Far upstream element-binding protein 1 facilitates hepatocellular carcinoma invasion and metastasis

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Abstract

Previous research suggests that far upstream element-binding protein 1 (FUBP1) plays an important role in various tumors including hepatocellular carcinoma (HCC). However, the role of FUBP1 in liver cancer remains controversial, and the regulatory pathway by FUBP1 awaits to be determined. This study aims to identify the role of FUBP1 in HCC progression. Our result shows that the high level of FUBP1 expression in HCC predicts poor prognosis after surgery. Overexpression of FUBP1 promotes HCC proliferation, invasion, and metastasis by activating transforming growth factor-β (TGF-β)/Smad pathway and enhancing epithelial-mesenchymal transition (EMT) in vitro and in vivo. Inhibitor of Thrombospondin-1 (LSKL) could inhibit HCC proliferation and invasion in vitro and in vivo by blocking the activation of TGF-β/Smad pathway mediated by thrombospondin-1 (THBS1). Our study identified the critical role of FUBP1-THBS1-TGF-β signaling axis in HCC and provides potentially new therapeutic modalities in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third leading cause of cancer-related deaths (1). Majority of HCC patients are not suitable for routine surgical resection at the time of diagnosis (2). Despite recent advances in systemic treatment for HCC and some improvement in the survival of HCC patients, the overall prognosis remains dismal (2,3). HCC has a high rate of recurrence and is highly resistant toward therapeutic intervention, including sorafenib (4). About 19% to 25% of HCC cases that had undergone radical resection recurred within 1 year and over 70% of the cases recurred within 5 years. Our exploration of effective new therapies has largely been hampered by our limited understanding of the molecular mechanisms underlying recurrence and metastasis of HCC (5).

Far upstream element-binding protein 1 (FUBP1), also known as human DNA helicase V (HDH V), is a single-stranded DNA-binding protein encoded by the FUBP1 gene, and it regulates the expression of target genes by binding to the far upstream element (FUSE) in their upstream noncoding sequences (6).
promote c-myc transcription. On the other hand, when c-myc transcription reaches its peak, FUBP1 replaces FUBP3, forms a complex with FIR (FBP Interacting Repressor) and inhibits c-myc transcription (7), serving as a negative feedback mechanism. If needed, FIR expression is decreased, thereby releasing the inhibitory effect and allowing a new round of c-myc transcription.

Given the important regulatory roles of FUBP1 on gene expression, many studies have been carried out to investigate the biological functions of FUBP1 in different types of cancers. Mutation in FUBP1 gene is related to poor prognosis of oligodendrogliomas, oligoastrocytomas, astrocytoma, glioblastoma, and other nervous system neoplasms (8–10). Upregulation of FUBP1 is associated with proliferation of chordoma (11), clear cell renal cell carcinoma (12), and esophageal squamous cell carcinoma (13) and affects the prognosis of patients. High expression of FUBP1 promotes the proliferation of Hodgkin’s lymphoma and mediates tumor cell adhesion (14). However, other studies have also shown that the abnormal expression of FUBP1 may not be related to the regulation of c-myc gene in bladder and prostate cancers (15).

FUBP1 has also been found to play an important role in liver cancer. Early in 2008, by examining 12 pairs of liver cancer and pericancerous tissues, FUBP1 levels were found to be higher in HCC tissues (16). In addition, FUBP1 was found to directly promote proliferation and migration of liver cancer cells by inducing stathmin expression and correlated with poor clinical outcome (17). Furthermore, FUBP1 was found to inhibit apoptosis and promote HCC cell proliferation by inhibiting p21 (18,19).

The earlier studies suggest that FUBP1 plays an important role in various tumors including HCC. However, the role of FUBP1 in liver cancer remains controversial, and the regulatory pathway by FUBP1 awaits to be determined. Therefore, further studies are required to explore the effects of FUBP1 on HCC and investigate the underlying mechanism related to the role of FUBP1 in the genesis and invasion of HCC and invasion.

### Materials and methods

#### Patients and follow-up

Four hundred and fifty-one HCC patients who diagnosed HCC (20) and underwent surgery between January 2011 to December 2011 were recruited. The inclusion criteria were as follows: (i) no prior cancer treatment, (ii) pathologically diagnosed HCC with complete resection of all tumor nodules with margins confirmed free of cancer by histologic examination and (iii) availability of complete clinicopathologic and follow-up data. The Barcelona Clinic Liver Cancer (BCLC) staging system was used to assess tumor stage. Tumor differentiation was determined according to the Edmondson grading system. This study was approved by the Clinical Trial Ethics Committee of Zhongshan Hospital and all other four clinical centers. Approval for research protocol and use of human subjects was obtained from the Research Ethics Committee of Zhongshan Hospital. Informed consent was obtained from each patient. Follow-up ended in August 2017. Time to recurrence (TTR) was defined as the interval between surgery and the diagnosis of any type of recurrence including intra- or extrahepatic recurrence as identified by MR or CT. OS was defined as the interval between treatment and death due to any cause or the last observation date (21).

#### Tissue microarray and immunohistochemistry

The construction of Tissue microarray (TMA) and immunohistochemistry protocol were described previously (22). Briefly, after TMA or paraffin section rehydration and microwave antigen retrieval, slides were incubated with primary antibodies overnight at 4°C. Then, secondary antibody incubation was performed at 37°C for 30 min. Staining was performed with DAB (diaminobenzidine) and Mayer's hematoxylin. We included negative control slides without the primary antibodies, in all assays. Result of immunohistochemical staining was independently assessed by two pathologists (23).

#### Cell lines and culture conditions

Huh-7, PLC/PRF/5, and SMCC-7721 cell lines were purchased from the Cell Bank of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) on 09/2015 and preserved at Shanghai Liver Cancer Institute. MHCC97L, MHCC97H, and HCCLM3 cell lines were established and preserved at Shanghai Liver Cancer Institute since 2001 (24,25). Cells were cultured as previously described in Dulbecco’s modified Eagle medium (DMEM, Gibco, Thermo, New York, US) containing 10% fetal bovine serum (FBS, Gibco, Thermo, New York, US) and incubated at 37°C in a humidified atmosphere with 5% CO2 (26). Cell lines are tested by nineteen short tandem repeat (STR) loci plus the gender determining locus every 6 months, and last tested 06/2016 by Genetic Testing Biotechnology (GTB), China. In detail, STR and gender determining locus, Amelogenin, were amplified using the commercially available FX20 Kit from ACGU. The cell line sample was processed using the ABI Prisma® 3130 XL Genetic Analyzer. Data were analyzed using GeneMapper® ID-X v1.5 software (Applied Biosystems). Appropriate positive and negative controls were run and confirmed for each sample. Cell lines were authenticated using Short Tandem Repeat (STR) analysis as described in 2012 in ANSI Standard (ASN-0002) by the ATCC Standards Development Organization (SDO) and Capes-Davis et al.‘s database (27).

#### RNA isolation, qRT-PCR, and western blot analyses

Total RNA was extracted using Trizol reagent (Life tech, Thermo New York, USA), and cDNA was synthesized using PrimeScript™ RT reagent kit (Takara, Dalian, China) according to the manufacturer’s instructions. Target genes were quantified using SYBR® Premix Ex Taq™ II (Takara, Dalian, China) and DNA amplification was carried out using ABI 7900 system (ABI, Thermo, Shanghai, China). The relative quantities of target gene mRNAs compared to an internal control were determined using the ΔCq method. Reverse transcription polymerase chain reaction (RT-PCR) conditions were as follows: 30s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. B-Actin was used as an internal control. Western blot analysis was carried out as previously described (5). Primer sequences are listed Supplementary Methods 1.

#### FUBP1 expression or shRNA constructs and transfection

FUBP1 expression constructs or shRNA and standard lentivirus packaging systems were prepared by GeneChem Co. Ltd., Shanghai, China. Cells were transfected with HitransG reagent (GeneChem, Shanghai, China) according to the manufacturer’s instructions. In short, target cells were infected with filtered lentivirus plus 10 μl/ml of HitransG for 24 h to generate stable cell lines. HCC cells were infected with these viruses or control virus and then selected against puromycin for 3 days before use in assays. Target FUBP1 shRNA sequences are listed Supplementary Methods 2 (28).

#### Cell proliferation, colony formation, and invasion assays

FUBP1 overexpressed or shRNA knockdown or vector control HCC cells were counted and seeded into a 96-well plate at 1000 cells/100 μl/well. Every 24 h, the absorbance for cell proliferation analysis was measured with cell counting kit 8 (CCK-8, (Dojindo, Shanghai, China)) following the
FUBP1 promotes HCC growth and metastasis in vitro and in vivo through epithelial-mesenchymal transition

To investigate the expression level of FUBP1 in various HCC cell lines that carry different metastatic potential, we performed qRT-PCR and western blot for FUBP1 in six representative HCC cell lines, including HCCLM3, MHCC97L, MHCC97H, PLC/PRF/5, SMMC7721, and Huh-7. Our results showed that FUBP1 expression was higher in highly invasive HCCLM3, MHCC97H, and MHCC97L cell lines than PLC/PRF/5, SMMC7721, and Huh-7 cell lines with lower invasive capacity (21, 23, 26, 29) (Supplementary Figure 1E, available at Carcinogenesis Online). First, we depleted FUBP1 cell expression by using specific shRNA against FUBP1 in highly invasive MHCC97H and HCCLM3 cell lines with high basal FUBP1 expression (Supplement Figure 1F, available at Carcinogenesis Online). We chose previously reported shRNA (shFUBP1) and a GeneChem-designed shRNA (shFUBP1-0) to exclude off target effects. FUBP1 depletion led to decreased cell proliferation in both paired adjacent liver tissues. FUBP1 mRNA expression data of 384 HCC tumors and 50 adjacent liver tissues were analyzed by online bioinformatics tool UCSC Xena (http://xena.ucsc.edu). Results showed that the expression level of FUBP1 was higher in tumor tissues than the adjacent liver tissues (P < 0.01, Supplementary Figure 1A, available at Carcinogenesis Online). Patients with higher level of FUBP1 had significantly lower postoperative overall survival rate (P = 0.01165, log-rank test statistics = 6.363, Supplementary Figure 1B, available at Carcinogenesis Online).

To further study the relationship of FUBP1 expression with HCC disease prognosis in a larger scale, a TMA containing 451 tissue samples from patients undergoing curative resection was generated, and the TMA slide was stained for FUBP1 by performing immunohistochemistry with a validated FUBP1 antibody. FUBP1 expression status was stratified into four different categories exemplified by negative, low expression, medium expression, and high expression, by using previously described standards (23) (Figure 1A). The basic clinical information of 451 HCC patients enrolled is described in Supplementary Table 1, available at Carcinogenesis Online. Statistical analysis showed that high expression of FUBP1 correlated positively with HBV infection background and microvascular invasion. High FUBP1 expression predicted significantly shorter median OS in HCC patients compared to those patients with low FUBP1 expression by Kaplan–Meier analysis (48.27 months versus 56.5 months, *P < 0.01). Also, patients with high FUBP1 displayed significantly shorter TTR compared to patients with low expression (44.44 months versus 56.4 months, **P < 0.01). 5-year (58.0% versus 73.1%) overall survival is lower in patients with high FUBP1 expression than those with low FUBP1 expression. And 5-year (42.8% versus 65.1%) overall survival is lower in patients with high FUBP1 expression than in the low expression group (Figure 1B, C).

Univariate analysis showed that both TTR (HR = 1.90 [1.41, 2.55], *P < 0.01) and OS (HR = 1.84 [1.39, 2.43], **P < 0.01) correlated with FUBP1 expression status. Multivariate analysis revealed that FUBP1 expression status was an independent indicator for both TTR (HR = 2.38 [1.75, 3.24], *P < 0.01) and OS (HR = 2.01 [1.48, 2.72], **P < 0.01). Collectively, these results suggest that FUBP1 might be a useful prognostic biomarker for HCC (Supplementary Tables 2 and 3, available at Carcinogenesis Online).

These results indicate that high FUBP1 expression is prone to recurrence and predicts poor prognosis after operation in HCC patients.

FUBP1 promotes HCC growth and metastasis in vitro and in vivo through epithelial-mesenchymal transition

The expression and clinical significance of FUBP1 in HCC was analyzed by University of California Santa Cruz (UCSC) Xena online bioinformatics analysis tool. Statistical analyses were performed using SPSS 21.0 software (IBM, Armonk, NY). Independent t-test was used to compare continuous variables with normal distribution. Chi-squared test and Fisher’s exact probability test were used to compare discrete variables. Skewed variable data are analysed with nonparametric Mann-Whitney test or Wilcoxon signed-rank test. The relationships between FUBP1 expression and TTR or OS were analyzed using Kaplan-Meier survival curves and log-rank tests, respectively. A P value <0.05 was considered statistically significant (22).

Results

FUBP1 was upregulated in HCC, and expression of FUBP1 in tumor tissue and predicts poor prognosis after operation

To determine the expression of FUBP1 in HCC patients, we first investigated the expression of FUBP1 in HCC tumor tissues and
Figure 1. (A) TMA containing tissue samples of 451 patients who underwent curative resection in our hospital was immunostained. FUBP1 expression status was stratified. (B) Kaplan–Meier analysis revealed significantly shorter median OS in patients with high FUBP1 compared to those with low FUBP1 (48.27 months versus 56.5 months, **P < 0.01). (C) Kaplan–Meier analysis shows that patients with high FUBP1 had significantly shorter TTR compared with patients with low expression (44.44 months versus 56.4 months, **P < 0.01). (D) CCK-8 test, (E) Colony formation assay, and (F) Transwell assay indicated that cell lines with overexpression of FUBP1 had enhanced ability of proliferation, clone generation, and invasion. After shRNA-mediated FUBP1 knockdown, the ability of proliferation, clone generation, and invasion had significantly reduced. (G,H) qPCR and Western blot confirmed that FUBP1 overexpression induces upregulation of N-cadherin and vimentin and inhibits E-cadherin.
cell lines measured by CCK-8 detection (Figure 1D). Conversely, we also overexpressed FUBP1 in Huh-7 cell line that expressed low FUBP1 and found that FUBP1 overexpression promotes Huh-7 cell proliferation. In accordance with these findings, FUBP1 depletion decreased HCC cell colony formation, whereas its overexpression promoted it (Figure 1E). Additionally, we also examined the cell migration and invasion phenotype upon FUBP1 depletion or overexpression by using transwell assay (Figure 1F). Consistently, FUBP1 downregulation decreased the migration/invasion ability of HCC cells, whereas its overexpression led to enhanced ability. Previous studies have demonstrated EMT as an important mechanism inducing tumor invasion. Our findings motivated us to examine the effect of FUBP1 on EMT-related proteins in HCC cells. To this end, we used qRT-PCR and western blot analyses. Our results showed that FUBP1 overexpression induces upregulation of N-cadherin and vimentin and inhibits E-cadherin expression (Figure 1G, H). Next, we injected these paired HCC cells onto the contralateral flanks of nude mice and monitored tumor growth in vivo. Consistently with our in vitro cell line proliferation data, FUBP1 depletion in two independent HCC cell lines led to decreased HCC tumor growth, whereas its overexpression promoted tumor clone formation ($P < 0.05$). In addition, we also observed increased lung metastasis upon FUBP1 overexpression (Figure 2). These results suggest that overexpression of FUBP1 might promote the proliferation, invasion, and metastasis of HCC by promoting EMT.

**Expression profile identifies FUBP1-induced EMT was mediated by TGF-β signaling pathway**

Next, we aimed to understand the underlying molecular mechanism by which FUBP1 promotes invasion and metastasis of HCC cells. For this purpose, we performed gene expression profiling test on FUBP1 overexpressed and depleted cells. We found 1474 differentially expressed gene between MHCCCL3-shFUBP1 vs MHCC-vector, 3320 differentially expressed gene between MHCC97H-shFUBP1 versus MHCC97H-vector, and 1934 differentially expressed gene between Huh-7-oeFUBP1 vs Huh-7-vector. We were specifically interested in those genes that fit into the following criteria: (i) FUBP1 depletion and overexpression causes reciprocal regulation on gene expression and (ii) those genes that were commonly regulated in multiple HCC cell lines. Using these criteria, we made intersection of differentially expressed gene between three groups. Totally, 31 genes were found to be significantly regulated by FUBP1 (Figure 3A, B). KEGG analysis identified 11 upregulated and 7 downregulated cancer-related pathways that have significant relationship with FUBP1 ($P < 0.05$). Among them, TGF-β signaling pathway was one of the most significantly enriched pathways regulated by FUBP1 ($P < 0.01$, FDR = 2.22, Figure 3C).

Among the 31 differentially expressed genes, FUBP1 positively regulated protein levels of THBS1, ANXA3, FTSJ3, FOXP2 and negatively regulated ADH6, NLRX1 levels (Figure 3B). KEGG analyses identified that TGF-β signaling pathway is the most significantly enriched pathway regulated by FUBP1 (Figure 3C).

**FUBP1 activates TGF-β/Smad pathway through enhancing THBS1 transcription**

Among genes, THBS1 (Thrombospordin-1, TSP-1) is the most significant differentially expressed gene and also has been reported to be a key regulator to inactivate TGF-β signaling pathway (35-37). Previous studies demonstrated that THBS1 promotes tumor progression in prostate cancer (38), melanoma (39), and other cancers. In addition, high expression of THBS1 may mediate drug resistance of breast cancer to chemotherapeutic drugs (40). As for HCC, previous studies have shown that high expression of THBS1 predicted poor prognosis (41). It is also essential to activate TGF-β/Smad pathway (30,42–45).

To confirm the significance of regulation relationship, we performed qPCR in 50 HCC patients’ tumor tissue and found that there was the most significant correlation between FUBP1 expression and THBS1 (Pearson $R = 0.53$, Spearman $R = 0.64$, **$P < 0.001$, Figure 3D) than other genes (Figure 3D). Though the negatively regulated gene ADH6 also has significant correlation with FUBP1 (Pearson $R = -0.45$, ***$P = 0.001$, Figure 3D), the gene ADH6 has not been reported to be relative with HCC invasion and metastasis. And then we used Level 3 expression data of 371 HCC patients from TCGA as validation group to validate the result of correlation analyses. The validation result confirmed the positive correlation between FUBP1 and THBS1 (Pearson $R = 0.324$, **$P < 0.0001$, Figure 3E), but the correlation between FUBP1 and ADH6 was reverse from our microarray and PCR result (Pearson $R = -0.231$, $P < 0.0001$, Figure 3E). We consider that might due to ADH6 alcohol-related enzyme which has close relationship with western HCC patients, whose etiology was different from Chinese patients. So, we focus on the importance of regulation of THBS1 by FUBP1. We performed luciferase reporter assay to examine whether THBS1 was transcriptionally regulated. The result shows that THBS1 transcriptional activity was significantly enhanced after FUBP1 was overexpressed compared with the control group and reduced after FUBP1 was knockdown, which indicates that THBS1 was transcriptionally regulated by FUBP1 (Figure 3F).

We further used western-blot and confirmed that FUBP1 knocking-down reduces the expression of THBS1, thus inhibit the TGF-β-mediated phosphorylation of Smad2/3 and suppress HCC cell EMT. Meanwhile, the overexpression of THBS1 can rescue protein expression and phosphorylation caused by FUBP1 depletion in HCC cells (Figure 3G), suggesting that THBS1 is an important downstream target of FUBP1. FUBP1 activates TGF-β/Smad pathway through enhancing THBS1 transcription (Figure 3G).

**LSKL, a specific THBS1 inhibitor, reduces HCC cell proliferation and invasion in vitro and in vivo by inhibiting TGF-β/Smad2/3-dependent epithelial-mesenchymal transition**

Aiming to find the solution for FUBP1/THBS1 high expression induced EMT, we therefore investigated the effects of LSKL (Peptide antagonist of Thrombospordin-1) on THBS1/TGF-β/Smad2/3 signaling axis in HCC cells. LSKL is a peptide derived THBS1 inhibitor, was reported to inhibit TGF-β activation by THBS1, and delayed the progression of hepatic damage and fibrosis (30).

Highly metastatic potential HCCLM3 HCC cell line with high basal expression of FUBP1 was treated with LSKL and phenotypes were examined. First, LSKL treatment led to decreased HCC cell proliferation, colony formation, and cell invasion ($P < 0.01$, Figure 4A–C). Next, LSKL treatment led to decreased tumor growth in vivo (treat vs control : 1.49 ± 0.10g versus 2.00 ± 0.13 g, **$P < 0.01$, Figure 4D), corresponding with decreased lung metastasis (pathological score treat vs control 0.86 ± 0.16 versus 2.85 ± 0.24, **$P < 0.01$, Figure 4D). Consistent with these findings, immunohistochemical staining of HCC tissues from mice showed that tumor EMT was significantly inhibited in the LSKL-treated group compared to control, vehicle-treated group (Figure 5A, B).
Figure 2. (A) Representative image of xenograft tumors derived from cells infected with lentivirus encoding either FUBP1 or shFUBP1 (n = 6). (B) Tumor weight was significantly increased or decreased after FUBP1 was upregulated or knocked down, respectively (P < 0.01). (C) Representative image for lung metastasis and tumor thrombus. (D) The proportion and pathological score of lung metastasis was increased after FUBP1 overexpression (P < 0.01).
Mechanistically, our results demonstrated that phosphorylation of Smad2/3 was increased when FUBP1 was overexpressed and significantly reduced when treated with LSKL. The expression levels of N-cadherin and vimentin were also inhibited (**P < 0.01, Figure 5C, D).

These evidence shows that LSKL could inhibit HCC proliferation and invasion in vitro and vivo through blocking activation of THBS1/TGF-β/smad pathway mediated by FUBP1 expression.

**Discussion**

Our work provides strong evidence that the expression level of FUBP1 is higher in tumor tissues than adjacent liver tissues. Statistical analysis showed that high expression of FUBP1 correlates with HBV infection background and often with microvascular invasion. High expression of FUBP1 in HCC tumor predicts poor prognosis after surgery.

We used three HCC cell lines with different metastasis potential and manipulated FUBP1 expression by overexpression or knockdown. Overexpression of FUBP1 enhances HCC proliferation, invasion, and metastasis. In contrast, its depletion led to decreased cell proliferation, colony formation, and invasion. In consistence with our in vitro data, FUBP1 overexpression promoted tumor formation in vivo, whereas its depletion diminished tumor formation. Mechanistically, we showed that FUBP1 controls SMAD2/3/TGF-β pathway hyperactivation through transcriptionally enhance THBS1 expression. By treating cells with a specific THBS1 inhibitor LSKL, we found that LSKL could robustly decrease cell proliferation, colony formation, and cell invasion. Consistently, LSKL treatment led to decreased HCC tumor growth in xenograft models. More importantly, LSKL could also suppress tumor cell invasion. Therefore, our results suggest that FUBP1-THBS1 can serve as a novel therapeutic signaling axis that could be targeted in HCC.

LSKL was originally reported in 1999 by Solange et al. (46). Latency-associated peptide (LAP) is a short peptide in the inactive TGF-β protein complex. LSKL is a key sequence in LAP, which can combine with the K412RFK415 sequence of TGF-β activator protein THBS1, thus performing a vital role in the activation of TGF-β protein complex (30). The short peptide LSKL, when used alone, can be considered as an inhibitor that effectively inhibits the activation of THBS1 on TGF-β protein complex (30). In an in vivo experiment carried out by Hiroki et al. in 2003, LSKL was observed to have the ability to inhibit the progression of liver fibrosis by blocking the activation of TGF-β pathway (30). It was also reported that LSKL exhibited potential inhibitory
Figure 4. (A) CCK-8 test, (B) Colony formation assay, and (C) Transwell assay indicated that the ability of proliferation, clone generation, and invasion was significantly attenuated after treatment with LSKL. (D) Representative image and tumor weight of xenograft tumors treated with LSKL. (E) Representative image for lung metastasis. Rate of tumor metastasis was effectively controlled ($P < 0.01$).
Figure 5. (A) Representative image for immunohistochemical staining of xenograft tumors. (B) Pathological score for immunohistochemical staining showed that tumor EMT was significantly inhibited in LSKL-treated group. (C) Western blot and (D) qPCR results demonstrated that phosphorylation of Smad2/3 is increased after FUBP1 was overexpressed and significantly reduced when treated with LSKL. The expression of N-cadherin and vimentin was also inhibited by LSKL.
effect on bone tumors. In addition, multiple subsequent studies have shown that LSKL is a potent inhibitor that blocks the activation of THBS1 on TGF-β complex (31,47–49). Our results confirmed that LSKL could block FUBP1 overexpression-mediated EMT of HCC cells by inhibiting the activation of THBS1 on TGF-β. In this study, we further verified the inhibitory effect of LSKL on the proliferation and invasion of HCC cells both in vitro and in vivo. Following the processing of HCCLM3 cell line with high expression of FUBP1 by LSKL, the proliferation rate of HCCLM3 cell line was reduced, and transwell invasion assay indicated weakened invasion ability of HCCLM3 cell line. Consistently, intraperitoneal injection of LSKL led to decreased HCC tumor formation and lung metastasis in xenografts. Meanwhile, EMT in LSKL-treated group was significantly inhibited as observed by immunohistochemical staining of HCC tissues from mice HCC xenografts.

The results showed that FUBP1 transcriptionally enhanced the protein expression of THBS1, thereby activating TGF-β/Smad pathway and mediating EMT of HCC cells. LSKL could effectively block the activation of THBS1 in TGF-β/Smad pathway, resulting in the inhibition of tumor proliferation and metastasis. Our results in this paper suggest that THBS1 may be a vital therapeutic target for the treatment of HCC. Further studies need to be carried out to examine the efficacy of LSKL in preclinical and clinical settings, including safety, dosage, dosage form, route of administration, etc. However, findings in this study contribute to further understanding of the mechanism of metastasis and recurrence of HCC, providing some new ideas and inspiration for the treatment of HCC.

Hepatitis B virus infection and cirrhosis are common in patients with HCC in China (50). Previous studies have demonstrated that FUBP1 protein also acts not only as a DNA-binding protein but also as a RNA-binding protein. In addition, FUBP1 also participates in the regulation of intracellular signaling pathways during viral infection (51–53). The first part of our study was to explore the correlation between the expression of FUBP1 and the clinicopathological characteristics of patients. It was noticeable that patients with high FUBP1 expression were accompanied by hepatitis B virus infection. Activation of TGF-β/Smad pathway and EMT were essential for tissue fibrosis and repair. The evidence above suggests that FUBP1 protein might be involved in signal regulation during the development of hepatitis-cirrhosis-hepatocellular carcinoma (54–57). This hypothesis may need to be further tested.

Meanwhile, this study discovered for the first time the inhibitory effects of LSKL peptide on cell proliferation, invasion, and metastasis of HCC. However, the underlying mechanism by which this peptide inhibitor works remains to be determined. In addition, it will be critical to study the dosage, dosage form, application scope, safety, and efficacy of LSKL so that it can be safely tested out in patient-related clinical studies of HCC.

Conclusions
High-level FUBP1 expression in HCC predicts poor prognosis after surgery. The overexpression of FUBP1 promotes HCC proliferation, invasion, and metastasis by activating TGF-β/Smad pathway and enhancing EMT in vitro and in vivo. LSKL could inhibit HCC proliferation and invasion in vitro and in vivo by blocking the activation of TGF-β/Smad pathway mediated by THBS1. Our study identified the critical role of FUBP1-THBS1-TGF-β signaling axis in HCC and provides potentially new therapeutic modalities in HCC.

Ethics approval and consent to participate
This study was approved by the Clinical Trial Ethics Committee of Zhongshan Hospital and all other four clinical centers. Approval for research protocol and use of human subjects was obtained from the research ethics committee of Zhongshan Hospital. Informed consent was obtained from each patient.

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Authors’ contributions
Pei-Yao Fu and Bo Hu contributed to the concept and design. Xiao-Lu Ma and Wei-Guo Tang contributed to the development of methodology. Zhang-Fu Yang, Hai-Xiang Sun and Min-Cheng Yu contributed to data acquisition and analysis. Ao Huang, Jin-Wu Hu and Chen-Hao Zhou contributed to the writing, review, and/or revision of the manuscript. Jia Fan, Yang Xu and Jian Zhou contributed to the administrative, technical, or material support.

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