functions of the kinase-defective Ephb6 receptor in cell adhesion and migration. J Biol Chem 2005; 280: 29355-29363.