Follistatin Like 5 (*FSTL5*) inhibits epithelial to mesenchymal transition in hepatocellular carcinoma

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Abstract

Background: Epithelial to mesenchymal transition (EMT) is a key process in determining distant metastasis and intra-hepatic dissemination of hepatocellular carcinoma (HCC). Follistatin (*FST*) family members are considered to be an attractive therapeutic targets and prognostic indicators in cancers. As a derivative of *FST*, Follistatin Like 5 (*FSTL5*) may play a similar role in HCC cells. This study aimed to investigate the expression and function of *FSTL5* in HCC and its role in EMT.

Methods: *FSTL5*, E-cadherin and vimentin in HCC, and paracancerous tissues were detected by immunohistochemistry. Correlation of *FSTL5* expression with overall survival was assessed. The proliferation and invasion of HCC cell lines SK-Hep1 and MHCC-LM3 were analyzed by cell counting kit-8 and Transwell assays. The expression of *FSTL5*, E-cadherin, and vimentin in HCC cells was examined by polymerase chain reaction and Western blot analysis. *T*-test was used to analyze the difference in proliferation and invasion ability between groups. The Spearman rank correlation test was used to detect the correlation between the expression of *FSTL5* and E-cadherin or vimentin.

Results: The expression of *FSTL5* in HCC was lower than that in paracancerous tissues (9.97% *vs.* 82.55%, $\chi^2 = 340.15$, $P < 0.001$). Patients with high *FSTL5* expression had a better prognosis ($\chi^2 = 8.22$, $P = 0.004$) and smaller tumor diameter ($\chi^2 = 45.52$, $P < 0.001$), less lymph node metastasis ($\chi^2 = 5.58$, $P = 0.02$), earlier tumor node metastasis stage ($\chi^2 = 11.29$, $P = 0.001$), a reduced number of tumors ($\chi^2 = 5.05$, $P = 0.02$), lower alpha-fetoprotein value ($\chi^2 = 24.36$, $P < 0.001$), more probability of hepatitis carrying ($\chi^2 = 40.9$, $P < 0.001$), and better liver function grade ($\chi^2 = 5.21$, $P = 0.02$). Immunohistochemistry showed that *FSTL5* expression in HCC tissues was positively correlated with E-cadherin expression ($r = 0.38$, $P < 0.001$) and negatively correlated with vimentin expression ($r = -0.385$, $P < 0.001$). Furthermore, over-expression of *FSTL5* up-regulated the expression of E-cadherin and down-regulated the expression of vimentin in SK-Hep1 (negative control [NC] *vs.* *FSTL5*-interfering group [Lv-*FSTL5*]: E-cadherin [$t = 45.03$, $P < 0.001$], vimentin [$t = 67$, $P < 0.001$]) and MHCC-LM3 (NC *vs.* Lv-*FSTL5*: E-cadherin [$t = 50$, $P < 0.001$], vimentin [$t = 72.75$, $P < 0.001$]) cells at mRNA level. The same as protein level. In addition, the over-expression of *FSTL5* inhibited the proliferation (NC *vs.* Lv-*FSTL5*: SK-Hep1, 3 d [$t = 7.324$, $P = 0.018$], 4 d [$t = 6.23$, $P = 0.021$], 5 d [$t = 10.21$, $P = 0.003$]; MHCC-LM3, 3 d [$t = 4.32$, $P = 0.037$], 4 d [$t = 7.49$, $P = 0.012$], 5 d [$t = 9.3661$, $P = 0.009$]) and invasion (NC *vs.* Lv-*FSTL5*: SK-Hep1, $t = 21.57$, $P < 0.001$; MHCC-LM3, $t = 18.04$, $P < 0.001$) of HCC cells.

Conclusions: Down-regulation of *FSTL5* may contribute to EMT of HCC, and *FSTL5* is a potential target in the treatment of HCC.

Keywords: Hepatocellular carcinoma; Follistatin-related protein; Epithelial to mesenchymal transition; Prognosis; Disease-free survival

Introduction

Hepatocellular carcinoma (HCC) ranks the sixth in incidence and the third in mortality among malignant tumors globally.[1-3] Surgery is still the first choice for the treatment of HCC. The postoperative recurrence rate in HCC patients is still high, and most recurrent HCC is not suitable for radical resection.[4] Tumor metastasis is the most important factor in recurrence. Recent studies have shown that epithelial to mesenchymal transition (EMT) plays an important role in tumor invasion and metastasis.[5] Cells which undergo EMT are more likely to be separated from the original tissue for distant metastasis. EMT is a key process in determining distant metastasis and intra-hepatic dissemination of HCC. So, identifying molecules that can inhibit EMT could help reduce the tendency for metastasis of HCC. Nowadays, although great efforts have been made to understand the biological mechanism of liver cancer, morbidity and mortality are still frustrating. Therefore, it is urgent to find better

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treatment strategies to supplement the present approach. The best way is to study the molecular mechanism of the occurrence and development of liver cancer.

Follistatin Like 5 (FSTL5) is an extracellular matrix secretory protein with a molecular structure similar to that of follistatin (FST). Follistatin Like (FSTL) family proteins is involved in cell migration, proliferation, differentiation, and organ development. FST can bind to the activin protein of transforming growth factor β (TGF-β) family, which can promote apoptosis and inhibit the proliferation of hepatocytes.[6] In addition, *FST* can bind directly to *TGF-β3* and inhibit EMT in mammary epithelial cells.[7] Furthermore, *FST* could inhibit the expression of the *Snail*, *Zeb*, and *twist1* to suppress EMT in bovine ovules.[8] Therefore, the members of the *FSTL* family are considered to be an attractive therapeutic targets and prognostic indicators in cancers. As a derivative of *FST*, *FSTL5* may play a similar role in HCC cells. *FSTL5* may be one of the important molecules in the regulation of hepatocarcinogenesis and development, especially in the process of EMT. However, the expression and function of *FSTL5* in HCC remain unclear. This study aimed to investigate the expression and function of FSTL5 in HCC and its role in the regulation of EMT.

Methods

Ethical approval

The study was approved by the Ethics Committee of Bengbu Medical College (No. 2011035) and informed consent was obtained from all of the patients.

Patients and tissue specimens

A total of 321 HCC patients treated in the Department of Hepatological Surgery from January 2012 to December 2018 were included and tissue specimens were collected after radical resection. All patients underwent radical surgery, and no other comprehensive treatments such as chemotherapy and targeted therapy were performed before surgery. All specimens included cancer tissue and paracancerous tissue, and patient information including age, sex, lymph node metastasis, tumor size, tumor number, tumor stage, postoperative survival time, overall survival, hepatitis carrier, liver function as assessed by Child-Pugh classification, and pre-operative alpha-fetoprotein were collected. The follow-up period ranged from 6 to 90 months.

Immunohistochemistry

Paraffin sections were routinely dewaxed and hydrated, incubated with 3% H_2O_2 at 37°C for 10 min to inhibit endogenous peroxidase activity, and then incubated in citrate buffer solution at 95°C for 20 min for antigen retrieval. The sections were then incubated with rabbit anti-human monoclonal antibodies for *FSTL5* (1:1000; Proteintech, Rosemont, IL, USA), E-cadherin (1:5000; Cell Signaling Technology, MA, USA) and vimentin (1:5000; Cell Signaling Technology), and streptavidin-peroxidase immunohistochemical staining kit and 3,3′-diaminobenzidine chromogenic reagent (Maixin Co., Fuzhou, China). The sections were counterstained with hematoxylin for microscopic examination. Positive staining for E-cadherin, vimentin, and *FSTL5* was indicated by yellowish to tan granules in the cytoplasm or non-cytoplasm. A total of ten high-density visual fields were randomly selected, and 100 cells were counted in each field. Based on the proportion of stained cells, the number of positive cells ≤10% was scored 0; 10% to 50% was scored 1 point; >50% was scored 2 points. Based on staining intensity, the score was 0 for unstained, 1 for light yellow, 2 for yellow, and 3 for tan. The results of the two scores were multiplied: 0 to 3 was denoted negative or low expression, and ≥4 was denoted positive or high expression.

Cell culture

SK-Hep1 and MHCC-LM3 HCC cell lines were purchased from Shanghai Academy of Life Sciences of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in Dulbecco's modification of Eagle's medium containing 5% fetal bovine serum at 37°C in an incubator with 5% CO_2 and saturated humidity.

A total of 50×10^4 SK-Hep1 and MHCC-LM3 cells were seeded into each well of six-well plates. When the cell density reached 60%, the cells were infected by lentiviral vector over-expressing *FSTL5* (Genecopoeia, Guangzhou, China), and the cells were screened using puromycin (2 μg/mL). HCC cell line stably expressing *FSTL5* was obtained 2 weeks later. Puromycin (2 μg/mL) was routinely added to stabilize the expression of *FSTL5*.[9]

Western blot assay

Cells were lysed in radio-immunoprecipitation assay buffer containing 1 mmol/L phenylmethanesulfonyl fluoride on ice for 10 min and the supernatant was collected following centrifugation at 4°C and 1000×*g* for 5 min. The protein concentration was calibrated by bicinchoninic acid method and 20 μg protein per well were separated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membrane. The membrane was incubated in phosphate buffer solution tween containing 5% skimmed milk powder for 1 h, and then incubated overnight with antibodies against E-cadherin, vimentin, FSTL5, and glyceraldehydes phosphatedehydrogenase (GAPDH) (1:5000; Cell Signaling Technology) at 4°C. Next the membranes were incubated with goat anti-rabbit antibody (1:5000 diluted) at room temperature for 2 h, then washed and exposed to a chemiluminescence instrument.

Quantitative real-time polymerase chain reaction

Trizol reagent (Invitrogen, USA) was used to extract total ribonucleic acid (RNA) from the cells. RNA was reverse transcribed into complementary deoxyribonucleic acid following the instructions of the PrimeScript reverse transcription reagent kit (Takara Bio Inc, Ohtu, Japan). Real-time polymerase chain reaction (PCR) was performed in accordance with the instructions of Synergy BrandsSynergy Brands Premix Ex Taq TM II kit (Takara Bio Inc). The sequences of primers (synthesized by Shanghai

Bioengineering Co, Ltd, Shanghai, China) were as follows: E-cadherin: upstream, 5′-ATTCTGATTCTGCTGCTT G-3′, downstream, 5′-AGTAGTCATAGTCCTGGTCTT-3′; vimentin: upstream, 5′-GAAGAA CTTG CCGTT GAAG-3′, downstream, 5′-GAAGGTGACGAGCCATTTC-3′; *GAPDH*, upstream, 5′-GGAGCGAGATCCC TCCAA AAT-3′, downstream, 5′-GGCTGGTCATACTT CTCAT GG-3′; *FSTL5*: upstream, 5′-TGAAGTGCACAGAGC TGCTT-3′, downstream, 5′-AGCATATTTTCT TGCT GTATTC-3′. The expression of target genes was calculated using the $2^{-\Delta\Delta ct}$ method.

Cell counting kit-8 assay

A total of 5000 cells in the logarithmic growth phase were seeded into 96-well plate and cultured for 1, 2, 3, 4, and 5 days, respectively. Next, 10 μL of cell counting kit-8 reagent was added to each well and the absorbance at 450 nm was measured.

Transwell assay

Matrigel (Thermo Fisher Scientific, Darmstadt, Germany) was coated onto the membrane of a transwell chamber. The cells were seeded into the upper chamber and medium containing 10% fetal bovine serum was added to the lower chamber. After 40 h, the cells on the upper membrane surface were lightly removed. Membrane was fixed with methanol and then stained with crystal violet and visualized under microscopy. A total of ten different visual fields were photographed using a microscope.

Data analysis

All the experiments were repeated three times. The measured data were expressed as mean ± standard deviation, and the count data were expressed using the percentile system. The Chi-squared test was used to analyze the relationship between *FSTL5* and clinical parameters. The Spearman rank correlation test was used to detect the correlation between the expression of *FSTL5* and E-cadherin or vimentin. The *t* test was used to analyze the difference between groups. $P < 0.05$ indicated significant difference. SPSS 19.0 statistical software (SPSS Inc, Chicago, IL, USA) was used to process data.

Results

Relationship between FSTL5 and HCC patient survival

Immunohistochemistry (IHC) showed that 56 HCC cancer specimens were positive and 265 were negative for *FSTL5* expression. In the corresponding paracancerous tissues, 289 were positive and 32 were negative for *FSTL5* expression. The expression of *FSTL5* was significantly lower in HCC cancer specimens than in paracancerous tissues (9.97% *vs.* 82.55%, $\chi^2 = 340.15$, $P < 0.001$). In addition, survival analysis showed that patients with high *FSTL5* expression had significantly longer survival than those with low *FSTL5* expression ($\chi^2 = 8.22$, $P = 0.004$) [Figure 1].

Relationship between FSTL5 and HCC indexes

The early tumor stage ($\chi^2 = 11.29$, $P = 0.001$), small tumor diameter ($\chi^2 = 11.29$, $P = 0.001$), a reduced number of tumors ($\chi^2 = 45.52$, $P < 0.001$), lower alpha-fetoprotein value ($\chi^2 = 24.36$, $P < 0.001$), more probability of hepatitis carrying ($\chi^2 = 40.9$, $P < 0.001$), and less lymph node metastasis ($\chi^2 = 5.58$, $P = 0.02$) were correlated with high expression of *FSTL5* ($P < 0.05$) [Table 1].

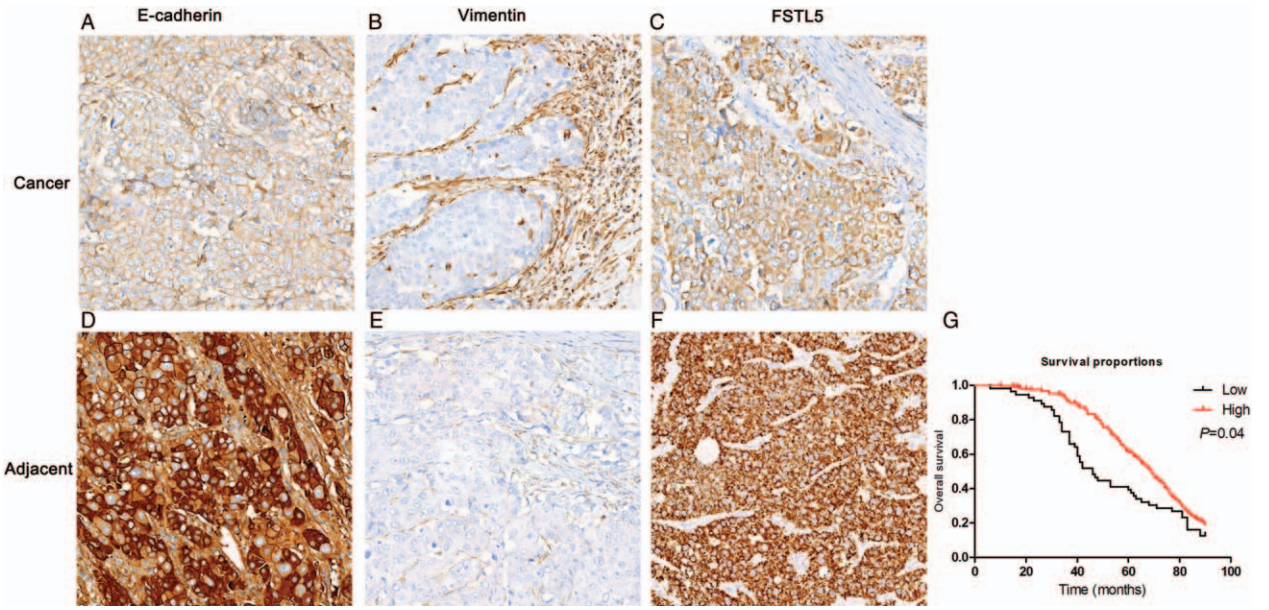

Figure 1: Immunohistochemistry staining of *FSTL5* and epithelial to mesenchymal transition markers E-cadherin and vimentin in HCC tissues (original magnification ×200), and the relationship between *FSTL5* expression and the overall survival of HCC patients. (A) Low expression of E-cadherin in HCC tissues. (B) High expression of vimentin in HCC tissues. (C) Low expression of *FSTL5* in HCC tissues. (D) High expression of E-cadherin in corresponding paracancerous tissues. (E) Low expression of vimentin in corresponding paracancerous tissues. (F) High expression of *FSTL5* in corresponding paracancerous tissues. (G) Kaplan-Meier curve showing overall survival of patients with HCC ($n = 321$, $P = 0.004$, log-rank test); Low: Low expression of FSTL5; High: High expression of FSTL5. *FSTL5*: Follistatin Like 5; HCC: Hepatocellular carcinoma.

Table 1: The correlation of *FSTL5* expression with clinical indexes of hepatocellular carcinoma cell, *n* (%).

Variable	*FSTL5* expression		χ^2	*P*
	High ($n=56$)	Low ($n=265$)		
Age			0.05	0.82
≤50 years	41 (40.3)	190 (190.7)		
>50 years	15 (15.7)	75 (74.3)		
Gender			0.11	0.75
Man	37 (38.0)	181 (180.0)		
Female	19 (18.0)	84 (85.0)		
Lymph node metastasis			5.58	0.02
Yes	43 (35.2)	159 (166.8)		
No	13 (20.8)	106 (98.2)		
Tumor diameter			45.52	<0.001
≥5 cm	10 (17.1)	177 (80.9)		
<5 cm	46 (38.9)	88 (184.1)		
Tumor number			5.05	0.02
1	40 (45.9)	223 (217.1)		
≥2	16 (10.1)	42 (47.9)		
TNM stage			11.29	0.001
I–II	29 (39.4)	197 (186.6)		
III–IV	27 (16.6)	68 (78.4)		
AFP			24.36	<0.001
≤200 μg/L	31 (15.9)	60 (75.1)		
>200 μg/L	25 (40.1)	205 (189.9)		
Hepatitis carrying			40.9	<0.001
Yes	42 (52.5)	259 (248.5)		
No	14 (3.5)	6 (16.5)		
Liver function of Child-Pugh			5.21	0.02
A	27 (34.5)	171 (163.5)		
B	29 (21.5)	94 (101.5)		

FSTL5: Follistatin Like 5; TNM: Tumor node metastasis; AFP: Alpha-fetoprotein.

Table 2: Correlations of *FSTL5* expression with E-cadherin and vimentin in hepatocellular carcinoma cell.

Immunoreactivity	*FSTL5* expression		*r*	*P*
	Low	High		
E-cadherin expression			0.38	<0.001
Low	197	15		
High	68	41		
Vimentin expression			−0.39	<0.001
Low	48	35		
High	217	21		

FSTL5: Follistatin Like 5.

Relationship between FSLT5 and EMT in HCC tissues

To analyze the role of *FSTL5* in EMT of HCC, we assessed the correlation between *FSTL5* expression and EMT markers E-cadherin and vimentin in HCC tissues. IHC showed that high expression of *FSTL5* in HCC was accompanied by high expression of E-cadherin and low expression of vimentin ($r=0.38$, $P<0.001$), while low expression of *FSTL5* was accompanied by low expression of E-cadherin and high expression of vimentin ($r=-0.385$, $P<0.001$) [Figure 1 and Table 2]. These data suggest that *FSTL5* is implicated in the regulation of EMT in HCC.

FSLT5 over-expression inhibits EMT of HCC cells

To confirm that *FSTL5* regulates HCC EMT, we detected changes in the expression of EMT markers at the protein and mRNA levels in HCC cells with the over-expression of *FSTL5* by Western blotting and quantitative real-time PCR, respectively. We found that over-expression of *FSTL5* up-regulated the expression of E-cadherin and down-regulated the expression of vimentin in SK-Hep1 and MHCC-LM3 cells, both at protein and mRNA (HCC-LM3, negative control [NC] *vs.* *FSTL5*-interfering group [Lv-*FSTL5*]: *FSTL5* [$t=31.36$, $P<0.001$], E-cadherin [$t=50$, $P<0.001$], vimentin [$t=72.75$, $P<0.001$];

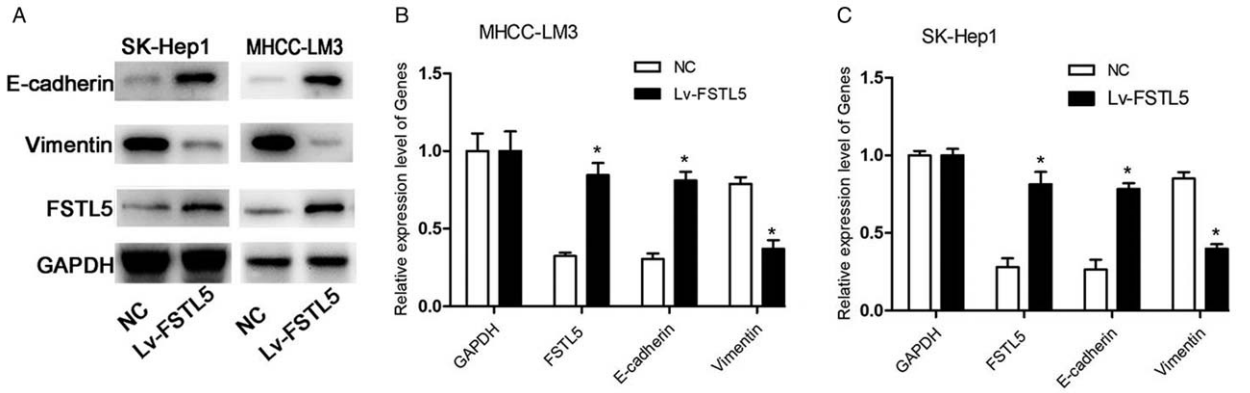

Figure 2: The expression of E-cadherin and vimentin in hepatocellular carcinoma (HCC) cells with the over-expression of *FSTL5*. (A) Western blotting showed that the expression of E-cadherin increased and the expression of vimentin decreased after the over-expression of *FSTL5*. (B, C) *qRT-PCR* analysis showed that the expression of E-cadherin mRNA increased and the expression of vimentin mRNA decreased after *FSTL5* over-expression. MHCC-LM3, NC *vs.* Lv-*FSTL5*: *FSTL5* ($t = 31.36$, $P < 0.001$), E-cadherin ($t = 50$, $P < 0.001$), vimentin ($t = 72.75$, $P < 0.001$); SK-Hep1, NC *vs.* Lv-*FSTL5*: *FSTL5* ($t = 60.85$, $P < 0.001$), E-cadherin ($t = 45.03$, $P < 0.001$), vimentin ($t = 67$, $P < 0.001$). $^{*}P < 0.001$. *FSTL5*: Follistatin Like 5; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; NC: Negative control; Lv-*FSTL5*: *FSTL5*-interfering group; qRT-PCR: Quantitative real-time polymerase chain reaction; EMT: Epithelial to mesenchymal transition.

SK-Hep1, NC *vs.* Lv-*FSTL5*: *FSTL5* [$t = 60.85$, $P < 0.001$], E-cadherin [$t = 45.03$, $P < 0.001$], vimentin [$t = 67$, $P < 0.001$]) levels [Figure 2]. These results indicate that *FSTL5* can inhibit EMT in HCC.

FSTL5 inhibits the proliferation of HCC cells

To investigate the inhibitory effects of *FSTL5* on HCC cells, we assessed changes in the proliferation ability of HCC cells with the over-expression of *FSTL5*. Cell counting kit-8 assay showed that *FSTL5* over-expression inhibited SK-Hep1 (NC *vs.* Lv-*FSTL5*: 1 day [$t = 0.65$, $P = 0.581$], 2 days [$t = 0.56$, $P = 0.632$], 3 days [$t = 7.324$, $P = 0.018$], 4 days [$t = 6.23$, $P = 0.021$], 5 days [$t = 10.21$, $P = 0.003$]) and MHCC-LM3 (NC *vs.* Lv-*FSTL5*: 1 day [$t = 0.46$, $P = 0.911$], 2 days [$t = 0.53$, $P = 0.712$], 3 days [$t = 4.32$, $P = 0.037$], 4 days [$t = 7.49$, $P = 0.012$], 5 days [$t = 9.3661$, $P = 0.009$]) cell proliferation [Figure 3].

FSTL5 inhibits the invasion ability of HCC cells

Furthermore, we investigated the invasion of HCC cells with the over-expression of *FSTL5*. Transwell assay showed that HCC cells with the over-expression of *FSTL5* had significantly lower cell invasion (SK-Hep1, NC *vs.* Lv-*FSTL5*: $t = 21.57$, $P < 0.001$; MHCC-LM3, NC *vs.* Lv-*FSTL5*: $t = 18.04$, $P < 0.001$) [Figure 4].

Discussion

EMT is a process of dedifferentiation and transformation of epithelial cells into mesenchymal cells. During EMT, the expression of E-cadherin and β-catenin is decreased, while the expression of vimentin, fibronectin, N-cadherin, and matrix metalloproteinases is up-regulated. In addition, the expression of EMT related transcription factors such as snail, slug, twist, and Zeb increases.[10] Cells which undergo EMT are more likely to be separated from the original tissue for distant metastasis, and develop chemotherapeutic drug resistance and even tumor stem cell-like characteristics.[11-13] EMT is a key process in

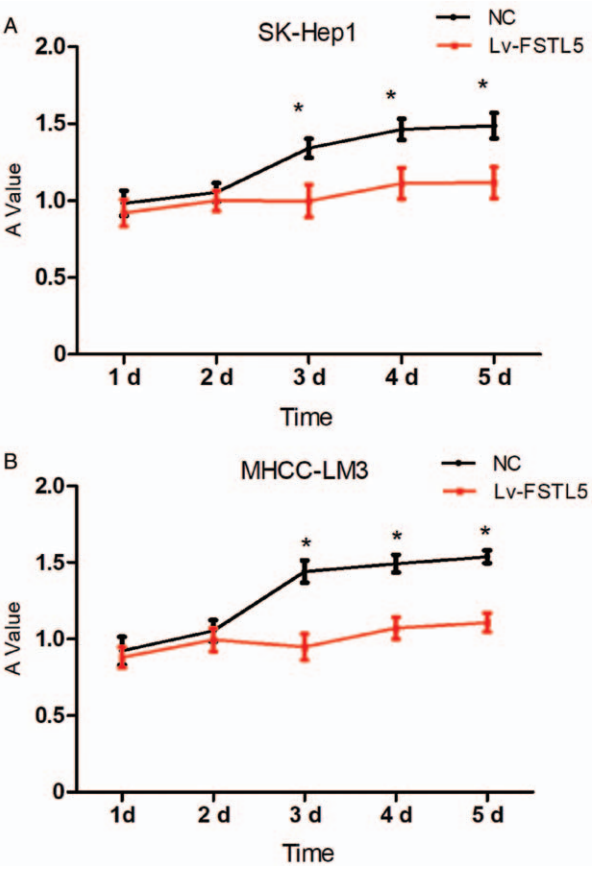

Figure 3: CCK-8 assay of the proliferation ability of hepatocellular carcinoma (HCC) cells after the over-expression of *FSTL5*. (A) The proliferation of SK-Hep1 cells (NC *vs.* Lv-*FSTL5*: 1d [$t = 0.65$, $P = 0.581$], 2d [$t = 0.56$, $P = 0.632$], 3d [$t = 7.324$, $P = 0.018$], 4d [$t = 6.23$, $P = 0.021$], 5d [$t = 10.21$, $P = 0.003$]). (B) The proliferation of MHCC-LM3 cells (NC *vs.* Lv-*FSTL5*: 1d [$t = 0.46$, $P = 0.911$], 2d [$t = 0.53$, $P = 0.712$], 3d [$t = 4.32$, $P = 0.037$], 4d [$t = 7.49$, $P = 0.012$], 5d [$t = 9.3661$, $P = 0.009$]). $^{*}P < 0.05$. CCK-8: Cell counting kit-8; *A*: Absorbance, the optical density at 450 nm; d: Day; NC: Negative control; Lv-*FSTL5*: *FSTL5*-interfering group.

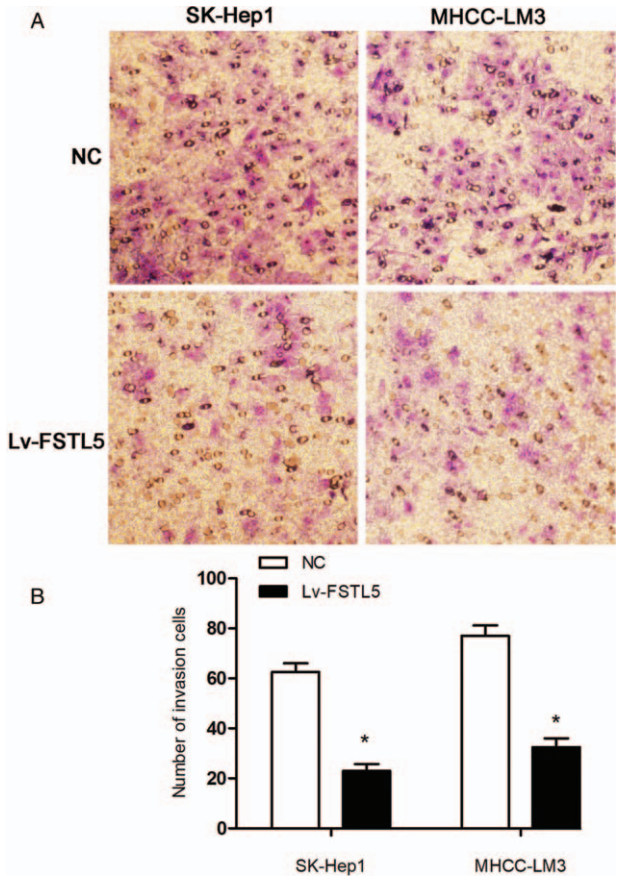

Figure 4: Transwell assay of the invasion ability of hepatocellular carcinoma (HCC) cells after the over-expression of *FSTL5*. (A) The invasion of SK-Hep1 and MHCC-LM3 cells observed under microscope (original magnification × 200, Crystal Violet staining). (B) Quantitative analysis of the number of invaded cells. SK-Hep1, NC *vs.* Lv-*FSTL5*: $t = 21.57$, $P < 0.001$; MHCC-LM3, NC *vs.* Lv-*FSTL5*: $t = 18.04$, $P < 0.001$. $^{*}P < 0.001$. *FSTL5*: Follistatin Like 5; NC: Negative control; Lv-*FSTL5*: *FSTL5*-interfering group.

determining distant metastasis and intra-hepatic dissemination of HCC.[14] Therefore, identifying molecules that can inhibit EMT could help reduce the tendency for metastasis of HCC.

Several members of *FSTL* family such as *FSTL1* and *FSTL3* have been investigated for their role in HCC. *FSTL3* demonstrated low expression in HCC and inhibited *TGF-β* signaling, while *FSTL1* was highly expressed in Hep3B HCC cell line and related to EMT in HCC.[15,16] It was reported that higher expression of *FSTL5* was associated with worse prognosis of *Wnt*/sonic hedgehog (*SHH*)-independent neuroblastomas.[17] However, the role of *FSTL5* in HCC remains largely unclear. In this study, we found that the expression of *FSTL5* in HCC tissues was lower than that in paracancerous tissues, and the expression level of *FSTL5* was negatively correlated with the prognosis of HCC patients. Patients with high expression of *FSTL5* had a longer survival than patients with low expression. However, we only included 321 patients who were followed up, and detected the corresponding HCC tissue samples of these patients. Our number of cases is not very large, and we will continue to increase the number of detection cases in the future to study the value of FSTL5 in evaluating the prognosis of patients with HCC.

To analyze the relationship between *FSTL5* and EMT, we detected the expression of *FSTL5* and EMT markers in HCC tissues. IHC analysis showed that the expression of *FSTL5* was positively correlated with the expression of E-cadherin and negatively correlated with the expression of vimentin. Moreover, using HCC cell lines we demonstrated that the over-expression of *FSTL5* down-regulated the expression of vimentin and up-regulated the expression of E-cadherin, confirming that *FSTL5* could inhibit EMT. Furthermore, we found that the over-expression of *FSTL5* inhibited the proliferation and invasion of HCC cells, consistent with the inhibitory effects of *FSTL5* on EMT. Different from other researchers, we found low expression of *FSTL5* in HCC, while some researchers found high expression of *FSTL1* and low expression of *FSTL3* in HCC. Similarly, we all found that *FSTL* family molecules are closely related to EMT in HCC. EMT is an important process of invasion and metastasis of HCC. So we think that *FSTL* family plays an important role in the process of EMT of HCC.

Previous studies have shown that interference with *FSTL5* expression in lung cancer can activate the expression of Yes-associated protein (YAP), and increase the sensitivity of Kirsten Rat Sarcoma (*KRAS*) mutated lung cancer cells to Exportin 1 (*XPO1*) inhibitors.[18] YAP is closely related to invasion, metastasis, and EMT in many kinds of tumors. In colon cancer cells, YAP can promote the expression of stroma markers such as vimentin and inhibit the expression of epithelial markers such as E-cadherin and occludin. In addition, YAP directly binds to the promoters of slug and vimentin to regulate EMT.[19,20] In addition, interference with YAP can inhibit proliferation, invasion, and EMT of HCC cells. YAP can directly bind to the promoter of *p53* and increase chemotherapeutic sensitivity of HCC cells.[21] The expression of YAP was higher in HCC tissues and lower in paracancerous tissues.[22] Our previous results showed that over-expression of *FSTL5* can regulate the proliferation and apoptosis of HCC via Wnt/β-catenin pathway, which may be regulated by YAP.[9] Combined with the results of this study, we speculate that *FSTL5* can regulate EMT in HCC through Wnt/β-catenin/YAP pathway, but further investigations are needed to reveal the molecular mechanism. For example, the rescue experiment on the regulation of EMT process of HCC YAP has not been studied. This is what we will study later.

This study speculates that *FSTL5* is closely related to prognosis in HCC, and plays an important role in regulating invasion, metastasis, and EMT in HCC. Targeting *FSTL5* may represent a new direction for the treatment of liver cancer.

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Conflicts of interest

None.

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