Pathology & Oncology Research (2020) 26:541–549
https://doi.org/10.1007/s12253-018-0562-x

ORIGINAL ARTICLE

Receptor Tyrosine Kinase EphB3: a Prognostic Indicator in Colorectal Carcinoma

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Received: 13 June 2018 / Accepted: 20 November 2018 / Published online: 7 December 2018

Abstract

Although EphB3 expression is down-regulated in colorectal cancer (CRC) cells compared with normal intestinal epithelial cells, the relationship between EphB3 expression and clinicopathological parameters in CRC is unclear. We examined EphB3 expression in 128 CRC tissue specimens and in 19 adenoma specimens using immunohistochemistry. The relationships between EphB3 expression and clinicopathological parameters, *KRAS* mutations, *BRAF* V600E mutation, MSI and survival were evaluated using Spearman's rank correlation and Kaplan–Meier survival analyses, respectively. CpG methylation in the EphB3 promoter was examined in four human CRC cell lines and tissues. EphB3 was strongly expressed in all normal intestinal epithelial cells (128/128) and adenoma cells (19/19). In CRC tumor cells, EphB3 expression was negative or weak in 41.4% (53/128), moderate in 26.6% (34/128), and strong in 32.0% (41/128) of samples. EphB3 expression was negatively associated with invasive depth ($P = 0.016$, $r_s = -0.213$), lymph node metastasis ($P = 0.000$, $r_s = -0.490$), and TNM stage ($P = 0.000$, $r_s = -0.390$), and was positively associated with poor differentiation ($P = 0.001$, $r_s = 0.290$), *BRAF* V600E mutation ($P = 0.008$, $r_s = 0.235$), and longer overall survival ($P < 0.001$). In multivariate analysis, EphB3 expression ($P = 0.007$) and lymph node metastasis ($P < 0.001$) were independent prognostic factors for poor survival. Hypermethylation of the EphB3 promoter was detected in cell lines and CRC tissues. EphB3 is down-regulated in CRC compared to normal mucosa. Hypermethylation of CpG island is contributed to downregulation of EphB3 in CRC. EphB3 expression in tumor cells may be a useful prognostic indicator for patients with CRC.

Keywords Colorectal cancer · EphB3 · Hypermethylation · Lymph node metastasis · TNM stage

Introduction

Tyrosine kinase receptors and their ligands play important roles in regulating the function of normal cells, and they also contribute to oncogenesis. Eph proteins (erythropoietin-producing human hepatocellular receptors) are the largest subfamily of receptor tyrosine kinases. Eight type A (A1–8) and six type B (B1–6) Eph receptors have been described, and their ligands (ephrins) are similarly classified into A (A1–5) and B (B1–3) subtypes [1].Since EphA1 was first identified in 1987 [2], many Eph family members have been implicated in pathophysiological processes in multiple cell types and organs. For example, interactions between cell surface Eph receptors and their ephrin ligands results in bidirectional signaling important for developmental procedures such as axon pathfinding, neural crest cell migration, and boundary formation between segmented structures [3, 4], as well as many other processes important for normal physiology and homeostasis [5].

Recent evidence suggests that Eph and ephrin genes are upregulated or downregulated in many cancers, leading to tumor promotion and suppression, respectively

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[6–10]. For example, EphA2 regulates tumor initiation, neovascularization, and metastasis in a wide range of epithelial and mesenchymal cancers. EphA2 is expressed at high levels in colorectal cancer (CRC) and is significantly associated with liver metastasis, lymphatic vessel invasion, and clinical stage in CRC [11]. Similarly, high EphA2 mRNA and protein expression are associated with poor overall survival (OS) in advanced CRC. In contrast, EphA2 expression in ovarian cancer is more varied. Human ovarian cancer cell lines commonly used in research may express high or low EphA2 levels, whereas benign ovarian masses and ovarian tumors of low malignant potential express low levels, and most (75.9%) invasive ovarian carcinomas express high levels [12]. In one study, EphA2 overexpression was significantly associated with high tumor grade, advanced stage, and short median survival [7]. High expression of EphA2 has also been detected in other human cancers, where it has tumor-promoting functions [6, 13–15].

In contrast to EphA2, EphA7 acts as a tumor suppressor in several human cancers [15–17]. EphA7 has the same major structural features as the other members of the Eph family. We previously reported that EphA7 is downregulated in CRC due to hypermethylation of CpG islands in the promoter region [8]. We found that EphA7 transcript was significantly reduced in CRC compared to normal mucosas. EphA7 Promoter hypermethylation and reduced expression is also observed in human germinal center B cell lymphoma [18], esophageal squamous cell carcinoma [16], and prostate cancer [17]. EphA7 is a tumor suppressor in follicular lymphoma. A soluble splice variant of EphA7 interferes with EphA2 and blocks oncogenic signals in lymphoma cells. By fusing splice of EphA7 and the anti-CD20 antibody, a directly target therapy to lymphomas was tested [10].

Like other Eph receptors, EphB3 (also known as EK2, ETK2, HEK2, and TYRO6) [19] plays important roles in organ development, particularly the nervous system [20]. Several studies have examined the roles of EphB3 in human cancer. EphB3 expression is upregulated in squamous cell lung cancers due to gene amplification [21], and Ji et al. showed that EphB3 upregulation in non-small-cell lung cancer (NSCLC) correlates with tumor size, differentiation, and metastasis [22]. In NSCLC cell lines, EphB3 overexpression accelerates cell proliferation and migration and promote xenograft tumorigenicity, whereas down-regulation inhibits cell proliferation and migration and suppresses tumor growth and metastasis in vivo [22]. Interestingly, Li et al. reported that the ligands of EphB3, ephrin-B1 and ephrinB2, are down-regulated in NSCLC, resulting in reduced tyrosine phosphorylation of EphB3. These authors found that EphB3 overexpression and elevated kinase activity inhibited NSCLC cell migration and metastasis [23].

The function of EphB3 in CRC has been explored recently. EphB3 and ephrinB1 are expressed in complementary domain in the intestinal epithelium and inversely controlled β-catenin/TCF signaling [24]. Overexpression of EphB3 in HT-29 CRC cells suppresses growth and induces apoptosis in vitro and in vivo [9]. A CRC cell type-specific transcriptional enhancer has been identified at the 5′-flanking region of the human EphB3 gene [25]. Inactivation of EphB3 in CRC cells was shown to result from dysfunction of this enhancer element as a consequence of defective Notch signaling.

Materials and Methods

Colon Cancer Cell Lines

The CRC cell lines HCT116, HT29, SW480, and SW620 were obtained from the cell resource center of the Shanghai Institute of life Sciences, Chinese Academy of Sciences and were cultured in RPMI 1640 medium (HyClone, Thermo Scientific, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin G, and 100 μg/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Patients and Tissue Samples

Tissue samples (including normal mucosa, tumor, and adenoma) were obtained from 128 patients with primary CRC who underwent surgical resection at Affiliated Jiangyin Hospital, China, between January 1, 2010 and December 30, 2016. All patients were treated with surgical resection of the primary CRC at initial diagnosis, and distant metastases were resected when detected. None of the patients were treated with preoperative chemotherapy or radiotherapy. Follow-up data were collected from the patients' records and telephone interviews. Tumors were classified histopathologically in accordance with the World Health Organization classification. The median follow-up period was 27.9 months (range 16–67 months). OS was calculated as the time between the date of surgery and date of death. Detailed clinicopathological parameters are shown in Table 1. The study protocol was approved by the Ethics Committee of Affiliated Jiangyin Hospital, China.

Table 1 Relationship between EphB3 expression and clinicopathological parameters

	EphB3 expression			*P*	r_s
	0/1	2	3		
	53 (41.4%)	34 (26.6%)	41 (32.0%)		
Gender					
Male	29	29	22	0.820	−0.020
Female	24	5	19		
Age (years)					
< 55	10	4	8	0.984	0.002
≥ 55	43	30	33		
Location					
rectum	27	15	21	0.699	0.034
colon	18	10	11		
Sigmoid	8	9	9		
Depth of invasion					
Tis/T1/T2	7	8	14	0.016	−0.213
T3/T4	46	26	27		
Lymph node					
N0	20	15	32	0.000	−0.490
N1	18	10	8		
N2	15	9	1		
Differentiation					
Poor	12	2	2	0.001	0.290
Moderate	41	32	36		
Well	0	0	3		
TNM stage					
I/II	18	23	32	0.000	−0.390
III/IV	35	11	9		
KRAS					
Wild	28	22	23	0.673	−0.038
Mutation	25	12	18		
BRAF V600E					
Wild type	52	32	34	0.008	0.235
Mutation	1	2	7		
MSI					
MSS/MSI-L	48	30	33	0.168	0.123
MSI-H	5	4	8		

Immunohistochemistry (IHC)

Tissue specimens were formalin-fixed, paraffin-embedded, and cut into 4-μm-thick sections according to standard protocols. Briefly, sections were baked at 65 °C for 1 h and cooled to room temperature. Sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 for 10 min at room temperature. Antigen retrieval was performed by autoclaving the sections in 10 mM citrate buffer (pH 6.0) at 120 °C for 2 min. The sections were then washed with phosphate-buffered saline (PBS, pH 7.3),cooled to 30 °C,and incubated at 4 °C overnight with a polyclonal anti-EphB3 antibody (Abgent, San Diego, CA, USA) diluted 1:600 in antibody diluent solution (Zymed, Invitrogen). After washing, the sections were incubated with a secondary antibody (Dako REAL EnVision Detection System; Dako, UK) for 30 min at room temperature. Finally, color was developed with 3,3′-diaminobenzidine and the nuclei were lightly counterstained with hematoxylin. Sections were visualized with a microscope. The specificity of EphB3 antibody was tested using a peptide absorption test in ovarian cancer before [12].

Evaluation of IHC Staining

The stained slides were evaluated independently by two pathologists and differences were resolved by discussion. EphB3 expression was scored on a semi-quantitative 0–3 scale that took into account the percentage of stained cells and the staining intensity in tumor and normal cells. The intensity was scored as: 0, none; 1, weakly positive (weak light yellow); 2, moderately positive (medium brown-yellow); and 3, strongly positive (dark brown). The percentage of cells stained was scored as: 0, none; 1, <25%; 2, 25–50%, and 3, >50%. The two scores were summed and assigned a final score of 0, 1, 2 or 3: 0/1 = negative or weak (total score 0–2); 2 = moderate to positive (total score 3–4), and 3 = strong positive (total score 5–6).

EphB3 Expression in CRC Cell Lines

For IHC, cells were grown on glass coverslips to 70% confluence, washed with PBS, and fixed with cold 75% ethanol for 10 min on ice. Cells were incubated in 0.3% H_2O_2 for 10 min, and then at 4 °C overnight with anti-EphB3 polyclonal antibody (1:600 in Antibody Diluent). After washing with PBS, the cells were incubated with secondary antibody (Dako, UK) for 20 min at room temperature. Color was developed with 3,3′-diaminobenzidine and nuclei were lightly counterstained with hematoxylin. Cells were visualized with a microscope.

Methylation-specific PCR of CpG in EphB3. Genomic DNA was extracted from CRC cells using the phenol/chloroform method. DNA was bisulfate converted using an EpiTec Fast DNA Bisulfite Kit (Qiagen, Germany) according to the manufacturer's protocol. Methylation-specific PCR (MSP) primer sets were designed using MethPrimer (www.urogene.org/methprimer) as follows: For methylated DNA, EphB3-MSP-Mf (5′–3′) GGTTCGGAGGTAGGTAGTTC; EphB3-MSP-Mr (5′–3′) AAACTCTAAAAAAC GTTCGTC. The PCR product length was 74 bp. For unmethylated DNA, EphB3-MSP-Uf (5′–3′)

TGGTTTGGAGGTAGGTAGTTTG; EphB3-MSP-Ur (5′–3′) CCAAAACTCTAAAAAACATTCA. The PCR product length was 78 bp. PCR products were subjected to electrophoresis with 1.5% agarose gel.

KRAS and *BRAF* Mutations in CRC Tissues

KRAS mutations in exon 2 (12 and 13 codon) and *BRAF* V600E mutation were detected using the Human *KRAS* Gene 7 mutation Fluoresence Polymerase Chain Reaction Diagnostic Kit (AmoyDx, Xiamen, China) and Human *BRAF* Gene V600E Mutation Fluorescence Polymerase Chain Reaction Diagnosis Kit (AmoyDx, Xiamen, China) in CRC tissues, in accordance with the manufacturer's instructions. Purified genomic DNA from CRC paraffin fixed tissues was applied for reactions on a LightCycler 480 real-time PCR instrument (LC480, Roche Diagnostics). The presence of mutant alleles was determined by the cycle threshold value.

MSI in CRC Tissues

MSI status was evaluated with five microsatellite markers (NR21, BAT26, NR24, BAT25, and MONO27) using fluorescence-based PCR kit (Yuanqi Bio, Shanghai, China). DNA samples from CRC tissues and normal tissues were amplified in a 20 ml volume reaction. PCR products were analyzed by a genetic analyzer (Applied Biosystems 3500, ABI). The data were analyzed using GeneMapper 4.1 software. Patients were categorized as MSI-H if any two or more of the five markers showed positivity, MSI-L if only one marker showed positivity, and MSS if all markers showed negativity.

Statistical Analysis

Correlations between EphB3 expression and clinicopathological parameters were evaluated using Spearman's rank correlation test. Kaplan–Meier survival curves were constructed to assess OS, and differences were analyzed using the log-rank test. Multivariate analysis of each independent risk factor was performed using the Cox proportional hazards model. All statistical analyses were performed using SPSS software (SPSS 16.0, Chicago, IL, USA). A two-sided P value of less than 0.05 was considered statistically significant.

Results

Expression of EphB3 in CRC Cell Lines

Expression of EphB3 in the CRC cell lines HCT116, HT29, SW480, and SW620 was examined by IHC. EphB3 was negatively stained in HCT116, weakly in HT29, and SW480, while was strongly expressed in SW620 (Fig. 1a, b, c and d).

Expression of EphB3 in CRC Tissues

EphB3 expression was analyzed in 128 specimens of CRC tissue. Staining of cancer cells and normal intestinal epithelial cells was examined in the same section from each patient. EphB3 expression was also examined in 19 adenoma samples from the parts of patients. The protein was generally detected as brown particles in the cytoplasm, with occasional uniform brown staining (Fig. 2). Strong positive staining of EphB3 (score 3) was observed in all normal epithelial cells (128/128, 100%, Fig. 2a) and adenoma samples (19/19, 100%, Fig. 2b). Of the 128 CRC samples, EphB3 was negative/weakly positive (score 0/1) in 53 (41.4%, Fig. 2c), moderately positive (score 2) in 34 (26.6%, Fig. 2d, e), and strongly positive (score 3) in 41 (32.0%, Fig. 2f) samples.

Relationships between EphB3 Expression and Clinicopathological Parameters

Correlations between clinicopathological parameters and EphB3 expression are shown in Table 1. The expression level was negatively associated with the depth of tumor invasion ($P = 0.016$, $r_s = -0.213$), lymph node metastasis ($P = 0.000$, $r_s = -0.490$), and TNM stage ($P = 0.000$, $r_s = -0.390$). Negative or weak expression was more common in poorly differentiated tumors ($P = 0.001$, $r_s = 0.290$). There were no significant associations between EphB3 expression and gender, age, or tumor location. The relationship between EphB3 expression and *KRAS* mutations, *BRAF* V600E mutation (Fig. 3) and MSI (Fig. 4) was analyzed as well. No association was found between EphB3 expression and *KRAS* mutations and MSI. Interestingly, we found that EphB3 expression was positively associated with *BRAF* V600E ($P = 0.008$, $r_s = 0.235$).

Survival Analyses and Prognostic Value of EphB3 Expression

The prognostic value of EphB3 expression in the 128 CRC patients was assessed using the Kaplan–Meier method with a log-rank test. Patients with high EphB3 expression (scores 2 and 3) exhibited longer OS than patients with negative or weak expression (0/1)($P < 0.001$, Fig. 5). In multivariate analysis, expression of EphB3 ($P = 0.007$) and lymph node metastasis

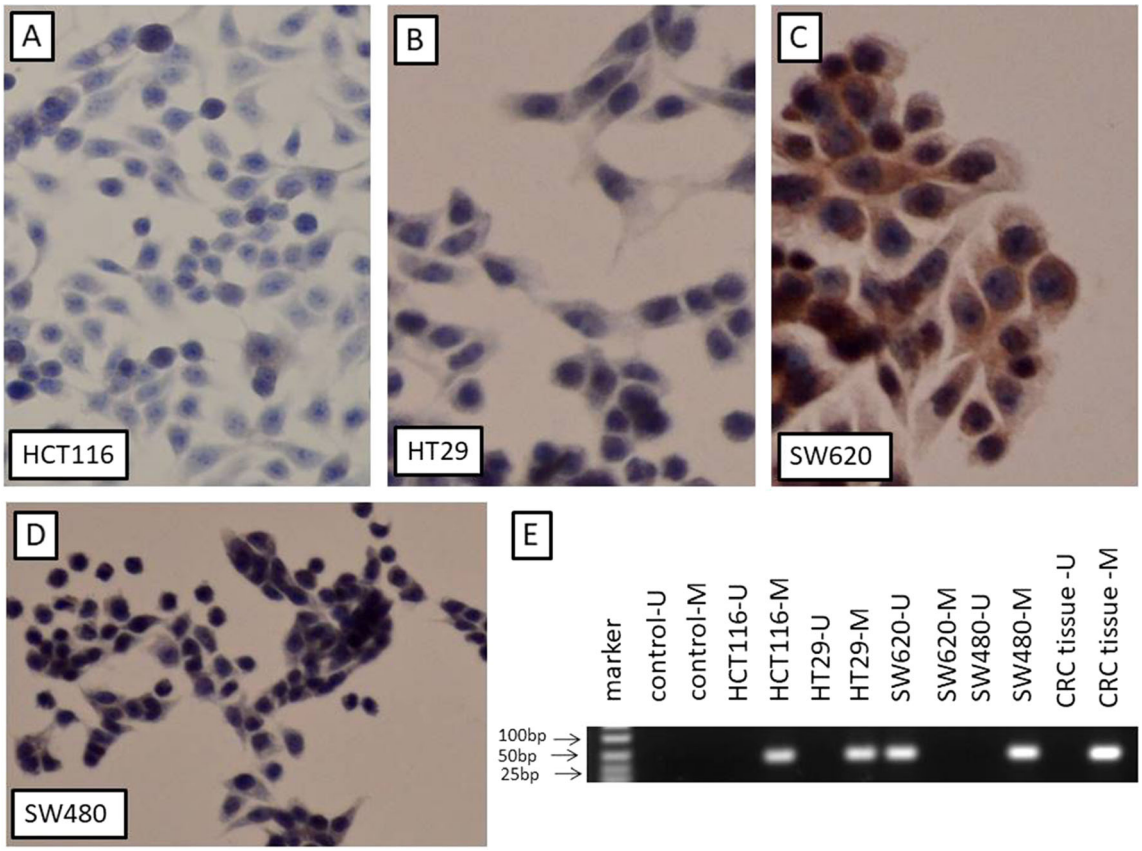

Fig. 1 Expression and methylation status of EphB3 in CRC cell lines. A: Negative staining of EphB3 in HCT116. B: Weak staining of EphB3 in HT29. C: Strong staining of EphB3 in SW620. D: Weak staining of EphB3 in SW480. E: Methylation status of EphB3 was checked in CRC cell lines by using MSP. PCR products were subjected to electrophoresis with 1.5% agarose gel. Unmetylated DNA was not found in blank control, HCT116, HT29, and SW480, but was detected in SW620. Methylated DNA was not found in blank control and SW620, while was detected in HCT116, HT29, and SW480. Methylated EphB3 was also detected in a CRC tissue sample

($P < 0.001$) were independent prognostic factors for poor survival of patients with CRC (Table 2).

Methylation Status of EphB3 in CRC Cells

The methylation status of CpG islands in the EphB3 gene was examined in the CRC cell lines HCT116, HT29, SW480, SW620 and CRC tissue samples using methylation-specific PCR (MSP). As shown in Fig. 1e, unmethylated DNA was not and methylated DNA was detected in HCT116, HT29, and SW480. Methylated EphB3 was also detected in CRC tissue samples. The methylated DNA was not detected and unmethylated DNA was detected in SW620. These data indicate that, in most of CRC cell lines, EphB3 expression is regulated by CpG methylation in the promoter region.

Discussion

In the present study, we examined EphB3 expression in CRC tissues and analyzed its relationship with clinicopathological parameters and survival. We found that most (87/128, 68%) CRC specimens showed down-regulation of EphB3 expression (negative/weak/moderate staining) in CRC cells compared with normal intestinal epithelial cells (strong staining). EphB3 expression was negatively associated with depth of tumor invasion, lymph node metastasis, TNM stage, differentiation, and was positively associated with *BRAF* V600E mutation and longer OS.

Several mechanisms may be responsible for the down-regulation of EphB3 in CRC, including chromosome deletion, gene mutation, promoter hypermethylation, histone modification, and microRNA modulation. Ronsch et al. found that class I and III histone deacetylases and loss of active chromatin features contribute to epigenetic silencing of EphB3 in CRC [26]. Jagle et al. identified a CRC cell type-specific transcriptional enhancer in the 5′-flanking region of the human EphB3 gene [25]. They also showed that EphB3 enhancer activity and expression in intestinal stem cells are combinatorially controlled by Wnt/β-catenin, Notch, and MAPK signaling and the stem-cell factor ASCL2. A deficiency in Notch activity was shown to contribute to impairment of the

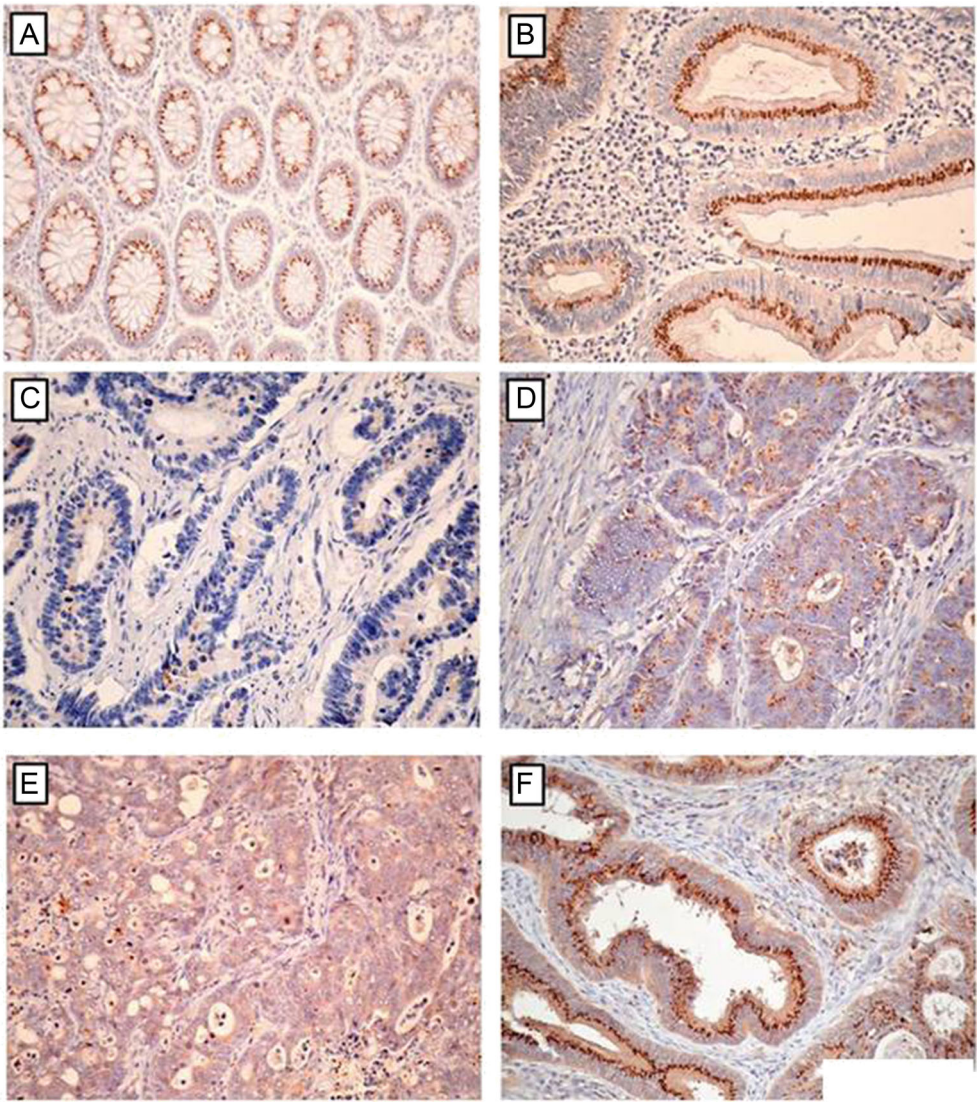

Fig. 2 Expression of EphB3 in normal mucosa and CRC tissue. A: EphB3 was strongly expressed (score 3) in normal mucosa cells as dark brown particles. B: EphB3 was strongly expressed (score 3) in adenoma cells as dark brown particles. C: EphB3 was negatively expressed (score 0) in cancer cells. D: EphB3 was weakly expressed (score 1) in cancer cells as weak light yellow or small amount of dark brown particles. E: EphB3 was moderately positive (score 2) as medium brown-yellow in cytoplasm. F: EphB3 was strongly expressed (score 3) in cancer cells as dark brown particles. (magnification, 400×)

enhancer function. EphB3 expression and Notch activity are positively correlated in human CRC tissues [25]. These results suggested that EphB3 is silenced in CRC through decommissioning of the transcriptional enhancer. In the present study, we detected methylation of CpG islands in the EphB3 promoter region of CRC cell lines and small number of paraffin fixed tissue samples, indicating that CpG hypermethylation is likely to contribute to the down-regulation of EphB3 expression in these cells. In addition, we found that status of methylation of EphB3 was related EphB3 expression level in colon cancer cell lines. EphB3 was strongly expressed in SW620 that was detected unmethylated DNA. EphB3 was negatively expressed in HCT116 that was detected methylated DNA. EphB3 was weakly expressed in HT29 that was detected both unmethylated and methylated DNA.

The tumor-suppressive function of EphB3 in CRC has been well documented. Chiu et al. demonstrated that stable transfection of EphB3 into human HT-29 CRC cells inhibited growth in monolayer cultures, anchorage-independent growth in soft agar, and xenograft growth in nude mice [9]. Notably, EphB3 overexpression induced cytoskeletal reorganization and functional changes favoring mesenchymal–epithelial transformation. Batlle et al. reported that loss of an *Ephb3* allele induced invasive colorectal carcinoma in $Apc^{min/+}$ mice [27]. Jagle et al. showed that Notch signaling-mediated reduction in EphB3 enhancer activity and silencing of EphB3 expression causes cell cycle arrest and induces apoptosis in CRC cells [25]. A tumor suppressor role for EphB3 has also been reported in other human cancers. Li et al. found that EphB3 suppresses NSCLC metastasis via the kinase RACK1, which mediates the assembly of a ternary signal complex comprising protein phosphatase 2A, RACK1, and Akt [23].

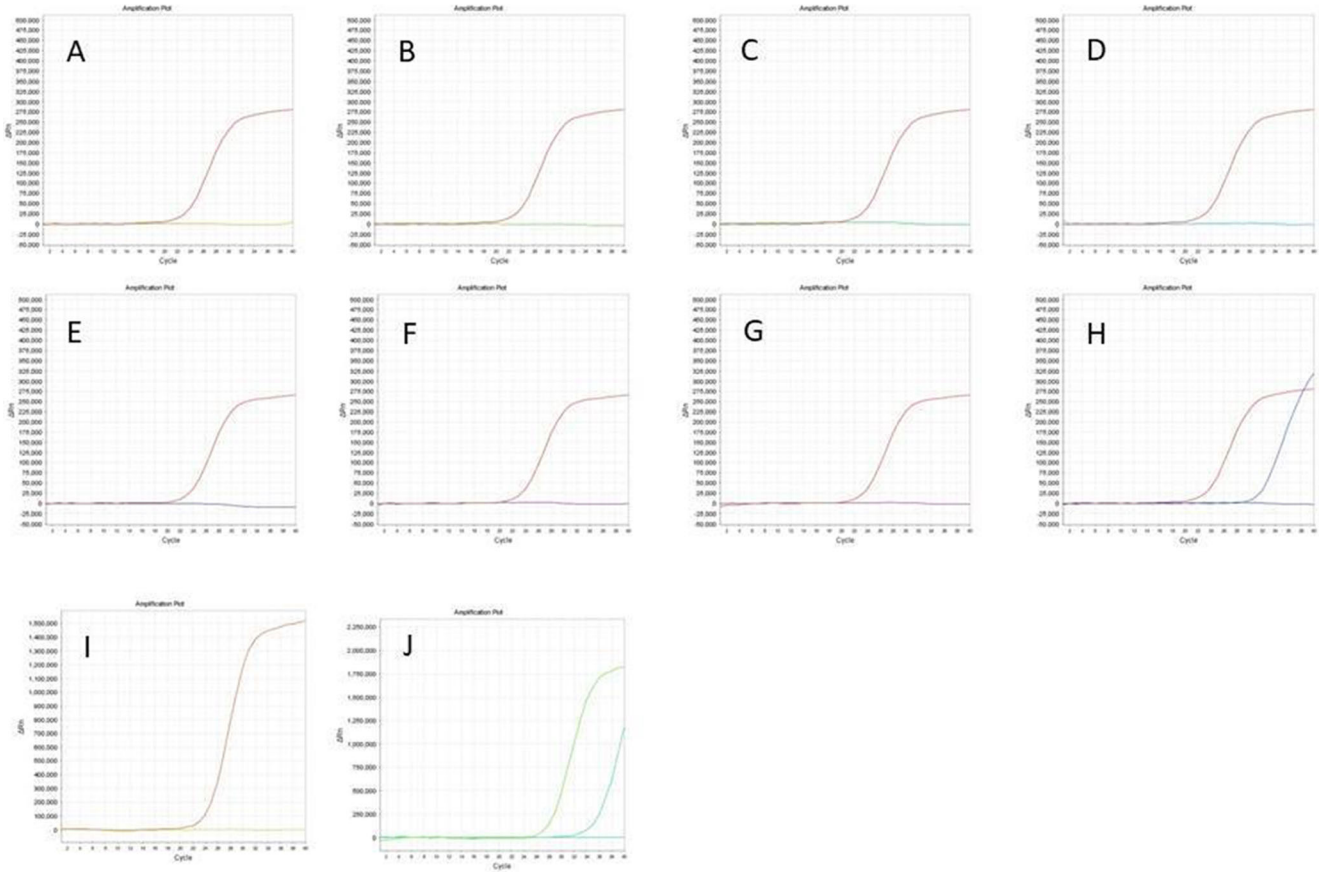

Fig. 3 Representative examples of detection of *KRAS* mutations and BRAF V600E. Wild types of *KRAS* (A-G) and mutation (H). Wild type of *BRAF* (I) and mutation (J)

The survival of CRC patients is highly variable, even at the same disease stage. At present, CRC prognosis is based primarily on pathological assessment of the depth of primary tumor invasion and the presence of lymph node metastasis. Thus, identification of molecular biomarkers that are crucial to tumor biology and could

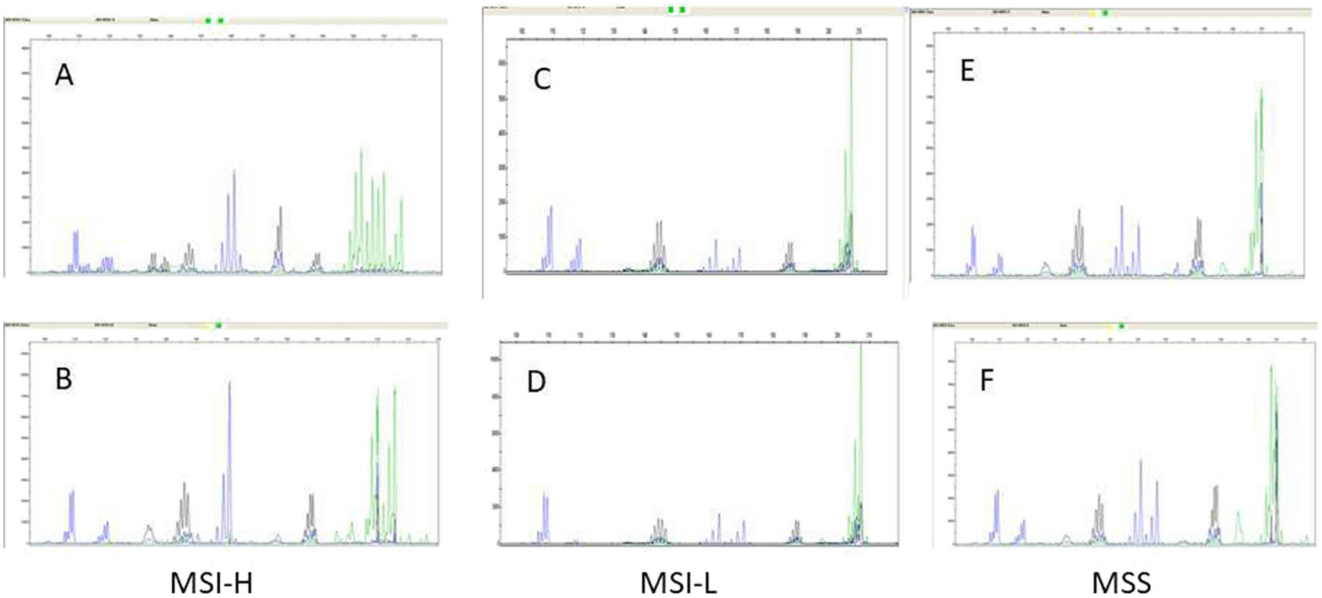

Fig. 4 Representative examples of MSI in colorectal cancers. Five microsatellite markers were detected in CRC tumor (A, C, and E) and matched normal tissues (B, D, and F). MSI-H was detected in A and B; MSI-L in C and D; MSS in E and F

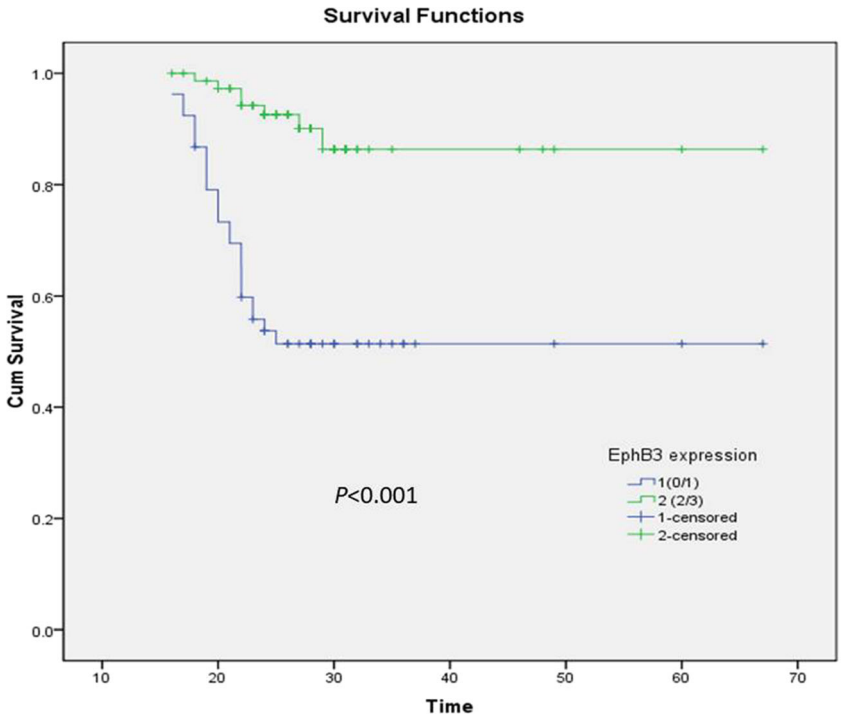

Fig. 5 Overall survival analysis using the Kaplan-Meier method. Log-rank test revealed that patients with high EphB3 expression (scores 2 and 3) exhibited longer OS than patients with negative or weak expression (0/1)($P < 0.001$)

serve as diagnostic and prognostic markers would greatly assist in the design of individualized therapy for CRC patients. In the present study, longer OS was significantly associated with high EphB3 expression by univariate analysis, and both EphB3 expression and lymph node metastasis were confirmed to be independent prognostic factors for poor survival by multivariate analysis. Our data therefore suggest that EphB3 is aprognostic indicator in CRC.

Conclusion

We found that EphB3 expression is reduced in CRC compared with normal intestinal mucosa and adenoma, and that hypermethylation of the EphB3 promoter is involved in the down-regulation. Our results confirm that EphB3 is a tumor suppressor in CRC and suggest that EphB3 expression may be a useful prognostic indicator for patients with this disease.

Table 2 Multivariate analyses of various potential prognostic factors in CRC patients by the Cox proportional hazards regression model

Variables	HR	95% CI	P
age	0.997	0.960–1.035	0.876
depth	1.358	0.670–2.751	0.395
lymph	5.903	2.728–12.772	0.000
differentiation	1.620	0.641–4.092	0.308
TNM	1.445	0.292–7.145	0.651
EphB3	0.276	0.108–0.706	0.007

Acknowledgements The authors wish to thank the patients who participate in this study. This study was supported by grants from the National Basic Research Priorities Program 973 Project (2014CB744504) from the Ministry of Science and Technology of China and the National Natural Science Foundation of China (81371611).

We thank Anne M. O'Rourke, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflicts of interest.

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