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Original Article

Decreased nuclear expression of FTO in human primary hepatocellular carcinoma is associated with poor prognosis

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Abstract: Fat mass and obesity-associated protein (FTO) has been well known for a pivotal role in regulation of fat mass, adipogenesis and body weight. In recent years, increasing studies revealed a strong association between FTO and various types of cancer. Its role in human hepatocellular carcinoma, however, remains unclear. We aimed at investigating the expression pattern and clinical significance of FTO in hepatocellular carcinoma. We found that FTO mRNA levels were significantly lower in hepatocellular carcinoma tissues. Immunohistochemical analysis showed the expression of FTO was reduced in the nuclei in hepatocellular carcinoma, and was associated with AFP level ($P < 0.001$), tumor size ($P < 0.001$), metastasis ($P = 0.025$) and vascular invasion ($P < 0.001$). Patients with decreased FTO expression had a shorter overall and tumor-free survival time ($P = 0.004$ and $P = 0.006$) than those with normal FTO expression. Cox's proportional hazard regression model revealed that reduced expression of FTO was a risk factor associated with the prognosis of HCC patients ($P = 0.022$). These results indicated that decreased FTO expression is correlated with clinicopathological factors, implying that FTO could be a vital predictor of poor outcome in HCC patients and serves as a novel biomarker for HCC.

Keywords: FTO, hepatocellular carcinoma, expression pattern, clinicopathological significance

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and ranked third in the cancer-related mortality worldwide [1, 2]. Among the numerous therapy methods available, tumor resection is the best choice. However, it is regretful that most HCC patients are already in the late stage of tumor development when diagnosed, so the surgical cure rates remains disappointing. Furthermore, even though these patients receive other treatments, like chemotherapy, radiotherapy and targeted therapy, the prognosis of patients with HCC still is poor because of the high rate of intrahepatic recurrence and metastasis [3]. Thus, there is an urgent need to focus on discovering an effective predictive and diagnostic target to improve the prognosis of HCC.

The fat mass and obesity-associated gene (FTO), as a genome-wide association studies-identified obesity susceptibility gene, shows a strong relationship with risk of obesity due to the multiple single-nucleotide polymorphisms (SNPs) in intron 1 [4-6]. A set of previous studies have demonstrated the critical role of FTO in the regulation of fat mass, adipogenesis, and body weight [7, 8]. In addition, FTO SNPs have also been well-known for the strong association with the increased risk of various types of cancer, including leukemia, glioblastoma, breast cancer, prostate cancer, kidney cancer, pancreatic cancer, and endometrial cancer [9-12], implicating the pathogenetic role of FTO in cancer development. For example, in leukemia and glioblastoma, FTO reveals an increased expression pattern and regulates the tumorigenesis of cancer cells [13, 14]. Furthermore, FTO has

even been reported to be associated with clinicopathological factors and the prognosis of tumors [15, 16].

N^6-methyladenosine (m^6A) epitranscriptional modification has recently gained much attention because of its regulatory functions in cancer development, such as proliferation, migration, and invasion [17]. In recent years, FTO was defined as the first N^6-methyladenosine (m^6A) demethylase of eukaryotic messenger RNA (mRNA) [18], and the function of FTO in adipogenesis and tumorigenesis is partly linked to the m^6A demethylase activities [13, 14, 18]. The m^6A modification is the most abundant internal modification in eukaryotic mRNA. As an m^6A "eraser", FTO can remove the m^6A modification and modulate the stability of mRNA, which finally leads to alteration of the pathogenesis in various types of cancer [13, 14].

Although there are increasing reports of FTO today, the expression pattern and clinical significance of FTO remain elusive in hepatocellular carcinoma. We hypothesized that FTO expression could play an important role in the development and progression of HCC. To address this question, we evaluated the expression patterns of FTO in HCC tissues, and determined its association with clinicopathological factors and the prognosis of patients with HCC.

Materials and methods

Patients and sample

All HCC and corresponding peritumor tissues samples, and follow-up information were provided by the Chronic Liver Disease Biological Sample Bank, Department of Hepatobiliary Surgery, Zhongshan Hospital, Xiamen University. Before undergoing hepatectomy, these patients never received any preoperative treatment. All the procedures for sample collection were approved by the ethics committee of the Zhongshan Hospital of Xiamen University, and written informed consent was obtained from all patients. In order to determine whether FTO is the major factor influencing progression of hepatocellular carcinoma but not related obesity, we excluded patients who suffer from fatty liver disease, and the patients whose BMI was more than 28. We used 68 pairs of paraffin-embedded HCC and matched adjacent normal tissues and a further 61 pairs of matched tissue mRNA from HCC patients.

Cell culture

HepG2, BEL-7402, SMMC-7721, PLC/PRF/5, SK-Hep-1 and LO2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences, and MHCC-97h and HCC-LM3 cells were obtained from Zhongshan Hospital of Fudan University. The cells were cultured in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% CO_2.

Real-time PCR analysis

Total RNA from different cell lines, HCC tissues, and adjacent normal liver tissues was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using the GoScriptTM Reverse Transcription System Kit (Promega, Madison, WI). Quantitative real-time PCR (qRT-PCR) was performed in the Lightcycle 96 Real-Time PCR System (Roche) using FastStart Universal SYBR Green Master (Rox) (Roche). The following primers were used for amplification of FTO: sense primer, 5'-GCTGCTTATTTCGGGACCTG-3' and antisense primer, 5'-AGCCTGGATTACCAATGAGGA-3'. GAPDH was amplified as an internal control using sense primer, 5'-CGACCACTTTGTCAAGCTCA-3' and antisense primer, 5'-GGAGAGTCAACGGGCATATAG-3'. Comparative quantitation was determined using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

HCC and adjacent normal tissues were fixed with 10% formalin, embedded in paraffin, and then 3-μm-thick sections were made. These sections were deparaffinized, hydrated, and soaked in 3% H_2O_2 at room temperature for 1 hour. After blocking nonspecific binding proteins, the slides were incubated with a FTO polyclonal antibody at 4°C in a moist chamber overnight. The slides were sequentially incubated with a biotinylated secondary antibody and then streptavidin-peroxidase conjugate, each for 30 min at room temperature. Finally, 3,5-diaminobenzidine (DAB) was used for color development followed by hematoxylin counterstaining.

Immunostaining evaluation

Two clinical pathologists who were unaware of the clinical data evaluated the immunostained sections. About 90% of the evaluating results

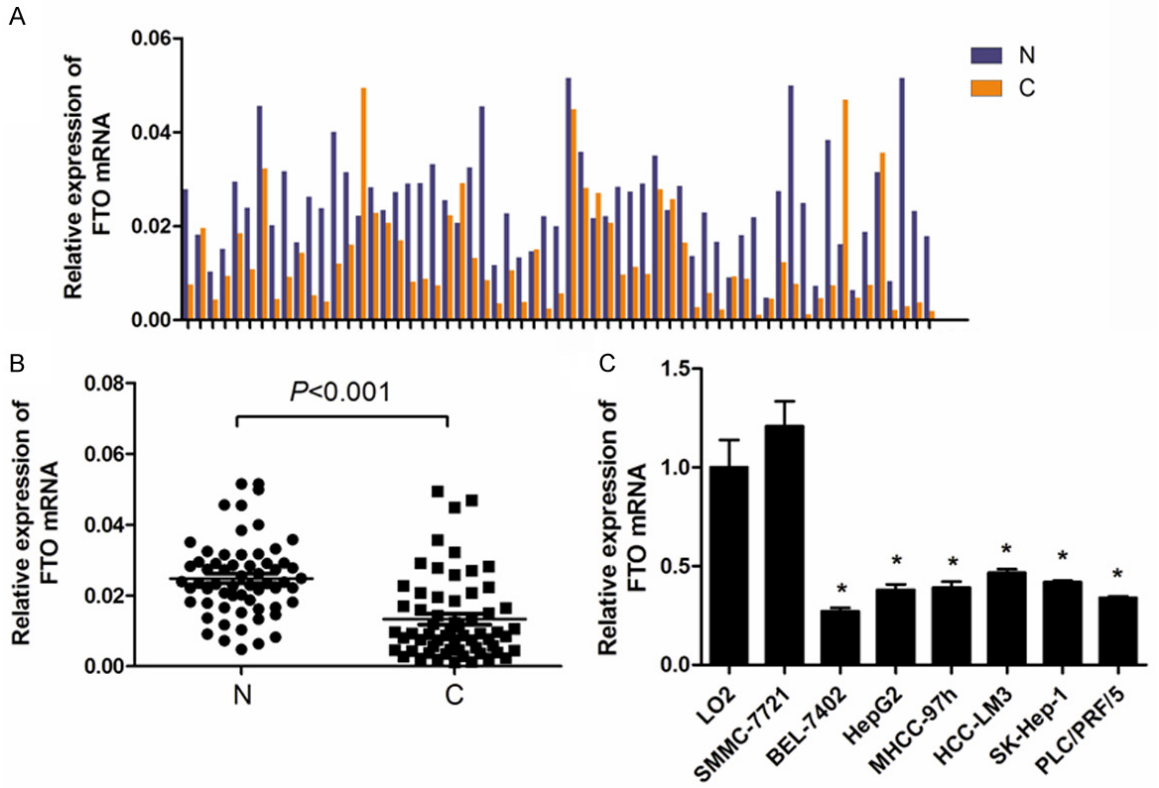

Figure 1. The expression of FTO was reduced both in hepatocellular carcinoma (HCC) tissues and HCC cell lines. A. Comparison of FTO mRNA Expression between 61 paired hepatocellular carcinoma tissues and adjacent normal tissue. The FTO mRNA level in adjacent normal liver tissue was higher than in hepatocellular carcinoma tissue (51 of 61 = 83.61%). B. Data were analyzed with two related-samples Wilcoxon's non-parametric test. C. FTO mRNA expression in BEL-7402, HepG2, MHHC-97h, HCC-LM3, SK-Hep-1, and PLC/PRF/5 were significantly lower than in LO2 cells, a normal human liver cell line ($P < 0.05$). N: adjacent normal tissue; C: cancer tissue.

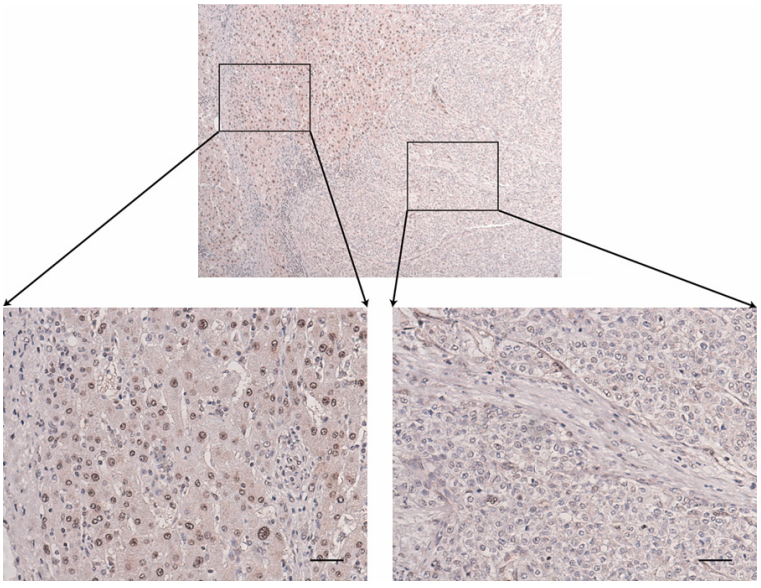

Figure 2. Decreased nuclear expression of FTO protein in hepatocellular carcinoma (HCC). Immunohistochemical staining showed that FTO was mainly located in the nuclei and was strongly expressed in adjacent non-tumor tissue, whereas hepatocellular carcinoma tissues showed a weak or undetectable expression of FTO in the nuclei.

were consistent between two pathologists. As for the inconsistent part, these sections were revaluated to reach an agreement. If a consensus could not be reached after the revaluation, a third pathologist was consulted to make the final decision. As previously described [19], five random 200 × microscopic fields were examined per slide, and 100 cells were evaluated per filed. The expression of FTO was classified into five groups according to the proportion of nuclear positive-staining cells: 0 = negative; 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; 4 = ≥ 76%. The nuclear staining intensity was defined as follow: 0 = negative; 1 = weak; 2 = moderate; 3 = strong. Finally,

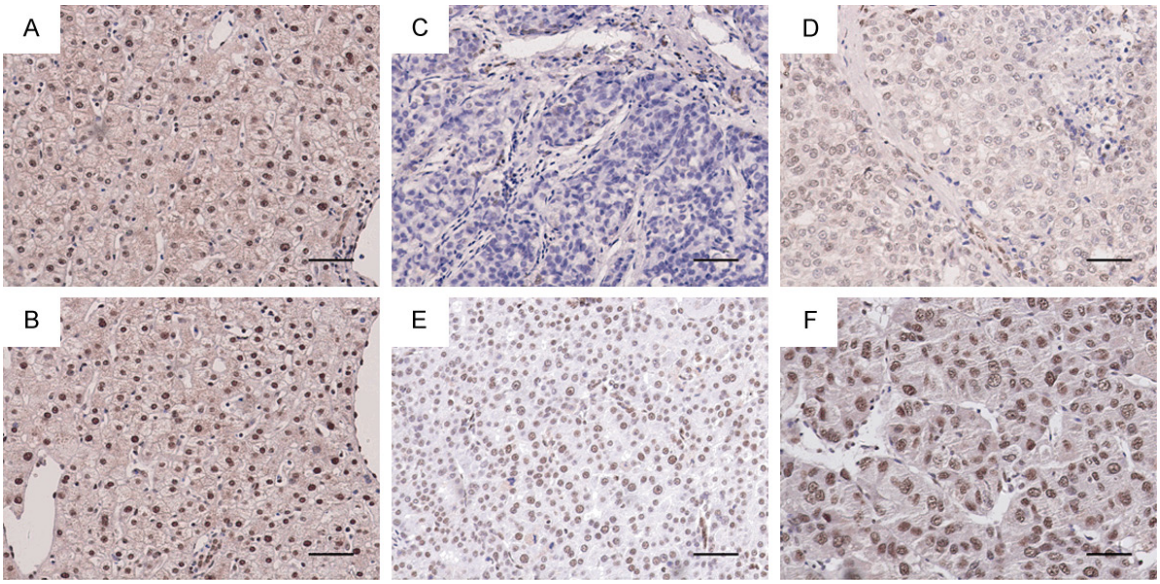

Figure 3. FTO expression analysis in normal liver and hepatocellular carcinoma (HCC). (A, B) FTO was heavily expressed in the nuclei of normal liver (regarded as normal expression); FTO nuclear staining intensity in HCC tissues was classified as: (C), negative (D), pale yellow (E), medium yellow (F), tawny.

the percentage and intensity scores were then multiplied to obtain a total score for each sample. We also performed 15 cases of normal liver tissue sections; the evaluation was carried out in the same manner as the cancer samples. These scores were all not < 8, so we regarded a score of 8-12 as normal expression and 0-7 as reduced expression in the present study respectively.

Statistical analysis

Data were analyzed by using SPSS version 17.0 for Windows (IBM Corporation, New York, USA). The two related-samples Wilcoxon's non-parametric test was performed to compare the levels of FTO expression between HCC specimens and adjacent no-tumor liver tissues in each patient. The chi-squared test and Fisher's exact tests were used to examine possible correlations between FTO expression and clinicopathological factors. Survival rates were calculated using the Kaplan-Meier method, and measured from the day of surgical resection until death from any cause. Differences in survival curves were analyzed by the log-rank test. Multivariate analysis was used to evaluate the risk factors associated with postoperative survival. Differences were considered significant when the *P*-value was < 0.05.

Results

Reduced expression of FTO in HCC tissues

Total RNA was extracted from 61 pairs of hepatocellular carcinoma tissues and adjacent non-tumor liver tissues. We used real-time PCR to detect the mRNA expression of FTO. As shown in **Figure 1A** and **1B**, the expression of FTO mRNA was obviously decreased in hepatocellular carcinoma tissues in comparison to adjacent non-tumor liver tissues (50 of 61 = 81.97%, $P < 0.001$). To further detect the expression of FTO in HCC cell lines, we chose seven HCC cell lines (SMMC-7721, BEL-7402, HepG2, MHHC-97h, HCC-LM3, SK-Hep-1, and PLC/PRF/5) and LO2, a normal liver cell line. The results demonstrated that the expression of FTO in LO2 was obviously higher that in the HCC cell lines except for SMMC-7721 (**Figure 1C**). These results imply the expression of FTO mRNA is reduced in hepatocellular carcinoma (HCC).

Decreased nuclear localization of FTO in HCC tissues

In order to determine the localization and the expression pattern of FTO in hepatocellular carcinoma, immunohistochemical staining was used to analyze the in-situ localization of FTO

Table 1. FTO abnormal expression correlates with clinical-patholological factors of HCC patients

Clinicopathological factors	Reduced expression (%)	n	χ^2	*P*
Age (year)				
<55	14 (45.2)	31	1.965	0.161
≥55	23 (62.2)	37		
Gender				
Male	30 (53.6)	56	0.09	0.764
Female	7 (58.3)	12		
AFP (ug/L)				
<400	14 (35.9)	39	12.64	<0.001*
≥400	23 (79.3)	29		
HBV DNA copies (cps/mL)				
<1000	10 (50.0)	20	0.222	0.895
≥1000	27 (56.3)	48		
Liver cirrhosis status				
Yes	23 (52.3)	44	0.23	0.632
No	14 (58.3)	24		
Tumor size (cm)				
<5	8 (28.6)	28	12.81	<0.001*
≥5	29 (72.5)	40		
Differentiation				
Well to moderate	31 (51.7)	60	1.549	0.213
Poor	6 (75.0)	8		
Metastasis				
Yes	22 (68.8)	32	5.01	0.025
No	15 (41.7)	36		
Vascular invasion				
Yes	27 (73.0)	37	11.27	<0.001*
No	10 (32.3)	31		

Abbreviations: AFP, alpha-fetoprotein; HBV, hepatitis B virus. *representative statistically significant (*P* < 0.05).

protein in 68 paired hepatic tumor specimens and adjacent non-tumor liver tissues. In adjacent normal liver tissues, FTO was highly accumulated in the nuclei, whereas 66.18% of the liver tumors showed undetectable or weak expression of FTO protein (**Figure 2**).

Association of nuclear FTO protein expression with clinicopathological factors of HCC patients

As shown in **Figure 3A** and **3B**, the expression of FTO in the nuclei in normal liver tissues was defined as normal. We also classified the FTO nuclear staining intensity in HCC tissues as undetectable (**Figure 3C**), pale yellow (**Figure 3D**), medium yellow (**Figure 3E**) and tawny (**Figure 3F**).

We next investigated the correlation between nuclear FTO protein expression and clinicopathological factors. Reduced FTO expression was detected at a higher rate in patients with AFP level ≥ 400 ug/L (23 of 29, 79.3%) than in patients with AFP level < 400 ug/L (14 of 39, 35.9%) (*P* < 0.001). Moreover, decreased expression of FTO was associated with HCC tumor size (*P* < 0.001). In addition, reduced FTO expression was more frequent in patients with metastasis (22 of 32, 68.8%) than those without metastasis (15 of 36, 41.7) (*P* = 0.025). Furthermore, tumors with vascular invasion had a higher rate of decreased FTO expression (*P* < 0.001). However, no significant correlation between reduced FTO expression and patient age, gender, hepatitis B virus (HBV) level, liver cirrhosis and tumor differentiation was observed (*P* > 0.05, **Table 1**).

Correlation of decreased FTO expression with the prognosis in HCC

The correlation of nuclear FTO protein expression with clinical outcome in HCC patients was analyzed by using the Kaplan-Meier method. Patients with reduced FTO expression had a shorter overall survival time than those with normal FTO expression (**Figure 4A**, *P* = 0.004). Patients with reduced FTO expression also had a shorter tumor-free survival time than those with normal FTO expression (**Figure 4B**, *P* = 0.006). We then use the Cox proportional hazard regression model to determine whether the decreased nuclear FTO expression was associated with patients' prognosis. The results revealed that reduced expression of FTO was a risk factor associated with the prognosis of patients with HCC [Exp(B) = 3.111, with 95% confidence interval = 1.177-8.224; *P* = 0.022, **Table 2**]. In addition, patients' prognosis was correlated with metastatic status (*P* = 0.005), whereas no significant association with age, gender, liver cirrhosis status, tumor size, and differentiation were observed (*P* > 0.05).

Discussion

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death world-

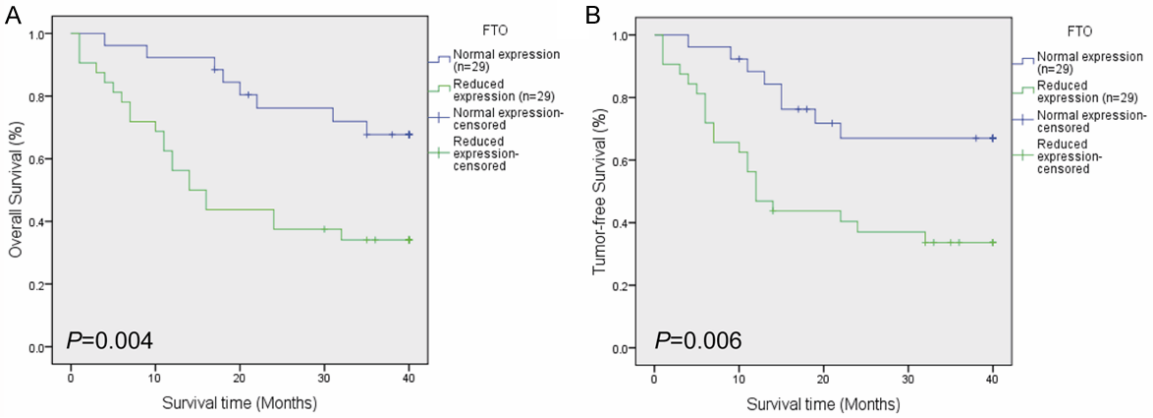

Figure 4. FTO expression is correlated with the survival of HCC patients. Kaplan-Meier analysis of 58 HCC patients according to FTO expression. Patients with reduced expression of FTO had a shorter overall survival time (A. P = 0.004) and tumor-free survival time (B. P = 0.006) than patients with normal expression of FTO.

Table 2. Cox regression model for prediction of 58 patients with hepatocellular carcinoma (HCC)

Factor	SE	*P*	Exp(B)	95% CI	
				Lower	Upper
Age (years)	0.413	0.879	1.065	0.474	2.395
Gender	0.486	0.472	0.705	0.272	1.826
Liver cirrhosis status	0.442	0.115	0.498	0.209	1.185
Tumor size (cm)	0.503	0.349	1.601	0.597	4.291
Differentiation	0.639	0.920	1.066	0.304	3.732
Metastasis	0.435	0.005*	0.293	0.125	0.688
FTO reduced expression	0.496	0.022*	3.111	1.177	8.224

SE, Standard Error; CI, confidence internal; FTO, Fat mass and obesity-associated protein. *representative statistically significant (P < 0.05).

wide, especially in China. Although we have made progress in the field of molecular biology of HCC, the survival of HCC patients is rarely improved. Most patients with HCC, no matter whether they receive surgical resection or not, will suffer from intra- or extra-hepatic metastasis, which leads to a poor prognosis because no effective treatments are available currently [3]. Therefore, further investigations of HCC-relative biomarkers would facilitate our understanding of the tumorgenesis and progression of HCC, and enable precise diagnosis, improving the survival of HCC patients as a whole.

Abnormal expression pattern of FTO has been reported in various types of cancer, such as leukemia, brain tumor, breast cancer and gastric cancer, where it is overexpressed [20]. In our study, to our surprise, we found a downregulated mRNA level of FTO in HCC by performing real-time PCR, which is opposite to the previous investigations reported. Organ specificity should be taken into the consideration in the evaluation of FTO expression in tumor tissues. Li et al. first demonstrated an oncogenic role of FTO in cancer through in vivo animal model studies. They showed that overexpression of FTO significantly promoted human acute myeloid leukemia (AML) cell survival and proliferation, and inhibited human AML cell differentiation and apoptosis [13]. According to immunohistochemical staining, we found that FTO was highly localized to the nuclei in non-tumor liver tissues, whereas 66.18% of HCC specimens showed weak or undetectable nuclear accumulation of FTO protein in hepatocellular carcinoma.

Our current study used a series of 68 archived clinical hepatic tumor specimens to evaluate the association between the reduced expression of FTO and clinicopathologic features of HCC patients. We showed for the first time that decreased nuclear expression of FTO protein in HCC specimens was associated with patients' AFP level, tumor size, metastasis, and vascular invasion. In contrast, there was no significant correlation between reduced FTO expression with age, gender, HBV DNA copies, liver cirrhosis status, or differentiation. Furthermore, in our survival analysis, patients with decreased expression of FTO had a shorter overall survival time and tumor-free survival time, predicting an underlying independent prognostic role of FTO in HCC. However, these results were ob-

tained in a local cohort, and further confirmation in other populations of HCC patients is needed.

Our investigation has indicated that the expression of FTO is correlated with poor prognosis in HCC. However, to the best of our knowledge, the biologic function of FTO in hepatocellular carcinoma remains unclear. Currently, studies are focused on the potential links between m^{6}A and hepatocellular carcinoma. Ma et al. reported that METTL14, a m^{6}A methyltransferase, suppresses liver cancer metastasis through mo-dulating the primary microRNA 126 process in an m^{6}A-dependent manner [21]. In addition, Chen et al. showed that METTL3 represses SOCS2 expression by an m^{6}A-YTHDF2-dependent mechanism, promoting liver cancer progression as a whole [22]. As an m^{6}A "eraser", FTO has been reported to promote demethylation activity. The level of m^{6}A in total mRNA increases after FTO knockdown, whereas m^{6}A in total mRNA notably decreases after overexpression of FTO [14]. A number of reports also showed a relationship between RNA demethylation activity of FTO and tumors, demonstrating the oncogenic role of FTO in tumorigenesis and development of various types of cancer through an m^{6}A-dependent mechanism [23-25]. For instance, Li et al. reported that FTO negatively regulates a set of tumor suppressor genes, such as ASB2 and RARA by post-transcriptionally modulating the quantities of m^{6}A in target mRNA, contributing to cell proliferation and drug response [13]. However, we speculated that FTO plays an anti-tumor role in HCC based on the decreased nuclear expression and the association we found between reduced expression of FTO and clinicopathologic factors. Thus, our next stage of work will emphasize biologic functions of FTO in hepatocellular carcinoma and whether it affects tumorigenesis through an m^{6}A-dependent mechanism.

In summary, downregulation of FTO was found in HCC samples in comparison to adjacent normal liver tissues. In addition, our present work revealed that the nuclear reduced FTO expression had crucial clinicopathologic significance and related to survival time of HCC patients. Our results strengthen the notion that decreased nuclear expression of FTO is associated with poor prognosis in human HCC, suggesting that FTO could be a new biomarker and a potential therapeutic target for the diagnosis and treatment of HCC.

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Disclosure of conflict of interest

None.

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