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ORIGINAL PAPER

Expression of EphA2 and E-cadherin in Gastric Cancer: Correlated with Tumor Progression and Lymphogenous Metastasis

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Abstract In this study, gastric cancer progression was correlated with the over-expression of erythropoietin-producing hepatocellular (Eph)A2 receptor and down-expression of epithelial cadherin (E-cadherin). Immunohistochemistry of EphA2 and E-cadherin were performed on these tumor samples from 165 primary lesions of gastric cancer. The results showed that expression of EphA2 was obviously increased in gastric cancer tissues ($P<0.01$), which was positively correlated with the depth of cancer invasion, tumor-node-metastasis (TNM) stage and lymph node metastasis ($P<0.05$). Meanwhile, the expression of E-cadherin was significantly reduced ($P<0.01$), which was negatively correlated with the depth of cancer invasion, grade of tumor differentiation, TNM stage and lymph node metas tasis ($P<0.05$). The correlation between EphA2 and E-cadherin expression was negative ($r=-0.198$, $P=0.011$). In conclusion, either the over-expression of EphA2 or the down-expression of E-cadherin is correlated with cancer progression and lymphogenous metastasis in gastric cancer, suggesting that both of them may play an important role in tumor progression and metastasis.

Keywords EphA2 · E-cadherin · Gastric cancer · Immunohistochemistry

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Abbreviations

Eph	erythropoietin-producing hepatocellular
E-cadherin	epithelial cadherin
RTK	receptor tyrosine kinase
PBS	phosphate buffered solution
DAB	diaminobenzidine tetrachloride
TNM	tumor-node-metastasis
MMP	matrix metalloprotease

Introduction

Gastric cancer, one of the most common cancers, is the main cause leading to death [1]. Invasion, metastasis and recurrence are the major causes of death in gastric cancer patients and the key factors affecting clinical treatment and prognosis [2]. Therefore, it is significant to study the mechanism underlying development, invasion and metastasis.

EphA2, a member of the Eph family of RTKs, is primarily found in adult human epithelial cells and is located on chromosome 1p36.1 [3]. The EphA2 is thought to down-regulate cell growth and migration in normal epithelium [4]. Interestingly, EphA2 protein was highly expressed in many cancers including esophageal [5], prostate [6], lung [7], ovarian [8] and urinary bladder carcinomas [9], the expression of which was consistent with the progression of these carcinomas. E-cadherin, one kind of cell adhesion molecules in cadherin family, plays an important role in maintaining the integrity and polarity of epithelial cells in the presence of Calciumions [10]. It is thought that the loss of E-cadherin expression or function in cancer is the main reason to disrupt the epithelial cell-cell contacts, causing metastasis and invasiveness of tumors [11–13]. The EphA2 subcellular localization and phosphor-

ylation are dependent on E-cadherin expression in breast cancer cells, which suggests that the down-expression of E-cadherin changes neoplastic cell growth and cell adhesion via effects on EphA2 [14].

However, there is no large sample report about EphA2 and E-cadherin expression in patients with gastric cancer. In this study, we investigated the expression of EphA2 and E-cadherin in gastric cancer, and studied the correlation between the clinicopathologic features and the expression of EphA2, E-cadherin, further providing the relevance of EphA2 and E-cadherin in gastric cancer.

Materials and Methods

Subjects and Samples

165 patients, including 107 males and 58 females, aged between 34–75 years (mean age 56.4 years) were collected during January 2004 to December 2006. All these patients were surgically treated in Xiangya Hospital affiliated to Central South University, Hunan, China. Neither chemotherapy nor irradiation was performed prior to tumor resection. The samples of cancer tissue, paratumoral tissue (2 cm distance from the cancer) and normal gastric mucosa (6 cm distance from the cancer) were collected from each patient during operation. After then, the samples were fixed in 10% formaldehyde solution, embedded in paraffin wax subsequently. All these samples were diagnosed as adenocarcinoma through pathologic examination.

Immunohistochemical Methods

The cross serial sections, 4-μm thick, were collected from samples. Immunohistochemical staining was performed according to manufacturer's instructions of the Histostain®-Plus kits (Zymed, Carlsbad, USA). Then, the sections were deparaffinized, rehydrated and incubated with fresh 0.3% hydrogen peroxide in methanol for 10 min at 37°C. The sections were autoclaved in citrate buffer at 100°C for 2 min for antigen retrieval. The serial sections were incubated with rabbit EphA2 polyclonal antibody (sc-924, dilution 1:200, Santa Cruz, CA, USA) or rabbit E-cadherin polyclonal antibody (sc-7870, dilution 1:200, Santa Cruz, CA, USA) at 4°C overnight. The sections were washed with PBS, incubated with biotinylated anti-rabbit IgG as second antibody for 15 min at 37°C and then incubated with streptavidin-conjugated horseradish peroxidase for 15 min at 37°C (Zymed, Carlsbad, USA). The immune reaction was demonstrated with DAB. The sections were counterstained with hematoxylin, dehydrated, and mounted. PBS was used to substitute primary antibody in negative control, with no evident detectable staining. Furthermore, EphA2 positive breast cancer and E-cadherin positive normal breast cell were treated as positive controls in this study.

Evaluation of EphA2 and E-cadherin Immunohistochemical Staining

The intensity of immunohistochemical staining was evaluated in five areas of each section for correlation and confirmation of tissue analysis. The sections were scored by two independent investigators without the knowledge of patient outcome (double-blinded).

For EphA2 staining, the sections were scored according to the percentage of immunoreactive cells (0 point for 0–5%, 2 points for 6–50%, 3 points for more than 50%) and the staining intensity (1 point for weak intensity, 2 points for moderate intensity, 3 points for strong intensity). Specimens were categorized into four groups based on the total scores of the positive staining cells and expression intensity negative expression group (−), ≤5% cells were stained, regardless of intensity; weak expression group (+), 1–2 points; moderate expression group (++), 3–4 points; and strong expression group (+++), 5–6 points [15]. In these groups, the moderate and strong ones (++ or +++) were considered to be EphA2 over-expressed and noted as positive results for statistical analysis [16].

For E-cadherin staining, the immunoreactivity of sections was graded semi-quantitatively as described previously [17, 18]: negative staining (−); cytoplasmic staining (+); heterogeneous staining (tumors composing of both normal and abnormal staining areas) (++); and normal membranous staining (+++). All specimens without membranous E-cadherin expression, including negative (−), cytoplasmic (+) or heterogeneous (++) ones were classified as negative for further statistical analysis [19].

Statistical Analysis

Wilcoxon rank sum test, χ^2 test, Spearman rank test were used for statistical analysis. $P<0.05$ (two-tailed) were considered to be statistically significant. Statistical analyses were performed with SPSS software (version 13.0, SPSS, Inc., Chicago, USA).

Results

Expression of EphA2 and E-cadherin in Gastric Cancer, Paratumoral and Normal Gastric Mucosa Tissues

Positive EphA2 immunostaining was diffusely distributed throughout the cytoplasm. The immunostaining results of EphA2 were shown in Table 1. The positive rate of EphA2

expression was 61.2% (101/165) in gastric cancer tissues, 30.3% (50/165) in paratumoral tissues and 23.0% (38/165) in normal gastric mucosa tissues. Wilcoxon rank sum test indicated that the expression of EphA2 in gastric cancer tissues was significantly higher than that in other tissues ($P<0.01$). The immunohistochemical staining of EphA2 was showed in Fig. 1.

Positive E-cadherin immunostaining was intensely distributed in the epithelial cell membrane. The immunostaining results of E-cadherin were also shown in Table 1. The positive rate of E-cadherin expression was 30.9% (51/165) in gastric cancer tissues, 53.9% (89/165) in paratumoral tissues, 80.6% (133/165) in normal gastric mucosa tissues. Wilcoxon rank sum test indicated that the expression of E-cadherin in cancer tissues was significantly lower than that in other tissues ($P<0.01$). The immunohistochemical staining of E-cadherin was also shown in Fig. 1.

Correlation Between EphA2, E-cadherin Expression and Clinicopathologic Features

The correlation between the clinicopathological features of gastric cancer patients and expressions of EphA2, E-cadherin was summarized in Table 2. There was a significant correlation between over-expression of EphA2 and depth of invasion ($P=0.030$), TNM stage ($P=0.013$), lymph node metastasis ($P=0.009$), but no significant correlation with patients' age, gender, tumor size and differentiation ($P>0.05$). Down-expression of E-cadherin was significant correlated with depth of invasion ($P=0.007$), tumor differentiation ($P=0.027$), TNM stage ($P=0.006$) and lymph node metastasis ($P=0.031$), but not correlated with patients' age, gender and tumor size.

Correlation Between EphA2 and E-cadherin Expression in Gastric Cancer Tissues

The Spearman rank test suggested that the immunostaining expression of EphA2 protein was inversely correlated with that of E-cadherin in gastric cancer tissues ($r=-0.198$, $P=0.011$, Table 3).

Discussion

In this study, we investigated the expression of EphA2 and E-cadherin in 165 patients with gastric cancer. We detected the expression of these two proteins in the paratumoral cancer and normal gastric mucosa tissues with immunohistochemistry. We found that EphA2 was over-expressed while E-cadherin was down-regulated in gastric adenocarcinoma compared with that in paratumoral tissues and normal gastric mucosa tissues. Moreover, such expression patterns of EphA2 and E-cadherin were associated with tumor progression and lymphogenous metastasis in gastric cancer. This large clinical sample study suggested that the expression between EphA2 and E-cadherin was inversely correlated.

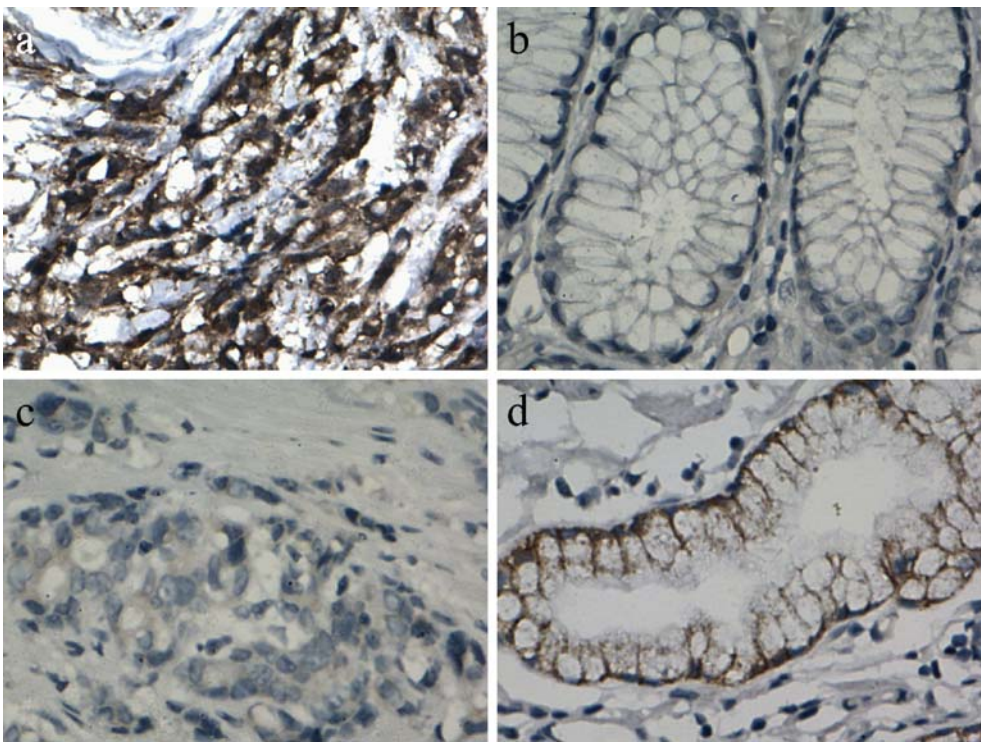

Fig. 1 EphA2 and E-cadherin immunohistochemical staining. **a** positive cytoplasmic expression of EphA2 in gastric cancer (SP method, ×400); **b** negative cytoplasmic expression of EphA2 in normal gastric mucosa (SP method, ×400); **c** negative membranous expression of E-cadherin in gastric cancer (SP method, ×400); **d** positive membranous expression of E-cadherin in normal gastric mucosa (SP method, ×400)

Table 1 Expression of EphA2 and E-cadherin in gastric cancer, paratumoral and normal gastric mucosa tissues

Groups	*n*	EphA2				E-cadherin			
		−	+	++	+++	−	+	++	+++
Cancer tissues	165	29	35	53	48	26	40	48	51
Paratumoral tissues	165	53	62	31	19	19	25	32	89
Normal gastric mucosa	165	76	51	25	13	5	11	16	133

Table 3 The correlation between EphA2 and E-cadherin expression in gastric cancer

EphA2	E-cadherin				Total
	−	+	++	+++	
−	1	3	6	19	29
+	4	7	19	5	35
++	9	22	13	9	53
+++	12	8	10	18	48
Total	26	40	48	51	165

In our study, the over-expression of EphA2 was significantly related to depth of invasion, TNM stage and lymph node metastasis, which suggested that the high expression of EphA2 may contribute to progression and metastasis of gastric cancer. It has been reported that the EphA2 is always over-expressed in many cancers as a powerful oncoprotein [5–9]. Some studies have shown that EphA2 is involved in the regulation of tumor cell growth, migration, invasion, and angiogenesis [16, 21–24]. The exact mechanisms for over-expression of EphA2 in carcinoma are still unclear. Some studies suggested that it is due to the increased protein stability and post-transcriptional level regulation. In normal cells, stable cell–cell contacts cause ligand mediated EphA2 autophosphorylation which promotes EphA2 degradation by combining EphA2 and its downstream adaptor protein c-Cbl. While, unstable cell–

Table 2 Correlation between EphA2, E-cadherin expression and clinicopathologic features

Clinicopathological factors	*n*	EphA2			E-cadherin		
		Positive	Negative	*P*	Positive	Negative	*P*
Age (years)		101	64		51	114	
<50	76	49	27	0.427	21	55	0.400
≥50	89	52	37		30	59	
Gender							
Male	107	67	40	0.615	31	76	0.465
Female	58	34	24		20	38	
Tumor size (cm)							
<5	97	56	41	0.273	35	62	0.086
≥5	68	45	23		16	52	
Depth of invasion [a]							
T1	31	14	17	0.030	17	14	0.007
T2	38	21	17		13	25	
T3	65	41	24		14	51	
T4	31	25	6		7	24	
Histologic type							
Well	49	26	23	0.150	22	27	0.027
Moderately	55	32	23		16	39	
Poorly	61	43	18		13	48	
TNM stage [a]							
I	33	13	20	0.013	17	16	0.006
II	34	20	14		13	21	
III	60	39	21		15	45	
IV	38	29	9		6	32	
Lymph node metastasis							
Positive	98	68	30	0.009	24	74	0.031
Negative	67	33	34		27	40	

[a] Both depth of tumor invasion and TNM stage: according to 1997 tumor-node-metastasis (TNM) classification of malignant tumors by the International Union Against Cancer [20].

cell contacts decreased EphA2 binding ability with its membrane-anchored ligands, causing decreased ligand-mediated degradation which might contribute to EphA2 over-expression in tumor cells [25, 26]. The study on the EphA2-associated phosphatase indicates that EphA2 accumulates on the surface of malignant cells. EphA2 is a kind of prominent tyrosine-phosphorylated protein in normal epithelial cells. However, the EphA2 in tumor cells accumulates on the cell surface without tyrosine phosphorylation [27, 28].

Similar to EphA2, the down-expression of E-cadherin is significantly correlated with depth of invasion, tumor differentiation, TNM stage and lymph node metastasis. It was reported that down-expression of E-cadherin was associated with an aggressive clinicopathologic phenotype that induced invasiveness and metastasis in gastric cancer [29, 30]. E-cadherin detection may be helpful in evaluating the degree of malignancy in gastric cancer. The mechanisms for reduced E-cadherin function in carcinoma are complicated and included hereditary mutation, promoter hypermethylation, transcriptional repression and posttranslational modification [31–34]. During tumor progression, some proteases such as MMPs and calpain lead to truncated E-cadherin, which cleave E-cadherin directly to form a soluble fragment that can increase invasion and inhibit cell aggregation [35, 36].

We found that the expression of EphA2 protein was inversely correlated with that of E-cadherin in a large sample of gastric cancer patients. Saito *et al* found a significant correlation between the expression of EphA2 and E-cadherin in colorectal cancer [37]. However, the exact correlation between EphA2 and E-cadherin in gastric cancer is still unclear. Several studies have reported that the localization and phosphorylation of EphA2 in mammary epithelial cells is dependent on E-cadherin-mediated adhesion [14]. E-cadherin is required for EphA2 receptor localization at cell–cell contacts. Without E-cadherin, EphA2 will localize to the perinuclear region which further blocking the combination with ephrin. Therefore, E-cadherin is required for the membrane localization of Eph receptors and their membrane-bound ligands [38].

In conclusion, the over-expression of EphA2 and down-expression of E-cadherin are consistent with the progression and lymphogenous metastasis of gastric cancer. The detection of EphA2 and E-cadherin can be used to evaluate the degree of malignancy in gastric cancer.

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