

GEFT protein expression in digestive tract malignant tumors and its clinical significance

YUANYUAN WANG, BING ZHANG, GE GAO, YINPING ZHANG and QINGXIN XIA

Department of Pathology, The Affiliated Cancer Hospital of Zhengzhou University,
Henan Cancer Hospital, Zhengzhou, Henan 450008, P.R. China

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Abstract. Guanine nucleotide exchange factor T (GEFT), a member of the Rho guanine nucleotide exchange factor family, is expressed in a variety of tumors. In the present study, the expression and clinical significance of GEFT in malignant digestive tract tumors was assessed. Tumor and adjacent control samples from 180 patients were tested. Positive GEFT expression rates were 80, 83.33 and 86.67% in esophageal squamous carcinoma (ESCC), gastric carcinoma (GC) and colorectal cancer (CRC), respectively. GEFT expression was associated with diffuse type carcinoma according to the Lauren classification ($\chi^2=12.525$, $P=0.002$) and tumor-node-metastasis (TNM) stages III/IV ($\chi^2=4.033$, $P=0.045$) in GC, and with vessel carcinoma embolus ($\chi^2=7.890$, $P=0.005$) and lymph node metastasis ($\chi^2=5.455$, $P=0.020$) in CRC, but was not associated with other clinicopathological parameters. Patients with high levels of GEFT protein expression had a less favorable outcome compared with patients with low levels of GEFT expression in patients with CRC ($\chi^2=3.876$, $P=0.049$). However, a significant association was not found between GEFT expression and overall survival in patients with ESCC ($\chi^2=0.040$, $P=0.842$) or GC ($\chi^2=0.501$, $P=0.479$). The rate of human epidermal growth factor receptor 2 upregulation in patients with GC was 13.33% and it was associated with nerve invasion ($\chi^2=4.005$, $P=0.045$) and TNM stages III/IV ($\chi^2=5.600$, $P=0.018$). Mismatch repair protein (MMRP) defect was observed in six cases, and the *KRAS* mutation rate was 26.67% in patients with CRC. GEFT expression was significantly correlated with MMRP ($r=-0.285$, $P=0.027$) and *KRAS* mutation in patients with CRC ($r=0.697$, $P<0.001$). These findings revealed frequent GEFT upregulation

in malignant digestive tract tumors, which may have promoted tumor development. GEFT expression in CRC may be associated with microsatellite instability and *KRAS* mutation status, suggesting that GEFT may be a potential therapeutic target for patients with CRC.

Introduction

Guanine nucleotide exchange factor T (GEFT) is a member of the Rho guanine nucleotide exchange factor family, and is capable of activating RhoA, Rac1 and Cdc42 by catalyzing the exchange of Rho-bound GDP for GTP (1). GEFT is highly expressed in excitable tissues such as the brain, heart and muscle, and it modulates the myogenic vs. adipogenic cell fate decision of progenitor mesenchymal cells, thus regulating muscle regeneration, myogenesis and adipogenesis (2).

GEFT is located on chromosome 12q13.3, a region frequently amplified in sarcomas (3). High copy numbers of the gene for *GEFT* were observed in rhabdomyosarcoma samples in microarray-based comparative genomic hybridization (4,5). Immunohistochemical analyses suggested that GEFT protein levels were upregulated in rhabdomyosarcoma samples, and was associated with disease aggressiveness and metastasis (6). Furthermore, the mRNAs encoding p63RhoGEF and GEFT, which are derived from the same gene, were both present in the same individual cells (7). Notably, GPR116 regulates cell motility and morphology through the p63RhoGEF-RhoA/Rac1 pathway in the breast carcinoma cell line MDA-MB-231 cells *in vitro* (8). In these cells, p63RhoGEF mediated the formation of a single polarized lamellipodium which is required for chemotactic migration (9). GEFT protein levels are also increased during differentiation of neuroblastoma cells, where exogenous GEFT expression promotes neurite outgrowth (10). Therefore, GEFT is expressed in a variety of tumors and may be involved in their occurrence and development.

Malignant digestive tract tumors, including gastric malignancy, intestinal malignant tumor and malignant tumor of the esophagus are increasingly prevalent, and seriously threaten patient health (11). Esophageal squamous cell carcinoma (ESCC) is a potentially lethal malignancy with a 15-34% 5-year survival rates (12). Despite improvements in imaging, surgical techniques and chemoradiation therapy, effective treatment of patients with ESCC remains challenging (13). In addition, gastric cancer (GC) constitutes a major cause of

Correspondence to: Dr Qingxin Xia, Department of Pathology, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, 127 Dongming Road, Zhengzhou, Henan 450008, P.R. China
E-mail: tudou414135404@163.com

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cancer-associated death worldwide, particularly in developing countries (14). The incidence of GC is particularly common in Eastern Asia, particularly in China (15). Amplification and/or upregulation of human epidermal growth factor receptor-2 (HER2, also known as ERBB2) is observed in 6.1-23.0% of GCs. *HER2* functions as a proto-oncogene and encodes a transmembrane receptor tyrosine kinase expressed in different types of solid tumors (16-18). However, the prognostic value of HER2 status in GC remains controversial.

Colorectal cancer (CRC) comprises the third most common malignancy in adults worldwide, accounting for 1.36 million cases, after lung cancer (1.8 million) and breast cancer (1.6 million) (19). The mechanism underlying the development of CRC involves two distinct pathways: Chromosomal (85%) or microsatellite instability (MSI) (15%) (20,21). MSI is a molecular fingerprint of a deficient DNA mismatch repair (MMR) system. The inheritance of a germline mutation in one of the MMR genes *MLH1*, *MSH2*, *MSH6* or *PMS2* causes MSI (22-25). Analysis of the encoded MMR proteins (MMRP) by immunohistochemistry (IHC) or MSI testing is used to evaluate MSI (26). *KRAS*, a member of the RAS family of GTPases, is a small GTPase that is also frequently mutated in a wide range of different types of cancer, including CRC (27). Numerous studies have confirmed that patients with *KRAS* mutations do not benefit from anti-epidermal growth factor receptor therapy (28-30).

To identify the key factors involved in the occurrence and development of digestive tract tumors, the expression of GEFT in digestive tract tumors on a global scale was assessed. In addition, the association between GEFT expression and the clinicopathological parameters of these patients was determined.

Materials and methods

Patients and tissue specimen. A total of 180 formalin-fixed paraffin-embedded tumor samples (60 ESCC, 60 gastric adenocarcinoma and 60 colorectal adenocarcinoma) were included in the present study. In addition, 180 matched control samples were selected from normal mucosal tissues ≥ 5 cm away from the tumor. The 60 ESCC samples consisted of 43 males and 17 females, aged 43-81 years with a median age of 65 years. The GC samples were from 46 males and 14 females, aged 31-79 years with a median age of 61 years. The CRC samples were obtained from 27 females and 33 males, with an age range of 38-87 years and a median age of 61 years. The sections were immersed in hematoxylin for 3 min and in eosin for 5 sec at room temperature for hematoxylin and eosin and staining (H&E). H&E sections were independently analyzed by two senior gastrointestinal pathologists. All patients were categorized according to the 7th American Joint Committee on Cancer Tumor-Node-Metastasis (TNM) stage (31). All samples were collected after surgery at the Department of Pathology, Henan Cancer Hospital (Zhengzhou, China) between August and November 2016. Clinical follow-up information was obtained by telephone from the surgical date till May 2019. The total follow-up period was up to 33 months post-surgery. Based on the hematoxylin and eosin slides, two representative fields were selected for each tumor sample. Tissue

samples containing the selected fields were embedded in paraffin blocks. Each area was confirmed to contain $\geq 70\%$ tumor cells. Sample blocks were sectioned (4 μ m) for further use in IHC analysis. The purpose of the present research was explained to the participants, who all signed a written consent prior to the study. The present study was approved by the Institutional Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University.

Experimental reagents. Rabbit polyclonal antibody against human GEFT (1:150; cat. no. ab127690; Abcam) and mouse monoclonal antibody against human HER2 (1:500; cat. no. ab134182; Abcam) were used for IHC. Antibodies against MMRPs, which included MLH1 (cat. no. GT218907), MSH2 (cat. no. GT210507), MSH6 (cat. no. GT219507) and PMS2 (cat. no. GT215907) were purchased as ready-to-use working stocks (Gene Tech Biotechnology Co., Ltd.). The secondary antibody used was purchased as ready-to-use working stocks (cat. no. SM802; Dako; Agilent Technologies, Inc.) which included dextran and peroxidase-conjugated goat secondary antibodies against rabbit and mouse immunoglobulins.

IHC staining procedure. IHC staining was performed using the EnVision system (Dako; Agilent technologies, GmbH). The tissues were fixed in 10% formalin at room temperature for 24 h and embedded in paraffin. Serial sections (4- μ m thick) were deparaffinized in xylene and rehydrated in a descending series of alcohol solutions (100, 95, 80 and 70%) for 5 min each. The samples were placed in citrate buffer (pH 6.0) for antigen retrieval at 95°C for 15 min and subsequently immersed in 3% hydrogen peroxide in methanol for 10 min at room temperature to inhibit endogenous peroxidase activity. The sections were incubated overnight at 4°C with each primary antibody. After a washing in PBS, the sections were incubated with the secondary antibody at room temperature for 30 min. For negative controls, each antibody was replaced by phosphate buffered saline. GEFT expression was evaluated using semi-quantitative scores as follows: 0, ≤ 5 ; 1, 6-35; 2, 36-65; and 3, 66-100%. Staining intensity was scored as follows: 0, no staining; 1, buff; 2, yellow; and 3, brown. A final staining score was obtained by multiplying the expression and intensity scores as follow: -, 0; +, 1-3; 2+, 4-6; and 3+, 7-9. Samples scored as 0 were negative, a score of 1-3 (1+) represented weak expression and a score of 4-9 (2+/3+) represented significantly increased positive expression (6). Samples were visualized on an Olympus BX-41 light microscope (Olympus Corporation, magnification, x400). All samples were evaluated independently by two pathologists.

For determination of HER2 expression, the scoring criteria used was adapted from Hofmann *et al* (32): 0, Absence of color or reaction in $<10\%$ of neoplastic cells; 1+, weak and/or incomplete coloring of the membrane in $>10\%$ of neoplastic cells; 2+, moderate and/or incomplete coloring of the membrane in $>10\%$ of neoplastic cells; and 3+, strong and complete and/or incomplete coloring of the membrane in $>10\%$ of neoplastic cells. The scores were stratified as follows: 0 and 1+, HER2-negative; 2+, indeterminate; and 3+, HER2-positive. Samples scored as 2+ were analyzed using fluorescence *in situ* hybridization (FISH) to confirm HER2 expression.

FISH. Tissues were analyzed using FISH using a Path Vysion kit (cat. no. 02j01-030; Abbott Pharmaceutical Co. Ltd.). The *HER-2* DNA probe is a 190 kb SpectrumOrange™ directly labeled fluorescent DNA probe specific for the *HER-2/neu* gene locus (17q11.2-q12). The Chromosome Enumeration Probe (CEP) 17 DAN probe is a 5.4 Kb SpectrumGreen directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17(17p11.1-q11.1). The 4 μ m tissue sections were deparaffinized in xylene, and placed in 100% alcohol for 5 min, twice at room temperature. Slides were subsequently immersed in pretreatment solution at 80°C for 30 min, and then in protease solution at 37°C for 10 min (both from Abbott Pharmaceutical Co., Ltd.). Tissue sections were fixed in 10% neutral buffered formalin solution at room temperature for 10 min and dried at 45-50°C for 2-5 min. The tissues were denatured in denaturation solution at 72°C for 5 min. To each section, 1 μ l probe, 7 μ l hybridization mix (50% formamide, 2x saline sodium citrate, 10% dextran sulphate) and 2 μ l deionized water was added and allowed to hybridize overnight at 37°C. The sections were immersed in post-hybridization solutions (2x saline sodium citrate/0.3% NP-40) at room temperature and then at 72°C for 2 min each. Slides were air dried in the dark and mounted in 10 μ l DAPI/antifade mounting agent (Abbott Pharmaceutical Co. Ltd.). Slides were imaged with a fluorescence microscope (Olympus, BX53; Olympus Corporation). In each slide, three areas were randomly identified, and the average *CEP17* and *HER2* copy number in 20 nuclei at x1,000 magnification was determined. *HER2/CEP17* copy number ratio >2.0 was defined as a positive result.

PCR-capillary electrophoresis. The PCR-capillary electrophoresis method was used to detect MSI in CRC. DNA was extracted from the samples using the QIAamp DNA Mini kit (Qiagen GmbH). MSI detection used five microsatellite sites: *BAT25*, *BAT26*, *D2S123*, *D5S346* and *D17S250*, as recommended by the National Cancer Institute (33). The primer sequences were as follows: *BAT25* forward, 5'TCG CCTCCAAGAATGTAAGT3' and reverse, 5'TCTGGATTT TAACATATGGCTC3'; *BAT26* forward, 5'TGACTACTT TTGACTTCAGCC3' and reverse, 5'AACCATTCACAT TTTTAAACC3'; *D2S123* forward, 5'AAACAGGATGCC TGCCTTTA3' and reverse, 5'GGACTTTCCACCTATGGG AC3'; *D5S346* forward, 5'ACTCACTCTAGTGATAAA TCGGG3' and reverse, 5'AGCAGATAAGACAAGTATTAC TAG3'; and *D17S250* forward, 5'GGAAGAATCAAATAG ACAAT3' and reverse, 5'GCTGGCCATATATATATTTAA ACC3'. A reaction mixture was composed of: 2 μ l each of both forward and reverse primers, 10 μ l DNA polymerase (Gene Tech Biotechnology Co. Ltd.), 1-8 μ l (50-100 ng) template DNA and deionized water to a final reaction volume of 20 μ l. The PCR thermocycling conditions were as follows: Denaturation, 42°C, 5 min followed by 94°C, 5 min; 40 cycles of 94°C for 15 sec, 55°C for 25 sec, and 72°C for 50 sec; and a final extension step at 72°C for 10 min. The PCR products were subsequently separated by capillary electrophoresis on an ABI 3500XL Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The results were analyzed using GeneMapper version 4.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). According to the MSI test results,

patients with CRC could be divided into three groups: High frequency MSI (MSI-H), where two or more genes showed instability; low frequency MSI (MSI-L), where only one locus showed gene deletion; and microsatellite stable (MSS), with no gene loss.

Quantitative PCR (qPCR). Extracted DNA was assayed by qPCR using the human *KRAS* gene mutation detection kit (Amoy Diagnostics, Co., Ltd.). *KRAS* mRNA expression was analyzed using TaqMan probes (Amoy Diagnostics, Co., Ltd.; sequences not provided). Assays were performed in triplicate on an ABI Prism 7000 cyclor (Applied Biosystems; Thermo Fisher Scientific, Inc.). β -actin was used as the internal control forward, 5'CCTTCAACACCCCAGCCA3' and reverse 5'ACC CCTCGTAGATGGGCAC3'. The thermocycling conditions were: 95°C for 5 min; followed by 15 cycles of 95°C for 25 sec, 64°C for 20 sec and 72°C for 20 sec; 31 cycles of 93°C for 25 sec, 60°C for 35 sec and 72°C for 20 sec; and a final extension step of 72°C for 10 min. The FAM and HEX signals were collected during the 60°C step. *KRAS* gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method according to the manufacturer's protocol.

Statistical analysis. SPSS version 21.0 (IBM Corp.) was used to analyze all statistical data. Statistical significance was determined using a χ^2 or Fisher's exact test. The correlations between GEFT/*HER-2*/MMRP/*KRAS* expression and the clinicopathological factors were determined using the same methods. The correlation between GEFT and *HER-2*/MMRP/*KRAS* was determined using Spearman's rank correlation. Kaplan-Meier and log-rank methods were used to calculate overall survival (OS) rates, and the OS curves were compared with the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GEFT expression is elevated in patients with ESCC. Positive GEFT protein expression rate in ESCC samples was 80%, which was significantly higher than the normal control samples (100% negative; $\chi^2=80.000$, $P < 0.001$). Representative images of GEFT protein expression in ESCC is shown in Fig. 1A, and the negative control samples are shown in (Fig. 1B). In the 60 cases of ESCC, the GEFT (1+) expression rate was 56.67% (34/60) and the GEFT (2+) expression rate was 23.33% (14/60). There were no cases of GEFT (3+) expression. The GEFT-negative expression rate was 20% (12/60) (Table I).

Association between the clinicopathological parameters of ESCC with GEFT expression. Table II shows the association between GEFT expression and ESCC clinicopathological factors. Although the expression of GEFT was significantly higher in tumor tissues compared with the control samples, GEFT levels were not associated with any of the assessed clinicopathological parameters (all $P > 0.05$; Table II).

Association between GEFT expression and OS in patients with ESCC. Of the 60 patients with ESCC included in the present study, two patients were lost to follow-up, 38 cases

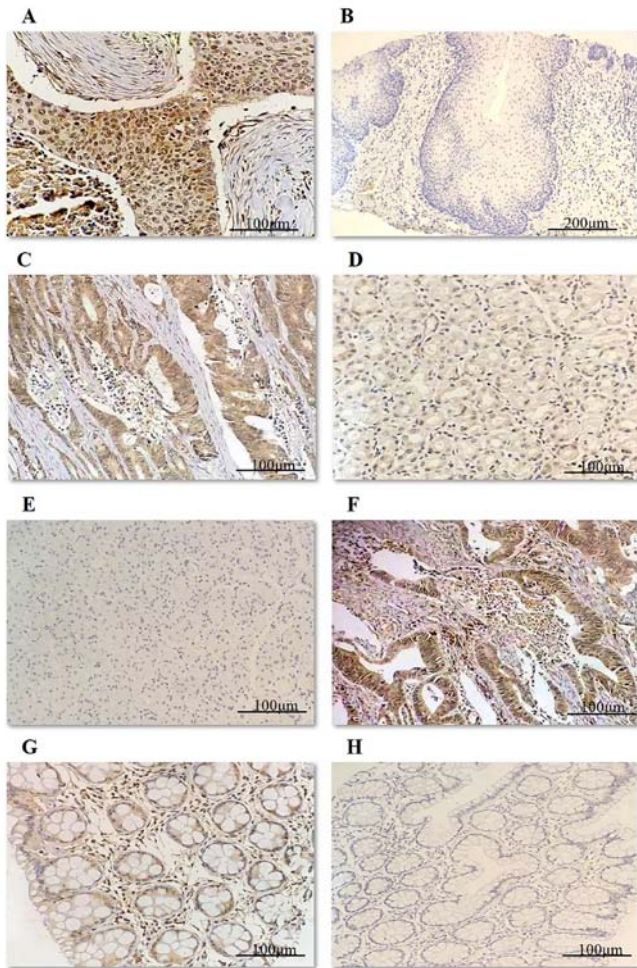


Figure 1. Immunohistochemical staining for GEFT in ESCC, GC and CRC shows positivity in the cytoplasm. (A) GEFT-protein positivity in ESCC (2+). Scale bar, 100 μ M. (B) GEFT-protein negativity in normal esophageal mucosa. Scale bar, 200 μ M. (C) GEFT-protein positivity in GC (3+). Scale bar, 100 μ M. (D) GEFT-protein positivity in normal gastric mucosa (1+). Scale bar, 100 μ M. (E) GEFT-protein negativity in normal gastric mucosa. Scale bar, 100 μ M. (F) GEFT-protein positivity in CRC (3+). Scale bar, 100 μ M. (G) GEFT-protein positivity in normal intestinal mucosa (1+). Scale bar, 100 μ M. (H) GEFT-protein negativity in normal intestinal mucosa. Scale bar, 100 μ M. GEFT, guanine nucleotide exchange factor T; ESCC, esophageal squamous carcinoma; GC, gastric carcinoma; CRC, colorectal cancer; 1+, weak expression; 2+/3+, significantly increased positive expression.

survived and 20 died. The collective OS time of all the patients ranged from 9-33 months, with a median overall survival time of 32 months. Among the 14 patients with GEFT overexpression (2+/3+), five patients died while nine survived. In the patient group with lower GEFT expression (-/1+), 44 patients were available for follow-up. Of those patients, 15 died and 29 survived. There was no significant association between GEFT expression and OS ($\chi^2=0.040$, $P=0.842$; Fig. 2A).

GEFT expression is increased in patients with GC. GEFT protein expression in 60 GC tissues and 60 normal gastric mucosa samples was observed by IHC. The positive GEFT expression rate in GC tissues was 83.33%, which was significantly higher ($\chi^2=61.788$, $P<0.001$) compared with the normal control samples. Of the control samples, seven expressed GEFT protein (1+), but the rest were negative. Fig. 1C

shows positive GEFT-protein expression in GC (3+); and Fig. 1D and E show 1+ and negative GEFT-protein expression, respectively, in normal gastric mucosa samples. Among the 60 GC tissues samples, the GEFT (1+) expression rate was 28.33% (17/60), the GEFT (2+) expression rate was 38.33% (23/60) and the GEFT (3+) expression rate was 16.67% (10/60). The GEFT-negative expression rate was 16.67% (10/60) in GC tissues (Table I).

Association between the clinicopathological parameters of GC and GEFT expression. Table III shows the association between GEFT expression and the clinicopathological features of gastric adenocarcinoma. GEFT expression was associated with Lauren stage ($\chi^2=12.525$, $P=0.002$) and TNM stage ($\chi^2=4.033$, $P=0.045$; Table III), but was not associated with any of the other clinicopathological parameters. The expression of GEFT protein was highest (80%) in the diffuse Lauren stage classification type, lowest (25%) in the intestinal type and 60% in the mixed type. GEFT protein was moderately expressed (2+/3+) in 40.74% (11/27) of TNM stage I/II samples, whereas expression (2+/3+) was lower (66.67%, 22/33) in TNM stage III/IV samples (Table III).

Association between GEFT expression and OS in patients with GC. Of the 60 patients with GC enrolled in the study, 5 cases were lost during follow-up, 35 patients survived, and 20 patients died. The collective OS time of all the patients ranged from 12-33 months, with a median overall survival time of 32 months. In the GEFT overexpression group (2+/3+), survival data for 30 patients were collected. Among them, 12 patients died and 18 survived. In the low GEFT expression group (-/1+), survival data for 25 patients were acquired of which eight patients died and 17 survived. Although the survival rates of patients with GC with higher GEFT expression was lower compared with patients with lower GEFT expression (Fig. 2B), there was no significant association between GEFT expression and survival in patients with GC ($\chi^2=0.501$, $P=0.479$).

HER2 expression in patients with GC. HER2 expression and the mRNA and protein levels in GC are shown in Fig. 3. In the present study, HER2 protein expression was 3+ in 5 of the 60 cases of GC, 2+ in 18 cases, 1+ in 20 cases and 0 in 17 cases. The expression of HER2 protein was absent in all of the normal control samples (Table IV). In cases where the sample was scored as 2+, FISH was used to detect *HER2* gene amplification. Of these samples, 3 of 18 showed *HER2* gene amplification, whereas normal control gastric mucosa samples were negative for *HER2* amplification (Table IV). Therefore, the HER2+ rate in the 60 GC samples was 13.33% (8/60) (Table IV).

The association between HER2 protein expression and the clinicopathological features in patients with gastric adenocarcinoma is shown in Table III. HER2 expression was associated with nerve invasion ($\chi^2=4.005$, $P=0.045$) and TNM stage ($\chi^2=5.600$, $P=0.018$), but was not associated with the other clinicopathological parameters. The expression of HER2 protein was higher in cases with nerve invasion (24.14%) compared with cases without nerve invasion (3.22%). HER2 expression was absent in TNM stage I/II

Table I. GEFT protein expression in ESCC, GC and CRC samples.

GEFT	ESCC		GC		CRC	
	Tumor samples n (%)	Control samples n (%)	Tumor samples n (%)	Control samples n (%)	Tumor samples n (%)	Control samples n (%)
-	12 (20.00)	60 (100.00)	10 (16.67)	53 (88.33)	8 (13.33)	59 (98.33)
1+	34 (56.67)	0 (0.00)	17 (28.33)	7 (11.67)	19 (31.67)	1 (1.67)
2+	14 (23.33)	0 (0.00)	23 (38.33)	0 (0.00)	27 (45.00)	0 (0.00)
3+	0 (0.00)	0 (0.00)	10 (16.67)	0 (0.00)	6 (10.00)	0 (0.00)
χ^2	80.000		61.788		87.896	
P-value	<0.001 ^a		<0.001 ^a		<0.001 ^a	

^aP<0.01. GEFT, guanine nucleotide exchange factor T; ESCC, esophageal squamous carcinoma; GC, gastric carcinoma; CRC, colorectal cancer.

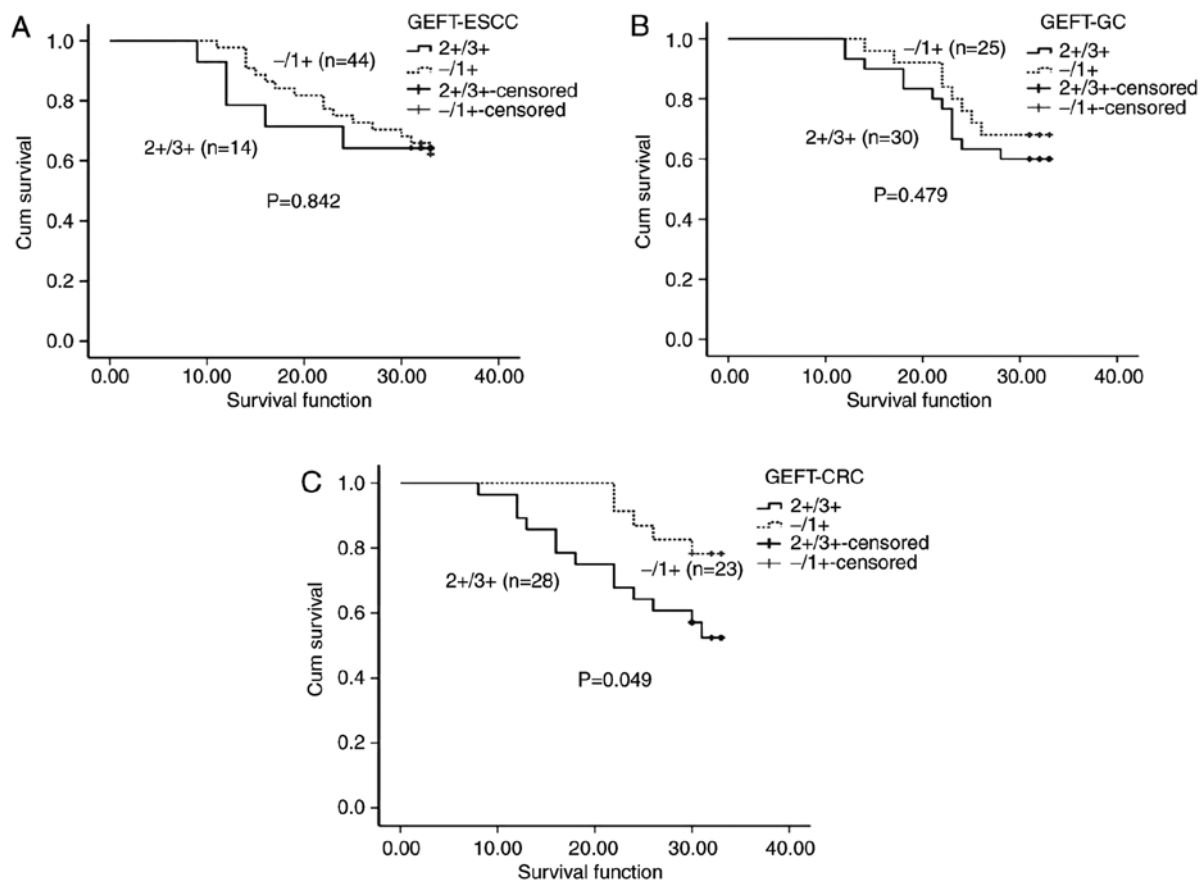


Figure 2. Kaplan-Meier survival curves comparing survival time for patients with upregulated GEFT expression with GEFT protein downregulation. There was no significant association between GEFT expression and overall survival in patients with (A) ESCC ($\chi^2=0.040$, $P=0.842$) and (B) patients with GC ($\chi^2=0.501$, $P=0.479$). (C) Patients with CRC with upregulated expression of GEFT (2+/3+) following surgery had a significantly reduced overall survival time compared with patients with low GEFT levels (-/1+) ($\chi^2=3.876$, $P=0.049$). GEFT, guanine nucleotide exchange factor T; ESCC, esophageal squamous carcinoma; GC, gastric carcinoma; CRC, colorectal cancer.

samples, and its expression was relatively lower in TNM stage III/IV samples (24.24%, 8/33). Of the 8 HER2 positive GC samples, 3 samples were scored as 1+ GEFT expression, 1 case was scored as 2+ GEFT expression, 1 case was scored as 3+ GEFT expression, and 3 cases scored as negative for GEFT expression. No significant correlation was identified between the levels of GEFT and HER2 expression ($r=0.197$, $P=0.132$) (Table V).

GEFT is highly expressed in patients with CRC. In the 60 CRC samples, 52 samples were positive for GEFT protein expression, with 6 samples rated as strongly positive (3+), 27 rated as positive (2+) and 19 rated as weakly positive (1+). The overall GEFT protein expression rate was 86.67% (52/60) which was significantly higher than the control group ($\chi^2=87.896$, $P<0.001$), where only 1 of 60 normal intestinal mucosa samples showed weakly positive GEFT expression (1+) (Table I). Fig. 1F

Table II. Association between GEFT protein expression and clinicopathologic features in patients with esophageal squamous cell carcinoma.

Variable	Cases	GEFT		χ^2 value	P-value
		-/1+ (%)	2+/3+ (%)		
Sex				<0.001	1.000
Male	43	33 (76.74)	10 (23.26)		
Female	17	13 (76.47)	4 (23.53)		
Age, years				0.497	0.481
≥ 60	45	33 (73.33)	12 (26.67)		
<60	15	13 (86.67)	2 (13.33)		
Location				0.542	0.462
Upper esophagus	5	5 (100.00)	0 (0.00)		
Middle/lower esophagus	55	41 (74.55)	14 (25.45)		
Tumor diameter, cm				0.001	0.982
≤ 3	17	13 (76.47)	4 (23.53)		
>3	43	33 (76.74)	10 (23.26)		
Histological type				3.828	0.281
Ulcerative type	28	21 (75.00)	7 (25.00)		
Medullary type	21	19 (90.48)	2 (9.52)		
Constrictive type	9	4 (44.44)	5 (55.56)		
Fungating type	2	2 (100.00)	0 (0.00)		
Tumor differentiation				0.836	0.361
Moderate/well	46	34 (73.91)	12 (26.09)		
Poor	14	12 (85.71)	2 (14.29)		
Depth of invasion				0.023	0.879
T1/T2	12	9 (75.00)	3 (25.00)		
T3/T4	48	37 (77.08)	11 (22.92)		
Nerve invasion				0.586	0.444
Yes	13	11 (84.62)	2 (15.38)		
No	47	35 (74.47)	12 (25.53)		
Vessel carcinoma embolus				2.402	0.121
Yes	28	24 (85.71)	4 (14.29)		
No	32	22 (68.75)	10 (31.25)		
TNM stage				0.106	0.744
I and II	32	24 (75.00)	8 (25.00)		
III and IV	28	22 (78.57)	6 (21.43)		
Lymph node metastasis				0.034	0.854
Yes	27	21 (77.78)	6 (22.22)		
No	33	25 (75.76)	8 (24.24)		

GEFT, guanine nucleotide exchange factor T; TNM, Tumor-Node-Metastasis.

shows GEFT-protein positivity in CRC (3+), and Fig. 1G and H show GEFT protein expression (1+) and negative expression in normal intestinal mucosa, respectively.

Association between the clinicopathological parameters of CRC and GEFT expression. Table VI shows the association between GEFT expression and the clinicopathological factors of CRC. GEFT protein expression was associated with vessel carcinoma embolus ($\chi^2=7.890$, $P=0.005$) and lymph node

metastasis ($\chi^2=5.455$, $P=0.020$), but was not associated with any of the other clinicopathological parameters. Among the samples with vessel carcinoma embolus (23/32) or lymph node metastasis (21/30), high GEFT protein expression (2+/3+) was significantly higher than in samples without vessel carcinoma embolus (10/28) or lymph node metastasis (12/30).

Association between GEFT expression and OS in CRC. Survival data were collected for 51 of the 60 patients with CRC

Table III. Association between GEFT protein expression, HER2 expression and clinicopathologic features in patients with gastric cancer.

Variable	Cases	GEFT 2+/3+ (%)	P-value	HER2+ (%)	P-value
Sex			0.297		0.142
Male	46	27 (58.70)		4 (8.70)	
Female	14	6 (42.86)		4 (28.57)	
Age, years			0.693		0.898
≥60	35	20 (57.14)		4 (11.43)	
<60	25	13 (52.00)		4 (16.00)	
Tumor diameter			0.373		0.644
≥5 cm	34	17 (50.00)		5 (14.71)	
<5 cm	26	16 (61.54)		3 (11.54)	
Lauren stage			0.002 ^b		0.065
Diffuse type	20	16 (80.00)		3 (15.00)	
Intestinal type	20	5 (25.00)		5 (25.00)	
Mixed type	20	12 (60.00)		0 (0.00)	
Tumor differentiation			0.053		0.577
Moderate/well	21	8 (38.10)		4 (19.05)	
Poor	39	25 (64.10)		4 (10.26)	
Mucinous adenocarcinoma			0.807		0.811
Yes	21	12 (57.14)		2 (9.52)	
No	39	21 (53.85)		6 (15.38)	
Depth of invasion			0.481		1.000
T1/T2	5	2 (40.00)		1 (20.00)	
T3/T4	55	31 (56.36)		7 (12.73)	
Nerve invasion			0.035		0.045 ^a
Yes	29	20 (68.97)		7 (24.14)	
No	31	13 (41.94)		1 (3.22)	
Vessel carcinoma embolus			0.925		0.256
Yes	47	26 (55.32)		8 (17.02)	
No	13	7 (53.85)		0 (0.00)	
TNM stage			0.045 ^a		0.018 ^a
I and II	27	11 (40.74)		0 (0.00)	
III and IV	33	22 (66.67)		8 (24.24)	
Lymph node metastasis			1.000		0.150
Yes	49	27 (55.10)		8 (16.33)	
No	11	6 (54.55)		0 (0.00)	
Distant metastasis			0.386		1.000
Yes	2	0 (0.00)		0 (0.00)	
No	58	33 (56.90)		8 (13.80)	

^aP<0.05, ^bP<0.01. GEFT, guanine nucleotide exchange factor T; TNM, Tumor-Node-Metastasis; 2+/3+, significantly increased positive expression.

in the present study. The remaining 9 cases were lost during follow-up. Of these cases, 34 patients survived, and 17 patients died. The collective OS time ranged from 8-33 months, with a median overall survival time of 30 months. GEFT protein upregulation (2+/3+) was detected in 33 patients with CRC, but survival data was available from only 28 patients, with 5 patients lost to follow-up. In patients with increased GEFT expression, 12 patients died and 16 survived. In the low GEFT expression group (-/1+), survival state information was collected for

23 patients. In this group, 5 patients died and 18 survived. The survival rates of patients with CRC with GEFT upregulation was lower compared with patients with lower GEFT expression ($\chi^2=3.876$, $P=0.049$; Fig. 2C). Additionally, patients with CRC with upregulated expression of GEFT had a less favorable outcome compared with patients with low expression of GEFT.

MMRPs are expressed in patients with CRC. The expression of the four CRC-associated MMRPs, including MLH1, MSH2,

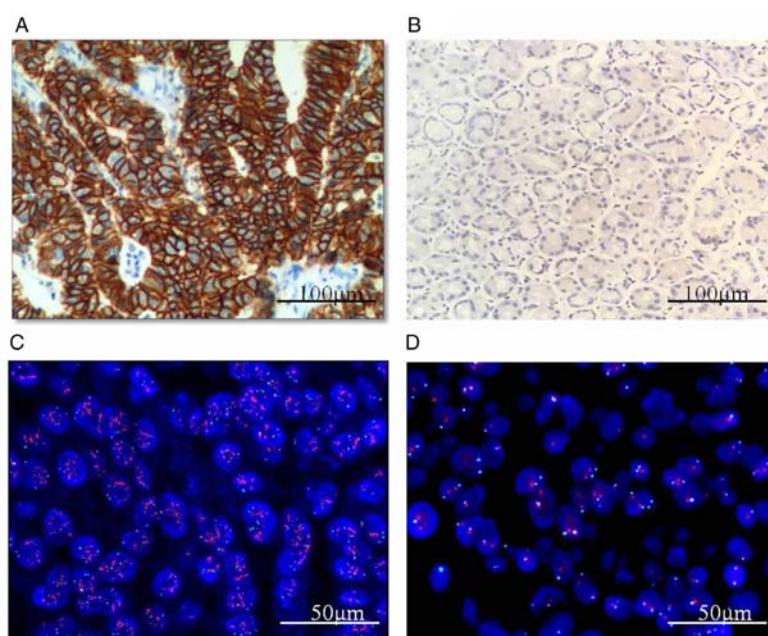


Figure 3. HER2 expression in GC. (A) Expression of HER2 protein was found to be 3+ based on the immunohistochemical staining. (B) Expression of HER2 protein was negative in normal gastric mucosa. (C) *HER2* gene (red) expression was amplified in GC, as shown using fluorescence *in situ* hybridization. (D) *HER2* gene expression was absent in normal gastric mucosa. Chromosome 17 centromere locus (CEP17) is shown in green. HER2, human epidermal growth factor receptor-2; GC, gastric carcinoma.

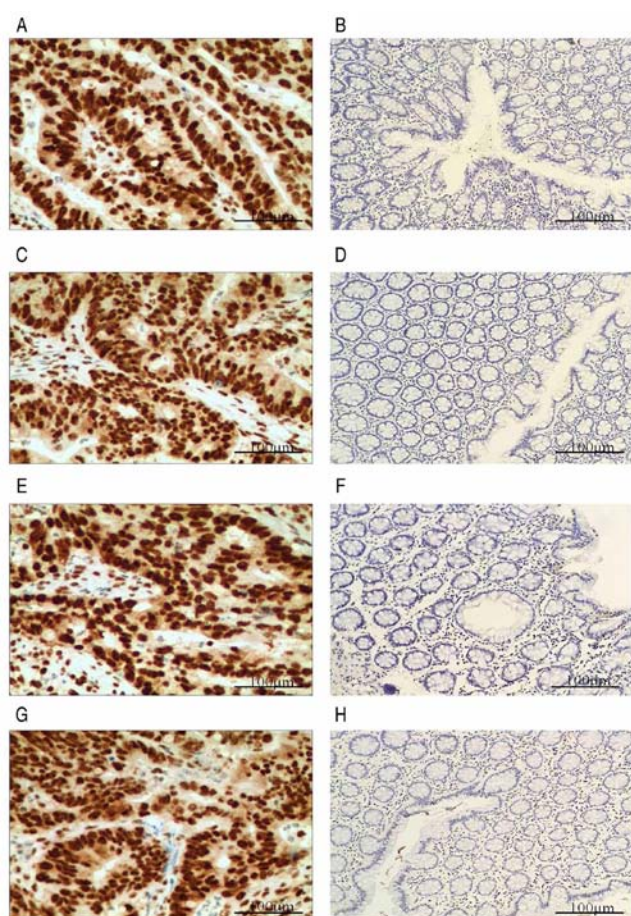


Figure 4. Immunohistochemical staining for mismatch repair protein in colorectal cancer shows positivity in the nucleus. MLH1 expression in (A) CRC tissue and (B) normal intestinal mucosa. MSH2 expression in (C) CRC tissue and (D) normal intestinal mucosa. MSH6 expression in (E) CRC tissue and (F) normal intestinal mucosa. PMS2 expression in (G) CRC tissue and (H) normal intestinal mucosa.

MSH6 and PMS2, were evaluated by IHC. Fig. 4 shows IHC staining of MMRP in CRC. Loss of at least one MMRP was defined as MMRP-defective (MMR-D) and no MMRP loss was defined as MMRP-intact (MMR-I). In the present study, 6 cases of CRC were MMR-D and 54 were MMR-I. Among the MMR-D group, 4 samples displayed loss of PMS2, 1 showed loss of MSH2 and MSH6, and 1 exhibited loss of PMS2 and MLH1 (Table VII).

Comparison of clinicopathological data of the MMR-D and MMR-I groups in colorectal adenocarcinoma. The association between the MMR-D and MMR-I groups in colorectal adenocarcinoma is shown in Table VI. MMR-D incidence was lower in patients with a tumor maximal diameter ≥ 5 cm ($\chi^2=5.208$, $P=0.022$; Table VI). However, there was significant difference between MMR-D and MMR-I groups in any of the other clinicopathological variables assessed in patients with CRC (Table VI). In the 6 MMR-D cases of CRC, 4 cases were GEFT expression negative, and 2 cases were GEFT expression (2+). The association between GEFT and MMRPs was further examined by correlational analyses, which identified a significant inverse correlation ($r=-0.285$, $P=0.027$) (Table V).

MSI in colorectal adenocarcinoma. Among the 60 CRC specimens, 6 samples were MSI-H, 1 was MSI-L and 53 were MSS. Analysis of the microsatellite sites showed that among the MSI-H group, 3 samples exhibited *Bat25* and *Bat26* mutations, 1 sample showed *Bat25*, *Bat26* and *D5S346* mutations, 1 sample showed *Bat25* and *D17S250* mutations and 1 sample exhibited *Bat25* and *D2S123* mutations. Only one sample is MSI-L with *Bat25* mutations (Table VIII). Using the PCR test results as the standard, the positive conformity rate in the present study was 83.33% (5/6) and the negative conformity rate was 98.15% (53/54). The coincidence rate between PCR

Table IV. HER2 protein and gene expression in the GC samples.

A, Negative	
Variable	n (%)
IHC-	17 (28.34)
IHC 1+	20 (33.33)
IHC 2+, FISH-	15 (25.00)
Total	52 (86.67)
B, Positive	
Variable	n (%)
IHC 2+, FISH+	3 (5.00)
IHC 3+	5 (8.33)
Total	8 (13.33)

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

and IHC was 96.67% (58/60), and the consistency check result (κ) was 0.815 ($P < 0.001$). The sensitivity of IHC was 83.33% (5/6) and the specificity was 98.15% (53/54) (Table IX). These results demonstrate that analysis via PCR and IHC are well associated.

KRAS gene mutations in patients with CRC. Mutations in the *KRAS* gene were detected by qPCR in 16 of the 60 CRC samples (in codon 12 in 13 cases and in codon 13 in 3 cases), with a mutation rate of 26.67%. Specific mutation types and the incidence rates are listed in Table X.

Comparison of clinicopathological data of *KRAS* gene mutations in patients with CRC. The association between *KRAS* mutations and clinicopathological features in CRC is listed in Table VI. The *KRAS* mutation rate was higher in poorly differentiated CRC (62.50%) compared with well/moderately differentiated samples (21.15%, $\chi^2 = 4.131$, $P = 0.042$). No difference in *KRAS* mutation status was identified with any of the other clinicopathological variables assessed. Among the 16 *KRAS* gene mutation samples of CRC, 1 sample was scored as 1+, for 8 samples were scored as 2+, 4 samples were scored as 3+, and 3 samples were scored as negative for GEFT protein expression. Further investigation of the association between GEFT expression and *KRAS* mutation by correlation analyses demonstrated a significant correlation ($r = 0.697$, $P < 0.001$) (Table V).

Discussion

GEFT was initially identified in 2003 by Guo *et al* (3), and it regulates cellular processes by catalyzing GDP/GTP exchange on Rho GTPases, including Rac1, Cdc42 and RhoA. These Rho GTPases are essential for cytoskeletal dynamics and are particularly important in migration of cancer cells (34,35). Although GEFT is expressed in a variety of tumors, its expression in

Table V. Association between GEFT and HER-2/MMRP/KRAS.

GEFT	HER2		MMRP		KRAS mutation	
	+	-	MMRD	MMRI	Yes	No
-	3	7	4	4	3	5
1+	3	14	0	17	1	16
2+	1	22	2	25	8	19
3+	1	9	0	6	4	2
r-value	0.197		-0.285		0.697	
P-value	0.132		0.027 ^a		<0.001 ^b	

^a $P < 0.05$, ^b $P < 0.01$. GEFT, guanine nucleotide exchange factor T; MMRD, mismatch repair protein-deficient; MMRI, mismatch repair protein-intact.

malignant digestive tract tumors has not been studied, to the best of our knowledge. The present study found that GEFT protein expression was higher in malignant digestive tract tumors compared with normal tissues. GEFT expression in ESCC samples was higher compared with normal squamous epithelium, but weaker than in tumor samples from patients with GC and CRC. The GEFT-positive samples primarily included samples with 1+ and 2+ GEFT protein expression. However, an association between GEFT expression and the clinicopathological factors of ESCC was not found. In the ESCC cohort, the median survival time was 32 months, which is close to the maximum survival time (33 months). This is the result of a short follow-up time, and thus the majority of patients were still alive. As such, the survival time in the present study is not really indicative of overall survival. This was also true for the CRC and GC cohorts. The association between GEFT protein expression and OS were further analyzed, and there was no significant association between GEFT expression and OS in ESCC. Therefore, increased GEFT protein expression in ESCC samples may not represent an important factor in the pathogenesis of esophageal cancer.

In GC tissues, GEFT protein expression was higher compared with the gastric mucosa. According to Lauren staging, GEFT protein expression was the highest in the diffuse type and lowest in the intestinal type. Several studies have demonstrated that the Lauren stage was closely associated with certain clinicopathological characteristics such as age, sex, tumor size, location, grade, invasion depth, lymphovascular invasion and prognosis of GC (36,37). Patients with intestinal type GC were predominantly older, male, had a smaller tumor size, had relatively well differentiated tumors, and tumors which has less tumor invasion depth and less lymphovascular invasion (37). Compared with proximal, middle and whole stomach, the incidence of intestinal type GC was the highest in distal stomach (37). Specifically, patients with intestinal-type tumors exhibited more favorable outcomes compared with patients with diffuse-type tumors (38-40). The data from the present study suggested that increased GEFT protein expression in diffuse-type samples may be associated with a poor prognosis. Consistently, the expression of GEFT protein was higher in TNM stage III/IV samples (66.67%) compared with

Table VI. Association between GEFT protein expression, MMRD, *KRAS* mutations and clinicopathologic features in patients with colorectal cancer.

Variable	Cases	GEFT 2+/3+ (%)	P-value	MMRD (%)	P-value	KRAS mutation (%)	P-value
Sex			0.983		0.299		0.907
Male	33	18 (54.54)		5 (15.15)		9 (27.27)	
Female	27	15 (55.56)		1 (3.70)		7 (25.93)	
Age, years			0.875		1.000		0.071
≥60	34	19 (55.88)		3 (8.82)		6 (17.65)	
<60	26	14 (53.85)		3 (11.54)		10 (38.46)	
Tumor diameter			1.000		0.022 ^a		0.836
≥5 cm	40	22 (55.00)		1 (7.50)		11 (27.50)	
<5 cm	20	11 (55.00)		5 (25.00)		5 (25.00)	
Location			0.523		0.510		0.801
Right hemicolon	12	5 (41.67)		2 (16.67)		3 (25.00)	
Sigmoid colon	15	8 (53.33)		2 (13.33)		5 (33.33)	
Rectum	33	20 (60.61)		2 (6.06)		8 (24.24)	
Tumor differentiation			1.000		1.000		0.042 ^a
Moderate/well	52	29 (55.76)		5 (9.62)		11 (21.15)	
Poor	8	4 (50.00)		1 (12.50)		5 (62.50)	
Mucinous adenocarcinoma			1.000		1.000		0.214
Yes	7	4 (57.14)		1 (14.29)		0 (0.00)	
No	53	29 (54.72)		5 (9.43)		16 (30.19)	
Depth of invasion			0.668		0.919		1.000
T3	14	7 (50.00)		2 (14.29)		4 (28.57)	
T4	46	26 (56.52)		4 (8.70)		12 (26.09)	
Nerve invasion			0.312		0.693		1.000
Yes	3	3 (100.00)		1 (33.33)		1 (33.33)	
No	57	30 (52.63)		5 (8.77)		15 (26.32)	
Vessel carcinoma embolus			0.005 ^b		1.000		0.785
Yes	32	23 (71.87)		3 (9.36)		9 (28.13)	
No	28	10 (35.71)		3 (10.71)		7 (25.00)	
TNM stage			0.077		0.546		0.755
I and II	32	21 (65.62)		2 (6.25)		8 (25.00)	
III and IV	28	12 (42.86)		4 (14.29)		8 (28.57)	
Lymph node metastasis			0.020 ^a		1.000		0.559
Yes	30	21 (70.00)		3 (10.00)		9 (30.00)	
No	30	12 (40.00)		3 (10.00)		7 (23.33)	
Distant metastasis			0.712		1.000		0.744
Yes	11	5 (45.45)		1 (9.09)		2 (18.18)	
No	49	28 (57.14)		5 (10.20)		14 (28.57)	

^aP<0.05, ^bP<0.01. GEFT, guanine nucleotide exchange factor T; MMRD, mismatch repair protein-deficient; TNM, Tumor-Node-Metastasis; 2+/3+, significantly increased positive expression.

TNM stage I/II samples, suggesting that GEFT may play an important role in the poor prognosis of these patients with GC. Therefore, the association between GEFT protein expression and OS was further analyzed. Although the survival rates of patients with GC with elevated GEFT expression was lower compared with patients with low GEFT expression, there was no significant association. However, increased sample sizes and longer follow-up time after surgery may be required to

fully describe the association between GEFT and prognosis in patients with GC.

Numerous studies have implicated HER2 in the development of various types of cancer. HER2 expression was detected in 6.1-23.0% of GCs cases (16-18). Similarly, the HER2-positive rate in the 60 GC samples in the present study was 13.33%. However, the prognostic value of HER2 status in GC is still controversial. Some studies report that HER2

Table VII. Expression of the four MMRPs in the colorectal cancer samples.

n	PMS2	MSH2	MSH6	MLH1
4	-	+	+	+
1	+	-	-	+
1	-	+	+	-
54	+	+	+	+

MMRP, mismatch repair protein.

Table VIII. MSI state in the colorectal cancer samples.

MSI	n	Bat26	Bat25	D5S346	D2S123	D17S250
MSI-H	3	+	+	-	-	-
	1	+	+	+	-	-
	1	-	+	-	-	+
	1	-	+	-	+	-
MSI-L	1	-	+	-	-	-
MSS	53	-	-	-	-	-

MSI, microsatellite instability; H, high; L, low; MSS, microsatellite stable.

Table IX. MSI results in CRC.

Variable	MMR-D, n	MMR-I, n	Total
MSI-H	5	1	6
MSI-L	1	0	1
MSS	0	53	53
Total	6	54	60

MSI, microsatellite instability; CRC, colorectal cancer; H, high; L, low; MSS, microsatellite stable; MMR, mismatch repair protein; D, deficient; I, intact.

overexpression is an adverse prognostic factor (41-44), but others suggest that there is no association between HER2 expression and survival rate (17,45-48). In the present study, HER2 protein expression was higher in tumors with nerve invasion, and significantly lower in TNM stage I/II samples compared with TNM stage III/IV samples. These findings corroborate another report showing that HER2 overexpression was associated with poor prognosis in patients with GC (44). Because both GEFT protein and HER2 upregulation was associated with GC TNM stage in the present study, further analysis of the correlation between GEFT and HER2 expression was performed. However, there was no significant correlation between these two proteins.

The high mortality rate of CRC, another common digestive tract tumor, stems from its metastatic potential (49). In the present study, GEFT protein expression levels were

Table X. *KRAS* mutations in colorectal cancer.

Detected region	Mutation	Base change	Incidence	Mutation rate (%)
Codon 12	G12V	35G>T	1	1.67
	G12D	35G>A	10	13.33
	G12R	34G>C	1	1.67
	G12S	34G>C	1	1.67
Codon 13	G13D	38G>A	3	5

significantly higher in patients with CRC compared with the control samples. Furthermore, GEFT protein expression was higher in CRC samples with vessel carcinoma embolus or lymph node metastasis. These results suggested that the GEFT protein may promote CRC metastasis. The data in the present study demonstrate that the survival rates of patients with CRC with GEFT overexpression was lower compared with patients with lower GEFT expression. These patients also had a less favorable outcome compared with that of the low GEFT expression group. Therefore, GEFT may contribute to the poor prognosis of patients with CRC by promoting metastasis.

Several studies have reported that MSI is present in ~15% of patients with CRC. The results of the present study showed that 10% of samples lost MMRP. Among the 60 CRC specimens, 6 were MSI-H, 1 was MSI-L and 53 were MSS. In comparison, the MSI rate in patients with CRC was ~11.67%. MSI status has been evaluated as a prognostic and predictive biomarker in patients with CRC. Specifically, patients diagnosed with CRC that are MSI-positive present with an improved prognosis when not treated with 5-fluorouracil chemotherapy following surgery (50). MSH CRCs are frequently located in the proximal colon (50-53), are poorly differentiated, and have mucinous or medullary histology. Additionally, MSH incidence is high in individuals <50 or >70 years old, but the incidence is low in patients between these ages (54). In the present study, the incidence of MMR-D was lower in patients with a maximal tumor diameter ≥5 cm, but was not associated with any other clinicopathological parameters in patients with CRC. However, there was a significant inverse correlation between GEFT and MMRP expression. Therefore, it was speculated that MSH/L may be more common in patients with CRC with GEFT expression, though increased sample size and further experiments are required to validate this hypothesis.

KRAS is a small GTPase and a member of the RAS family. Activated KRAS promotes the regulation of cellular proliferation through the receptor tyrosine kinase MAPK/PI3K signaling cascades (55). The rate of *KRAS* gene mutations in CRC is 35-45% (28,56,57). In the present study, the rate of mutation was 26.67% in patients with CRC, which was notably lower than previously reported. Although *KRAS* gene mutation status in patients with CRC is used to inform treatment options, particularly in the selection of targeted therapeutic drugs such as cetuximab, the association between *KRAS* gene mutations and the prognosis of patients with CRC has not been definitively determined. In the present study, the *KRAS* mutation rate was higher in poorly differentiated CRC compared

with well/moderately differentiated tumors, although a difference in *KRAS* mutation status between other clinicopathologic features was not identified. This suggests that patients carrying tumors with *KRAS* mutations may exhibit shorter overall survival. Furthermore, a significant correlation between GEFT expression and *KRAS* mutation in CRC was identified. Notably, as both GEFT and *KRAS* proteins are small GTPases, both may be similarly involved in the development of CRC.

The systematic study described here demonstrated, for the first time, that the GEFT protein is expressed in malignant digestive tract tumors. The results indicate that GEFT protein expression is higher in ESCC, GC and CRC tumors compared with normal adjacent tissues. GEFT may predominantly act as a tumor promoter in adenocarcinomas of the stomach, as suggested by associations with diffuse tumor type and TNM stages III/IV in GC. In GC, HER2 was overexpressed and was associated with nerve invasion and TNM stages III/IV. Therefore, HER2 likely promoted the incidence of GC. Patients with CRC with upregulated protein expression of GEFT frequently had vessel carcinoma embolus or lymph node metastasis, and had a less favorable outcome. GEFT may contribute to poor prognosis of patients with CRC by promoting metastasis. The importance of MSI state and *KRAS* mutation status in patients with CRC was demonstrated. Furthermore, GEFT protein expression was associated with MSS and *KRAS* mutations. GEFT expression and *KRAS* mutations, therefore, may synergistically promote the incidence of CRC.

In conclusion, GEFT may be an oncogenic factor in malignant digestive tract tumors, particularly in CRC. However, the specific underlying mechanisms involved in the progression of these tumors remain to be elucidated.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the author on reasonable request.

Authors' contributions

QX conceived and designed the experiments. YW performed the experiments, analyzed the data, and contributed to the writing of the manuscript. BG, GG and YZ contributed to the sample collection.

Ethics approval and consent to participate

The purpose of the present research was explained to the participants, who all signed a written consent prior to the study. The present study was approved by The Institutional Ethics

Committee of the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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