Original Article
High expression of EphA4 is associated with invasion and lymph node metastasis in colorectal carcinomas

Jia Feng[1], Qing Zhang[1], Qin Jin[1], Haibin Liang[2], Haruhiko Sugimura[3], Shu Zhang[1], Jiandong Wang[4]

[1]Department of Pathology, [2]General Surgery, Affiliated Hospital of Nantong University, Nantong, Jiangsu, China;
[3]Department of Pathology, Hamamatsu University School of Medicine, Handayama, Hamamatus, Japan;
[4]Department of Pathology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, China

Abstract: Eph receptors and ephrin ligands have been implicated in development of embryo, especially in vascular and nervous system. Increasing evidence suggests that Eph/ephrin signaling plays important roles in carcinogenesis. EphA4 is a member of Eph receptors and its role has been found in development of nerve and in tumorigenesis. Till now, EphA4 expression and its clinical significance in colorectal carcinoma have not been well investigated. In the present study, a set of 102 colorectal carcinoma tissue specimens were subjected to immunohistochemistry by using a specific polycolonal antibody for EphA4. Expression of EphA4 protein was more often detected in colorectal carcinoma than in normal mucosa ($P<0.001$). High expression of EphA4 protein in colorectal carcinomas was positively associated with tumor size ($P=0.008$, $r_s=0.260$), depth of invasion ($P=0.004$, $r_s=0.286$), lymph node metastasis ($P=0.013$, $r_s=0.244$), and TNM stage ($P=0.005$, $r_s=0.279$), and was negatively associated with age of patients ($P=0.027$, $r_s=-0.219$). No significant relationship between expression of EphA4 and sex and tumor location was found. The survival analyses showed that patients whose carcinoma exhibited high expression of EphA4 had a poor outcome ($P=0.006$). Our results suggest that EphA4 play a role in the invasion and lymph node metastasis of colorectal carcinoma. The EphA4 protein expression level may be used as a potential prognostic marker in colorectal carcinoma. Furthermore, the EphA4 gene may provide a novel target for therapy of colorectal carcinoma.

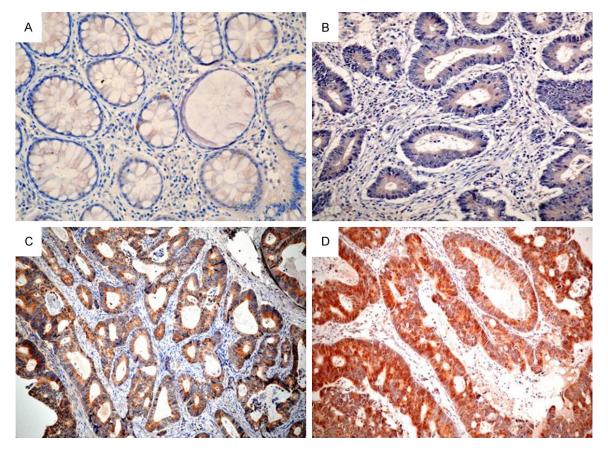**Keywords:** Colorectal carcinoma, EphA4, receptor tyrosine kinase

Introduction

Colorectal cancer was ranked third in morbidity and mortality in the United States in 2011 [1]. In China, colorectal cancer was ranked second in women and third in men in morbidity and mortality in 2011 [2]. Although substantial progress has been made in surgical techniques and postoperative chemotherapy in recent years, the prognosis for colorectal cancer is still not satisfactory. The search for new molecular targets for early diagnosis, rational therapy, and prognosis is a current research goal. The Eph receptors were named for the first member of EphA1 and found in an erythropoietin-producing human hepatocellular carcinoma cell line. They are the largest subfamily of the receptor tyrosine kinases (RTKs) and include at least 14 distinct receptors and eight distinct ligands [3]. Eph receptors are divided into EphA and EphB group based on the sequence homology of their extracellular domains and their affinity to bind corresponding ligands, ephrinA and ephrinB. Initial studies indicated that Eph receptors and ephrin ligands were key players in many developmental processes, including embryo patterning, angiogenesis, and axon guidance [4-7]. It is now clear that the Eph molecules also play roles in adult tissues under physiological conditions and in disease states such as cancer [8-13]. Eph receptors and their ligands are frequently overexpressed in different types of cancer. EphA4 is previously designated Cek8 in chicken, Tyro1 in rat, and Sek1 in mouse, which is preferentially expressed in the embryonic and adult nervous system. Martone et al mapped the distribution of EphA4 in the adult rat brain and spinal cord using a polyclonal antibody raised against a synthetic carboxy-terminal peptide [14]. The function of the EphA4 receptor was explored by Dottori et al through creation of a null mutant mouse [15].

Figure 1. Representative examples of immunohistochemistry of EphA4 in normal mucosa and carcinoma. A: Negative expression of EphA4 in normal mucosa cells (score 0). B: Low expression of EphA4 in colorectal carcinoma cells (score 1). C: Moderate expression of EphA4 in colorectal carcinoma cells (score 2). D: High expression of EphA4 in colorectal carcinoma cells (score 3).

Table 1. Expression of EphA4 protein in colorectal carcinoma and normal mucosa

	EphA4 protein expression			P value
	0/1	2	3	
Normal mucosa	78 (76.5%)	21 (20.6 %)	3 (2.9%)	<0.001
Colorectal carcinoma	15 (14.7%)	39 (38.2%)	48 (47.1%)	

Their results demonstrate a critical role for EphA4 in establishing the corticospinal projection. Expression of Ephs and ephrins is frequently altered in human tumors. Emerging evidence suggests their strong involvement in tumor biology, including in metastasis, invasion, and prognosis.

Overexpression of EphA4 was found in gastric cancer [16] and pancreatic ductal adenocarcinoma [17]. Oshima et al found that high expression of EphA4 mRNA in colorectal cancer is correlated with liver metastasis [16]. More recently, de Marcondes et al demonstrated the PI3K/AKT, Wnt/beta-catenin, and ERK1/2 signaling pathways downstream of EphA4 receptor activation and being dependent on EphA4, which plays an important role in the regulation of events related with the EMT development and regulates the aggressive phenotype of irradiation survivor colorectal cancer cells [18, 19]. The expression of EphA4 protein and its clinical significance have not been well studied in colorectal carcinomas. In the present study, we detected the expression of EphA4 protein in a set of colorectal carcinoma samples, and analyzed the relationship between EphA4 expression and clinicopathologic parameters.

Materials and methods

Specimens

All the tissue samples in our study were collected from 102 patients with colorectal carcinoma, as part of a study approved by the

Table 2. Correlation between expression of EphA4 and clinicopathological parameters

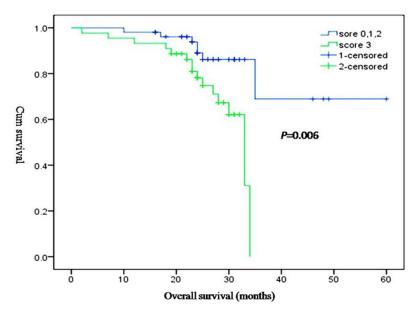|	No.	EphA4 protein expression			P	r_s
		0/1	2	3		
Overall	102	15 (14.7%)	39 (38.2%)	48 (47.1%)		
Sex						
Male	72	9 (12.5%)	27 (37.5%)	36 (50.0%)	0.281	-0.108
Female	30	6 (20.0%)	12 (40.0%)	12 (40.0%)		
Age (years)						
≤55	12	0 (0.00%)	3 (25.0%)	9 (75.0%)	0.027	-0.219
>55	90	15 (16.7%)	36 (40.0%)	39 (43.3%)		
Location						
Rectum+Sigmoid colon	57	6 (10.5%)	27 (47.4%)	24 (42.1%)	0.669	0.043
Colon	45	9 (20.0%)	12 (26.7%)	24 (53.3%)		
Tumor size (cm)						
≤5	87	15 (17.2%)	33 (37.9%)	39 (44.8%)	0.008	0.260
>5	15	0 (0.00%)	3 (20.0%)	12 (80.0%)		
Depth of wall invasion						
T1+T2	27	6 (22.2%)	15 (55.6%)	6 (22.2%)	0.004	0.286
T3+T4	75	9 (12.0%)	24 (32.0%)	42 (56.0%)		
Tumor differentiation						
Well	6	0 (0.0%)	3 (50.0%)	3 (50.0%)	0.368	0.090
Moderately	78	9 (11.5%)	36 (46.2%)	33 (42.3%)		
Poorly	18	3 (16.7%)	3 (16.7%)	12 (66.6%)		
Lymph node metastasis						
No	45	9 (20.0%)	21 (46.7%)	15 (33.3%)	0.013	0.244
Yes	57	6 (10.5%)	18 (31.6%)	33 (57.9%)		
TNM stage						
I+II	48	9 (18.7%)	24 (50.0%)	15 (31.3%)	0.005	0.279
III+IV	54	6 (11.1%)	15 (27.8%)	33 (61.1%)		

Research Ethics Board of Affiliated Hospital of Nantong University, China. These patients had undergone surgery in the Hospital between 2009 and 2014 without any preoperative therapy. These patients consisted of 72 males and 30 females, and the age range was from 43 to 87 years (median age, 67.5 years). Tumor-node-metastasis (TNM) staging was evaluated based on the World Health Organization (WHO) classification of Tumors of the Digestive System (4th edition, 2010).

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 4 μm thickness. All the sections were deparaffinized using xylene, dehydrated by an ethanol gradient, and then rehydrated with deionized water. Heat-mediated antigen retrieval was accomplished by autoclave treatment (120°C for 2 min in 1 mmol/L EDTA, pH 8.0), and then followed by cooling at room temperature. Incubation with a monoclonal antibody raised against the human EphA4 (dilution 1:200, Abgent, San Diego, CA, USA) was performed overnight at 4°C. After washing with pH 7.4 phosphate-buffered saline (PBS), the sections were then incubated with secondary antibody (Dako, Cambridge, UK) for 30 min at room temperature. Color development was performed with 3, 3'-diaminobenzidine. Nuclei were counterstained with hematoxylin. The gastric carcinoma specimen that highly expressed EphA4 was used as positive control. The negative control was carried out by replaced primary antibody of EphA4 with PBS.

The immunostaining results were evaluated independently by two pathologists. The different results were verified by consensus. EphA4 immunoreactivity was scored on a scale of 0 to

Figure 2. Kaplan-Meier plots of overall survival show patients with increased expression of EphA4 had a shorter survival than those with negative expression. (group 1: score 0, 1 and 2 of EphA4; group 2: score 3 of EphA4).

3. The score of EphA4 expression was as follows: 0, no reactivity or faint staining; 1+, faint or weak staining; 2+, moderate staining; 3+, strong staining in >10% of tumor cells. The expression of the EphA4 protein in colorectal carcinomas was categorized into low expression (score 0~1), moderate expression (score 2), and high expression (score 3).

Statistical analysis

The correlation between EphA4 expression and clinicopathological parameters was evaluated by a Spearman's rank correlation test. Kaplan-Meier survival analysis was used to examine the relationship between categorical groups and survival in the univariate analysis. All statistical analyses were performed by use of SPSS 15.0 software (SPSS, Chicago, IL, USA). A two-side *P*-value <0.05 was considered statistically significant.

Results

Expression of EphA4 in colorectal carcinoma and normal mucosa

The expression of EphA4 protein was evaluated in 102 cases colorectal carcinoma tissue specimens, which including normal mucosa and carcinoma cells (**Figure 1**). As shown in **Table 1**, the expression of EphA4 was negative-

ly or weakly detected in 76.5% (score 0/1), moderately detected in 20.6% (score 2), and strongly detected in 2.9% (score 3) of normal mucosas, while negatively or weakly detected in 14.7%, moderately detected in 38.2%, and strongly detected in 47.1% of colorectal carcinomas. The expression of EphA4 was significantly increased in colorectal carcinoma cells comparing with normal mucosa cells (*P*<0.001).

The significance of increased expression of the EphA4 protein in colorectal carcinoma

The relationship between the expression level of EphA4 protein and clinicopathological parameters was analyzed. High expression of EphA4 was associated with age (*P*=0.027, r_s=-0.219), tumor size (*P*=0.008, r_s=0.260), depth of invasion (*P*=0.004, r_s=0.286), lymph node metastasis (*P*=0.013, r_s=0.244), and TNM stage (*P*=0.005, r_s=0.279). No significant relationship between the expression of EphA4 and sex, tumor location, and tumor differentiation was found (**Table 2**).

High EphA4 protein expression is associated with poor survival in patients with colorectal carcinoma

We examined the association of EphA4 protein expression with clinical outcomes. Colorectal carcinoma patients who underwent surgery at Affiliated Hospital of Nantong University, China between 2009 and 2014 were followed up. The data for 96 from 102 patients who were involved in immunohistochemistry of EphA4 are available. The follow-up period was 2-60 months and the median follow-up period was 25 months. The Kaplan-Meier univariate survival analysis showed patients with high EphA4 expression (score 3 of EphA4) had shorter overall survival than those with low EphA4 expression (score 0,1,and 2 of EphA4) (log-rank test, *P*=0.006; **Figure 2**).

Discussion

The role of EphA4 in development of nervous system has been well documented. Till now, expression of EphA4 was only investigated in several types of human cancer including gastric cancer, lung cancer, and pancreatic ductal adenocarcinoma [16, 17, 20, 21]. Mariko et al detected the expression of EphA4 mRNA and protein in gastric cancer cell lines, fresh specimens, and paraffin-embedded tumor specimens by using semi-quantitative real-time reverse transcript PCR and immunohistochemistry [16]. They found that overexpression of EphA4 mRNA was observed in 73% of gastric cancer cell lines, and 42% of gastric cancer tissues. The increased expression of EphA4 protein was observed in 48% of gastric cancer tissues. The expression of EphA4 protein was significantly associated with depth of invasion and recurrence. Patients with EphA4 positive cancer had significantly shorter overall survival periods. More recently, another group reported their research of EphA4 in gastric cancer [20]. The expression of EphA2, EphA4, and ephrinA1 were evaluated immunohistochemically in 222 patients with gastric adenocarcinoma. High expression of EphA2, EphA4 and ephrinA1 significantly correlated with the depth of invasion, metastatic lymph nodes, pathological stage, and distant metastasis or recurrent disease. High expression of EphA2, EphA4, and ephrinA1 were significantly associated with poorer disease-specific survival. Liizumi et al performed expression of profile analysis of pancreatic ductal adenocarcinoma cells (PDAC) using a genome-wide cDNA microarray combined with laser microdissection and identified EphA4 up-regulated in PDAC cells [17]. To investigate the biological function of EphA4 in PDAC cells, they knocked down its expression by siRNA, which drastically attenuated PDAC cells viability. Their results suggested that EphA4 is likely to promote cancer cell growth. Oshima et al studied the expression of EphA4 mRNA in colorectal cancers and found that high expression of EphA4 mRNA might promote liver metastasis [22]. Guimaraes de Marcondes et al studied the role of EphA4 signaling in radiation-induced EMT-like phenotype in colorectal cancer cells [18, 19]. Using the progeny of the human colorectal cancer HT-29 cells that were submitted to irradiation, they demonstrated that the PI3K/AKT, Wnt/beta catenin signaling

pathways and ERK1/2 downstream of EphA4 receptor activation, which plays an important role in the regulation of events related with the EMT development. Their data show that progeny of HT-29 radiation survivor cells having increased EphA4 activity, which induced cell migration, invasiveness by disorganization of adherens junction of E-cadherin. Those data indicate that EphA4 gene may have roles as a cancer promote gene. Very interestingly, Saintigny et al carried out a gene expression assay in 2 independent cohorts of lung cancer by qPCR and western blot, and identified EphA4 and ephrinA1 expression were associated with an improved outcome in patients with lung adenocarcinoma. The biological effects of EphA4 in lung cancer cell lines were assayed following overexpression and knockdown. EphA4 overexpression reduced cell migration and invasion but did not affect cell cycle, apoptosis, or drug sensitivity. Their data indicated that EphA4 may act as a tumor suppressor in some cancers [21]. We previously detected expression and clinical significance of EphA5 in colorectal carcinomas [23]. We found that EphA5 is reduced in colorectal cancer, which is associated with lymph node metastasis, advanced TNM stage, and poor prognosis. Our data indicate that EphA5 receptor may be a tumor suppressor in colorectal carcinoma.

In this study, we evaluated the expression of EphA4 protein in surgical specimens of colorectal carcinomas and found that EphA4 was overexpressed in tumors comparing with adjacent normal mucosa. High expression of EphA4 was more often detected in younger patients, tumors with big size, deep depth of wall invasion, lymph node metastasis, and advanced TNM stage. The survival analyses showed that patients whose carcinoma exhibited high expression of EphA4 had a poor outcome. To our knowledge, this is first time explore expression and clinical significance of EphA4 protein in colorectal carcinoma. Our data suggest that EphA4 protein plays roles in promoting invasiveness, progression and metastasis of colorectal carcinoma.

In addition, we noticed that EphA4 protein was strongly expressed in 2.9% (score 3) of normal mucosa, though it was strongly expressed in 47.1% of colorectal carcinoma. This may be interpreted that the function of EphA4 gene is

complicated in addition to be as an oncogene. The function of EphA4 in colorectal cancer and other types of cancer is worth being intensively studied.

In summary, our results indicate that EphA4 may play a role in invasion and metastasis of colorectal carcinoma. The EphA4 protein expression may be used as a potential prognostic marker in colorectal carcinoma.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jiandong Wang, Department of Pathology, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, China. E-mail: jd_wang@outlook.com; Dr. Shu Zhang, Department of Pathology, Affiliated Hospital of Nantong University, Xi Si Road, Nantong 226001, Jiangsu, China. E-mail: ntfyzhangshu@163.com

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