Original Article

Mitochondrial single-stranded DNA binding protein 1 (SSBP1) high expression as a potential biomarker and association with poor prognosis in hepatocellular carcinoma (HCC)

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Background: Single-stranded DNA binding protein 1 (SSBP1) is a DNA binding protein found in mitochondria, encoded by nuclear genes. SSBP1 plays a crucial role in responding to mitochondrial DNA (mtDNA) damage and maintaining genome stability, and it is linked to cancer occurrence and progression, but its role in hepatocellular carcinoma (HCC) is still unclear. Therefore, the aim of this research was to investigate the expression of SSBP1 and its potential clinical significance in HCC.

Methods: RNA-seq data and clinical information of HCC samples and normal liver samples were downloaded from The Cancer Genome Atlas (TCGA). The expression of SSBP1 in HCC and its correlation with clinical pathological indicators, prognosis, immune cells, and infiltration were analyzed using R software, while the diagnostic value of SSBP1 in HCC was evaluated. Using the R software, we conducted Gene Ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and gene set enrichment analysis (GSEA) on the SSBP1 expression in HCC. Immunohistochemistry (IHC) detected SSBP1 expression in 31 HCC tissue pairs. Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) quantified protein and mRNA levels in 6 fresh HCC tissue. Protein expression and distribution of SSBP1 in HCC cell lines were analyzed using qRT-PCR, Western blotting, and Immunofluorescence techniques.

Results: SSBP1 mRNA expression was significantly higher in HCC tissues compared to normal tissues ($P<0.001$) in both matched and unmatched samples. SSBP1 expression was correlated with gender and M stage ($P<0.05$), but not with other factors. High SSBP1 expression was identified as an independent risk factor for overall survival (OS) in HCC patients [hazard ratio =1.713, $P=0.01$]. Immunocell infiltration analysis showed a negative correlation between SSBP1 expression and level of naive B cells, but a positive correlation with memory B cells and macrophages ($|$Spearman's r$|$ >0.2, $P<0.05$). The diagnostic value of SSBP1 mRNA expression for early diagnosis prognosis of HCC (area under the curve >0.50). The GO enrichment analysis of SSBP1 revealed that it was enriched for mitochondrial biological functions. KEGG analysis showed that SSBP1 was associated with multiple DNA replication, mismatch repair and homologous

recombination pathways. GSEA analysis showed that the first three pathways strongly related to the high expression of SSBP1 were DNA repair, myc-targets-v1 and reactive oxygen species signaling pathways. Validation through IHC and Western blotting high SSBP1 protein expression in HCC tissue, as well as qRT-PCR and Western blotting results showed high expression in HCC cell lines. Immunofluorescence experiments indicated the localization of SSBP1 in the mitochondria of HCC cells.

Conclusions: High expression of SSBP1 is an independent risk factor for poor prognosis in HCC patients and has good diagnostic value.

Keywords: Mitochondrial single-stranded DNA binding protein 1 (mitochondrial SSBP1); The Cancer Genome Atlas (TCGA); hepatocellular carcinoma (HCC); biomarker; prognosis

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor globally and the second leading cause of cancer-related deaths (1). It accounts for 90% of liver cancers and is the most common form of primary liver cancer (2). The treatment of HCC has become complex due to drug resistance and high tumor recurrence rates. In the early stages, it is treated with surgery, while in the advanced stages, chemotherapy, immunotherapy, and oncolytic virus therapy can be used (3). The prognosis for patients with HCC remains relatively poor due to the high rates of recurrence and metastasis. This indicates that current treatment regimens for advanced HCC have not achieved the expected outcome (3). Therefore, there is an urgent need to develop new molecular biomarkers for early diagnosis and prognosis assessment of HCC in order to improve treatment efficacy, reduce recurrence rates, and extend survival time.

Currently, human proteomic research has identified three simple single-stranded DNA binding (SSB) proteins, including human single-stranded DNA binding protein 1 (*hSSB1*), human single-stranded DNA binding protein 2 (*hSSB2*), and human mitochondrial single-stranded DNA-binding protein (*mtSSB*) (4). Among them, mtSSB is also known as mitochondrial single-strand DNA binding protein 1 (SSBP1) (5). SSBP1 is the only known SSB protein in eukaryotes that is specifically localized to the mitochondrial nucleoid (6). The SSBP1 gene contains seven exons, encoding a 212-amino acid protein, with the translational initiation site located within exon 2 (7). SSBP1 consists of an N-terminal DNA binding domain and a C-terminal helical domain. The N-terminal DNA binding domain includes an oligonucleotide/oligosaccharide-binding (OB) fold structure, which can bind to a single-stranded DNA (ssDNA) thereby regulating various important physiological activities in cells (8). SSBP1 is an essential molecule in the synthesis of mitochondrial DNA (*mtDNA*), functioning as a housekeeping gene that plays a critical role in mitochondrial biogenesis. Additionally, it is part of a ssDNA binding complex that contributes to the maintenance of genomic stability (9). At the mtDNA replication fork, SSBP1 interacts functionally with DNA polymerase γ (pol γ) and mtDNA helicase (also known as Twinkle) to promote mtDNA replication (10). Maintaining proper *mtDNA* levels is critical for normal development and health. Deficiencies

Highlight box

Key findings

- We tentatively conclude that mitochondrial single-stranded DNA binding protein 1 (*SSBP1*) may be a mitochondria-localized oncogene in hepatocellular carcinoma (HCC).

What is known and what is new?

- Some studies have found that *SSBP1* is abnormally highly expressed in various cancers such as glioblastoma, gastric cancer, osteosarcoma, colorectal cancer and is significantly associated with poor prognosis.
- We verified that *SSBP1* is localized to the mitochondria and found that high expression of *SSBP1* is an independent risk factor for poor prognosis in HCC.

What is the implication, and what should change now?

- *SSBP1* has a good diagnostic value for HCC, and it may be a potential molecular marker for diagnosis and prognosis judgment of HCC patients, as well as a potential target for immunotherapy.

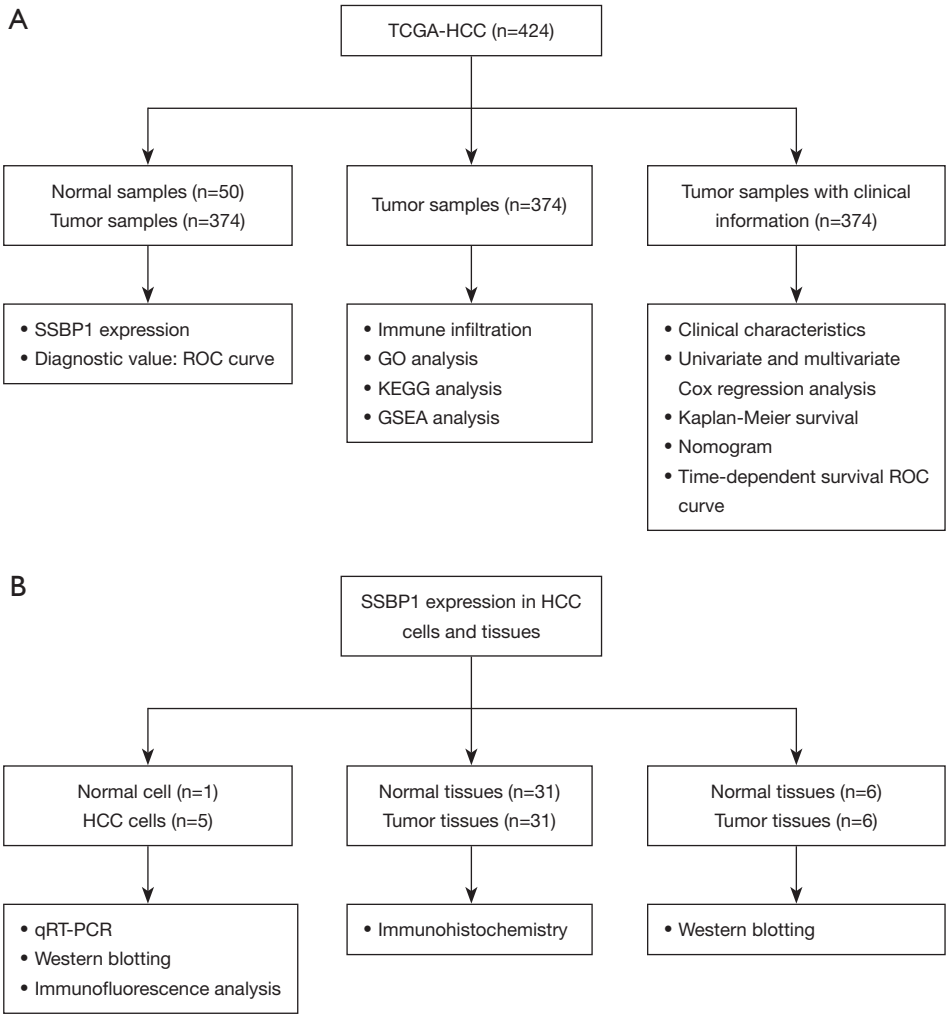

Figure 1 Flowchart of this study. (A) Analysis of the transcriptome sequencing data and clinical data from TCGA database by bioinformatics methods. (B) Analysis of the expression of SSBP1 in HCC cells and tissues by different experiments. TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma; SSBP1, single-stranded DNA binding protein 1; ROC, receiver operating characteristic; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

in the maintenance of *mtDNA* give rise to a diverse array of mitochondrial disorders. These disorders are molecularly characterized by either *mtDNA* depletion or the presence of *mtDNA* deletions, resulting in compromised oxidative phosphorylation (OXPHOS) in the tissues that are affected (11). Abnormal expression of SSBP1 will directly lead to abnormal mtDNA copy number or gene mutation, lead to instability of mtDNA and ultimately cause the occurrence and progression of malignant tumors (12). Some studies have found that SSBP1 is abnormally highly expressed in various cancers such as glioblastoma, gastric cancer, osteosarcoma, colorectal cancer and is significantly associated with poor prognosis (13-15). Since SSBP1 is abnormally highly expressed in other cancers, it could potentially also be elevated in HCC. However, the biological function and prognostic significance of SSBP1 in HCC remain unclear.

In this study, we explored the changes of *SSBP1* mRNA and protein expression levels in HCC and their prognostic significance, aiming to provide a theoretical basis for clinical diagnosis and prognosis assessment of HCC patients. The flowchart of this study is shown in *Figure 1*. We present this article in accordance with the MDAR and TRIPOD reporting checklists (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1196/rc).

Methods

Data collection and preprocessing

The mRNA-SeqV2 expression levels, which integrate level 3 data from the Illumina GA and HiSeq platforms, for patients with HCC were obtained from The Cancer Genome Atlas (TCGA) public dataset (http://www.cbioportal.org/) utilizing R version 4.3.3 with the Cgdsr package. The RNA sequencing data were subsequently converted into transcripts per million (TPM), and the gene expression data in TPM format underwent log2 transformation for further analysis. The methodologies employed for the procurement of biospecimens, total RNA extraction, and mRNA sequencing have been previously detailed by The Cancer Genome Atlas Research Network (16). Level 3 TCGA data (lihc_tcga_rna_seq_v2_mrna) were retrieved from the TCGA database. Furthermore, clinical information, overall survival (OS) data, and disease-free survival (DFS) data were also acquired for subsequent analysis. The dataset comprised 374 HCC patients, who were monitored over a follow-up period ranging from 0 to 115 months. For the purposes of survival analysis and follow-up, the date of surgery was designated as the commencement of the follow-up period. The endpoint event was defined as either the date of the last visit (in cases where no death occurred) or the time until death. Patients who succumbed to causes unrelated to HCC, such as other diseases or accidents, were excluded from the research cohort.

Analysis of the expression and clinical relevance of SSBP1 in HCC samples

Using the R4.3.3 package "survival" to analyze the differential expression of *SSBP1* mRNA in 374 cases of HCC samples and 50 cases of normal samples. The patients were divided into high- and low-expression groups based on the median expression level. A total of 342 mRNA expression profiles were extracted after removing cases that missing survival time and status, and remaining cases were applied for further survival analysis. A logistic regression model was employed to analyze the correlation between *SSBP1* expression and clinical features such as age, gender, TNM stage, histological type, and pathological stage.

Analysis of the expression of SSBP1 in different clinical stages of HCC

Using the gene expression profiling interactive analysis (GEPIA), a one-way analysis of variance was performed to compare the expression differences of the *SSBP1* gene among 374 HCC patients in different clinical and pathological stages. The "Stage Plot" plate was utilized to automatically generate violin plots based on the pathological stage of HCC patients.

Immune infiltration analysis

The infiltration levels of 28 immune cells in each sample were assessed by using single sample gene set enrichment analysis (GSEA) based on the R4.3.3 package [gene set variation analysis (GSVA)]. Additionally, the ESTIMATE algorithm was also used for calculated the Immune Score, Stromal Score, and ESTIMATE Score of each sample-based on the "estimate" package in R4.3.3. The correlations between *SSBP1* expression and the infiltration of immune cells were analyzed by Spearman method (17).

Prognostic analysis

The relationship between *SSBP1* expression and OS and DFS of patients was analyzed using the survival package in R4.3.3 software. Meanwhile, OS and DFS data of HCC patients were predicted by performing the Kaplan-Meier curves according to the data from GEPIA. The survival curves of low and high *SSBP1* expression groups were compared by the log-rank test (17). Furthermore, Cox analysis was used to identify the risk factors influencing OS. Clinical data with a significance level of $P<0.1$ from the univariate Cox analysis were further subjected to multivariate Cox analysis. The R package "Nomogram" was utilized to construct a prediction model for estimating 1-, 3-, and 5-year survival rates. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were calculated to evaluate the diagnostic efficacy of *SSBP1* using the "pROC" package.

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and GSEA analysis

GO enrichment analysis was performed to investigate the biological process (BP), molecular function (MF), and cellular component (CC) of SSBP1. KEGG enrichment analysis was used to explore the signaling pathways associated with HCC. The biological enrichment for GO and KEGG analyses was achieved using the "Cluster Profiler" R package ($P<0.05$, FDR <0.05). Finally, to gain

insight into the BP and predict the potential signaling pathways of *SSBP1* expression in HCC, we divided HCC samples into high- and low-expression groups based on the median expression of SSBP1 in HCC and performed GSEA analysis.

Cell culture

Five kinds of human HCC cell lines (HepG2, Hep3B, Huh7, SMMC-7721, Bel-7402), and a human noncancerous hepatic cell line (L-02) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Bel-7402 and L-02 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Carlsbad, USA). HepG2, Hep3B, Huh7, and SMMC-7721 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (BI, Kibbutz Beit Haemek, Israel) under 5% carbon dioxide at 37 ℃. The medium was changed every 2–3 days, and trypsin was added for digestion and passage when the cells were 80% confluent. All cells were authenticated via STR profiling. Mycoplasma status was checked often using the Luciferase Mycoplasma Detection Kit (Mlbio, Shanghai, China).

Patients and tissue sample

Between January 2019 and December 2023, HCC samples were collected from the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Yunnan University, which was under the supervision of the Board and Ethical Committee of Yunnan University. The patients included 24 males and 7 females with a mean age of 52.1 years (range, 35.0–72.0 years). The Clinical Trial Registration number is ChiCTR2200102016. All HCC patients provided written informed consent for their enrollment in this study, and the study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The present study was approved by the Ethics Committee of Yunnan University (No. IRB-2022-KMYXLL12).

Immunohistochemical (IHC) staining and evaluation

Paired HCC tissues and adjacent liver tissues (n=31) were collected from the Hepatological Surgery Department of the First Affiliated Hospital of Yunnan University, which was under the supervision of the Ethics Committee of Yunnan University. Detection of SSBP1 protein expression levels was conducted by IHC using the streptavidin-peroxidase method in HCC specimens. SSBP1 primary antibody (ab224053, Abcam, Cambridge, USA) was added dropwise and incubated overnight at 4 ℃. The slides were stained using the Maxvision TM3 HRP-Polymer IHC Kit (KIT-5220, MXB Biotechnologies, Fujian, China) (17). Negative controls were subjected to staining with isotype-matched control immunoglobulin G (IgG). The intensity of IHC staining was evaluated utilizing ImageJ software to determine the positive rate of tumors. Subsequently, the immunoreactivity scores (IRS) were computed in accordance with the methodology established in our prior research (18). All tests were performed in triplicate.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis for SSBP1 transcripts

Total RNA was isolated from the cell lines and tissues using TRIzol (Invitrogen, Waltham, USA). The 1 μg total RNA was reverse transcribed into cDNA using qPCR RT Master Mix (Toyobo, Shanghai, China). One-step qRT-PCR reaction assays were conducted utilizing the ABI StepOnePlus™ Real-Time PCR System with Tower (Life Technologies, Waltham, USA) using the LightCycler FastStart DNA Master SYBR Green I Kit (Vazyme, Nanjing, China). The primers for *SSBP1* gene (NM_001256510.1) were as follows: forward primer 5'-ACTGGGTGATGCAGTCAAAAG-3' and reverse primer 5'-TGCTTGTCGCCTCACATTATT-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; NM_002046.7) served as an internal control gene for the quantification of SSBP1 mRNA levels in cells (forward primer 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse primer 5'-GGCTGTTGTCATACTTCTCATGG-3'). The qRT-PCR data were analyzed using the double delta Ct method. All experiments were conducted in triplicate to ensure reliability of the results.

Protein isolation and Western blotting

Fresh HCC samples and adjacent liver tissues (n=6) were obtained for Western blotting analysis from the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Yunnan University. Protein samples were obtained from tissue lysates or cell lysates, the proteins were quantified by a BCA protein quantification kit (Beyotime, Shanghai, China). After denaturing the proteins, they

were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for protein separation and nitrocellulose membranes (Millipore, Burlington, USA) for protein blotting. Subsequently, the membranes were blocked for 1 h at room temperature and incubated with SSBP1 primary antibody (ab224053, Abcam) or GAPDH primary antibody (ab9485, Abcam) overnight at 4 ℃. Bound antibodies were detected using the Chemiluminescence Imaging System (Bio-Rad, Carlsbad, USA) and fluorochrome-labelled secondary antibodies. The relative expression level of the SSBP1 protein is quantified by calculating the ratio of the accumulated optical density (AOD) of the target band to that of the internal reference band. Semi-quantitative analysis was performed with ImageJ software. All tests were performed in triplicate.

Immunofluorescence analysis

We performed cellular localization analysis of SSBP1 protein using cellular immunofluorescence. First, HepG2, Hep3B, SMMC-7721, and Bel-7402 cells in good growth conditions were seeded in 6-well plates. On the second day after cell adherence, the mitochondria were stained with Mito Tracker Red, and the cells were fixed, permeabilized and blocked. After incubation with secondary antibodies the next day, the nuclei were stained with DAPI reagent and observed under a fluorescence microscope and photographed. Green fluorescence indicates SSBP1 protein, red fluorescence marks mitochondria, and blue fluorescence represents nuclei.

Statistical analysis

The statistical results were constructed using SPSS 24.0 and GraphPad Prism 9.0 software. Quantitative data are expressed as the mean ± standard deviation (SD). Comparative analyses between two groups were performed utilizing either paired or unpaired Student's *t*-test, contingent upon the normality of the data distribution and the equality of variances. P<0.05 was used as the threshold for significance in all analyses.

Results

SSBP1 is upregulated in HCC tissues and significantly associated with clinical stages

Based on TCGA data, comparing the expression of SSBP1 in 374 HCC samples and 50 normal samples, it was found that *SSBP1* is upregulated in HCC (*Figure 2A*). Further comparison of the expression of SSBP1 in 50 paired HCC samples also revealed increased expression of *SSBP1* in HCC tissues (*Figure 2B*). These results suggest that *SSBP1* is upregulated in HCC tissues. Using GEPIA database, a univariate analysis the expression of the *SSBP1* gene across clinical stages in 374 HCC patients was conducted (*Figure 2C*), and using the R software analyzed the differences during staging (*Figure 2D*). The results indicate a significant association between the expression of *SSBP1* and the clinical staging of HCC patients (F=3.2, P=0.02). The differences between stage I and stage II, as well as between stage I and stage III were statistically significant.

The correlation of SSBP1 mRNA expression with clinical pathological parameters in HCC patients

In the TCGA database, there were 171 cases of low *SSBP1* mRNA expression and 171 cases of high expression. According to the analysis of *SSBP1* mRNA expression in relation to age, gender, TNM staging, pathological type, and pathological staging, the expression of *SSBP1* mRNA is significantly associated with the gender (P=0.02) and M staging (P=0.03) of HCC patients, while it showed no significant correlation found with age, tumor grade, T staging, N staging, and pathological staging (all P>0.05) (*Table 1*).

The impact of SSBP1 mRNA expression on HCC patients' survival

The Kaplan-Meier curves show that the OS of HCC patients with high *SSBP1* mRNA expression is lower than that of patients with low *SSBP1* mRNA expression [hazard ratio (HR) =1.8, log-rank P=0.001], and this difference is statistically significant (*Figure 3A*). Furthermore, although the DFS of HCC patients with high *SSBP1* mRNA expression is lower than that of patients with low *SSBP1* mRNA expression, the difference between the two groups is not statistically significant (HR =1.3, log-rank P=0.07), indicating that the impact of high *SSBP1* mRNA expression versus low expression on HCC patient DFS is not significant (*Figure 3B*). This suggests that high *SSBP1* mRNA expression is a risk factor for the prognosis, with high *SSBP1* expression indicating a shortened OS time for HCC patients.

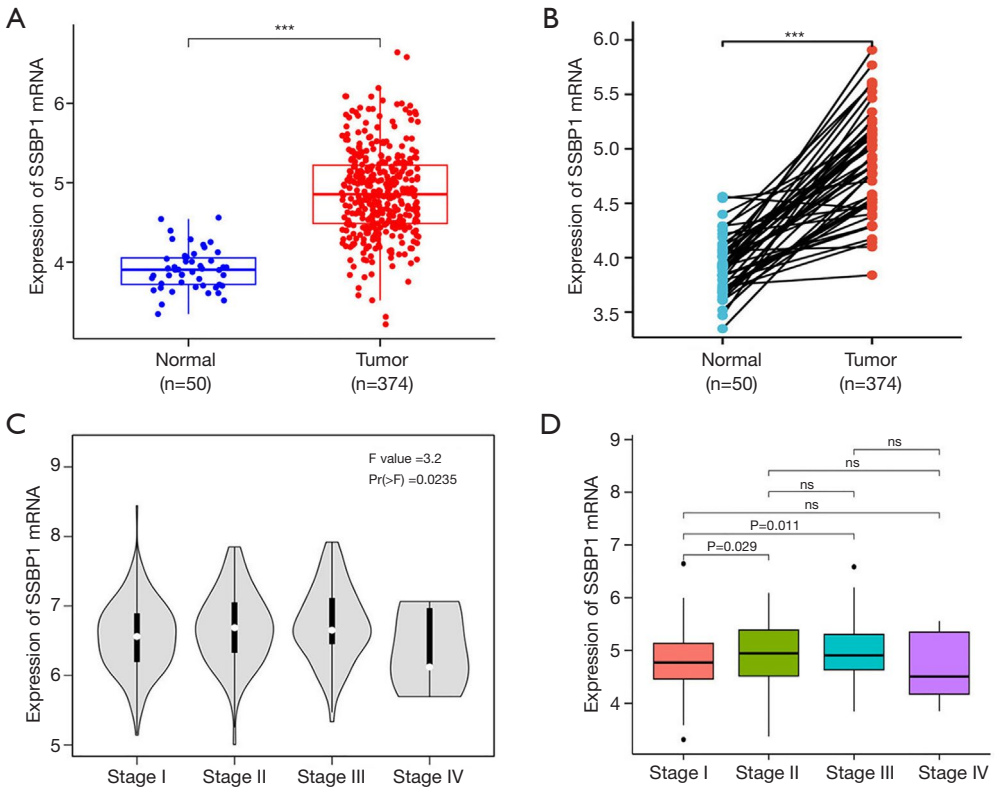

Figure 2 The relationship between the expression of SSBP1 mRNA and clinical stages of HCC. (A) Expression of SSBP1 mRNA in normal samples and HCC samples. (B) Expression of SSBP1 mRNA in paired HCC samples. (C) Expression of SSBP1 mRNA in different clinical stages of HCC. (D) The different expression of SSBP1 mRNA in different clinical stages of HCC. ***, P<0.001. SSBP1, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; ns, no significance.

Prognostic value of clinical and pathological parameters in HCC patients

The results of univariate Cox regression model showed that the expression of *SSBP1* (HR =1.933, P=0.003), pathological stage (HR =1.879, P<0.001), T stage (HR =1.816, P<0.001), and M stage (HR =3.924, P=0.02) were all significantly correlated with the OS (*Figure 4A*). Factors with P<0.1 were included in the multivariate Cox regression analysis. The expression level of *SSBP1* (HR =1.713, P=0.01) was identified as an independent risk factor for the prognosis of HCC patients (*Figure 4B*).

According to the multivariate Cox regression analysis results, a nomogram model integrating the risk score and these clinical indicators was constructed to predict the 1-, 3-, and 5-year survival rates of HCC patients (*Figure 4C*). The expression level of *SSBP1* has a significant impact on the survival, with higher *SSBP1* expression associated with lower survival rates. This indicates that the nomogram predictive model we constructed holds certain clinical value, enabling early monitoring and timely intervention to improve the quality of life for HCC patients.

Analysis of immune cell infiltration in HCC tissues

We then explored the relationships between the tumor immune microenvironment and HCC. The expression of *SSBP1* shows a significant correlation with the infiltration of three types of immune cells (|Spearman's r| >0.2, P<0.05). Specifically, *SSBP1* expression shows a negative correlation with the infiltration of naive B cells (r=–0.3, P=0.01), while it is positively correlated with memory B cells (r=0.28, P=0.02) and macrophages (r=0.44, P<0.001) (*Figure 5A-5C*).

Analysis of the diagnostic value of SSBP1 in HCC

We plotted the ROC curves to evaluate the diagnostic

Table 1 The correlation between *SSBP1* mRNA expression and the clinicopathological features of HCC patients

Clinical characteristics	Total	Expression of *SSBP1* mRNA, n (%)		χ^2	P
		Low expression	High expression		
Age (years)				1.170	0.28
<60	170	90 (52.9)	80 (47.1)		
≥60	172	81 (47.1)	91 (52.9)		
Gender				5.361	0.02
Male	232	106 (45.7)	126 (54.3)		
Female	110	65 (59.1)	45 (40.9)		
Grade				1.581	0.66
G1	45	25 (55.6)	20 (44.4)		
G2	167	86 (51.5)	81 (48.5)		
G3	118	55 (46.6)	63 (53.4)		
G4	12	5 (41.7)	7 (58.3)		
T stage				2.821	0.58
T0	1	1 (100.0)	0 (0.0)		
T1	170	86 (50.6)	84 (49.4)		
T2	86	39 (45.3)	47 (54.7)		
T3	75	41 (54.7)	34 (45.3)		
T4	10	4 (40.0)	6 (60.0)		
N stage				2.269	0.32
N0	250	119 (47.6)	131 (52.4)		
N1	3	2 (66.7)	1 (33.3)		
NX	89	50 (56.2)	39 (43.8)		
M stage				6.635	0.03
M0	259	121 (46.7)	138 (53.3)		
M1	4	1 (25.0)	3 (75.0)		
MX	79	49 (62.0)	30 (38.0)		
AJCC stage				1.214	0.75
Stage I	168	85 (50.6)	83 (49.4)		
Stage II	84	38 (45.2)	46 (54.8)		
Stage III	84	45 (53.6)	39 (46.4)		
Stage IV	6	3 (50.0)	3 (50.0)		

SSBP1, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; AJCC, American Joint Committee on Cancer.

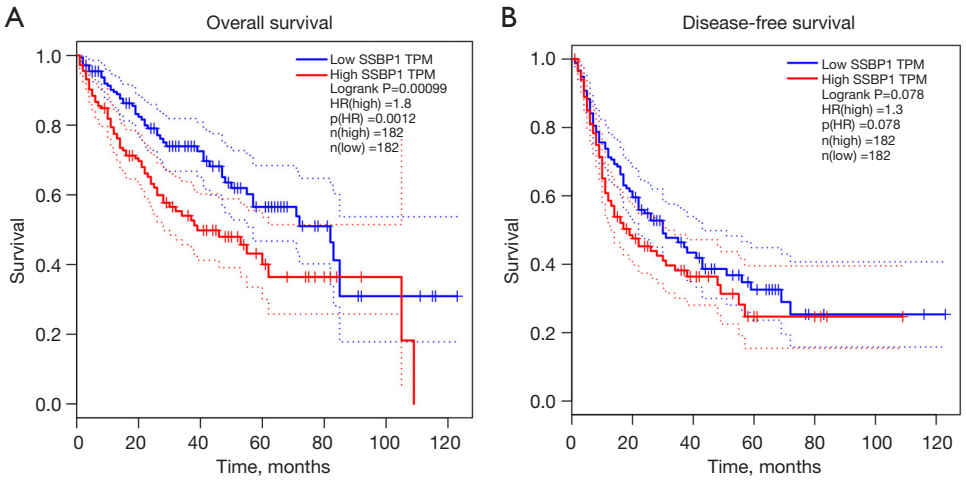

Figure 3 The relationship of *SSBP1* mRNA expression with the survival times of HCC patients. (A) Patients exhibiting high levels of SSBP1 (indicated in red) demonstrate a reduced OS in comparison to those with low levels of SSBP1 (indicated in blue). (B) Patients with high SSBP1 expression (red) experience a decreased DFS relative to their low SSBP1 expression counterparts (blue). SSBP1, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; OS, overall survival; DFS, disease-free survival; TPM, transcripts per million; HR, hazard ratio.

value of *SSBP1* expression in HCC. *SSBP1* expression levels are helpful for diagnosing HCC (AUC =0.954), T1/T2 stage tumor tissues (AUC =0.948), M0/M1 stage tumor tissues (AUC =0.959), and pathological stage I/II tumor tissues (AUC =0.947) (*Figure 6A-6D*). This indicates that *SSBP1* expression has good value for the early diagnosis of HCC. In addition, *SSBP1* expression is also effective in evaluating patients' 1-, 3-, and 5-year OS (AUC =0.664, 0.642, 0.627, respectively), disease-specific survival (DSS) (AUC =0.562, 0.623, 0.581, respectively), and progression-free interval (PFI) (AUC =0.599, 0.603, 0.639, respectively) (*Figure 6E-6G*).

GO, KEGG, and GSEA enrichment analyses

Enrichment analyses of GO, KEGG, and GSEA were performed to study the biological function of *SSBP1*. According to GO analysis, *SSBP1* is a key molecule in mtDNA synthesis, mainly involved in the BP associated with regulation of helicase activity, mtDNA replication, mitochondrial morphogenesis, and mtDNA metabolic process. The main CCs are nucleoid, mitochondrial nucleoid, and mitochondrial matrix. The main MF of *SSBP1* is single stranded DNA-binding (*Figure 7A*). In addition, the KEGG enrichment analysis indicated that *SSBP1* were mainly enriched in the signaling pathways related to mismatch repair, DNA replication and homologous recombination (*Figure 7B*). Finally, we explored the biological function of SSBP1 in HCC through GSEA analysis, the results of GSEA showed that the first three pathways related to high expression of *SSBP1* were DNA repair, myc-targets-v1 and reactive oxygen species signaling pathways (*Figure 7C*).

High expression of SSBP1 protein in human HCC tissues

To validate the results of the TCGA database analysis, we examined the expression of SSBP1 in HCC by IHC. We analyzed the expression of SSBP1 protein in 31 pairs of HCC tissues and adjacent liver tissues. The results showed that the positive signal of SSBP1 in HCC tissue samples was significantly stronger than that in adjacent liver tissues (*Figure 8A*). Further quantitative analysis of the positive cell rate of IHC showed that the expression of SSBP1 in HCC tissues was significantly higher than that in adjacent liver tissues ($P<0.01$) (*Figure 8B*).

Meanwhile, we collected 6 fresh HCC tissue samples and adjacent noncancerous liver tissues from the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Yunnan University. Firstly, we found that the mRNA expression level of SSBP1 in HCC tissues was significantly elevated compared to their corresponding adjacent liver

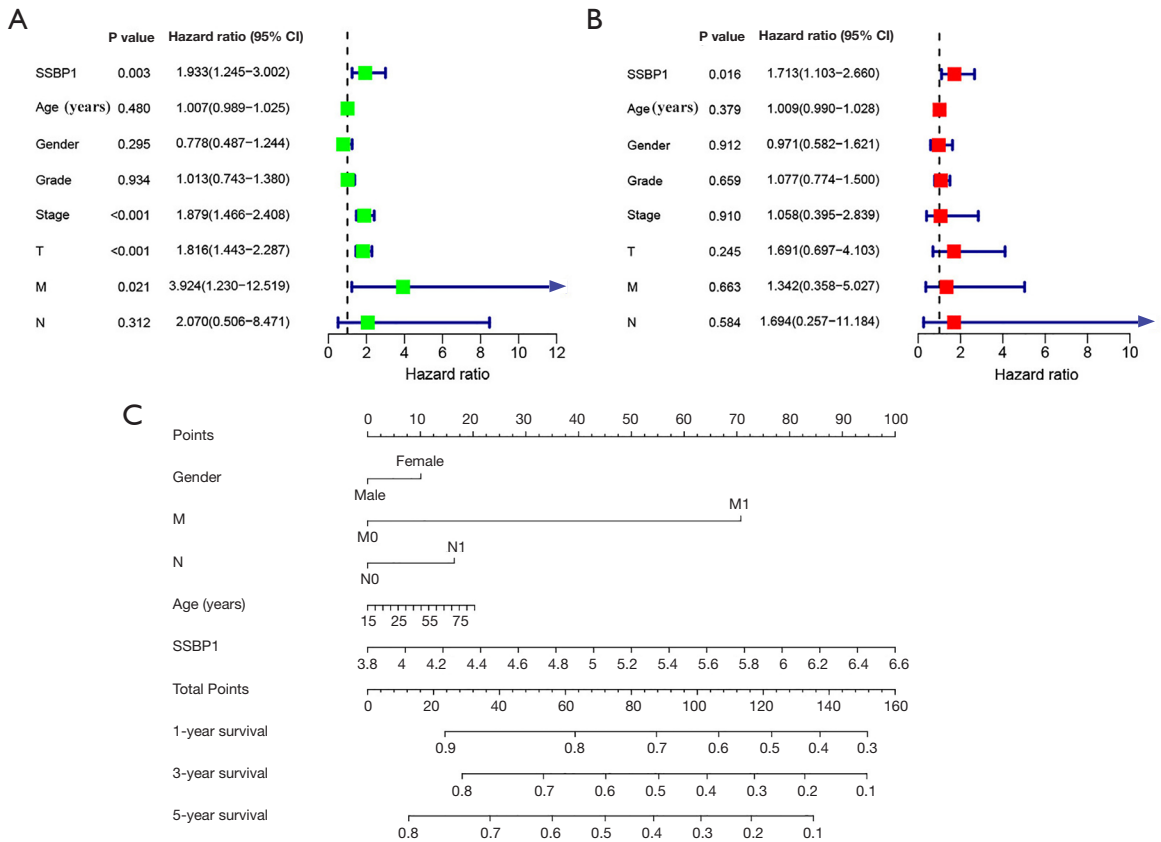

Figure 4 Construction of a nomogram. (A) Univariate Cox regression model to assess the prognostic relevance of the risk score and clinical variables in the TCGA cohort. (B) Evaluation through a multivariate Cox regression model to determine the prognostic importance of the risk score and clinical variables in the TCGA cohort. (C) A nomogram that integrates the risk score, age, and T stage to forecast the survival probabilities at 1, 3, and 5 years. Cox, cox proportional hazards; TCGA, The Cancer Genome Atlas; *SSBP1*, single-stranded DNA binding protein 1.

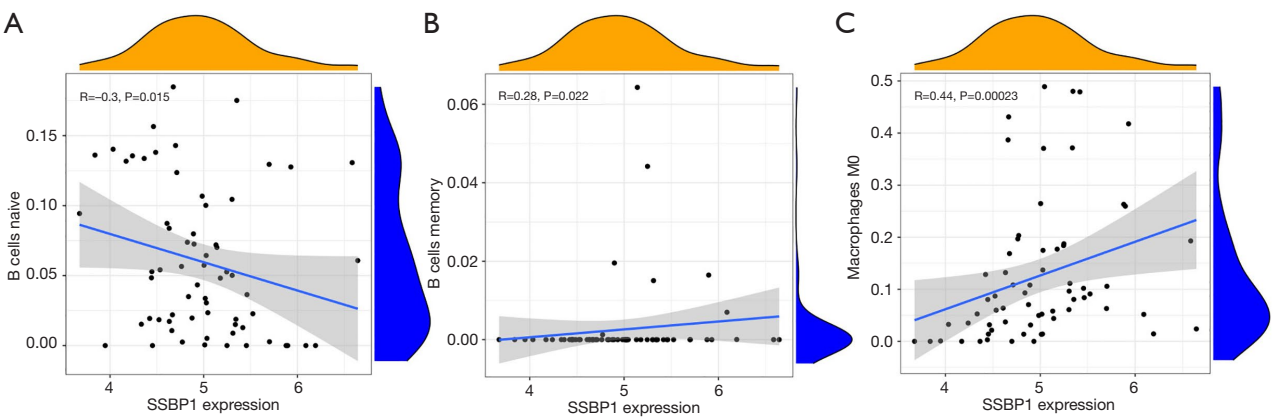

Figure 5 Correlation analysis of *SSBP1* gene with immune cell infiltration in HCC. (A) The correlation between *SSBP1* expression and the degree of infiltration of naive B cells. (B) The correlation between *SSBP1* expression and the degree of infiltration of memory B cells. (C) The correlation between *SSBP1* expression and the degree of infiltration of macrophages. *SSBP1*, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma.

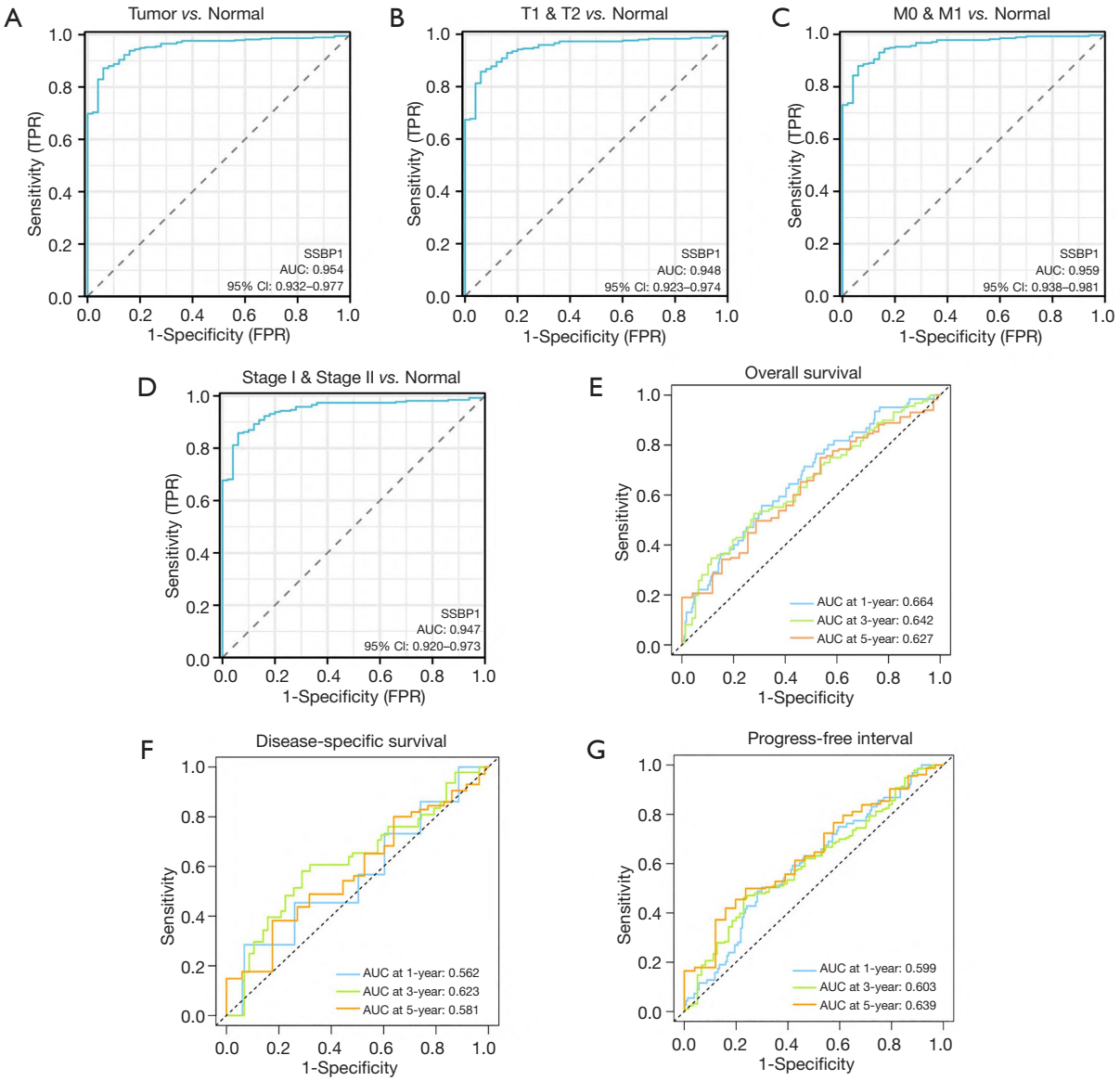

Figure 6 ROC curve analysis of *SSBP1* gene in HCC diagnosis. (A) ROC curve analysis of *SSBP1* for HCC diagnosis. (B) ROC curve analysis of tumor tissues in T1/T2 stage. (C) ROC curve analysis of tumor tissues in M0/M1 stage. (D) ROC curve analysis of tumor tissues in pathological stage I/II. (E) ROC curve analysis of OS. (F) ROC curve analysis of DSS. (G) ROC curve analysis of PFI. *SSBP1*, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic; OS, overall survival; DSS, disease-specific survival; PFI progression-free interval; TPR, true positive rate; FPR, false positive rate; AUC, area under the curve.

tissues (P<0.001) (*Figure 8C*). Next, total protein of tissues were extracted, and Western blotting was performed to detect the expression level of SSBP1 protein. The results showed that the expression level of SSBP1 protein in HCC tissues was significantly higher than in their corresponding adjacent liver tissues (*Figure 8D*). Quantitative analysis of SSBP1 protein expression revealed a significant increase in SSBP1 expression in liver cancer tissues compared to their corresponding adjacent tissues (P<0.001) (*Figure 8E*). This is consistent with the bioinformatics results, suggesting that SSBP1 may play a promoting role in the occurrence and development of HCC.

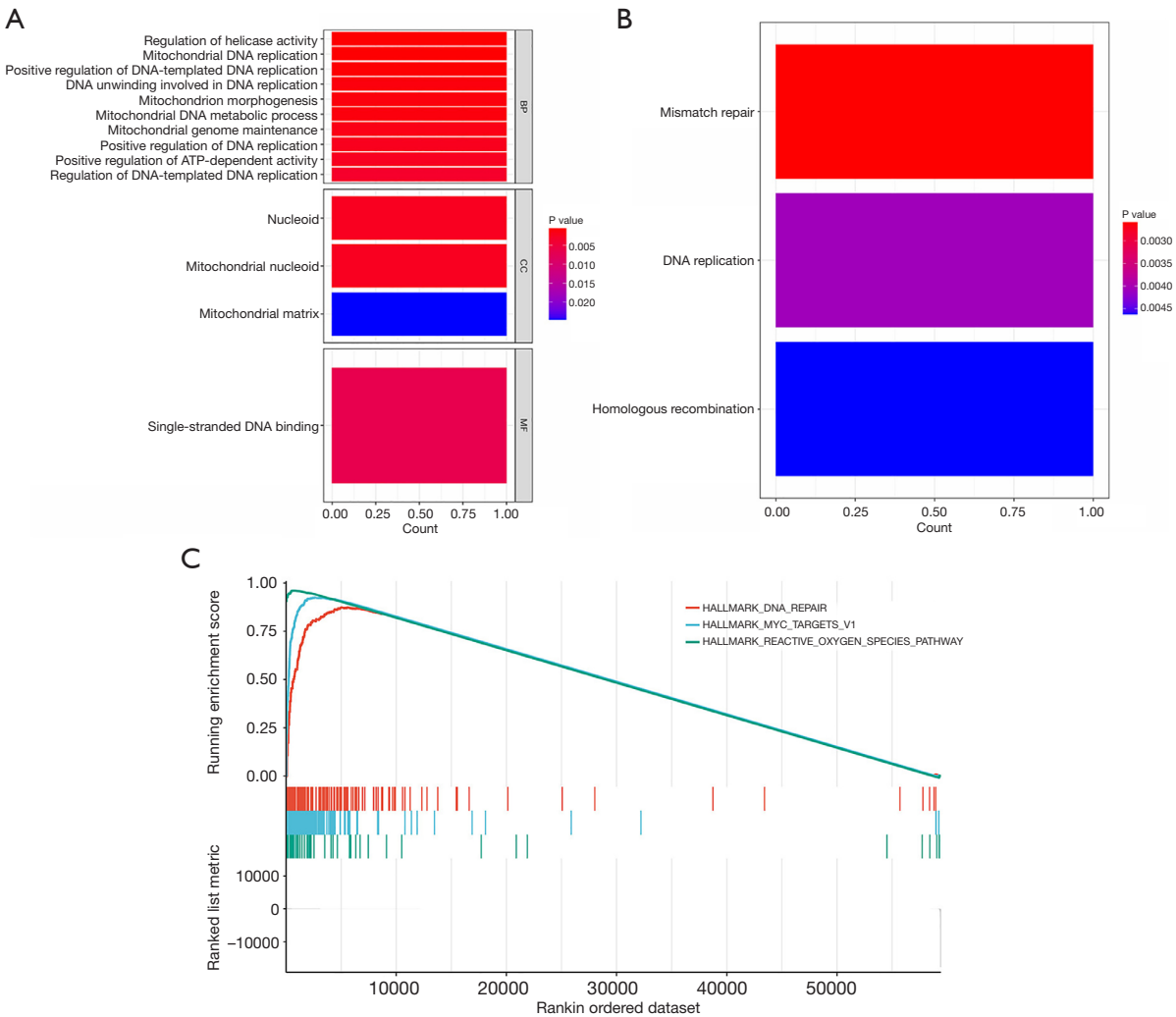

Figure 7 The results of the biological function of *SSBP1* in HCC through enrichment analysis. (A) Boxplot of GO enrichment analysis, including BP, CC, and MF. (B) Boxplot of KEGG signaling enrichment analysis. (C) Curve diagram of GSEA. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; *SSBP1*, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; BP, biological process; CC, cellular component; MF, molecular function; GSEA, gene set enrichment analysis.

High expression of SSBP1 mRNA and protein in HCC cell lines

We performed qRT-PCR and Western blotting to detect the mRNA and protein expression levels of SSBP1 in a noncancerous hepatic cell line (L-02) and five different kinds of HCC cell lines. The results showed that the mRNA and protein levels of SSBP1 in the HCC cell lines HepG2, Hep3B, Huh7, SMMC-7721, and Bel-7402 were significantly higher than those in human noncancerous hepatic cell line (L-02) (*Figure 9A*,*9B*).

SSBP1 protein is localized in the mitochondria of different HCC cell lines

Immunofluorescence analysis of HepG2, Hep3B, SMMC-7721, and Bel-7402 cells showed that the green fluorescence of SSBP1 completely overlapped with the red fluorescence of the mitochondria, confirming the localization of human SSBP1 protein to the mitochondria (*Figure 9C*).

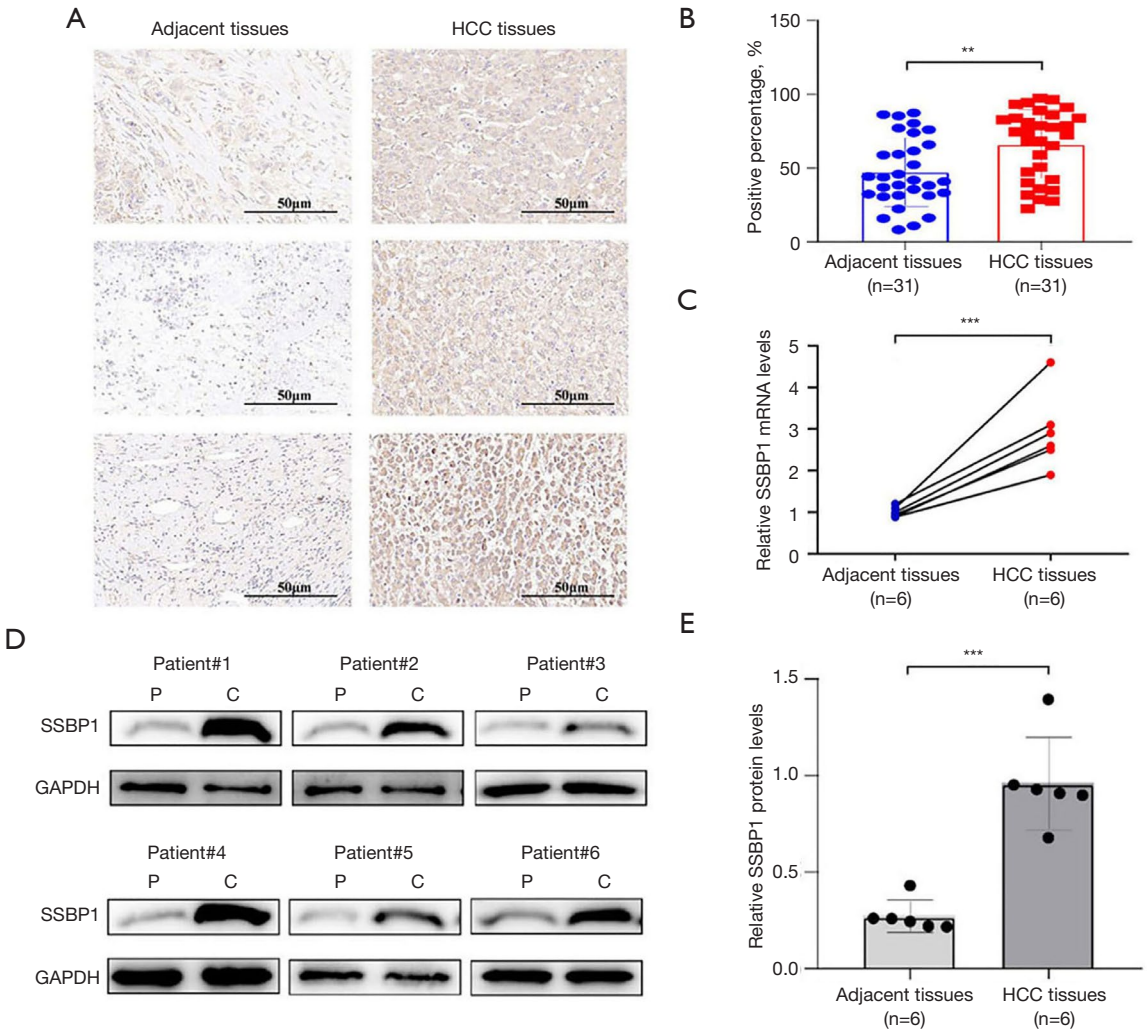

Figure 8 Human SSBP1 is significantly upregulated in HCC tissues. (A) IHC of adjacent liver tissues versus cancerous tissues from HCC patients. (B) Quantitative analysis of immunohistochemical detection. (C) mRNA expression levels of SSBP1 in 6 pairs of HCC tissue samples using qRT-PCR analysis. (D) Protein expression levels of SSBP1 in 6 pairs of HCC tissue samples using Western blot analysis. (E) Relative quantification of SSBP1 protein expression levels. **, P<0.01; ***, P<0.001. SSBP1, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; qRT-PCR, quantitative reverse transcription polymerase chain reaction; P, paired adjacent liver tissues; C, HCC tissues.

Discussion

The liver is a highly oxygen-consuming and metabolically active organ, being one of the most mitochondria-rich organs in the human body, highlighting the vital importance of mitochondrial function for hepatocytes (19,20). Mitochondria are unique organelles with their own mtDNA and genetic patterns, replicating and maintaining independently of the cell's nuclear genes (21). Mitochondria serve as critical sites for energy conversion and metabolic processes in eukaryotic organisms. Dysfunction of these organelles is associated with a range of diseases, including various forms of cancer (22). Furthermore, solid tumors are characterized by hypoxic conditions and disturbances in mitochondrial metabolism, which are linked to metabolic reprogramming that contributes to the progression of the disease (22). SSBP1 is a key molecule in the synthesis of mtDNA, a housekeeping gene involved in mitochondrial biogenesis, and a ssDNA binding complex that maintains the stability of the genome. Hence, abnormal expression

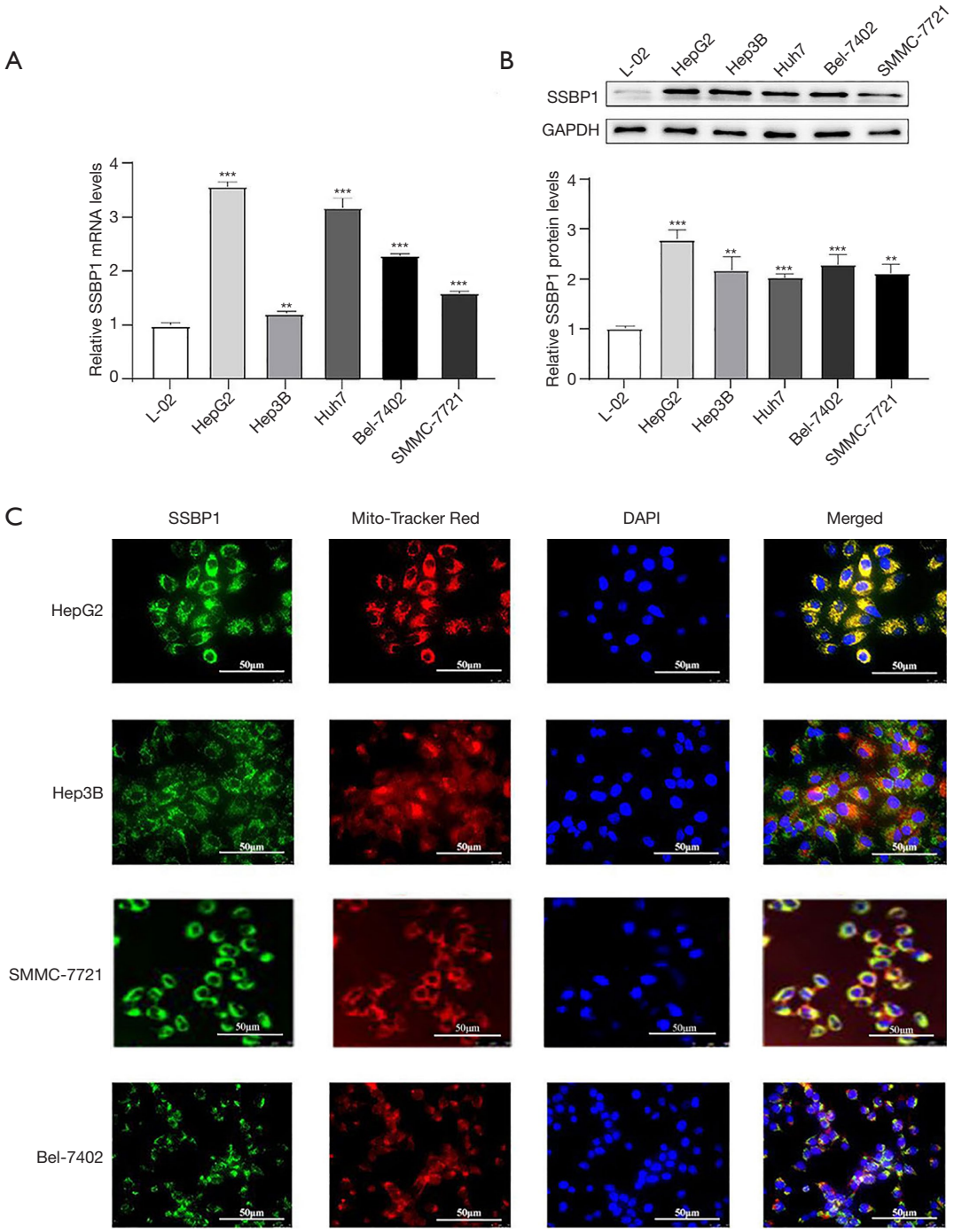

Figure 9 Expression analysis of SSBP1 in different HCC cell lines. (A) The mRNA expression level of SSBP1 in HCC cell lines. (B) The expression level of SSBP1 protein in HCC cell lines. (C) Cellular localization of SSBP1 protein in HCC cell lines. The mitochondria were stained with Mito Tracker Red, while the nuclei were stained with DAPI reagent. Green fluorescence indicates the SSBP1 protein, red fluorescence marks the mitochondria, and blue fluorescence represents the nuclei. **, P<0.01; ***, P<0.001. SSBP1, single-stranded DNA binding protein 1; HCC, hepatocellular carcinoma; DAPI, 4',6-diamidino-2'-phenylindole.

of SSBP1 is closely associated with the occurrence, development, and prognosis of various malignant tumors (12-15). This study found that the expression of *SSBP1* was high in patients with HCC and was significantly related to worsening prognosis. Meanwhile, *SSBP1* has a good diagnostic value for HCC, and it may be a potential molecular marker for diagnosis and prognosis judgment of HCC patients as well as a potential target for immunotherapy.

We firstly identified the high expression of SSBP1 in HCC tissue through the TCGA database. Secondly, we evaluated the correlation between the mRNA expression levels of SSBP1 and the clinical pathological features as well as the prognosis of survival in HCC patients. The constructed Nomogram prediction model showed that the expression level of SSBP1 significantly impacted the survival of HCC patients, suggesting that high SSBP1 expression could serve as a molecular marker associated with poor prognosis. The continuous interaction between tumor cells and the tumor microenvironment (TME) plays a crucial role in the occurrence, progression, metastasis, and response to treatment of tumors (23). In the context of immune cells being important factors in the TME, we found that SSBP1 is widely involved in immune cell infiltration (12). In our study, we also observed a significant positive correlation between SSBP1 expression levels and the infiltration of two immune cell types in HCC tissues, namely memory B cells and macrophages, while showing a significant negative correlation with naive B cells. Within the TME of HCC, SSBP1 may have an important regulatory role. This indicates that SSBP1 may promote immune activity in the TME of HCC. We also found that the diagnostic value of SSBP1 as a biomarker was statistically significant for diagnosing HCC, T staging, M staging, pathological staging, OS, DSS, and PFI based on the area AUC. SSBP1 has shown promising effectiveness in the diagnosis and prognostic assessment of HCC. Therefore, the expression level of SSBP1 has the potential to be used as a new molecular marker for the early diagnosis and prognosis of HCC. The GO enrichment analysis of SSBP1 revealed that it was enriched for mitochondrial biological functions. KEGG analysis showed that SSBP1 was associated with multiple DNA replication, mismatch repair and homologous recombination pathways. Through GSEA analysis, we explored the biological function of SSBP1 in HCC. Functional analysis showed that the first three pathways strongly related to the high expression of SSBP1 were DNA repair, myc-targets-v1 and reactive oxygen species signaling pathways. These results suggest that SSBP1 has a potential application value in HCC.

Additionally, evidence of high SSBP1 expression was found in HCC tissues and cell lines either by high-sensitivity PCR molecular diagnosis, Western blotting or IHC. Immunofluorescence indicated that SSBP1 is localized to the mitochondria. The result is consistent with previous literature report (24). Based on bioinformatics analysis, clinical tissue samples analysis, and cell line expression analysis, we tentatively conclude that SSBP1 may be a mitochondria-localized oncogene in HCC. This study has laid the experimental foundation for future research on the relationship between SSBP1 and mitochondrial function regulation in HCC.

This study conducted preliminary research on the expression and prognosis of SSBP1 in bioinformatics, with only a small portion of experimental validation of SSBP1 expression and a lack of more in-depth mechanistic experiments. Subsequent prospective clinical studies will explore the important role of SSBP1 in HCC.

Conclusions

In summary, our study suggests that the expression level of *SSBP1* is significantly increased in both HCC cells and tissues, and high expression level of *SSBP1* is an independent risk factor for poor prognosis. Meanwhile, *SSBP1* has a good diagnostic value for HCC, and it may be a potential molecular marker for diagnosis and prognosis judgment of HCC patients as well as a potential target for immunotherapy.

Acknowledgments

None.

Footnote

Reporting Checklist: The authors have completed the MDAR and TRIPOD reporting checklists. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1196/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-1196/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Yunnan University (No. IRB-2022-KMYXLL12). All patients provided written informed consent for their enrollment in this study.

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