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High FITM2 expression promotes cell migration ability of hepatocellular carcinoma by regulating the formation of caveolae and indicates poor patient survival

Yan Chen[1]*, Li-juan Ji[1]*, Yan Wang[1] and Xiang-Feng Guo[2]

[1]Department of Gastroenterology, Weifang Yidu Central Hospital, Weifang and

[2]Department of Hepatobiliary Surgery, Shanxian Central Hospital, Heze, Shandong, China

*These authors contributed equally

Summary. Background. Hepatocellular carcinoma (HCC) is among the most malignant tumors with high recurrence and low 5-year survival rate. Lipid metabolism is essential in tumor metastasis, although how altered lipid metabolism promotes HCC progression has not been well elucidated. Fat Storage Inducing Transmembrane Protein 2 (FITM2) is a gene involved in lipid homeostasis and cytoskeletal organization; however, its role in regulating tumor biological behavior has not been evaluated.

Methods. In this study, immunohistochemistry was performed to evaluate the expression of FITM2 in HCC. Univariate and multivariate analysis was performed to identify the prognostic factors. RNA interference wound healing and transwell experiments were performed to analyze the biological role of FITM2. Western blot analysis was performed to investigate the potential downstream signaling.

Results. The results revealed that FITM2 was highly expressed in the intratumoral tissues of HCC. Expression of intratumoral FITM2 was associated with microvascular invasion. FITM2 is an independent risk factor of HCC disease-free survival and overall survival. *In vitro* studies revealed that knockdown of FITM2 significantly inhibited the migration ability of HCC cells. FITM2 promotes HCC cell migration by regulating the expression of caveolin-1 and promoting the formation of caveolae. These results indicate that high intratumoral expression of FITM2 is associated with poor HCC prognosis, which may be applied to develop a new adjuvant therapy.

Corresponding Author: Dr. Xiang-Feng Guo, Department of Hepatobiliary Surgery, Shanxian Central Hospital,1 Wenhua Road, Shanxian, Heze, 274300, Shandong, China. e-mail: xiangfengguo01s@163.com

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Introduction

Liver cancer is among the most aggressive and fatal malignant tumors worldwide (Bray et al., 2018; Villanueva, 2019). Although many patients have been effectively diagnosed and treated in the early stage of the disease, the recurrence rate of the disease is still high (Brown et al., 2019; Zhu et al., 2020). Most patients are diagnosed with advanced stages of the disease, accompanied by local or distal metastasis. Several tyrosine kinase inhibitors and immune checkpoint inhibitors (ICI) have been developed and applied to treat advanced hepatocellular carcinoma (HCC) (Abou-Alfa et al., 2006; Faivre et al., 2020; Kudo et al., 2018; Liu et al., 2019). However, tyrosine kinase inhibitors may promote tumor metastasis and have severe adverse reactions (Rimassa et al., 2019; Zhang et al., 2012). Also, there are no postoperative adjuvant therapies to reduce tumor recurrence after curative resection of HCC to date (Zhu et al., 2020). Therefore, identifying more and targetable candidates for the treatment of HCC and reducing tumor relapse is urgently needed.

In recent years, studies have focused on the relationship between lipid metabolism and tumor metastasis (Iwamoto et al., 2018; Luo et al., 2017; Pascual et al., 2017). The results revealed that aberrant lipid metabolism reprogramming promoted tumor metastasis in ovarian cancer and colorectal cancer (Aguirre-Portoles et al., 2017; Sevinsky et al., 2018). Fatty acids can participate in the structural synthesis of phospholipids on cancer cell membranes and induce important signal transduction (such as PI3K-Akt mTOR). Meanwhile, ATP and the nicotinamide adenine dinucleotide phosphate (NADPH) used by cancer cells were mainly produced by fatty acid β-oxidation (Iershov

et al., 2019). Therefore, the synthesis and decomposition of lipids may play an essential role in tumor metastasis. However, the detailed mechanism still needs further elucidation.

FITM2 plays a vital role in forming lipid droplets (LDs) which are storage organelles at the center of lipid and energy homeostasis (Gross et al., 2011; Yang et al., 2012). FITM2 can facilitate nascent LD protrusion and bud from the endoplasmic reticulum toward the cytoplasm. FITM2 also plays a role in the regulation of cell morphology and cytoskeletal organization (Bai et al., 2011). Studies have also revealed that FITM2 was involved in the pathogenesis of colon adenocarcinoma (Yang et al., 2019). And recent data have shown that FITM2 was essential in maintaining cell fitness to interferon-γ (IFNγ) exposure and promoting cancer-intrinsic evasion of killing by T cells (Lawson et al., 2020). However, the role of FITM2 in the development and progression of HCC has not been studied.

In this study, we have explored the expression pattern of FITM2 in tumor tissues and peritumor normal tissues of hepatocellular carcinoma. The prognostic value of FITM2 in HCC patients was also evaluated. Further, we assessed the potential biological role of FITM2 in HCC cell lines and studied the downstream signaling mechanism. Our results provided evidence of lipid metabolism in the progression of HCC and may be applied to develop a new adjuvant therapy.

Materials and methods

Patients

The Ethics Committee of Weifang Yidu Central Hospital approved the study. The patient was enrolled in our hospital and received curative tumor resection from January 2014 to January 2015. Tumors were obtained 0.5 cm from the margin of the tumor and adjacent normal tissues, which was believed to be the most representative of tumor biological characteristics. Tumor samples were obtained immediately after complete removal and formalin-fixed paraffin-embedded. Two senior pathologists confirmed pathological diagnosis. The follow-up duration varied from 3 months to 58 months.

IHC

Immunohistochemistry was performed according to the manufacturer's manual (SP-9001, Zsbio). The tissue microarray section was first roasted at 70°C for 1 h and then immersed in xylene for 20 min. After that, the section was immersed in methanol in different gradients, including 100%, 95%, 85%, and 75%. The section was then washed three times with PBS at intervals of 10 mins. The section then underwent microwave heat antigen retrieval at pH 6.0 citrate buffer and was allowed to stand at room temperature for at least 2 hours to cool to room temperature. The section was then washed three times with PBS at intervals of 10 mins. The section was then immersed in endogenous peroxidase blocking solution for 10 mins and then washed with PBS. After that, the section was blocked with normal goat serum at room temperature for 15 mins and then incubated with the primary antibody at a dilution of 1:250 at 4°C overnight. The section was then washed with PBS three times and sequentially incubated with biotin-labeled secondary antibody and horseradish enzyme-labeled streptomycin. Finally, the section was incubated with DAB at room temperature for 5 mins for color development. The FITM2 antibody used for immunohistochemistry was obtained from Biorbyt (St Louis, USA, Catalog Number: orb183696). The expression of FITM2 was evaluated according to an H score method with the following calculating formula: (% cells of 1 + intensity score × 1) + (% cells of 2 + intensity score × 2) + (% cells of 3 + intensity score × 3).

Cell culture and RNA interference

Human HCC cell lines including Huh7 and PLC/PRF/5 were kindly obtained from Liver Institute of Fudan University and cultured in DMEM medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin combination. RNA interference was performed according to the manufacture's manual (Thermo Fisher Scientific, lot: L3000-015). The siRNA fragments were synthesized by Genepharma. In brief, cells were cultured to 60% confluence, and the culture medium was replaced with serum-free DMEM medium 3h before transfection. 5 μl of siRNA fragments (20 μM) and 5 μl of Lipo3000 transfectamine reagent were diluted in the RNAase-free Opti-MEM medium respectively and incubated at room temperature for 5 minutes. After that, the siRNA medium was added to the Lipo3000 transfectamine reagent medium drop by drop and incubated at room temperature for 10 minutes. Then, the medium was added to the cells and incubated at 37°C, 5% CO_2 for 4 h. Four hours later, the culture medium was replaced with DMEM medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin combination. Cells were cultured for a further 48h before the following experiments.

qPCR

RNA extraction was performed according to a Trizol method, and reverse transcription was performed according to Takara's manual. qPCR was performed according to the manufacturer's manual (Yeason, Shanghai, China, 11201ES03). GAPDH was applied as the housekeeping gene, and the following primers were used:

FITM2-Upward: 5'- GCAACGTCCTCAACGTGTA TT -3'; FITM2-Downward: 5'- GCCCGTGTAGTG TTCGATGTT -3'; GAPDH-Upward: 5'- AATGGACAA

CTGGTCGTGGAC -3'; GAPDH-Downward: 5'- CCC TCCAGGGGATCTGTTTG -3'

Western blot

Western blot analysis was performed as previously described (Hu et al., 2020). Total protein was extracted with RIPA lysis buffer with protease inhibitor; 30 μg of total protein subjected to Western blot were separated using 10% SDS-PAGE and electro-transferred onto polyvinylidene difluoride membranes (Millipore, Billerica). Membranes were blocked with 5% skim milk and then incubated with the primary antibody. The Caveolin-1 antibody was obtained from Abcam (ab2910), and the GAPDH antibody was obtained from zsbio (TA-08). The FITM2 antibody used for western blot was obtained from bioss (Beijing, CHN, Catalog Number: bs-23312R).

Table 1. Relationship between intratumoral FITM2 expression and clinicopathologic features.

Variable		Intratumoral FITM2		p
		Low expression (n=45)	High expression (n=45)	
Age(years)	<50	9	10	0.796
	≥50	36	35	
Gender	Male	8	9	0.788
	Female	37	36	
Cirrhosis	Presence	29	30	0.824
	Absence	16	15	
AFP	<20 ng/ml	15	16	0.824
	≥20 ng/ml	30	29	
HBsAg	Negative	9	11	0.612
	Positive	36	34	
Tumor number	single	32	34	0.634
	Multiple	13	11	
Tumor size	<3cm	18	7	0.010
	≥3cm	27	38	
Microvascular invasion	Absence	21	11	0.028
	Presence	24	34	

Wound healing

Wound healing assay was performed as previously described (Liu et al., 2016). In brief, cells were seeded in 6-well plate and grown to 100 % confluence. Three straight lines were drawn at the bottom of the plate to mark the position of measurement. The wound was performed with a 200 μl pipette, and the cell debris was washed 3 times with PBS. Wound closure was measured at the same position 48h later, and the data were analyzed with ImageJ software.

Transwell

Transwell analysis was performed as previously described (Liu et al., 2016). A total of 50,000 cells cultured in serum-free medium were seeded on the upper chamber, and the lower chamber contained DMEM medium supplemented with 20 % FBS. 48h later, the upper chamber was collected, and the unmigrated cells were wiped with cotton swabs. The migrated cells were fixed with 20 % methanol and dyed with 0.1 % crystal violet at room temperature for 15 minutes. Each transwell chamber was observed under a microscope at a magnification of 100. Three visual fields were randomly selected to calculate the number of liver cancer cells passing through the basement membrane, and the average value was taken for statistical analysis.

Immunofluorescence

Immunofluorescence staining and confocal microscopy were performed as previously described (Cai et al., 2017). Cytoskeleton organization was examined by immunofluorescence. Phalloidin-TRITC (P1951, Sigma Aldrich) was used to label the cytoskeleton of PLC/PRF/5 cells (Stained in red), while DAPI was used to label the nucleus (C1002, Beyotime, stained in blue). The immunofluorescence staining was observed under the confocal fluorescent microscope (×600

Table 2. Univariate and multivariate analysis of factors associated with recurrence -free survival and overall survival of HCC following surgical resection.

Clinicopathological factors	Recurrence -free survival				Overall survival			
	Univariate P	Multivariate			Univariate P	Multivariate		
		Hazard ratio	95% CI	p		Hazard ratio	95% CI	p
Age <50 vs ≥50	0.726				0.841			
Gender female vs male	0.840				0.865			
Cirrhosis no vs yes	0.694				0.455			
AFP <20 vs ≥20	0.614				0.571			
HBsAg negative vs positive	0.547				0.266			
Number single vs multiple	0.517				0.337			
Tumor size <3cm vs ≥3cm	0.092				0.046	2.127	0.988-4.578	0.054
MVI absence vs presence	0.001	2.312	1.274-4.194	0.006	0.000	3.551	1.649-7.646	0.001
Intratumoral FITM2	0.002	1.899	1.125-3.207	0.016	0.024			

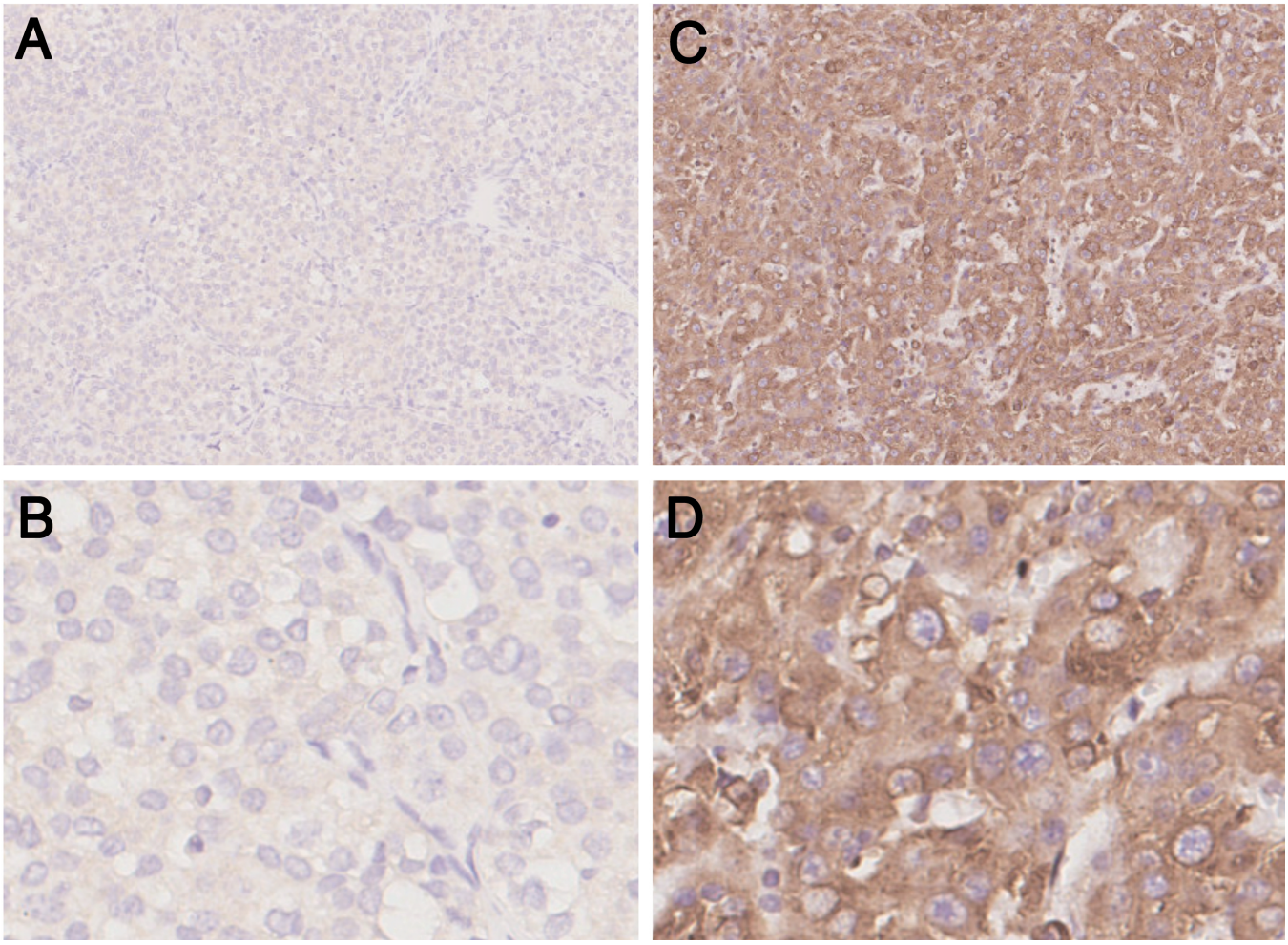

Fig. 1. Typical pictures of intratumoral FITM2 expression in HCC samples. **A, B.** Typical pictures of low FITM2 expression in HCC samples. **C, D.** Typical pictures of high FITM2 expression in HCC samples. A, C, x 50; B, D, x 200.

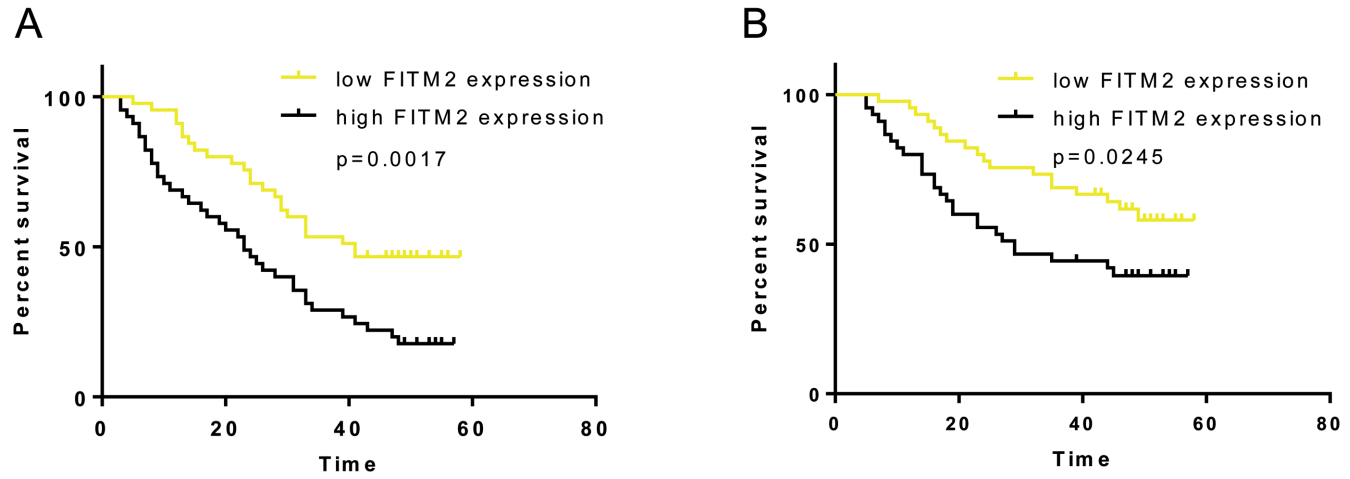

Fig. 2. High intratumoral expression of FITM2 indicates poor patient prognosis. **A.** High intratumoral expression of FITM2 indicates poor patient recurrence-free survival. N=45 for each group; p=0.0017. **B.** High intratumoral expression of FITM2 indicates poor patient overall survival. N=45 for each group; p=0.0245.

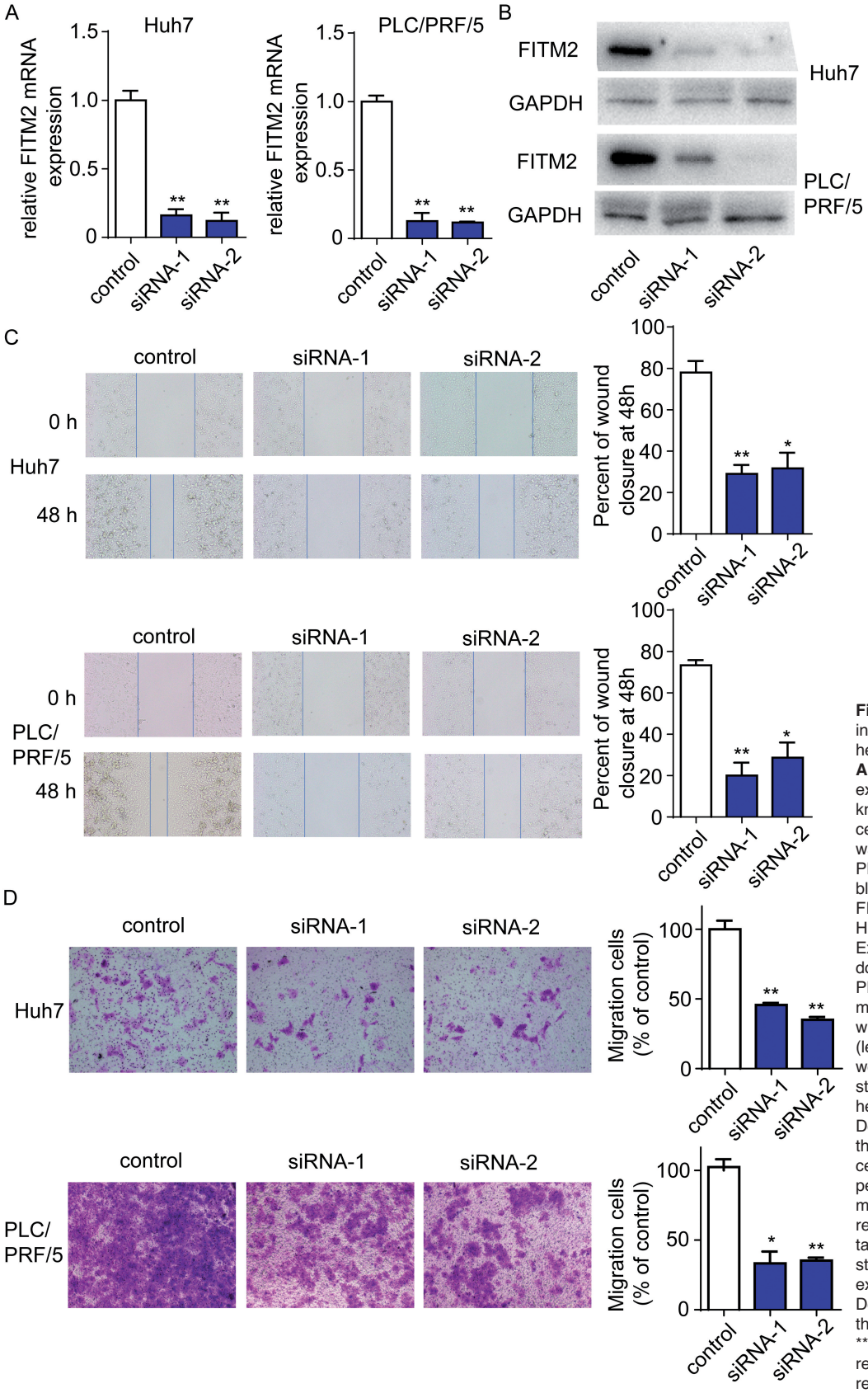

Fig. 3. Downregulation of FITM2 inhibits the migration ability of hepatocellular carcinoma cells. **A.** qPCR analysis of the expression of FITM2 in control and knockdown Huh7 and PLC/PRF/5 cell lines. Expression of FITM2 was downregulated in Huh7 and PLC/PRF/5 cell lines. **B.** Western blot analysis of the expression of FITM2 in control and knockdown Huh7 and PLC/PRF/5 cell lines. Expression of FITM2 was downregulated in Huh7 and PLC/PRF/5 cell lines. **C.** Cell migration ability was measured with wound healing experiment (left: representative pictures of the wound healing experiment; right: statistical analysis of the wound healing experiment results). Downregulation of FITM2 inhibited the wound closure ability of HCC cells. **D.** Transwell assay was performed to measure the cell migration ability (left: representative pictures of the tanswell experiment; right: statistical analysis of the transwell experiment results). Downregulation of FITM2 inhibited the migration ability of HCC cells. **: p<0.01; *: p<0.05; each column represents three independent replicates.

magnification, Olympus).

Statistics

SPSS version 25.0 software was applied for statistical analysis. Graphpad Prism 6.0 was applied to draw the survival analysis plots. Chi-square analysis was performed to analyze the relationship between clinical characteristics and the FITM2 expression. Risk factors were evaluated with univariate and multivariate analysis. Kaplan–Meier method was applied to draw the disease-free survival and overall survival curves. $P<0.05$ was considered statistically significant.

Results

High intratumoral FITM2 expression indicates poor HCC survival and correlates with microvascular invasion

To evaluate the potential role of FITM2 in the progression of HCC, the expression of FITM2 in HCC tumor sections was first analyzed. As shown in Fig. 1, the expression of FITM2 varies from low (Fig. 1A,B) to relatively high (Fig. 1C,D). To further evaluate the prognostic role of FITM2, we collected the specimens of a cohort of patients who have received curative tumor resection in our hospital. The tumor diameter of these patients was less than 5 cm, and no metastasis or tumor thrombus existed. The specimens were then subjected to IHC analysis to determine the expression of FITM2. The expression of FITM2 in the tumor sections was quantified with the H score method. The patients were divided into high and low FITM2 expression groups according to the median H score value. As shown in Fig. 2, high intratumoral FITM2 indicates poor patient recurrence-free survival and poor patient overall survival.

The relationship between FITM2 expression and clinicopathological factors was then evaluated. As shown in Table 1, high intratumoral FITM2 expression correlates with high microvascular invasion (MVI) rate and larger tumor size, while no correlation was observed between FITM2 expression and other clinicopathological factors including age, gender, cirrhosis, α fetoprotein (AFP), HBsAg and tumor number. The prognostic role of FITM2 was then evaluated with univariate and multivariate analysis. As shown in Table 2, microvascular invasion and high FITM2 expression were identified as prognostic factors for recurrence-free survival, while tumor size, microvascular invasion, and high FITM2 expression were identified as prognostic factors for overall survival. Further multivariate analysis revealed that MVI and intratumoral FITM2 were independent prognostic factors for patient recurrence-free survival, while MVI was an independent prognostic factor for patient overall survival.

Downregulation of FITM2 expression inhibits the migration ability of Huh7 and PLC/PRF/5 HCC cell lines

Whether FITM2 regulates the biological behavior of tumor cells was further analyzed by downregulating the expression of FITM2 in Huh7 and PLC/PRF/5 HCC cell lines. As shown in Fig. 3A,B, downregulation of FITM2 was successfully obtained. While high FITM2 expression correlates with a high MVI rate, whether FITM2 promotes HCC progression by regulating the cell migration ability of HCC was then evaluated. Wound healing results revealed that the wound closure at 48h was significantly inhibited after FITM2 downregulation in both Huh7 and PLC/PRF/5 cell lines (Fig. 3C). Transwell migration assay revealed that the migrated cells were significantly decreased after FITM2 downregulation (Fig. 3D).

Downregulation of FITM2 expression correlates with reduced caveolae related protein expression and malformation of the cytoskeleton

FITM2 was previously reported to be related to cell

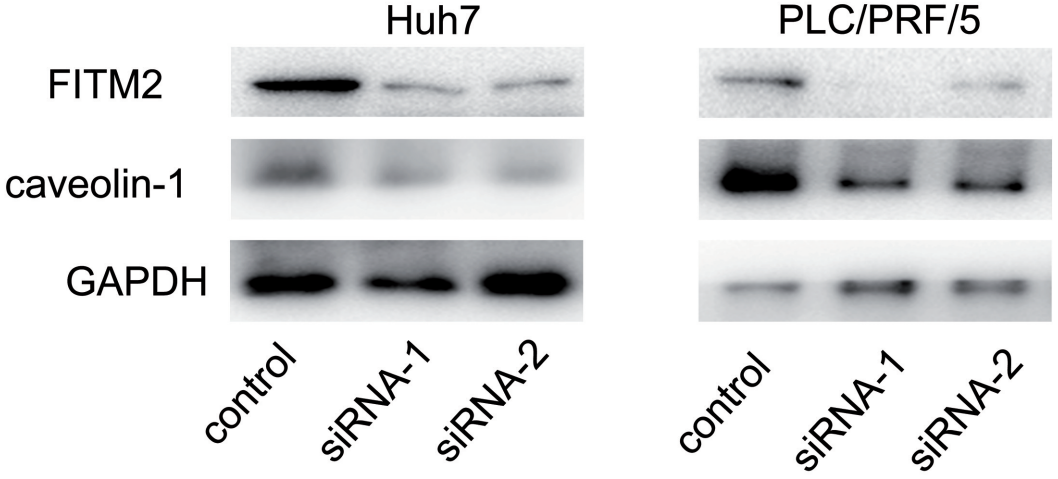

Fig. 4. FITM2 promotes HCC progression by regulating the formation of caveolae. Downregulation of FITM2 correlates with reduced expression of caveolin-1 in Huh7 (left) and PLC/PRF/5 (right) hepatocellular carcinoma cell lines.

morphology and cytoskeletal organization regulation, but the detailed mechanism has not been fully understood. RNA interference study indicates that FITM2 promotes cell migration ability (Fig. 3), and therefore, we hypothesized that FITM2 promotes cell migration by regulating cell morphology changes or cytoskeletal reorganization. As FITM2 was a protein associated initially with lipid metabolism, we analyzed whether FITM2 regulates cell migration by regulating the formation of caveolae which is formatted by lipid and protein. As shown in Fig. 4, knockdown of FITM2 dramatically inhibited the expression of caveolin-1, which is the marker gene of caveolae. These results revealed that FITM2 promotes HCC cell migration by regulating the expression of caveolin-1 and the formation of caveolae.

Discussion

Aberrant lipid metabolism exerts a vast influence on the development of cancer (Currie et al., 2013; Liu et al., 2017; Yi et al., 2018; Pope et al., 2019). Significantly increased activity and coordinated expression of several lipogenic enzymes in tumor cells contribute to neoplastic lipogenesis and promote tumor progression (Luo et al., 2017). For example, fatty-acid synthase (FASN), ATP citrate lyase (ACLY), and proprotein convertase subtilisin/kexin type 9 (PCSK9) were all well-known lipid metabolism-regulating genes that also play essential roles in the proliferation, migration, and metastasis of several malignant tumors including breast cancer, pancreatic cancer, liver cancer as well as melanoma (Jones and Infante, 2015; Piao et al., 2015; Menendez and Lupu, 2017; Granchi, 2018; Wen et al., 2019). FITM2 is a gene related to lipid droplets biosynthesis and fat storage (Yang et al., 2012). Recent studies have also revealed that a single nucleotide polymorphism of FITM2 correlates with deafness. The study also revealed that FITM2 is required to maintain cell fitness after exposure to interferon-γ (IFNγ) (Shakir et al., 2018; Lawson et al., 2020). These studies imply that FITM2 participates in various cellular activities while its role in the development and progression of HCC has not been elucidated.

In this study, we found that FITM2 is expressed in HCC tissues, and high intratumoral FITM2 indicates poor patient recurrence-free survival and poor patient overall survival. High intratumoral FITM2 expression correlates with high microvascular invasion (MVI) rate and larger tumor size. Univariate analysis identified FITM2 as a prognostic factor for recurrence-free survival and overall survival, while multivariate analysis revealed that intratumoral FITM2 was an independent prognostic factor for patient recurrence-free survival. These results, for the first time, revealed a prognostic role of FITM2 in the progression of HCC and linked FITM2 regulated cellular processes with HCC tumor metastasis and progression.

As FITM2 correlates with MVI, we further explored whether FITM2 regulates cell migration at the cellular level and found that downregulation of FITM2 expression inhibited tumor wound healing and migration. These results indicate that FITM2 promotes HCC progression by promoting cell migration. As FITM2 was previously reported to correlate with the formation of lipid droplets, we then examined whether FITM2 promotes cell migration by mediating the formation of caveolae on the cell surface. The expression of caveolin-1 was significantly downregulated after downregulating the expression of FITM2. These results revealed that FITM2 promotes HCC cell migration by regulating the formation of caveolae.

Overall, these results for the first time establish a role for FITM2 in the progression of HCC and as a prognostic factor of HCC. The results also link lipid metabolism with the formation of caveolae and cell migration. Our findings may be applied to develop a new adjuvant therapy for HCC patients after surgical treatment and lower the recurrence rate of HCC.

Author Contributions. XF.G and Y.C participated in conception and design of the study; Y.C, LJ.J and S.W participated in acquisition and analysis of data; XF.G and Y.C participated in drafting the manuscript and figures.

Compliance with Ethical Standards

Funding. No funding information was applicable.

Conflict of Interest. The authors declare no conflict of interest.

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Yidu Central Hospital and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent. Informed consent was obtained from all individual participants included in the study.

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