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Original Article

FBI-1 mRNA in normal mucosa is an independent prognostic factor in colorectal cancer patients

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Abstract: Although several studies provide evidence that FBI-1 is an important gene regulator in colorectal cancer (CRC), it is noteworthy that, to our knowledge, no analysis of the correlation between FBI-1 expression and prognosis in CRC has been reported. Using real-time RT-PCR, we detected FBI-1 mRNA in 161 CRC patients (primary tumor, along with the corresponding normal mucosa), 36 liver metastases, and analyzed the relationship of its expression with clinicopathological features. Colon cancer cell lines were used to study FBI-1 function. Our study found that FBI-1 was significant up-regulated in tumor tissue (2.621 ± 0.157) compared with the corresponding normal mucosa (1.620 ± 0.165, $P < 0.0001$). FBI-1 in normal mucosa was a prognostic factor ($P = 0.039$, RR 0.431, 95% CI 0.194-0.958), independent of gender, age, stage, and differentiation. High levels of FBI-1 mRNA were related with good survival. Patients with complications had a higher primary tumor FBI-1 expression than those without complications (3.400 ± 0.332 vs. 2.516 ± 0.241, $P = 0.032$). Suppression of FBI-1 in colon cancer cell lines could repress proliferation of cancer cells. In conclusion, FBI-1 mRNA is overexpressed in CRC, and takes part in the development of CRC. FBI-1 mRNA in normal mucosa is an independent prognostic factor. Our findings give further support to the concept of "field cancerization", and hint that when we study a biomarker, we should not only focus on the tumor tissue but also the corresponding normal mucosa.

Keywords: Colorectal cancer, FBI-1, prognosis, biomarker, field cancerization

Introduction

Colorectal cancer (CRC) is a major public health problem, being the third most commonly diagnosed cancer and the fourth cause of cancer death worldwide [1]. The TNM (Tumor size, lymph Node involvement, and distant Metastasis) staging system is the current standard for determining the prognosis of patients with CRC. Recently, some of the prognostic biomarkers for CRC have been proposed such as, chromosomal instability, microsatellite instability, RAS mutation, BRAF mutation, mRNA and microRNA expression profile. Although these factors have shown potential in this field, there is insufficient evidence to recommend routine clinical use of any of these biomarkers [2]. Therefore, discovery of robust prognostic and predictive biomarkers in CRC patients is imperative.

Factor that binds to the inducer of short transcripts of human immunodeficiency virus-1 (FBI-1) is a recently characterized proto-oncogenic transcription factor, also named as LRF (leukemia/lymphoma related factor) or Pokemon (POK erythroid myeloid ontogenic factor) [3-5]. More and more evidence has shown that FBI-1 is a master regulator of oncogenesis, and represses transcription of a variety of important genes, including ARF, c-fos, and c-myc oncogenes and extracellular matrix genes [6]. FBI-1 is up-regulated in ovarian cancers, and a higher level of FBI-1 is significantly associated with advanced stages, shorter overall survival, and disease-free survival [7]. Compared with adjacent non-tumorous liver tissue, FBI-1 is significantly overexpressed in hepatocellular carcinoma (HCC), and is correlated with multiple tumor nodes, Edmondson-Steiner grade, and poor prognosis in patients with HCC [8].

FBI-1 mRNA is overexpressed in CRC cancer tissue compared with the adjacent normal mucosa. FBI-1 silencing inhibits proliferation of colon cancer [9]. ETS-1 is a genome-wide effector of RAS/ERK signaling in epithelial cells, and is involved in the development and progression of cancer [10, 11]. More recently, FBI-1 has been shown to enhance colon cancer cell proliferation, invasion, and metastasis via the ETS-1 signaling pathway activity [12].

Although several studies provide evidence that FBI-1 is an important gene regulator in CRC, it is noteworthy that no analysis of correlation between FBI-1 expression and patient prognosis in CRC has been reported. In this study, we detected the mRNA level of FBI-1 in 161 CRC patients and 36 liver metastases, and found that FBI-1 was overexpressed in cancer tissue, and that FBI-1 in normal mucosa was an independent prognostic factor in CRC patients.

Materials and methods

Patients and samples

The study included 161 patients with CRC and 36 CRC patients with liver metastases that were diagnosed and treated at Linköping University Hospital between 1980 and 2009. The eligible patients were required to be primary CRC without preoperative radiotherapy and/or chemotherapy. For each patient, samples from primary tumor and the corresponding normal mucosa (taken from morphologically normal colonic or rectal tissues more than 10 cm from cancer) were collected. The samples were examined for the presence of tumor cells and usually tumor cells were at least 80%, as determined at the Department of Pathology at Linköping University Hospital. All specimens were flash-frozen in liquid nitrogen and then stored at -80°C. The patient's gender, age, tumor site, TNM stage, differentiation, and complications (including infection, ileus, anastomic leak, etc.) were obtained from surgical and pathological records from the hospital. No information was available about age in 4 patients, location in one patient, stage in 11 patients, and differentiation in 6 patients. The survival analysis was based on overall survival.

Cell culture and transient transfection

Human colon cancer cell lines KM12SM and KM12L4A used in this study were a kind gift from Dr. Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX). The cell lines were cultured in Eagle's Minimal Essential Medium (EMEM, ATCC®30-2003), supplemented with 1% PEST (Invitrogen, Carlsbad, CA), 1.5 mM L-glutamine (Invitrogen), 2% vitamin solution (Invitrogen), and 10% FBS (Invitrogen). The cells were incubated at 37°C with 5 % CO_2. All cell lines were routinely tested as negative for mycoplasma. FBI-1 siRNA ON-TARGET plus SMARTpool (GE Healthcare Dharmacon Inc.) and ON-TARGET *plus* Non-targeting Pool (Negative control) (GE Healthcare Dharmacon Inc.) were transfected at a final concentration of 50 nM using the Thermo Scientific DharmaFECT Transfection Reagents (GE Healthcare Dharmacon Inc.) according to the manufacture's protocol.

RNA extraction and cDNA preparation

According to the manufacturer's instructions, total RNA of CRC, the corresponding normal mucosa and colon cancer cell lines was extracted using the TRizol reagent (Sigma-Aldrich, St. Louis, MO) and RNeasy extract Kit (QIAGEN). The concentration, purity, and integrity of RNA was measured by NanoDrop (Thermo Scientific, Wilmington, DE) and Bioanalyzer Agilent (Agilent Technologies, Santa Clara, CA). For reverse-transcription, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was utilized. Briefly, a total of 550 ng RNA was mixed with 3.0 μL 10×RT Buffer, 1.2 μL 25×dNTP Mix (100 mM), 3.0 μL 10×RT Random Primers, 1.5 μL 50 U/μL MultiScribe™ Reverse Transcriptase without RNase inhibitor, and Nuclease Free Water in a final volume of 20 μL. The program was as follows: 25°C 10 min, 37°C 120 min, 85°C 5 min, 4°C 120 min (MJ Research PTC-200 Thermo cycler).

Real-time PCR

The relative expression levels of FBI-1 were determined by qRT-PCR in Applied Biosystems 7900 HT Fast Real-Time PCR System and normalized to GAPDH. Primers and probes were TaqMan™® Gene Expression Assays for FBI-1 (Hs00252415_s1) and GAPDH (4352934E) (Applied Biosystems). Fifteen μL PCR mix included 1 μL RT product, 7.5 μL TaqMan Fast Universal PCR Master Mix (2×No AmpErase UNG), 0.75 μL 2×TaqMan gene Assay, 5.75 μL Nuclease Free Water. The reactions were incu-

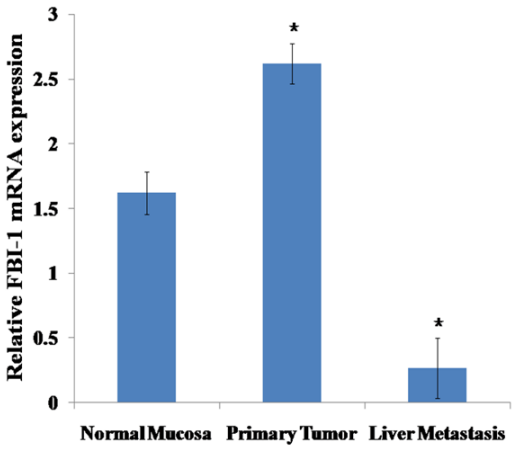

Figure 1. The mRNA level of FBI-1 in normal mucosa, primary tumor, and liver metastasis. Compared with the corresponding normal mucosa, FBI-1 was significantly up-regulated in primary tumor (2.621 ± 0.157 vs. 1.620 ± 0.165, $P < 0.0001$) and down-regulated in liver metastasis (0.270 ± 0.232, $P = 0.0002$).

bated in a 96 well plate at 95°C for 20 sec, followed by 40 circles of 95°C for 3 sec and 60°C for 30 sec. In addition, ddH20 as the no-template control was analyzed for every plate. All reactions, including no-template control, were performed in triplicates.

Western blot

The cells were seeded in 6-well plates and were transfected with FBI-1 siRNA and negative control respectively, and incubated for 48 h. Cells were trypsinized, and the protein was extracted by lysis buffer (Sigma, St Louis, MO) and stored at -20°C. The protein concentration was determined by the colorimetric Bradford protein assay reagent (Pierce, Woburn, MA). Equal amounts of protein (25 μg) were loaded, separated by electrophoresis for 50 min at 200 V, and transferred to PVDF membrane (Amersham Bioscience/GE Healthcare, Piscataway, NJ) for 55 min at 100 V. The membranes were blocked with 5% milk powder in TBS containing 0.1% Tween-20 for 1 hour at room temperature and incubated with the primary antibody FBI-1 (Abcam, ab70208) (1:200) overnight at 4°C. The membranes were washed and subsequently incubated with the secondary HRP-conjugated polyclonal goat anti-rabbit (1:2000, DAKO Cytomation, Glostrup, Denmark) for 1 hour at room temperature. Protein bands were detected using ECL plus Western Blotting Detection System (Amersham Bioscience/GE Healthcare, Piscataway, NJ). Anti-β-actin (1:5000, Cell Signaling Technology, Danvers, MA) was used as a loading control.

MTT assay

Cell proliferation was measured by the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay. KM12SM and KM12L4A cells were plated at a density of 5×10^3 cells/well in 96-well plate, and incubated for 24 h. The cells were then transfected with FBI-1 siRNA or negative control at a final concentration of 50 nM and incubated for 24 h. After discarding the transfection medium, adding 200 μL culture medium, the cells were continued incubating for 24, 48, 72 hours prior to MTT analysis. The final DMSO concentration was less than 0.1%. Then, 20 μL of 5 mg/mL solution of MTT in PBS was added to each well. The plates were incubated for another 4 h at 37°C. The precipitate was solubilized in 100% DMSO 150 μL/well, and shaken for 10 min. Absorbance of each well was measured on a microplate reader (Anthos ht III, Anthos Labtec Instruments GmbH, Wals, Austria) at a wave length of 490 nm. Each test was repeated in triplicate.

Statistical analysis

The values for the mRNA level were transformed to $\log_2$ values and the data were normally distributed. Paired *t*-test, or student's *t*-test, or one-way ANOVA (two sided) method was performed using SPSS software 17.0 (SPSS, Inc., Chicago, IL). The Kaplan-Meier method was applied to calculate the survival curve. Cox's Proportional Hazard model was used to estimate the relationship between FBI-1 and patient survival in univariate and multivariate analyses. $P < 0.05$ was considered as statistical significance. All values were divided at a cut-off point of 25%, to distinguish between low and high FBI-1 expression level.

Results

Expression of FBI-1 mRNA in normal mucosa, primary tumor, and liver metastasis in CRC patients

Among 161 CRC patients, compared with the corresponding normal mucosa, 108 (67%) tumor tissues showed FBI-1 mRNA overexpression, of which 76 (47%) tumor tissues had > 2 times higher expression. Fifty-three (33%)

Table 1. Relationship between FBI-1 of normal mucosa, primary tumor, and clinicopathological variables in CRC patients

Characteristics	No	Normal mucosa FBI-1		Primary tumor FBI-1	
		Mean ± SE	*P* value	Mean ± SE	*P* value
Gender			0.008		0.421
Male	103	1.294 ± 0.200		2.527 ± 0.191	
Female	58	2.199 ± 0.274		2.792 ± 0.275	
Age (years)			0.546		0.481
< 70	52	1.799 ± 0.299		2.789 ± 0.317	
≥ 70	105	1.585 ± 0.200		2.547 ± 0.183	
Location			0.154		0.590
Colon	102	1.823 ± 0.209		2.679 ± 0.199	
Rectum	58	1.337 ± 0.260		2.501 ± 0.262	
Stage			0.109		0.362
I	20	1.250 ± 0.461		2.740 ± 0.449	
II	77	2.048 ± 0.235		2.950 ± 0.242	
III	35	1.112 ± 0.349		2.761 ± 0.261	
IV	18	1.461 ± 0.486		2.042 ± 0.400	
Differentiation			0.841		0.400
Well	19	1.807 ± 0.479		2.847 ± 0.409	
Moderately	94	1.533 ± 0.216		2.466 ± 0.222	
Poorly	42	1.683 ± 0.322		2.945 ± 0.283	
Complications			0.488		0.032
Yes	28	1.772 ± 0.373		3.400 ± 0.332	
No	48	1.440 ± 0.292		2.516 ± 0.241	

SE, standard error; Varied number in the different variables is due to missing data.

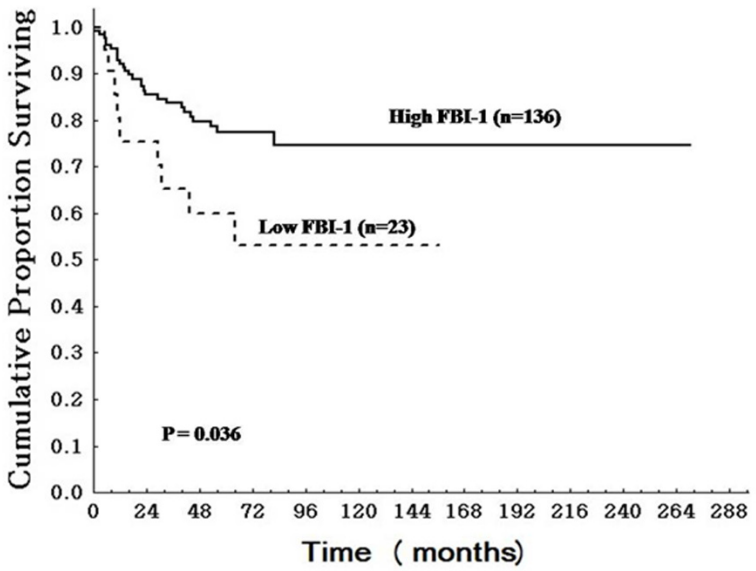

Figure 2. FBI-1 mRNA in normal mucosa was an independent prognostic factor.

tumor tissues showed low FBI-1 mRNA expression, of which 27 (17%) had > 2 times lower expression. Compared with the corresponding normal mucosa, FBI-1 was significantly up-regulated in tumor tissue (2.621 ± 0.157 vs. 1.620 ± 0.165, $P < 0.0001$, **Figure 1**), while FBI-1 was down-regulated in liver metastasis (0.270 ± 0.232 vs. 1.620 ± 0.165 $P = 0.0002$, **Figure 1**). The difference between primary tumor and liver metastasis was also significant ($P < 0.0001$).

FBI-1 mRNA level in relation to clinicopathological variables in CRC

We further analyzed the relationship between FBI-1 mRNA in normal mucosa, primary tumor with gender, age, tumor site, TNM stage, differentiation, and with complications. FBI-1 in normal mucosa was related to gender, i.e., it was higher in female (2.199 ± 0.274) than that in male (1.294 ± 0.200, $P = 0.008$). FBI-1 had no relationship with other clinicopathological variables including age, tumor site, TNM stage, differentiation and complications ($P > 0.05$, **Table 1**). We also examined the relationship of FBI-1 mRNA in primary tumor with clinicopathological variables, the patients with complications had a higher FBI-1 expression than that patients without complication (3.400 ± 0.332 vs. 2.516 ± 0.241, $P = 0.032$, **Table 1**). There was no other significant difference ($P > 0.05$, **Table 1**).

FBI-1 mRNA in normal mucosa was an independent prognostic factor

We examined FBI-1 mRNA in relation to patient survival, and found that FBI-1 mRNA in normal mucosa was an independent prognostic factor. The higher the level of FBI-1 mRNA the better the survival ($P = 0.036$, **Figure 2**).

Table 2. Multivariate analysis of the FBI-1 mRNA in normal mucosa, gender, age, stage, and differentiation in relation to patient overall survival

Variables	n	Hazard ratio	95% CI	*P* value
FBI-1 mRNA				0.039
Low	22	1.000	-	
High	123	0.431	0.194-0.958	
Gender				0.755
Male	91	1.000	-	
Female	54	0.880	0.395-1.962	
Age (years)				0.336
< 70	47	1.000	-	
≥ 70	98	1.506	0.655-3.464	
Stage				< 0.001
I	20	1.000	-	
II	77	1.376	0.293-6.459	
III	33	3.983	0.862-18.398	
IV	15	11.830	2.421 - 57.797	
Differentiation				0.158
Well	18	1.000	-	
Moderately	87	1.700	0.370-7.809	
Poorly	40	3.267	0.660-16.173	

Total 145 cases.

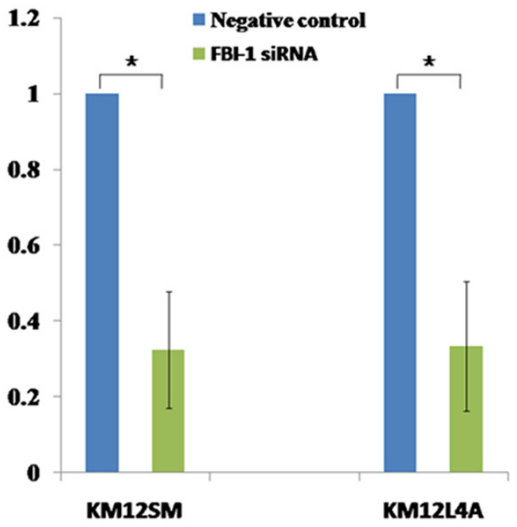

Figure 3. FBI-1 siRNA transfection could decrease FBI-1 mRNA in colon cancer cell lines KM12SM and KM12L4A (*P < 0.01).

Even in multivariate analysis including gender, age, stage and differentiation, the relationship between FBI-1 and survival still remained significant (P = 0.039, RR 0.431, 95% CI 0.194-0.958, **Table 2**). We also analyzed the relationship of FBI-1 mRNA level in primary tumor with survival, and could not find any significant difference (P > 0.05, data not show).

Suppression of FBI-1 could repress proliferation of colon cancer cell lines

FBI-1 siRNA transfection could reduce mRNA and protein level in colon cancer cell lines. Compared with the negative control, FBI-1 mRNA was reduced to 32.4% in KM12SM cell lines (P = 0.001), and 33.3% in KM12L4A cell lines (P = 0.002, **Figure 3**). FBI-1 siRNA transfection could significantly reduce expression of the 80 kDa FBI-1 protein (**Figure 4**). Proliferation of KM12SM and KM12L4A cell lines was significantly suppressed at 48 h and 72 h after FBI-1 siRNA transfection (P < 0.01, **Figure 5**).

Discussion

In this study, we detected the mRNA level of FBI-1 in CRC patients and found that FBI-1 was overexpressed in tumor tissue compared with that in the corresponding normal mucosa. FBI-1 in normal mucosa was higher expression in female than in male, and was an independent prognostic factor, with a high level of FBI-1 mRNA being related to better survival. The patients with complications had higher primary tumor FBI-1 expression than patients without complication.

The mRNA level of FBI-1 was up-regulated in tumor tissue compared with corresponding normal mucosa; this was in accordance with other studies. Zhao et al. [9] detected mRNA and protein in 46 CRC patients and found that FBI-1 mRNA and protein were up-regulated in tumor tissue than that in normal mucosa, and FBI-1 could regulate the colon cancer proliferation. Except that primary tumor FBI-1 had relationship with complications, we could not find any other relationship of FBI-1 in tumor with clinicopathological variables mentioned in this study, including gender, age, tumor site, TNM stage, and differentiation, hinted that FBI-1 took part in the development but not progression of CRC. But in another study with 66 CRC samples, higher expression rate of Pokemon (FBI-1) was associated with lymph node metastasis and higher Duke's stage [13]. This discrepancy

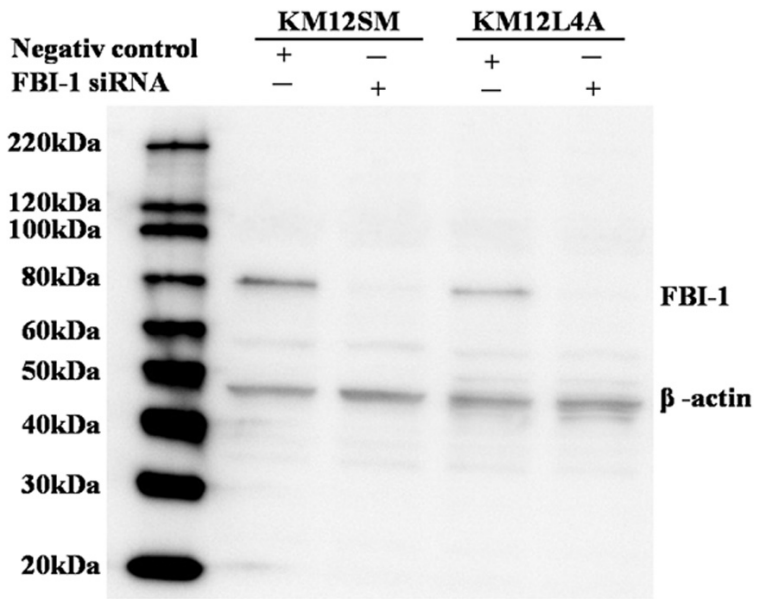

Figure 4. FBI-1 protein was decreased after FBI-1 siRNA transfection.

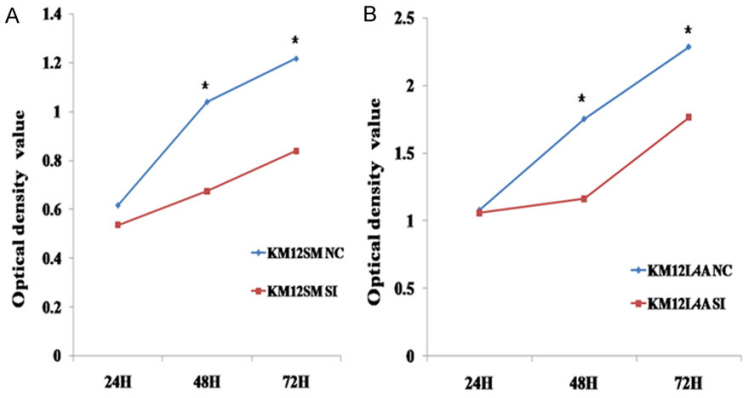

Figure 5. Suppression of FBI-1 could repress proliferation of colon cancer cells (A. KM12SM cell line; B. KM12L4A cell line; NC, negative control; SI, FBI-1 siRNA; *$P < 0.01$).

might be partly due to mRNA and protein not being parallel in the CRC. As we know that microRNA function as posttranscriptional gene regulators, microRNA-137 could repress FBI-1 expression in HCC [14]. However, to date there are no reports about FBI-1 mRNA being regulated by miRNA in CRC. The other possible reason might be due to the small sample size, so further study is needed to verify the mRNA and protein level with clinicopathological parameters.

FBI-1 acting as a prognostic factor has been found in HCC patients. FBI-1 was elevated significantly in HCC tissues compared with adjacent non-tumorous liver tissues, and increased FBI-1 expression had poor prognosis [8]. More interestingly, we found that the mRNA level of FBI-1 in normal mucosa was a prognostic factor, whereas high FBI-1 mRNA had a good survival independent of gender, age, stage and differentiation. FBI1 in normal mucosa was correlated with patient's survival, one possible reason was that basic level of FBI-1, as a transcription factor, could predict the disease progression but the other possible reason was that genes were deregulated in normal-appearing colon mucosa of human cancer patients [15]. These findings imply that higher FBI-1 mRNA level in normal mucosa has a certain role in early carcinogenesis.

In carcinogenesis, the "field cancerization" concept developed from the phenomenon that survivors of cancers are prone to suffer from other malignancies of the same tissue type near the primary cancer [16]. Recent studies have shown several changes, such as increased occurrence of chromosomal aberrations or aberrant DNA methylation in the normal colon mucosa adjacent to colon cancer [17]. In CRC patients, O6-methylguanine-DNA methyltransferase (MGMT) methylation was frequently detected from the grossly normal colonic mucosa even 10 cm away from the tumor [18]. Hypermethylation of certain molecular markers was observed in normal-appearing tissue of patients with CRC, and demonstrated the field cancerization in CRC [19]. Not only our lab but also others had found the prognostic factors in normal mucosa. The PINCH gene located at chromosome 2q12.2, and PINCH expression in adjacent normal mucosa was related to survival in CRC patients [20, 21]. More recently, high Ki67 expression in distant mucosa was statistically related to

worse locoregional control and disease-specific survival in T1-2N0 oral squamous cell carcinoma patients [22]. These findings are interesting since as a biomarker, we paid more attention to the tumor tissue, and overlooked the corresponding normal mucosa. These observations hinted that we should not only focus on the tumor tissue but also the corresponding normal mucosa due to field cancerization in CRC.

The roles and mechanism of FBI-1 in CRC have been explored step by step. We found that FBI-1 siRNA transfection could repress proliferation of colon cancer and this was in agreement with previous studies [12, 13]. Although the p14ARF-Mdm2-p53 pathway was important for the FBI-1 [23], there might be another FBI-1 pathway in CRC. A recent observation found that FBI-1 was able to enhance colon cancer proliferation, invasion, and metastasis via ETS-1 signaling pathway activity [12].

In conclusion, FBI-1 is overexpressed in CRC, and takes part in the development of CRC. The mRNA level of FBI-1 in normal mucosa is an independent prognostic factor, with high levels of FBI-1 mRNA being related to improved survival. Our findings give further support of the concept of "field cancerization", and demonstrate that when we study a biomarker, we should not only focus on the tumor tissue but also the corresponding normal mucosa due to the field cancerization in CRC.

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Disclosure of conflict of interest

None.

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