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ORIGINAL RESEARCH

High Expression of Follistatin is a Risk Factor Predicting Poor Overall Survival in Hepatocellular Carcinoma Patients

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Background: Hepatocellular carcinoma (HCC) is a malignant tumor originating from hepatocytes, characterized by high mortality rates. Follistatin (FST), an inhibitor of follicle-stimulating hormone, correlates with poor prognosis in some cancers, but its role in HCC is unclear. This study intends to clarify FST's impact on HCC prognosis and clinical characteristics.

Materials and Methods: FST expression was compared between HCC and adjacent tissues using Clinical Proteomic Tumor Analysis Consortium (CPTAC) and The Cancer Genome Atlas (TCGA) databases. 78 HCC patients were dichotomized into FST-high and FST-low groups based on the median expression of FST assessed by immunohistochemistry. Kaplan-Meier analysis evaluated recurrence-free (RFS) and overall survival (OS). Univariate/multivariate Cox regression identified OS risk factors; logistic regression analyzed FST's association with clinical features.

Results: FST was upregulated in HCC tissues. High FST correlated with Larger tumor size (OR = 2.030, P = 0.025), Microvascular invasion (OR = 1.933, P = 0.037), Elevated AFP (OR = 2.046, P = 0.024). Patients in the FST-high group had shorter mean OS (53.189 ± 6.37 vs. 94.832 ± 7.739 months, P = 0.0084). Multivariate Cox confirmed FST as an independent OS predictor (HR = 3.88, P = 0.0003), alongside tumor size (HR = 4.33, P = 0.0025) and gender (HR = 0.309, P = 0.0421).

Conclusion: FST is upregulated in HCC tissues and serves as an independent prognostic factor for poor overall survival. High FST expression was strongly correlated with aggressive clinicopathological features, including larger tumor burden, microvascular invasion (MVI), and elevated AFP.

Keywords: hepatocellular carcinoma, follistatin, prognosis

Introduction

Hepatocellular carcinoma (HCC) is a malignant tumor characterized by insidious onset, high recurrence rate, and poor prognosis.[1] According to 2022 global cancer statistics, incidence and mortality rates of liver cancer in all genders rank 6th and 3rd among all cancers respectively,[2] and rank 4th and 2nd in China respectively,[3] indicating a particularly heavy disease burden. Although many patients are diagnosed at an advanced stage, surgical resection is an effective curative treatment for early cases.[4] However, high postoperative recurrence rate remains a major challenge in HCC treatment, compromising the overall survival of these patients.[5] Meanwhile, despite continuous improvements in the latest treatment methods, the 5-year relative survival rate for liver cancer patients of all races is as low as 22% in the USA during.2014 −2020.[6] A myriad of factors contribute to the poor prognosis in HCC patients, including advanced age, male gender, cirrhosis, and high tumor burden.[7] Furthermore, the insufficient understanding of molecular risk factors severely hinders the management of post-surgical patients, as inappropriate therapeutic decisions may fail to halt disease progression or even compromise residual liver function. Currently, alpha-fetoprotein (AFP) is the most widely used biomarker for HCC;

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however, its clinical utility is often limited by unsatisfactory sensitivity and specificity, because elevated AFP level can also be found in other benign liver diseases, such as hepatitis C or cirrhosis,[8–11] leaving a significant number of patients without effective prognostic indicators. Therefore, it is imperative to identify novel prognostic biomarkers in HCC, which facilitates the development of precise prediction models and offers new insights for therapeutic intervention.

Follistatin (FST) is a secreted single-chain glycoprotein,[12] first isolated from porcine follicular fluid, which specifically inhibits pituitary secretion of FSH.[13] Its main biological function is to antagonize the transforming growth factor-β (TGF-β) superfamily signaling pathways, including molecules such as activins, myostatin, TGF-βs, and bone morphogenetic proteins (BMPs).[14,15] Accumulating evidence suggests that FST is associated with tumor prognosis. Pretreatment serum FST levels can effectively predict the efficacy of PD-1/L1 inhibitor therapy in non-small cell lung cancer patients.[16] On the other hand, FST can suppress metastasis of HER2-negative breast cancer in mouse models.[17] In prostate cancer, serum FST levels show a high predictive value for patients with bone metastasis.[18,19] In the field of HCC, some studies have preliminarily explored the role and clinical significance of FST. Tomoda et al studied serum FST levels in HCC patients and found that patients with high serum FST levels had significantly shorter overall survival, larger tumor size, and higher portal vein cancer embolus incidence compared to the low serum FST level group.[20] Choi et al made similar findings, reporting higher serum FST levels in HCC patients compared to non-HCC patients, and that serum FST level independently predicted poor prognosis.[21] However, circulating FST levels can be influenced by many systemic metabolic factors, such as endometriosis or polycystic ovary syndrome,[22,23] thus may not accurately reflect the local biological behavior within the tumor. Consequently, whether in situ FST expression within the tumor microenvironment serves as a more direct prognostic indicator and how it potentially drives carcinogenic mechanisms remain largely unknown.

In this study, we investigated FST's predictive role for the prognosis of HCC patients, by exploring the impact of its expression levels in tumor tissue. Using the Cancer Genome Atlas (TCGA) and the Clinical Proteomic Tumor Analysis Consortium (CPTAC), we investigated the difference in FST expression between HCC tumor tissue and adjacent tissue. Subsequently, we determined the FST expression level in tumor tissues of 78 previous HCC patients after immunohistochemical staining. We then performed survival analysis using the Kaplan-Meier method to explore the impact of FST expression levels on recurrence-free survival (RFS) and overall survival (OS). Finally, Cox proportional hazards regression models and logistic regression were applied to analyze the clinical risk factors through which FST causes poor prognosis in HCC patients, and to determine the impact of FST expression on different clinical factors, to better serve HCC treatment and prognosis prediction.

Materials and Methods

Analysis of FST Expression in HCC Based on Public Databases

To validate FST expression levels in HCC tissues, publicly available databases of liver cancer protein expression data were used, and statistical graphs were generated using online tools. The Cancer Genome Atlas (TCGA) database is from a research project of the US National Cancer Institute. We used the online tool GEPIA2 (http://gepia2.cancer-pku.cn/) to access and obtain mRNA expression sequencing data from the TCGA liver cancer sample database (LIHC), comprising 369 tumor tissues and 160 adjacent tissues, and generated a box plot. The Clinical Proteomic Tumor Analysis Consortium (CPTAC) database is also from a US National Cancer Institute project. We used the online tool UALCAN (https://ualcan.path.uab.edu/) to access and obtain FST expression data from 165 paired HCC tumor and adjacent tissues in CPTAC and generated a box plot.

Immunohistochemical Staining

Source of Pathological Samples

A tissue microarray (TMA) containing tumor tissues from patients who underwent hepatic resection for HCC at our center between November 2014 and May 2019, with postoperative pathological confirmation of HCC and subsequent follow-up, was collected. Importantly, all eligible patients during the study period were consecutively enrolled to minimize potential selection bias.

Inclusion criteria: (1) Age over 18 years old; (2) The patient was diagnosed with liver cancer at our center and underwent liver cancer resection surgery for the first time; (3) The postoperative pathological diagnosis was "hepatocellular carcinoma".

Exclusion criteria: (1) Patients who have previously undergone liver cancer resection surgery; (2) Patients with other types of tumors.

78 patients were enrolled. Clinical information related to liver cancer treatment was extracted from patient medical records, including age, gender, tumor size, tumor number, TNM stage, pathological grade, AFP level, capsule integrity, degree of cirrhosis, and hepatitis B surface antigen status, no HCV or other virus infections were found in these patients. Outcome information, including patient death, time of death, cause of death, recurrence status, recurrence time, and last follow-up time, was extracted from follow-up data. Sample collection was conducted with informed patient consent and approved by the hospital's Ethics Review Committee (the Fifth Affiliated Hospital of Sun Yat-sen University), strictly following the Declaration of Helsinki.

Immunohistochemical Staining Procedure

Immunohistochemistry (IHC) was performed to detect FST protein expression in HCC tissues. Briefly, pathological slides were baked in a 70°C oven for 15 min, immersed in xylene twice for 15 min each in a fume hood, then sequentially immersed in 100%, 95%, 90%, 80%, 70%, and 60% ethanol for 5 min each, and finally immersed in distilled water twice for 5 min each. After drying, an appropriate amount of 3% H_2O_2 was added and incubated at room temperature for 15 min. After washing off H_2O_2, the slides were immersed in citrate-based antigen retrieval buffer, heated to boiling in a microwave oven, and repeated twice. After cooling, 5% BSA was added dropwise and blocked at 37°C for 30 min. After washing, the slides were incubated overnight at 4°C with a 1:200 dilution of rabbit anti-FST antibody (Catalog # ab157471, Abcam, Cambridge, UK). After washing off the antibody, an appropriate amount of biotin-labeled goat anti-mouse IgG was added dropwise and incubated at 37°C for 30 min, followed by an appropriate amount of SABC and incubation at 37°C for 30 min. An appropriate amount of 3,3′-diaminobenzidine (DAB) chromogen solution (Catalog #: AR1027, Boster Biological Technology Co., Ltd., Wuhan, China) was added dropwise, and color development was observed under a microscope. Development was terminated (typically 1–2 min) when sufficient brown-yellow staining was observed under the microscope, and rinsed thoroughly with water. An appropriate amount of hematoxylin was added dropwise for counterstaining for 1 min, and the staining solution was washed off. The slides were immersed in a 1% hydrochloric acid in anhydrous ethanol solution for 2 seconds, then rinsed extensively with running water. The slides were dried at 37°C, then sequentially re-immersed following the reverse order of the dewaxing-to-hydration steps for 2 min each, air-dried, and an appropriate amount of neutral resin was added for mounting, waiting for the resin to solidify. Images of the mounted chip were acquired using an optical microscope (100x magnification). The 3% H_2O_2, 5% BSA blocking solution, secondary antibody, SABC, and DAB chromogen solution used in the above steps were all from the SA1021 - Mouse IgG SABC IHC Staining Kit (Boster Biological Technology Co., Ltd., Wuhan, China). All stained images were independently evaluated by two experienced pathologists who were blinded to the clinical information. Quantitative analysis was performed using Image-Pro Plus 6.0 (Media Cybernetics, USA), and the average of the two independent assessments was used as the final score. The inter-observer agreement was high, indicating good reproducibility of the scoring. Brown-yellow stained areas were selected, their Integrated Optical Density (IOD) and area were measured, and the average IOD per unit area for the stained part of each slice was calculated to reflect the relative expression level of FST protein.

Western Blotting Assay

Sample Loading, Protein Electrophoresis and Transfer

10 randomly selected postoperative pathological tissues of HCC patients were collected. Protein samples from the tumor tissues and adjacent tissues were extracted respectively using the RIPA method and quantified using BCA method. 20μg protein sample was added in a Nuclease-free EP tube, and diluted into 20μL using loading buffer. Prepared protein samples were loaded into the wells of the stacking gel of a 10% SDS-PAGE gel. Electrophoresis was performed at 80

V for 30 min and 120 V for 45 min for protein separation. Proteins were then transferred to a PVDF membrane under conditions of 250 mA for 3 h.

Non-Specific Antigen Blocking

After transfer, the PVDF membrane was incubated with sufficient 5% skim milk solution as blocking buffer at room temperature with gentle agitation for 1 h.

Primary Antibody Incubation

The blocking solution was removed, and the membrane was briefly rinsed with TBST buffer to remove residual blocking solution. Rabbit anti-FST primary antibody (lot: ab157471, Abcam, Cambridge, UK) diluted at 1:1000 was added. The membrane was gently agitated to ensure full contact with the antibody solution and incubated overnight at 4°C on a shaker, for 8–12h. The next day, the primary antibody was removed, and the membrane was washed at room temperature with ample 1× TBST buffer for 5 min, repeated three times.

Secondary Antibody Incubation

After thoroughly removing the wash buffer, anti-rabbit IgG secondary antibody diluted at 1:2000 was added at 1 mL per membrane. The membrane was gently agitated for full coverage and incubated at room temperature for 1 h on a shaker. The secondary antibody was then collected, and the membrane was washed at room temperature with ample 1× TBST buffer under gentle agitation for 5 min, repeated three times.

Membrane Trimming and Chemiluminescence

With reference to the band positions of the protein molecular weight marker (Pageruler 19919), the regions of the PVDF membrane containing the FST band (approximately 35 kDa) and the internal reference β-actin band (approximately 37 kDa) were trimmed. The membrane pieces were placed face-up in a detection cassette and covered with a high-sensitivity ECL chemiluminescent substrate (product No.: 180–5001, Tanon™ High-sig ECL Western Blotting Substrate, China). After incubation for 1 min, excess substrate was removed, and the membrane was imaged using an automated fluorescence/chemiluminescence image analysis system (Tanon, Shanghai, China). The relative gray value of the FST band was calculated as the ratio of the FST band gray value to the β-actin band gray value (FST/β-actin) to compare the relative expression levels of FST protein between the two cell groups.

Statistical Analysis Methods

In this study, continuous variables conforming to a normal distribution are expressed as mean ± standard deviation, while those not conforming to a normal distribution are expressed as median. The *t*-test was used for hypothesis testing of continuous variables. Categorical variables are expressed as number (n) or percentage (n%) and were compared using the χ^2-test. Survival curves were plotted using the Kaplan-Meier method and compared using the Log rank test. Cox proportional hazards regression analysis was used to identify clinical factors affecting overall survival (OS). Univariate logistic regression analysis was used to assess the impact of FST expression level on clinical factors, the continuous IOD_mean value was first converted to a Z-score. The Z-score was calculated using the formula Z-score = $\frac{(X-\bar{X})}{S}$ (where X is the variable value, $\bar{X}$ is the sample mean, and S is the sample standard deviation) for the FST level (IOD_mean), and this was used as the independent variable in univariate logistic regression analysis. All statistical analyses were performed using SPSS 25.0 (IBM, New York, USA). Statistical graphs were generated using GraphPad Prism 9.4.1 (GraphPad Software, USA). In all hypothesis tests, $P < 0.05$ was considered statistically significant.

Results

Increased Expression Level of FST in HCC Tissues From CPTAC and TCGA Database

To determine the expression level of FST in HCC tissues, we first analyzed the data from the TCGA and CPTAC databases. The results revealed that FST expression levels were elevated in tumor tissues compared to adjacent liver tissues, regarding both its protein (CPTAC, Figure 1A) and mRNA expression level (TCGA, Figure 1B) within the HCC tissues.

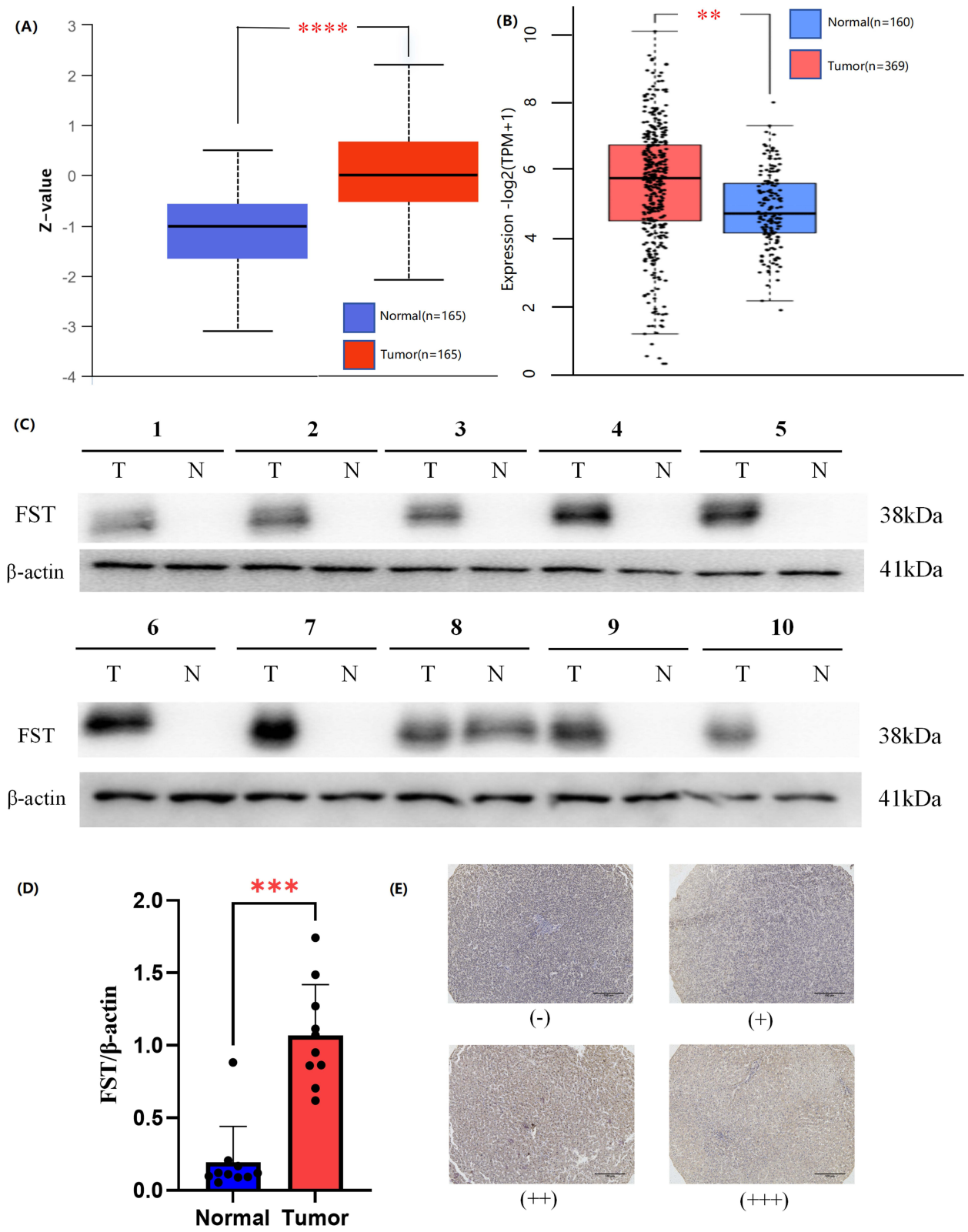

Figure 1 FST expression in HCC tissues. (**A** and **B**) FST expression differences between HCC tumor tissues and adjacent normal liver tissues. (**A**) FST protein expression data from the CPTAC database, **** P < 0.0001; (**B**) FST mRNA expression data from the TCGA database, ** P < 0.01. (**C** and **D**) Western blotting image of tumor tissues and adjacent normal liver tissues from 10 patients. "T" represents tumor tissues, "N" represents adjacent normal liver tissues; paired *t*-test showed significant differences of FST expression between tumor and normal tissues (***P = 0.0004). (**E**) Immunohistochemical staining of FST in HCC pathological slides. (-) ~ (+++) represents 4 intensity levels of FST staining representing negative, weak positive, moderate positive and strong positive. The scale bar in the figure indicates a length of 100 μm.

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Table 1 Baseline Clinical Features of All Included HCC Patient

Clinical Factor	FST Low (n=39)	FST High (n=39)
Age [n (%)]		
<50 years	16 (41.0)	11 (28.2)
≥50 years	23 (59.0)	28 (71.8)
Gender [n (%)]		
Male	34 (87.2)	31 (79.5)
Female	5(12.8)	8(20.5)
TNM Stage [n (%)]		
I, II	32 (82.1)	31 (79.5)
III, IV	7(17.9)	7(20.5)
MVI [n (%)]		
No	31 (79.5)	28 (71.8)
Yes	8 (20.5)	11 (28.2)
Pathology differentiation grade[m (%)]		
Mid-high	29 (74.4)	30 (76.9)
Low	10 (25.6)	9 (23.1)
Integrality of the capsule[n (%)]		
Yes	29 (74.4)	28 (71.8)
No	10 (25.6)	11 (28.2)
Cirrhosis[n (%)]		
No	16 (41.0)	23 (59.0)
Yes	23 (59.0)	16 (41.0)
HBsAg[n (%)]		
Negative	5(12.8)	8(20.5)
Positive	34 (87.2)	31 (79.5)
AFP[n (%)]		
<400ng/mL	26 (66.7)	22 (56.4)
≥400ng/mL	13 (33.3)	17 (43.6)
Number of tumors[n (%)]		
One	26 (25.6)	24 (28.2)
More than one	13 (74.4)	15 (71.8)
Size of tumor[n (%)]		
<5cm	32 (82.1)	31 (79.5)
≥5cm	7 (17.9)	8 (20.5)

Abbreviations: TNM stage, based on the eighth edition of the AJCC (American Joint Committee on Cancer) tumor staging criteria; MVI, microvascular invasion; HbAg, hepatitis B surface antigen; AFP, alpha-fetoprotein.

Western Blotting Validated Increased Expression of FST in HCC Tissues From Our Center

To confirm the expression of FST between para-tumoral and tumoral tissues in HCC, we next performed Western Blotting in the post-surgical samples collected at our center. The result validated that FST was increased in the HCC tissues (Figure 1C and D).

To further validate the impact of FST expression level on patient prognosis in HCC, we next performed IHC staining of FST to determine its expression levels on the HCC tissue microarray from 78 patients. General characteristics of all included patients were shown in Table 1.

After scoring the IHC stained paraffin-embedded slices, we quantified their FST expression levels. The typical images show the different staining status of FST within HCC tissues, indicating the scores from negative to strong positive (Figure 1E).

Table 2 Univariate Logistic Regression Analysis FST Expression and Various Clinical Factors

Clinical Factor	Z-score of FST (IOD_mean)			
	OR	HR 95% CI		P value
		Lower	Upper	
Age(years):<50 vs≥50	1.02	0.638	1.67	0.945
Gender: male vs female	1.09	0.572	1.88	0.780
TNM stage: I-II vs III-IV	1.24	0.707	2.13	0.423
MVI: no vs yes	1.93	1.11	3.91	**0.0367***
Pathology differentiation grade:mid-high vs low	1.39	0.842	2.44	0.200
Integrality of the capsule:yes vs no	0.885	0.491	1.46	0.652
Cirrhosis:no vs yes	0.653	0.362	1.06	0.117
HBsAg:negative vs positive	0.861	0.496	1.59	0.595
AFP (ng/mL):<400 vs≥400	2.05	1.18	4.07	**0.0235***
Number of tumors: one vs more than one	1.18	0.708	1.95	0.510
Size of tumor(cm): <5 vs≥5	2.02	1.17	4.03	**0.025***

Note: Bold values indicate statistical significance ($p < 0.05$), *: $P < 0.05$.

Abbreviations: TNM stage, based on the eighth edition of the AJCC (American Joint Committee on Cancer) tumor staging criteria; MVI, microvascular invasion; HbAg, hepatitis B surface antigen; AFP, alpha-fetoprotein.

High Expression of FST Was Significantly Correlated with Increased Tumor Size, MVI and AFP Level in HCC Patients

To further validate whether FST expression was correlated with any clinicopathological factors, we performed univariate logistic regression analysis, shown in Table 2. The results revealed that higher FST expression was significantly correlated with increased tumor size [OR = 2.02, 95% CI 1.17–4.03, P = 0.0252], enhanced microvascular invasion [OR = 1.93, 95% CI 1.11–3.91, P = 0.0367], and higher AFP level [OR = 2.05, 95% CI 1.18–4.07, P = 0.0235] were statistically significant. Notably, 22 patients in the high FST group presented low AFP level (< 400 ng/mL).

Subsequently, using the median of the average optical density value (IOD_mean) in the IHC staining areas as the cutoff, we divided the samples into the FST low-expression group (FST low group) and the FST high-expression group (FST high group). The baseline data of the two groups are summarized below (see Table 3). The differences in clinical factors such as age, gender, TNM stage, microvascular invasion (MVI) status, pathological grade, capsule integrity, cirrhosis status, hepatitis B status, tumor number, and tumor size between the two groups were not statistically significant (P > 0.05), suggesting that the baseline data of the two patient groups were comparable.

High Expression of FST Was Correlated with Poor Clinical Outcomes

Results of survival analysis based on the clinical data of the two groups were shown in Figure 2. Regarding recurrence-free survival (RFS), the median RFS was 56.04 ± 9.439 months in the FST low group and 27.96 ± 8.936 months in the FST high group. Comparison of their survival curves using the Log rank test and univariate Cox model showed no statistically significant difference between the two groups [HR = 1.960, 95% CI 0.7972–4.820, $\chi^2 = 2.15$, P = 0.143] (Figure 2A). For overall survival (OS), the mean postoperative OS was 94.832 ± 7.739 months in the FST low group and 53.189 ± 6.37 months in the FST high group. Comparison of their overall survival curves using the Log rank test and univariate Cox model showed a statistically significant difference between the two groups [HR = 2.301, 95% CI 1.228–4.312, $\chi^2 = 6.943$, P = 0.008] (Figure 2B). These results indicated that FST expression level in HCC tumor tissue adversely affected patient overall survival, with high FST expression leading to shortened OS.

To further analyze which clinicopathological factors were potentially influenced by FST to affect overall survival, we included clinical factors such as age, gender, TNM stage, MVI status, pathological grade, capsule integrity, cirrhosis status, hepatitis B status, tumor number, and tumor size, together with FST level, and performed Cox proportional

Table 3 Correlation of FST Expression with Different Clinical Factors in HCC Patients

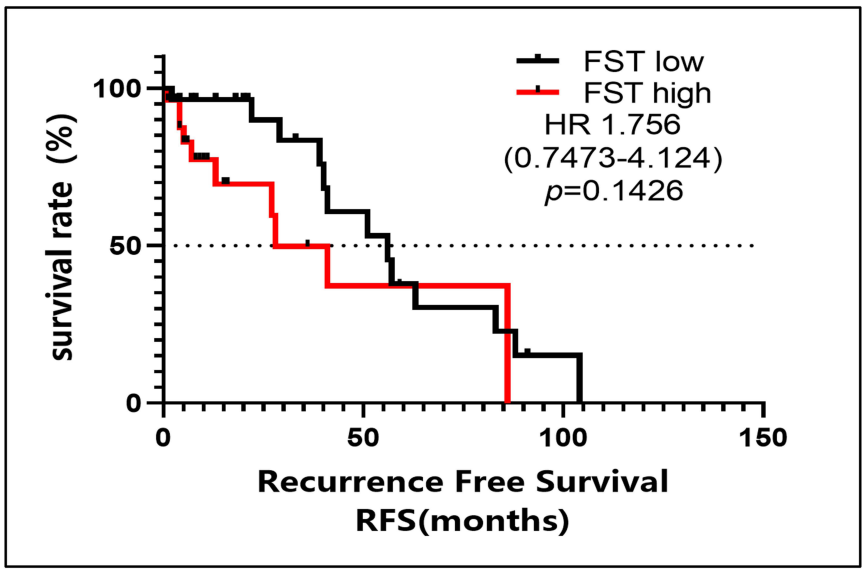
Clinical Factor	FST Low (n=39)	FST High (n=39)	P value
Age [n (%)]			0.341
<50 years	16 (41.0)	11 (28.2)	
≥50 years	23 (59.0)	28 (71.8)	
Gender [n (%)]			0.545
Male	34 (87.2)	31 (79.5)	
Female	5 (12.8)	8 (20.5)	
TNM Stage [n (%)]			>0.999
I, II	32 (82.1)	31 (79.5)	
III, IV	7(17.9)	7(20.5)	
MVI [n (%)]			>0.999
No	31 (79.5)	28 (71.8)	
Yes	8 (20.5)	11 (28.2)	
Pathology differentiation grade [m (%)]			0.599
Mid-high	29 (74.4)	30 (76.9)	
Low	10 (25.6)	9 (23.1)	
Integrality of the capsule [n (%)]			>0.999
Yes	29 (74.4)	28 (71.8)	
No	10 (25.6)	11 (28.2)	
Cirrhosis [n (%)]			0.174
No	16 (41.0)	23 (59.0)	
Yes	23 (59.0)	16 (41.0)	
HBsAg [n (%)]			0.545
Negative	5 (12.8)	8 (20.5)	
Positive	34 (87.2)	31 (79.5)	
AFP [n (%)]			0.485
<400ng/mL	26 (66.7)	22 (56.4)	
≥400ng/mL	13 (33.3)	17 (43.6)	
Number of tumors [n (%)]			0.814
One	26 (25.6)	24 (28.2)	
More than one	13 (74.4)	15 (71.8)	
Size of tumor[n (%)]			>0.999
<5cm	32 (82.1)	31 (79.5)	
≥5cm	7 (17.9)	8 (20.5)	

Notes: The statistical differences of each factor in the table were all tested by the chi-square test ($\chi 2$ test).
Abbreviations: TNM stage, based on the eighth edition of the AJCC (American Joint Committee on Cancer) tumor staging criteria; MVI, microvascular invasion; HbsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein.

hazards regression analysis, shown in Table 4. TNM stage [HR = 3.36, 95% CI 1.57–6.70, P = 0.0009], pathological grade [HR = 2.76, 95% CI 1.40–5.24, P = 0.0024], tumor number [HR = 3.05, 95% CI 1.55–5.82, P = 0.0008], tumor size [HR = 3.66, 95% CI 1.93–6.92, P = 0.0008] and FST level [HR = 2.29, 95% CI 1.22–4.44, P = 0.0116] showed statistically significant associations with OS. To further eliminate the influence of confounding factors, we conducted a multivariate Cox regression analysis. The results are shown in Table 5. In conclusion, gender [HR = 0.309, 95% CI 0.0858–0.869, P = 0.0421], tumor size [HR = 4.33, 95% CI 1.69–11.4, P = 0.0025], and FST level [HR =3.88, 95% CI 1.89–8.32, P = 0.0003] were finally included in the model, which showed statistically significant associations with OS.

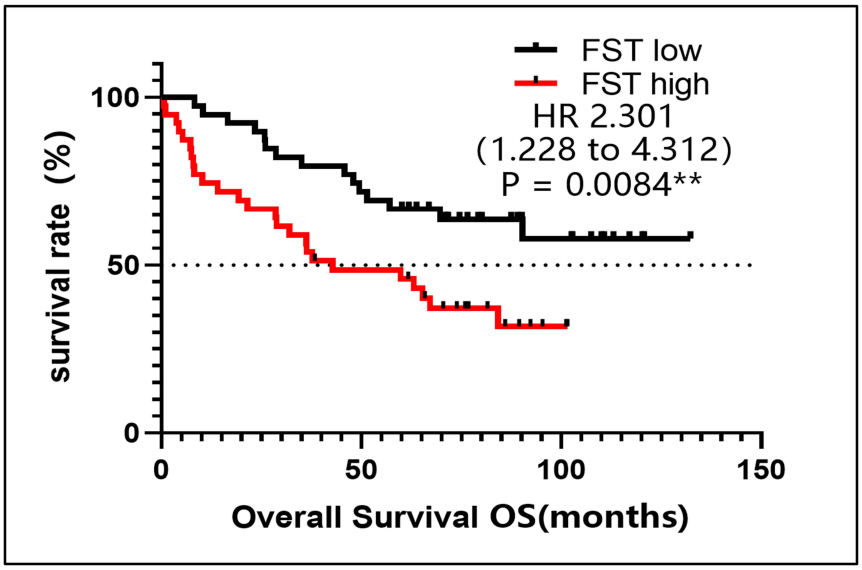
Discussion

Despite advances in early diagnosis and curative treatments such as hepatic resection and radiofrequency ablation, the long-term survival of HCC patients remains unsatisfactory, largely due to high rates of postoperative recurrence.[2,7] In this study, to address the urgent need for novel biomarkers, we provided the first evidence that higher intratumoral FST expression increased aggressive tumor phenotypes, including larger tumor size, microvascular invasion, and higher AFP



number at risk

low	26	13	6	4	0
high	27	8	4	2	0



number at risk

low	39	32	26	24	24
high	39	23	16	14	14

Figure 2 Survival analysis of different groups in FST level. (**A**) Comparison of recurrence-free survival (RFS) between HCC patients with different FST expression level. Median RFS was 56.04 ± 9.439 months in the FST low group and 27.96 ± 8.936 months in the FST high group, P = 0.143 (Log rank test), HR = 1.960, 95% CI 0.7972–4.820 (from univariate Cox model). (**B**) Comparison of overall survival (OS) between HCC patients with different FST expression levels. Mean OS was 94.832 ± 7.739 months in the FST low group and 53.189 ± 6.37 months in the FST high group, P = 0.008 (Log rank test). HR = 2.301, 95% CI 1.228–4.312 (from univariate Cox model).
Note: ** P < 0.01.

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Table 4 Univariate Cox Regression Analysis of 78 HCC Patients

Clinical factor	Overall Survival			
	HR	HR 95% CI		P value
		Lower	Upper	
Age(years): <50 vs≥50	1.13	0.602	2.20	0.706
Gender: male vs female	0.471	0.141	1.18	0.154
TNM stage: I-II vs III-IV	3.36	1.57	6.70	***0.0009
MVI: no vs yes	1.60	0.737	3.17	0.205
Pathology differentiation grade:mid-high vs low	2.76	1.40	5.24	**0.0024
Integrality of the capsule: yes vs no	1.04	0.497	2.03	0.910
Cirrhosis: no vs yes	0.967	0.517	1.81	0.915
HBsAg: negative vs positive	1.57	0.673	4.58	0.346
AFP (ng/mL): <400 vs ≥400	1.35	0.711	2.52	0.345
Number of tumors: one vs more than one	3.05	1.55	5.82	***0.0008
Size of tumor(cm): <5 vs ≥5	3.66	1.93	6.92	****<0.0001
FST level: low vs high	2.29	1.22	4.44	*0.0116

Note: Bold values indicate statistical significance (p < 0.05), *:P<0.05. **:P<0.01. ***:P<0.001. ****:P<0.0001.
Abbreviations: TNM stage, based on the eighth edition of the AJCC (American Joint Committee on Cancer) tumor staging criteria; MVI, microvascular invasion; HbsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein.

Table 5 Multi-Variate COX Regression Analysis of 78 HCC Patients

Clinical Factor	Overall Survival			
	HR	HR 95% CI		P value
		Lower	Upper	
Age(years): <50 vs≥50	1.28	0.628	2.69	0.504
Gender: male vs female	0.310	0.0858	0.869	*0.0421
TNM stage: I-II vs III-IV	2.02	0.629	6.48	0.234
MVI: no vs yes	0.710	0.295	1.59	0.420
Pathology differentiation grade:mid-high vs low	1.94	0.809	4.45	0.127
Integrality of the capsule: yes vs no	1.33	0.575	2.95	0.488
Cirrhosis: no vs yes	1.17	0.530	2.57	0.703
HBsAg: negative vs positive	2.24	0.794	7.87	0.162
AFP (ng/mL): <400 vs ≥400	0.515	0.223	1.16	0.114
Number of tumors: one vs more than one	2.15	0.776	5.68	0.129
Size of tumor(cm): <5 vs ≥5	4.33	1.69	11.4	**0.0025
FST level: low vs high	3.88	1.89	8.32	***0.0003

Note: Bold values indicate statistical significance (p < 0.05), *:P<0.05. **:P<0.01. ***:P<0.001.
Abbreviations: TNM stage, based on the eighth edition of the AJCC (American Joint Committee on Cancer) tumor staging criteria; MVI, microvascular invasion; HbsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein.

level. Furthermore, our data demonstrated that elevated FST expression serves as an independent prognostic factor for overall survival (OS). These findings highlight the potential of FST as a useful biomarker for OS prediction and suggest that it may play a critical role in the malignant progression of HCC. While FST is a strong predictor of OS, its role as an independent predictor of recurrence was not confirmed in this cohort and requires further study.

To validate the prognostic value of FST at the protein level, we performed IHC staining on tissues from 78 HCC patients. Consistent with our hypothesis, patients in the high FST expression group exhibited significantly shorter OS compared to the low expression group (53.19 ± 6.37 months vs. 94.83 ± 7.74 months, P = 0.008), confirming that intratumoral FST protein level is a good predictor of patient survival. Regarding RFS, although a distinct trend towards

shorter recurrence intervals was observed in the high expression group, the difference did not reach statistical significance (P = 0.143). Nevertheless, the Kaplan-Meier curves clearly demonstrate a separation between the two groups, suggesting a potential biological impact. Crucially, multivariate Cox regression analysis demonstrated that high FST expression remained an independent risk factor for shortened OS after adjusting for confounding variables such as TNM stage and pathological grade, indicating that FST captures specific malignant biological behaviors not fully encompassed by traditional staging systems.

A notable finding in our study was the significant positive correlation between FST overexpression and larger tumor size, which comprehensively reflects the biological characteristics of the primary liver tumor and can predict patient prognosis better than many other clinical indicators.[24–26] Therefore, various mainstream HCC staging systems, such as the American Joint Committee on Cancer (AJCC) pathological stage, Barcelona Clinic Liver Cancer (BCLC) stage, and China Liver Cancer (CNLC) stage, consider tumor size as one of the most important factors in HCC staging. Therefore, it indicates that FST could be a potent driver of tumor proliferation. Tumor growth is not only determined by the cancer cells themselves but also by their interaction with the tumor microenvironment (TME). In prostate cancer, FST secreted by cancer-associated fibroblasts (CAFs) was shown to enhance the proliferation and survival of tumor cells via paracrine signaling.[27] Similarly, in lung cancer, FST contributes to a pro-tumorigenic niche that supports rapid tumor expansion.[28] In the context of HCC, we believe that FST, highly expressed by tumor cells, may create a positive feedback loop with stromal cells (such as CAFs), thereby accelerating the mitotic rate and inhibiting apoptosis, ultimately resulting in the larger tumor burden observed in our high-expression cohort.

Beyond tumor burden, the robust association observed between high FST expression and microvascular invasion (MVI) is of particular clinical significance. MVI is a histological marker of early vascular invasion and is fundamentally linked to pathological angiogenesis, which are linked to intrahepatic dissemination and early recurrence in HCC and poor prognosis.[29,30] Previous research in thymic epithelial tumors has demonstrated that high tissue FST levels can significantly enhance angiogenesis within the TME.[31] Biologically, FST is a known antagonist of activins and BMPs, both of which play complex roles in regulating vascular endothelial cell quiescence and stability.[14] By antagonizing these signals, FST may promote a chaotic, immature vascular network that facilitates tumor cell intravasation. Clinically, this suggests that FST-driven vascular remodeling might contribute to resistance against anti-angiogenic therapies. Since Tyrosine Kinase Inhibitors (TKIs) like Sorafenib and Lenvatinib target VEGF receptors,[32] FST-mediated alternative angiogenic pathways could theoretically act as an escape mechanism, rendering standard TKI treatments less effective in FST-high patients.

Furthermore, the correlation with elevated AFP levels suggests that FST enhances the invasive and metastatic potential of HCC. AFP is a classic serum marker for HCC diagnosis and prognosis monitoring.[33] High AFP levels are classically associated with poor differentiation and a "stem-like" cell state.[34,35] In brief, FST can promote epithelial-mesenchymal transition (EMT) and maintain stemness features in HCC cells, thereby increasing cell motility and facilitating the colonization of cancer cells at distant sites within the liver.

Collectively, our findings underscore the potential of intratumoral FST expression not only as a prognostic biomarker but also as a versatile tool for clinical management in HCC. In terms of diagnosis and risk stratification, assessing FST expression via biopsy or post-surgical pathology could serve as a valuable complement to traditional markers like AFP. For patients with AFP-negative HCC, high tissue FST levels could alert clinicians to a "high-risk" phenotype prone to early recurrence, prompting more intensive postoperative surveillance. Regarding therapeutic guidance, the link between FST, MVI, and angiogenesis provides a rationale for precision medicine. Patients with high FST expression might harbor tumors with active vascular remodeling and potential resistance to standard anti-angiogenic TKIs. For these patients, combination therapies involving immune checkpoint inhibitors or novel agents targeting the TGF-β/Activin axis might be more beneficial than TKI monotherapy. Finally, from a drug development perspective, FST represents a promising therapeutic target. Strategies to neutralize FST (eg., using monoclonal antibodies) or to restore the signaling of its antagonized ligands (such as Activins) could theoretically inhibit tumor proliferation and normalize tumor vasculature. Future preclinical studies are warranted to evaluate the efficacy of FST-targeted interventions in reversing the aggressive phenotypes observed in this study.

However, several limitations of this study should be acknowledged. First, this was a single-center retrospective study with a relatively small cohort size. This limited statistical power likely explains why the difference in RFS did not reach statistical significance, despite the substantial numerical difference in median RFS between the high and low expression groups and the strong correlation between FST and MVI. Moreover, our study focused on the clinical significance of FST protein expression in tissue samples. While we proposed potential mechanisms such as FST-mediated angiogenesis or TKI resistance based on the significant associations with MVI and tumor size, these hypotheses lack validation through in vitro or in vivo functional experiments in this study. Last but not least, as the study was limited to patients who underwent hepatic resection, selection bias may exist, and the prognostic value of FST in patients with advanced, unresectable HCC remains to be confirmed. Future multi-center prospective studies with larger sample sizes and mechanistic investigations are warranted to validate our findings.

Conclusion

This study systematically evaluated the prognostic value of intra-tumoral FST protein expression in hepatocellular carcinoma (HCC). We confirmed that FST is significantly upregulated in HCC tissues and serves as an independent prognostic factor for poor overall survival. Crucially, high FST expression was strongly correlated with aggressive clinicopathological features, including larger tumor burden, microvascular invasion (MVI), and elevated AFP. These findings indicated FST as a promising biomarker for risk stratification and highlight the FST signaling axis as a potential therapeutic target for HCC.

Data Sharing Statement

All data supporting the findings of this study are available within the manuscript and from the corresponding author (Baojia Zou) upon reasonable request.

Ethics Statement

Patient samples included in the study were on a retrospective basis and all patients signed an informed consent form when first admitted to the hospital. Patient data confidentiality was strictly maintained. The study was conducted in compliance with the Declaration of Helsinki. All protocols for experiments concerning human samples were approved by the Research Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University (reference number 2025SQ259).

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Disclosure

All authors declare no conflicts of interest in this work.

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