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Downregulation of *EphA1* in colorectal carcinomas correlates with invasion and metastasis

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The Eph gene family has important roles in the developmental processes and may also be involved in the initiation, progression, and metastasis of certain types of cancers. In the present study, quantitative real-time reverse-transcriptase PCR was performed to detect the expression of *EphA1* transcript in 5 colon cancer cell lines and 75 colorectal carcinomas. Immunohistochemical staining was used to check the expression of *EphA1* protein in 20 colorectal adenomas and in 111 colorectal carcinomas specimens. *EphA1* protein expression was not completely consistent with transcript expression. *EphA1* protein was expressed in all adenomas and reduced in 54% colorectal cancers. Reduced expression of *EphA1* protein occurred more often in male patients ($P=0.028$) and in patients with poor differentiation ($P=0.027$), greater depth of wall invasion ($P=0.003$), lymph node metastasis ($P=0.034$), and advanced tumor stage ($P=0.003$). Patients with reduced *EphA1* expression had a poor overall survival ($P=0.059$). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively). Our data indicate that the *EphA1* may play different roles during the different stages of colorectal carcinoma progression.
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Keywords: *EphA1*; colorectal carcinoma; invasion; metastasis; survival

Colorectal carcinoma is one of the most common malignant tumors. There are estimated 112 340 new cases of colon cancer and 41 420 new cases of rectum cancer in USA, and estimated 52 180 cases of colorectal cancer patients died in 2007. The incidence and mortality rate of colorectal cancer rank the third in all types of cancers both of male and female patients.[1] The search for new molecular targets of early diagnosis, rational therapy, and prognosis is the current research hot spot.

Eph receptors, the largest subfamily of the receptor tyrosine kinases, are divided into two subfamilies, EphA and EphB, based on the sequence homology of their extracellular domains and their affinity to bind corresponding ligands, EphrinA and EphrinB (Eph Nomenclature Committee, 1997).[2] The Eph family of receptor tyrosine kinases has important roles in diverse biological processes including nervous system development,[3–5] angiogenesis,[6] and vascular system development.[7] A number of Eph receptors and their ephrins ligands are implicated in carcinogenesis.[8–16] *EphA1*, the first member of Eph receptors tyrosine kinase, was isolated from erythropoietin-producing hepatocellular carcinoma cell lines and is located on chromosome 7q34.[17] It is widely expressed in normal tissues including lung, small intestinal, kidney, bladder, thymus, and colon.[18] The expression level of *EphA1* in human cancers is variable. Overexpression of *EphA1* was observed in certain types of tumors including ovarian carcinoma,[19] and head and neck squamous carcinoma.[20] Reduced expression of *EphA1* was detected in prostate cancer cell lines,[21] breast carcinoma cell lines,[12] and basal-cell carcinomas and squamous-cell carcinoma specimens of the skin.[22] There was a marginal study of *EphA1*

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expression in colorectal carcinoma specimens, particularly, with respect to clinicopathologic parameters. In this study, we performed quantitative real-time reverse-transcriptase PCR (RT-PCR) and immunohistochemistry to detect the expression of *EphA1* mRNA and protein in a set of colorectal carcinomas and adenomas. And the association of *EphA1* expression levels with clinicopathologic parameters of colorectal carcinomas was analyzed. To our knowledge, this is the first description of the role of *EphA1* in colorectal progression and prognosis.

Materials and methods

Five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 were used in the present study. The cells were routinely cultured in Dulbecco's modified Eagle's medium (NISSUI Pharmaceutical Co., Tokyo, Japan), supplemented with 1 mmol/l L-glutamine, 10% fetal bovine serum (FBS; Life Technologies Inc.), and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

All the tissue samples in our study were collected from 111 patients with colorectal carcinoma and 20 patients with colorectal adenomas, as part of a study approved by the Research Ethics Board of the Nanjing Jinling Hospital, Clinical School of Medical College of Nanjing University. These patients had undergone surgery in Nanjing Jinling Hospital between 2004 and 2006 without any preoperative therapy. Among the 111 cases of colorectal carcinomas, 75 fresh tissue samples including cancer tissues and matched normal mucosas were immediately frozen in liquid nitrogen after the resection and then stored at −80°C for the preparation of the total RNA. Formalin-fixed and paraffin-embedded tumor tissues were sectioned at 4 μm thickness and stained with hematoxylin and eosin for the pathological identification. The patients included 66 men and 45 women. Ages ranged from 23 to 84 (median age: 59 years). The distribution of the tumors by sites of origin was as follows: the cecum and ascending colon, 26 tumors; the transverse colon, 4 tumors; the sigmoid colon, 14 tumors; and the rectum, 67 tumors. The clinicopathologic variables of the 111 patients of colorectal carcinoma were shown in Table 1. The tumor stage was classified according to the TNM classification of World Health Organization of 2007.

Quantitative Real-Time RT-PCR

The total RNA was extracted using the RNA extraction reagent TRIzol (Invitrogen, CA, USA) according to the manufacturer's protocol. Single-strand cDNA was synthesized using 2 μg total RNA with an oligo(dT) primer. Quantitative real-time RT-PCR was performed to detect the *EphA1* transcript expression in colorectal carcinoma on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). The experiments were run in triplicate. The sense primer, antisense primer, and TaqMan probe for detection of *EphA1* were designed according to the *EphA1* mRNA sequence (GenBank accession number: NM_005232). The sense primer is 5′-ATCTTTGGGCTGCTGCTTGG-3′ and the antisense primer is 5′-GCTTGTCCTCTCGATCCACA TC-3′. The PCR products are 127 bp long. The TaqMan probe is 5′-(FAM) CGGTCACGCTGC CTCTGCTGCC (Eclipse)-3′. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control (GenBank accession number: NM_002046). The sense primer is 5′-CCAGGTGGTCTCCTCTGACTT-3′ and the antisense primer is 5′-GTTGCTGTAGCCAAATTCGTT GT-3′. The PCR products are 130 bp long. The probe is 5′-(FAM) AACAGCGACACCCACTCCTCCACC (Eclipse)-3′. The values of *EphA1* mRNA expression were normalized using the *GAPDH* expression. The primers and probes for *EphA1* and *GAPDH* were synthesized by TaKaRa Biotechnology Inc. (Dalian, China).

The reaction mixture consisted of 3.0 μl 10 × buffer; 3.0 μl 2.0 μmol/l deoxy-ribonucleoside triphosphates (dNTPs; Invitrogen); 3.0 μl 3.0 μmol/l sense primer; 3.0 μl 3.0 μmol/l antisense primer; 1.0 μl 3.0 μmol/l fluorescence probe; 0.20 μl 5 U/μl Takara ExTaq Hotstart Taq (TaKaRa Biotechnology), 0.6 μM 5-carboxy-X-rhodamine reference dye (Invitrogen), 2.0 μl cDNA template, and distilled water for a total volume of 30 μl. The PCR cycling conditions were used as follows: 2 min at 95°C, followed by 40 amplification cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 1 min.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples used for immunohistochemistry were sectioned at 2 μm thickness. All the sections were deparaffinized using xylene, dehydrated by gradient ethanol, and then rehydrated with deionized water. Heat-mediated antigen retrieval was run 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 COOH terminus of the human *EphA1* receptor (dilution 1:100, ABGENT, San Diego, USA) was performed overnight at 4°C. After washing with phosphate-buffered saline (pH 7.4), the sections were then incubated with secondary antibody (Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3′-diaminobenzidine. Nuclei were counterstained with hematoxylin.

Table 1 Correlation between expression of EphA1 transcript and clinicopathologic parameters in 75 colorectal carcinomas

	Case number	*N/T>2*	*N/T0.5-2*	*N/T<0.5*	P-*value*
Overall	75	28	17	30	—
Sex					
Male	44	17	10	17	0.952
Female	31	11	7	13	
Age (years)					
≤55	36	13	8	15	0.96
>55	39	15	9	15	
Location					
Rectum+sigmoid colon	57	23	13	21	0.556
Colon	18	5	4	9	
Tumor size (cm)					
≤5	49	18	11	20	0.98
>5	26	10	6	10	
Depth of wall invasion					
Mucosa+submucosa	4	3	1	0	0.278
Muscularis propria	19	7	2	10	
Subserosa+serosa	52	18	14	20	
Tumor differentiation					
Well+moderate	48	15	13	20	0.36
Poor+mucinous	27	13	4	10	
Lymph node metastasis					
Negative	46	18	9	19	0.72
Positive	29	10	8	11	
Clinical stage (TNM)					
I	20	9	2	9	0.617
II	25	8	7	10	
III+IV	30	11	8	11	
Stage (Dukes)					
A+B	45	17	9	19	0.78
C	30	11	8	11	

N/T, normal–tumor; TNM, tumor, node, metastasis.

The immunostaining results were evaluated independently by three pathologists. The different results were unified by consensus. The score of *EphA1* expression was made semiquantitatively by assessing the percentage of stained cells and the staining intensity in both tumor tissue and normal mucosa. The percentage of positive cells was rated as follows: 0 score for 0–5%, 1 score for 6–25%, 2 scores for 26–50%, and 3 scores for more than 50%. The staining intensity was rated as follows: 0 score for no staining, 1 score for weak staining, 2 scores for moderate staining, and 3 scores for strong staining. The scores from the percentage and intensity were added to an overall score. The expression of the *EphA1* protein in colorectal carcinomas was categorized into downregulation, upregulation, and no difference by comparing the overall score in tumor tissue *vs* matched normal mucosa.

Methylation-Specific PCR

Genomic DNA was modified by sodium bisulfite, as described by Clark *et al*.[23] Primers were designed to discriminate between methylated and unmethylated alleles after sodium bisulfite treatment. Primer sequences were chosen for the regions containing frequent CpG near the 3′-end of the primers to provide maximum discrimination between methylated and unmethylated DNA. Aliquots (2 μl) were amplified in a 30-μl reaction mixture consisting of 1 × buffer (10 mM Tris-HCl, 2.0 mM $MgCl_2$, 50 mM KCl, pH 8.3), 1 U Takara ExTaq Hotstartaq, 260 μM dNTPs, and 0.3 μM of the primer sets. The PCR reaction involved 2 min at 95°C, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and finally 10 min at 72°C. The methylation-specific primers were 5′-ATTCGGGTTATTGTTTTAGGTT TC-3′ (forward) and 5′-GAAAATCGATACCTTC CTTAACG-3′ (reverse). The PCR products were 129-bp long. Unmethylation-specific primers were 5′-ATTTGGGTTATTGTTTTAGGTTTTG-3′ (forward) and 5′-ACAAAAATCAATACCTTCCTTAACAC-3′ (reverse). Primer sets for detection of methylated and unmethylated DNA were located at the same sites of genomic sequence (forward primer was located at −35 to −12 from translation start site; reverse primer was located at 71–93; Figure 4). The

PCR products were 131-bp long. The PCR products were separated on 8% nondenaturing polyacrylamide gel, followed by ethidium-bromide staining.

Statistical Analysis

The χ^2-test was adopted to determine differences among intergroup variables by use of SPSS 15.0. software (SPSS, Chicago, IL, USA). Kaplan–Meier survival analysis was used to examine the relationship between categorical groups and survival for univariate analysis. A *P*-value <0.05 was considered statistically significant.

Results

Expression of the *EphA1* Transcript in Colon Cancer Cell Lines and in Colorectal Carcinomas

RT-PCR was performed to detect the expression of *EphA1* transcript in colon cancer cell lines DLD1, HT29, HCT116, SW480, and SW620. *EphA1* mRNA was detected in all the colon cancer cell lines (Figure 1). Quantitative real-time RT-PCR was used to detect the expression of *EphA1* transcript in 75 fresh specimens of colorectal carcinomas. The ratio of normal–tumor (N/T) was based on the relative expression level of *EphA1* transcript in paired normal mucosa and the tumor tissues of the same patient. The results were classified into three groups according to the ratio of the two: downregulation (N/T > 2), upregulation (N/T < 0.5), and no difference (N/T 0.5–2). Down and upregulation of *EphA1* transcript were observed in 37% (28 of 75) and 40% (30 of 75) of the specimens, respectively (Table 1). The association of expression of *EphA1* transcript with the sex, age, tumor site, size, depth of wall invasion, differentiation, clinical stage, lymph node metastasis, and Dukes stage were analyzed. No any significant difference between the *EphA1* transcript expression and these clinicopathologic parameters was found. The data were summarized in Table 1.

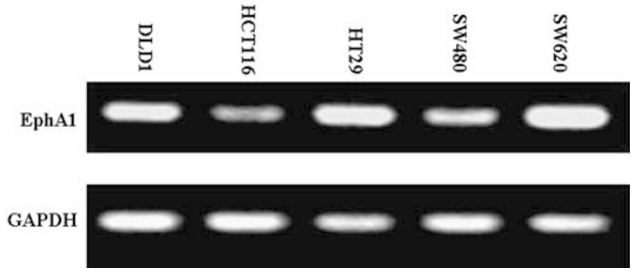

Figure 1 Expression of *EphA1* in colon cancer cell lines DLD1, HCT116, HT29, SW480, SW620. Housekeeping gene *GAPDH* was used as an internal control.

Expression of *EphA1* Protein in Colorectal Carcinomas and Adenomas

The *EphA1* protein was detected in most of the normal mucosa cells (Figure 2a①). The adenoma cells expressed *EphA1* protein diffusely (Figure 2a②). A heterogeneous *EphA1*-staining pattern between cells was observed in carcinoma tissue sections. The up and downregulations of the *EphA1* protein were observed in 31% (34 of 111) and 54% (60 of 111) cases of colorectal carcinomas, respectively (Table 2; Figure 2a③④). The immunostaining of *EphA1* was observed as particles in cytoplasm or distributed homogenously in cytoplasm (Figure 2b⑤⑥).

The Significance of Reduced Expression of the *EphA1* Protein in Colorectal Carcinoma

The expression of *EphA1* protein was significantly related to sex, depth of wall invasion, differentiation, lymphatic metastasis, and clinical stage. The reduced expression of *EphA1* was more often occurred in poorly differentiated colorectal carcinomas and mucinous adenocarcinomas than in well- and moderately differentiated cases ($P=0.027$). The patients with reduced *EphA1* protein had deeper serosa and subserosa invasiveness than those without *EphA1* downregulation ($P=0.003$). Colorectal carcinomas with reduced *EphA1* expression had more advanced tumor stage ($P=0.003$) and lymph node metastasis ($P=0.034$). Reduced expression of the *EphA1* protein was more often detected in male than in female patients ($P=0.028$). There was no significant association with other clinicopathologic variables (Table 2).

Reduced *EphA1* Protein Expression is Associated with Poor Survival in Patients with Colorectal Carcinoma

We examined the association of *EphA1* protein expression with clinical outcome. The Kaplan–Meier survival analysis showed patients with reduced *EphA1* expression had shorter survival than those with high *EphA1* expression (log-rank test, $P=0.059$; Figure 3①). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P=0.034$ and 0.015, respectively; Figure 3②③).

Detection of Methylated *EphA1* DNA in Colon Cancer Cell Lines

Five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 were checked for methylation status at promoter-associated region of *EphA1* by methylation-specific PCR. Methylated DNA of *EphA1* was detected in all five tested colon cancer cell lines (Figure 4).

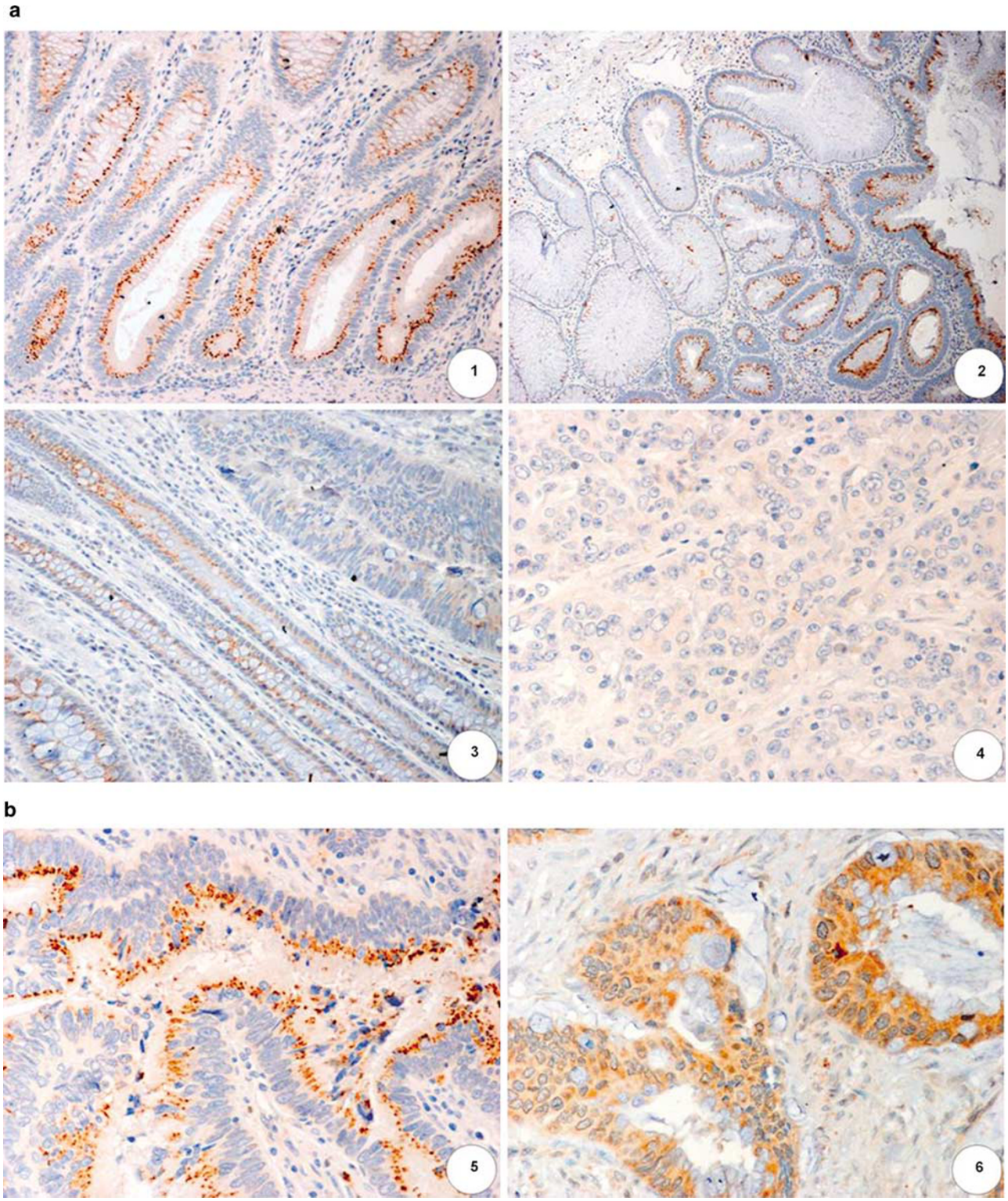

Figure 2 (**a**) Immunohistochemical staining of *EphA1* in colorectal carcinomas. ① The expression of *EphA1* in normal mucosa (EnVision, original magnification ×200). ② Upregulation of *EphA1* in adenoma cells (right lower) compared with normal mucosas (left upper; EnVision, original magnification ×100). ③ Downregulation of *EphA1* in carcinoma cells (right upper) compared with normal mucosas (left lower; EnVision, original magnification ×200). ④ Lost expression of *EphA1* in poorly differentiated carcinoma cells (EnVision, original magnification ×400). (**b**) ⑤ Strong immunoreactivity was detected in moderately differentiated carcinoma cells. The subcellular localization of *EphA1* protein revealed the accentuation of golgiosome (EnVision, original magnification ×400). ⑥ *EphA1* protein was expressed homogeneously in cytoplasm (EnVision, original magnification ×400).

Table 2 Correlation between EphA1 protein expression and clinicopathologic parameters in 111 colorectal carcinomas

	Case number	*Downregulation*	*No difference*	*Upregulation*	P-value
Overall	111	60	17	34	—
Sex					
Male	66	37	14	15	0.028
Female	45	23	3	19	
Age (years)					
≤55	49	31	8	10	0.109
>55	62	29	9	24	
Location					
Rectum+sigmoid colon	81	40	12	29	0.144
Colon	30	20	5	5	
Tumor size (cm)					
≤5	80	40	14	26	0.352
>5	31	20	3	8	
Depth of wall invasion					
Mucosa+submucosa	10	3	3	4	0.003
Muscularis propria	24	7	3	14	
Subserosa+serosa	77	50	11	16	
Tumor differentiation					
Well+moderate	80	37	15	28	0.027
Poor+mucinous	31	23	2	6	
Lymphatic metastases					
Negative	66	29	12	25	0.034
Positive	45	31	5	9	
Clinical stage (TNM)					
I	28	7	5	16	0.003
II	37	22	7	8	
III+IV	46	31	5	10	
Stage (Dukes)					
A+B	65	29	12	24	0.06
C	46	31	5	10	

N/T, normal–tumor; TNM, tumor, node, metastasis.

Discussion

The Eph gene family is the largest subfamily of receptor tyrosine kinase including at least 16 receptors and 9 ligands. The role of Eph family in developing processes has been well documented. The interaction of Eph receptors and their ligands controls the cells repulsion and movement during tissue patterning in embryonic development. However, the roles of Eph and Ephrin proteins in tumorigenesis are not clearly established. The Eph family was initially known as a putative oncogene based on their overexpression in certain types of human cancers. The *EphA2* receptor is overexpressed in colorectal,[24] gastric,[25] ovarian,[11] and esophageal squamous-cell carcinoma.[26] The *EphA4* receptor is overexpressed in pancreatic ductal adenocarcinoma,[27] and *Ephrin B1* is overexpressed in ovarian carcinoma.[28] More recently, increasing data have shown that some members of Eph receptors and Ephrin ligands have roles of tumor suppressor. A representative example is that certain EphB subfamily proteins, including *EphB2*, *EphB4*, suppress colorectal cancer progression through Wnt signal pathway.[9,10,29]

The *EphA1* receptor is widely expressed in human normal tissues. However, its expression levels in different types of human tumors are greatly diverse, and its role in tumorigenesis is still very vague. In the present study, we described the expression of the *EphA1* transcript and protein in colorectal carcinomas and analyzed the association of expression of *EphA1* with clinicopathologic parameters.

The *EphA1* transcript was detected in all five colon cancer cell lines DLD1, HCT116, HT29, SW480, and SW620 by RT-PCR (Figure 1). The expression levels of *EphA1* mRNA in five colon cancer cell lines were different. There is a CG-rich region around *EphA1* translation start site (Figure 4a), the methylation, and unmethylation-specific primer sets were designed by using web software MethPrimer (http://www.urogene.org//methprimer/). Methylated and unmethylated DNA

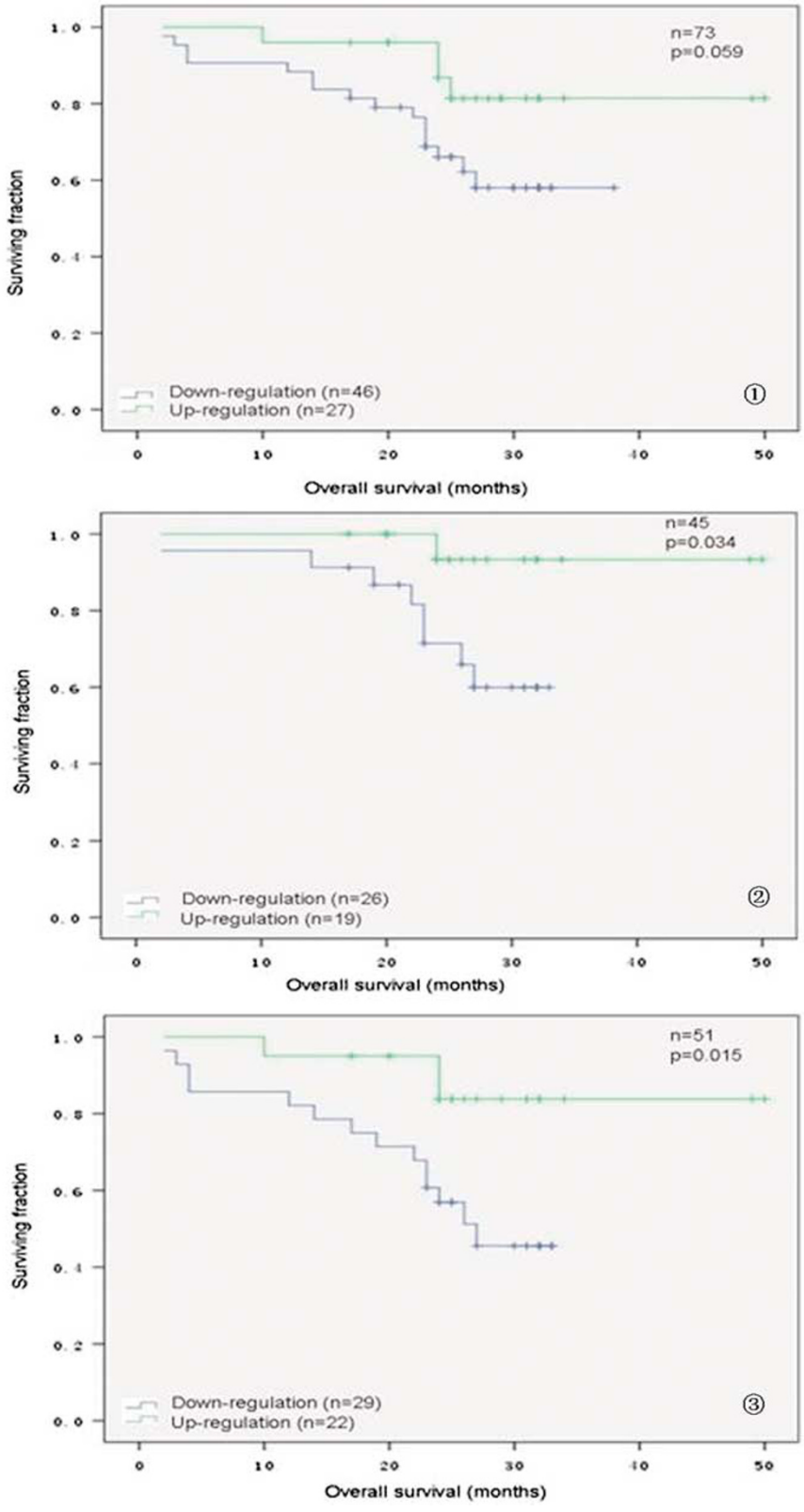

Figure 3 ① Kaplan–Meier plots of overall survival showed patients with *EphA1* downregulation had a shorter survival than those with *EphA1* upregulation ($P = 0.059$). ②③ Kaplan–Meier plots of overall survival showed reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P = 0.034$ and 0.015, respectively).

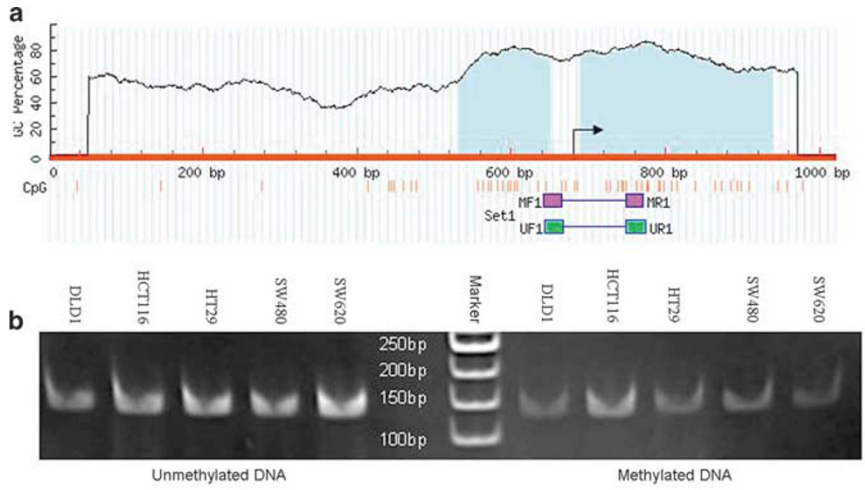

Figure 4 (**a**) Schematic show of the promoter-associated CpG island in *EphA1* and the location of PCR primer sets for specific detection of methylation and unmethylation *EphA1* DNA. The arrow showed the translation start site of *EphA1*. (**b**) Methylated and unmethylated DNAs of *EphA1* were detected in colon cancer cell lines.

of *EphA1* was detected in all tested five colon cancer cell lines (Figure 4b). To explore the methylation status of the promoter-associated CpG island of *EphA1* in tissue DNA of colorectal carcinomas and the association of the methylation with clinicopathologic parameters will be our next project.

The expression of *EphA1* transcript was downregulated in 37% and upregulated in 40% tested samples. No significant relation between the *EphA1* transcript expression and clinicopathologic parameters was found (Table 1). The *EphA1* protein was detected in most of the normal mucosa cells and diffusely expressed in adenoma cells (Figure 2). However, in the colorectal carcinomas, the *EphA1* expression was showed heterogeneity in carcinoma cells both of intra- and inter-samples. Our data suggest that *EphA1* protein was partly lost in the transition from adenomas to adenocarcinomas. Reduced expression of *EphA1* protein occurred more often in male patients ($P=0.028$) and in patients with poor differentiation ($P=0.027$), greater depth of wall invasion ($P=0.003$), lymph node metastasis ($P=0.034$), and advanced tumor stage ($P=0.003$). These data show that *EphA1* may be involved in the progression of colorectal carcinomas. Although *EphA1* has a closer homologous sequence and more similar structure to *EphA2* than any other Eph receptor,[30] our results show that there are great different features between them in terms of protein expression and its relation to clinicopathologic parameters in colorectal carcinomas. *EphA2* expression is present at the cytomembrane of the normal colorectal epithelium. However, *EphA2* immunoreactivity in colorectal carcinoma cells was diffusely distributed throughout the cytoplasm, with little staining of the cytomembrane.[24] In this study, different subcellular localization of EphA1 was found. In normal mucosa gland and adenoma cells, the immunostaining of EphA1 was showed dense brown particles in the Golgi's body. This staining pattern was only showed in parts of well-differentiated carcinoma cells. In poorly differentiated carcinoma and parts of well-differentiated carcinoma cells, the EphA1 was diffusely stained in cytoplasm. The staining pattern of EphA1 in skin ulcers was also altered, in which EphA1 was expressed in keratinocytes adjacent to the rim of the ulcer with an intense cytoplasmic staining, but with a membranous staining in those distant from the rim of the same ulcer.[22] The mechanism for this altered EphA1 immunostaining pattern is unknown. In addition, overexpression of *EphA2* is associated with metastasis and stage of the cancer.[24] However, in this study, reduced expression of *EphA1* occurred more often in patients with advanced tumor stage and lymph node metastasis. Our data suggest that *EphA1* and *EphA2* play different roles in the progression of colorectal carcinomas.

The expression of *EphA1* protein was not completely consistent with the transcript expression in 75 samples, in which only 35% (26 of 75) showed consistency. This pattern was also reported on *EphB4* expression in breast carcinomas,[31] *EphA2* expression in bladder carcinomas,[32] and *EphA7* expression in hepatocellular carcinomas.[18] The post-transcription regulation mechanisms interpreted *EphB4* and *EphA2* differential expression between protein and transcript. Upregulation of *EphA7* mRNA in hepatocellular carcinomas may be attributable to higher vascularization in the investigated tumor, resulting in intercellulalr cross-contaminations. In the present study, *EphA1* staining was not observed in stromal cells, vascular endothelial cells, or lymphocytes; we postulate that post-transcription, post-translation regulation mechanisms, or quick degradation of unstable *EphA1* protein are the reasons of inconsistent expression of *EphA1* mRNA and protein.

Follow-up information was available in 73 patients with a follow-up duration of 2–50 months (median time: 25 months), including 46 cases with reduced *EphA1* expression and 27 cases with *EphA1* upregulation. The overall survival rate in patients with *EphA1* downregulation was shorter than that in patients with *EphA1* upregulation (log-rank test, $P = 0.059$). Reduced *EphA1* expression in patients over 55 years or with rectal cancers and sigmoid colon cancers is associated with a poor overall survival ($P = 0.034$ and 0.015, respectively). The protective roles of *EphA1* protein in aged colorectal patients and patients with rectal cancers and sigmoid colon cancers are more obvious.

In summary, reduced expression of the *EphA1* protein in colorectal carcinomas is related to invasiveness, differentiation, metastasis, stage, and prognosis. Our data implicate that *EphA1* receptor may play different roles in the different stage of progression of colorectal carcinomas.

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