doi:10.4149/neo_2014_065

Far upstream element-binding protein 1(FUBP1) expression differs between human colorectal cancer and non-cancerous tissue

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Received November 18, 2013 / Accepted January 30, 2014

Presented study aimed to detect the expression of far upstream element-binding protein 1 (FUBP1) in clinical samples of colorectal carcinoma (CRC) and explore the correlations of their expression with the clinicopathological characteristics of CRC.

The streptavidin-perosidase (SP) method of immunohistochemistry was used to detect the expression of FUBP1 in 34 cases of colorectal cancer and their surrounding surrounding normal tissue, 30 cases of adenoma tissue. Using fluorescent quantitative reverse transcription polymerase chain reaction (qPCR), the expression of FUBP1 mRNA was measured in colorectal cancer and its surrounding normal tissue from 32 patients. FUBP1 protein expression level was detected by the Western blot method in 32 pairs of colorectal cancer tissue and surrounding normal tissue, and 30 cases of adenoma tissue.

The positive rate of FUBP1 was detected through histochemistry in colorectal carcinomas (82.3%) which was higher than that in colorectal adenomas (46.7%) and surrounding normal tissues (20.5%). The relative amount of FUBP1 mRNA by qPCR method in colorectal carcinoma tissues (0.2703±0.1118) was higher than that of surrounding normal tissues (0.1898±0.0635; P<0.05). The Western blot showed that FUBP1 was mainly expressed in colorectal carcinoma tissues (0.6499±0.1473),which barely expressed in adenoma tissues (0.3756±0.1377; P<0.05) and surrounding normal tissues (0.1675±0.0613; $P < 0.05$).

FUBP1 expression differs among colorectal tissues, which is overexpressed in colorectal carcinoma tissue. Further studies are needed to explore the role of FUBP1 in the pathogenesis of colorectal carcinoma.

Key words: FUBP1, colorectal carcinoma, immunohistochemistry, qPCR, Western blot

The colorectal cancer (CRC) is one of the most common malignancies in the world [1]. Worldwidely, CRC is the 3rd of all malignant tumors and is the 4th leading cause of cancer-related deaths. CRC is diagnosed in over one million persons annually [2]. As early as 1990, Fearon and Vogelstein pointed out that the development of colorectal cancer is a multi-step process and that it involves the tumor-suppressor gene mutation deactivation and the accumulation of oncogene activation [3]. Over the past two decades, various inputs to cancer research have promoted the diagnosis and treatment of the colorectal cancer. The early colorectal cancer patients with 5-year survival rate is about 90%. However the overall survival of patients with advanced and metastatic still has not been obviously increased, which is only 15%. As a result, many investigations have focused on the early diagnosis and prognosis of colorectal cancer, and a variety of biomarkers that could predict the survival of CRC has been reported recently [4-6]. It is well-known that c-myc is an important proto-oncogene and activation for c-myc will cause cells malignant transformation tendency. The far upstream element-binding protein 1 (FUBP1) plays an important role due to its transcriptional activity on the oncogene c-*myc* [7]. In the following years the studies found that the detection of a variety of tumor cell lines and tumor tissues including kidney cancer, bladder cancer, leukemia, ovarian cancer, breast cancer, osteosarcoma cells, cervical cancer showed these cells contained FUBPs family members and that the FUBP1 had high expression in the dividing cells, which is consistent with the c-myc expression [8-13]. To investigate the role of FUBP1 in the occurrence and development of CRC, we employed qPCR, Western blot and immunohistochemical methods to detect the expression of FUBP1 in clinical samples of CRC and then analyzed the correlations of their expression with the clinicopathological features of CRC.

Patients and methods

Patients and tumor samples. From April 2011 to March 2012, fresh tissue samples were collected from 32 cases of CRC from the Department of Surgery, at General Hospital of Jinan Military Command of Chinese People's Liberation Army (PLA), and Affiliated Hospital of Taishan Medical University. The clinical samples were obtained from surgically removed and pathologically confirmed CRC. The patients included 18 men and 14 women and their mean age was 61.5 years old. None of the patients received preoperative radiotherapy or chemotherapy. The pathological classifications of CRC were based on sex, age, grade, tumor location, lymph node metastasis and distant metastasis. The matched surrounding normal tissue was obtained from a segment of the resected specimens that were the farthest from the tumor (>5 cm). Tissue samples were immediately snap-frozen in separate vials using liquid nitrogen and stored at –80°C for the PCR and Western-blot. There were another 30 adenoma tissues removed from biopsy by colonoscopy for the Western-blot. The collection of clinical samples were approved by the Ethics Committee of General Hospital of Jinan Military Command of Chinese PLA and all patients gave their written informed consent. For immunohistochemistry we collected archived wax lumps of tissues from the pathology department of the same hospital from December 2011 to June 2012. They contained the colorectal cancer tissue 34 cases (19 men, 15 women; mean age, 60.87 years) and surrounding normal tissues in the colorectal cancer from tumor tissue >1.5 cm which have none cancer confirmed by pathology, and 30 cases of adenoma tissues.

Immunohistochemistry. Two-micrometer-thick tissue sections were cut from the paraffin blocks containing tumor tissue, surrounding normal tissue and adenoma tissue, mounted on the object slide and subjected to immunohistochemistry using the standard streptavidin–peroxidase technique. The antibody used here was mouse anti-human FUBP1 monoclonal antibody (diluted 1:800; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The method followed the instructions of Sp – 9000 immunohistochemical staining kits and ZLI – 9033 concentrated DAB kit (ZSGB- Biotechnology Inc., Beijing, China). In each analysis, positive controls were used consisting of CRC samples previously shown to stain with the FUBP1 antibody. PBS in place of the primary antibody was used as a negative control.

Real-time quantitative PCR. We extracted the Total RNA from the tissues (50-100 mg) using Trizol (TaKaRa Inc., Japan) following the manufacturer's protocol. The cDNA synthesis was carried out by reverse transcription using a cDNA synthesis kit (PrimeScript® RT reagent Kit , TaKaRa Inc., Japan) following the manufacturer's protocol. PCR amplification of FUBP1 and GAPDH was done with SYBR® *Premix Ex Taq*™ (TaKaRa Inc., Japan) with cDNA synthesized from the tissues. The primers used were as follows: FUBP1 (TaKaRa Inc., Japan): 5´-GGAACTCCAATGGGA CCATACAAC-3´ (forward) and 5´-AGTGAGCGTAATAA GCAGCCCAAG-3´ (reverse), amplicon 199 bp; GAPDH (Sangon Biotech(Shan ghai)Co.,Ltd) : 5´-GAAGGTGAAGGTCGGAGTC-3´ (forward) and 5´-GAAGATGGTGATGGGATTTC-3´ (reverse) amplicon 220 bp. Amplification conditions were as follows: Initial denaturation: 95°C for 5 min, and the PCR proceeded for 40 cycles: 95°C for 5 s, 60°C for 20 s. The expression level of FUBP1 was expressed as $2^{-\Delta Ct}$, where ΔCt = Ct (FUBP1) – Ct (GAPDH). PCR products were visualized and photographed under ultraviolet light by 2% gel electrophoresis.

Western blot. Tissues were washed with ice-cold PBS prior to disruption on ice for ten minutes with Total Protein Lysis buffer supplemented with protease inhibitors (Thermo Fisher Scientific, CA, USA). Equal amounts of protein were separated by SDS-PAGE, transferred to Polyvinylidene Fluoride membranes (Thermo Fisher Scientific, CA, USA), which were activated by formaldehyde and then were blocked for 2 hours at 4°C. The primary antibody, mouse anti-human FUBP1 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), was diluted 1:800 in 5% BSA and incubated with the membrane overnight at 4°C. The secondary antibody (Thermo Fisher Scientific, CA, USA) which was marked by horseradish peroxidase (HRP) was incubated for 4 hours at 4°C and visualised using electrochemiluminescence (ECL) developer. By using the ImageJ processing software (National Institutes of Health, USA) we analyzed the densitometric of band intensity.

Scoring and statistical analyses. Double-blind method was used to observe 5 horizons by two observers independently. Cores were scored for staining intensity of FUBP1-immunoreactive cells (0, no staining; 1, mild; 2, moderate; 3, strong) and percentage of the cells staining positively (0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%). These scores were multiplied to give final scores of 0–12. A staining index of <3 was deemed to negative while ≥3 was positive (3-5, weakly positive; 6-8, moderate positive; 9-12, intense positive) [14]. Statistical analysis was performed by using SPSS17.0 software (SPSS, Inc., Chicago, IL, USA) and P <0.05 was considered significant. All the measurement data were expressed as mean (χ) ± standard deviation(s). The differences between the CRC tissues and the surrounding normal tissues were analyzed using χ^2, a paired samples *t*-test, or One – Way ANOVA (one – way Analysis of Variance), while categorical data were studied using the independent samples *t*-test or Rank-Sum test.

Results

Immunohistochemistry. The FUBP1 staining was detected in the nucleus. Immunohistochemical staining found that FUBP1 immunoreactivity was detected mainly in the nucleus of colorectal carcinoma cells but in the cytoplasm in the adjacent normal colorectal tissues (Fig. 1). The positive rates of FUBP1 in the tissues of colorectal carcinoma, surrounding normal tissues and adenoma tissues were 82.3% (28/34), 20.5% (7/34), and 46.7% (14/30), respectively. The difference in the positive rates of FUBP1 between the

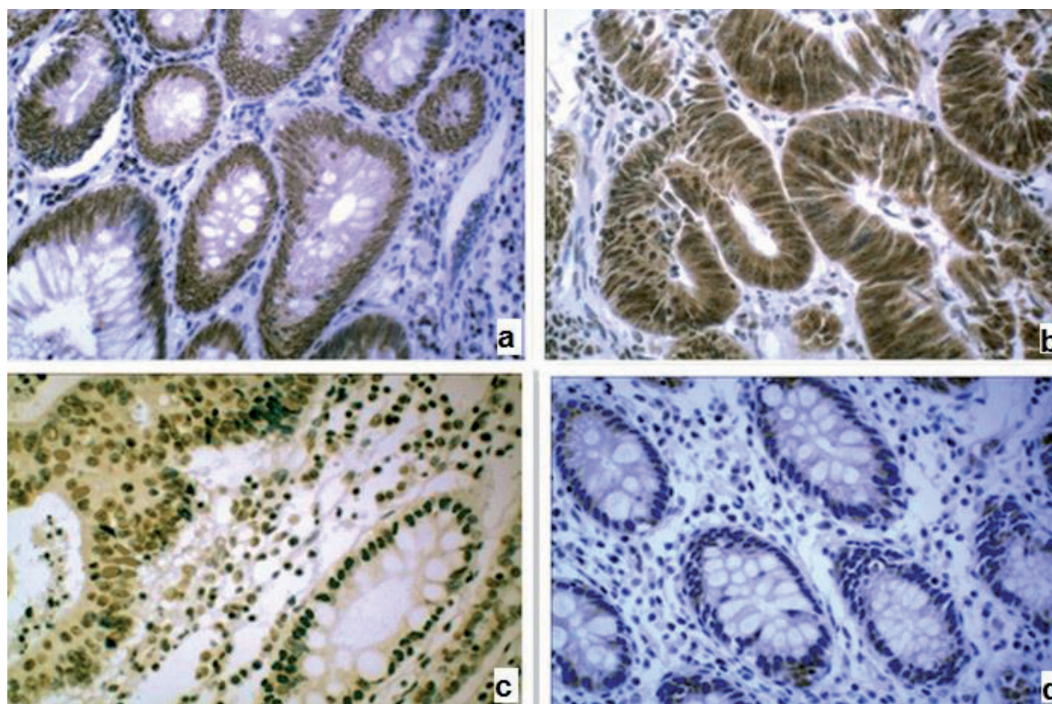

Figure 1. Immunohistochemical staining for FUBP1 in tissues of adenoma tissues, colorectal carcinoma tissues and surrounding normal tissues. a. FUBP1-positive staining in the nuclei in adenoma tissues; b. FUBP1-positive staining in the nuclei in colorectal carcinoma tissues; c. FUBP1-positive staining in the nuclei in colorectal carcinoma tissue and negative staining in surrounding normal tissues; d. FUBP1-negative staining in surrounding normal tissues.

colorectal carcinoma tissues and the other two tissues was statistically significant (all $P < 0.05$) (Table 1). There were no significant correlations between FUBP1 expression and sex, age, grade, tumor location, lymph node metastasis or distant metastases (Table 2).

qPCR. We performed qPCR analysis to detect the expression of FUBP1 at mRNA level in 32 pairs of matched clinical samples. A melting curve analysis was performed after completion of the PCR (Fig. 2). The relative amount of FUBP1 mRNA with qPCR method in colorectal carcinoma tissues (0.2703±0.1118) was higher than that of surrounding normal tissues (0.1898±0.0635; $P<0.05$) (Table 3). There were no significant correlations between FUBP1 expression and sex, age, grade, tumor location, lymph node metastasis or distant metastases (Table 4). Some samples of the electrophoresis assay results are shown in Fig. 3.

Table 1. FUBP1 expression in the colorectal carcinoma, Surrounding normal and adenoma tissue (cases, %)

Group	n	-	+	++	+++	Rate (%)
colorectal carcinoma①	34	6	9	11	8	82.3
Surrounding ormalue②	34	27	6	1	0	20.5
adenoma tissue③	30	16	11	2	1	46.7

①, ②: P=0.000; ①, ③: P=0.003; ②, ③: P=0.027

Table 2. Correlation between FUBP1 expression and clinicopathological features of colorectal carcinoma

Features	n	FUBP1 experssion		P value
		positive	Negtive	
Sex				0.564
Male	19	15	4	
Female	15	13	2	
Age group (yr)				0.753
< 60	15	12	3	
> 60	19	16	3	
LN involvement				0.142
Yes	15	14	1	
No	19	14	5	
Distant metastasis				0.549
Yes	9	8	1	
No	25	20	5	
Dukes stage				0.634
A+B	20	17	3	
C+D	14	11	3	
Differentiation				0.111
High and medium	25	19	6	
Low	9	9	0	
Tumor location				0.231
Right colon	13	12	1	
Left colon	21	16	5	

LN: lymph node

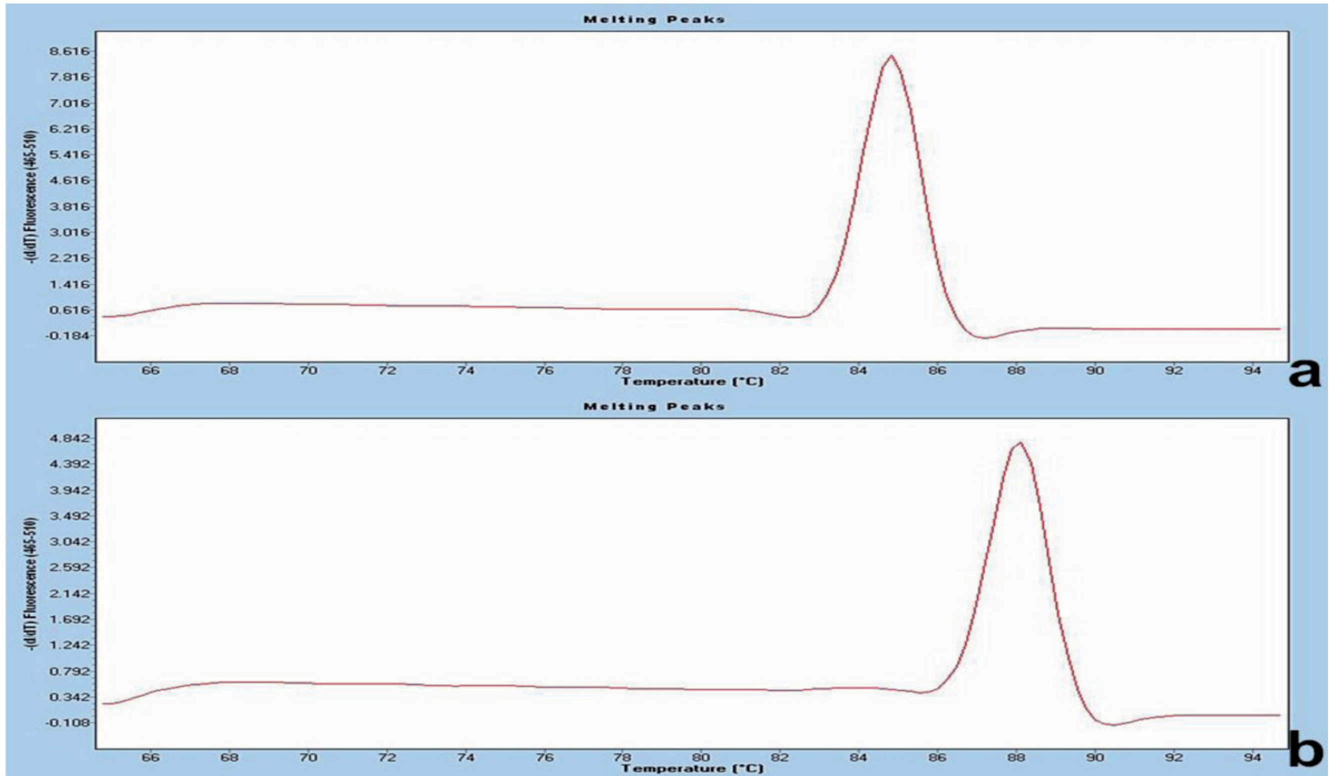

Figure 2. Typical amplification and melting curves of real-time RT-PCR for GAPDH (a) and FUBP1 (b). The sharp peak in each panel denotes the melting temperature of GAPDH (84.83°C) and FUBP1 (88.11°C).

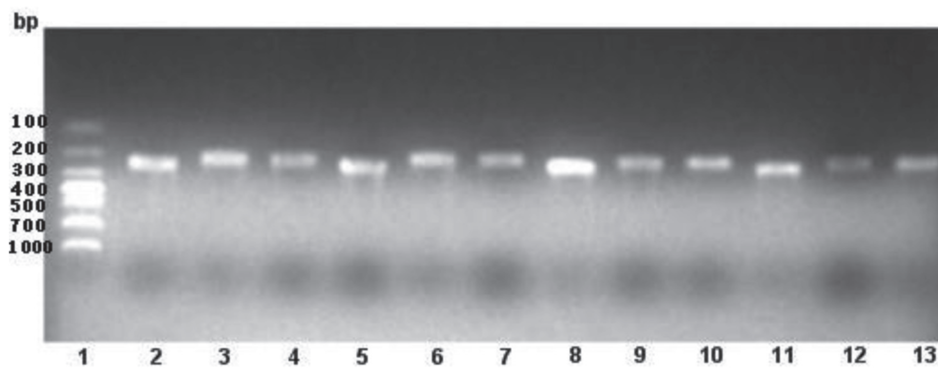

Figure 3. RT-PCR gel for GAPDH/FUBP1 mRNA in various colorectal tissues. (1). Marker; (2.5.8.11) RT-PCR products of GAPDH from colorectal carcinoma tissues; (3.6.9.12) RT-PCR products of FUBP1 from colorectal carcinoma tissues. (4.7.10.13); RT-PCR products of FUBP1 from surrounding normal tissues.

Table 3 Comparison of the relative amount of FUBP1 mRNA in colorectal carcinoma and surrounding normal tissue (mean ± SD)

Group	n	Mean ± SD	*t*	*P*	95% CI of the Difference	
					Lower	Upper
colorectal carcinoma	32	0.2703±0.1118				
Surrounding normal tissue	32	0.1898±0.0635	2.179	0.046	0.00149	0.13461

CI: Confidence Interval

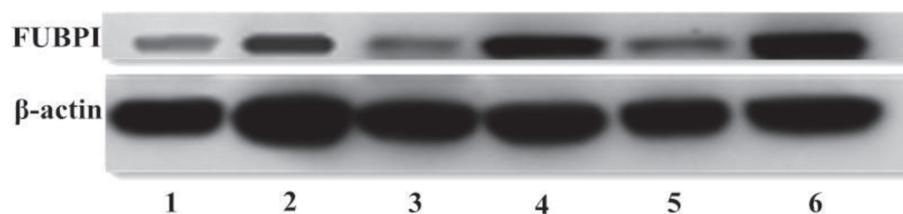

Figure 4. Western blot gel for β-actin/FUBP1 protein in various colorectal tissues. (1, 3, 5) Western blot products from Surrounding normal tissues; (2) Western blot products from colorectal carcinoma tissues; 4, 6) Western blot products from colorectal carcinoma tissues.

Western blot. FUBP1 protein expression level was detected by Western blot method in 32 pairs of colorectal cancer tissue and surrounding normal tissue, 30 cases of adenoma tissue. The FUBP1 was mainly expressed in colorectal carcinoma tissues (0.6499±0.1473), barely expressed in surrounding normal tissues (0.1675±0.0613; $P < 0.05$) and adenoma tissue (0.3756±0.1377; $P<0.05$) (table 5). Results of a typical experiment are shown in Fig. 4. Protein FUBP1 is found to be strongly expressed in tumor tissue and less in control tissue.

Discussion

FUBP1, a single-stranded DNA binding protein, is considered to be the ancestor of FUBPs family. Furthermore, being the most important transcription factor in FUBPs, FUBP1 has the functions of DNA helicase and RNA helicase [15], and can be combined with non-coding region of single-stranded far- upstream element (FUSE). Studies confirmed that many genes in cells contain FUSE sequences, in which the c-myc are the frequently researched and the most proto-oncogenes, whose activation will increase the malignant tendency of cells. FUBP1 play its regulatory function mainly by involving in FUSE / FUBP / FUBP Interacting Repressor (FIR) / Transcription Factor IIH (TFIIH) system. In this system, FUBP and FIR are a pair of functionally opposite and mutually collaborative proteins [16]. Through the FUSE identification in certain genes, like c-myc, FUBP activates the c-myc transcription and reaches the peak state by TFIIH stimulation. Meanwhile, under normal circumstances, FIR can be recruited by FUBP in combined FUSE / FUBP / TFIIH to play its control effect on the excessive transcription of c-myc so as to maintain the c-myc transcription to basic or relatively stable levels.

Numerous studies found that the detection of a variety of tumor cell lines and tumor tissues including kidney cancer, bladder cancer, leukemia, ovarian cancer, breast cancer, osteosarcoma cells, cervical cancer showed these cells contained FUBPS family members, and the FUBP1 had high expression in the dividing cells, which was consistent with the c-myc expression. The research on the digestive tract tumors showed that FUBP1 is an important Hepatic Cellular Cancer (HCC) tumor protein and induces tumor proliferation through direct or indirect inhibition of inhibitory factors and apoptosis genes in cell cycle [17-19]. In the earlier stage, we studied 30 primary hepatocarcinoma tissues and surrounding normal tissues by immunohistochemistry and PCR. It has been found that the expression of both FUBP1 and c-myc is higher than that of surrounding normal tissues, and they are positively correlated [20]. FUBP1, as candidate indicators for early detection and diagnosis of colon cancer, can be combined with carcino-

Table 4. Correlation between FBUP1 mRNA and clinicopathological features of colorectal carcinoma(mean ± SD)

Features	n	Mean ± SD	*t*	*p*
Sex			0.725	0.481
Male	18	0.2852±0.0943		
Female	14	0.2896±0.0839		
Age group (yr)			-0.231	0.820
< 60	9	0.2522±0.0162		
> 60	23	0.2708±0.1775		
LN involvement			-0.432	0.929
Yes	14	0.2844±0.0461		
No	18	0.2949±0.1163		
Distant metastasis			-0.392	0.701
Yes	8	0.2848±0.1181		
No	24	0.2879±0.0855		
Dukes stage			0.323	0.771
A+B	19	0.2827±0.0639		
C+D	13	0.2946±0.1304		
Differentiation			0.638	0.534
High and medium	24	0.2995±0.1061		
Low	8	0.2666±0.0581		
Tumor location			-0.693	0.545
Right colon	12	0.2514±0.0419		
Left colon	20	0.3086±0.1061		

Table 5. Comparison of the relative amount of FUBP1 protein in colorectal carcinoma and Surrounding normal tissue(mean ± SD)

Group	n	Mean ± SD	*F*	*P*
colorectal carcinoma①	32	0.6499±0.1473	76.372	0.000
Surrounding normal tissue②	32	0.1675±0.0613		
adenoma tissue③	30	0.3756±0.1377		

①、②: P=0.000; ①、③: P=0.000; ②、③: P=0.000

embryonic antigen (CEA), CA199, CA72-4 or CA242 to be used as diagnostic and prognostic indicators, the physicians should take the CEA as the first choice [21]. Kuramitsu Y, et al [22] once used two-dimensional gel and mass spectrometry to compare the efficacy of gemcitabine (GEM) in treatment of sensitive pancreatic cancer cell lines (KLM1R) and gemcitabine-antagonistic pancreatic cancer cell lines (KLM1), and they found FUBP1 is down-regulated protein and that FUBP2 protein is up-regulated ones [22]. In this study, the immunohistochemistry, qPCR and Western blot were used to detect FUBP1 localization and expression intensity in colorectal cancer, adenoma and adjacent normal tissues, and we found: FUBP1 could be detected in various colorectal tissues, and its had different expression intensities in the cytoplasm and nucleus mRNA of different tissues. The cytoplasmic expression predominated for FUBP1 in adenoma and adjacent normal cells, while the nuclear expression predominated in colorectal cancer cells and the cytoplasmic expression was relatively weakened. The cytoplasmic, nuclear expressions in colorectal carcinoma tissues and adenoma, adjacent normal tissues of colorectal cancer are 82.3% (28/34), 20.5% (7/34), and 46.7% (14/30),respectively. And the difference is statistically significant (all $P < 0.05$). Meanwhile, the FUBP1 expression was found to be not significantly correlated with the age, sex, tumor location and degree of differentiation, lymph node metastasis and distant metastasis in studies about colorectal carcinoma tissues, which may be caused by small sample size or other influencing factors or unknown. This suggests that the abnormal expression of FUBP1 may occur during precancerous lesions and can be increasingly significant with the progression of the disease, which also plays a certain role in the occurrence and development of colorectal cancer, but this role is unobvious in invasion and metastasis. FUSE is a cis-acting element located on proto-oncogene c-myc and can be recognized by FUBP, while FUBP can be combined with FUSE, FIR, TFIIH to form a "molecular servo system" to regulate the c-myc gene transcription in a way of feedback. After p38, TGF-β and other regulatory factors affect FUBP1 expression, the proto-oncogene c-myc transcription in this system is further affected, thus the slight changes of c-myc is sufficient to cause the abnormal cell growths [23]. In addition, since the FUBP1 expression can affect the c-myc transcription, some studies show that it can achieve the effect of treating c-myc dependent tumor by influencing the FUBP1 expression. Huth et al. have confirmed that benzoylanthranilic acids can identify FUBP1 specifically and accurately. And benzoylanthranilic acids can combine with the DNA binding domain of FUBP1 and regulate FUBP1, thus inhibiting the c-myc activity [24]. Jang M et al. have studied and found that when cells receive the apoptosis stimulation, due to the effect of cysteinyl aspartate specific proteinase, FUBP1 content in the cell nucleus is reduced, thus the contents of c-myc and a variety of targeted protein are decreased obviously. Therefore, after apoptosis induction, carcinogenic effect of c-myc is suppressed, as the result of FUBP1 fracture mediated by cysteinyl aspartate specific proteinase [25]. It is also found that the expression of FUBP1 is different in various colorectal tissue, which can further enrich the pathogenesis of colorectal cancer, and at the same time provide a new idea for diagnose and treatment of colorectal cancer.

Conclusion

FUBP1 expression differs among colorectal tissues, which is overexpressed in colorectal carcinoma tissue. Further studies are needed to explore the role of FUBP1 in the pathogenesis of colorectal carcinoma.

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