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Original Article

EphA3 promotes malignant transformation of colorectal epithelial cells by upregulating oncogenic pathways

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ABSTRACT

Ephrin Type-A Receptor 3 (EphA3) belongs to the ephrin receptor subfamily of the protein tyrosine kinase family, and plays an important role in embryogenesis and neurogenesis. This study aimed to investigate the role of EphA3 in promoting malignant transformation of colorectal epithelial cells, and explore underlying molecular mechanisms. Colorectal cancer tissue specimens from 68 patients were analyzed for EphA3 expression. EphA3 expression levels were manipulated in rat colon epithelial cell lines. We found that EphA3 expression level in tumor tissues was associated with patient age ($P = 0.015$), tumor differentiation ($P = 0.001$), and lymph node metastasis ($P = 0.039$). Overexpression of EphA3 and its constitutively active mutants promoted colony formation, migration and invasion, and tumorigenicity of colon epithelial cells in nude mice. The cDNA and lncRNA microarray profiling data revealed that differentially expressed genes and lncRNAs in EphA3 or mutant-transfected cells were associated with cell proliferation, invasion and angiogenesis. Our findings reveal the mechanisms underlying the oncogenic activities of EphA3 in colorectal cells, which could provide novel targets for the prevention, early diagnosis, and treatment of colorectal cancer.

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide, with over 1.2 million new cancer cases and more than 600,000 cancer-related deaths estimated globally in 2008 [1–4]. The 5-year survival rate of CRC with late-stage is only 12% [5]. Both genetic and environmental factors are involved in the development of CRC but the underlying mechanisms remain elusive [6,7]. Identification of novel diagnostic biomarkers and effective therapeutic targets for CRC is urgently needed.

Erythropoietin-producing hepatocellular (Eph) receptors, the largest known family of tyrosine kinase receptors, mediate cell compartmentalization and directional cell migration during embryonic development [8]. Eph receptor subfamily consists of 16 members that are divided into two groups based on sequence identity: EphA1–10 and EphB1–6, which play important roles in cancer invasiveness [9,10]. The action of EphA3 in cancer seems contradictory: although tumor suppressor properties of EphA3 have been reported [8,11,12], EphA3 functions as an oncogene in different solid and hematopoietic tumors [13,14], and has been implicated in maintaining tumor-initiating cells in glioblastoma and leukemia [13,15]. A previous study demonstrated that EphA3 is the sixth most frequently mutated gene in CRC [14]. However, the role of EphA3 in CRC has been poorly investigated.

In this study, we employed microarray, proteomics and bioinformatics analyses to investigate the role of EphA3 in malignant transformation of colorectal epithelial cells. Our data demonstrate the role of EphA3 in upregulating angiogenesis-related pathways in CRC and provide novel insight into the mechanisms of CRC tumorigenesis.

Materials and methods

Tissue specimens

Primary tumor specimens of colorectal cancer and adjacent normal mucosa were harvested from 68 patients who had undergone colectomy for pathologically diagnosed CRC at the Affiliated Tumor Hospital of Harbin Medical University (Harbin, China) between March 2012 and March 2013. All tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C. None of the patients received adjuvant chemoradiotherapy before surgery. The study protocol was approved by the Ethics Committee of Harbin Medical University, and all patients provided written informed consent.

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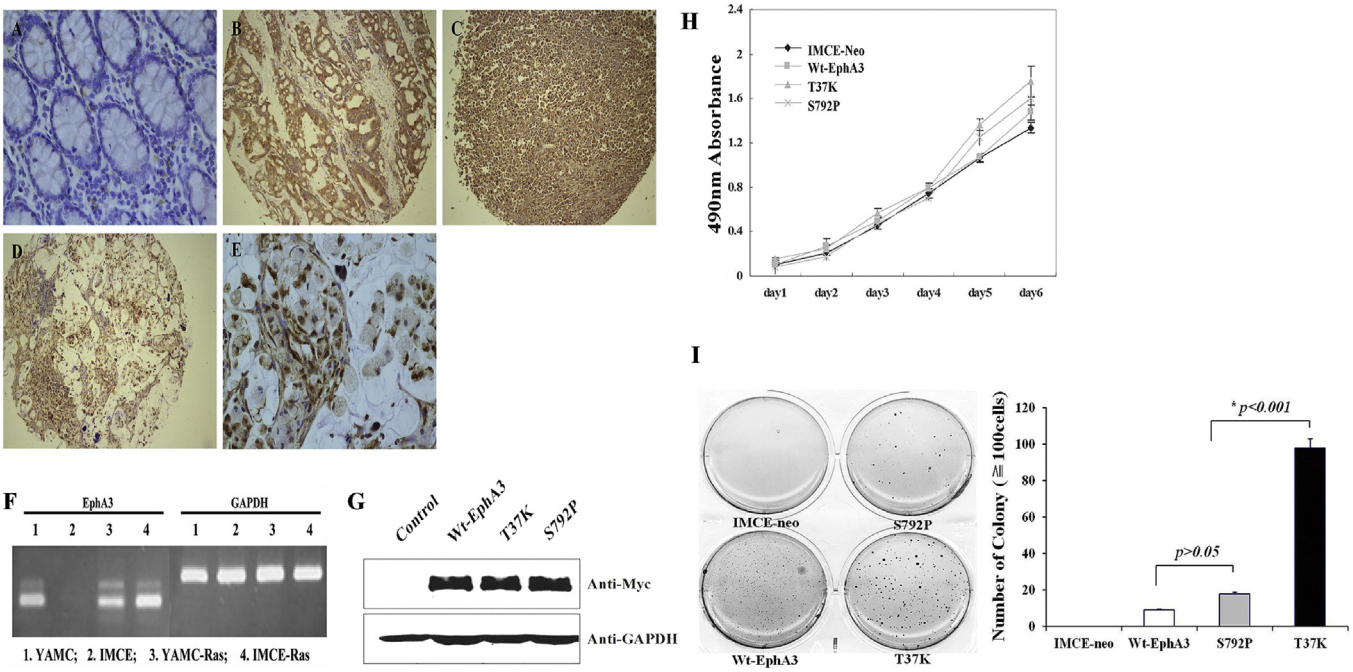

Fig. 1. EphA3 was overexpressed in CRC tissues and promoted colony formation of colorectal epithelial cell cells. Immunohistochemistry staining of EphA3 in normal mucosae (A), differentiated adenocarcinoma (B,C), mucinous adenocarcinoma (D), and signet-ring cell carcinoma (E). RT-PCR analysis of EphA3 mRNA level in different cells (F). Western blot analysis of EphA3 protein in IMCE cells stably transfected with EphA3 wild-type and mutant constructs (G). MTT assay of IMCE cells stably transfected with EphA3 wild-type and mutant constructs (H). Colony formation assay of IMCE cells stably transfected with EphA3 wild-type and mutant constructs (I).

Immunohistochemistry

Paraffin-embedded tissue blocks from 68 patients were retrieved from the Pathology Department, and 5 μm thick sections were prepared for standard immunohistochemistry with EnVision™ immunohistochemistry methods. Briefly, the tissue sections were deparaffinized in xylene and re-hydrated through an ethanol gradient. Samples were blocked in 20% normal serum, and incubated overnight at 4 °C with a primary antibody against EphA3 (sc-920, Santa Cruz Biotechnology; Santa Cruz, CA, USA) at a dilution of 1:100. The sections were washed with phosphate-buffered saline (PBS) and then incubated with biotin- or fluorescence-labeled secondary antibody at 4 °C for 2 h. The color reaction was processed with 3,3′-diaminobenzidine (DAB) solution, and then reviewed under a light microscope or inverted fluorescence microscope.

Cell culture

Rat colorectal YAMC, IMCE, YAMC-Ras, and IMCE-Ras cell lines were kindly provided by Dr. Zhenfeng Zhang of Ireland Cancer Center, Case Western Reserve University (Cleveland, OH, USA). These cell lines were generated from colonic epithelia of F1 immorto-*APC*$^{min/+}$ mouse hybrids [16]. The cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5 U/ml murine interferon gamma (IFN-γ), 100 U/ml penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium acid in a humidified incubator with 5% CO_2 at 37 °C.

Reverse transcription PCR

Total RNA was isolated from colorectal epithelial cell lines using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using Superscript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The primers were as follows: EphA3 5′-ACTGGAATGGATTGTCAGCTCTCCATCCTCCTCCTTCTC-3′ and 5′-ACTCTCGAGTCAcagatcctcttctgagatgagtttttgttc(Myc)GAACACGGGAACTGGGCCATTCTTTGATTGCG-3′; GAPDH 5′-GCCAAAAGGGTCATCATCTC-3′ and 5′-GTAGAG GCAGGGATGATGTTC-3′. PCR conditions were initial denaturation at 95 °C for 2 min, followed by 30 cycles of amplification at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 15 min.

Western blot analysis

Whole cell lysates were prepared and quantified using standard protocols, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in blocking buffer and incubated with monoclonal anti-Myc antibody (Cell Signaling Technology, Danvers, MA, USA). The membranes were stripped and incubated with monoclonal anti-GAPDH antibody (Cell Signaling) to confirm equal loading.

Plasmid construction and transfection

A plasmid carrying mutated EphA3 cDNA was constructed using a GeneArt® Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's instructions, and named pcDNA3.1-EphA3-Myc. The expression vector carrying EphA3-T37K cDNA (impacts the ligand domain) was constructed using primers 5′-TCAATCTACTGGATTCAAAAAAAATTCAAGGGGA-3′ and 5′-TTTTGAATCCAGTAGATTGACTTCATTGGA-3′, the expression vector carrying EphA3-S792P (impacts the

Table 1
Association of EphA3 expression with clinicopathological features of colorectal cancer patients.

Character	N	EphA3		*p* value
		Positive (n = 49)	Negative (n = 19)	
Age (yrs.)				
<60	21	11	10	0.015
≥60	47	39	9	
Sex				
Male	52	37	15	0.76
Female	16	12	4	
Gross type				
1	30	20	10	0.28
2	22	15	7	
3	16	10	4	
Differentiation				
Well	0	0	0	0.001
Moderate	26	13	13	
Poor	42	36	6	
LN metastasis				
Positive	14	7	7	0.039
Negative	54	42	12	
Distant metastasis				
Positive	8	5	3	0.52
Negative	60	44	16	