

SPINDOC is Highly Expressed in Pan-Cancer Samples and Can Promote the Proliferation, Invasion and Migration of Hepatocellular Carcinoma Cells by Activating Wnt/ β -Catenin Signaling Pathway

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Purpose: As a novel genetic biomarker, little information is available about the possible role of *SPINDOC* in different malignant tumors and in hepatocellular carcinoma (HCC).

Methods: Based on The Cancer Genome Atlas (TCGA) database, the expression level of *SPINDOC* in pan-cancer and hepatocellular carcinoma samples was first determined using bioinformatics analysis. The potential relationship between the expression level as well as the clinical characteristics and the molecular mechanisms through which *SPINDOC* can promote the proliferation, invasion and migration of HCC cells was evaluated. In addition, cell-based studies and in vivo experiments were used to verify the bioinformatics analysis results.

Results: Bioinformatics analysis showed that *SPINDOC* expression was significantly increased in 18 human malignancies and the gene expression level was positively correlated with poor clinical prognosis in kidney renal papillary cell carcinoma (KIRP), pheochromocytoma and paraganglioma (PCPG) and liver hepatocellular carcinoma (LIHC). The main type of genetic variation of *SPINDOC* was amplification, and the increase of *SPINDOC* mRNA expression level was directly related to the amplification of this gene. The expression level of *SPINDOC* in patients with primary HCC was positively correlated with poor clinical prognosis, as well as the clinical grade and stage of carcinoma. Gene set enrichment analysis (GSEA) analysis showed that high expression of *SPINDOC* could activate Wnt/ β -catenin signaling pathway. Moreover, in vitro and in vivo experiments showed that *SPINDOC* gene silencing significantly inhibited the proliferation, migration and invasion of Huh7 and SK-HEP-1 cells and decreased the expression of SPIN1, Wnt1, β -catenin and cyclin D1 but increased the expression of AXIN2.

Conclusion: *SPINDOC* is highly expressed in pan-cancer and HCC samples. This factor can effectively promote the invasion and migration of hepatocellular carcinoma (HCC) cells by activating Wnt/ β -catenin signaling pathway, and thus can serve as a potential therapeutic target for HCC management.

Keywords: *SPINDOC*, SPIN1, Wnt/ β -catenin, pan-cancer, hepatocellular carcinoma

Introduction

Liver cancer is a lethal malignancy, among which hepatocellular carcinoma (HCC) constitutes the main type. HCC displays the characteristics of high malignancy and rapid metastasis and has recurrent rate after treatment.^{1,2} The clinical symptoms of early HCC have been found to be insidious, and approximately 70% to 80% of patients have metastasized tumors when they are initially diagnosed. Although there are several treatment options such as surgical liver resection, radiotherapy as well as chemotherapy available for the management of this malignancy, the prognosis of HCC patients still remains very poor.¹⁻³ Targeted therapy represented by sorafenib as an important treatment modality for HCC has

achieved promising clinical effects to some extent.⁴ However, there is an urgent need to identify novel and efficient targeted therapy biomarkers to improve clinical treatment outcomes.

Spindlin1 (*SPIN1*) is an important member of the SPIN/SSTY gene family.⁵ A number of previous studies have shown that *SPIN1* can promote tumor progression by activating Wnt signaling pathway in breast cancer, non-small cell lung cancer and glioma.^{6–8} Based on *SPIN1*'s tumor-promoting effects in breast cancer,⁹ gastric cancer¹⁰ and liver cancer,¹¹ *SPIN1* is considered function as a tumor-promoting factor. As an interaction partner of *SPIN1*, *SPIN1* docking protein (*SPINDOC*) plays an important biological role by binding to *SPIN1* protein.¹² *SPINDOC* can block the potential interaction between *SPIN1* and methylated histone tails, thus inhibiting the activation of *SPIN1* on the various downstream pathways and molecules. *SPINDOC* has been considered as a negative regulator of *SPIN1*. However, it was also found that *SPINDOC* was highly expressed in a variety of tumors, and the mutant *SPINDOC* could activate the Wnt signaling cascade.¹³ Since *SPINDOC* has been poorly studied in cancer, the role of this factor in cancer remains unclear. Therefore, we have designed this study to investigate the potential role of *SPINDOC* HCC progression.

Bioinformatics analysis was used to observe the expression of *SPINDOC* and to decipher the relationship between the expression level of *SPINDOC* in pan-cancer and HCC samples of different age, gender, tumor clinical grade, prognosis stage, etc. Subsequently, we employed the Gene Set Enrichment Analysis (GSEA) method to observe the pathway of *SPINDOC* enrichment in the tumors to elucidate the underlying mechanisms through which *SPINDOC* can promote the progression of HCC. Additionally, these results were further verified by in vitro experiments in two different HCC cell lines and xenograft mouse model.

Materials and Methods

Data Sources and Bioinformatics Analysis

The mRNA expression and clinical data of *SPINDOC* in 33 tumors were obtained from UCSC Xena online database (<https://xena.ucsc.edu/>). We only extracted the tumor types from the tumors with the number of normal control samples greater than or equal to 1 for analysis, and the specific number of samples has been shown in [Supplementary Table S1–3](#). Genetic variation information from biological portal (<https://www.cbioportal.org/>).^{14,15} “Liver” and “Liver Hepatocellular Carcinoma (TCGA, Firehose Legacy, 442)” were selected in “Query”, and then the gene change characteristics of *SPINDOC* was searched. The results of *SPINDOC* mutation frequency, mutation type and CAN (copy number change) in TCGA database were observed in the module “Tumor Type Summary”. The cancer samples were then divided into low and high groups according to the median value of the *SPINDOC* expression level. We used the gene set enrichment analysis (GSEA) method to compare the various gene expression profiles of HCC specimens with *SPINDOC* low expression as well as *SPINDOC* high expression. CIBERSORT was then used to examine the relative fractions of the 22 infiltrating immune cell types in each tumor sample and to determine the variations among the various immune cells present in the different groups. In addition, the “survival” Receiver Operating Characteristic (ROC) software package verified the validity of *SPINDOC* expression levels in evaluating patient outcomes by calculating Area Under Curve (AUC).^{16,17}

Cell Culture

HCC cell lines Huh7, HepG2, SK-HEP-1 and immortalized human liver cell line LO2 were obtained from School of Life Sciences, Sun Yat-sen University (Guangzhou, CHN). The cells were cultured in dulbecco's modified eagle medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% penicillin and streptomycin (Sigma, USA) at 37°C with 5% of CO₂.

Cell Transfection

Huh7 cells were transfected with siRNA NC and *SPINDOC* siRNA-1, *SPINDOC* siRNA-2, *SPINDOC* siRNA-3 (OLIGOBIO, CHN) by liposome transient transfection method. According to the Lipofectamine™ RNAiMAX specification (Invitrogen, USA), the cell transfection concentration was in the range of 40%–50%. The siRNA concentration used was 30 nmol/L. The transfection was observed under fluorescence microscope. shRNA targeting *SPINDOC* and shRNA-NC (OLIGOBIO, CHN) were stably transfected into Huh7 cells. Spindoc-plvx-shRNA2-ZSGreen-T2A-Puro was introduced

into 293T cells to package the lentivirus. 293T cells were co-transfected with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Eight hours after transfection, they were replaced with the complete culture medium. After 48 h of culture, the cell supernatant rich in lentivirus particles was collected and concentrated to obtain the concentrated lentivirus with high titer. The virus titers were thereafter measured and calibrated in 293T cells. siRNA sequences for *SPINDOC* are shown below: *SPINDOC*-siRNA-1 (Sense, 5'-GCCGCACCAUGGCCCUAAATT-3' and anti-sense, UUUAGGGCCAUGGUGCGGCTT); *SPINDOC*-siRNA-2 (Sense, 5'-CGUUCUCAUUUACAUCUGUTT-3' and anti-sense, ACAGAUGUAAAUGAGAACGTT); *SPINDOC*-siRNA-3 (Sense, 5'-GAGUGACCCAACAGGAGAATT-3' and anti-sense, UUCUCCUGUUGGGUCACUCTTT).

Quantitative Real-Time PCR

Total RNA was extracted by Trizol (Invitrogen, USA) method and RNA concentration was then determined. According to the transcription response kit instructions (Invitrogen, USA), 1 μ L of RNA was taken from each sample for reverse transcription reaction to synthesize cDNA. Using cDNA as the template, PCR reaction was performed using 20 μ L system for gene detection of each sample. The expression of *SPINDOC* was calculated by the $2^{-\Delta\Delta C_t}$ method. The target-specific primer sequences for the RT-PCR used were as follows: *SPINDOC* (forward primer, GAGGTGACGCTGAAATGC and reverse primer, ACTCCTGCTCCCAAGACA); GAPDH (forward primer, GGAGCGAGATCCCTCCAAAAT and reverse primer, GGCTGTTGTCATACTTCTCATGG).

Western Blot Analysis

The proteins were extracted by using Radio-Immunoprecipitation Assay (RIPA) (Beyotime Institute of Biotechnology, CHN) lysis buffer adding with PMSF (KeyGEN, CHN) and protease inhibitor cocktail. Forty micrograms of protein lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (KeyGEN, CHN) and transferred to a polyvinylidene fluoride (PVDF) membrane (KeyGEN, CHN). After blocking with skimmed milk for 1 hour, the membrane was incubated with the specific primary antibodies overnight. The membranes were then washed and incubated with secondary antibodies (ZSGB-BIO, CHN) at room temperature for 2 hours. Antibodies against Wnt1 (1500, ab85060), cyclin D1 (1100, ab16663), *SPINDOC* (1:50, ab220962) were purchased from Abcam (UK). β -catenin (1:500, 610153), GAPDH (1:5000, 60004-1-Ig) were purchased from BD sciences (USA). AXIN2 (1:50, K006954P) and SPIN1 (1:50, K006346P) were purchased from Solarbio (CHN).

Flow Cytometry

After the transfected Huh7 and SK-HEP-1 cells were harvested, fluorescein isothiocyanate (FITC) and Annexin V (BestBio, CHN) were used to stain the cells according to the manufacturer's protocol (FITC Annexin V Apoptosis Detection Kit, BD Biosciences, USA) and a flow cytometric analysis was performed.

Colony Formation Assay

Twenty-four hours after transfection, the cells were digested with 0.25% trypsin (BestBio, CHN), neutralized and centrifuged. The cells were then inoculated at a density of 300 cells/2mL/well in a 6-well plate (Corning, USA) and incubated at 37°C in a 5% CO₂ incubator (ShangHai KunCheng Scientific Instrument Co., LTD, CHN). On the 5th day, 500 μ L FBS was added to each well in the plate and the colony formation was closely observed. When the colony formation was visible to the naked eye, the supernatant was discarded, the cells were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet dye (Beijing Dingguo Changsheng Biotechnology, CHN), and the colony number of >50 cells was counted.

MTT Colorimetric Assay

MTT colorimetric assay was used to measure the cell viability. The cells (1×10^4) were seeded in 96-well plates (Corning, USA). Huh7 and SK-HEP-1 cells were inoculated with 96-well plates in an incubator (ShangHai KunCheng Scientific Instrument Co., LTD, CHN) containing 5% CO₂ and saturated humidity at 37°C for 24 h. The complete medium was added and the cells were cultured for 48 h, after which the supernatant was removed. The wavelength of 490 nm was

measured by the microplate analyzer (Olympus, JPN) to determine the absorbance *A*, and the inhibition rate of the cell proliferation was calculated.

Invasion and Migration Experiments

Matrigel matrix glue (BD Biosciences, USA) was diluted in the serum-free medium at 1:6, and 50 μ L was evenly spread into the upper chamber of Transwell chamber (Corning, USA). The chamber was placed in a 24-well plate and incubated at 37°C for 4h to cause it to gelatinize. The cell density was adjusted to 2×10^5 cells/mL, and 100 μ L per well was inoculated into the upper chamber of transwell chamber. The cells in the upper chamber were removed after 24h, fixed with methanol and 0.1% crystal violet (Beijing Dingguo Changsheng Biotechnology, CHN) for 20min, stained for 10min, and washed with PBS twice. The count was carried out under an inverted optical microscope (Olympus, JPN).

Scratch Wound-Healing Assay

The Huh7 and SK-HEP-1 cells were seeded in 6-well culture plates (Corning, USA) at 95–100% confluence. The cells were harvested 48 hours after transfection. The scratch was created using a 200 μ L pipette tip. PBS was used to wash the plates for three times to remove the cellular debris. Images for the wound closure were photographed at the different times (12, 24, 36 and 48 h) by microscope (Olympus, JPN).

Animal Experiments

Female BALB/c nude mice (4–6 weeks of age) were purchased from GemPharmatech Co., Ltd. [Experimental Animal Production License No: SCXK (Su) 2019–0007]. After successful implantation of tumor, nude mice were placed in experimental Animal Center of Guangxi University of Chinese Medicine. The mice were divided into sh-NC group and *SPINDOC*-shRNA group. *SPINDOC*-shRNA and negative control (sh-NC) were stably transfected into Huh7 cells. The cells infected with fluorescent virus were sub-cultured to the required number and collected. In total, 1×10^7 transfected cells were suspended in 100 μ L serum-free RPMI 1640 culture medium and were injected into the axilla of nude mice. After inoculation, the nude mice were raised in a sterile environment. The long diameter and short diameter of the tumor were measured with vernier calipers at an interval of 2–3 days. The tumor volume was calculated according to the formula $0.5 \times \text{length} \times \text{width}^2$ and the growth curve was drawn. When subcutaneous tumor volume $> 50 \text{mm}^3$, the tumor formation reached the standard size. Until day 33, all the mice were sacrificed and tumor xenografts were collected and weighed. All the animal experiments were approved by the Animal Experiment Ethics Committee of Guangxi University of Chinese Medicine (DW20220310-032). National Institutes of Health guide for the care and use of laboratory animals was strictly followed by us.

Immunohistochemistry

Fresh nude mouse tumor specimens were converted into wax blocks by routine fixation, and standardized operation was carried out according to the kit instructions (Beijing Dingguo Changsheng Biotechnology, CHN). The primary antibody was added and incubated at 4°C overnight. The specific information of primary antibodies used is as follows: Wnt1, 1:500, ab85060, Abcam, (UK); cyclin D1, 1:100, ab16663, Abcam, (UK); *SPINDOC*, 1:50, ab220962, Abcam, (UK); β -catenin, 1:500, 610153, BD sciences, (USA); AXIN2, 1:50, K006954P, Solarbio, (CHN); SPIN1, 1:50, K006346P, Solarbio, (CHN). The blocks were washed with PBS 3 times, 2min each, and biotin-labeled sheep anti-rabbit IgG solution (1:100, Z021006Y, ZSGB-BIO, CHN), was added followed by incubation at room temperature for 30min. Thereafter, DAB chromogenic solution was added after PBS washing, and the blocks were placed under the microscope for observation. The reaction time was controlled, and the blocks were then washed with the distilled water to stop the reaction. Hematoxylin (Beijing Dingguo Changsheng Biotechnology, CHN) was added for redyeing, followed by washing, routine dehydration, spin drying, and observation of the results under the microscope after sealing with the neutral adhesive.

Hematoxylin-Eosin (HE) Staining

The tissue sections were washed with the distilled water and stained with hematoxylin (Beyotime, CHN). The staining time was controlled (about 5min) according to the condition of the tissue and dye. After rinsing with the distilled water, 1% hydrochloric acid ethanol (Beyotime, CHN) was added. After rinsing with the distilled water, eosin staining was performed (about 2s~8s). After rinsing with the distilled water again, anhydrous ethanol (Beyotime, CHN) was added to dehydrate, and neutral gum (Beyotime, CHN) was added. After sealing, the tablets were observed under a microscope and photographed (Olympus, JPN).

Statistical Analysis

Bioinformatics analysis was performed based on R programming (version 4.0.2, <https://www.r-project.org/>), statistical analysis was performed by using SPSS statistical software (version 22.0, Chicago, USA) and GraphPad Prism V7.0 software (GraphPad Software, Inc., California, USA). All the data were expressed as mean \pm standard deviation (SD), and *t*-test was used to compare the mean values between the two groups. The values of more than two groups were compared using analysis of variance (ANOVA). Chi-square test was used to analyze the relationship between SPINDOC expression and clinical features. Kaplan Meier method was used for survival analysis, and long rank test was used for comparison. When $P < 0.05$, the difference was considered as statistically significant.

Results

SPINDOC Expression in Pan-Cancer Samples Was Correlated with Adverse Clinical Outcomes

A total of 24 tumor types were included in the statistical analysis by analyzing specific tumor types with a normal sample number greater than or equal to 1 ([Supplementary Table S1](#)). *SPINDOC* expression was elevated in 18 of these tumors compared with the adjacent normal tissues ($P < 0.05$) ([Figure 1A](#) and [B](#)) and there was no difference in expression as compared to the normal controls in the other 6 tumors ([Supplementary Table S2](#)). We divided the expression level of *SPINDOC* into high and low groups and observed the effect of different expression levels on the prognosis of patients in 24 tumor types. *SPINDOC* was highly expressed in KIRP (Kidney renal papillary cell carcinoma), PCPG (Pheochromocytoma and Paraganglioma), ([Figure 1D](#) and [E](#)) and Liver hepatocellular carcinoma (LIHC) ([Figure 3A](#)) resulted in the poor prognosis. The findings of univariate analysis indicated that *SPINDOC* can be used as a potential risk factor affecting the prognosis of patients in LIHC, KIRP and PCPG tumor types ([Figure 1C](#) and [Supplementary Table S3](#)). These results indicated that *SPINDOC* can act as a tumor-promoting factor in a variety of human tumors. To investigate the potential association between *SPINDOC* and primary liver cancer, we decided to further analyze its oncogenic role in HCC models.

Genetic Variation and Methylation Analysis of SPINDOC

We observed the genetic changes of *SPINDOC* in LIHC samples from the TCGA cohort, and the results showed that *SPINDOC* was genetically altered in 8.9% (34/382) of HCC samples. High mRNA expression (7.74%) displayed the highest frequency among various variations, followed by mutation (0.86%), and amplification and deep deletion both accounted for 0.29% ([Figure 2A](#)). According to the type, site and number of cases of *SPINDOC* gene mutation, the most common *SPINDOC* gene mutation was missense mutation (0.61%) occurred in R197C, and the type was copy number deletion. The second was missense mutation (0.28%) in R53T, with two alleles. The mutation of P247Hfs*147 was intra-frame insertion or deletion (0.09%), and the type was two alleles. Missense mutation occurred in D234G (0.07%), and the type was copy number increase ([Figure 2B](#)). The increase of *SPINDOC* mRNA expression in the tumors was mainly caused by gain and amplification ([Figure 2C](#)). In terms of the relationship between mutation and survival prognosis of patients, mutation was found to lead to poor prognosis of patients ($P < 0.01$) ([Figure 2D](#)). From the methylation data of *SPINDOC* in liver cancer, the mRNA expression level of *SPINDOC* was found to be significantly negatively correlated with methylation level (Cor = -0.68, $P = 3.98E-52$) ([Figure 2E](#)). The above indicated that the increased mRNA expression level of *SPINDOC* was related to the amplification of this gene and was negatively correlated with the methylation level.

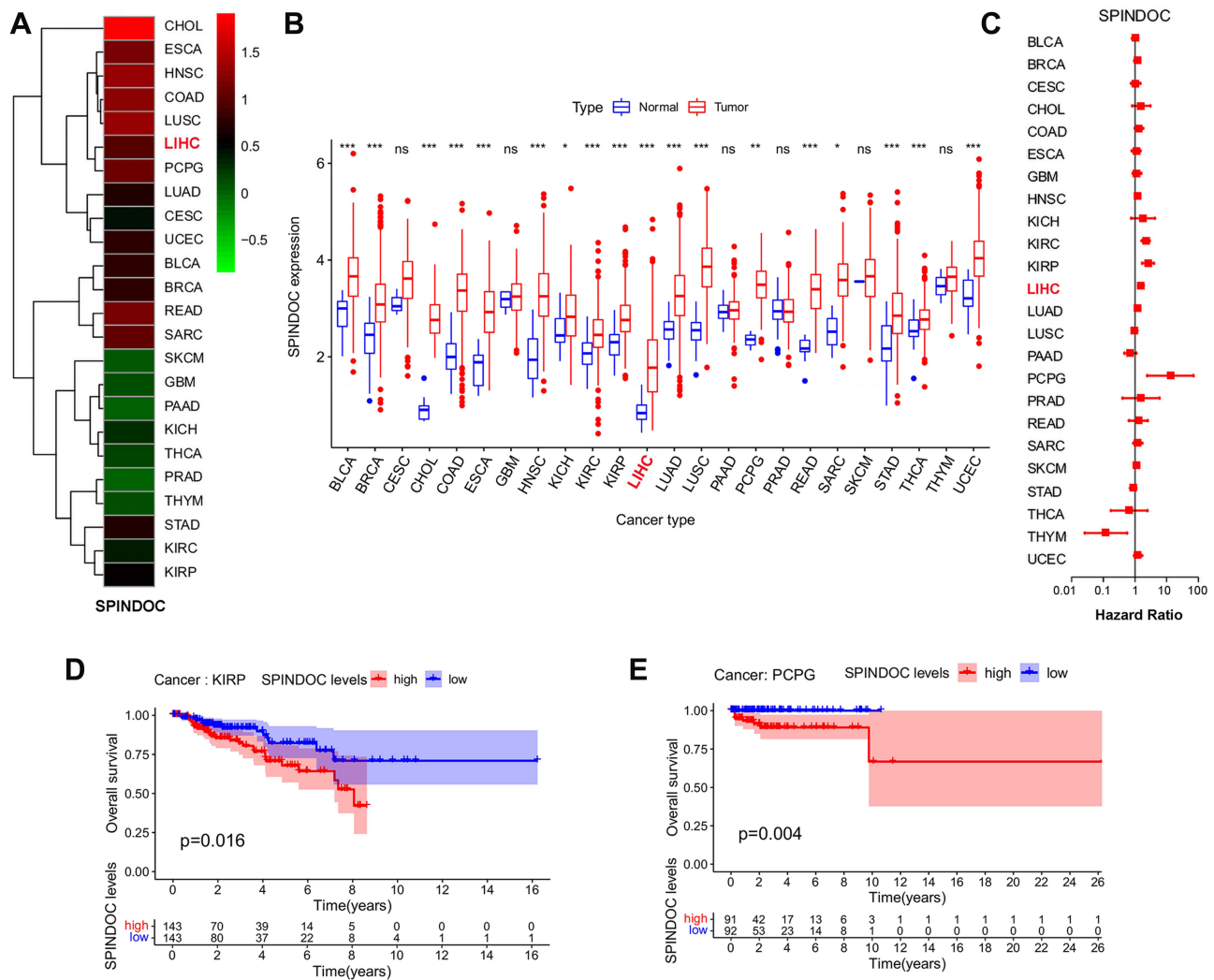


Figure 1 Expression of SPINDOC in pan-cancer samples and its effect on prognosis. **(A and B)** SPINDOC expression was elevated in 18 types of malignant tumors compared with normal tissue. **(C)** Univariate analysis showed that SPINDOC could be a risk factor for prognosis in LIHC, KIRC and PCPG tumor. **(D and E)** High expression of SPINDOC can lead to poor prognosis in patients with KIRC and PCPG tumors. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **Abbreviations:** SPINDOC, SPINI docking protein; ns, no significant.

SPINDOC Was Highly Expressed in HCC Samples and Was Correlated with Adverse Clinical Outcomes

We observed that high *SPINDOC* expression was associated with poor overall survival in patients with HCC in above analysis ($P < 0.001$) (Figure 3A). To further clarify the potential relationship between *SPINDOC* high expression and clinical characteristics of patients, we analyzed *SPINDOC* expression level with patients' gender (male, female), clinical Stage (Stage I, II, III, IV), histological Grade (Grade G1, G2, G3, G4) and T Stage (T1, T2, T3, T4). We observed that the *SPINDOC* was highly expressed in patients younger than 60 years old ($P = 0.015$) (Figure 3B), and the expression level of *SPINDOC* increased with an increase in the clinical stage, histological grade and T stage ($P < 0.01$) (Figure 3C–E), thereby indicating that the high expression of *SPINDOC* was closely related to the poor clinical prognosis. The AUC of ROC curve at *SPINDOC* expression levels was observed to be 0.64 (Supplementary Figure S1A), thereby indicating a relatively better diagnostic performance. Multiple ROC curves showed that the prediction value of *SPINDOC* expression for 1-year, 3-year and 5-year survival was 0.71, 0.64 and 0.597, respectively (Supplementary Figure S1B).

Thereafter, in the univariate analysis of clinical characteristics and the possible correlation between *SPINDOC* gene expression and the survival status, as well as the poor prognosis of the patient was related to the relatively higher

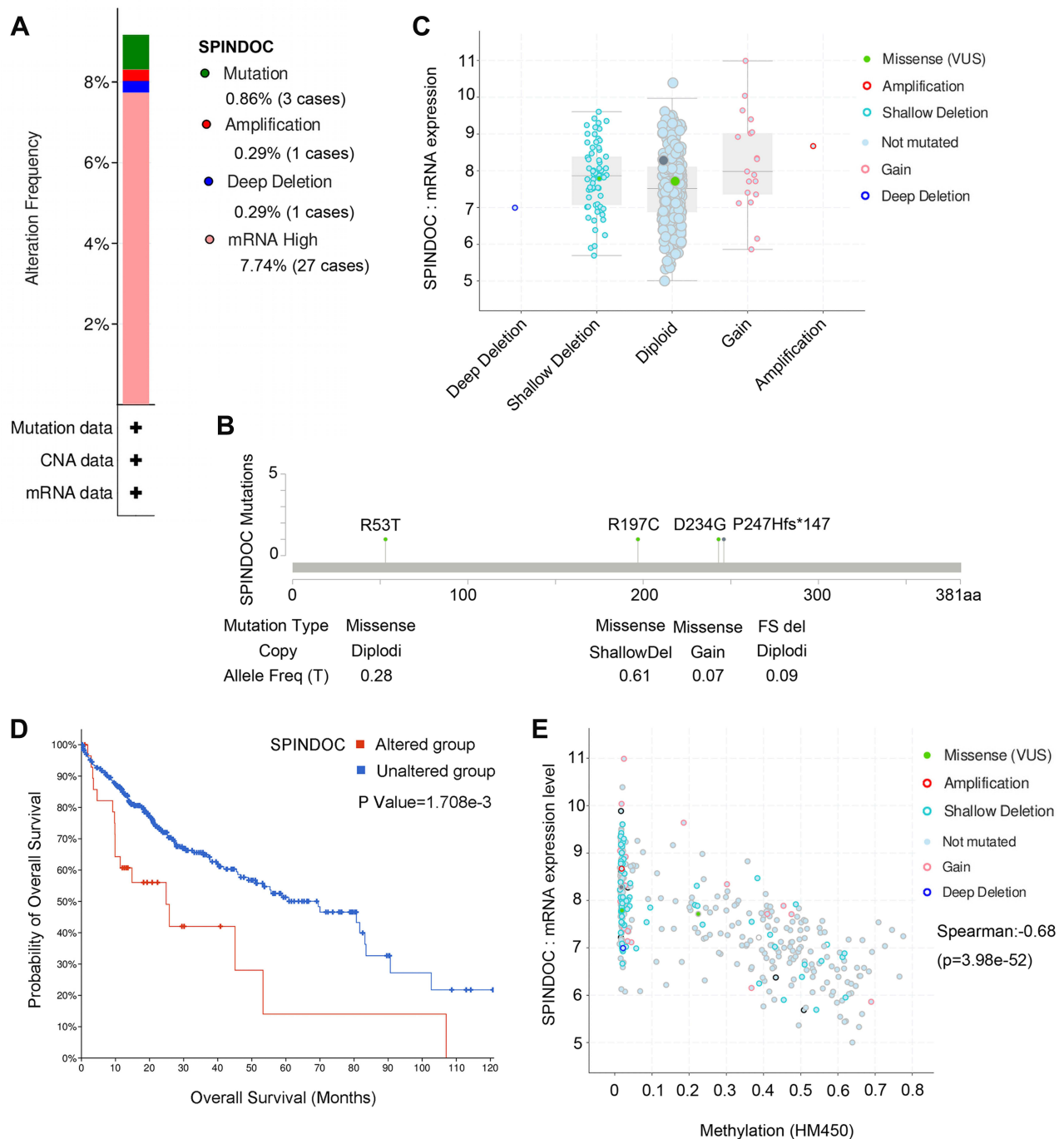


Figure 2 Genetic variation and methylation analysis of SPINDOC. **(A)** Types and frequency of genetic changes in SPINDOC. **(B)** Types, sites and proportions of SPINDOC mutations. **(C and D)** mRNA expression of SPINDOC is associated with mutations, and mutations lead to poor prognosis in patients. **(E)** Relationship between SPINDOC gene expression and methylation.

Abbreviations: SPINDOC, SPINI docking protein; CAN, copy-number alterations; HM450, HumanMethylation450.

expression of *SPINDOC* (HR: 1.10; $P=4.89E-05$), and the clinical stage of the tumor (HR: 1.865; $P=8.07E-07$), T stage (HR: 1.804; $P=4.73E-07$) and M stage (HR: 3.850; $P=0.023$). In multivariate analysis, the high expression of *SPINDOC* (HR: 1.076; $P=0.005$) was found to be significant as an independent predictor for patients with primary hepatocellular carcinoma (Table 1). We also analyzed whether there were differences in immune cell invasion between *SPINDOC* high and low expression groups in the liver cancer tissues, and CIBERSORT analysis showed that there were no substantial

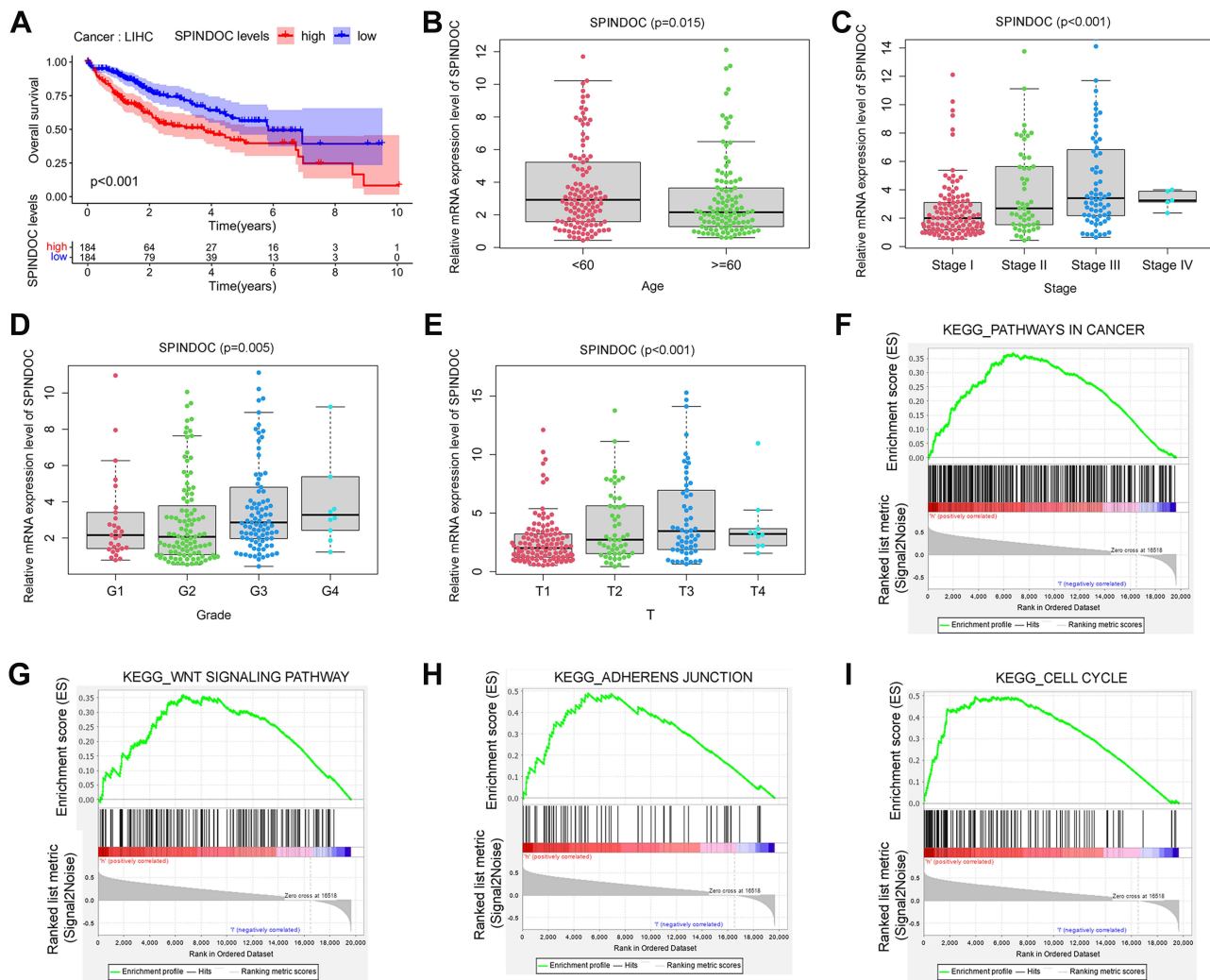


Figure 3 The gene expression level of *SPINDOC* in HCC and its potential effects on the clinical outcome of patients. **(A)** Patients with high *SPINDOC* expression displayed a poor prognosis; **(B)** *SPINDOC* was highly expressed in HCC patients younger than 60 years old; **(C)** The expression of *SPINDOC* increased with the clinical stage, **(D)** histological grade and **(E)** T stage of HCC. **(F–I)** GSEA analysis to identify the various cancer-related pathways where *SPINDOC* was enriched. **(F)** tumor-related pathways, **(G)** WNT signaling cascade, **(H)** adhesion junction, **(I)** cell cycle.

Abbreviations: *SPINDOC*, SPIN1 docking protein; HCC, hepatocellular carcinoma; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; T, tumor; WNT, wingless-type.

differences in immune cell invasion between *SPINDOC* high–low expression groups in liver cancer tissues ([Supplementary Figure S1C](#)).

GSEA Revealed the Potential Effects of *SPINDOC* on the Various Oncogenic Signaling Pathways That Can Regulate Tumor Proliferation and Metastasis

To explore the molecular mechanisms underlying the ability of *SPINDOC* in promoting the occurrence and development of HCC, we used the gene set enrichment analysis (GSEA) method to compare the various gene expression profiles of HCC specimens with *SPINDOC* low expression as well as *SPINDOC* high expression. According to the median of *SPINDOC* expression level, they were divided into *SPINDOC* low expression group and *SPINDOC* high expression group. GSEA analysis showed that high *SPINDOC* expression was significantly related to the various cancer-related signaling pathways, such as those of WNT, cell cycle, adhesion junctions, etc. thereby suggesting that these pathways might play a potential role in the tumorigenesis and metastasis of *SPINDOC* ([Figure 3F–I](#), [Supplementary Table S4](#)).

Table I Univariate and Multivariate Analysis of the Clinical Characteristics of HCC Patients and the Possible Correlation Between SPINDOC Gene Expression and Patient's Survival Status

Variables	Univariate Analysis				Multivariate Analysis			
	HR	P-value	95% CI		HR	P-value	95% CI	
SPINDOC	1.100	4.89E-05	1.050	1.151	1.076	0.005	1.022	1.131
Age	1.005	0.591	0.987	1.023	1.011	0.271	0.991	1.031
Gender	0.780	0.301	0.487	1.249	1.073	0.790	0.641	1.796
Grade	1.017	0.914	0.746	1.387	1.043	0.807	0.745	1.459
Stage	1.865	8.07E-07	1.456	2.388	0.962	0.939	0.361	2.562
T	1.804	4.73E-07	1.434	2.270	1.771	0.205	0.732	4.287
M	3.850	0.023	1.207	12.281	1.578	0.503	0.415	5.998
N	2.022	0.328	0.494	8.276	1.855	0.494	0.316	10.895

Abbreviations: HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; SPINDOC, SPIN1 docking protein; T, tumor; N, nodes; M, metastases.

SPINDOC Levels Were Significantly Up-Regulated in HCC Cells, and Its Knockdown Can Inhibit the Proliferation and Promote Apoptosis

The cell-based experiments were next used to verify the different biological functions of *SPINDOC* in hepatocellular carcinoma cells. We employed qRT-PCR to detect the expression level of *SPINDOC* in the normal liver cell lines (LO2) and the different HCC cell lines (Huh7, HepG2, SK-HEP-1). The results showed that mRNA and protein expression levels of *SPINDOC* in Huh7, HepG2 and SK-HEP-1 cells were significantly increased compared with those in LO2 cells, and among all the cell lines tested, Huh7 and SK-HEP-1 showed the maximal expression ($P < 0.01$) (Figure 4A and B). We designed the corresponding primers to knock out the *SPINDOC* gene and selected the target sequence and Huh7 and SK-HEP-1 cell lines with lowest expression for the next step.

We used MTT assay to determine the cell viability of Huh7 and SK-HEP-1 and it was found that *SPINDOC* gene knockout could significantly reduce the survival rate of Huh7 and SK-HEP-1 cells at 48 and 72 h, respectively ($P < 0.01$) (Figure 4G). Additionally, we also used colony formation experiments to further analyze the proliferation of Huh7 and SK-HEP-1 cells. The findings showed that the colony forming ability of Huh7 and SK-HEP-1 cells was significantly reduced after *SPINDOC* gene was effectively knocked out (Figure 4C and F). Moreover, *SPINDOC* gene knockout promoted a substantial increase in the apoptosis rate of HCC cells (Figure 4D and E). The above results suggested that *SPINDOC* can functionally promote the proliferation of HCC cells.

SPINDOC Can Effectively Promote the Migration and Invasion and Can Activate the WNT/ β -Catenin Signaling Pathway in HCC Cells

To study the possible effects of *SPINDOC* on the migration and invasion of HCC cells, wound healing assay and transwell invasion assays were employed. The analysis of wound healing assay clearly showed that knocking down the expression of *SPINDOC* gene can significantly inhibit the wound healing ability (Figure 5A and B). In addition, knockout of *SPINDOC* gene markedly reduced the invasion of HCC cells (Figure 5C and D) and the number of migrating cells (Figure 5E and F). In summary, these results indicated that *SPINDOC* can significantly promote the migratory and invasive abilities of both Huh7 and SK-HEP-1 cells.

In addition, previous GSEA analysis has revealed that high *SPINDOC* expression was significantly related to the various cancer-related signaling pathways, such as Wnt signaling cascade. Hence, after knocking down *SPINDOC* gene, we used Western blot analysis to detect the expression of the various key proteins involved in the Wnt/ β -catenin signal pathway. These proteins included Wnt1 that can activate Wnt pathway, Wnt signaling pathway activator marker β -catenin, downstream target genes of β -catenin such as cyclin D1, and AXIN2 that negatively regulates this pathway. At the same time, in view of the close relationship

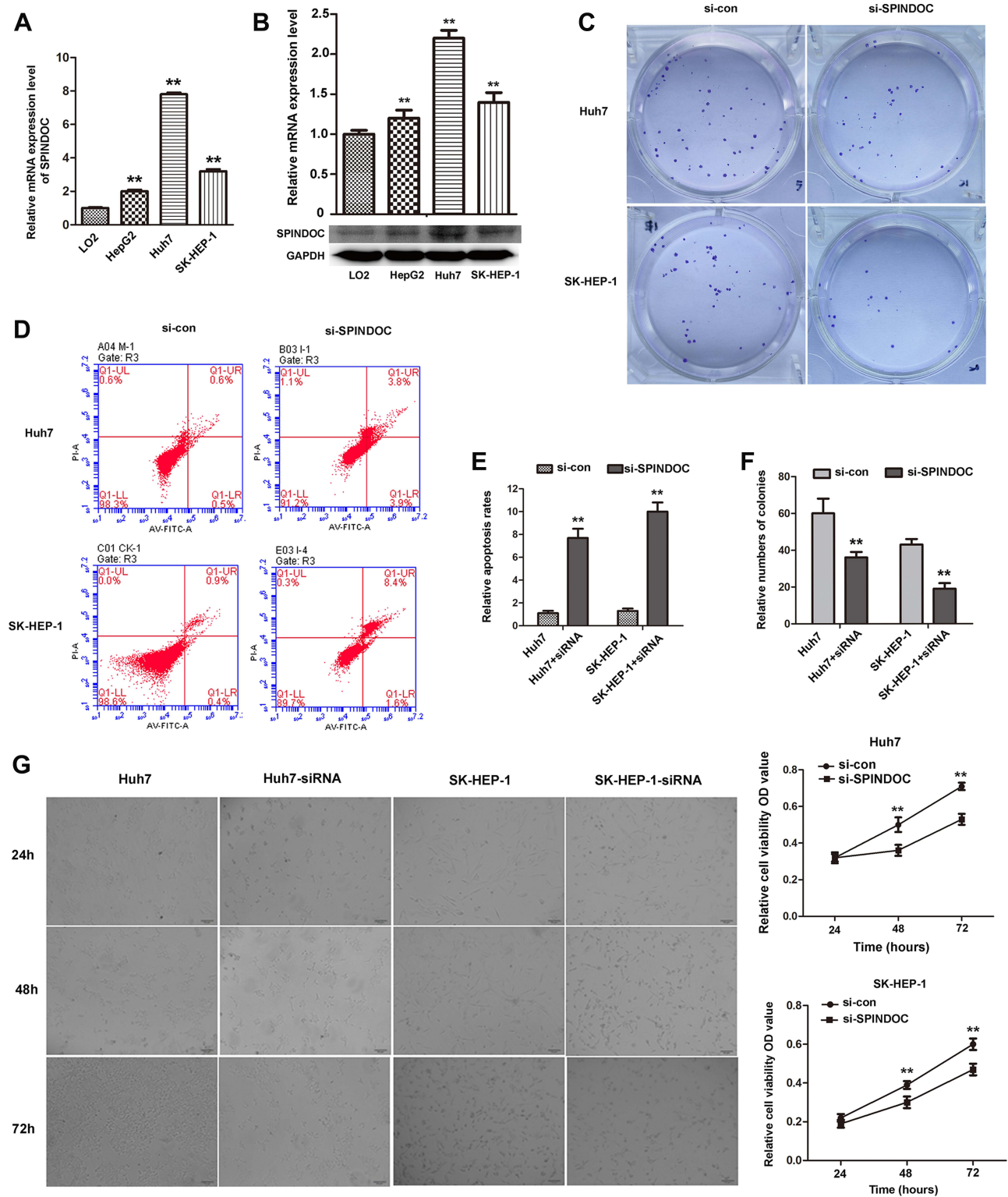


Figure 4 mRNA and protein expressions of SPINDOC in HCC cells. The proliferation and apoptosis ability of HCC cells after SPINDOC gene was knockdown. (**A** and **B**) mRNA and protein expressions of SPINDOC was markedly up-regulated in HCC cells; (**C** and **F**) Knockdown of SPINDOC gene can significantly attenuate colony forming ability of HCC cells; (**D–E**) Knockdown of SPINDOC can promote apoptosis of HCC cells. (**G**). Knockout of SPINDOC gene significantly reduced the survival rate of HCC cells at 48h and 72h respectively. ** $P < 0.01$.

Abbreviations: SPINDOC, SPIN1 docking protein; HCC, hepatocellular carcinoma; OD value, optical density value; si-con, siRNA-control; si-SPINDOC, siRNA-SPINDOC.

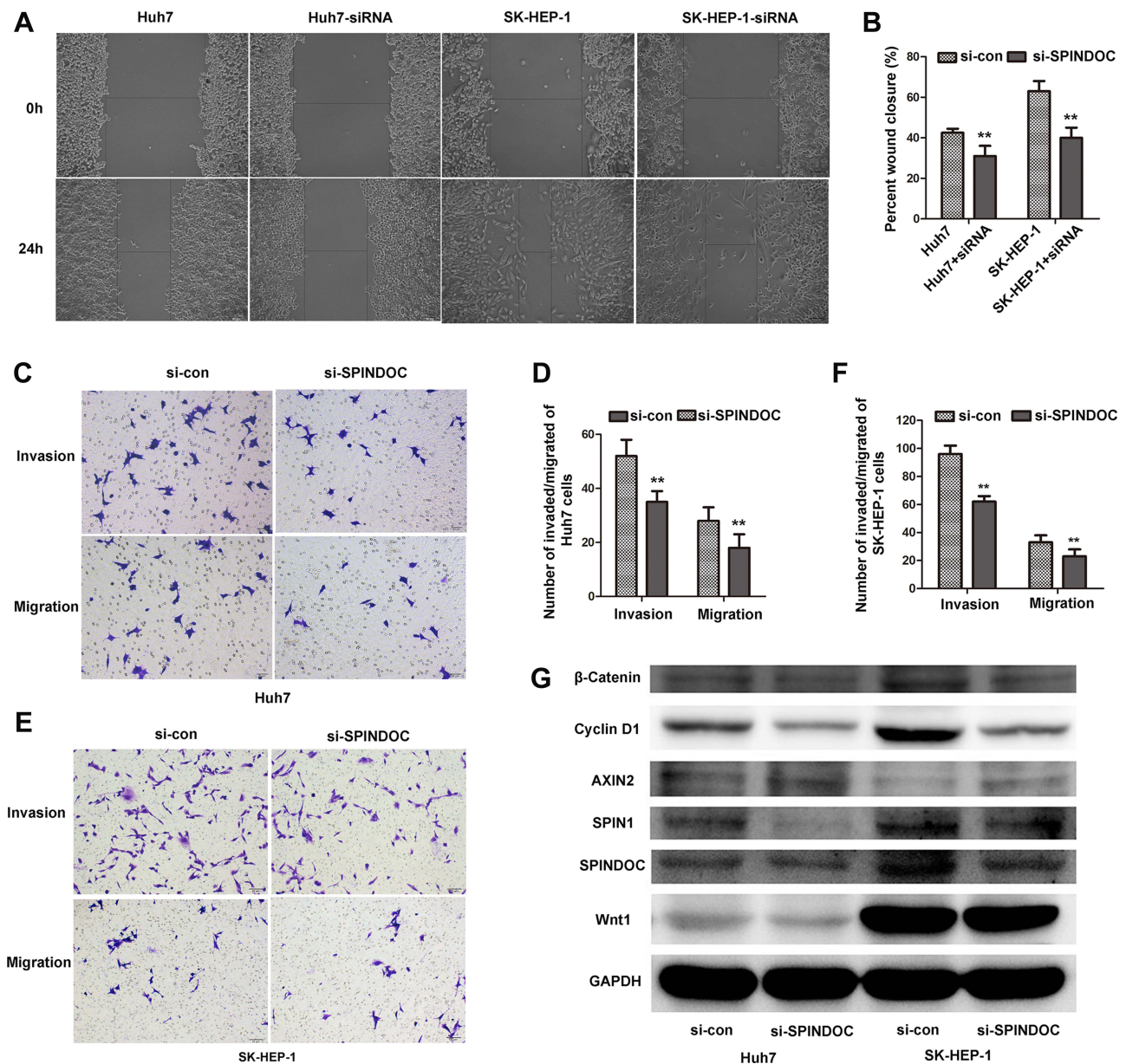


Figure 5 SPINDOC can promote the migration as well as invasion of HCC cells and can activate the Wnt/ β -catenin signaling pathway. (A and B) Knocking down the expression of SPINDOC gene can inhibit the wound healing; (C–F) reduce the invasion and migration ability of HCC cells; (G) The expression of SPIN1, Wnt1, β -catenin and cyclin D1 in HCC cells after SPINDOC gene knockdown decreased, but the expression of AXIN2 was found to be increased. $**P < 0.01$.

Abbreviations: SPINDOC, SPIN1 docking protein; HCC, hepatocellular carcinoma; SPIN1, Spindlin1; AXIN2, axis inhibition protein 2; Wnt1, wingless-type 1; si-con, siRNA-control; si-SPINDOC, siRNA-SPINDOC.

between *SPIN1* and *SPINDOC*, we also analyzed the expression of *SPINDOC* also. The results showed that the expression of SPIN1, Wnt1, β -catenin and cyclin D1 proteins in Huh7 and SK-HEP-1 cells after *SPINDOC* gene knockdown decreased markedly, but the expression of AXIN2 was increased (see Figure 5G and Supplementary Figure S1 D–J). These results clearly demonstrated that *SPINDOC* can effectively promote the proliferation, migration and invasion of HCC cells by activating Wnt/ β -catenin signaling pathway.

Knockdown of *SPINDOC* Inhibited HCC Growth and Wnt/ β -Catenin Signaling Pathway in Xenograft Mouse Model

Finally, we verified these results in a xenograft mouse HCC model. We selected stably transfected Huh7 cell lines for subcutaneous inoculation of the nude mice. The results showed that subcutaneous tumors grew in both the control group

and the *SPINDOC* knockout group. The subcutaneous tumors in the *SPINDOC* knockout group were significantly smaller than those in the control group on day 26 ($P=0.016$), and there was a significant statistical difference observed on day 29 ($P=0.009$) (Figure 6A–C). In addition, compared with the control group, the HE staining pathological sections of the tumor showed loose arrangement and obvious necrosis after knocking down of *SPINDOC* (Supplementary Figure S1J). Immunohistochemical staining showed that the expression of *SPINDOC* (Figure 6D and J), *SPINI* (Figure 6E and K), Wnt1 (Figure 6F and L), β -Catenin (Figure 6G and M), Cyclin D1 (Figure 6H and N) in *SPINDOC* knockout group was significantly lower than that in control group, whereas the expression of AXIN2 (Figure 6I and O) was markedly higher than that in control group.

Discussion

In this study, we reported an important oncogenic factor *SPINDOC* that has not been extensively studied in tumors. We observed the role and mechanism of *SPINDOC* in pan-cancer and HCC samples. We found that *SPINDOC* is highly expressed in a variety of malignant tumors and primary liver cancer, as an oncogenic factor can effectively promote the proliferation, invasion and migration of HCC by activating Wnt/ β -catenin signaling pathway.

It has been reported by Bae et al that *SPINDOC* is located at chr11 and consists of 1945bp. *SPINDOC* can block the potential interaction between *SPINI* and methylated histone tails, it was thereafter named based on its function in the complex.¹² *SPINDOC* can interfere with the binding of *SPINI* to the chromatin and negatively regulate spin1-mediated transcriptional activity, thereby largely neutralizing *SPINI*'s activation of its target genes.¹² Spindlin1 (*SPINI*) is the main member of the SPIN/SSTY gene family. This gene family is highly conserved and has been found to be expressed in the mature gametes and early embryos.¹⁸ *SPINI* is a tumor-domain protein, which contains three tandem repeats of tumor domains and can specifically recognize trimethylation of the fourth lysine of histones (H3K4me3) to bind to the histones. The human *SPINI* gene is located in interphase nuclei and mitotic chromosomes and encodes proteins involved in the cell cycle regulation which can influence epigenetic regulation.^{19,20} *SPINI* plays a role in early embryonic development and tumorigenesis by modulating the activation of PI3K/AKT pathway. *SPINI* has been found to display tumor-promoting effects in a variety of tumors by activating Wnt/ β -catenin signaling pathway.²¹

It is speculated that *SPINDOC* is a transcription corepressor of *SPINI* due to its tumor-promoting effect,^{12,13} but the results also show a paradoxical effect. As an inhibitor of *SPINI*, *SPINDOC* controls the chromatin load of *SPINI*, and overexpression of *SPINDOC* can lead to an increase in *SPINI* level. At the same time, *SPINDOC* knockout can lead to *SPINI* instability.¹³ These findings suggest a potential synergistic rather than antagonistic relationship between *SPINDOC* and *SPINI*. In addition, previous data has shown that *SPINDOC* was highly expressed in a variety of tumors. However, it is not clear whether *SPINDOC* can function as a tumor suppressor or a tumor progenitor in tumors? With this question, we observed the expression of *SPINDOC* in pan-cancer and liver cancer samples and its potential relationship between the tumors and *SPINDOC*.

In this study, the bioinformatics analysis was used to observe the potential expression of *SPINDOC* in pan-cancer and HCC based on the TCGA database. The results showed that the expression of *SPINDOC* in variety of tumors and in HCC was significantly higher than that in normal controls ($P<0.05$). Additionally, the findings of bioinformatics analysis indicated that the expression of this factor in variety of tumors and in HCC was positively correlated with the poor prognosis of patients. In view of the high incidence and hazard degree of liver cancer in the population, we chose liver cancer for further analysis. Based on this observation, we speculated that this factor might act as a tumor-promoting factor in variety of tumors and HCC. Thereafter, analysis of the possible relationship between the *SPINDOC* gene expression and the patient's age, gender, clinical grade and stage of tumors revealed that the expression *SPINDOC* in was positively correlated with young patients, and clinical grade, stage and lymphatic stage of the tumor, thereby indicating that a high expression of this factor in HCC can significantly promote the clinical progression of tumors. In other words, *SPINDOC* can significantly promote the proliferation, invasion and metastasis of HCC cells. In order to explore the underlying mechanisms through which *SPINDOC* can promote the progression of HCC, we used the GSEA method to observe the different pathways of *SPINDOC* enrichment in the tumors. It was found that *SPINDOC* was enriched in a wide variety of oncogenic pathways, such as those of Wnt, cell cycle and cellular adhesion pathways, which can regulate the proliferation, invasion and metastasis of the tumors.

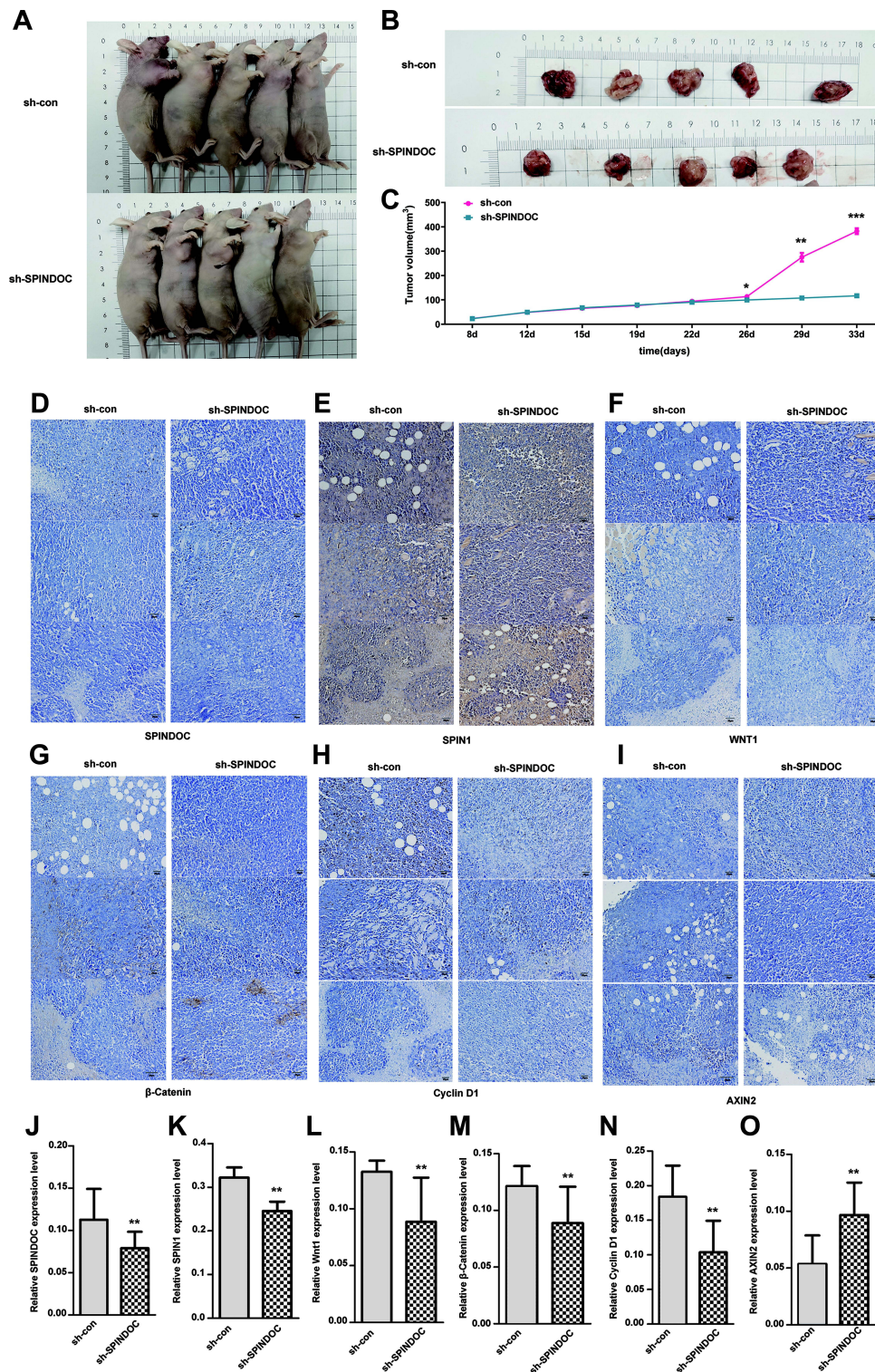


Figure 6 Knockdown of SPINDOC inhibited HCC growth and Wnt/ β -catenin signaling pathway in nude mice. **(A and B)** Subcutaneous tumor growth was observed in both the control group and SPINDOC knockout group. **(C)** The growth of subcutaneous tumor in the nude mice after SPINDOC knockout was significantly smaller than that in the control group after 29 days (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). **(D–I)** Immunohistochemical staining showed that protein expression levels of SPINDOC. **(D and J)** SPINDOC, **(E and K)** SPIN1, **(F and L)** Wnt1, **(G and M)** β -catenin, **(H and N)** cyclin D1 in SPINDOC-knockout group were lower than those in the control group, while the protein expression levels of AXIN2 **(I and O)** were higher than those in control group. ** $P < 0.01$.

Abbreviations: SPINDOC, SPIN1 docking protein; HCC, hepatocellular carcinoma; SPIN1, Spindlin1; AXIN2, axis inhibition protein 2; Wnt1, wingless-type 1; sh-con, shRNA-control; sh-SPINDOC, shRNA-SPINDOC; Scale bars: 20 μ m.

A number of previous studies have reported that overexpression of *SPINDOC* can effectively reduce Wnt signaling, while *SPINDOC* mutants can activate Wnt signaling. These results suggested that the various phenotypic differences may result from gene disruption and alteration of genetic content.²² We also analyzed the genetic variation of *SPINDOC*. *SPINDOC* was found to be genetically altered in 8.9% (34/382) of the samples. High mRNA expression (7.74%) and mutation (0.86%) were the main types of *SPINDOC* genetic changes observed in HCC. *SPINDOC* gene had the most missense mutation (0.61%) in R197C, and the type was copy number deletion. Based on the proportion of different mutations in the genetic alteration of *SPINDOC*, we speculated that *SPINDOC* itself might have the function of activating the Wnt/ β -catenin signaling pathway in HCC, independent of the small number of *SPINDOC* mutations at the overall level. In addition, amplification of genetic alteration of *SPINDOC* resulted in increased mRNA levels of *SPINDOC*, while methylation resulted in decreased mRNA levels of *SPINDOC*.

Thereafter, various in vitro experiments were used to verify the above hypothesis. Firstly, we found that mRNA and protein expression levels of *SPINDOC* were significantly higher in HCC cells as compared to the normal liver cells. Secondly, based on the results of cell proliferation, colony formation and apoptosis assay, we observed that the low expression of *SPINDOC* can markedly inhibit the proliferation and promote the apoptosis of HCC cells, thus indicating that *SPINDOC* can essentially function as an oncogene. In the subsequent scratch, invasion and migration assays, it was found that low expression of *SPINDOC* can markedly inhibit the invasion and migration, thereby indicating that this oncogenic factor also has a significant impact on the invasion and metastasis of HCC cells. These results indicated that *SPINDOC* was highly expressed in HCC, and it can promote the proliferation, invasion and migration of HCC cells, and can act as a potential tumor promoting factor. Subsequently, we also observed the regulatory effect of *SPINDOC* on Wnt signaling pathway.

The Wnt signaling cascade is highly conserved in different species and plays a vital role in regulating the early development, organ formation, tissue regeneration and other important physiological processes. An abnormal activation of this signaling pathway can induce the occurrence of a variety of cancers, including HCC.^{23,24} Wnt/ β -catenin cascade can be activated by autocrine or paracrine Wnt ligand and effectively cause β -catenin accumulation. This causes β -catenin in the cytoplasm to enter into the nucleus and bind to the *LEF/TCF* transcription factor family to initiate the transcription of the various downstream target genes (e.g c-myc, cyclin D1).²⁵

SPINI has been shown to promote tumor progression by activating the Wnt/ β -catenin signaling system, and the activation of the Wnt/ β -catenin signaling pathway can promote tumor progression by stimulating the transcription of multiple oncogenic factors.^{20,21} Moreover, previous studies have shown that overexpression of *SPINDOC* in the *SPINDOC*-*spin1* complex can inhibit the activation of the Wnt signaling pathway, but this study also suggested that mutated *SPINDOC* can activate the Wnt signaling system.¹³ Thus, *SPINDOC*-*SPINI* complex may act as the transcriptional co-repressor of the Wnt signaling system, or other unknown proteins and may bind to the C-terminal region of *SPINDOC* to inhibit the transcriptional activation. When *SPINDOC* is mutated and cannot bind to *SPINI*, it can act as a transcriptional activator of Wnt signaling.¹³

To observe the potential effect of *SPINDOC* expression changes on Wnt/ β -catenin signaling pathway in HCC, we knocked down *SPINDOC* expression and detected the key factors downstream of Wnt/ β -catenin signaling pathway. The results showed that with the decrease of *SPINDOC*, the expression of Wnt1 which can activate Wnt signaling pathway, Wnt signaling pathway activation marker β -catenin, and the downstream target genes such as cyclin D1 decreased, whereas that of AXIN2, which can negatively regulate this pathway was increased. These findings suggested that the decrease of *SPINDOC* might block Wnt signaling pathway. In view of the close relationship between *SPINI* and *SPINDOC*, we also analyzed the expression of *SPINI*. The results showed that the expression of *SPINI* decreased with the decrease of *SPINDOC*. This result was consistent with the previous findings that the overexpression of *SPINDOC* can lead to the elevation of *SPINI* level.¹³ Based on these results, we believe that *SPINDOC* can itself act as an oncogenic factor that can activate Wnt/ β -catenin signaling pathway in HCC cell lines.

Limitations

This study was associated with certain limitations. First, since no mutant group was set in the experiment, we were unable to determine whether the mutant *SPINDOC* could also activate the Wnt/ β -catenin signaling pathway. Second, PCR and WB analysis of *SPINDOC*, *SPINI* and the factors related to the activation of Wnt/ β -catenin signaling pathway

in the tissues obtained from xenograft mouse model was not performed, so the activation effect of *SPINDOC* on Wnt/ β -catenin signaling pathway in HCC could not be confirmed from the multiple perspectives.

Conclusion

SPINDOC was highly expressed in a variety of tumors and primary HCC models. This gene can effectively promote the proliferation, invasion and migration of HCC cells by activating the Wnt/ β -catenin signaling cascade. Therefore, this oncogenic factor can form the basis of a potential therapeutic target for the management of HCC.

Abbreviations

KIRP, Kidney renal papillary cell carcinoma; PCPG, Pheochromocytoma and Paraganglioma; LIHC, Liver hepatocellular carcinoma; HCC, Hepatocellular Carcinoma; GSEA, Gene Set Enrichment Analysis; *SPINDOC*, *SPIN1* docking protein; TCGA, The Cancer Genome Atlas; FITC, Fluorescein isothiocyanate; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma.

Consent for Publication

All the patients that were involved in the study have given their consent to publish their individual data.

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Disclosure

The authors report no conflicts of interest in this work.

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