

Reduced expression of EphA5 is associated with lymph node metastasis, advanced TNM stage, and poor prognosis in colorectal carcinoma

Shudong Gu^{1,2}, Jia Feng³, Qin Jin³, Wei Wang³ and Shu Zhang³

¹Department of Oncology, Affiliated Hospital of Nantong University, Nantong, ²Department of Tumor Biological Treatment, The Third Affiliated Hospital of Soochow University, Changzhou and ³Department of Pathology, Affiliated Hospital of Nantong University, Nantong, Jiangsu, China

Summary. Colorectal carcinoma (CRC) is the third most common cancer and a major cause of morbidity and mortality throughout the world. The prognosis of patients has improved markedly over the last 15 years because of the introduction of new therapy including molecular target drugs. To comprehensively understand the molecular process of carcinogenesis of colorectal carcinoma is essential for the diagnosis, prognosis and treatment. EphA5 is a member of the Eph family and plays a critical role in carcinogenesis of lung cancer, prostate cancer, and breast cancer. The expression profile and the role of EphA5 in colorectal carcinoma have not been well investigated till now. In this study, a set of colorectal carcinoma specimens was subjected to immunohistochemical assay using an EphA5 specific antibody. The relationship between the expression of EphA5 and clinicopathological parameters was statistically analyzed. EphA5 was positively expressed in all tested normal mucosa specimens (120/120, 100%) and partly in colorectal carcinoma specimens (70/120, 58.3%). The loss of EphA5 protein was associated with depth of wall invasion ($P=0.002$), poor tumor differentiation ($P<0.001$), lymph node metastasis ($P<0.001$), and advanced TNM stage ($P<0.001$). The survival analysis showed that patients with reduced expression of EphA5 had a poor overall survival ($P=0.017$). Our data indicate that EphA5 receptor may be a tumor suppressor in colorectal carcinoma and it

may be a new therapeutic target for colorectal carcinoma.

Key words: EphA5, Receptor tyrosine kinase, Colorectal carcinoma

Introduction

Colorectal carcinoma (CRC) is the third most common cancer worldwide with more than one million patients diagnosed each year (Siegel et al., 2016), and remains the second most common cause of cancer-related death in North America and Western Europe. In China, the incidence of colorectal cancer is increasing, and it is the leading cause of cancer-related death as well (Chen et al., 2016). Most colorectal carcinoma arises from premalignant adenoma that requires years or even decades to progress to invasive disease. The development of CRC is a complicated and incompletely understood process which is determined by environmental and genetic factors, including the activation of multiple oncogenes and the inactivation of tumor suppressor genes. That deficient DNA mismatch repair genes or mutation of the APC (adenomatosis polyposis coli) tumor suppressor gene lead to activation of Wnt/beta-catenin signaling have been identified to be the corresponding molecular events, respectively. Thus identification of the precise molecular biomarkers which can serve as diagnostic and prognostic markers for CRC is extremely desirable.

Protein phosphorylation is an important intracellular communication mechanism for regulation of cellular

functions. Receptor tyrosine kinases are located at the cellular membrane. Erythropoietin-producing hepatocellular carcinoma (Eph) receptors are the largest superfamily of receptor tyrosine kinases, which include at least 14 distinct receptors (EphA1-8 and EphB1-6) and 8 ligands (ephrinA1-6 and ephrinB1-3) (Astin et al., 2010; Pasquale, 2010). Increasing evidence indicates that the Eph/ephrin signaling pathways are involved in both human physiological and pathological conditions (Hafner et al., 2006; Chen et al., 2008; Astin et al., 2010; Fu et al., 2010; Herath and Boyd, 2010; Li et al., 2015; Staquicini et al., 2015). Eph receptors activated by ephrin ligands play important roles in axon guidance and cell migration during development of the nervous system (Gao et al., 1998; Olivieri and Miescher, 1999). Aberrant expression of Eph/ephrin has been detected in human tumor tissues, which suggests their strong involvement in tumor metastasis, invasion, and prognosis (Surawska et al., 2004; Wang et al., 2013; Koshikawa et al., 2015; Staquicini et al., 2015; Dunne et al., 2016; Youngblood et al., 2016).

EphA5, also named as CEK7, EHK1, Hek7, and TYRO4 is located on chromosome 4q13.1. EphA5 was first identified from a chicken embryo expression library using anti-phosphotyrosine antibodies (Siever and Verderame, 1994). Similar to other Eph members, EphA5 has a single transmembrane spanning domain, an extracellular domain containing an immunoglobulin-like motif, a Cys-rich region and two fibronectin type-III repeats, and an intracellular region with a juxtamembrane domain. There are three isoforms of EphA5 cDNA produced by two alternative splices in primary transcripts. EphA5 is almost exclusively expressed in the nervous system and involved in its development (Bruce et al., 1999; Kimura et al., 2011; Li et al., 2015). Aberrant expression of EphA5 was found in several human cancers including breast cancer, prostate cancer and lung cancer (Fu et al., 2010; Li et al., 2015; Staquicini et al., 2015). To our knowledge, no study has been focused on colorectal cancer. In the present study, a set of colorectal carcinoma specimens was subjected to immunohistochemical assay using an EphA5 specific antibody. The relationship between the expression of EphA5 and clinicopathological parameters and outcome of patients was statistically analyzed.

Materials and methods

Patients and tissue specimens

A total of 120 surgically resected specimens of colorectal carcinoma were collected from the archives of the Department of Pathology, Affiliated Hospital of Nantong University. All patients underwent complete excision of primary tumor from January 2012 until December 2015, and had not received preoperative chemotherapy. The patients included 66 males and 54 females, with a median age of 61.4 years (age range from 32 to 85). Routine histological examination was

performed with hematoxylin and eosin staining. All carcinomas were classified in accordance with the criteria of the WHO (World Health Organization), Pathology and Genetics Tumors of the Digestive System (4th edition, 2010). The protocol used in this study was approved by the Research Ethics Board of the Affiliated Hospital of Nantong University, China. The detailed clinicopathological parameters of patients are listed in Table 1.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples were sectioned for immunohistochemistry at 4 μ m thickness. All the sections were deparaffinized using 100% xylene, dehydrated by an ethanol gradient, and then rehydrated with deionized water according to standard protocols. Heat-induced antigen retrieval was performed 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 polyclonal antibody raised against the human EphA5 (Abgent, San Diego, CA, USA) at a dilution 1:500, and 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 REAL EnVision Detection System, Dako, UK) for 30 min at room temperature. Color development was performed with 3,

Table 1. Correlation between the expression of EphA5 and clinicopathological parameters in 120 colorectal carcinomas

Parameters	No.	EphA5 expression		P-value
		-	+	
Sex				
male	66	30	36	0.457
female	54	20	34	
Age (years)				
≤55	22	12	10	0.232
>55	98	38	60	
Location				
rectum+sigmoid colon	82	30	52	0.114
colon	38	20	18	
Depth of wall invasion				
mucosa+submucosa	8	0	8	0.002
muscularis propria	20	4	16	
subserosa+serosa	92	46	46	
Tumor differentiation				
well	16	4	12	<0.001
moderately	82	28	54	
poor	22	18	4	
Lymph node metastasis				
yes	40	26	14	<0.001
no	80	24	56	
TNM stage				
I	22	2	20	<0.001
II	58	22	36	
III+VI	40	26	14	

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3'-diaminobenzidine (DAB). Nuclei were lightly counter stained with hematoxylin. Two pathologists independently assessed the immunostained slides. Any difference in immunohistochemical scores was resolved by a consensus.

Evaluation of EphA5 expression

Immunohistochemical staining of EphA5 was assessed by two pathologists in a blinded fashion without knowledge of the clinicopathological parameters. The staining intensity was evaluated as: 0=negative, 1=weak, 2=moderate, and 3=strong. According to the percentage of positive staining cells, the staining density was expressed semi-quantitatively as follows: 0, less than 5%; 1, 5 to 25%; 2, 25 to 50%; or 3, more than 50%.

The sum of these two parameters was calculated for each section. The scores of 0-2 were considered as negative expression while 3-6 were defined as positive expression of EphA5.

Follow-up

Follow-up data were collected from the patient's records and or telephone interview of patients. Overall survival (OS) was defined as the time from surgery to date of patient's death. The median period of follow-up time was 25.5 months (range from 7 to 38 months).

Statistical analysis

Data were analyzed using SPSS 15.0 software

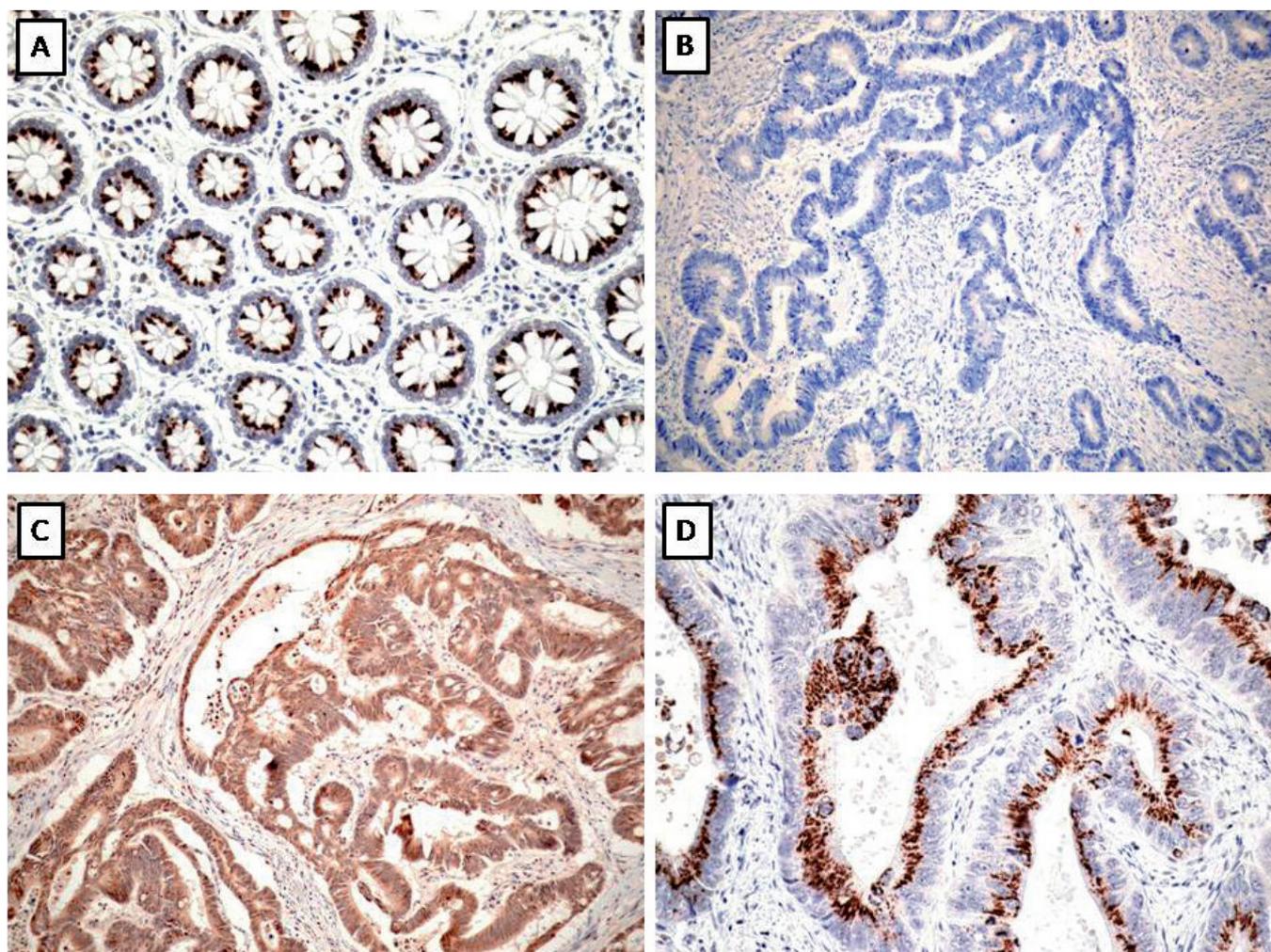


Fig. 1. Immunohistochemical staining of EphA5 in colorectal carcinomas. **A.** EphA5 protein was positively stained in normal mucosa cells as brown particles in cytoplasm. **B.** Negative staining of EphA5 in colorectal carcinoma. **C.** The positive expression of EphA5 was homogenously stained in cytoplasm of colorectal carcinoma cells. **D.** The positive expression of EphA5 was stained in cytoplasm of colorectal carcinoma cells as particles in cytoplasm. A, $\times 200$; B-D, $\times 400$.

(SPSS, Chicago, IL, USA). Comparisons of the clinicopathological variables and expression of EphA5 protein were conducted using the χ^2 test. The relationship between expression of EphA5 and patients overall survival was evaluated using the univariate Kaplan–Meier survival method and statistically checked with the log-rank test. All statistical results were considered significant when $P < 0.05$.

Results

Expression of EphA5 protein in colorectal carcinomas and normal mucosa

The immunostaining of EphA5 protein was shown as particles in cytoplasm or distributed homogeneously in cytoplasm (Fig. 1A,C,D). The positive staining of EphA5 was observed in all normal mucosa cells (100%).

A heterogeneous EphA5 staining pattern inter-specimens was detected in colorectal carcinoma tissue sections (Fig. 1B–D). Positive expression of EphA5 was found in 58.3% in colorectal carcinomas. Loss of the expression of EphA5 protein was detected in 41.7% of colorectal carcinoma cells compared with normal mucosa cells from same patients (Fig. 2A). The EphA5 protein was positively expressed in mucinous adenocarcinoma cells (Fig. 2B), and in Signet-ring cell carcinoma (Fig. 2C). Loss of expression of EphA5 protein was found in poorly differentiated colorectal carcinoma cells (Fig. 2D).

Association of EphA5 protein with clinicopathological parameters

The relationship between the expression of EphA5 protein and clinicopathological parameters was analyzed

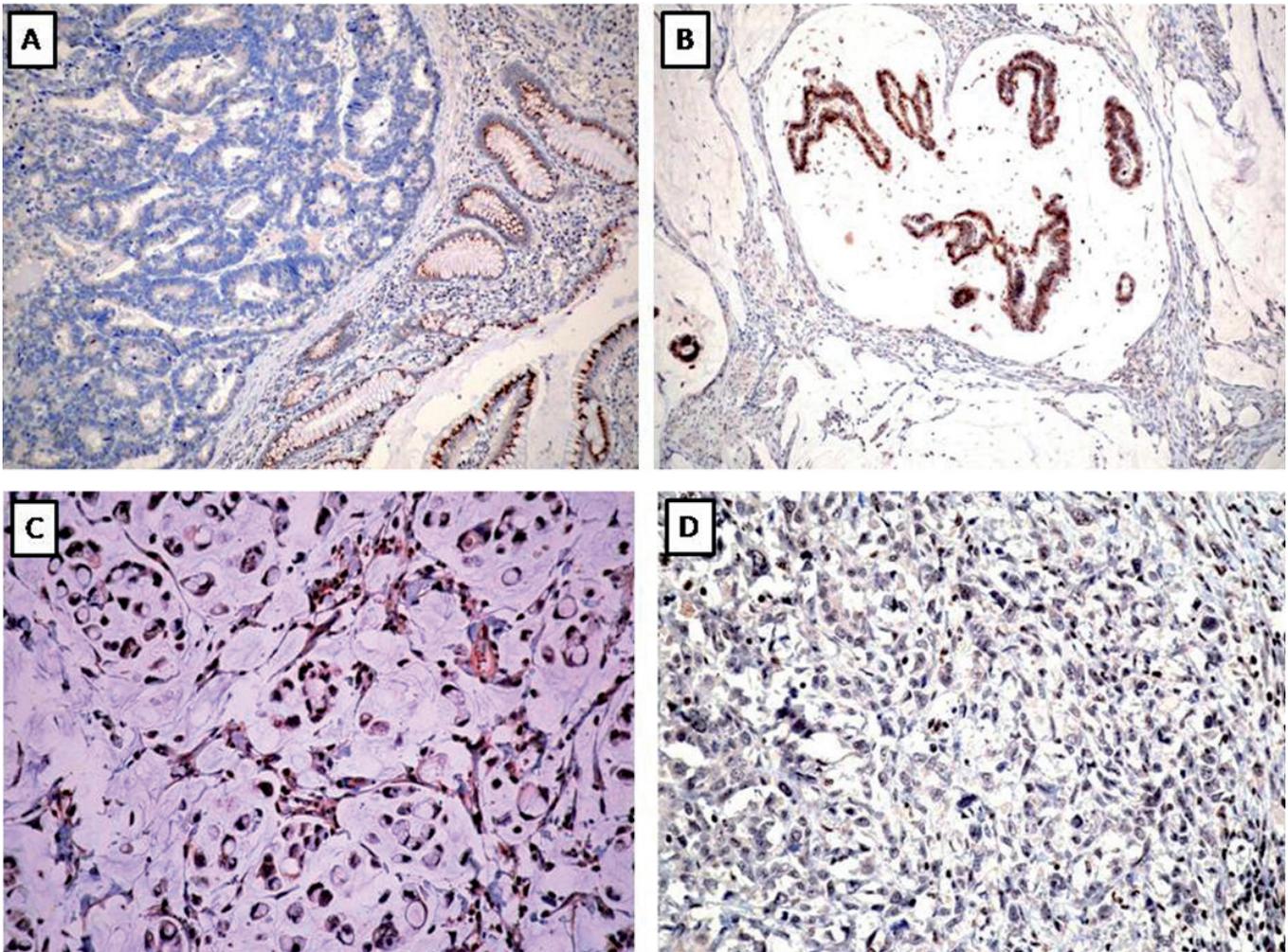


Fig. 2. A. The EphA5 protein was positively expressed in normal cells and negatively expressed in colorectal carcinoma cells. B. The EphA5 protein was positively expressed in mucinous adenocarcinoma cells. C. The EphA5 protein was positively expressed in Signet-ring cell carcinoma. D. Loss of expression of EphA5 protein in poorly differentiated colorectal carcinoma cells. A, B, $\times 200$; C, D, $\times 400$

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(Table 1). The expression of EphA5 protein was significantly related to depth of wall invasion ($P=0.002$), tumor differentiation ($P<0.001$), lymph node metastasis ($P<0.001$) and TNM stage ($P<0.001$). The patients with reduced expression of EphA5 had deeper invasion than those with EphA5 positive expression. The reduced expression of EphA5 more often occurred in poorly differentiated colorectal carcinomas than in moderate or well differentiated tumors. Patients with reduced EphA5 protein in colorectal carcinomas had more advanced tumor stage and lymph node metastasis.

Patients with reduced EphA5 protein had poor overall survival

We examined the association of EphA5 protein expression with the overall survival in 105 patients. The Kaplan-Meier survival analysis showed that patients with reduced expression of EphA5 had shorter overall survival than those with positive EphA5 protein expression (log-rank test, $P=0.017$, Fig. 3).

Discussion

EphA5 receptor, identified from chicken embryo expression library is well documented in the development of nervous system. EphA5 protein is widely distributed in the central nervous system, and the distribution of the neuronal staining is not uniform. Data suggested that EphA5 plays a role in neural plasticity, cell-cell recognition, and topographical orientation of neuronal systems (Bruce et al., 1999). EphA5 and its ligand ephrina5 are involved in the guidance of retinal, cortical and hippocampal axons during development (Gao et al., 1998).

Aberrant expression of EphA5 receptor was reported

in several types of human cancer. Fu et al detected the expression of EphA5 transcript in human breast cancer cell lines (Fu et al., 2010). They found that EphA5 was expressed in the galactophore cell line HBL-100 and breast cancer cell lines ZR-75-30 and SKBR-3. In contrast, the expression of EphA5 was lost in breast cancer lines MCF-7, T47D, MDA-MB-231, Bacp37 and MDA-MB-435s. To investigate the mechanism underlying the silencing of EphA5 in breast cancer cell lines, they analyzed the methylation status of CpG island in promoter region of the EphA5 gene. Their results confirmed that hypermethylation of CpG island leads to the silencing of the EphA5 transcript in breast cancer cells. Furthermore, they determined the epigenetic silencing of the EphA5 gene in breast cancer specimens. EphA5 transcript was reduced in 67% of breast cancer samples, which is correlated with EphA5 gene hypermethylation. Li et al detected EphA5 expression in seven prostate cell lines, thirty-nine BPH and twenty-two primary prostate carcinomas (Li et al., 2015). Down-regulation or loss of EphA5 transcript and protein was detected 62% of prostate carcinomas, 5% of hyperplasias, and all 6 prostate carcinoma cell lines. Methylation of EphA5 promoter region was found in 71% of carcinoma samples, 8% of hyperplasias, and all 6 prostate carcinoma cell lines. More recently, Staquicini et al identified EphA5 is a functional molecular target in human lung cancer by using the high throughput combinatorial screening of a phage displayed random peptide library (Staquicini et al., 2015). They confirmed that EphA5 is a novel molecular target of lung cancer and a regulator of IR-induced cell cycle checkpoint and DNA damage repair.

In this study, we investigated the expression of EphA5 protein in a set of colorectal carcinoma specimens. To our knowledge, this is the first time to analyze the expression of EphA5 protein and its relationship with clinicopathological parameters in colorectal carcinoma. We found that EphA5 reduced in 41.7% colorectal carcinoma cells in comparison to normal mucosa. The possible molecular mechanisms lead to loss of EphA5 in colorectal carcinoma including genetic and epigenetic disorder. The genomic mutations, deletion, and hypermethylation of CpG island at the promoter region of the EphA5 gene will be assayed in the next study.

Despite progressive clinical advances that have been carried out in the last decade to decrease or prevent metastasis, the survival of patients has not greatly changed. It is therefore a clinical challenge to develop alternative therapeutic strategies to improve the survival of these patients. Although significant advances have been made in the treatment of metastatic colorectal cancers, in particular the introduction of novel chemotherapies and targeted agents, including oxaliplatin and/or irinotecan-based regimens in combination with angiogenesis or epidermal growth factor receptor (EGFR) inhibitors, patient outcomes are still very poor (Sunakawa et al., 2016; Zhou et al.,

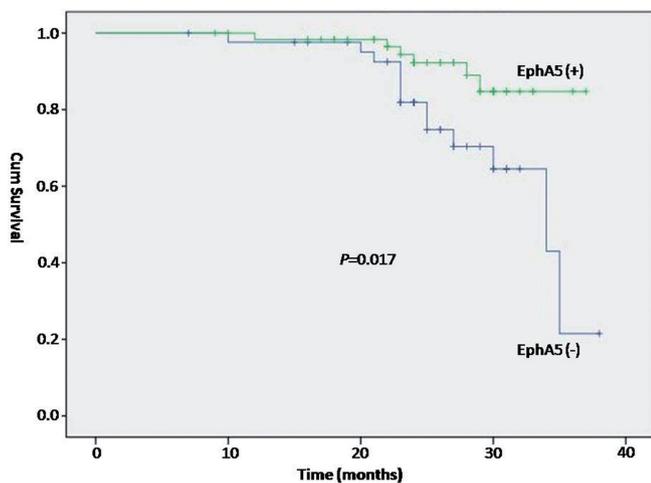


Fig. 3. Overall survival analysis using the Kaplan-Meier method. Log-rank test revealed that patients with negative expression of EphA5 showed significantly the poorer survival outcomes.

2016). Approximately 60% of patients receiving curative resection will undergo local recurrence or distant metastasis, and 85% of patients will relapse within the first 2.5 years after surgery (Dong et al., 2015; Tokunaga et al., 2015). Thus, a thorough understanding of the molecular mechanisms of colorectal cancer metastasis is urgently required to facilitate early diagnosis of individuals with a high risk of metastasis, and it is therefore a clinical challenge to develop alternative therapeutic strategies to improve the survival of these patients. Our data show that EphA5 protein expression in colorectal carcinoma is associated with depth of wall invasion, lymph node metastasis, and TNM stage. Patients with reduced expression of EphA5 had a poor overall survival. Our results suggest that the molecular mechanisms underlying EphA5 involved in invasion and metastasis need to be intensively investigated. EphA5 may be used as a new molecular marker for prediction of lymph node metastasis, prognosis and for the guiding clinical management of colorectal carcinoma.

In summary, EphA5 is reduced in colorectal carcinomas compared with normal mucosa. Loss of the expression of EphA5 is associated with the depth of wall invasion, lymph node metastasis, advanced tumor TNM stage and a poor prognosis. Our data show that EphA5 receptor may be a tumor suppressor in colorectal carcinoma and it may be a new therapeutic target for colorectal cancer.

Conflicts of interest. The authors declare that they have no conflict of interest.

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