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Original Article

FTL: a novel predictor in gastric cancer

Lixiang Zhang*, Zhangming Chen*, Aman Xu

*Department of Gastrointestinal Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China. *Equal contributors.*

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Abstract: Background: Ferritin light chain (FTL) is a functionally distinct composition of ferritin, which is over-expressed and play an important role in multiple human malignancies. Methods: Matched normal and cancerous tissues in 60 gastric cancer (GC) patients were detected for FTL expression using immunohistochemistry staining. The relationship betweent FTL expression and clinicopathological features was analyzed by chi-square test, and the Kaplan-Meier curves and Cox regression analyses were used to calculate the overall survival (OS) characteristics and recurrence-free survival (RFS). Results: FTL protein was significantly higher in GC tissues than adjacent tissues. Further, FTL expression was not correlative with gender, age, tumor size, tumor location, CEA and AFP level (P>0.05). However, the associations between FTL expression and depth of tumor invasion (P=0.014), differentiation grade (P=0.001), lymph node metastasis (P<0.001) and TNM stage (P<0.001) were significant. Additionally, patients of GC with low FTL expression had longer OS (P=0.019) and RFS (P=0.011), and FTL was an independent prognostic factor for both RFS and OS. Conclusions: FTL was an independent risk factor for both RFS and OS, indicating that FTL could be considered as a prognostic factor for GC patients.

Keywords: Ferritin light chain, gastric cancer, overall survival, recurrence-free survival

Introduction

Gastric cancer (GC) is one of the most prevalent digestive tract cancers worldwide and has a high mortality, especially in developing countries [1]. The patients of GC are commonly diagnosed at advanced stage because of the asymptomatic performance of early gastric cancer, and the five-years survival rate of gastric cancer is remained poor [2]. Therefore it is important to find the appropriate prognostic markers which can help to improve diagnosis and therapy of gastric cancer patients, this will possibly increase the rate of survival.

As we all know, ferritin is critical to iron homeostasis, it play an important role of our human body, however, Related studies showed that elevated serum and tissue ferritin were linked to coronary artery disease and some solid neoplasms [3-6]. Recently, the connection between the ferritin and breast cancer have been studied worldwide, compared with control group, ferritin expression levels are significantly higher in breast cancer patients, besides, ferritin levels are associated with advanced malignant phenotype, such as advanced TNM stage, increased metastatic ability and poor survival [7], suggesting that ferritin protein may contribute to the growth of malignant tumor cells.

Ferritin is made up of two functional elements, the ferritin heavy chain (FTH) and ferritin light chain (FTL) subunits, FTH have ferroxidase activity ($Fe2+ \rightarrow Fe3+$), whereas FTL levels were previously reported to modulate the rate of HeLa cell proliferation [8] and and glioblastoma multiform (GBM) cell [9], some researcher considered that FTL may play an important role in the development of some tumors. Here, this study will explore that whether FTL is a prognostic factor of GC patients.

Materials and methods

Ethics

This study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University. Written informed consent was obtained from each patient.

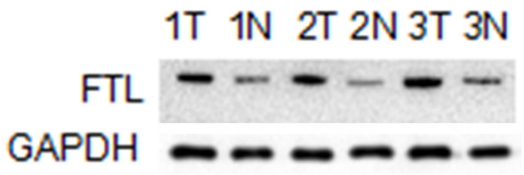

Figure 1. 3 fresh gastric cancer samples and the corresponding normal tissue. T: tumors. N: normal tissue.

Patient sample

Patients, who were diagnosed as gastric cancer and underwent total or subtotal gastrectomy in the First Affiliated Hospital of Anhui Medical University between October 2011 and December 2011, were analyzed retrospectively in this study. The patients who accepted preoperative chemo-or radiotherapy and were diagnosed with iron metabolism-related disease such as hypercholesterolemia, were excluded from this study. In the end, 60 patients were included in this study, and the information was collected on basic patient characteristics, including preoperative blood tests and follow-up results. Overall, 23 (38.3%) patients were males and 37 (61.7%) were females, the median age of patients was 61 years old (range, 29-80). Enrolled patients were prospectively followed-up until October 2016 through telephones and outpatient visit. The median follow-up month was 30 (range, 1.5-60), recurrence or metastasis was assessed by computed tomography or positron emission tomography computed tomography. The disease progression in the GC patients was classified using the guidelines outlined in the seventh edition of the American Joint Committee on cancer (AJCC) about tumor-node-metastasis (TNM) staging [10].

In addition, we collect 3 fresh gastric cancer samples and the corresponding normal tissue, and these samples were frozen temporarily in liquid nitrogen and stored in -80 temperature fridge until Western blot use, similarly, the patients have no preoperative adjuvant therapy or iron metabolism related disease.

Immunohistochemistry (IHC) staining

The tissue specimens were formalin (10%) fixed and paraffin-embedded using standard technique, Construction of the IHC staining was performed as described previously [11], FTL monoclonal antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was applied overnight at 4 centigrade at a dilution of 1:50. The results were evaluated independently by two pathologists in a blinded fashion, if a disagreement occurred, the slides were re-examined to obtain a final consensus, scoring system was evaluated both staining intensity: (0, no stain; 1+, weak stain; 2+, moderate stain; 3+, strong stain) and the percentage of stained cells (1, <10%; 2, 10-30%; 3, 31-60%; 4, >60%), for semiquantitative analysis of immunoreactivity of FTL, immunoreactivity score (IS) was used as described [12], IS results were scored by multiplying percentage positivity of cells by the scores for staining intensity for each case. High expression of FTL was defined as detectable immunoreactions in the cytoplasm and nucleus with IS≥6.

Western blot analysis

Total proteins from gastric cancer samples and the corresponding adjacent tissue were extracted using RIPA lysis buffer (Beyotime, China). The protein was quantified by using the BCA protein assay (Beyotime, China). Then equivalent amount of proteins of each paired specimens were separated by SDS-PAGE on 10% polyacrylamide gels and electrotransferred to 0.45 mm polypropylene fluoride membranes (Millipore. USA). After blocking in 5% nonfat milk diluting with TBST (Tris-buffered saline with tween-20) for 1 h at room temperature, the membranes were incubated with mouse anti-FTL monoclonal antibody (1:500; Abcam) and mouse anti-GAPDH antibody (1:3000; Bioss) at 4°C overnight. In the second day, after washing three times in TBST solution per 10 min, membranes were incubated with peroxidase-conjugated affinipure goat anti-mouse IgG (1:5000; zsgb) as the secondary antibody for 1 h at room temperature. After washing 3 times with TBST per 10 min, the membranes were detected with the enhanced chemiluminescence system according to the manufacturer's instruction. The density of bands was quantified by image J software. FTL band intensities were normalized with GAPDH.

Statistical analyses

All statistical analyses were conducted using SPSS software (version 16.0). The relationship between FTL expression levels and clinicopathological features were explored and assessed by Pearson's chi-square tests or the Fisher test as appropriate. The Kaplan-Meier curves and Cox regression analyses were used to calculate the overall survival (OS) characteristics and

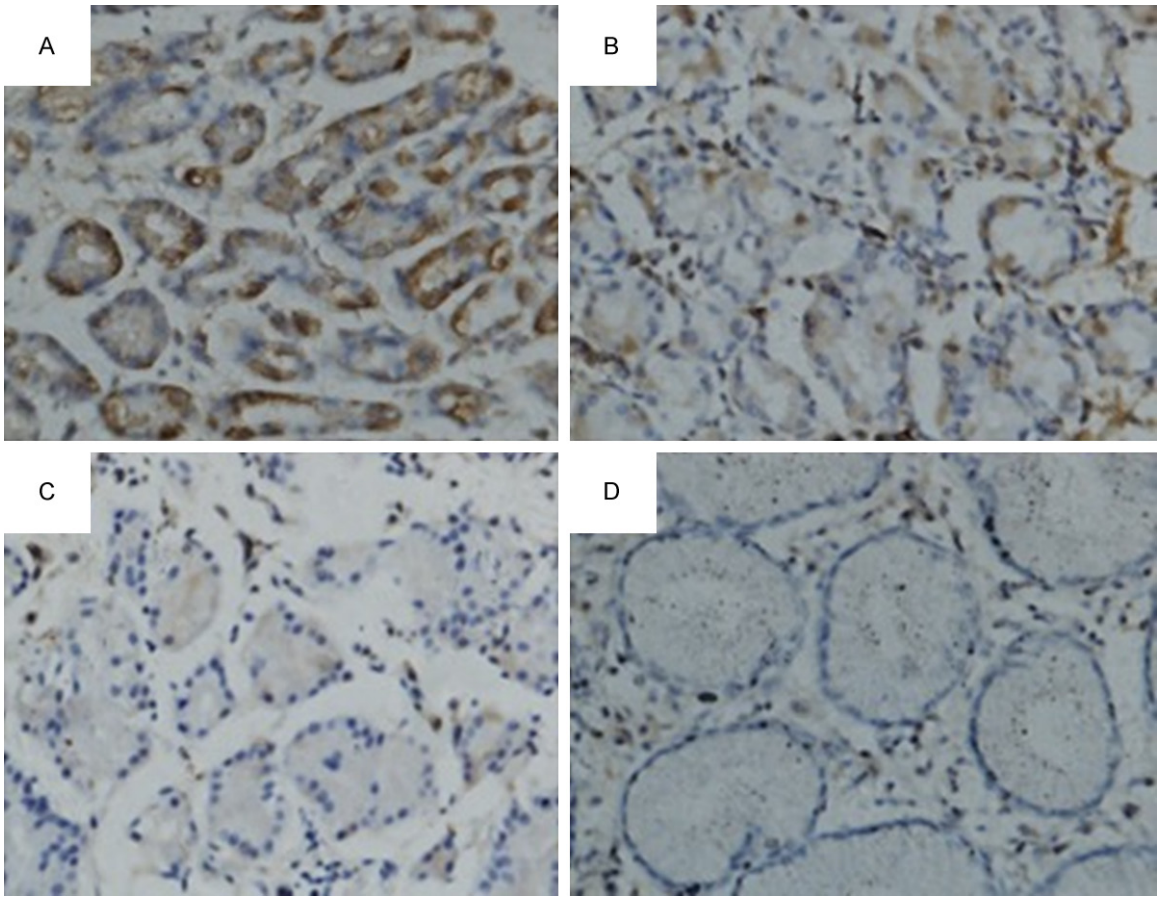

Figure 2. IHC staining of FTL protein in GC or in adjacent noncancer tissue. (A) Showed FTL staining was strong in GC tissue samples; (B-D) Showed moderate staining, weak staining and barely negative staining of FTL expression. (Original magnification, *400 in A-D).

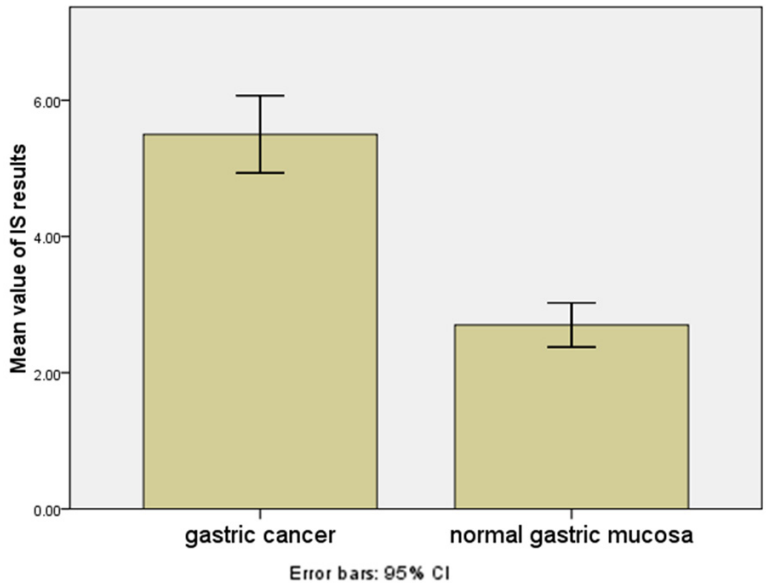

Figure 3. The IS results of gastric cancer and the paired normal gastric mucosa by IHC.

recurrence-free survival (RFS). Correlations between the staining intensity of FTL and TNM stage were determined using Sperman's rank correlation coefficient. For all tests, a 2-sided value of $P<0.05$ was considered to be statistically significant.

Results

FTL expression results of western blot

We evaluated the FTL expression levels of 3 fresh gastric cancer samples and the corresponding normal tissue by using Western blots (**Figure 1**).

Table 1. Relationship between the FTL expression level in tumor tissue and clinicopathologic characteristics

Patient-related factors	FTL expression		χ^2 value	*P* value
	Low (n=26)	High (n=34)		
Gender			1.187	0.206
Men	12	11		
Women	14	23		
Age (years)			0.554	0.457
<60	9	15		
≥60	17	19		
Tumor sizes			0.687	0.407
<5 cm	18	20		
≥5 cm	8	14		
Depth of invasion			7.330	0.014*
T1, T2	11	4		
T3, T4	15	30		
Lymph node metastasis			26.063	0.000*
Negative	20	4		
Positive	6	30		
TNM stage			40.147	0.000*
I-II	28	6		
III-IV	0	28		
Differentiation grade			12.319	0.001*
Well	17	7		
Poor	9	27		
CA199 (u/ml)			0.207	0.649
<40	19	23		
≥40	7	11		
CEA (u/ml)			1.297	0.378
<5	21	23		
≥5	5	11		
Tumor location			0.087	0.768
Cardia	14	17		
Body or antrum	12	17		

*Considered to be statistically significant.

Table 2. The correlation of the FTL staining intensity with TNM stage

			TNM stage			Total
			I	II	III	
Intensity	1	Count	2	3	0	5
	2	Count	8	16	21	45
	3	Count	1	2	7	10

FTL expression results of IHC

To determine the connection of clinic-pathologic features with FTL expression, matched normal and cancerous tissues in 60 gastric cancer (GC) patients were detected for FTL expression using immunohistochemistry staining. Strong staining of FTL protein was predominantly distributed in gastric cancer cells, by contrast, FTL protein was weak expression or barely detectable in the normal gastric epithelial cells. **Figure 2** shows 4 representative cases of tissues: A Showed FTL staining was strong. B, C and D showed moderate staining, weak staining and barely negative staining of FTL expression. IS results of FTL in gastric cancer is higher than the paired normal gastric mucosa (*P*<0.001, **Figure 3**).

The relationship between FTL expression and clinical pathological characteristics

The association is shown in **Table 1**, The FTL expression was not correlative with gender, age, tumor size, tumor location, CEA and AFP level (*P>0.05*). However, the associations between FTL expression and depth of tumor invasion (*P=0.014*), differentiation grade (*P=0.001*), lymph node metastasis (*P<0.001*) and TNM stage (*P<0.001*) were significant.

Sperman's correlation coefficient

To further study the potential roles of FTL protein in GC cancer progression. The FTL staining intensity is in positive correlation with TNM stage (r=0.295, *P*=0.022) (**Table 2**), indicating the higher FTL expression suggested more advanced pathological TNM stage.

Survival analysis of FTL expression

The overall 5-year survival rate of the low expression and the high expression of FTL was 61.5% and 32.4%, respectively, and the patients with low FTL expression had longer OS (P=0.019) and recurrence-free survival (P=0.011) (**Figure 4**), indicating the high FTL expression of gastric cancer patients had increased risk of overall mortality and recurrence.

Univariate and multivariate analysis

As indicated by univariate analysis, TNM stage, the depth of invasion, tumor size, lymph node metastasis, the way of surgical resection, FTL expression, CEA and CA199 level significantly

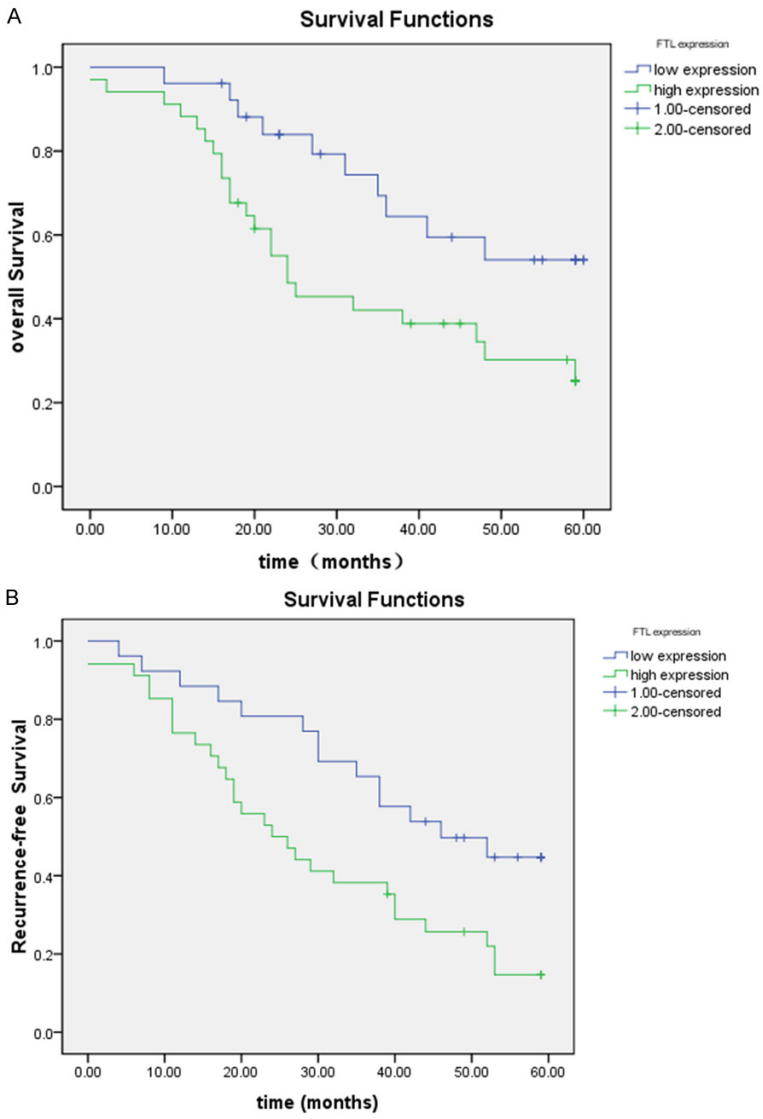

Figure 4. Kaplan-Meier survival of OS (A) and RFS (B) in the low FTL expression and high FTL expression.

impacted the recurrence-free survival ($P<0.05$, **Table 3**), while TNM stage, the depth of invasion, tumor size, lymph node metastasis, FTL expression, CEA and CA199 level ($P<0.05$) were positive prognostic factor for the overall survival. And multivariate survival analysis using the Cox proportional hazards model was adjusted to compare the prognostic value of FTL expression with CEA and CA199 level (**Table 4**), it showed that the FTL levels was an independent risk factor for the over survival (95% CI 1.246-5.696; $P=0.012$) and recurrence-free survival (95% CI 1.304-4.889; $P=0.006$).

Discussion

As everyone knows that ferritin is the primary iron storage protein, but recent studies have demonstrated that it may play an important role in the cell proliferation, angiogenesis, and immuno-suppression [13]. The aberrant expression of ferritin was reported in diverse disease [3, 14]. Some scholars explore the possible mechanism, excess iron can induce oxidative stress and DNA damage, at the same time, excess iron alters the distribution of T-lymphocyte subsets and suppresses the function of CD4 cells and monocytes, resulting in decreased immune function of human body [15], so the excess iron may have potential carcinogenesis. Ferritin consists of two distinct ferritin subunits, ferritin heavy chain (FTH) and ferritin light chain (FTL) subunits, researchers found ferritin heavy chain was related with the development of a variety of tumors and down-regulation of FTH may substantially increase the efficiency of cancer therapy [16, 17], as a similar subunit with FTH, whether the light chain also participate in the development of tumor caused strong interest. In some tumors, we found FTL expression was connected with the overall survival of the patients [5, 18-21], and FTL may play an important role as a potential diagnostic and prognostic bio-maker.

In our present study, we verified that FTL is frequently over-expressed in GC tissue by Western blot and immunohistochemical-staining. In the IHC, the expression of FTL in gastric cancer was up-regulated paired with noncancerous tissue ($P<0.001$), therefore FTL may be a potential diagnostic marker of GC patients. **Table 1** showed that the expression of FTL protein was

Table 3. Univariate analysis of RFS and OS

Factors	Univariate analysis of RFS			Univariate analysis of OS		
	χ^2	*HR* (95% *CI*)	*P*	χ^2	*HR* (95% *CI*)	*P*
TNM stage	11.622	2.989 (1.593, 5.609)	0.001	7.510	2.706 (1.328, 5.515)	0.006
I, II						
III, IV						
Differentiation grade	0.857	1.348 (0.716, 2.536)	0.354	2.574	1.817 (0.876, 3.767)	0.109
Well						
Poor						
FTL expression	6.411	2.244 (1.176, 4.280)	0.014	5.083	2.354 (1.118, 4.957)	0.024
Low						
High						
CA199 (u/ml)	6.372	2.316 (1.207, 4.447)	0.012	4.132	2.138 (1.028, 4.447)	0.042
<40						
≥40						
CEA (u/ml)	7.952	2.505 (1.323, 4.744)	0.005	7.543	2.636 (1.320, 5.264)	0.006
<5						
≥5						
LNM	6.259	2.361 (1.205, 4.628)	0.012	4.981	2.399 (1.112, 5.173)	0.026
Negative						
Positive						
T stage	10.78	5.677 (2.014, 16.003)	0.001	5.818	4.314 (1.315, 14.41)	0.016
T1, T2						
T3, T4						
Surgical type	7.741	3.226 (1.414, 7.362)	0.005	3.363	2.290 (0.945, 5.554)	0.067
Total						
Subtotal						
Location	0.543	0.795 (0.431, 1.465)	0.461	0.003	0.982 (0.496, 1.495)	0.959
Cardia						
Body/antrum						

Recurrence-free survival (RFS); overall survival (OS); lymph node metastasis (LNM).

Table 4. Multivariate analysis of RFS and OS

Factors	Multivariate analysis of RFS			Multivariate analysis of OS		
	χ^2	*HR* (95% *CI*)	*P*	χ^2	*HR* (95% *CI*)	*P*
FTL (low/high)	7.551	2.525 (1.304, 4.889)	0.006	6.386	2.664 (1.246, 5.696)	0.012
CEA (low/high)	1.197	1.573 (0.699, 3.542)	0.274	0.60	1.132 (0.419, 3.062)	0.807
CA199 (low/high)	4.261	2.316 (1.043, 5.141)	0.039	7.543	2.786 (1.094, 7.094)	0.032

Recurrence-free survival (RFS); overall survival (OS).

significantly associated with the depth of tumor invasion (*P=0.014*), differentiation grade (*P= 0.001*), lymph node metastasis (*P<0.001*) and TNM stage (*P<0.001*), moreover, Sperman's rank correlation analysis suggested the more TNM stage was positive correlated with staning intensity of FTL (P<0.05). We speculate that FTL may play an important role in promoting tumor invasion in gastric cancer, FTL overexpression is associated with more aggressive tumor behavior.

Currently, overall survival time and recurrence-free survival are critical elements to evaluate the prognosis of patients, our study revealed that the patients with low FTL expression had longer OS (*P*<0.05) and recurrence-free survival (*P*<0.05), indicating the high FTL expression

of gastric cancer patients had increased risk of overall mortality and recurrence. Moreover, in the univariate analysis of OS and RFS, FTL protein was positive prognostic factor like other clinical parameters, such as TNM stage, the depth of invasion, tumor size, lymph node metastasis, CEA and CA199 level (all P<0.05), indicating FTL might be used as a prognostic molecular bio-maker of GC patients. As we all know, CEA and CA199 are significant screening methods of recurrence of tumors, the multivariate cox model analysis verified that the FTL was an independent risk factor for both RFS and OS when combined with CEA and CA199, indicating that FTL has a prognostic value for us to assess the recurrence of GC patients.

In this study, FTL was frequently over-expressed in GC tissue by IHC, The M2 tumor-associated macrophages (TAMs) of GC tissue have an expression profile (FTL up-regulation and FTH down-regulation) that enhances iron release [22, 23], thus increase the iron load of body. This mechanism might be a reason why high FTL expression in tissue promote proliferation and growth of gastric cancer cells. Although we can not detect the level of FTL in gastric cancer tissue, we can acquire the serum ferritin of these patients, if they have a little bit higher level of serum ferritin combined with their untypical symptoms, they need more intensively care and followed-up. If needed future studies get the result that the excess iron can contribute the development of gastric cancer, maybe we can reduce the risk of GC by the use of chelating agents [24] which can binding iron to reduce its damage to human body. In addition, we may improve the survival of GC patients through targeted therapy against FTL. So future research about the mechanism of FTL in the process of GC progression is needed to further explore.

Conclusion

To our knowledge, this is the first study about the prognostic value of FTL in gastric cancer, we found that FTL protein was significantly higher in GC tissues than adjacent tissues by Western blot and immunohistochemical staining. Further, FTL expression was not correlative with gender, age, tumor size, tumor location, CEA and AFP level (P>0.05). However, the associations between FTL expression and depth of tumor invasion (P=0.014), differentiation grade (P=0.001), lymph node metastasis (P<0.001) and TNM stage (P<0.001) were significant. Additionally, patients of GC with low FTL expression had longer OS (P=0.019) and RFS (P=0.011), and FTL was an independent prognostic factor for both RFS and OS, indicating that FTL might be used as a prognostic molecular bio-maker of GC patients and may be a potential novel therapeutic target for GC.

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Disclosure of conflict of interest

None.

Address correspondence to: Aman Xu, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China. E-mail: 18788831253@163.com

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