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Original Article

lncRNA FTX predicts tumor progression and adverse prognosis in gastric cancer

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Abstract: Long noncoding RNAs (lncRNAs) have been found to be involved in tumorigenesis of different tumors. Expression patterns and prognostic significance of lncRNA FTX in gastric cancer remain unclear. This study found lncRNA FTX expression was significantly increased in gastric cancer tissues, compared to adjacent normal tissues. Moreover, lncRNA FTX expression was significantly associated with larger tumor size, deeper depth of invasion, advanced pathological stage, and lymphatic metastasis. lncRNA FTX expression was correlated with overall survival in gastric cancer. In addition, upregulation of lncRNA FTX promoted cell proliferation and invasion in gastric cancer cells. Overexpression of lncRNA FTX was associated with progression and prognosis of gastric cancer and may represent a potential molecular biomarker for predicting outcome of patients.

Keywords: Long noncoding RNA, FTX, gastric cancer, prognosis, tumor progression

Introduction

Gastric cancer is a common malignancy and the second leading cause of cancer-related deaths worldwide [1]. In most cases, gastric cancer is diagnosed at late stage, presenting with malignant proliferation, extensive invasion, and distant metastasis. Although significant progress has been achieved in therapy of early gastric cancer, outcomes for advanced stage gastric cancer are still rather poor [2]. Characteristic progressive tumorigenesis and distant metastasis may contribute to the overall poor prognosis for gastric cancer. Previously, TNM stage was considered as an indicator to predict prognosis of patients. Recent studies have revealed that this criteria alone is not sufficient for estimating clinical outcomes [3, 4]. Thus, it is very important to discover novel biomarkers for predicting prognosis in gastric cancer.

With fast development of the sequencing technique and completion of ENCODE (encyclopedia of DNA elements) project, it has been found that less than 2% of the mammalian genome are in protein-encoded regions, while others are in non-coding RNAs (ncRNAs) [5]. Among them are a new group of RNAs, known as long non-coding RNAs (lncRNAs). LncRNAs are more than 200 nt in length with no or limited protein-coding capacity. Mounting evidence has indicated that lncRNAs play important roles in regulation of multiple cellular processes, including stem cell pluripotency, cell growth, cell apoptosis, cell differentiation, and cell invasion [6-10]. Recent studies have demonstrated that lncRNAs are involved in the process of tumorigenesis and metastasis. For example, it was found that MALAT1 expression was increased in both hepatocellular carcinoma tissue samples and cell lines. Moreover, patients with high levels of MALAT1 had a relatively higher risk of tumor recurrence [11]. In another study, it was found that HOTAIR expression was higher in colorectal cancer tissues than in adjacent noncancerous tissues and higher HOTAIR expression was markedly correlated with liver metastasis in colorectal cancer patients [12]. In gastric cancer, Yang et al. found that lncRNA H19 was upregulated in tumor tissues and cells, compared with normal controls. Moreover, forced expression of H19 promoted cell growth [13]. More recently, Sun et al. reported that

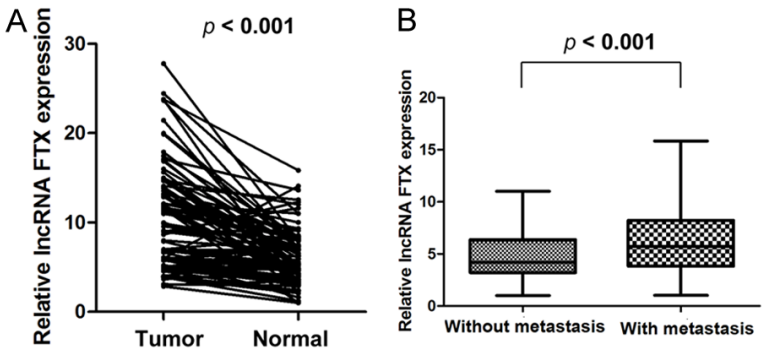

Figure 1. lncRNA FTX was significantly upregulated in gastric cancer tissues. A. Real-time PCR analysis showed increased expression of lncRNA FTX in tumor tissues than normal tissues ($P < 0.001$); B. Real-time PCR analysis showed higher expression of lncRNA FTX in tissues with distant metastasis than that without distant metastasis ($P < 0.001$).

Table 1. Correlation between clinicopathological parameters and lncRNA FTX expression in 93 gastric cancer patients

Characteristics	n	High expression	Low expression	*P* value
Age				0.420
< 60	37	21	16	
≥ 60	56	27	29	
Gender				0.989
Male	60	31	29	
Female	33	17	16	
Tumor size				< 0.001
< 4 cm	44	13	31	
≥ 4 cm	49	35	14	
Differentiation				< 0.001
Well	19	7	12	
Moderate	41	14	27	
Poor	33	27	6	
Lymph node invasion				0.189
Absent	33	14	19	
Present	60	34	26	
Distant metastasis				0.008
Absent	74	33	41	
Present	19	15	4	
TNM stage				0.015
I-II	34	12	22	
III-IV	59	36	23	

expression of GAS5 was significantly downregulated in gastric cancer and low expression of GAS5 was associated with adverse disease-free survival and overall survival of patients with gastric cancer. In addition, overexpression of GAS5 inhibited gastric cancer cell proliferation and induced apoptosis both *in vitro* and *in vivo* [14]. However, expression patterns and prognostic implications of lncRNA FTX in gastric cancer have not been reported. This study measured expression levels of lncRNA FTX in gastric cancer and adjacent normal tissues. Correlation of lncRNA FTX expression and clinicopathological characteristics and overall survival of gastric cancer patients was analyzed. Results indicate that lncRNA FTX is involved in progression of gastric cancer.

Materials and methods

Patients and tissue samples

Ninety-three paired gastric cancer tissues and adjacent normal tissues were obtained from patients that had undergone surgery at the Department of Gastroenterology, Affiliated Hospital of Jianghan University, between 2011 and 2013. All patients were pathologically diagnosed with gastric cancer and none of the patients received any treatment prior to surgery. Tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C until use. Stage of patients was evaluated according to the Seventh Edition of the AJCC Cancer Staging Manual. This study was approved by the Medical Research Ethics Committee of Renmin Hospital of Wuhan University. Informed consent was obtained from all participants.

RNA extraction and real-time PCR analysis

Total RNA was extracted from tissues samples using TRIZOL Reagent (Invitrogen, Carlsbad, CA), according to manufacturer protocol. For qRT-PCR, 1 μg RNA was reverse transcribed into cDNA using a Reverse Transcription Kit

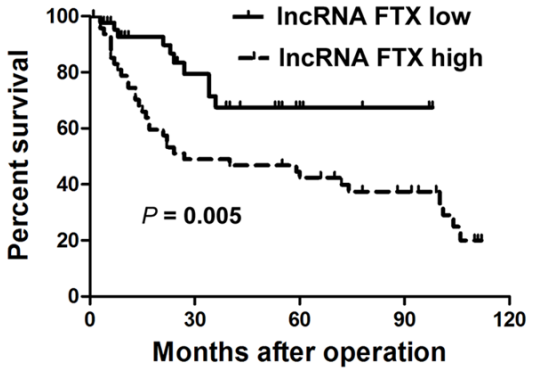

Figure 2. lncRNA-FTX expression associated with overall survival of gastric cancer patients, patients with high lncRNA FTX expression presented with worse overall survival ($P = 0.005$).

(Takara, Dalian, China). Real-time PCR amplifications were performed for 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds with 1.0 μL of cDNA using SYBR Premix ExTaq II kit (Takara, Dalian China) and an ABI7900 system (Applied Biosystems, Foster City, USA). Results were normalized to expression of GAPDH.

Primers (Invitrogen) were designed as follows: for human lncRNA FTX, the forward primer was 5'-CAAAGCTGGTCCTGTGCCTG-3' and reverse primer was 5'-ATTGAGTGTGGCATCACCTCC-3'; for human GAPDH, the forward primer was 5'-CCCACTCCTCCACCTTTGAC-3' and reverse primer was 5'-ATGAGGTCCACCACCCTGTT-3'. Relative quantification of RNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. Each sample was tested in triplicate.

Construction and transfection of expression vector for FTX

LncRNA FTX sequences were synthesized and subcloned into the pcDNA3.1 (Invitrogen, Shanghai, China) vector. pcDNA constructs or the empty vectors were transfected into AGS cells and cultured on six-well plates, according to manufacturer instructions. The empty vector was used as control. Expression level of lncRNA FTX was detected by qRT-PCR. Stably transfected clones were detected by G418 selection (Promega). A stable transfectant of the pcDNA3.1 empty vector was used as a control. For transfection, complexes of Lipofectamine 2000 (Invitrogen Corp, Carlsbad, USA) and one of the plasmids mentioned above was prepared, according to manufacturer instructions. Levels of lncRNA FTX expression after transfection were assayed by real-time PCR.

Colony formation assay

Gastric cancer cells were trypsinized to single-cell suspensions of 3×10^3 cells and plated in six-well plates in a complete culture medium containing 0.3% agar layered on top of 0.6% agar. Plates were incubated at 37°C in the presence of 5% CO_2 for 16 days. Colonies containing at least 50 cells were scored. Data are presented as mean ± standard deviation of five randomly scored fields.

Invasion assays

Cell invasion assays were performed using AGS cells. Cell culture was performed in Transwell chambers (Corning, NY, USA). Insert membranes were coated with diluted Matrigel (San Jose, CA, USA). Cells (1×10^5) were added to the upper chamber and were cultured for 48 hours. Finally, the insert membranes were cut and stained with crystal violet (0.04% in water; 100 mL) and migrated cells were counted under an inverted microscope and photographed.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 and GraphPad 5.0 software. Expression levels of lncRNA FTX in tumor tissues were compared with adjacent normal tissues by Wilcoxon test, while correlations between lncRNA FTX expression and clinicopathological characteristics were evaluated by Chi-square test. Overall survival analysis was conducted using the Kaplan-Meier method with log-rank test. The independent prognostic factor was analyzed by performing Cox multivariate proportional hazards model. Two-tailed $P < 0.05$ is considered statistically significant.

Results

Expression of lncRNA FTX significantly upregulated in gastric cancer

This study first determined lncRNA FTX expression levels in 93 paired human gastric cancer and adjacent normal tissues by real-time PCR.

Table 2. Univariate and Multivariate analysis of various potential prognostic factors in 93 gastric cancer patients

Characteristics	Univariate analysis		Multivariate analysis	
	HR[b] (95% CI[c])	*P*	HR[b] (95% CI[c])	*P*
Age	0.83 (0.70-1.29)	0.158	-	-
Gender	1.12 (0.81-1.73)	0.536	-	-
Differentiation	1.15 (0.72-1.49)	0.173	-	-
Tumor size	1.07 (0.81-1.42)	0.512	-	-
Tumor location	1.15 (0.83-1.42)	0.095	-	-
Lymph node invasion	1.12 (1.02-1.80)	0.112	-	-
Distant metastasis	1.58 (1.15-2.17)	0.008[a]	1.27 (1.09-1.97)	0.102
TNM stage	1.29 (1.07-2.01)	0.015[a]	1.16 (1.08-1.92)	0.127
lncRNA FTX level	1.91 (1.57-2.69)	0.005[a]	1.35 (1.19-2.37)	0.028[a]

[a]$P < 0.05$, [b]HR: hazard ratio, [c]CI: confidence interval.

As shown in **Figure 1**, after normalization to GAPDH expression levels, expression levels of lncRNA lncRNA FTX were markedly upregulated in tumor tissues, compared to normal tissues ($P < 0.001$). Moreover, expression of lncRNA FTX was more increased in patients with distant metastasis than those without metastasis ($P < 0.001$). These data indicate that abnormal lncRNA FTX expression may be involved in gastric cancer pathogenesis.

Relationship between lncRNA FTX expression and clinicopathological factors in patients with gastric cancer

Median level of lncRNA FTX expression was used as a cutoff value to divide all 93 patients into two groups. Gastric cancer patients that expressed lncRNA FTX at levels higher than the cutoff value were assigned to the high expression group (n = 48, lncRNA FTX expression level ≥ cutoff point) and those with expression lower than the cutoff value were assigned to the low expression group (n = 45, lncRNA FTX expression level < cutoff point). Next, this study analyzed association between expression of lncRNA FTX and clinicopathological parameters of gastric cancer patients. As shown in **Table 1**, lncRNA FTX expression was significantly associated with tumor size (< 4 cm *vs.* T ≥ 4 cm; $P < 0.001$), differentiation ($P < 0.001$), distant metastasis ($P = 0.008$), and TNM stage ($P = 0.015$). However, there was no correlation between lncRNA FTX expression levels and age (< 60 *vs.* ≥ 60, $P = 0.420$), gender (female *vs.* male, $P = 0.989$), and lymph node invasion ($P = 0.189$).

lncRNA FTX overexpression associates with poor prognosis in patients with gastric cancer

Kaplan-Meier analysis with the log-rank test was performed to determine expression of lncRNA FTX on survival of gastric cancer patients. As shown in **Figure 2**, patients with high level expression of lncRNA FTX tended to have worse overall survival than those with low level lncRNA FTX expression (log-rank test, $P < 0.001$). Moreover, to determine whether expression of lncRNA FTX was an independent prognostic factor for gastric cancer patients, univariate and multivariate analyses were carried out. Univariate analysis demonstrated that distant metastasis ($P = 0.013$), TNM stage ($P = 0.023$), and lncRNA FTX expression levels ($P = 0.005$) were significantly associated with overall survival of gastric cancer patients (**Table 2**). However, multivariate analysis, using the Cox proportional hazards model for all variables that were significant in univariate analysis, showed that only lncRNA FTX expression level was an independent prognostic factor for patients with gastric cancer ($P = 0.028$, **Table 2**).

lncRNA FTX promotes cell proliferation and invasion in gastric cancer cells

As lncRNA FTX was significantly upregulated in gastric cancer tissues, it was suspected that lncRNA FTX might be involved in regulating cellular biology. This study first detected expression levels of lncRNA FTX in six gastric cancer cell lines (MKN-45, MKN-48, AGS, BGC823, BGC803, SGC7901) and a normal gastric epithelial cell GES-1. Results showed that all gastric cancer cell lines displayed higher expression of lncRNA FTX, compared to normal cell GES-1 (**Figure 3A**). To analyze the effects of lncRNA FTX on gastric cancer cells, AGS cells were transfected with lncRNA FTX expressing vector to overexpress lncRNA FTX (**Figure 3B**). Ectopic expression of lncRNA FTX increased colony formation number of AGS cells, as demonstrated by colony formation assays (**Figure**

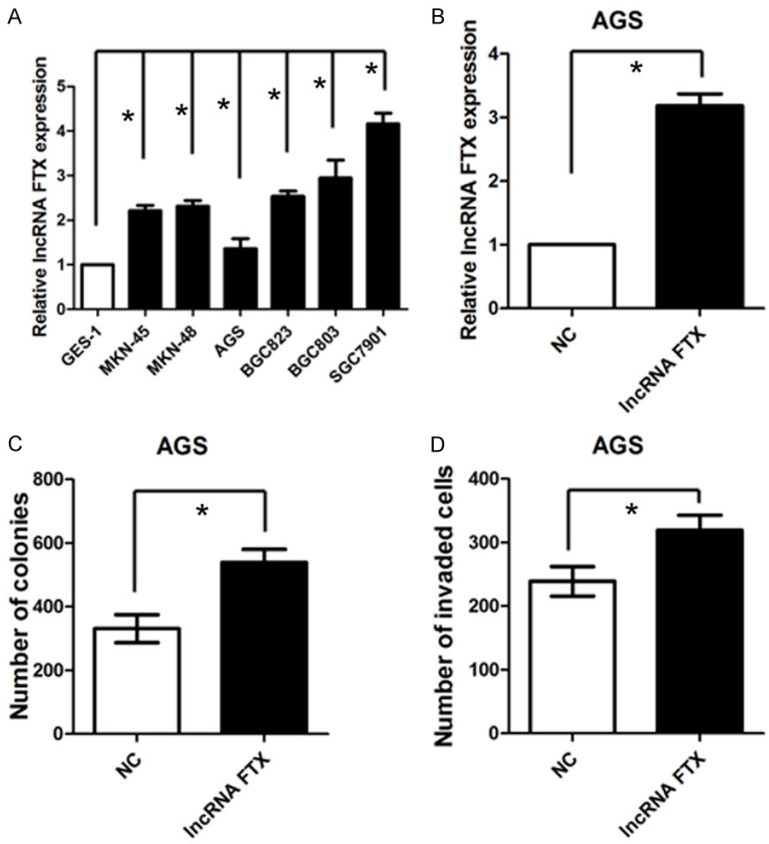

Figure 3. lncRNA FTX promotes cell proliferation and invasion in gastric cancer cells. A. Expression level of lncRNA FTX in gastric cancer cell lines (*P < 0.05); B. Real-time PCR confirmed upregulation of lncRNA FTX in AGC cells (*P < 0.05); C. Ectopic of lncRNA FTX promoted colony formation in AGS cells (*P < 0.05); D. Ectopic expression of lncRNA FTX promoted invasion of AGS cells (*P < 0.05).

3C). Next, the effects of lncRNA FTX expression on invasion of gastric cancer cells were explored. Transwell assay indicated that invasion ability markedly increased in AGS cells after ectopic expression of lncRNA FTX (**Figure 3D**). Data suggests that overexpression of lncRNA FTX could promote proliferation and invasion of gastric cancer cells.

Discussion

Increasing studies have demonstrated that dysregulated lncRNA expression may be a major contributor to tumorigenesis and that aberrant expression of specific lncRNAs could mark the spectrum of disease progression. These lnRNAs may serve as independent biomarkers for diagnosis and prognosis [15, 16]. More recently, lncRNAs have been found to be involved in gastric cancer progression [17, 18]. For example, Xu et al. reported that lncRNA FENDRR expression was associated with deeper tumor invasion, higher tumor stage, and lymphatic metastasis in gastric cancer. Moreover, upregulation of FENDRR inhibited tumor cell migration and invasion by downregulating FN1 and MMP2/MMP9 expression [19]. Hu et al. found that lncRNA GAPLINC (gastric adenocarcinoma predictive long intergenic noncoding RNA) was significantly upregulated in gastric cancer samples, compared to paired normal adjacent normal samples. Furthermore, lncRNA GAPLINC overexpression could promote gastric cancer cell proliferation and invasion [20]. lncRNA FTX is located at the upstream of XIST, which is within the X-inactivation center (XIC). It produces a spliced long non-coding RNA that can positively regulate expression of XIST [21]. Recent studies have indicated that lncRNA FTX plays important roles in the progression of colorectal cancer [22]. However, the status of lncRNA FTX expression in gastric cancer and its prognostic significance remain unclear. Thus, the aim of this study was to investigate correlation of lncRNA FTX expression with clinicopathologic features and prognosis of gastric cancer patients.

This study explored the clinicopathological role of lncRNA FTX in gastric cancer patients. Results demonstrated that lncRNA FTX expression was significantly upregulated in gastric cancer tissues, compared with that of adjacent normal tissues. Moreover, increased lncRNA FTX expression was associated with tumor size, differentiation, distant metastasis, and TNM stage, suggesting that upregulation of lncRNA FTX plays a critical role in gastric cancer pathogenesis. This is in accordance with reports concerning other tumor entities [22, 23]. In addition, results showed that patients with high lncRNA FTX expression had worse overall survival than those with low lncRNA FTX expres-

sion. More importantly, both univariate and multivariate survival analyses indicated that high lncRNA FTX expression was correlated with poor overall survival in gastric cancer patients, indicating that lncRNA FTX is an independent prognostic marker for patients with gastric cancer. Finally, this study determined the biological role of lncRNA FTX in gastric cancer. First, representative gastric cell lines were chosen and lncRNA FTX expression was measured in these cell lines. It was found that the SGC7901 presented with high levels of lncRNA FTX, whereas AGS cells presented with low expression of lncRNA FTX. Upregulation of lncRNA FTX promotes colony formation and invasion in AGS cells. Present data proves that lncRNA FTX might be an important modulator involved in gastric cancer progression.

In conclusion, the present results offer the first evidence that lncRNA FTX plays critical roles in progression of gastric cancer and overexpression of lncRNA FTX could independently predict poor overall survival of patients. Results imply that lncRNA FTX might be a potential marker for further risk stratification. However, further studies are necessary to explore the molecular mechanisms of lncRNA FTX in gastric cancer.

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Disclosure of conflict of interest

None.

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