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Original Article

The expressions of EphB4 and ephrinB2 in lung adenocarcinomas: a high level of the EphB4 protein is associated with lymph node metastasis

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Abstract: Eph tyrosine kinase receptors and their ephrin ligands are involved in normal development and tumorigenesis. Eph receptors are frequently over expressed in a wide variety of human malignant tumors, being associated with tumor growth, invasion, angiogenesis, and metastasis. This study aimed to evaluate the clinical significance of EphB4 and its ligand of ephrinB2 protein expressions in human lung adenocarcinomas. EphB4 and ephrinB2 protein expressions were assessed immunohistochemically on paraffin embedded tissues obtained from 93 patients with lung adenocarcinoma. Fifty-one out of 93 (54.8%) specimens were negative for EphB4 expression, and 42 out of 93 (45.2%) were positive for EphB4 expression. EphrinB2 expression was consistently negative in all tissues. EphB4 expression was significantly associated with tumor differentiation (P = 0.001), lymph node metastasis (P = 0.021), and Ki67 (P = 0.012). No significant relationship was found between EphB4 expression and gender, age, or ALK mutation. Our data show that EphB4 is differentially expressed in lung adenocarcinoma tissues. A high level of EphB4 protein expression was associated with lymph node metastasis in lung adenocarcinoma.

Keywords: EphB4, ephrinB2, lung adenocarcinoma, metastasis

Introduction

The erythroprotein-producing hepatoma amplified sequence (Eph) receptor tyrosine kinase (RTK) family is the largest family of RTKs, which includes 14 Eph receptors and 8 ephrin ligands. Eph receptors are divided into the A- or the B-type based on their interactions with ephrin ligands. The A class receptors preferentially bind A-type ligands, while the B class receptors bind B-type ligands. A special feature of Eph receptors and ephrins is the concept of bi-directional signaling [1]. The Eph/ephrin family is involved in developmental processes, particularly in the vascular and nervous systems [2-4]. Increasing evidence suggests that Eph receptors play roles in tumorigenesis, which has both tumor-promoting and tumor-suppressing activities depending on their expression pattern in different organs. Some of the Eph receptors are highly expressed in human cancers and act as an oncogene. For example, EphA2 is highly expressed in lung cancer and associated with brain metastasis and poor survival [5-7]. On the other hand, Eph receptors are down-regulated in certain cancers and function as a tumor-suppressor. EphA7 is down-regulated in colorectal cancer and prostate cancer with hypermethylation in the CpG island of the promoter region [8, 9].

EphB4, like all other Eph receptors, is a type I transmembrane protein that has prototypical RTK characteristics. The receptor protein includes an N-terminal multidomain extracellular region, a single transmembrane segment, and a cytoplasmic region. The EphB4 receptor is overexpressed in several tumor types, including colon cancer, prostate cancer, breast cancer, bladder cancer, and ovarian cancer [10-14]. The expressions of EphB4 and its ligand of ephrinB2 in lung adenocarcinoma have not

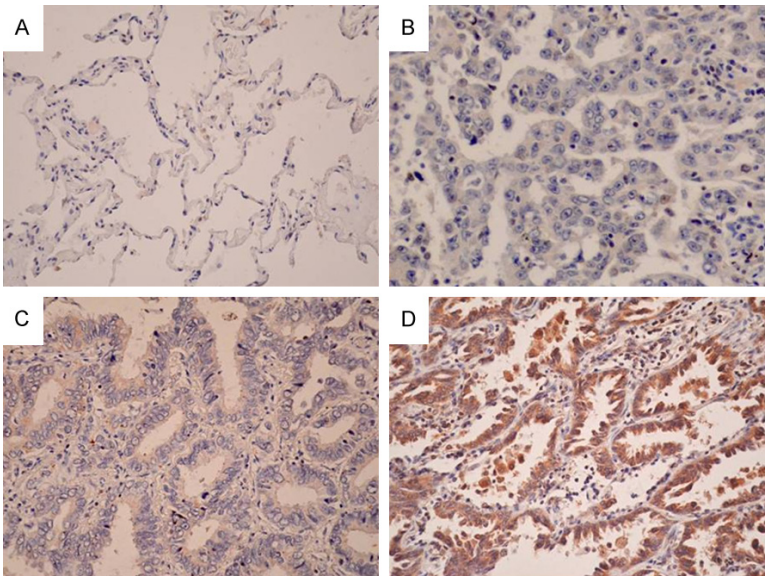

Figure 1. EphB4 and ephrinB2 expressions were immunohistochemically detected in lung adenocarcinoma tissues. A. Negative expression of EphB4 in normal lung cells. B. Negative expression of ephrinB2 in lung adenocarcinoma cells. C. Negative expression of EphB4 in lung adenocarcinoma cells. D. Positive expression of EphB4 in lung adenocarcinoma (×400).

been well investigated. In this study, we examined the EphB4 and ephrinB2 proteins in a set of lung adenocarcinoma tissues and statistically analyzed their associations with the patients' clinicopathological parameters.

Materials and methods

Patient and tissue samples

The study included 93 patients being treated for lung adenocarcinoma who underwent surgical resection at the Affiliated Jiangyin Hospital of Southeast University Medical College, China, from January to December 2014. The 93 patients included 39 men and 54 women with an average age of 60.7 years (median, 61 years; range, 32-90 years). This study was approved by institutional review board of the Affiliated Jiangyin Hospital of Southeast University Medical College. The histopathological classification of the specimens was done based on both the 2004 WHO classification system and the new adenocarcinoma classification by IASLC/ATS/ERS.

Immunohistochemical staining of EphB4, ephrinB2, Ki67, and ALK

Tissue specimens were fixed in phosphate-buffered 4% formaldehyde, paraffin embedded and processed for routine diagnosis. Briefly, the sections were baked at 65°C for 1 h and cooled to room temperature. The sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 for 10 min at room temperature. Antigen retrieval was performed by autoclaving the sections in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min. The sections were then washed with phosphate-buffered saline (PBS, pH 7.3), cooled to 30°C, and incubated at 4°C overnight with a polyclonal anti-EphB4 antibody (Abgent, San Diego, CA, USA), ephrinB2 (Abgent, San Diego, CA, USA) diluted 1:600 and Ki67 (Abcam, Cambridge, MA, USA) diluted 1:100 in antibody diluent solution (Invitrogen, Carlsbad, California, USA). After washing, the sections were incubated with a secondary antibody (Dako REAL EnVision Detection System; Dako, UK) for 30 min at room temperature. Finally, the color was developed with 3, 3'-diaminobenzidine and the nuclei were lightly counterstained with hematoxylin. The sections were then examined under a microscope. As a positive control, breast cancer tissue sections with known increased EphB4 positivity were used. Appropriate negative controls were performed by omitting the primary antibody and substituting it with an irrelevant anti-serum.

IHC for ALK was stained with the Ventana anti-ALK (D5F3) antibody and was run on the BenchMark ULTRA platform according to the protocol provided by Ventana Medical Systems, Inc. and Roche Diagnostics International, Inc (Arizona, USA).

The stained slides were evaluated independently by two pathologists and any differences were resolved by discussion.

Statistical analysis

Correlations between EphB4 expression and the patients' clinicopathological parameters were evaluated using a Chi-squared test. All

Table 1. The relationship between EphB4 expression and the clinicopathologic parameters

	EphB4		*p* value
	-	+	
	51	42	
Gender			
Male	24	15	0.298
Female	27	27	
Age (years)			
< 55	18	15	1.000
≥ 55	33	27	
Differentiation			
Poor	12	3	0.001
Moderate	24	36	
Well	15	3	
lymph node metastasis			
Negative	24	30	0.021
Positive	27	12	
T stage			
T1	24	27	0.170
T2	18	12	
T3 +T4	9	3	
TNM stage			
I	27	30	0.127
II	21	9	
III + IV	3	3	
Ki67			
< 20%	6	15	0.012
≥ 20%	45	27	
ALK mutation			
No	45	39	0.506
Yes	6	3	

statistical analyses were performed using SPSS software (SPSS 16.0, Chicago, IL, USA). A two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Expression of EphB4 and ephrinB2 in lung adenocarcinoma

To determine the level of EphB4 and ephrinB2 expression in lung adenocarcinoma, tumor tissues and adjacent normal tissue from lung adenocarcinoma surgical specimens were examined (**Figure 1**). EphB4 protein expression is confined to the neoplastic epithelium. Both membrane and cytoplasmic staining were seen, but the expression is absent in normal pulmonary alveoli cells. The ligand of ephrinB2 was consistently negative in all lung adenocarcinoma tissues.

The relationship between EphB4 expression and the clinicopathologic parameters

As shown in **Table 1**, fifty-one out of 93 (54.8%) specimens were negative for EphB4 expression, while 42 out of 93 (45.2%) were positive for EphB4 expression. EphB4 expression was significantly associated with tumor differentiation (*P* = 0.001), lymph node metastasis (*P* = 0.021), and Ki67 (*P* = 0.012) (**Figure 2**). No significant relationship was found between EphB4 expression and gender, age, or ALK mutation (**Figure 3**).

Discussion

Despite progress in locoregional and systemic therapies, patient survival from lung cancer remains a challenge. Receptor tyrosine kinases are frequently implicated in lung cancer pathogenesis, and some tyrosine kinase inhibition strategies have been effective clinically. Aberrant expression of the EGFR protein has been detected in lung cancer. The availability of EGFR small molecule TKIs for the treatment of lung adenocarcinoma has made targeted therapy the most popular treatment modality.

Eph receptors are the largest subfamily of receptor tyrosine kinase. Several Eph receptors have been investigated as targeted therapy in cancers. EphA2 was found highly expressed in NSCLC and not expressed in normal tissue. Lee *et al.* developed a novel method to deliver ephrinA1 for targeting EphA2 receptors on NSCLC cells [15]. The ligand protein ephrinA1 of the EphA2 receptor was conjugated with albumin mesospheres and intratumorally delivered to the mouse model bearing NSCLC. They demonstrated that ephrinA1 reduced tumor growth in mouse NSCLC xenograft models. Immunotherapy with chimeric antigen receptor (CAR)-engineered T cells is a breakthrough tumor treatment. Li *et al.* developed a specific CAR targeted to EphA2 highly expressed on NSCLC cells [16]. They demonstrated that these EphA2-specific T cells can cause tumor cell lysis. They concluded that EphA2-specific T-cell immunotherapy may be a promising approach for the treatment of NSCLC. Accumulating evi-

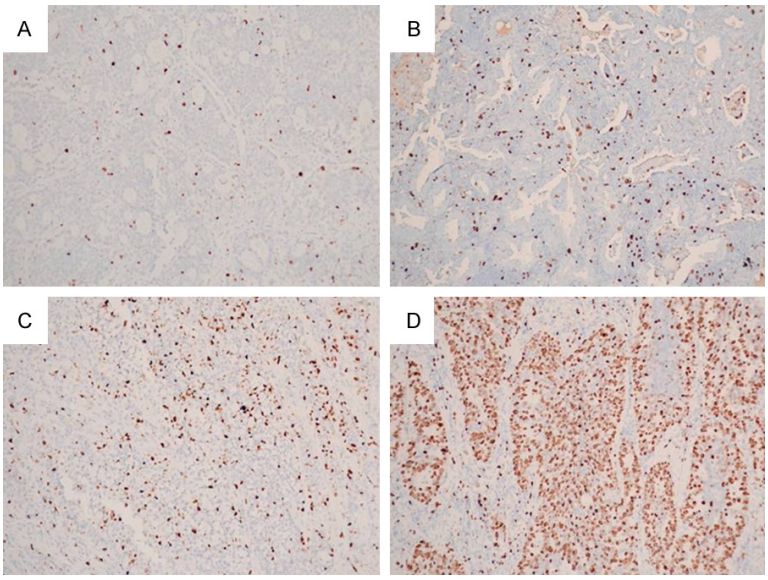

Figure 2. Representative examples of Ki67 expression in lung adenocarcinoma. A. 2% positive Ki67. B. 10% positive. C. 20% positive. D. 80% positive (×400).

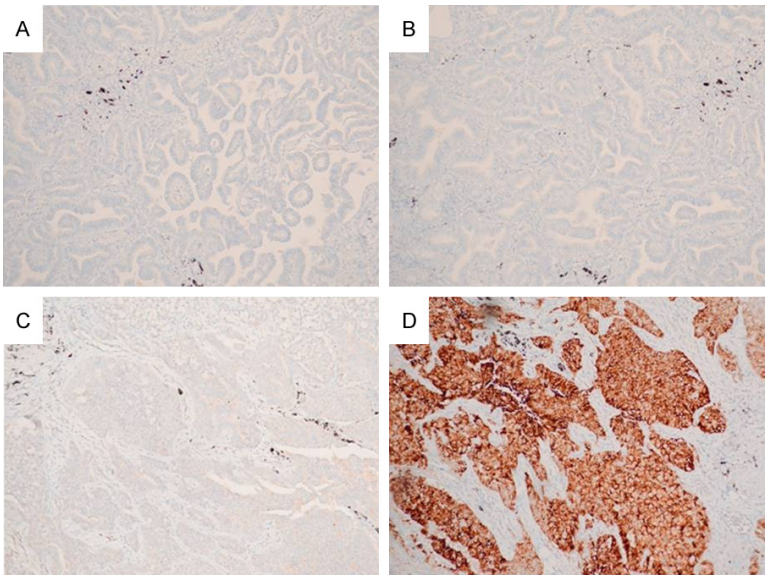

Figure 3. Representative examples of ALK expression in lung adenocarcinoma. A and B. A was a negative control of B tissue sample. B. Negative expression of ALK. C and D. C was a negative control of D tissues sample. D. Positive expression of ALK (×400).

dence suggests that EphB4 plays key roles in cancer progression, and therapies focusing on EphB4 have become potentially important components of various cancer treatment strategies [17-20]. Li *et al.* developed near-infrared fluorescence probes for EphB4 targeted imaging using an EphB4-specific human monoclonal antibody [18]. The probe was used to evaluate both the colon cancer cell line HT29 xenograft and the mAb131 (anti-EphB4) treated models. Their data indicate that this targeted probe can used as a specific contrast agent for the noninvasive imaging of EphB4 expression and can be used to predict the response of EphB4 targeted interventions. Wang *et al.* used a chitosan-stearic acid copolymer to encapsulate hollow gold nanospheres and a near-infrared fluorescent tracer. They conjugated on the surface of nanoparticles with a peptide (TNYL) specifically targeting EphB4 [19]. When combined with near-infrared laser irradiation, the nanoparticles induced more EphB4 positive tumor cells death *in vitro* and in *vivo*.

Before carrying out the therapeutic study of EphB4 in lung adenocarcinoma, we should investigate the expression of EphB4 in lung adenocarcinoma tissues. Although few groups have reported EphB4 expression in lung cancers, their data are limited due to the small number of lung cancer tissues used [21, 22]. In present study, we checked EphB4 and ephrin B2 expressions in 93 cases of lung adenocarcinoma tissue including both normal and tumor cells. We found that EphB4 was positively expressed in 45.2% of lung adenocarcinomas. No positive expression of ephrinB2 was found. Eph receptors and ephrin ligands typically reside on different cells, and a very special feature of the Eph/ephrin axis is that the complexes emanate signals bidirectionally [23]. Eph signaling controls cell morphology, adhesion, migration, and invasion by modifying the organization of the actin cytoskeleton and influencing the activities of integrins and intercellular adhesion molecules [23, 24]. The overex-

pression of EphB4 enhances anchorage-independent growth, migration, and invasion [25]. Our results suggest that a high expression of EphB4 is associated with lymph node metastasis. Ki67 is a marker of proliferation expressed exclusively during active phases of the cell cycle. It is commonly assessed by IHC in clinical settings to judge cell proliferative activity. We found that lung adenocarcinoma cells that highly express the EphB4 protein have a high expression of Ki67.

In summary, we investigated EphB4 and eprhinB2 in a set of lung adenocarcinoma tissues. Our data indicate that EphB4 is positively expressed in parts of lung adenocarcinoma, but no ephinB2 expression was detected. A high expression of EphB4 is associated with lymph node metastasis and Ki67.

Disclosure of conflict of interest

None.

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