

## Original Article

# LSF expression and its prognostic implication in colorectal cancer

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**Abstract:** Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of cancer death. Therapy failure was the first cause of death. LSF is a transcription factor regulating gene expression of angiogenesis, tumor invasion and proliferation, and is identified as a chemoresistant gene. Real-time PCR and Western blot to analyze mRNA and protein expression of LSF in 23 paired CRC samples. Immunohistochemistry was used to detect protein expression of LSF in 166 paired CRC samples. Both LSF mRNA and protein were upregulated in CRC. High LSF expression in CRC correlated with large tumor size, advanced pN stage, advanced AJCC stage and high Ki-67 index ( $P < 0.001$ ). High expression of LSF favored worse prognosis. 5-year survival rates of LSF high and low expression were 39.6% and 78.6%, respectively. The 5-year median OS were 34 months and 57 months, respectively. LSF is an important mediator in CRC tumorigenesis and progression, and LSF expression is an important index for and prognostic prediction.

**Keywords:** LSF, colorectal cancer, prognosis

## Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide. It is estimated that more than 1.2 million new individuals will develop colorectal cancer in one year [1]. With the widespread use of chemotherapy drugs and targeted therapy drug, the prognosis of CRC has been improved. Bujanda et al. compared two groups of consecutive CRC patients that were prospectively recruited: Group I included 1990 patients diagnosed between 1980 and 1994. Group II included 871 patients diagnosed in 2001. 17% of Group I patients received chemotherapy compared with 50% of Group II patients ( $P < 0.001$ ). The result shows that chemotherapy prolonged the overall median survival from 25 months to 73 months [2]. Targeted therapy, such as EGFR inhibitors and small-molecule tyrosine kinase inhibitors has been used for colorectal cancer. The New EPOC randomized controlled trial shown that compared with systemic chemotherapy only, systemic chemotherapy with cetuximab prolonged progression-

free survival of patients with resectable colorectal liver metastasis from 14.1 months to 20.5 months ( $P=0.030$ ) [3]. But the overall outcome of early and advanced colorectal cancer patients is not optimistic, and approximately 33% of colorectal cancer patients will die of cancer [4]. Colorectal carcinogenesis is a well characterized multistep progression model with accumulation of oncogenes and tumor-suppressor genes mutation or dysregulation [5]. The understanding of the molecular abnormality of colorectal cancer not only provides new therapeutic strategy for colorectal cancer, but also offers information about response to treatment in individual patients and gives references for personalized medicine [6].

LSF, also known as LBP-1c and TFCP2, was first identified as a transcriptional activator factor of the late Simian virus 40 (SV40) promoter in Hela cell [7]. Thereafter, it is found that LSF expresses in many cells as a ubiquitous transcription factor and plays lineage-specific and ubiquitous biological roles in mammalian cells

**Table 1.** Correlation of LSF expression and clinicopathological features in colorectal cancer

Characteristics	No. (166)	LBP		$\chi^2$	P-value
		Negative (96)	Positive (70)		
Age (years)				0.264	0.607
< 58	91	51	40		
≥ 58	75	45	30		
Gender				2.232	0.135
Male	98	52	46		
Female	68	44	24		
Location				0.552	0.457
Colon	68	37	31		
Rectum	98	59	39		
Tumor size (cm)				20.962	< 0.001
< 5 cm	98	71	27		
≥ 5 cm	68	25	43		
Gross feature				2.489	0.115
Non-infiltrating	104	65	39		
Infiltrating	62	31	31		
Differentiation				2.56	0.1097
Well/moderate	121	75	46		
Poor	45	21	24		
pT classification				2.536	0.111
T2, T3	90	47	43		
T4	76	49	27		
pN classification				24.213	< 0.001
N0	62	51	11		
N1, N2	104	45	59		
pM classification				2.37	0.124
M0	139	84	55		
M1	27	12	15		
AJCC stage				21.589	< 0.001
II	57	47	10		
III	81	37	45		
IV	27	12	15		
Nerve involved				2.709	0.100
No	92	48	44		
Yes	74	48	26		
Differentiation				3.761	0.052
Well/Moderate	122	76	46		
Poor	44	20	24		
Ki-67 expression				16.865	< 0.001
Negative	59	46	12		
Positive	108	50	58		

[8]. Besides on SV40 promoter, it can bind to the other viral promoter, such as TAR and TATA regions of HIV long terminal repeat, and regulates gene expression [9]. As mentioned previ-

ously, LSF also can play lineage-specific roles in mammalian cells. For example, it can bind to the promoter of  $\alpha$ -globin gene and uroporphyrinogen III synthase, and regulates erythropoiesis [10]. In the meantime, LSF is expressed in all mammalian cell type and plays an important role in cell cycle regulation [10]. Thymidylate synthase (TS) is the limiting enzyme in production of dTTP, which is required for DNA synthesis. And it is essential for G1/S transition after re-entry of quiescent cells into the cell cycle [11]. LSF can recognize the promoter of TS and induce its expression, and promote cell cycle progression. Recently, LSF was identified as an oncogene in hepatocellular carcinoma (HCC) [10]. Firstly, it is found to confer 5-Fu resistance on HCC cell lines by induction expression of TS gene [12]. Secondly, LSF activates osteopontin (OPN) expression and regulates invasion, angiogenesis, chemoresistance, and senescence of HCC cells [13]. Further works shows c-Met participates in the OPN mediated oncogene roles of LSF [14]. Fan RH, et al. also shown LSF could be activated by notch signaling and promoted HepG2 cell proliferation and invasion [15].

As yet, no studies have linked LSF to colorectal cancer. In present study, we evaluated the LSF mRNA and protein expression in CRC patient samples by

qPCR, Western blot and immunohistochemistry to investigate the expression status in CRC development, and the association with clinicopathological and biological variables.

## Materials and methods

### *Patients*

The patient samples for real time PCR (RT-PCR) analyses used in this study included the primary tumor and the corresponding normal mucosa from 21 patients. All samples, including 7 of 21 pairs used for western blot analysis, were flash-frozen and stored at -80°C. Paraffin-embedded material used for immunohistochemistry included 54 distant normal colorectal mucosa samples, which were histologically free from tumor (29 corresponding to the primary tumors, i.e. distant normal mucosa and primary samples from the same patients) and taken from the margin of distant resection, 166 primary colorectal adenocarcinomas. All patients were diagnosed and received surgical treatment at the Affiliated Hospital of Jiangnan University between 2003 and 2005. The patient's gender, age, tumor location, stage and differentiation were obtained from surgical and pathological records (**Table 1**). The study was approved by the Ethical Review Board of the Affiliated Hospital of Jiangnan University.

### *RNA extraction and cDNA preparation*

Total RNA was extracted using the RNAiso plus reagent (Takara, Dalian, China) according to the manufacturer's instructions. The concentration, purity and integrity of RNA were measured by NanoDrop 2000 (Thermo Scientific, Wilmington, DE). For the RT-PCR, the PrimeScript™ RT-PCR Kit (Takara, Dalian, China) was used for reverse transcription. The program was the following: 37°C 50 min, 85°C 5 s and 4°C 120 min (ABI). The cDNA samples were stored at -80°C.

### *Quantitative real-time PCR*

The relative expression levels of LSF mRNA in CRC patient material were determined by SYBR® Premix Ex Taq™ (Tli RNaseH Plus) in Applied Biosystems 7300 Real-Time PCR System and normalized to GAPDH according to the manufacturer's instructions. Primers were provided by Invitrogen (Shanghai, China). All samples were performed in triplicates. The PCR amplification program was the following: denature 95°C 20 sec, amplification and quantification program repeated 40 times 95°C 1 sec and 60°C 20 sec. In addition, ddH<sub>2</sub>O and a

minus RT product as the negative control were analyzed for every plate. LSF F, AGGAACTTGG-AGAACTTCCAGA; LSF R, CCCTCTAGCTGCTGATGCTC; GAPDH F, AGCCGCATCTTCTTTTGCCTC; GAPDH R, TGACCAGGCGCCCAATAC.

### *Western blot*

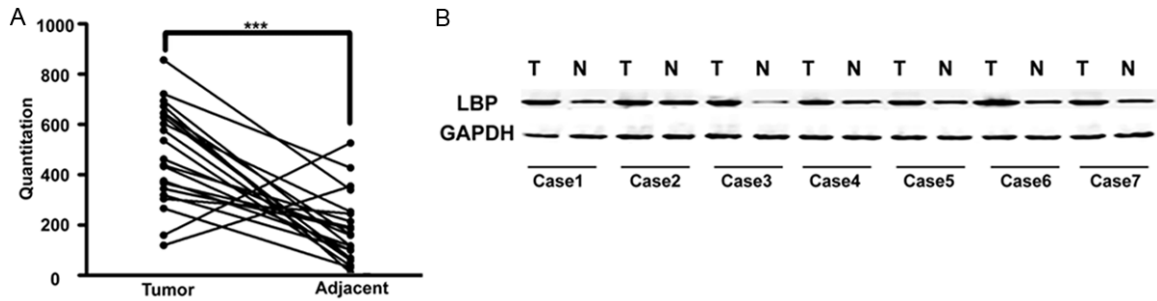
The primary antibodies used were anti-LSF (1:2,000, mouse monoclonal; BD Biosciences) and anti-GAPDH (1:1,000, mouse monoclonal; Santa Cruz Biotechnology). The LSF protein expression was determined by Western blot assay and the expression of GAPDH was used for loading control. Protein was extracted by lysis buffer containing 150 mM NaCl, 2% Triton, 0.1% SDS, 50 mM Tris pH8.0 and 10% Protease inhibitor cocktail (Sigma, St. Louis, MO) and stored at -20°C. The protein concentration was determined by the colorimetric BCA protein assay reagent (Pierce, Woburn, MA). Equal amounts of protein (50 µg) for each sample were loaded into polyacrylamide gels (Beiyuntian Biotechnology, Nantong, China) and separated by electrophoresis for 50 min at 200 V. The separated proteins were transferred to a PVDF transfer membrane (Amersham Bioscience/GE Healthcare, Piscataway, NJ) for 55 min at 100 V. Membranes were blocked with 5% milk in TBS supplemented with 0.1% Tween-20 for 1 h at room temperature and incubated with a primary antibody over night at 4°C. The membranes were washed and subsequently incubated with the secondary HRP conjugated polyclonal goat anti-mouse antibody (1:2000, DAKO Cytomation, Glostrup, Denmark) for 1 h at room temperature. Protein bands were detected using ECL plus Western Blotting Detection System (Amersham Bioscience/GE Healthcare, Piscataway, NJ).

### *Immunohistochemistry*

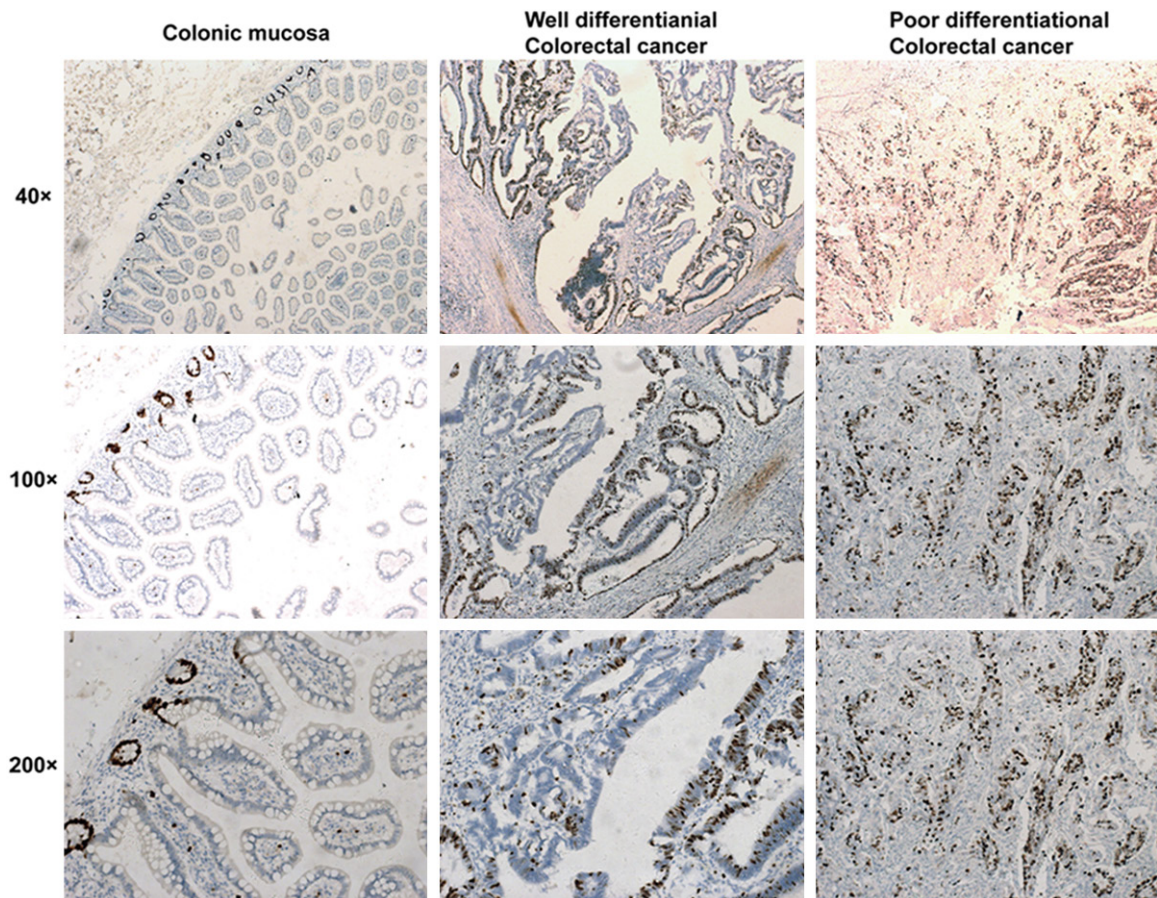
Sections from paraffin-embedded tissue blocks were incubated at 60°C for 12 h, then deparaffinized and hydrated in descending concentrations of ethanol and finally in ddH<sub>2</sub>O. To expose masked epitopes, the sections were microwaved in citrate buffer (pH 6.0) for 2×5 min, then kept at room temperature for 30 min, followed by a PBS-wash for 2 min. The activity of endogenous peroxidase was blocked in 3% H<sub>2</sub>O<sub>2</sub> in methanol and subsequently washed three times in PBS. After blocking with 1.5% blocking serum in PBS for 10 min, anti-LSF



## LSF expression and colorectal carcinoma



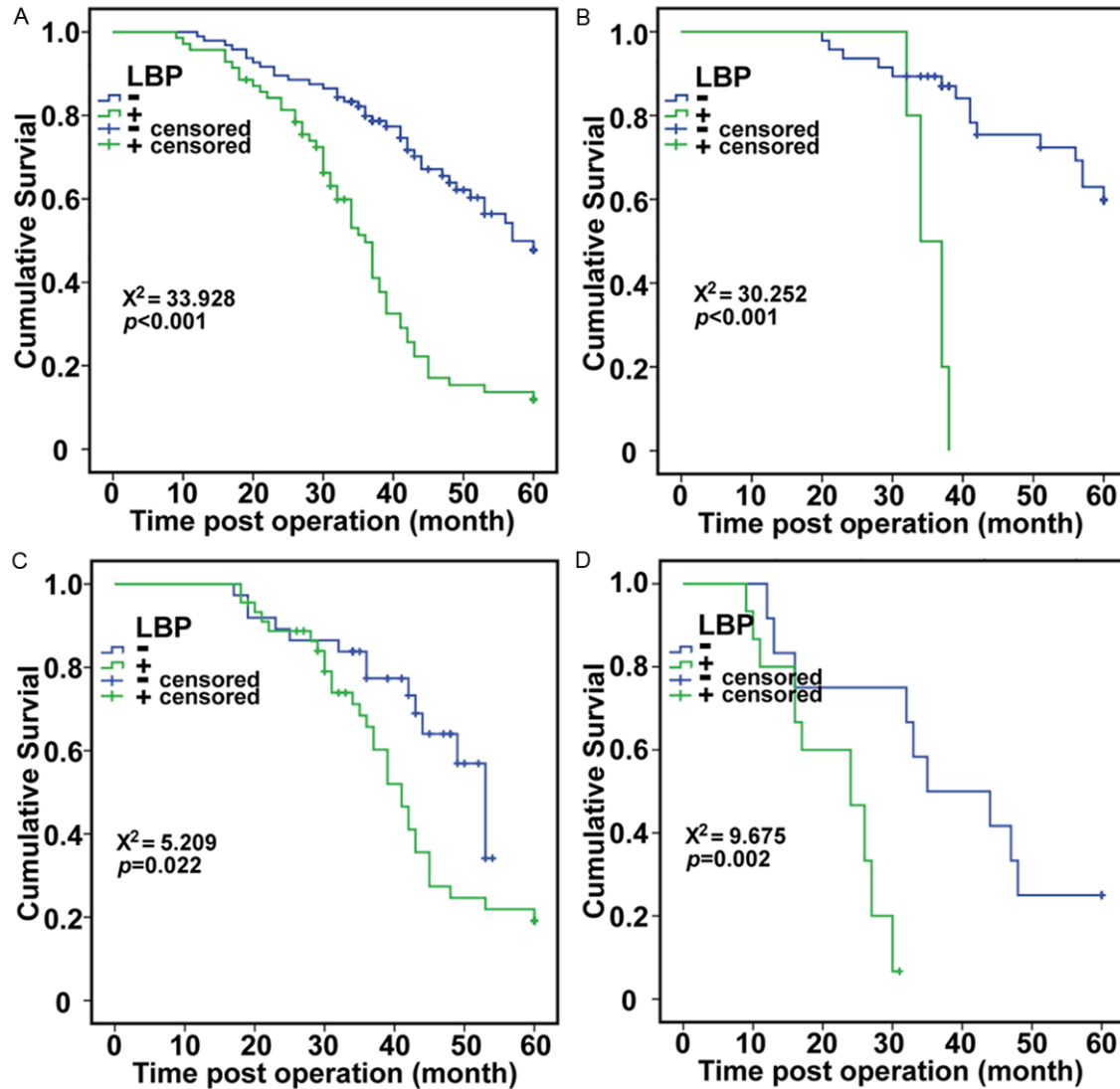
**Figure 1.** Elevated LSF expression in colorectal cancer. A. mRNA expression of LSF in colorectal cancerous tissues and adjacent tissues analyzed by Q-PCR; B. protein expression of LSF in colorectal cancerous tissues and adjacent tissues analyzed by western blot. T, colorectal cancerous tissue; N, adjacent noncancerous tissue.



**Figure 2.** IHC analysis of LSF in colon cancer.

(mouse monoclonal; BD Biosciences) was added at 1:150 in antibody diluent (DAKO, Cytomation, Glostrup, Denmark) and then incubated at 4°C over night. After washing with PBS, a biotinylated secondary anti-mouse antibody (DAKO Cytomation, Glostrup, Denmark) was added. After 30 min the sections were rinsed with PBS and AB enzyme reagent (ABC

Staining System, Santa Cruz Biotechnology), containing avidin, biotinylated horseradish peroxidase and PBS, was added to the slides. The AB enzyme reagent was rinsed off after 30 min with PBS. Peroxidase substrate containing 3, 3-diaminobenzidine chromogene, peroxidase substrate buffer and ddH<sub>2</sub>O, was added and incubated for 10 min and subsequently rinsed



**Figure 3.** Kaplan-Meier survival curves of patients with colorectal cancer. Patients with high LSF expression have a significantly worse overall 5-year survival. A. K-M curve of 166 CRC patients; B. K-M curve of 57 CRC patients in TNM stage II; C. K-M curve of 81 CRC patients in TNM stage III; D. K-M curve of 27 CRC patients in TNM stage IV.

with water. The sections were then counter-stained with haematoxylin. In all runs, negative controls were included, where PBS was used instead of the primary antibody. The degree of immunostaining was reviewed by two independent observers based on the proportion of positively stained cells, the intensity and location without knowledge of clinicopathological and biological information. The intensity of staining was classified according to the following criteria: 0 (negative staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining), and the staining patterns were graded as cytoplasmic or nuclear (**Figure 3**). For statistical analyses, negative and weak stained cases were

considered as low expressing group, whereas moderate and strong cases were considered as high expressing group. To avoid artificial effects, cells in areas with necrosis or with poor morphology were not counted.

#### Statistical analyses

All data were analyzed by the statistics program IBM SPSS21.0 Statistics. Student's t-test was used to test significance between LSF mRNA levels in the different sites of the samples. Chi-square test was applied to test the significance of the differences in the association of LSF expression with clinicopathological/biological

**Table 2.** Multi-variant analysis for poor prognosis of colorectal cancer

Characters	p	HR (95.0% CI)
LSF	0.000	3.734 (2.085, 6.688)
Age	0.998	0.999 (0.652, 1.533)
Gender	0.202	1.329 (0.858, 2.060)
Size	0.906	0.973 (0.624, 1.520)
Morphology	0.573	0.877 (0.555, 1.385)
Location	0.509	1.157 (0.750, 1.785)
T stage	0.434	1.211 (0.750, 1.956)
N stage	0.013	6.699 (1.492, 30.083)
M stage	0.000	52.707 (8.082, 343.722)
TNM stage	0.008	0.115 (0.023, 0.561)
Nerve involved	0.615	1.121 (0.719, 1.747)
Differentiation	0.583	1.148 (0.701, 1.882)
Ki67	0.084	1.562 (0.941, 2.594)

variables. Kaplan-Meier analysis was used to test the relationship between the LSF staining and the patient survival. Multivariate Cox regression analyses were used to evaluate the relationship of LSF staining and clinicopathological characteristics with patients' survival. All tests were two sided, and a *P*-value less than 0.05 was considered as significant.

## Results

### *Elevated LSF expression in colorectal cancer*

To determine the expression variation of LSF in colorectal cancer, we explore mRNA level of LSF in 21 paired cancerous/normal samples by quantitative real-time PCR (Q-PCR), and protein levels of LSF in 7 paired samples from 21 by Western Blot. Q-PCR analysis showed mRNA level of LSF elevated in 19 of 21 colorectal cancerous samples, and only decreased in 2 of 21 colorectal cancerous samples. The mRNA level of LSF in cancerous tissue ( $484.3 \pm 42.76$ ) compared with adjacent tissue ( $179.6 \pm 30.10$ ,  $P < 0.0001$ ) (**Figure 1A**). Similarly, Western blotting analysis was used to reveal protein expression of LSF in colorectal cancer (**Figure 2**). Compared with paired normal colon epithelial, colon cancer tissue expressed much more LSF. Q-PCR and western blot analyses have shown expression of LSF elevated during colorectal cancer carcinogenesis.

### *Correlation of LSF expression and clinicopathological features in colorectal cancer*

To determine relationship between LSF expression and clinicopathological features in colorec-

tal cancer, LSF expression was further evaluated by IHC in 166 paraffin-embedded colorectal cancer samples. As shown in **Figure 2**, LSF positive staining in colorectal normal mucosa was predominately located within the crypts, and decreased in the villus, which means LSF may participate cell proliferation and maintain mucosal homeostasis. In the colorectal cancer, LSF positive cells distributed randomly but were much higher than in normal mucosa. Also, we found LSF overexpression in poor differentiation samples was much higher than in well differentiation samples.

We further analyzed the link between LSF expression and the clinical characteristics of colorectal cancer in 166 paraffin-embedded, archival primary colorectal cancer tissues by IHC. As shown in **Table 1**, 70 of the total 166 CRC cases (42.2%) demonstrated high LSF expression, whereas 96 cases (57.8%) had low LSF expression. As shown in **Table 1**, a statistical analysis revealed a strong correlation between LSF expression and the clinicopathologic characteristics including tumor size, clinical stage, N classification, and Ki-67 staining ( $P < 0.001$ ), but no significant correlation with differentiation ( $P=0.052$ ).

### *Association between LSF expression and patient survival*

To evaluate prognosis value of LSF expression in colorectal cancer patients, Kaplan-Meiers' analysis and Cox regression model were performed to explore the effects of LSF expression and clinicopathological characteristics on patient survival. Kaplan-Meiers' analysis shown high LSF expression favored poor 5-year overall survival (OS) (78.57% vs. 39.59%). The median OS of high LSF expression patients was 36 months, and the median OS of LSF low expression patients was 57 months (**Figure 3A**). Interestingly, LSF high expression in each sub-TNM stage also predicted unfavorable prognosis (**Figure 3B-D**). Multivariate Cox regression model analysis shown N stage, M stage, TNM stage and LSF were independent prognostic factors influencing 5-year OS (**Table 2**). Our results indicate that LSF might be valuable marker for predict the outcome of colorectal cancer patients.

## Discussion

In this study, we clarified expression of LSF in colorectal cancer by real-time PCR, western blot and immunohistochemistry staining. All of



the results have shown LSF upregulated in colorectal cancer at both mRNA and protein levels. To identify the significance of LSF expression in colorectal cancer, we analyzed the relationship between expression of LSF and clinicopathologic characteristics, proliferation index Ki-67 expression and the overall outcome. Overexpression of LSF in colorectal cancer tissue correlated with large tumor size, lymph node metastasis, advanced TNM stage, higher Ki-67 expression and poor prognosis. TNM staging classification system is a classical method of prognostic evaluation for colorectal cancer patients [16]. However, a large discrepancy exists within the same stage. In this study, we also observed LSF high expression in the same TNM stage correlated with unfavorable prognosis. This indicates that LSF staining in colorectal cancer patient may be a method for prognostic prediction in the same TNM stage.

The transcription factor LSF is widely expressed in a variety of tissues and is involved in many biological events, including regulation of cellular and viral promoters, cell cycle, DNA synthesis, cell survival and Alzheimer's disease [10]. Recent studies establish an oncogenic role of LSF in HCC. LSF overexpression is detected in human HCC cell lines and in more than 90% cases of human HCC patients, compared to normal hepatocytes and liver, and its expression level showed significant correlation with the stages and grades of the disease [13]. Microarray studies reveal that LSF modulates specific genes regulating invasion, angiogenesis, chemoresistance and senescence [13]. In HCC cells, OPN is a direct downstream target of LSF [13]. OPN is important for regulating every step in metastasis [17]. In vitro, OPN can interact with CD44 receptor and augment proliferation, anchorage-independent growth, and invasion of HCC cells. Blocking OPN with anti-CD44 antibody can abrogate the oncogenic effects mediated by LSF [13]. In vivo, Inhibition of OPN abrogates LSF-induced tumorigenesis and metastasis [13]. In HCC, LSF induces OPN secretion, and the later activates c-Met via a potential interaction with its cell surface receptor CD44 [14]. Additionally, LSF activates two important cell survival-regulating pathways, MEK/ERK and NF- $\kappa$ B [13]. Ras/Raf/MEK/ERK signaling cascade is a key intracellular signaling pathway that regulates diverse cellular functions including cell cycle progression, cell proliferation, migration, survival and angiogenesis [18]. Blocking MAPK via small-molecule

inhibitors, such as CI-1040, PD 0325901, and AZD6244, has become an exciting approach in cancer therapeutics [18]. Recently, several small-molecule MEK inhibitors have been developed and are currently being tested in clinical trials for cancer treatment, including colorectal cancer, etc. [18]. NF- $\kappa$ B pathway has been shown to be activated in all cells by many stimuli, such as inflammatory factor TNF- $\alpha$ . NF- $\kappa$ B activation may regulate expression of diverse target genes and promotes cell proliferation, regulate immune and inflammatory response, and contribute to carcinogenesis, including colorectal cancer [19]. NF- $\kappa$ B activation might be involved in development of not only colitis-associated cancer, but also sporadic colorectal cancer. Alternatively, NF- $\kappa$ B activation is associated with hallmarks of cancer and confers resistance to chemotherapy [20]. Several NF-kappaB inhibitors have proven to be useful in coping with chemoresistance [20]. In this study, we found LSF overexpression in colorectal cancer, which means LSF is an oncogene in colorectal cancer and participates colorectal carcinogenesis. Analyzed with clinicopathological features, we found LSF overexpression correlated with TNM stage and proliferative index Ki-67 staining, which indicates LSF may participate in proliferation regulation and colorectal cancer development. However, the biological effect of LSF in colorectal is not clear, this needs to be explore further in the future.

In summary, we presented evidences for the first time that LSF overexpression in CRC occurred at both transcriptional and translational levels. The increasing LSF expression was evident throughout the clinical progression from normal tissue to malignant transformation and metastasis of CRC. In the archive CRC specimens, the high expression of LSF increased the propensity to develop lymph node metastasis, ectopic proliferation, high likelihood of therapy failure, and the probability of death from cancer after surgery. Additionally, further studies are needed to clarify the mechanism by which LSF is involved in the development and progression of CRC and whether LSF inhibition can reverse chemoresistance in CRC.

## Disclosure of conflict of interest

None.

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