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Low Expression of Fragile-Site Associated Tumor Suppressor Is Associated with Prognosis in Patients with Bladder Cancer

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Abstract

Fragile-site associated tumor suppressor (FATS) may be closely related to the occurrence and development of human tumors. However, the expression of FATS in bladder tumors has not been reported. In this study, we aimed to evaluate the expression of FATS in tumor tissues and determine its correlation with tumor characteristics and prognosis in patients with bladder cancer. The expression of FATS protein and FATS mRNA in bladder cancer tissues and adjacent tissues were detected by immunohistochemistry (IHC), Western blotting and reverse transcription-PCR (RT-PCR). Both IHC and Western blotting showed that the positive expression rate of FATS protein in bladder cancer tissues was significantly lower than that in adjacent tissues [(44.94% (71/158) vs. 91.01% (81/89), $P < 0.05$; (43.67% vs. 89.89%), $P < 0.05$, respectively]. RT-PCR revealed that the positive rate of FATS mRNA in bladder cancer tissues was 41.14% (65/158), which was significantly lower than that in adjacent tissues 87.64% (78/89). Low FATS protein expression was related to gender, tumor diameter, tumor grade, lymph node metastasis, and tumor stage in patients with bladder cancer. Patients with lower FATS protein expression in bladder cancer tissues had a significantly shorter disease-free survival (DFS) rate and overall survival (OS) rate than patients with higher FATS levels. Lower FATS protein expression was an independent risk factor for DFS and OS in bladder cancer patients. In summary, low FATS protein expression was closely related to the occurrence, development, and prognosis of patients with bladder cancer. These findings may contribute to novel therapeutic and diagnostic strategies for patients with bladder cancer.

Keywords: fragile-site associated tumor suppressor (FATS), bladder cancer, prognosis, diagnostic strategies

1. Introduction

Bladder cancer (Koutros et al. 2020; Peng et al. 2019) is the most common urinary malignant system tumour and the ninth most common worldwide cancer. Annual bladder cancer causes around 165,000 deaths each year. Bladder cancer has the highest urogenital tumour incidence rate in China and the 2nd highest prostate cancer in western countries (Lin et al. 2019; Yang et al. 2020). Bladder cancer, including in children, may occur at any age. The rate of incidence increases with age and the highest incidence among the 50 to 70-year olds (Linxweiler et al. 2020). Bladder cancer is 3-4 times more prevalent in men than in women (Steinberg et al. 2020). Bladder cancer development stage may be divided into two categories: Invasive NMIBC and Invasive Musculoskeletal Bladder Cancer (MIBC) (Shen et Al. 2019; Wang et Al. 2020). Most blood cancer patients are diagnosed late in life and the survival rate for five years is only approximately 50% (Guo et al. 2020).

Fragile-site associated tumor suppressor (FATS) is a newly discovered common fragile site gene,

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which was found by establishing a mouse model induced by DNA damage and studying the abnormal deletion of DNA copy number in tumor tissue on the whole genome scale (Ma *et al*. 2012; Zhang *et al*. 2011). As a new tumor suppressor gene, FATS is highly sensitive to DNA damage, and there are many single nucleotide polymorphism loci. There are abundant repeats in the introns and expression regulatory regions of FATS (Barnes *et al*. 2017; Palumbo *et al*. 2019). It has the typical gene characteristics of fragile sites. Due to the loss of heterozygotes at this site in many tumors, FATS may be closely related to the occurrence of human tumors (Hazan *et al*. 2016). A large number of studies have found that FATS not only has a significant gene deletion in the DNA of breast cancer and ovarian cancer patients, but also in a variety of tumor cells and tissues, and the expression level of FATS mRNA may decrease (Kuroki *et al*. 2006; Le *et al*. 2013). However, the expression of FATS in bladder tumors has not been reported.

Thus, in this study, we aimed to evaluate the expression of FATS in tumor tissues and determine its correlation with tumor characteristics and prognosis in patients with bladder cancer.

2. Materials and Methods

2.1 Patients and controls

Out of 225 patients admitted to the Dafeng City Hospital, the Yancheng City First People's Hospital, and the Shanghai Public Health Clinical Center from May 2014 to May 2019, 158 patients with detailed clinical evidence for bladder cancer were chosen. All registered patients received surgical resection and received a follow-up time of 1-5 years after surgery until 1 May 2020. The study was endorsed by the Ethics Committee of the Dafeng City People 's Hospital.

The inclusion criteria were, (1) the clinical and pathological diagnosis of bladder disease and (2) complete clinical data. Exclusion criteria included: (1) serious damage to liver and renal function; (2) other complicated solid tumours or malignant blood diseases, (3) first diagnosed with bladder cancer and radiation therapy, chemotherapy and biologic treatment, and (4) incomplete clinical data; and (5) loss of follow-up. The exclusion criteria were the following:

2.2 Baseline data and tissue sample collection

Base clinical data, including demographic characteristics, location of tumour, tumour sized, metastases of the lymph nodes, TNM and pathological differentiation have been obtained from medical records. Overall data from the follow up records were collected and DFS and OS were calculated. Survival data were collected from the follow-up records. The surgical resection of 89 individuals was used to collect adjacent tissue specimens to serve as control groups.

2.3 Immunohistochemistry (IHC)

IHC was used for the assessment of bladder cancer expression of FATS. Paraffine slices were added in the oven at a temperature of 60-65oC, baked for 60-120 minutes, and hydrochlorinated with xylene (10min / 3 times) at concentrations of graded ethanol (absolute = 3 times, 95%, 85%, and 75%). The parts have been carefully rinsed for 1 minute with distilled water and washed twice with PBS for 5 minutes each. A pressure cooker thermal solution was added to the antigen solution, boil for 3 minutes and then cooled in water distilled. An endogenous peroxidase blocker was added after rinsing with distilled water and PBS. It was incubated for 15 min at room temperature. The slices were then incubatoried with normal goat serum during 15 minutes at room temperature and the serum was poured out, but the slices were not washed. At 4 ° C, the slices were incubated overnight, the antimicrobial was decanting and the slices had been rinsed with PBS. Subsequently, biotin-labeling rabbites were added, the IgG anti-mouse solution was incubated, the reagent was poured and the slices were rinsed using PBS. Similarly, the solution was also added. Added and incubated at room temperature, for 5 min, the DAB colour development solution. A counter-stained hematoxyline with hydrochlorinated differentiation of 2 s with a saturated Li2CO3 anti-blue for 2 s. Hematoxyline was used. After drying and transparency, these sections were sealed. The positive areas FATS were observed and the proportion of positive areas calculated under optical microscopes.

The IHC results for positive cells in bladder cancer and adjoining tissues were evaluated and observed by two pathologist(s). In each field, 10 high-powered cells have been randomly selected and 100 tumour cells have been counted. The results of the staining were assessed: negatively, (not brownish-yellow positive tumour or glandular epithelial cell staining, weakly positive (+) positive cell percentage was below 25%, positive (+ +), a positive cell percentage between 25% and 50% and positive (+ + +), positive cell percentage was above 50 percent. To facilitate statistical analysis, low expression was defined as negative and weak positive, and high expression as positive and strong

positive.

2.4 Detection of FATS protein expression by Western blotting

Thirty minutes after the addition of RIPA and PMSF (100:1), the fresh tissue was ground and lysed on ice. The mixture was centrifugated at 4 ° C, 14.400 rpm during 15 minutes, and the overlapping was harvested with the BCA (Thermo Fisher / Pierce BCA Protein Assay Kit) kit to determine the total protein concentration. Total protein was mixed well and the protein sample (1:4) was boiled for 5 minutes at 100 ° C with a buffer load. Concentrated gum and gum were prepared and electrophoretic gel was carried out at 80 V in 1-2 h and 100-120 V for a period of 30-50 Min. During 2 hours the membrane washed with TBST and subsequently incubated with primary antibody (FATS, 1:1,000, Abcam) overnight in a shaker at a temperature of 5 percent milk .. Secondary anticorps diluted to 5% milk have been incubated for 2 hours on a shaker. Wash, infiltrate and detect membranes with solution (1:1) and the Image Lab software. A chemiluminescence imaging system has visualised protein bands. Protein bands

2.5 Detection of FATS mRNA expression by RT-PCR

The tissue was lysed with TRIZOL, the total RNA extracted and the NanoDrop spectrophotometer was used to determine the concentration and purity of the RNA solution at 260 nm and 280 nm. The sequences of the primers used were as follows: FATS: Forward: 5'-CATTCACATTCGCTGGAGTTA-3', Reverse: 5' CCTCTTGCTGCTTCCAGAAAATACT-3'; GAPDH-F:5'-CGGAGTCAACGGATT TGGTCGTAT-3'; Reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' Real-Time PCR (TaKaRa, RR820A) response system consisted of SYBR Premix Ex Taq II (2x), 10 µL; Forward Primer (10 µM), 1 µL; Reverse Primer (10 µM), 1 µL; cDNA template, 1-2 µL; dH2O, up to 20 µL. Thermocycling conditions were as follows: pre-denaturation at 95 ° C for 30 s, and recurrence of PCR on a gradient thermal cycler: 40 cycles at 95 ° C for 5 s, 60 ° C for 30 s, and extension at 60 ° C for 30 s. Quantity-One electrophoresis apparatus was used to examine the amplification of FATS by agarose gel electrophoresis. The absorbance (a) value of the belt and the reference were obtained, the data were expressed as the ratio (sample value / reference value) and the relative expression was calculated according to the ΔC(t) principle.

2.6 Statistical analysis

SPSS (Version 25; IBM SPSS stats, USA) and GraphPad prism 7.00 (GraphPad software) statistical analysis were pertaining to this analysis. The data are presented with medium ± defaults. The tumour and adjacent tissue expression of FATS and FATS mRNA was compared with the test of McNemar. The Kaplan-Meier curves are used to calculate DFS and OS. Using the log-rank tests, differeties between DFS and OS groups were determined. Univariate and multivariate regression analyses have evaluated DFS and OS factors. A $P < 0.05$ value was considered to be statistically significant.

3. Results

3.1 Baseline characteristics

The baseline characteristics of enrolled 158 bladder cancer patients were in Table 1. Among the enrolled patients, 105 (66.46%) were men, the number being significantly higher than that of 53 (33.54%) women. One hundred and twelve patients (70.89%) were over 60 years old. In 93 (58.86%) patients, the tumor was located in the triangle area. Tumor diameter was more than 2 cm in 94 cases (59.49%) and less than 2 cm in 64 cases (40.51%). The numbers of patients with high (G1), moderate (G2), and poor (G3) tumor differentiation were 46 (29.11%), 79 (50.00%), and 33 (20.89%), respectively. Forty-nine patients (31.01%) showed lymph node metastasis. Among the 158 patients, 125 (79.11%) and 33 (20.89%) patients had NMIBC and MIBC, respectively.

3.2 FATS protein and FATS mRNA expression in bladder cancer tissues and adjacent tissues

FATS protein expression in bladder cancer tissues and adjacent tissue was assessed by IHC and Western blot. IHC showed a significant reduction in positive expression for FATS protein in bladder tißue than in neighbouring tissue (44.94% (71.158) compared to 91.01% (81.089); $P < 0.05$; figure 1). Western blots have further confirmed that the expression of FATS protein in bladder cancer tissues was significantly lower (43.67% vs. 89.89%; P<0,05; Figure 2A – B).

RT-PCR was also used to evaluate the levels of FATS mRNA expression in bladder and neighbouring tissues. The results showed a positive FATS mRNA rates of 41.14% (65/158), which are significantly less than 87.64% (78/89) of adjacent tissue ($P < 0.05$, Figure2C) in bladder cancer tissue (P).).

3.3 Relationship between FATS protein expression and bladder cancer characteristics

The relationship between the expression of FATS protein and the characteristics of bladder cancer was assessed using a single factor analysis. Results showed that low FATS protein expression was not

related to the location or age of the tumour but related to gender, tumour diameter, tumour grade, lymph node metastases, and tumour stage (all P < 0.05; Table 2).

3.4 Association of FATS protein expression with DFS and OS in patients with bladder cancer

Low FATS patients had a lower DFS than high FATS expression patients (P < 0.001; Figure 3A). A multivariable analysis of the Cox regression in patients with different tumour stages (NMIBC and MIBC), has shown that the DFS is shorter for both NMIBC (P=0.001) and MIBC (P=0.039) patients (Figure 3B – C) than for the patients with high FATS expression.

With regard to operating system, patients with low FATS expression had considerably shorter operating systems than those with high FATS expression (P < 0.001; Figure 4A). The OS was also significantly different in NMIBC and MIBC patients compared to patients with low FATS and high FATS (P = 0.036; Figure 4B-C).

3.5 DFS and OS factors in bladder cancer patients

Cox regression testing was used to assess DFS and OS factors in bladder cancer patients. The Cox univariable analysis showed a correlation between sex (male), tumour diameter (> 2 cm), poor tumour differentiation (G3), metastatic lymph node, and low FATS protein expression and worse DFS. Posterior multivariate analysis also demonstrated the independent risk factors of the DFS in bladder cancer (table 3) in patients who had low expression of the FATS proteins, metastatic lymph node, the tumour (> 2 cm) and poor differentiation tube (G3).

For OS, univariate analysis of Cox has shown that low tumour FATS expression, tumour diameter (> 2 cm), metastase of the lymph node, or poor tumour differentiation (G3) are correlated with poor OS in bladder cancer patients. Multivarial analysis also showed the independence of risk factors of OS in patients with bladder cancer, both in tumourous tissual FATS and in lymph node metastases (Table 4).

4. Discussion

Multigenic and multi-stage synergies associated with genetic elements such as activation of oncogenes and inactivation of the tumour suppressor gene due to many environmental factors (Lee et al. 2019; Meisenberg et al. 2019). The development and development of tumour is the product. The common feature of the tumour cells is a genomic instability, demonstrated as an abnormal DNA damage repair mechanism, as well as disordered cell cycle regulation (Krenning et al. 2019). The damage to dNA is an important mechanism for malignant tumours and is highly sensitive to DNA damage at fragile sites of chromosomal tumour suppressors. (Jones et al. 2020). The incidence and development of tumours can be linked greatly to low expression or removal of these genes (Archambeau et al. 2019). An unstable area with 39 rare fragile sites and 88 common fragile sites, is a fragile site (Marquardt et al. 2020; Zheglo et al. 2019). In a normal cell, there are no fragile sites of chromosomes. When replication of DNA in cells is partially inhibited, in the mitosis metaphase a gap or broken region is formed and many cancers are detected (Bjerregaard et al. 2018; Blin et al. 2019).

In an earlier study, Zhang et al (Zhang et al. 2017) found that low levels of FATS are associated with breast cancer and that FATS is an independent predictor of longer DFS in patients. The study by Song et coll. (Song et al. 2015) showed the role of the signalling cascades of FATS-p53 in inhibiting pregnancy-related cancer and the possible use of FATS genotyping in breast cancer prevention. The expression of FATS protein was linked to development in patients suffering from NSCLC and was an independent predictor factor in another study (Zhang et al. 2019). The FATS protein detection is expected to serve as a new biomarker in the evaluation of the NSCLC forecast. However, FATS' biological and expression roles are not clear in bladder cancer.

Both FATS and FATS mRNA expressions have been found to be less in blood tissue than in adjacent tissues in this study. The expression of low FATS proteins in patients with bladder cancer has been related to male sex, tumour diameter, tumour grade, lymph node metastasis and tumour stage. Low FATS expression patients had shorter DFS and shorter OS in comparison to FATS expression patients. Moreover, both DFS and OS were significantly shorter in patients with NMIBC and MIBC, in patients with low expression FATS compared to patients with high expression FATS.

In terms of the effects of DFS and OS in bladder cancer patients, our study shows that low expression of proteins FATS and metastases of lymph nodes, tumour (> 2 cm) and poor tumour differentiation (G3) are separate risk factors for DFS in patients with bladder cancer, whereas both low tumour tissue FATS and metastases of lymph node are independent risk factors for OS in the thesis. Thus, the low expression of FATS protein was a major independent prognostic factor for bladder cancer patients.

This study had some limitations. First, although

this is the first report of the FATS-bladder cancer connexion, the number of samples was limited. To further verify the results, further samples and multiple centre research is needed. Second, FATS is a separate risk factor for the diagnosis of patients with bladder cancer. However, it is important to analyse more data, including the development of a diagnostic model, as a prognosis indicator for FATS.

5. Conclusion

In brief, our study showed that low FATS protein expression is closely associated with patients with bladder cancer, their development, and their pronostics. These findings can contribute to new therapeutic and diagnostic approaches for bladder cancer patients.

Recognition:

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Table 1. Baseline characteristics of enrolled bladder cancer patients

Characteristic	Bladder cancer patients (n = 158)
Gender	
Male	105（66.46）
Female	53（33.54）
Age（years）	
<60	46（29.11）
≥60	112（70.89）
Tumor location	
Trigonal area	29（18.35）
Bladder base	36（22.79）
Bladder body	93（58.86）
Tumor diameter（cm）	
≤ 2	64（40.51）
> 2	94（59.49）
Tumor grade	
G1	46 (29.11)
G2	79 (50.00)
G3	33(20.89)
Lymph node metastasis	
YES	49（31.01）
NO	109（68.99）
Tumor stage	
MIBC	33 (20.89)
NMIBC	125 (79.11)

MIBC muscle-invasive bladder cancer; NMIBC non-muscle-invasive bladder cancer

G1: high tumor differentiation; G2, moderate tumor differentiation; G3: poor tumor differentiation

Table 2. **Relationship between FATS protein expression and tumor characteristics**

Characteristic	n	low FATS protein expression	x^2	*P*
Gender, No (%)				
Male	105	57（54.29）	11.057	0.001
Female	53	14（26.42）		
Age（years), No (%)				
<60	46	19（41.30）	0.346	0.556
≥60	112	52（46.43）		
Tumor location				
Trigonal area	29	13 (44.83)	0.006	0.997
Bladder base	36	16 (44.44)		
Bladder body	93	42 (45.16)		
Tumor diameter (cm), No (%)				
≤ 2	64	9 (14.06）	41.442	< 0.001
> 2	94	62 (65.96）		
Tumor grade, No (%)				
G1	46	6 (13.04)	35.557	< 0.001
G2	79	53 (67.09)		
G3	33	12 (36.36)		
Lymph node metastasis, No (%)				
YES	49	31（63.27）	9.643	0.002
NO	109	40（36.70）		
Tumor stage, No (%)				
MIBC	33	26 (78.79)	19.317	< 0.001
NMIBC	125	45 (36.00)		

MIBC muscle-invasive bladder cancer; NMIBC non-muscle-invasive bladder cancer

G1: high tumor differentiation; G2, moderate tumor differentiation; G3: poor tumor differentiation

Table 3. **The analysis of Factors affecting DFS with univariate and multivariate Cox's proportional hazards regression model**

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	***P***	**OR (95% CI)**	***P***
Age (≥ 60y)	1.47(0.86-2.50)	0.159		
Gender (male)	1.81(1.07-3.06)	0.026	1.58(0.91-2.72)	0.102
Tumor location	0.98(0.74-1.31)	0.914		
G3	15.84(6.10-41.10)	< 0.001	6.66(2.30-19.24)	<0.001
Tumor diameter (> 2 cm)	6.64(3.39-12.97)	< 0.001	2.56(1.22-5.36)	0.013
Lymph node metastasis	3.83(2.41-6.08)	< 0.001	1.84(1.10-3.08)	0.020
MIBC	2.41(1.44-4.04)	0.001	1.66(0.94-2.91)	0.078
FATS protein expression (low)	2.91(1.81-4.66)	< 0.001	2.70(1.34-5.47)	0.006

MIBC muscle-invasive bladder cancer; G3: poor tumor differentiation

Table 4. **The analysis of Factors affecting OS with univariate and multivariate Cox's proportional hazards regression model**

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	***P***	**OR (95% CI)**	***P***
Age (≥ 60y)	1.28(0.72-2.27)	0.399		
Gender (male)	1.71(0.96-3.02)	0.067		
Tumor location	0.98(0.71-1.35)	0.887		
G3	7.20(2.70-19.21)	< 0.001	2.24(0.72-6.91)	0.162
Tumor diameter (> 2 cm)	4.27(2.16-8.43)	< 0.001	2.11(0.98-4.51)	0.055
Lymph node metastasis	3.91(2.35-6.53)	< 0.001	2.20(1.23-3.93)	0.008
MIBC	2.46(1.43-4.24)	0.001	1.40(0.77-2.56)	0.271
FATS protein expression (low)	3.51(2.05-6.02)	< 0.001	2.48(1.22-5.03)	0.012

MIBC: muscle-invasive bladder cancer; G3: poor tumor differentiation

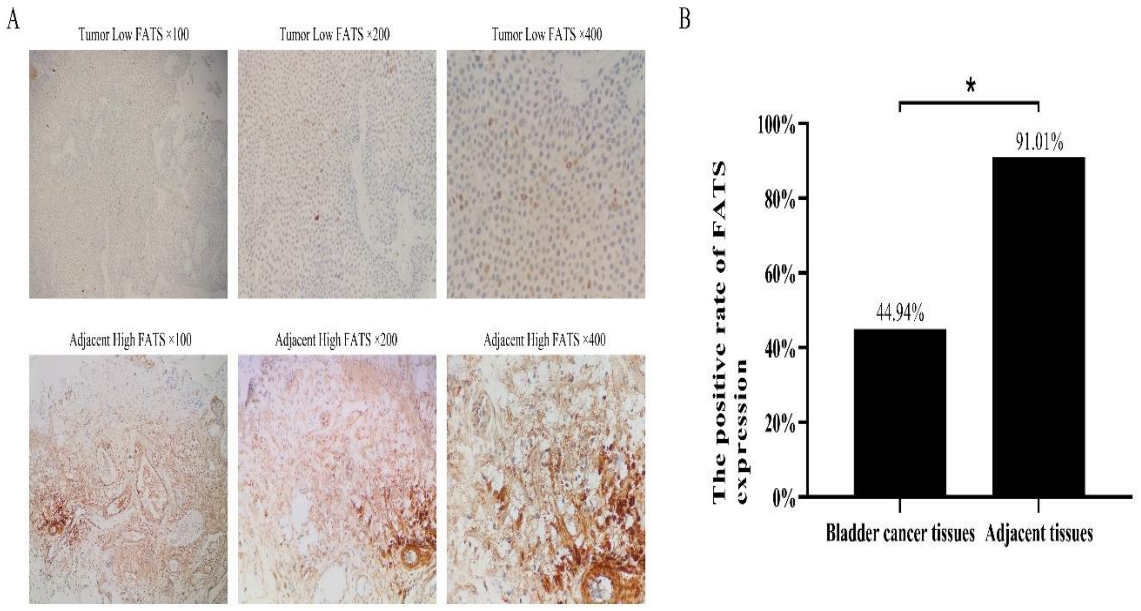

Figure 1. **Immunohistochemical analysis of FATS expression in bladder cancer tissues and adjacent tissues.** (A) Low FATS expression in bladder cancer tissues at 100×, 200×, and 400× magnification, High FATS expression in adjacent tissues at 100×, 200×, and 400× magnification; (B) The positive rate of FATS expression in bladder cancer tissues and adjacent tissues.

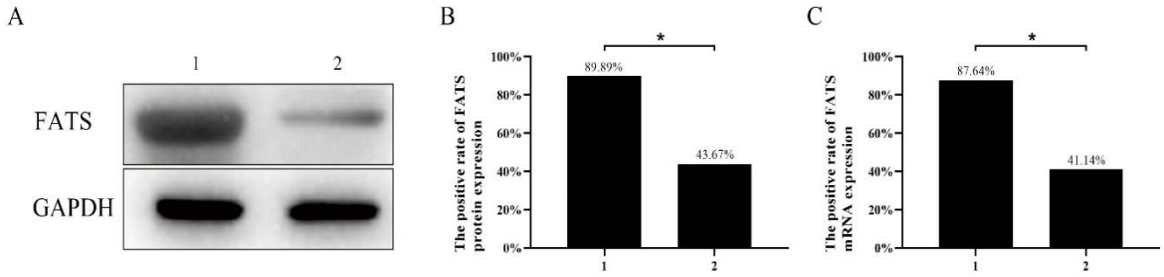

Figure 2. **Western blotting and RT-PCR analysis of FATS protein and FATS mRNA expression in bladder cancer tissues and adjacent tissues.**

(a) the positive FATS protein expression in bladder cancer tissue and adjacent tissues; (b) the positive rate of bladder cancer and adjacent tissues expression of FATS protein;(c) the beneficial rate of FATS mRNA expression in bladder cancer tissue and adjacent tissue. 1. Tissue group adjacent; 2. Group of bladder tissue cancer.

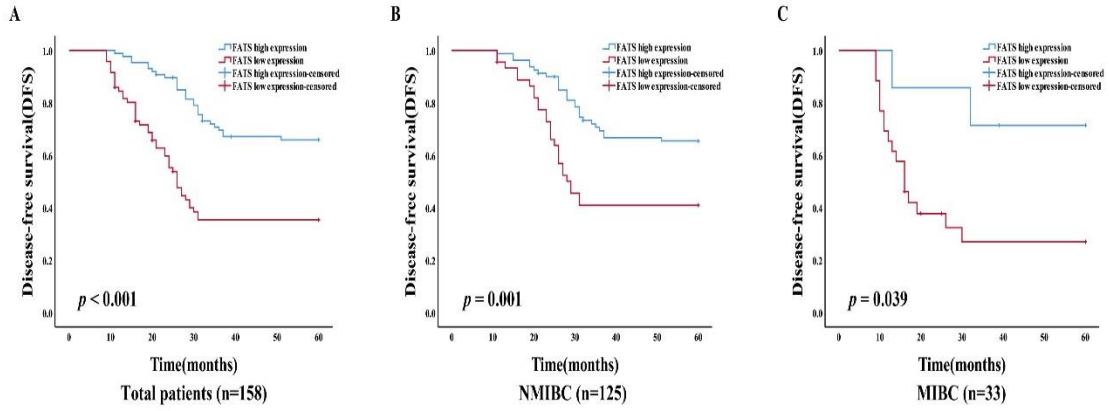

Figure 3. **Association of FATS protein expression with DFS in bladder cancer patients.**

(A) FATS protein expression with DFS in all bladder cancer patients; (B) FATS DFS protein expression in patients with NMIBC; (C) FATS protein expression with DFS in muscle-invasive bladder cancer patients (MIS).

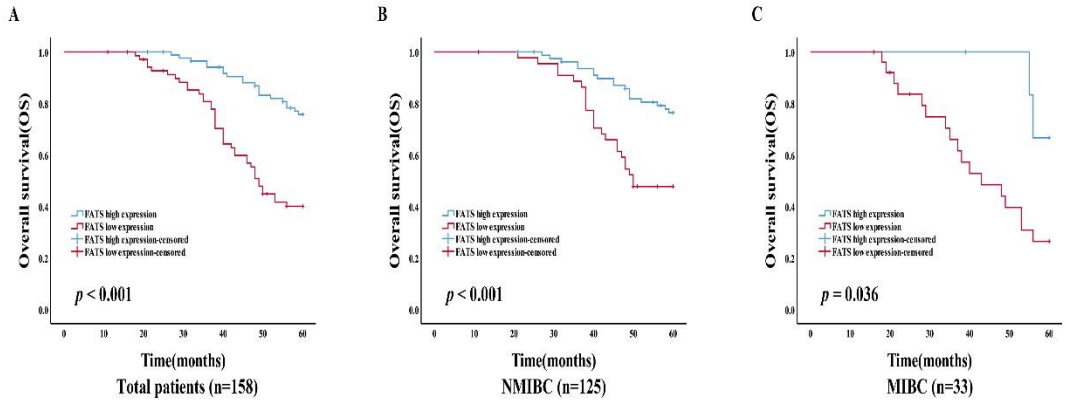

Figure 4. **Association of FATS protein expression with OS in bladder cancer patients.**

(A) FATS protein expression with OS in all patients suffering from the disease of bladder cancer; (b) FATS OS protein expression in patients with non-MUCBC; (c) FATS protein expression with OS in patients with MUCC.