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Original Research

Comprehensive Analysis of Prognostic Value and Immune Infiltration of Src Family Kinases in Hepatocellular Carcinoma

Dingjin Yao[1], Yiran Deng[1], Si Zhang[1], Limiao Liang[1], Li Zhang[1], Shuqiang Weng[2,*], She Chen[1,*]

[1]Key Laboratory of Glycoconjugate Research Ministry of Public Health, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, 200032 Shanghai, China
[2]Department of Gastroenterology and Hepatology, Shanghai Institute of Liver Diseases, Zhongshan Hospital, Fudan University, 200032 Shanghai, China
*Correspondence: weng.shuqiang@zs-hospital.sh.cn (Shuqiang Weng); shechen@fudan.edu.cn (She Chen)
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Abstract

Background: Src family kinases (SFKs) belong to the non-receptor protein tyrosine kinase family and are generally dysregulated in a variety of tumors. This study aimed to thoroughly investigate the mutation status, expression level, prognostic value and relationship with immune infiltration of SFKs in hepatocellular carcinoma (HCC). **Methods**: TIMER2.0, UALCAN, cBioPortal, Gene Expression Profiling Interactive Analysis (GEPIA) and Kaplan-Meier Plotter were used to analyze the differential expression, genetic alteration, prognostic value and immune cell infiltration of SFKs in HCC patients. Furthermore, we used quantitative real-time PCR (qPCR) and western blot (WB) analysis to measure SFKs mRNA and protein expression in matching specimens of normal tissue and HCC. We analyzed the biological effects of FYN in Huh7 cells and subcutaneous xenograft tumor model. We also studied the biological effects of SRC on Huh7 cells. **Results**: The mRNA expression levels of *LYN*, *SRC* and *SRM* were elevated in HCC tissues, whereas *FYN* was reduced. Approximately 10% genetic alterations rate of SFKs was observed in HCC. The mRNA levels of *BLK*, *BRK*, *FRK*, *FYN*, *LCK*, *LYN*, *SRC*, *SRM* and *YES* were correlated with clinical cancer stage. Elevated *FYN* mRNA levels in HCC were positively correlated with overall survival (OS), whereas *SRC* was negatively correlated with OS. All SFKs members in HCC were significantly associated with at least half of the six immune-infiltrating cells, including B cells, macrophages, dendritic cells, neutrophils, CD4+ T cells and CD8+ T cells. Furthermore, we confirmed that the protein expression level of FYN was decreased in patients with HCC and in a human hepatoma cell line. Overexpression of FYN suppressed Huh7 cell proliferation, migration, invasion, and tumorigenesis in xenograft nude mice. Knockdown of SRC inhibited Huh7 cell proliferation, migration and invasion. **Conclusions**: Dysregulated *FYN* and *SRC* expression in HCC is associated with poor prognosis and may be used as novel prognostic biomarkers in patients with HCC.

Keywords: hepatocellular carcinoma; Src family kinases; prognosis; immune infiltration; prediction biomarkers

1. Introduction

Primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer death worldwide in 2020 [1]. Hepatocellular carcinoma (HCC) comprises 75%–85% of primary liver cancer cases [1]. Although surgical resection with curative potential is the most effective treatment, the high recurrence and incidence of HCC metastasis have hindered improved survival [2]. The identification of novel prognostic and therapeutic predictors is of great clinical interest.

Src family kinases (SFKs) belong to the non-receptor protein tyrosine kinase family and eleven family members have been reported [3]. SFKs mostly share a similar structure: a *Src* homology (SH) 4 domain, a unique region, a SH3 domain, a SH2 domain, a catalytic (SH1) domain, and a C-terminal regulatory tail [4,5]. The four closely related group I enzymes consist of *FGR*, *FYN*, *SRC* and *YES*, and group II enzymes are made up of *BLK*, *HCK*, *LCK*, and *LYN*. Group III enzymes, which are distantly correlated with these two groups, include *BRK*, *FRK* and *SRM* [6]. SFKs are involved in signal transduction pathways triggered by a diverse set of cell surface receptors, including receptor tyrosine kinases, cytokines, immune cell receptors, growth factors, integrins and G-protein-coupled receptors [7–9]. Evidence suggests that changes in SKFs protein expression and/or kinase activity are related to the development of multiple types of cancer [10,11], such as colorectal cancer [12], breast cancer [13], gastric cancer [14], melanoma [15], ovarian cancer [16], lung cancer [17], hematological malignancies [18] and hepatocellular carcinoma [19]. However, the prognostic roles of SFKs and their functions in the immune microenvironment of HCC remain unknown.

The tumor microenvironment (TME) in HCC is a complex mixture of tumor cells within the extracellular matrix, combined with a complex mix of stromal cells (immune cells, angiogenic cells, cancer-associated fibroblastic cells) and the proteins they secrete [20]. Immune cells have been shown to play a vital role in HCC [21]. Immune cells in the HCC microenvironment are considered important determinants of clinical outcomes and response to im-

munotherapy [22]. Early tumor infiltration by immune cells such as CD8+ T and B cells is key to inhibit HCC development. However, the anticancer immune response generated by these cells is suppressed by the action of immunosuppressive cells, such as M2 macrophages, which are intrinsically linked to facilitate the HCC TME [23]. Modulation of TME signaling and molecules is a potential strategy for HCC prevention and treatment.

In this study, we systematically explored SFKs in HCC using bioinformatics analysis combined with verification experiments in HCC cell lines, tissues, and a mouse model. We found that dysregulated *FYN* and *SRC* expression in HCC is associated with poor prognosis. Overexpression of FYN suppressed Huh7 cell proliferation, migration, invasion, and tumorigenesis in xenograft nude mice. Knockdown of SRC inhibited Huh7 cell proliferation, migration and invasion. Certain SFKs members, such as *FYN* and *SRC* might sever as novel prognostic biomarkers for HCC.

2. Materials and Methods

2.1 Human Samples

Twenty pairs of HCC and matched peritumor liver tissues were collected from patients who underwent surgical resection and were pathologically diagnosed with HCC at Zhongshan Hospital, Fudan University (Shanghai, China), and used to detect the mRNA and protein expression levels of SFKs by quantitative real-time PCR (qPCR) and western blot (WB). The clinical characteristics of the 20 resected patients with HCC were shown in **Supplementary Table 1**. This study was approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University, and informed consent was obtained from each patient before use.

2.2 TIMER2.0

TIMER2.0 (http://timer.comp-genomics.org/) is a comprehensive tool that provides modules for systematic analysis of immune infiltrates and gene expression across multiple cancer types [24]. The Cancer Genome Atlas (TCGA) samples were used by this tool for data analysis. We compared the expression levels of SFKs between tumor and normal tissues in multiple cancers via the 'Gene_DE Module' of Cancer Exploration Component. Statistical significance evaluated by the Wilcoxon test was annotated by the number of stars ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). The 'sCNA (Somatic Copy Number Alterations) Module' and 'Gene Module' of Immune Associated Component were used to analyze the interrelated proportion of different sCNA states and the correlation between SFKs with the immune cell types, including B cells, macrophages, dendritic cells, neutrophils, CD4+ T cells and CD8+ T cells. Meanwhile, the'purity adjusted'option was selected and the relationship between gene expression level and tumor purity appeared on the left-most panel. Partial Spearman's correlation was used to perform association analysis and $p < 0.05$ was considered statistically significant.

2.3 UALCAN

UALCAN (http://ualcan.path.uab.edu/index.html) is a free web resource for analyzing cancer OMICS data (TCGA, CPTAC and CBTTC) [25]. UALCAN allows users to perform gene expression analysis and identify biomarkers, as well as graphs representing expression profiles and patient survival information. The transcriptional expression of SFKs and their relationship with HCC stage and prognosis were analyzed through this website using TCGA dataset. The significance of the difference in the transcriptional levels was assessed by Student's *t*-test, and *p*-value < 0.05 was considered statistically significant.

2.4 cBioPortal

cBioPortal (https://www.cbioportal.org/) is an open access tool for research, visualization, and analysis of multidimensional cancer genomic data [26]. The tool integrates data from 126 tumor genome studies, including large tumor studies such as TCGA, covering data from 28,000 samples, some of which also include clinical prognostic and phenotypic information. In this study, the genetic alterations in SFKs and their relationship with mRNA expression were obtained from cBioPortal based on the HCC dataset including 973 patients (TCGA, 372 samples; three studies, 601 samples).

2.5 Kaplan-Meier Plotter

The Kaplan-Meier plotter (https://kmplot.com/analysis/), an online tool that contains gene expression data and survival information of 21 cancer types, was used to assess the effect of SFKs mRNA levels on the overall survival (OS) of patients with HCC [27]. The resources used by this tool for HCC survival analysis were sourced from TCGA database. Information on the number-at-risk case, median mRNA expression level, hazard ratio (HR), 95% confidence interval (CI), and *p*-value were displayed on the Kaplan-Meier plotter web page. Statistical significance was considered when the *p*-value was < 0.05.

2.6 Gene Expression Profiling Interactive Analysis (GEPIA)

GEPIA (http://gepia.cancer-pku.cn/index.html) is an interactive web application that provides fast and customizable functions based on TCGA and Genotype Tissue Expression (GTEx) data [28]. OS analysis of SFKs based on gene expression was achieved through GEPIA using these samples (TCGA, 369 tumor samples, 50 normal samples; GTEx, 110 samples). The log-rank test was used for hypothetical tests. The median expression level was used to divide the high- and low-expression cohorts. The Cox proportional HR and 95% CI information were included in the survival plot. $p < 0.05$ was considered statistically significant.

2.7 Cell Culture and Transfection

The HCC cell line Huh7 was obtained from Shanghai Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences. Huh7 was cultured in DMEM medium (#D6429, Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA) at 37 °C in a humidified incubator with 5% CO_2.

Full-length cDNA of *FYN* or *SRC* were cloned into the empty lentiviral vector pCDH-CMV-MCS-EF1-Puro. Recombinant plasmids (psPAX2 and pMD2.G) were co-transfected into 293T cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). The lentivirus was collected from the complete medium two days after transfection.

To establish stably overexpressing cell lines, an appropriate amount of virus was added to Huh7 cells, followed by puromycin selection 48 h later. To establish knockdown cells, small interfering RNA (siRNA) targeting FYN (siFYN: GGUGGAUACUACAUUACCA) and SRC (siSRC: GAAUCUGAUCAACAGUUUAUU) was used in huh7 cells. The overexpression and knockdown efficiencies of FYN and SRC were verified by WB.

2.8 qPCR Analysis

Total RNA was extracted from 20 pairs of HCC and matched peritumor liver tissues using the RNA-easy Isolation Reagent (#R701-01, Vazyme, Nanjing, China). RNA was reverse-transcribed into cDNA using the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) kit (#R223-01, Vazyme, Nanjing, China). For qPCR, the ChamQ Universal SYBR qPCR Master Mix kit (#Q711-02, Vazyme, Nanjing, China) was used according to the manufacturer's instructions. DNA amplification and detection were performed using a 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sequences used are listed in **Supplementary Table 2**. Relative gene expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method and were normalized with *GAPDH*. Data were analyzed using GraphPad Prism (version 8.3.0, Dotmatics, Boston, MA, USA). $p < 0.05$ was considered as statistically significant using Student's *t*-test.

2.9 Western Blot

Total proteins were extracted in RIPA lysis buffer, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% nonfat milk for one hour at room temperature and then incubated with primary antibody (β-actin, #abs137975, Absin, Shanghai, China; FYN, #sc-365913, Santa Cruz, CA, USA; SRC, #2109, CST, Danvers, MA, USA) overnight at 4 ℃, followed by HRP-conjugated secondary antibody for one hour at room temperature. Immunoreactive bands on the membrane were detected using an enhanced chemiluminescence (ECL) reagent.

2.10 Cell Proliferation, Cell Migration and Invasion Assays

Huh7 cells were seeded in 96-well plates at 10^3 cells/well and observed for seven days. Ten μL CCK8 solution (#8-500T, FTC Life Science, Shanghai, China) was added to each well and the plate was incubated for one hour at 37 °C. The absorbance of each well was measured at a wavelength of 450 nm using a Microplate Reader (AMR-100, Allsheng, Hangzhou, China).

For the colony formation assay, Huh7 cells were seeded in 6-well plate at 10^3 cells per well. After incubation for two weeks, the cells were fixed with 4% paraformaldehyde (#P0099, Beyotime, Shanghai, China) and stained with 0.05% crystal violet (#C0121, Beyotime, Shanghai, China).

Transwell assays were performed in chambers with a pore size of 8 μm (#3422, Corning, NY, USA). For the cell migration assay, 1×10^4 Huh7 cells in 200 μL serum-free DMEM were placed in the upper chamber, and 600 μL DMEM with 20% FBS was added to the lower chamber. After incubation for 48 h, the cells on the bottom surface of the chamber were fixed in 4% paraformaldehyde, and stained with 0.05% crystal violet (Beyotime, Shanghai, China). For the cell invasion experiments, each chamber was precoated with 50 μL BD Matrigel mixture (diluted at 1: 5 with DMEM) for one hour at 37 °C. 5×10^4 Huh7 cells were added to the upper chamber and the subsequent steps were the same as those used in the cell migration assay. Five randomly selected fields were photographed using an IX71 inverted microscope (IX71, Olympus Corp, Tokyo, Japan), and the mean value was recorded.

2.11 Xenografts in Nude Mice

BALB/c-nude mice (4–6 weeks, male) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China) and maintained under pathogen-free conditions. To establish the subcutaneous xenograft tumor model, the mice were randomly divided into the control group (n = 6) and the FYN overexpression group (n = 6). Cell suspensions (5×10^6 cells) of Huh7 cells were subcutaneously injected into the flanks of each mouse and tumor size was measured with digital calipers every five days. About three weeks after injection, the mice were sacrificed and tumors were harvested. Tumor size was calculated using the formula: $V = 0.5 \times \text{length} \times \text{width}^2$. All experiments were approved by the Animal Ethics Committee of School of Basic Medical Sciences, Fudan University.

2.12 Statistical Analysis

Transcriptional expression levels of SFKs in HCC and matched normal tissues from the TIMER2.0 database were analyzed using the Wilcoxon test, and those from the UALCAN database and qPCR experiments were assessed using the Student's *t*-test. The Student's *t*-test was also used to analyze the relationship between the expression levels of

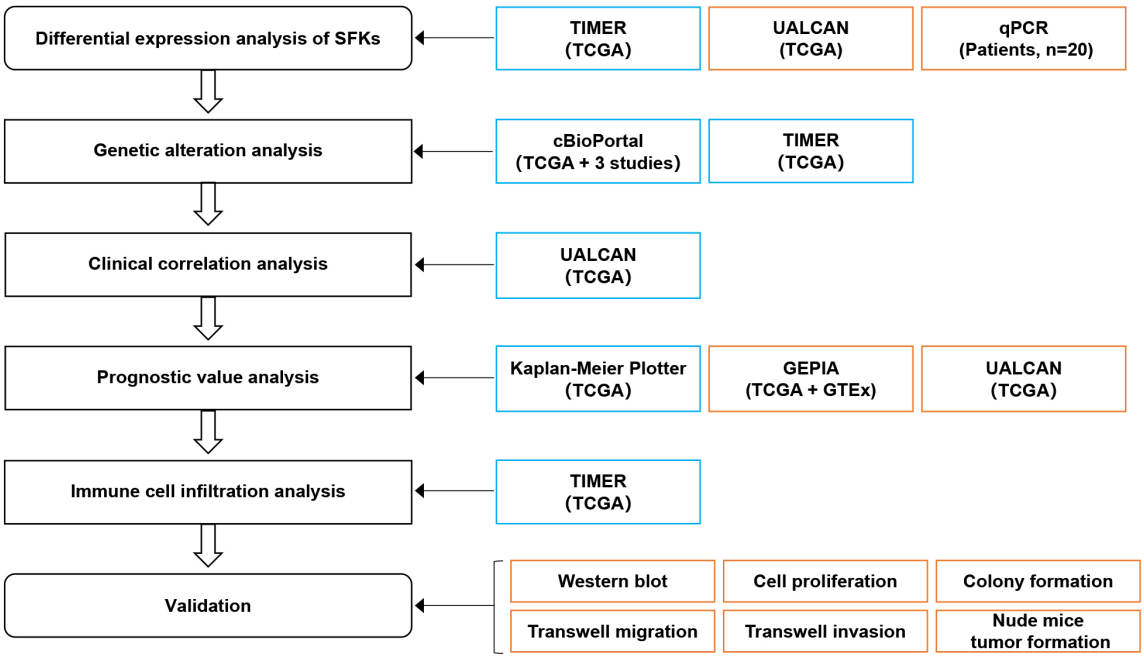

Fig. 1. Flow chart of the study. Blue, discovery cohort; Orange, validation cohort and experiments. The sample sources used by each analysis tool are shown under the tool.

SFKs and HCC stages. The effect of SFKs mRNA expression on the survival of patients with HCC was evaluated using the log-rank test. A partial Spearman's correlation was used to perform an association analysis between SFKs expression levels and immune cells. GraphPad Prism (version 8.3.0, Dotmatics, Boston, MA, USA) was used for statistical analysis and figure creation. Cell proliferation, migration, invasion, tumor volume, and tumor weight were analyzed using the Student's *t*-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1 The mRNA levels of SFKs in Various Types of Cancer Including HCC

The working flow chart depicted in Fig. 1 and the clinical characteristics of HCC patients in each dataset shown in **Supplementary Table 3**. We first used TIMER2.0 tool to explore the expression of SFKs in multiple cancers, including HCC (Fig. 2A). The data revealed that the transcriptional levels of SFKs were frequently abnormal in many types of cancer. In HCC, *LYN*, *SRC* and *SRM* were significantly elevated in HCC tissues compared to normal tissues, while *BLK*, *FGR*, *FYN* and *HCK* were reduced. Other members, including *BRK*, *FRK*, *LCK* and *YES*, showed no significant differences in HCC. We next utilized the UALCAN website to verify seven SFK genes with altered mRNA expression levels (Fig. 2B). The results indicated that the expression levels of *LYN*, *SRC* and *SRM* were significantly higher in HCC tissues than in normal tissues, and the expression levels of *FYN* were markedly lower. However, the changes in the expression levels of *BLK*, *FGR* and *HCK* were not statistically significant. These results were partly consistent with those obtained using TIMER2.0.

To further verify the above results, we detected the mRNA expression levels of SFKs in 20 pairs of HCC and matched peritumor liver tissues using qPCR (Fig. 3). The results revealed that the expression levels of *LYN*, *SRC* and *SRM* were upregulated, and *FYN* was downregulated, whereas other SFK members did not show notable differences in HCC. The qPCR results were largely in agreement with the common parts of the TIMER2.0 and UALCAN.

3.2 Genetic Alteration Analyses of SFKs Genes in HCC

We then investigated the genetic alterations of SFKs in patients with HCC using the cBioPortal and TIMER2.0 databases. As shown in Fig. 4A, alterations in the frequencies and types of SFKs were determined in 973 samples from four HCC studies. The SFKs mutations, amplifications, deep deletions, and multiple alterations in 973 HCC samples occurred at 10.08%, with frequencies of 3.08% (30 cases), 3.60% (35 cases), 3.19% (31 cases) and 0.21% (2 cases), respectively. The percentages of gene changes in individual SFKs in HCC were displayed in Fig. 4B, and the mutation frequency ranged from 0.4% to 4% (*BLK*, 3%; *BRK*, 0.7%; *FGR*, 0.6%; *FRK*, 0.8%; *FYN*, 1.3%; *HCK*, 0.4%; *LCK*, 0.6%; *LYN*, 4%; *SRC*, 0.7%; *SRM*, 1.1%; *YES*, 0.8%).

Next, the stacked bar plot from TIMER2.0 showed the relative proportion of different sCNA states of SFKs in HCC patients (Fig. 4C). Arm-level deletion and arm-level gain were the primary sCNA states, and arm-level deletions

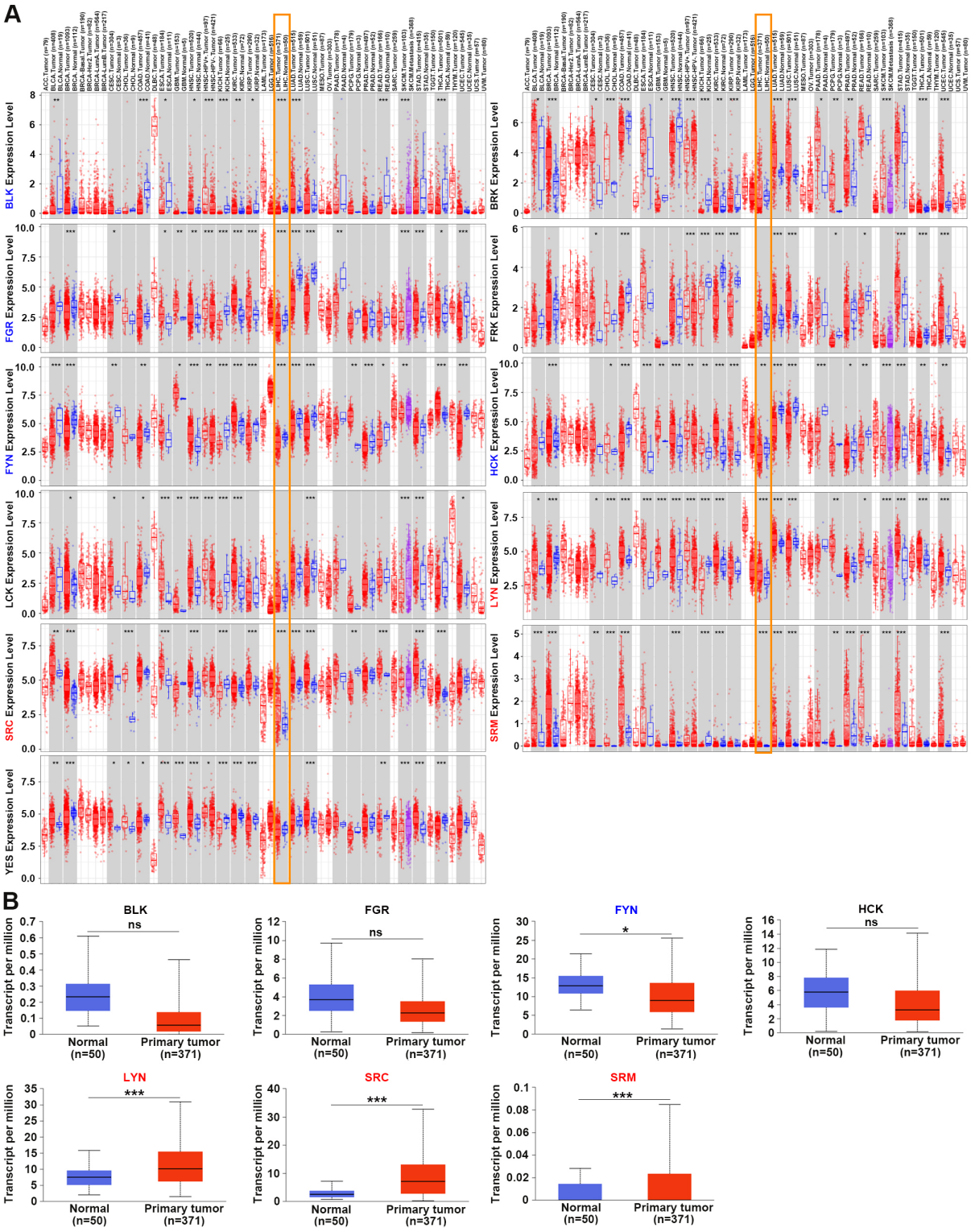

Fig. 2. The mRNA levels of Src family kinases (SFKs) in various types of cancer including Hepatocellular Carcinoma (HCC). (A) The mRNA expression data analysis from TIMER2.0 indicated that the mRNA levels of *BLK*, *FGR*, *FYN* and *HCK* were significantly down-regulated in HCC tissues, while *LYN*, *SRC* and *SRM* were up-regulated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Wilcoxon test. (B) Validation analysis from UALCAN on the relative mRNA level of *BLK*, *FGR*, *FYN*, *HCK LYN*, *SRC* and *SRM* in normal liver and HCC tissues. The expression levels of *FYN* were decreased in HCC tissues than that in normal liver tissues, while *LYN*, *SRC* and *SRM* were increased (Normal, n = 50; Primary tumor, n = 371). ns, not significant, *$p < 0.05$, ***$p < 0.001$, Student's *t*-test. Red, overexpression genes; Blue, low expression genes.

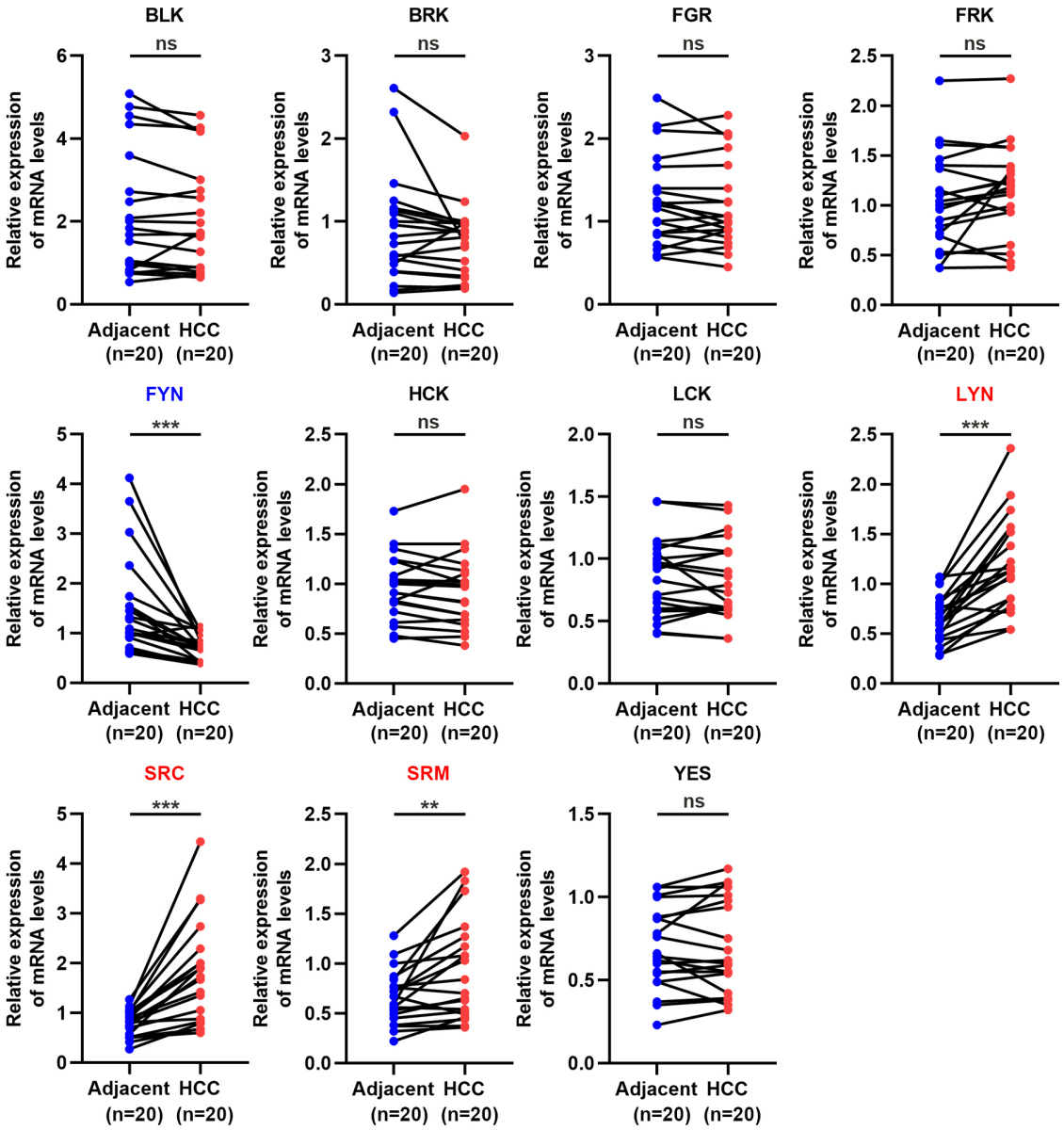

Fig. 3. The relative SFKs mRNA level in 20 paired HCC and adjacent nontumor tissues. Quantitative real-time PCR (qPCR) assay were employed to measure the mRNA levels in tissues. Relative expression of SFKs was normalized with *GAPDH*. Compared to adjacent normal tissues, the expression levels of *FYN* were reduced, but *LYN*, *SRC* and *SRM* were elevated in HCC. n = 20. ns, not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test. Red, overexpression genes; Blue, low expression genes.

were observed mainly in *BLK*, *FGR*, *FRK*, *FYN*, *LCK* and *YES*, whereas arm-level gain was detected in *BRK*, *HCK*, *LYN*, *SRC* and *SRM*.

Furthermore, we explored the relationship between gene alterations and mRNA expression in the 973 HCC samples (Fig. 4D). The decrease in the mRNA expression level of *FYN* might be partly caused by the deep or shallow deletion of genes. The gain or amplification of genes could explain the increased mRNA expression levels of *LYN*, *SRC* and *SRM*. Combined with the above data, we speculated that genetic alterations in SFKs might lead to changes in mRNA expression.

3.3 Correlations between SFKs Expression and Tumor Stage in HCC Patients

We then assessed the correlation between SFKs mRNA expression and pathological stage in HCC using the UALCAN website. As shown in Fig. 5, *SRC* was highly expressed in HCC stage 1–3 compared to that in normal tissues, and *BRK*, *FRK*, *FYN*, *LYN* and *SRM* were differentially expressed in several tumor stages. In comparison to normal tissues, the expression levels of *BLK*, *LCK* and *YES* were diverse in certain tumor stages, but there was no difference in the expression levels of *FGR* and *HCK* in different tumor stages.

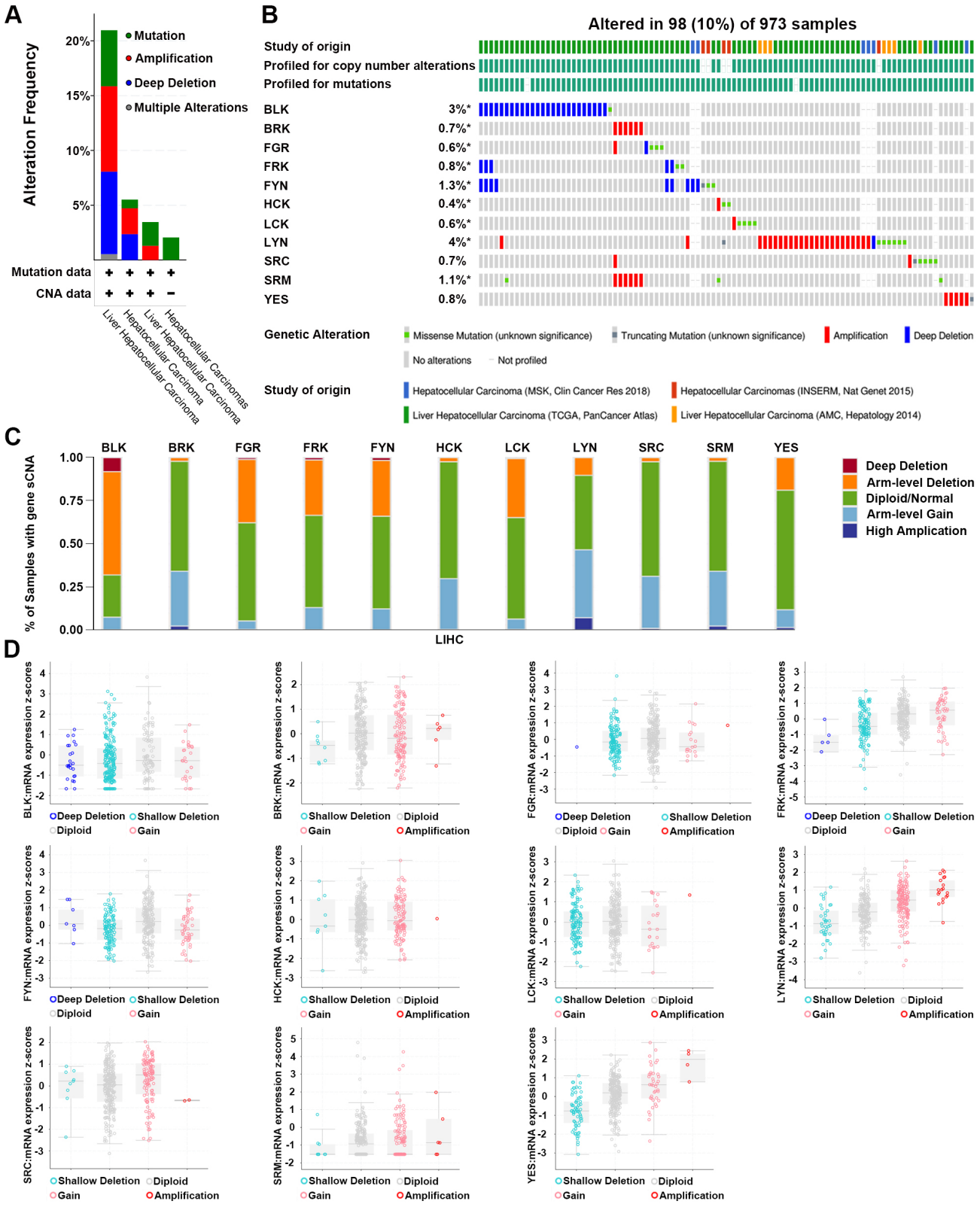

Fig. 4. Genetic alteration of SFKs genes in HCC. (A,B) The genetic alteration type and frequency of different expressed SFKs in different HCC datasets (cBioPortal). SFKs were altered in 98 samples of 973 patients with HCC, accounting for 10%, $*p < 0.05$. (C) The relative proportion of different sCNA status of SFKs members in The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) (TIMER2.0). (D) The boxplot of correlation between the genetic alteration of SFKs and its corresponding mRNA expression (cBioPortal).

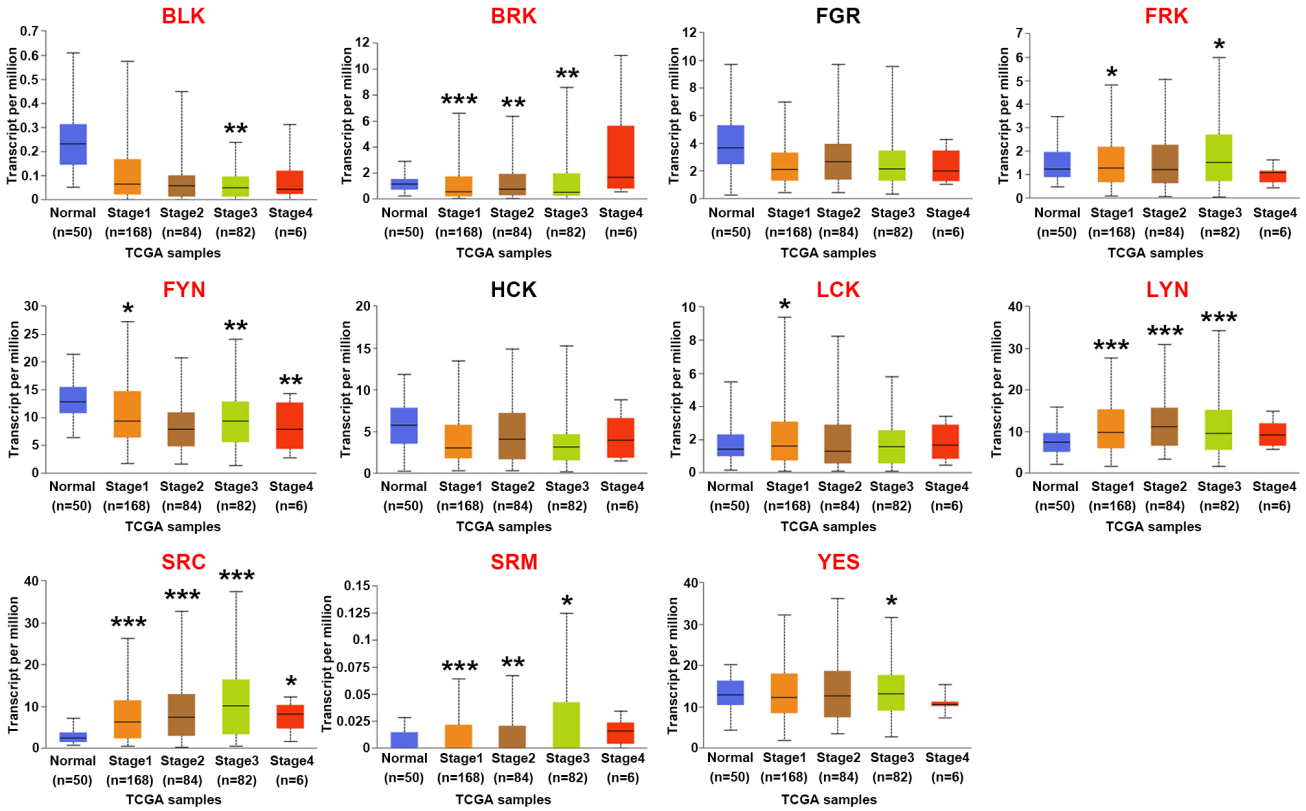

Fig. 5. Correlations between SFKs expression and tumor stage in HCC patients. Histogram (UALCAN) showed that *BLK*, *BRK*, *FRK*, *FYN*, *LCK*, *LYN*, *SRC*, *SRM* and *YES* were correlated with HCC clinical cancer stage. The gene names are shown in red. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's *t*-test.

3.4 Prognostic Values of SFKs in HCC

Furthermore, we analyzed the prognostic significance of SFKs expression in HCC using the Kaplan-Meier Plotter, GEPIA, and UALCAN websites. The OS curves were presented in Fig. 6A. Increased *BLK* (HR = 0.61, $p = 0.01$), *BRK* (HR = 0.68, $p = 0.033$), *FYN* (HR = 0.43, $p = 2.7E-06$), *LCK* (HR = 0.59, $p = 0.0034$), *SRM* (HR = 0.52, $p = 0.00021$), *YES* (HR = 0.68, $p = 0.028$) mRNA levels and decreased *SRC* (HR = 1.75, $p = 0.0023$) mRNA levels in HCC patients were strongly associated with better OS. However, the expression levels of *FGR* (HR = 1.18, $p = 0.4$), *FRK* (HR = 0.75, $p = 0.099$), *HCK* (HR = 1.23, $p = 0.24$) and *LYN* (HR = 0.77, $p = 0.15$) did not affect the OS of HCC patients.

GEPIA was used to verify the seven SFK genes that had an impact on prognosis (Fig. 6B). HCC patients with higher transcriptional levels of *SRC* were significantly associated with shorter OS ($p = 0.016$), whereas those with increased transcriptional levels of *FYN* ($p = 0.0034$) or *LCK* ($p = 0.044$) were markedly associated with better long-term OS. There was no significant correlation between the expression levels of the rest four genes and OS in patients with HCC. The results of *FYN*, *LCK*, *SRC* from the Kaplan-Meier Plotter and GEPIA were consistent.

UALCAN was used to further verify the influence of the mRNA expression levels of *FYN*, *LCK* and *SRC* on OS (Fig. 6C). Among these three genes, only *FYN* ($p = 0.043$) and *SRC* ($p = 0.00049$) mRNA expression levels were significantly associated with OS. The above results from the three websites indicated that the mRNA expression levels of *FYN* and *SRC* were remarkably correlated with prognosis in patients with HCC.

3.5 Correlation between SFKs Expression and Immune Cell Infiltration in HCC

Interaction network and functional enrichment analysis revealed that SFKs might be closely associated with tumorigenesis, progression, and the immune microenvironment (**Supplementary Fig. 1**; **Supplementary Table 4**; **Supplementary Table 5**). We further explored the relationship between SFKs and immune infiltration levels in HCC using the TIMER2.0. As shown in Fig. 7, the expression levels of *FGR*, *HCK*, *LCK*, *LYN* and *YES* were positively in connection with the infiltration of B cells, macrophages, dendritic cells, neutrophils, CD4+ T cells and CD8+ T cells. Additionally, *BLK* expression was positively associated with the infiltration of B cells, dendritic cells, CD4+ T cells and CD8+ T cells. *BRK* expression was positively correlated with the infiltration of macrophages, neutrophils and CD4+ T cells. *FRK* expression was significantly posi-

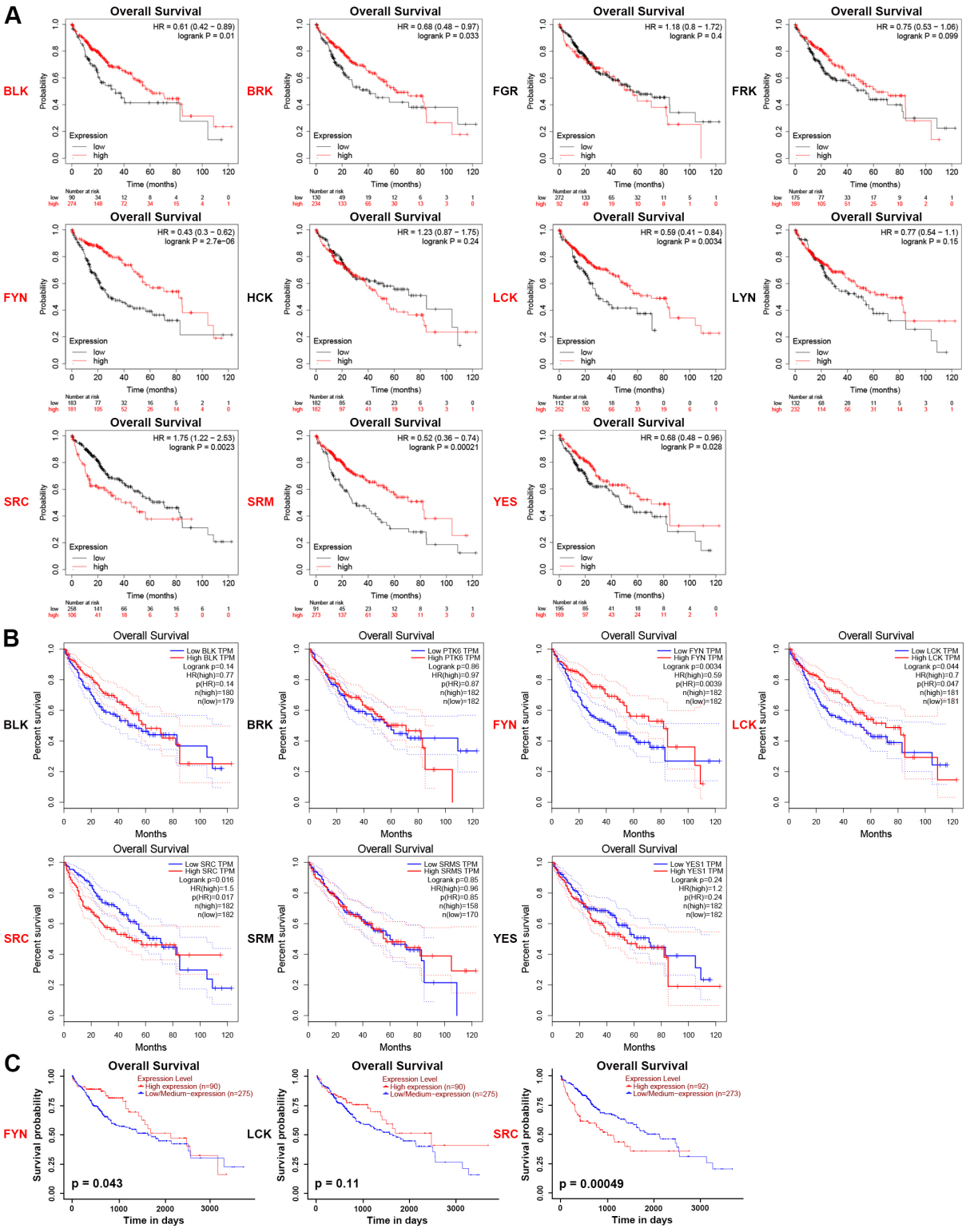

Fig. 6. Prognostic values of SFKs in HCC. (A) HCC patients with reduced mRNA expression levels of *BLK*, *BRK*, *FYN*, *LCK*, *SRM* and *YES* were significantly related to short overall survival (OS), while higher *SRC* mRNA expression was correlated with short OS. Cutoff: median (Kaplan-Meier plotter). (B) Low mRNA expression levels of *FYN* and *LCK* were significantly associated with short OS, conversely, high *SRC* mRNA expression was correlated with short OS in HCC patients. Cutoff: median (GEPIA). (C) Elevated *FYN* mRNA level in HCC was positively correlated with OS, while *SRC* was negatively correlated with OS (UALCAN). $p < 0.05$ (genes labeled with red) was considered statistically significant.

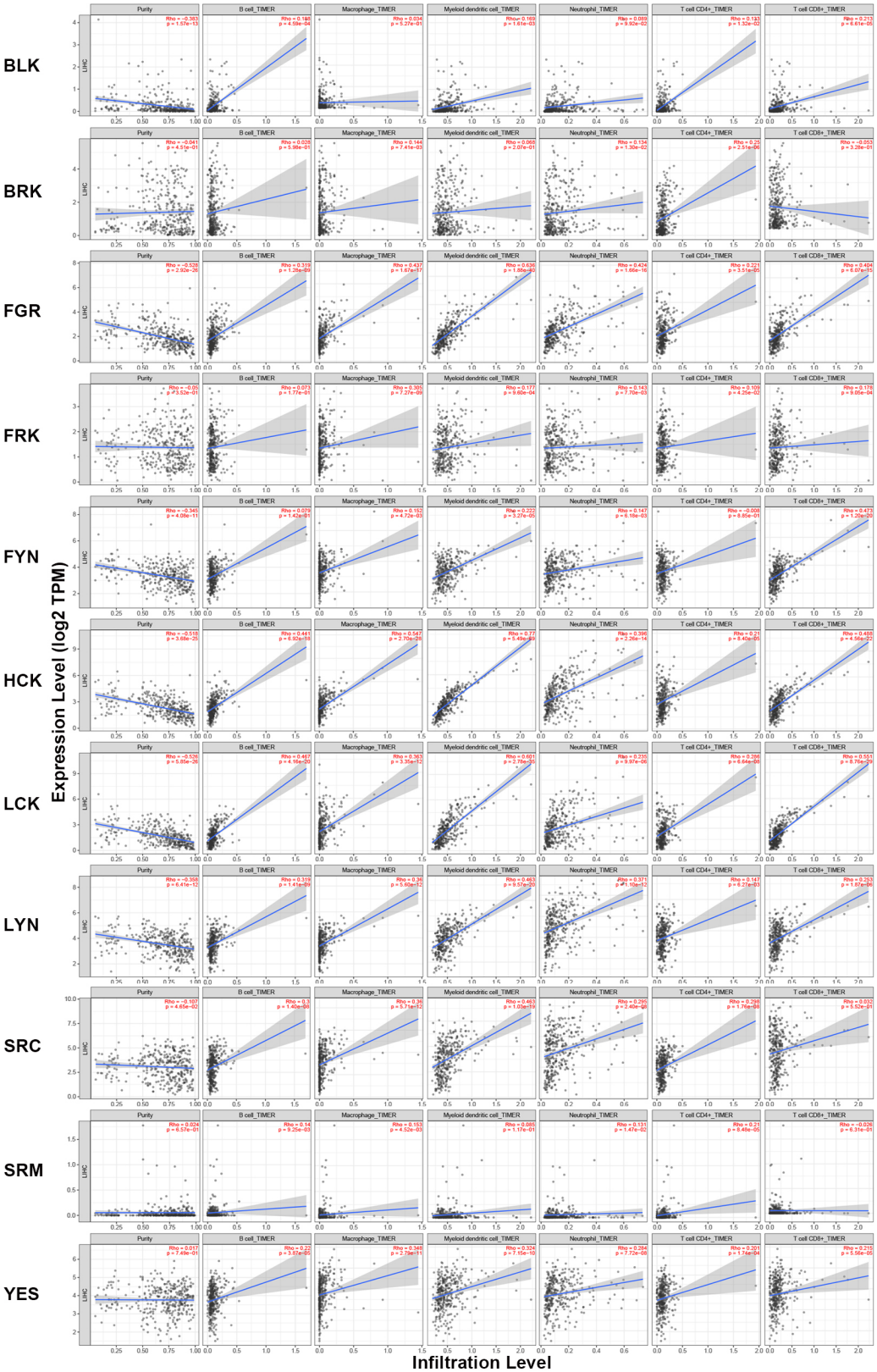

Fig. 7. Correlations between differentially expressed SFKs and immune cell infiltration (TIMER2.0). Spearman tests were used for analyzing the correlation coefficients (Rho). Positive correlation ($p < 0.05$, Rho > 0); Negative correlation ($p < 0.05$, Rho < 0); Not significant ($p > 0.05$).

tively correlated with the infiltration of macrophages, dendritic cells, neutrophils, CD4+ T cells and CD8+ T cells. *FYN* expression was positively connected with the infiltration of macrophages, dendritic cells, neutrophils and CD8+ T cells. *SRC* expression was positively related to the infiltration of B cells, macrophages, dendritic cells, neutrophils and CD4+ T cells. *SRM* expression was positively relevant to the infiltration of B cells, macrophages, neutrophils and CD4+ T cells. Taken together, the above results demonstrated that SFKs might play a dominant role in the immune infiltration of HCC.

3.6 Overexpression of FYN Suppresses Tumor Biological Behavior In Vitro and In Vivo

Multiple analysis tools combined with qPCR experiments suggested that among all SFK members, only *FYN* and *SRC* were dysregulated in expression and associated with OS, therefore, we mainly considered these two genes as candidate genes for predicting prognostic markers in HCC patients. The impact of *FYN* on HCC has rarely been reported, therefore, we first focused on identifying the effect of *FYN* on HCC *in vitro* and *in vivo*.

Western blot analysis revealed decreased protein expression levels in both HCC tissues (**Supplementary Fig. 2A**) and the human hepatoma cell line Huh7 (**Supplementary Fig. 2B**) by western blot analysis. We then generated FYN-overexpression or -knockdown Huh7 cell lines (Fig. 8A,B). Cell proliferation ability, detected by CCK-8 proliferation assay and colony formation assay, decreased in FYN-overexpression Huh7 cells and increased in FYN-knockdown Huh7 cells (Fig. 8C–F). Moreover, transwell migration and invasion experiments showed that overexpression of FYN inhibited cell migration and invasion, which was the opposite of FYN knockdown (Fig. 8G–J). Next, we examined the effects of FYN on HCC cell proliferation *in vivo*. In the subcutaneous xenograft tumor model, the tumor growth rate in mice injected with FYN-overexpression cells was significantly slower than that in mice injected with control Huh7 cells (Fig. 8K). FYN-overexpression Huh7 cells formed smaller tumors (365.5 $\pm$ 113.0 mm^3 vs. 945.0 $\pm$ 156.7 mm^3, Fig. 8L; 412.8 $\pm$ 64.79 mg vs. 918.3 $\pm$ 66.37 mg, Fig. 8M). Together, these data imply that overexpression of FYN, a potential tumor suppressor of HCC, suppresses tumor biological behavior *in vitro* and *in vivo*.

3.7 Knockdown of SRC Suppresses Tumor Biological Behavior In Vitro

Several studies have shown that *SRC* has a tumor-promoting effect on HCC. Here, we studied its function through tumor biological behavior experiments *in vitro*. We first generated SRC-overexpression or -knockdown Huh7 cell lines (Fig. 9A,B). CCK-8 proliferation and colony formation assays revealed that knockdown of SRC suppressed cell proliferation, while overexpression of SRC promoted cell proliferation (Fig. 9C–F). Transwell migration and invasion assays demonstrated that knockdown of SRC inhibited cell migration and invasion, conversely, overexpression of SRC promoted cell migration and invasion (Fig. 9G–J). These results suggest that knockdown of SRC, a potential tumor promoter of HCC, suppresses the biological behavior of tumors *in vitro*.

4. Discussion

Comprehensive analyses of SFKs in HCC have not yet been reported. In this study, we systematically explored SFKs in HCC through bioinformatics analysis combined with biological behavior experiments. Multiple analysis results showed that the expression levels of *FYN* were decreased in HCC tissues compared to normal liver tissues, while *LYN*, *SRC* and *SRM* were increased. Moreover, most SFK members were correlated with the HCC clinical cancer stage. *FYN* and *SRC* significantly correlated with OS in patients with HCC. These results suggested that SFKs play a critical roles in HCC progression.

Mechanically, the interaction network and functional enrichment analysis indicated that SFKs and the 20 related genes were closely related to multiple signaling pathways, such as the NF-κB and MAPK signaling pathways. Previous studies have suggested that the NF-κB signaling pathway mediated epithelial-mesenchymal transition (EMT) can promote the progression of HCC [29,30]. Activation of the MAPK signaling pathway is common in human HCC [31], and increased MAPK activity correlates with a more malignant HCC phenotype [32].

In the TME, chemokines can regulate immune cells, and perturbations in chemokine signaling often alter immune cell composition and localization [33]. Natural killer cells [34] and T cells [35] are important cells in the TME, and the regulation of these immune cells affects the progression of HCC. These findings indicate that SFKs play an important role in the regulation of immune cells and the progression of HCC. Consistently, we found that all SFKs were strongly associated with at least three types of immune cells in HCC. At least eight SFK members were significantly correlated with CD4+ T cells [36], CD8+ T cells [37], B cells [38] and dendritic cells [39], which were considered important players in impeding, inhibiting and killing HCC cells. In addition, ten SFK members were significantly connected with neutrophils [40] and macrophages [41], which have a dual role in cancer progression based on their polarization state. Immune-infiltrating cells in the TME have been shown to play a critical role in tumor development and impact clinical outcomes in patients with cancer [42]. We also found that SFKs and the 20 related genes were correlated with multiple immune-related signaling pathways, such as chemokine signaling pathway, natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, and B cell receptor signaling pathway. Therefore, SFKs presumably affect the progression of HCC by regulating

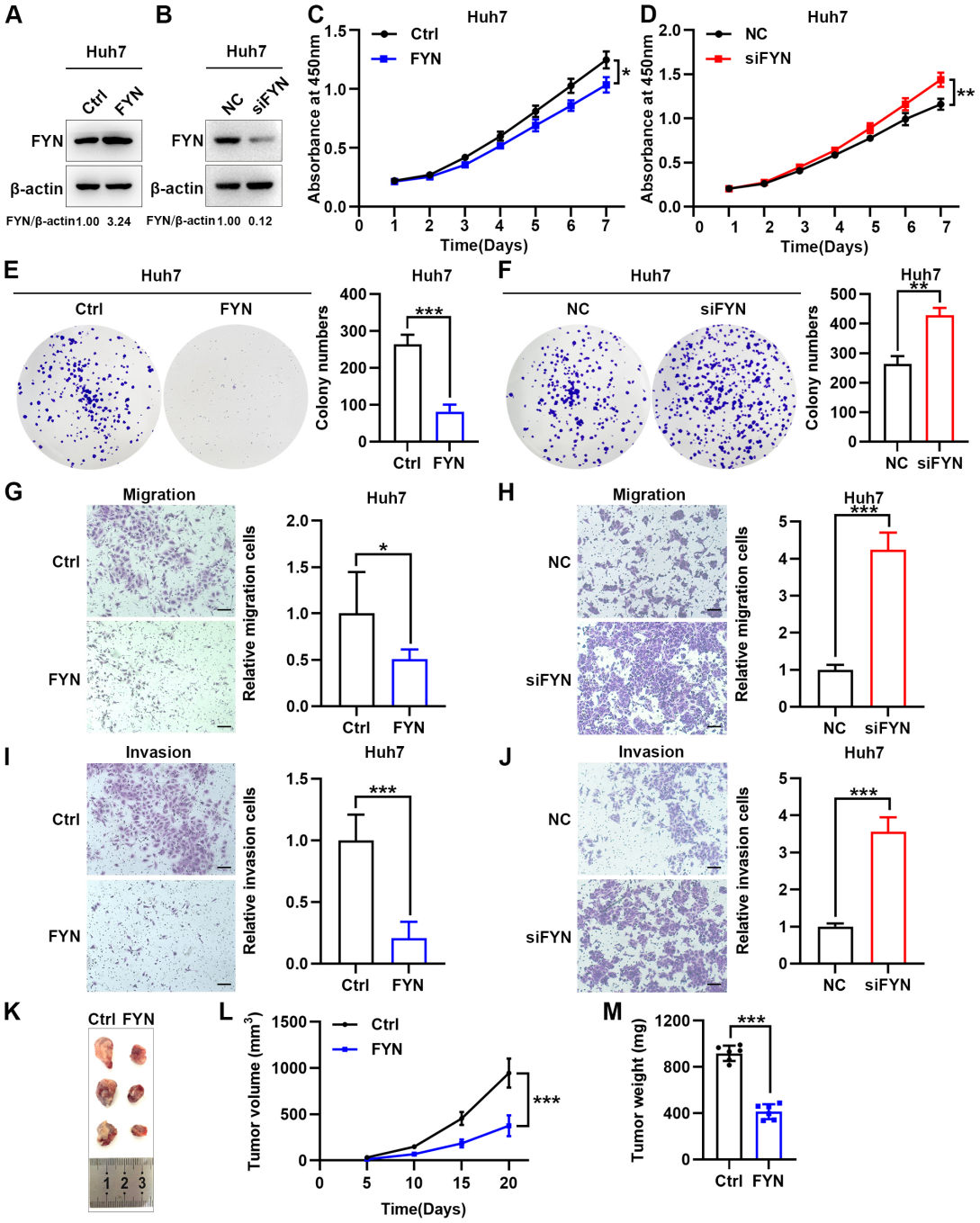

Fig. 8. Overexpression of FYN suppresses tumor biological behavior *in vitro* and *in vivo*. (A,B) FYN overexpression (A) and knockdown (B) efficiency verification at the protein level. FYN protein levels were detected by western blot and relative levels were normalized to β-actin. Ctrl, control group; FYN, overexpression group; NC, negative control group; siFYN, knockdown group. (C–F) Overexpression of FYN inhibited the proliferation ability of Huh7 cells. CCK-8 (C,D) and colony formation assays (E,F) were used to detect proliferationin ability of Huh7 cells. Results were expressed as mean ± SD; n = 3; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; Student's *t*-test. (G–J) Overexpression of FYN inhibited the migration and invasion of Huh7 cells. Transwell assays were performed to test the migration (G, H; left panel) and invasion (I, J; left panel) abilities of Huh7 cells. Scale bar, 100 μm. Quantification was achieved by ImageJ (version 1.53n, NIH, Bethesda, MD, USA) and shown in the right panels of G–J, respectively. Results were expressed as mean ± SD; n = 5; $*p < 0.05$, $***p < 0.001$; Student's *t*-test. (K) Overexpression of FYN suppressed tumor xenograft growth *in vivo*. Effects of FYN on tumor growth in subcutaneous xenograft tumor model. The tumors were excised on day 20. Three representative mice in each group were shown. n = 6. (L) The tumor volume progression of nude mice within the observation period (20 days). Results were expressed as mean ± SD; $***p < 0.001$; Student's *t*-test. (M) The weight of tumors excised on day 20 were lighter in the FYN overexpression group than that in the control group. Values were mean ± SD; n = 6; $***p < 0.001$; Student's *t*-test.

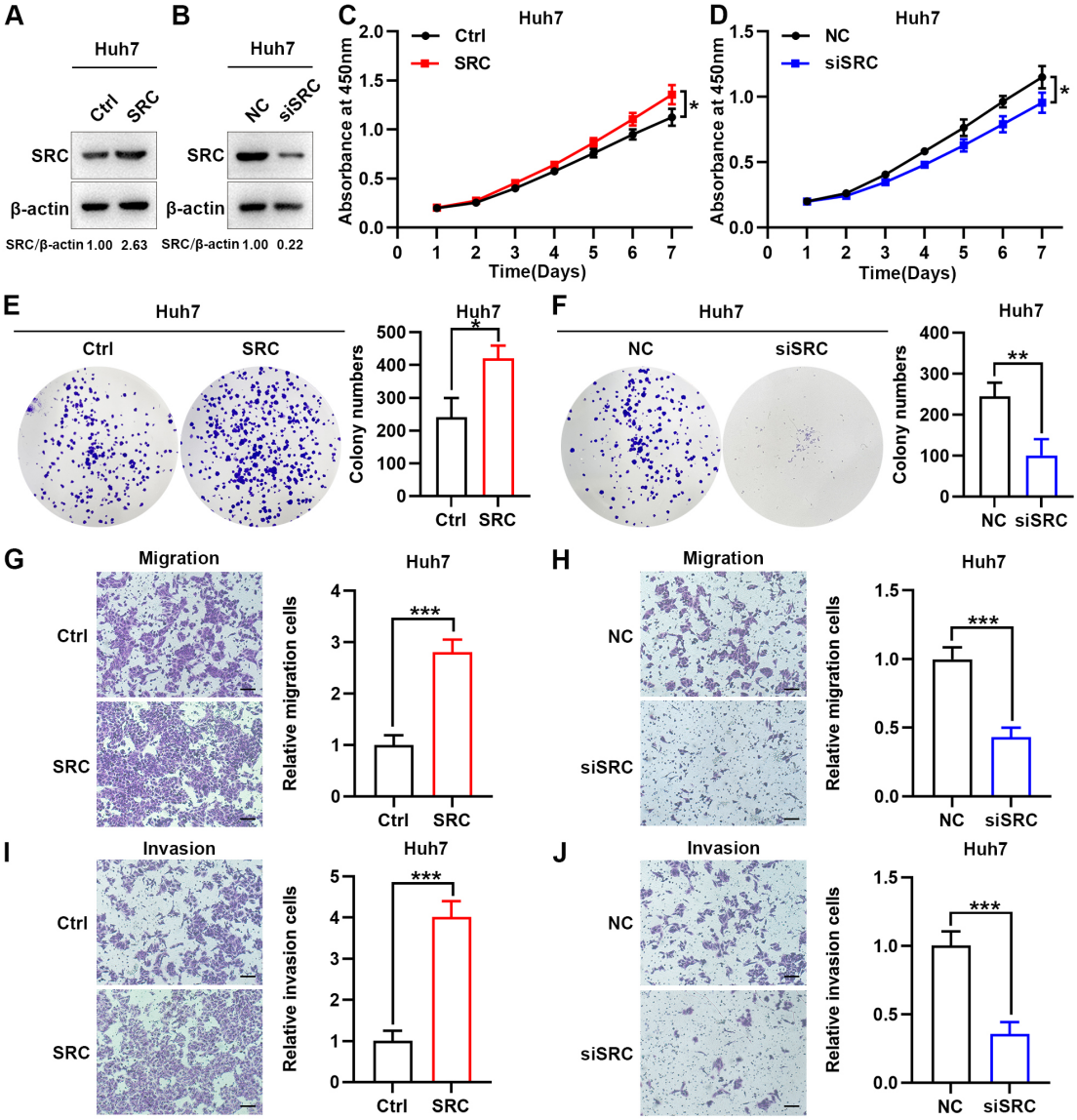

Fig. 9. Knockdown of SRC suppresses tumor biological behavior *in vitro*. (A,B) SRC overexpression (A) and knockdown (B) efficiency verification at the protein level. SRC protein levels were detected by western blot and relative levels were normalized to β-actin. (C–F) Knockdown of SRC inhibited the proliferation ability of Huh7 cells. CCK-8 (C,D) and colony formation assays (E,F) were performed to detect proliferationin ability of Huh7 cells. Results were expressed as mean ± SD; n = 3; *$p < 0.05$, **$p < 0.01$; Student's *t*-test. (G–J) Knockdown of SRC inhibited the migration and invasion of Huh7 cells. Transwell assays were achieved to test the migration (G, H; left panel) and invasion (I, J; left panel) abilities of Huh7 cells. Scale bar, 100 μm. Quantification was achieved by ImageJ and shown in the right panels of G–J, respectively. Results were expressed as mean ± SD; n = 5; ***$p < 0.001$; Student's *t*-test.

immune-infiltrating cells and may be potential targets for HCC immunotherapy.

FYN was ubiquitous in almost all cell types [4]. However, the role of *FYN* in cancer development appears tissue-specific. In a variety of cancers, including breast cancer [43], gastric cancer [44], colon cancer [45], lung cancer [46], and pancreatic cancer [47], *FYN* was upregulated and acted as a tumor promoter by promoting proliferation and migration or by affecting EMT [43–45,47]. In this study, we found that *FYN* is frequently lost in patients with HCC and is associated with poor prognosis. In agreement with this, *FYN* was downregulated in prostate cancer, and served as a new candidate prostate tumor suppressor gene [48]. Reduced *FYN* expression was caused by both chromosomal deletion and promoter hypermethylation, whereas decreased mRNA expression of *FYN* may be due to deep or shallow deletion of the gene. Besides *FYN*, other genes among SFKs also showed potential as novel prognostic biomarkers for HCC, which requires further investigation.

LYN has attracted widespread attention on multiple types of malignancies, such as chronic lymphocytic leukemia (CLL) [49], lung adenocarcinoma [50], colorec-

tal cancer [51], cervical cancer [52], renal cancer [53] and gastric cancer [54]. The impact of *LYN* on tumors may be involved in ERK1/2 activation, NF-κB signaling pathway, EGFR signaling pathway, AKT signaling pathway and shaping the tumor microenvironment [49–52,55], but the role of *LYN* in HCC has rarely been explored. Our study showed that the mRNA expression of *LYN* in HCC patients was dramatically elevated, and *LYN* was strongly expressed in HCC stages 1, 2 and 3. Nevertheless, the higher mRNA expression of *LYN* was not significantly associated with OS in patients with HCC. It is possible that *LYN* is involved in the occurrence and development of HCC by regulating its activity rather than its protein content. As seen in breast cancer, patients with high levels of phospho-LYN Y32 had worse OS than those with low levels of phospho-LYN Y32 [56]. Similar to *LYN*, *SRM*, also known as *SRMS*, was not correlated with OS in HCC patients. Currently, the prognostic significance of *LYN* and *SRM* in HCC is uncertain.

SRC, the most investigated member of SFKs, expressed in almost all cell types [4], has been confirmed to have an effect on the initiation and progression of many cancers. As for HCC, prior studies have revealed that *SRC* promoted HCC tumorigenesis by activating the downstream MAPK signaling pathway [19]. In addition, it was reported that *SRC* had an impact on HCC growth and metastasis by mediating aerobic glycolysis via the PI3K/AKT/mTOR signaling pathway [57]. Moreover, targeting *SRC* related signaling pathway has been found to inhibit HCC metastasis [58]. A similar tumorigenic effect of *SRC* on HCC was observed in this study. *SRC* was highly expressed in the first three stages of HCC compared with normal tissues. However, similar to LYN, the increased expression of SRC was not found significantly in stage 4, which could be due to: (1) the sample sizes (n = 6) were too small to truly reflect its expression level, or (2) the development of tumors was not linear [59]. Moreover, the increased mRNA expression of *SRC* in HCC patients was significantly associated with poor OS. It seems that *SRC* does participate in HCC tumorigenesis and progression, and could serve as a prognostic biomarker and therapeutic target for HCC.

Among all SFKs, FYN and SRC showed potential as prognostic markers. As mentioned above, FYN and SRC were expressed in almost all cell types, including blood cells, implying that their expression could theoretically be detected in plasma. Besides, it is feasible to stain tissues with FYN and SRC. However, whether there is a correlation between plasma level expression and tissue level expression, whether there is a correlation between plasma level expression and HCC prognosis, and the quantification between gene expression in tissues and prognosis need to be further investigation.

5. Conclusions

In summary, we systematically explored the expression, mutation, prognostic value and relationship with immune infiltration of individual SFKs in HCC through bioinformatics analysis combined with biological behavioral experiments. The expression levels of *FYN* and *SRC* were altered in HCC, and they were associated with clinical cancer stage, OS, immune cell infiltration, and tumor progression. *FYN* and *SRC* may be potential prognostic biomarkers for patients with HCC. Additionally, our research contributes to a novel perspective on the distinct roles of SFKs in HCC and provides new clues for the early detection, prognostic evaluation, and individualized treatment of HCC patients. However, owing to the limitations of online databases and the small sample size, further studies are required to confirm these findings.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

SC, DY and SZ conceived the study, wrote the manuscript and designed the figures and tables. DY, and YD performed experiments. LL, YD and LZ performed bioinformatic, statistical analyses and maintained the animals. SW provided the tissue samples, clinical data and related analysis. SC and DY revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Tissue samples for research were collected in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University (ethics number: Y2017290). The mouse experiments were conducted in accordance with the ARRIVE guidelines and approved by the Animal Ethics Committee of School of Basic Medical Sciences, Fudan University (ethics number: 20210302004). All methods were performed in accordance with relevant guidelines and regulations.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2805090.

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