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Original Article

Expression of EphB6 protein in breast invasive ductal carcinoma

Weimin Cai[1*], Hong Zhao[2*], Hong Xiao[1], Jiemin Zhang[1], Xiao Chen[3], Jiandong Wang[3], Hai Wang[3]

[1]Department of Pathology, Changshu Hospital, Affiliated to Suzhou University, Changshu 215500, China; [2]Department of Laboratory Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China; [3]Department of Pathology, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, China. [*]Equal contributors.

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Abstract: Erythropoietin-producing hepatocyte (Eph) receptor family constitutes the largest family of tyrosine kinase receptors in the human genome. Aberrant expression of Eph receptors has been reported in a variety of human cancer types. The EphB6 receptor is a distinctive member of the EphB subclass in that its kinase domain contains several alterations in the conserved amino acids and thus lacks catalytic activity. Interestingly, increased metastatic activity is associated with reduced EphB6 receptor expression in several tumor types, including breast cancer. In this study, we examined the expression of EphB6 protein in 76 cases of breast carcinoma by immunohistochemical staining with a polyclonal anti-EphB6 antibody. The relationship between EphB6 expression and pathological parameters was analyzed. EphB6 protein was strongly expressed in normal luminal cells of all tested samples (76/76, 100%) and differentially expressed in invasive ductal carcinomas inter-samples. EphB6 protein was negatively or weakly expressed in 30% (23/76), moderately expressed in 37% (28/76) and strongly expressed in 33% (25/76) invasive ductal carcinomas. The expression of EphB6 protein was associated with tumor grade, molecular subtype, Her2 expression and Ki-67. Our data showed that EphB6 protein was down-regulated in breast carcinoma, it may play a tumor suppressor in breast carcinoma and may be a potential target for therapy.

Keywords: EphB6, tumor grade, molecular subtype, breast carcinoma

Introduction

Breast cancer is the most common malignancy in women and represents 14% of cancer-related deaths in women. It has been estimated that there will be about 231840 new cases of breast cancer in the United States in 2014, and about 40290 women will die from this disease [1]. Breast cancer is a heterogeneous disease, which can be classified into biologically, morphologically and clinically meaningful entities. Even though there has been considerable progress in the early detection and surgical therapy of breast cancer, the mortality of breast cancer has not been decreased obviously. There is an urgent need to understand the molecular mechanism and pathways of breast cancer and for better and improved treatment of women diagnosed with breast cancer.

In the erythropoietin producing hepatocellular carcinoma (EPH) family of receptor tyrosine kinases (RTK), 16 members have been identified in humans to date. These EPH receptors are divided into class A and B based on their homology to each another as well their affinity for their ephrin ligands. EphB6 is a unique member of the Eph family in that its kinase domain contains several alterations in conserved amino acids and it is catalytically inactive [2]. Although EphB6 is expressed both in a variety of embryonic and adult tissues, the biological functions of this receptor are largely unknown [3-6].

The aim of the present study was to correlate the immunohistological expression of EphB6 in a cohort of patients with breast invasive ductal carcinoma. The study also determined the possible relationship between this immuno expres-

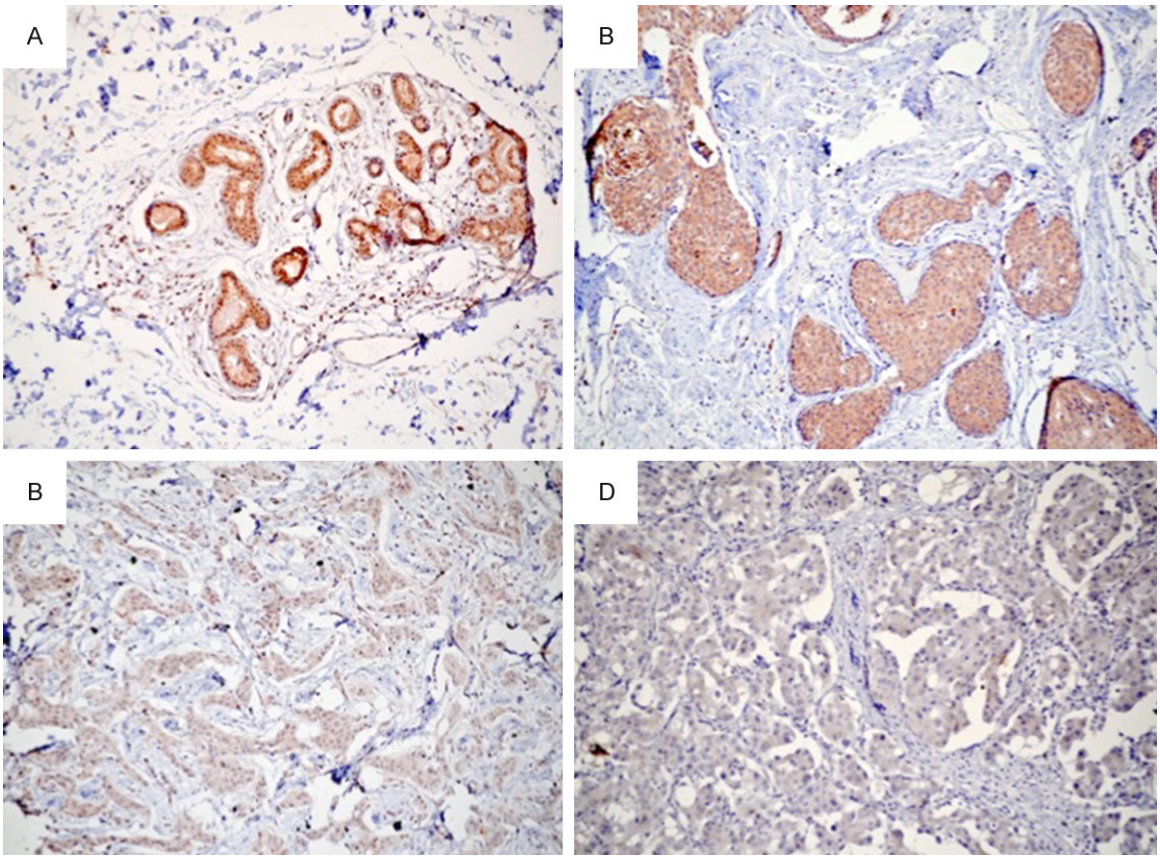

Figure 1. Expression of EphB6 in breast invasive ductal carcinoma. A: High expression in normal breast luminal cells (3+); B: High expression in invasive ductal carcinoma cells (3+); C: Moderate expression in invasive ductal carcinoma cells (2+); D: Weak expression in invasive ductal carcinoma cells (1+).

sion and tumor proliferation activity expressed by Ki-67 immuno expression.

Materials and methods

Patients and clinicopathological variables

The study cohort consisted of 76 patients with breast invasive ductal carcinoma (age range 30-72 years, mean age 49 years) who underwent surgery from 2008 to 2009 in the First People Hospital Changshu, China. All hematoxylin and eosin stained slides were reviewed by two gynecological pathologists to verify the diagnosis, histological grade and stage. None of the patients received preoperative chemotherapy or radiation therapy. This investigation was performed following approval from the Ethics Committee of the First People Hospital Changshu, China.

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 bal-

Table 1. Relationship between the expression of EphB6 and clinical pathological parameters in 76 breast cancer tissues

		EphB6 expression			*P* value
		0/1+	2+	3+	
No.		23	28	25	
Age					
	<50	10	14	13	0.827
	≥50	13	14	12	
Grade					
	1/2	6	16	14	0.05
	3	17	12	11	
Lymph node					
	Yes	14	13	10	0.336
	No	9	15	15	
Molecular					
Subtype	Luminal A/B	10	15	8	0.003
	Her2 positive	0	8	9	
	Triple negative	13	5	5	
Her2					
	0/1	16	11	9	0.022
	2	5	4	3	
	3	2	13	13	
Ki-67					
	≥20%	21	23	15	0.026
	<20	2	5	10	

sam. 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).

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 in breast normal and carcinoma cells

The expression of EphB6 protein was determined in breast carcinoma by immunohistochemical staining. As shown in **Figure 1**, EphB6 staining was predominantly localized in the cytoplasm. High expression of EphB6 was observed in 100% of normal breast luminal cells and differentially expressed in invasive ductal carcinoma cells.

EphB6 expression in breast carcinoma and its correlation with clinicopathological features

EphB6 protein was differentially expressed in 76 samples of invasive ductal carcinoma (**Table 1**). Twenty-three of 76 (30.3%) samples were negatively or weakly (0/1+) stained with anti-EphB6 antibody, 28 of 76 samples (36.8%) were moderately stained (2+) and 25 of 76 samples (32.9%) were strongly stained (3+). The expression of EphB6 was down-regulated in invasive ductal carcinoma cells compared with normal luminal cells.

The relationship between EphB6 expression and pathological parameters was analyzed (**Table 1**). Expression of EphB6 was significantly associated with grade (*P*=0.05), molecular subtype (*P*=0.003), and Her2 over-expression (*P*=0.022). EphB6 expression was reversely related to Ki-67 (*P*=0.026). No significant relationship was observed between the expression of EphB6 and age and lymph node metastasis.

Discussion

The biological function of the Eph/ephrin family was initially identified as a regulator of axonal guidance and cell migration in the nervous system. The Eph/ephrin family also plays an essential role in angiogenesis and embryogenesis. In general, Eph receptors possess intrinsic tyrosine kinase activities, but EphB6 has unique structural characteristics in the kinase domain. EphB6 has substitutions in the six invariant amino acids such as lysine in the ATP-binding site and aspartic acid in the phosphotransfer site in the conserved kinase domain. Therefore, EphB6 has no detectable intrinsic tyrosine kinase activity [7]. We previously detected the EphB6 protein in colorectal cancer and colorectal adenoma samples [2]. Decreased expres-

sion of EphB6 was found in colorectal cancer as compared with adenoma and normal tissues. The loss of EphB6 protein in colorectal cancer was positively associated with poor differentiation, lymph node metastasis and depth of invasion. Low levels of EphB6 protein expression are associated with a shorter mean duration of survival in colorectal cancer.

In the present study, the clinical significance of EphB6 expression in invasive ductal carcinoma was analyzed. The expression of EphB6 protein was decreased in breast carcinoma cells compared with normal luminal cells. High expression of EphB6 was significantly associated with low-grade, molecular subtype, Her2 overexpression and low Ki-67 expression. Invasive carcinomas of no special type and all other invasive breast carcinomas are routinely graded based on an assessment of tubule/gland formation, nuclear pleomorphism and mitotic count. Assessment of histological grade has become a significant powerful prognostic factor having a significant association with survival of patients with invasive breast carcinoma. High expression of EphB6 was more often detected in low-grade invasive ductal carcinoma, it may be a prognostic marker. The relation between expression of EphB6 and survival of patients should analyze in the feature. Analysis of gene expression arrays has resulted in the recognition of several fundamentally different subtypes of breast cancer. St Gallen international breast cancer conference expert panel defined subtype of breast cancer based on immunohistochemical expression of estrogen, progesterone receptor, Her2 and Ki-67 labeling index [8, 9]. In the 2013 proposed classification, breast cancer was subtyped as Luminal A-like, luminal B-like (Her2 negative), luminal B-like (Her2 positive), Her2 positive and Triple negative. In the present study, we found that low expression of EphB6 receptor was significantly associated with triple negative subtype. Expression of EphB6 was positively related to Her2 expression and negatively related to Ki-67 index. The interaction of EphB6 and Her2 should be explored in the next project. Our data show that EphB6 expression significantly associated with subtype of breast cancer and may be a potential target for therapy.

Metastatic dissemination drives the high mortality associated with breast cancer. Several studies showed that EphB6 receptor is as a suppressor in metastasis of certain cancers [10-13]. In this study, we did not find significant relation between EphB6 expression and metastasis of breast cancer. We noticed that 14 out of 23 cases (60.9%) occurred metastasis of lymph node when negative of weak expression of EphB6, 13 out of 28 cases (46.4%) occurred metastasis when moderate expression of EphB6 and 10 out of 25 cases (40%) occurred metastasis when strong expression of EphB6. No statistical difference between EphB6 expression and metastasis may be interpreted as small number of cases.

In summary, our results show that EphB6 was decreased in breast invasive ductal carcinoma cells compared with normal breast luminal cells. Expression of EphB6 protein is significantly associated with tumor grade and subtype. Our findings indicated EphB6 may be used as a prognostic marker and a potential therapeutic target of breast invasive ductal carcinoma.

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Disclosure of conflict of interest

None.

Address correspondence to: Hai Wang and Jiandong Wang, Department of Pathology, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, China. E-mail: drwh77@hotmail.com (HW); wang_jd@outlook.com (JDW)

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