# SPAG5, the upstream protein of Wnt and the target of curcumin, inhibits hepatocellular carcinoma

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**Abstract.** The inhibitory role of curcumin on sperm-associated antigen 5 (SPAG5) and its effects on the cancer-related Wnt classical signaling pathway has been previously demonstrated. Nevertheless, research on the modulatory role of curcumin on the Wnt signaling pathway by acting on SPAG5 has yet to be reported. The activation of the Wnt/β-catenin pathway is frequently observed in patients suffering from hepatocellular carcinoma (HCC), suggesting that small molecular drugs that target Wnt could present a promising therapeutic strategy. However, these drugs often result in substantial side effects. In the present study, the presence of SPAG5 in the cancer tissues of patients with HCC and cell lines was validated using immunohistochemistry, cellular immunofluorescence, reverse transcription-quantitative polymerase chain reaction, and western blot analyses. Subsequently, the effect of SPAG5 and the regulatory role of curcumin on SPAG5 and the Wnt/β-catenin pathway were examined using cell function tests, flow cytometry, and western blotting. Techniques of gene knockout and overexpression were employed. The findings revealed a significant overexpression of SPAG5 in the cancer tissues of patients with HCC. Both the mRNA and protein levels of SPAG5 in Huh7 and HCCLM3 cell lines were markedly elevated. Treatment with curcumin led to a decrease in SPAG5 expression, while also inhibiting cell migration and promoting apoptosis. Additionally, suppression of SPAG5 expression resulted in the decreased expression of β-catenin. Furthermore, curcumin was observed to reduce the expression of cyclin D1 in SPAG5-overexpressing cell lines. However, the degree of inhibition was diminished once SPAG5 expression was silenced. These initial findings indicate that SPAG5 may function as an upstream regulatory protein of the Wnt/β-catenin pathway, hence offering a potential alternative target for HCC. Moreover, as curcumin has the capacity to inhibit Wnt via suppressing SPAG5, it could potentially serve as a natural drug component for early intervention and treatment of HCC.

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*Abbreviations:* SPAG5, sperm-associated antigen 5; HCC, hepatocellular carcinoma; CTA, cancer testis antigen; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8

*Key words:* curcumin, sperm-associated antigen 5, Wnt/β-catenin pathway, hepatocellular carcinoma, cyclin D1

## Introduction

Primary liver cancer ranks as the sixth most prevalent cancer globally and the fifth most common malignant tumor in China, where it is also the second leading cause of cancer-related deaths (1,2). Comprising hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, and combined hepatocellular cholangiocarcinoma, primary liver cancer is dominated by HCC, which accounts for 75-85% of cases (1). The development of HCC has been closely linked with viral infections, cirrhosis, alcoholism, smoking, aflatoxin, exposure to harmful chemicals, and genetic factors (2). The disease prevalence significantly varies across the world, reflecting the differing distribution of these pathogenic factors, with 72% of cases occurring in Asia and >50% in China (3). Notably, sex-based disparities exist in the incidence and mortality rates of liver cancer. For instance, liver cancer is the second most lethal form of cancer in men and ranks sixth in women (4). The high mortality rate of liver cancer is primarily due to late diagnosis and a lack of early treatment, necessitating the exploration of new diagnostic and therapeutic approaches. This pursuit, particularly regarding the regulation of the tumor microenvironment and early drug intervention targets, is a promising direction for clinical diagnosis and treatment of liver cancer.

Sperm-associated antigen 5 (SPAG5), a member of the SPAG family, is a spindle-related protein primarily expressed in the testis and placenta that regulates spindle assembly and sister chromatid separation during the M phase of the cell cycle (5). Aberrations in SPAG5 have been implicated in irregular cell cycle regulation and DNA damage, both of which are closely associated with tumorigenesis (6,7). An elevated level of SPAG5 has been documented in numerous tumors (8-12), fostering an interest in its function as a cancer testis antigen (CTA) in cancer genesis, especially its role

in promoting cancer via the Wnt/β-catenin pathway. The Wnt/β-catenin pathway is activated in HCC patients, and the function of SPAG5 in HCC has been identified as being mediated through the same pathway (13). In patients with HCC, SPAG5 has been revealed to downregulate the expression of SCARA5 via the β-catenin/TCF4 pathway, thereby exacerbating the progression of the cancer (13). Separate studies by Jiang *et al* (14) and Liu *et al* (15) explored the influence of SPAG5 via the Wnt/β-catenin pathway in breast cancer and gastric cancer, respectively. In addition, Rebouissou *et al* (16) reported that ~95% of patients with HCC exhibited Wnt/β-catenin pathway activation. However, the biological role and clinical significance of SPAG5 in HCC remain unclear.

Wnt is a type of secreted glycoprotein that uses frizzled as its receptor (17,18). The classical Wnt signaling pathway involves β-catenin accumulation and subsequent entry into the nucleus to activate target gene transcription, thus promoting cancer development (19,20). Accordingly, the Wnt pathway has been considered a suitable therapeutic target. Despite this, targeted drugs have faced clinical trial challenges and have not been implemented clinically due to severe side effects (21). As a result, research has shifted towards exploring natural pharmaceutical components capable of inhibiting the Wnt/β-catenin pathway. For instance, genistein has been revealed to inhibit the Wnt/β-catenin pathway and regulate the expression of several Wnt/β-catenin antagonists through epigenetic modifications. Furthermore, myricetin has been demonstrated to reduce cytoplasmic and nuclear β-catenin levels, and curcumin has been observed to limit β-catenin nuclear translocation (22-24).

Curcumin, an active compound extracted from Curcuma longa, exhibits numerous pharmacological effects such as antioxidative, anti-inflammatory, free radical scavenging, and antitumor properties, and is employed in the treatment of cardiovascular diseases and digestive system diseases (25). The antitumor effect of curcumin has been widely researched, revealing a close association with the dysregulation of tumor cell proliferation, apoptosis, and angiogenesis signaling pathways (26). Curcumin, either in isolation or in combination with other drugs, impacts cancer-related signaling pathways (27-28).

There is existing evidence indicating that curcumin can downregulate the Wnt/β-catenin signaling pathway by inhibiting Wnt in HCC, a process partially mediated by the activation of autophagy (29-31). Furthermore, additional research has identified the inhibitory effect of curcumin on tumors associated with the Wnt/β-catenin pathway (32). Notably, it has been established that SPAG5 operates through the Wnt/β-catenin pathway (13). However, to date, no study has explored the curcumin-SPAG5-Wnt/β-catenin triad.

Based on the analysis of bioinformatics databases and previous research (33), the aim of the present study was to combine CTA and SPAG5 with the cancer-related Wnt/β-catenin signaling pathway to explore the regulatory effect of SPAG5 on the Wnt/β-catenin pathway. Confirmation of the hypothesis that curcumin inhibits the Wnt/β-catenin pathway by acting on SPAG5, was also attempted. The goal was to identify alternative methods and new targets for targeted therapeutic drugs aimed at the Wnt pathway, which currently cannot be used clinically due to their side effects. Equally important was the exploration of natural drug ingredients with lower toxicity and side effects for early intervention in HCC, enhancement of the effectiveness of other treatments, and alleviation of drug resistance.

## Materials and methods

*Gene expression analysis*. Differential analysis of SPAG5 expression in cancer tissue compared to adjacent tissue from 374 hepatocellular carcinoma (HCC) patient tissue samples was carried out using The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/). In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/kegg/kegg1.html) and Gene Ontology (GO https://david.ncifcrf.gov/home.jsp) database used for enrichment analysis. DESeq2 (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) (34) was used to analyze differentially expressed genes, with genes deemed differentially expressed if $|\log_2 FC| \geq 1$ and $P<0.05$. Additionally, differential gene expression analysis associated with SPAG5 was conducted.

*Patients and sample collection*. Tumor tissues from patients with HCC patients, along with corresponding non-tumor tissues, were procured from the Second People's Hospital of Hunan Province (Changsha, China), between March 2021 and December 2022. The clinical and demographic data of participants are outlined in Table I. The inclusion criteria were as follows: i) Age, ≥30 years; ii) patients with HCC who were undergoing surgical treatment; and iii) patients who had previously undergone a physical examination. The exclusion criteria included mental illness and liver and kidney dysfunction. Resected specimens were promptly frozen and stored at -80˚C for subsequent analysis. A team of pathologists confirmed the identification of tumor tissues and adjacent normal tissues. Each patient provided written informed consent, and the study was approved (approval no. DK2018002) by the Ethics Committee of the Second People's Hospital of Hunan Province.

*Cell culture*. The human HCC cell lines (Huh7 and HCCLM3) and a normal human hepatocytes line (MIHA) were procured from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). These cells were cultivated in DMEM (Dalian Meilun Biology Technology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS; Bioexplorer Life Sciences). The Huh7 cell line, derived from a Japanese male high-grade HCC, is hepatitis B virus-negative and is capable of producing cytoplasmic molecules such as Alb, ATT, and AFP (35). The HCCLM3 cell line, also known as human highly metastatic hepatoma cells, was developed via multiple rounds of *in vivo* selection in nude mice of the human hepatoma cell line MHCC97-H for its high lung metastatic potential (36). MIHA denotes normal human hepatocytes. The cells were incubated at 37˚C in an atmosphere containing 5% $CO_2$.

*Plasmid transfection*. SPAG5 siRNA plasmid was procured from Shanghai GeneChem Co., Ltd. The process of plasmid

Table I. Clinical data of patients with HCC.

| Sample number | Age | Sex | Clinicopathological diagnosis |
|---|---|---|---|
| 1 | 62 | Female | Medium differentiated liver cancer |
| 2 | 50 | Female | Medium-low differentiated liver cancer |
| 3 | 73 | Male | Medium differentiated liver cancer |
| 4 | 51 | Male | Medium-high differentiated liver cancer |
| 5 | 49 | Female | High differentiated liver cancer |
| 6 | 32 | Male | Medium differentiated liver cancer |
| 7 | 62 | Male | Medium differentiated liver cancer |
| 8 | 34 | Male | High differentiated liver cancer |
| 9 | 50 | Male | High differentiated liver cancer |
| 10 | 42 | Male | Medium differentiated liver cancer |
| 11 | 64 | Male | Medium differentiated liver cancer |
| 12 | 41 | Male | High differentiated liver cancer |
| 13 | 52 | Male | Medium differentiated liver cancer |
| 14 | 49 | Male | Medium differentiated liver cancer |
| 15 | 66 | Male | Medium differentiated liver cancer |

transfection commenced with seeding of cells in a six-well plate at a density of 2-3x$10^5$ and addition of 2 ml of complete medium. The cells were then placed in a carbon dioxide incubator at 37˚C overnight. Transfection reagents were then prepared. Solution A consisted of Opti-MEM (125 $\mu$l; Gibco; Thermo Fisher Scientific, Inc.) combined with Lipofectamine®3000 (3.75 $\mu$l per well; Invitrogen; Thermo Fisher Scientific, Inc.). Solution B comprised Opti-MEM (125 $\mu$l), P3000 (5 $\mu$l; Invitrogen; Thermo Fisher Scientific, Inc.), and 2.5 $\mu$g of the plasmid to be transfected per well. Solutions A and B were mixed in equal proportions and left to rest at room temperature for 15 min. Upon achieving a cell density of 70-90%, the complete medium was replaced with basal medium. The combined A + B solution was gradually added to each well, mixed gently in a cross direction, and then incubated at 37˚C in a carbon dioxide incubator. After 24 h, fluorescence expression was observed with a fluorescence microscope (Olympus Corporation). The sequence for SPAG5 siRNA was as follows: 5'-ccAUGCAACUGGAUUAUACAA-3'. The sequence for the scrambled siRNA was as follows: 5'-UUCUCCGAACGU GUCACGU-3' (used as the negative control).

*Cell Counting Kit-8 (CCK-8) assay*. Both Huh7 and HCCLM3 cells were seeded in 96-well plates (Zhejiang Sorfa Life Science Research Co., Ltd.) at a density of 5x$10^4$ cells/ml in DMEM medium supplemented with 10% FBS. Following incubation for 24 h at 37˚C in an atmosphere containing 5% $CO_2$, the cells were exposed to curcumin at varying concentrations (0, 12.5, 50, 100, 125 and 200 $\mu$M). After another 24-h incubation period, 10 $\mu$l CCK-8 (Dojindo Laboratories, Inc.) was added to each well and the cells were incubated for an additional 1 h at 37˚C. The absorbance at a wavelength of 450 nm was measured for each well using a microplate reader (Thermo Fisher Scientific, Inc.).

*Wound-healing assay*. Huh7 and HCCLM3 cells were seeded in six-well plates (Zhejiang Sorfa Life Science Research Co., Ltd.) at a density of 2x$10^5$ cells/ml and incubated overnight at 37˚C. Subsequently, when cell confluence reached 80-90%, a wound was created in the cell layer by manual scraping with a 200-$\mu$l pipette tip. The cells were rinsed with PBS (Dalian Meilun Biology Technology Co., Ltd.), then exposed to curcumin in serum-free medium at concentrations of 80 and 100 $\mu$M. Images were captured at 0 and 24 h post-wounding to observe the healing process by fluorescence microscopy.

*Immunohistochemistry*. Tissues were formalin-fixed (fixed in 4% buffered formaldehyde at room temperature for 24 h) and embedded in paraffin. The HCC tissue sections (thickness, 1 $\mu$m) underwent treatment with xylene and fractional ethanol, followed by antigen retrieval in 0.01 M citric acid buffer. Blocking was accomplished using 3% hydrogen peroxide (Fuzhou Maixin Biotech Co., Ltd.). Tissue sections were subsequently subjected to a 30-min incubation with 5-10% goat serum (cat. no. SL038; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature, followed by an overnight incubation at 4˚C with anti-SPAG5 monoclonal antibody (1:100; cat. no. 14726-1-AP; ProteinTech Group, Inc.). Subsequently, the sections were treated with HRP-conjugated goat anti-rabbit immunoglobulin G for 30 min at room temperature (1:200; cat. no. PR30011; ProteinTech Group, Inc). DAB was utilized for color rendering. Post-staining with hematoxylin (at room temperature for 3-5 min), images of the tissue sections were captured using an Olympus light microscope (Olympus Corporation). Scoring was independently performed by a pathologist (37).

*Western blot analysis*. Protein extraction of Huh7 and HCCLM3 cell was accomplished using a lysis buffer (Biosharp Life Sciences), followed by quantification using the BCA method. Proteins (50 $\mu$g) were separated on 8% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 $\mu$m; Abiowell). The PVDF

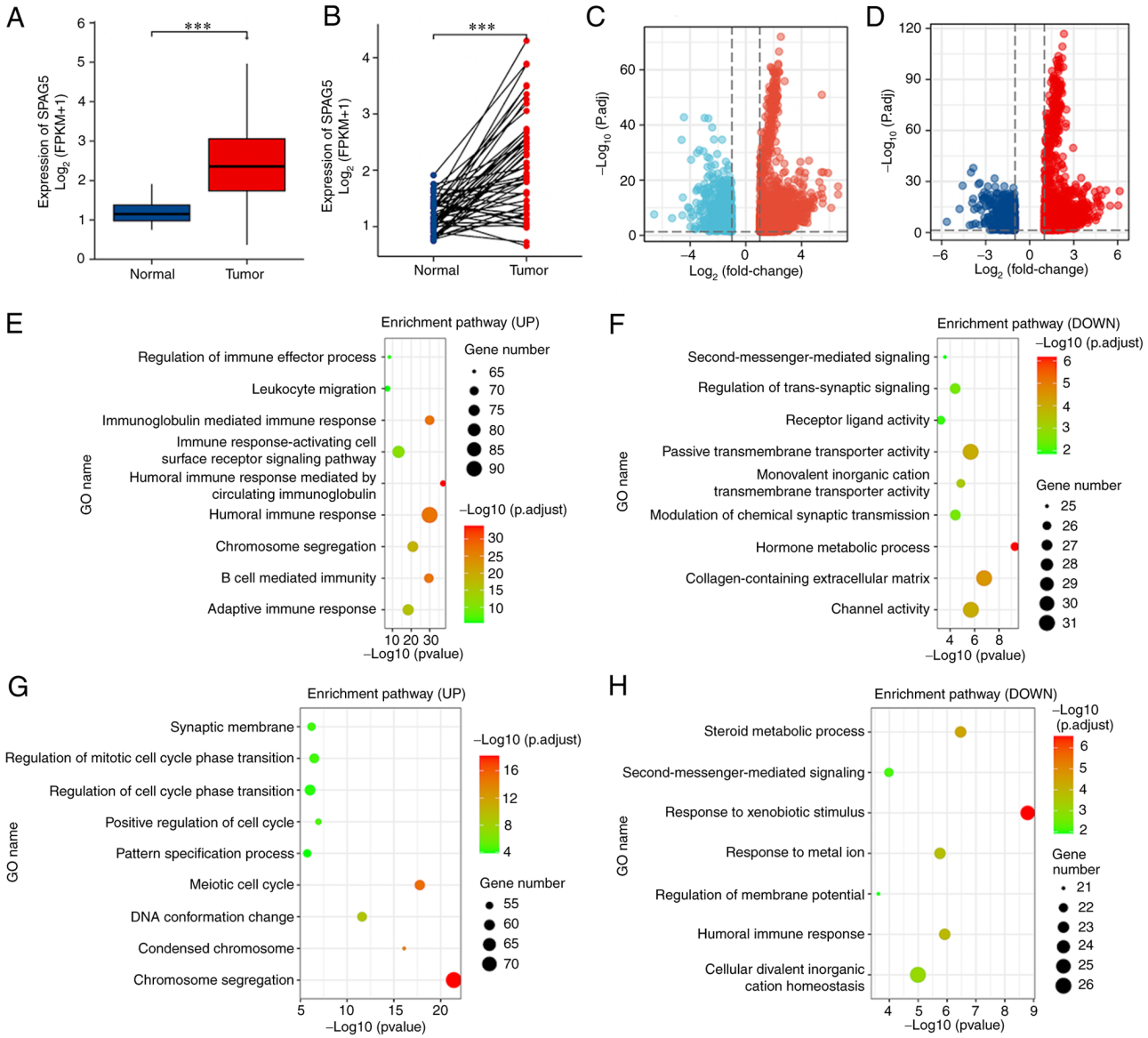


Figure 1. Analysis of SPAG5 expression from TCGA datasets. (A and B) The levels of SPAG5 expression in tumor tissues compared to normal tissues, extracted from TCGA. (C and D) Volcano plot of differentially expressed genes, between samples with high and low SPAG5 expression; blue represents downregulated genes, and red depicts upregulated genes. (E-H) Significantly enriched GO annotations and Kyoto Encyclopedia of Genes and Genomes pathways of SPAG5 in hepatocellular carcinoma. ***P<0.001. SPAG5, sperm-associated antigen 5; TCGA, The Cancer Genome Atlas; GO, Gene Ontology.

membranes were then blocked with 5% skim milk for 1.5 h at room temperature. Overnight incubation at 4˚C on a shaker followed, with antibodies against SPAG5 (1:20,000; cat. no. 14726-1-AP; ProteinTech Group, Inc.), cyclin D1 (1:20,000; cat. no. ab134175; Abcam), and GAPDH (1:20,000; cat. no. 10494-1-AP; ProteinTech Group, Inc.). Post-0.05% TBST washing, the membranes underwent incubation with goat anti-rabbit IgG-HRP (1:20,000; cat. no. PR30011; ProteinTech Group, Inc.) at 37˚C for 1 h. The membrane was visualized using an enhanced chemiluminescence (ECL) detection system (Biosharp Life Sciences). The band grayscale value is calculated using ImageJ (1.53a; National Institutes of Health).

*RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).* Total RNA was extracted from Huh7 and HCCLM3 cell using the Ultra-pure total RNA extraction Kit (Simgen Xinjing Biological). The RNA was subsequently reverse transcribed using the PrimeScript RT Reagent Kit (Novoprotein Scientific, Inc.) according to the manufacturer's instructions. For the quantitative polymerase chain reaction (PCR) analysis, qPCR was conducted using SYBR Premix Ex Taq (Novoprotein Scientific, Inc.) as per the manufacturer's instructions. The relative amounts of SPAG5, and GAPDH (internal control) mRNAs were determined using the Real-Time PCR System. The thermocycling conditions were as follows: 95˚C for 1 min, followed by 40 cycles at 95˚C for 20 sec, and 60˚C for 1 min. Relative gene expression was caculated using the $2^{-\Delta\Delta Cq}$ method (38). Primers used for the RT-qPCR assay were as follows: GAPDH forward, 5'-ACAGCCTCAAGATCATCAGC3' and reverse, 5'-GGTCATGAGTCCTTCCACGAT-3'; SPAG5 forward, 5'-CATCTCACAGTGGGATAACTAATAAAC-3' and reverse, 5'-CAGGGATAGGTGAAGCAAGGATA-3'.

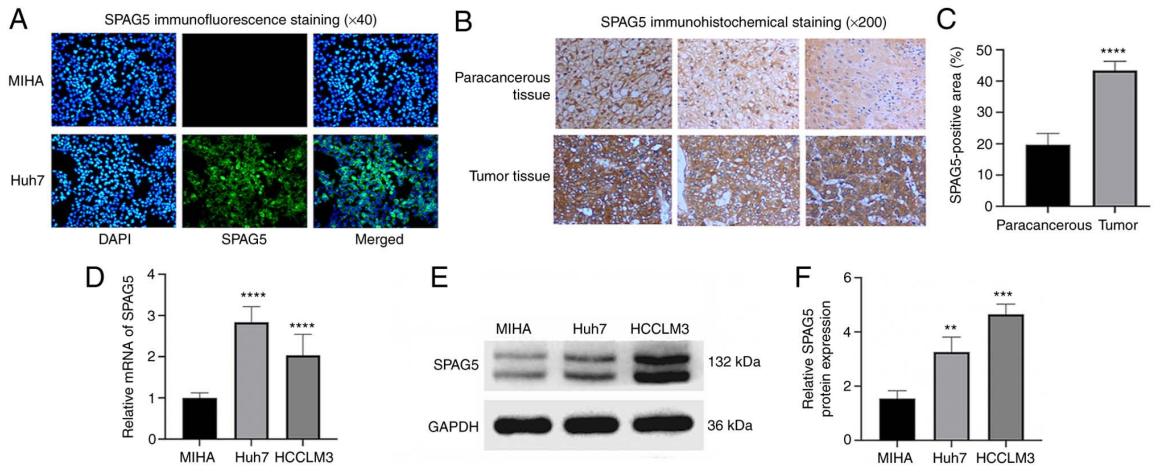


Figure 2. Differential expression of SPAG5 in hepatoma cells and normal hepatocytes and tissues. (A) The differential expression of SPAG5 in MIHA and Huh7 observed by fluorescence microscopy. The blue signal represents the nuclear DNA staining of DAPI, and the green signal represents the staining of SPAG5. (B and C) Immunohistochemical staining revealed SPAG5 expression in 15 patients. (D) Reverse transcription-quantitative PCR analysis of the relative mRNA levels of SPAG5 in MIHA, Huh7 and HCCLM3 cell lines. (E and F) Western blot analysis of SPAG5 expression in MIHA, Huh7 and HCCLM3 cell lines. $^{**}$P<0.01, $^{***}$P<0.001 and $^{****}$P<0.0001. SPAG5, sperm-associated antigen 5.

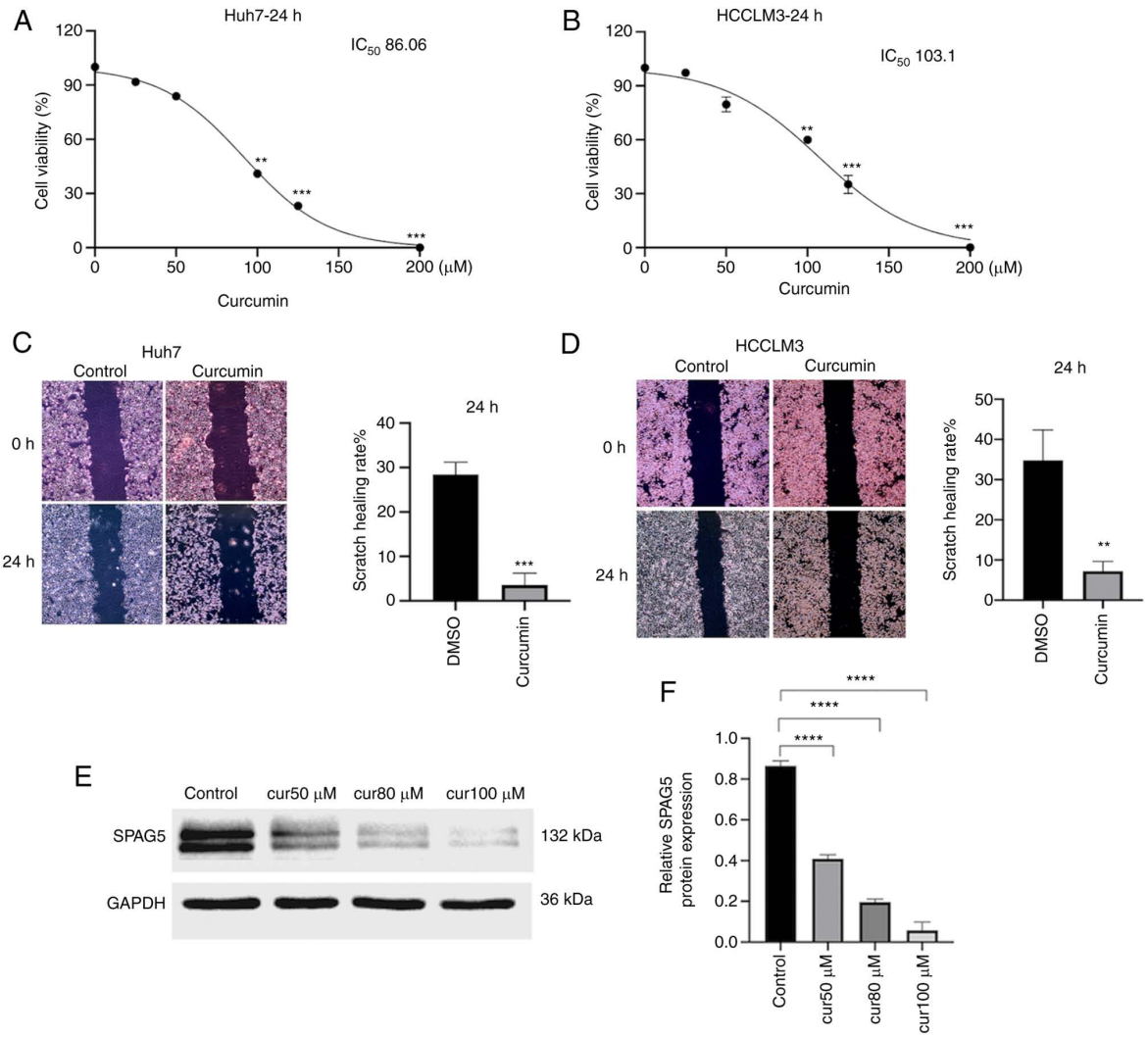


Figure 3. Curcumin inhibits the proliferation and migration of hepatoma cells. (A and B) Cell Counting Kit-8 analysis of the cell survival of HCCLM3 and Huh7 cells treated with various concentrations of curcumin. $^{**}$P<0.01 and $^{***}$P<0.001 vs. 0 $\mu$M curcumin. (C) The inhibitory effect of curcumin on cell scratch healing in Huh7 cells. (D) The inhibitory effect of curcumin on cell scratch healing in HCCLM3 cells. (E and F) Western blotting demonstrated that SPAG5 expression significantly decreased with the increase in curcumin concentration. Control, cells not treated with curcumin; Cur 50 $\mu$M, Huh7 cells treated with 50 $\mu$M curcumin; Cur 80 $\mu$M, Huh7 cells treated with 80 $\mu$M curcumin; Cur 100 $\mu$M, Huh7 cells treated with 100 $\mu$M curcumin. $^{**}$P<0.01, $^{***}$P<0.001 and $^{****}$P<0.0001. SPAG5, sperm-associated antigen 5.

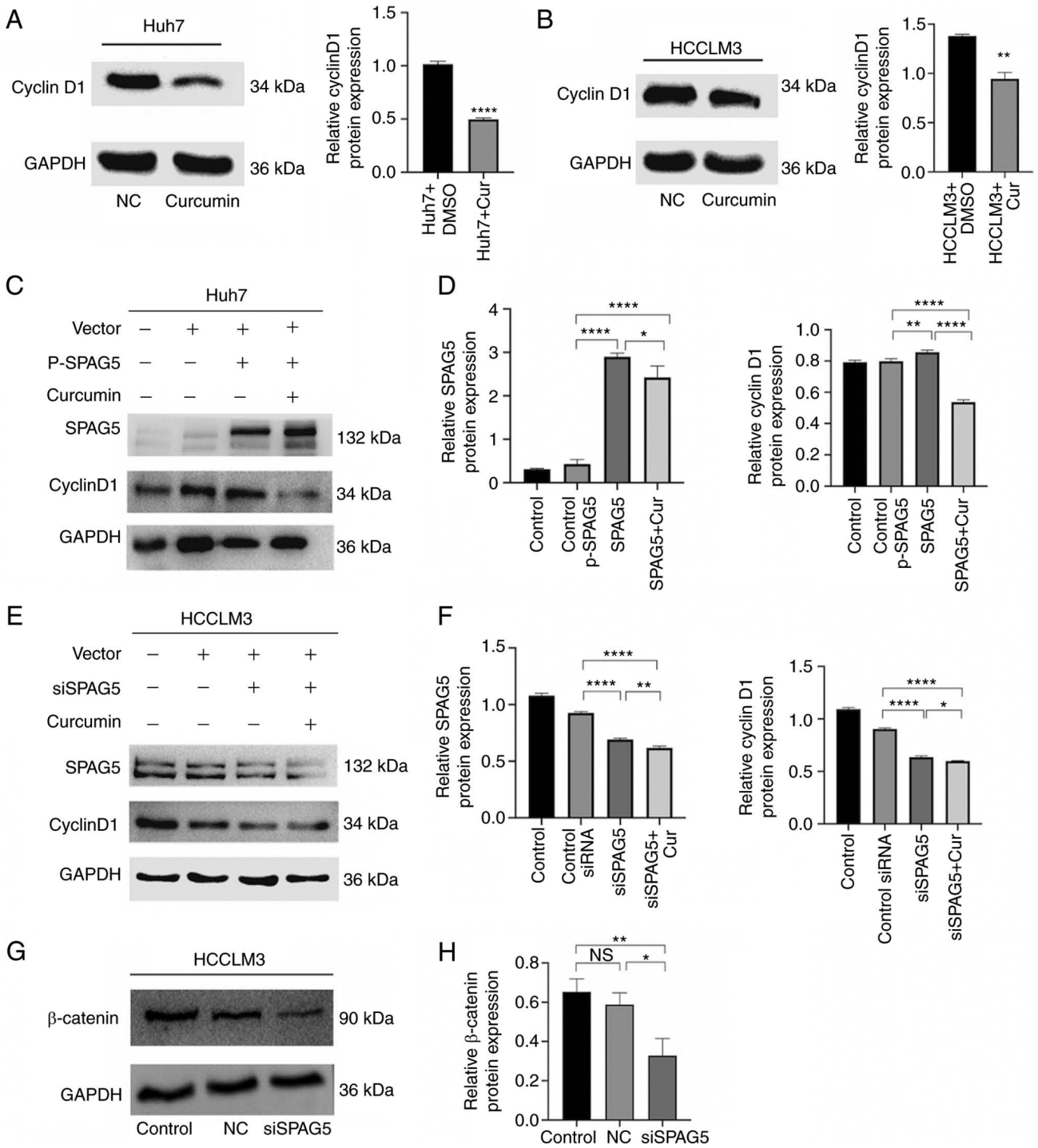


Figure 4. Inhibitory effect of curcumin on SPAG5 expression in hepatocellular carcinoma. (A and B) Western blotting experiments revealed that curcumin can suppress cyclin D1 expression in hepatoma cells. (C and D) Huh7 cells were transfected with P-SPAG5 plasmid and treated with curcumin, followed by the analysis of SPAG5 and cyclin D1 expression via western blotting. Control, Huh7 cells; Control p-SPAG5, cells transfected with a negative control expression plasmid; SPAG5, cells transfected with overexpression SPAG5 plasmids; SPAG5 + Cur, cells transfected with overexpression SPAG5 plasmids and the addition of curcumin. (E and F) HCCLM3 cells were transfected with siSPAG5 plasmid and treated with curcumin, leading to the analysis of SPAG5 and cyclin D1 expression via western blotting. Control, HCCLM3 cells; Control siRNA, cells transfected with a negative control expression plasmid; siSPAG5, cells transfected with SPAG5-knockdown plasmids; SPAG5 + Cur, cells transfected with SPAG5-knockdown plasmids and the addition of curcumin. (G and H) SPAG5-knockdown decreased β-catenin expression. Control, HCCLM3 cells; NC, cells transfected with a negative control expression plasmid; siSPAG5, cells transfected with SPAG5-knockdown plasmids. $^{*}$P<0.05, $^{**}$P<0.01 and $^{****}$P<0.0001. SPAG5, sperm-associated antigen 5; NS, not significant.

*Cell apoptosis analysis*. The apoptosis rate of the cells was assessed using Annexin V-APC/PI Apoptosis Kit (cat. no. E-CK-A117; Elabscience Biotechnology, Inc.) as per the manufacturer's instructions. Briefly, $5x10^5$ cells were harvested by centrifugation at 300 x g for 5 min at room temperature and rinsed twice with PBS. The cells were then resuspended in 500 $\mu$l binding buffer and incubated with 5 $\mu$l Annexin V-APC and 5 $\mu$l PI in the dark at room temperature for 15 min. Apoptotic events were subsequently detected using flow cytometry (BD Fortessa; BD Biosciences) Data

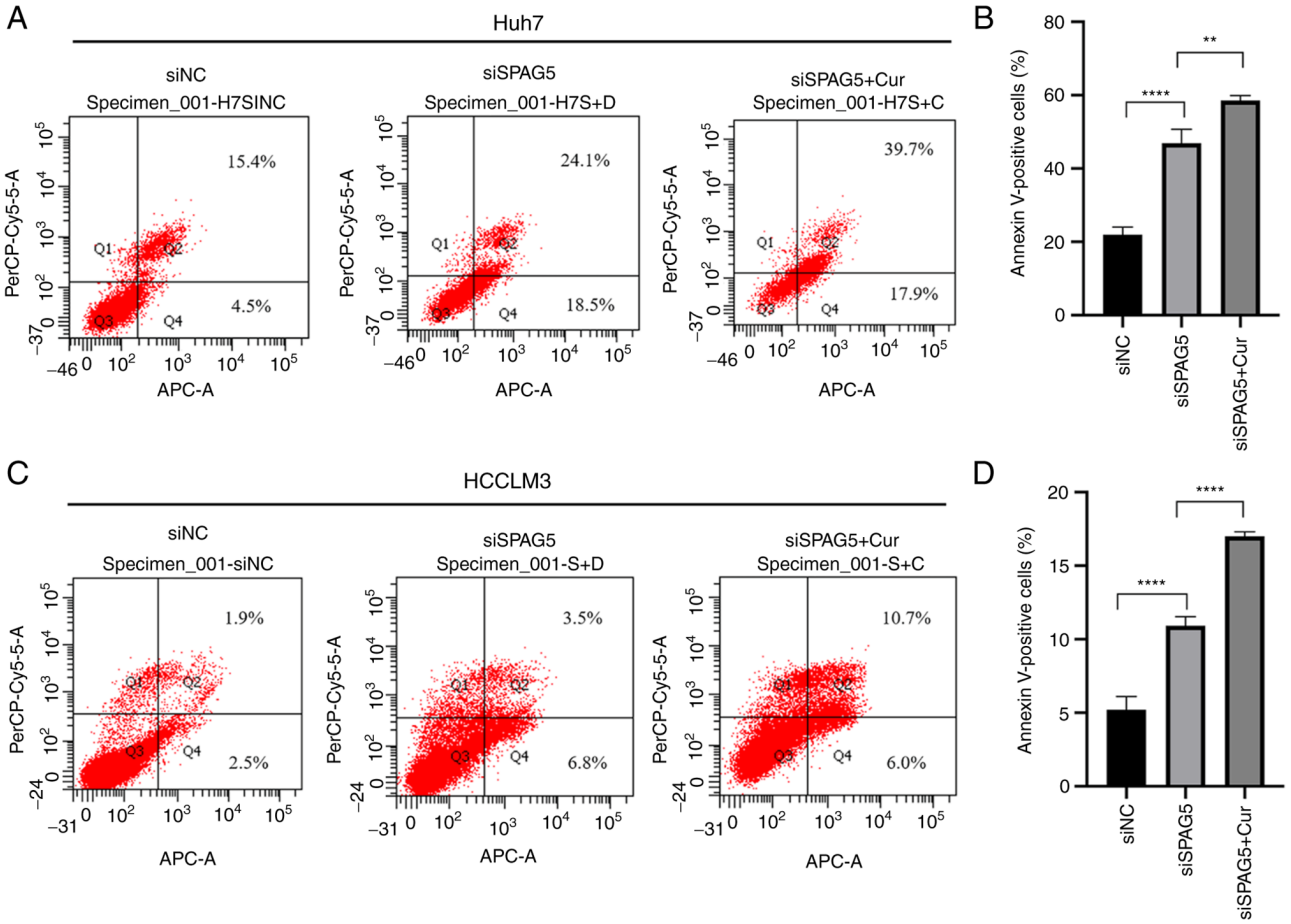


Figure 5. Effect of curcumin on apoptosis of hepatoma cell lines after SPAG5 knockdown. (A and B) siNC, Huh7 cells transfected with a negative control expression plasmid; siSPAG5, cells transfected with SPAG5-knockdown plasmids; siSPAG5 + Cur, cells transfected with SPAG5-knockdown plasmids and the addition of curcumin. (C and D) siNC, HCCLM3 cells transfected with a negative control expression plasmid; siSPAG5, cells transfected with SPAG5-knockdown plasmids; siSPAG5 + Cur, cells transfected with SPAG5-knockdown plasmids and the addition of curcumin. $^{**}$P<0.01 and $^{****}$P<0.0001. SPAG5, sperm-associated antigen; NC, negative control; Cur, curcumin.

analysis was used by FlowJo 10.8.1 (Becton Dickinson and Company).

*Statistical analysis*. The results, expressed as the mean ± SD, were derived from at least three independent experiments and analyzed using GraphPad Prism 8 (GraphPad Software, Inc.). Significant differences were determined using Student's paired t-test and two-tailed distribution. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Bioinformatics database analysis of SPAG5 expression and experimental verification in HCC*. The expression level of SPAG5 in the tissue of patients with HCC was analyzed via TCGA database. Compared with normal tissues, the tumor tissue of patients with HCC exhibited a significantly higher expression of SPAG5 (Fig. 1A and B; P<0.001). Functional enrichment analysis was carried out on transcriptome data from TCGA. Using the median value of SPAG5 expression as the cutoff point, the upper 50% was designated as the high expression group and the lower 50% as the low expression group. This revealed 14,858 differentially expressed genes, including 2,710 upregulated genes (with top genes being KIF18B, MCM10 and GINS1) and 12,148 downregulated genes (with top genes being SMR3A, MT1B and ANKFN1). DESeq2 was used to analyze differentially expressed genes, with genes deemed differentially expressed if $|\log_2 FC| \geq 1$ and P<0.05. Applying the same methodology but considering the top 25% as the high expression group and the bottom 25% as the low expression group, 13,213 genes were found to be differentially expressed, including 2,677 upregulated genes (top genes being AL139327, MAGEA4 and LGALS14) and 10,536 downregulated genes (top genes being SMR3A, BX322559 and TRARG1). As indicated in the volcano plot, low expression genes are in blue, and high expression genes are in red (Fig. 1C and D). A series of enrichment analyses, including KEGG and GO, were subsequently performed. The results of GO-KEGG enrichment analysis mainly implicated involvement in the 'humoral immune response', 'immunoglobulin-mediated immune response', and 'immune response-activating cell surface receptor signaling pathway'. Additionally, these genes were involved in the regulation of cell division activities, such as 'chromosome segregation', 'regulation of mitotic cell cycle phase transition', and 'positive regulation of cell cycle'. Notably, these genes also partook in 'response to metal ion', 'channel activity', 'second-messenger-mediated signaling', and 'response to xenobiotic stimulus' (Fig. 1E-H).

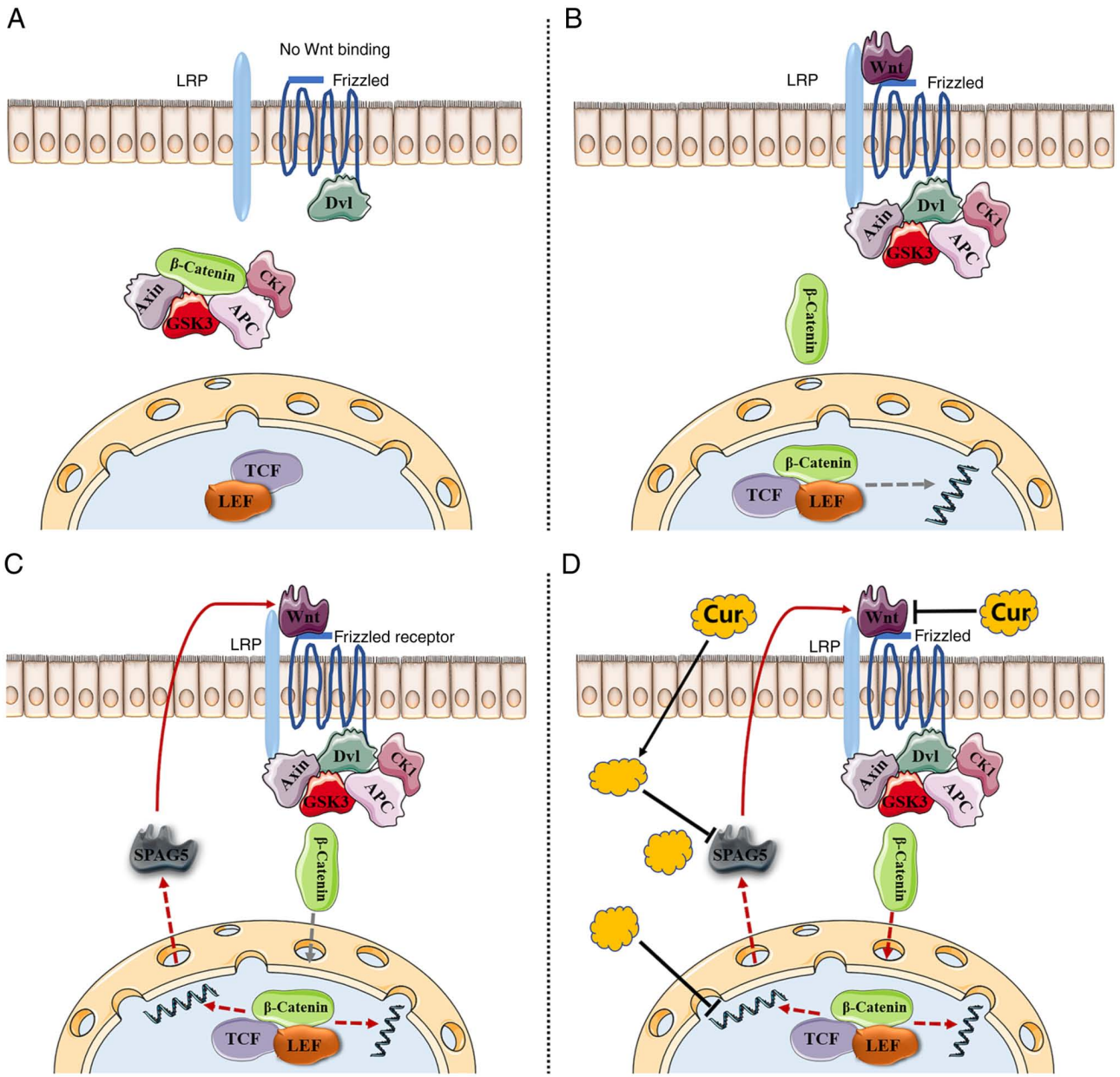


Figure 6. (A and B) Illustration of classical and non-classical Wnt/β-catenin signaling pathways. (C and D) Description of the regulatory role of SPAG5 in the Wnt signaling pathway, and the indirect regulation of Wnt through the effect of curcumin on SPAG5. SPAG5, sperm-associated antigen; Cur, curcumin.

Cellular immunofluorescence analysis results showed strong fluorescent expression of SPAG5 in Huh7 cells, while normal human hepatocyte line MIHA displayed no fluorescent expression (Fig. 2A). The expression of SPAG5 in cancer tissues and adjacent tissues from patients with HCC was investigated through immunohistochemical staining. This revealed significantly higher SPAG5 expression in cancer tissues than in adjacent tissues ($P<0.001$; Fig. 2B and C). To determine the expression of SPAG5 in HCC cells, RT-qPCR experiments were conducted using normal human hepatocyte line MIHA and two hepatocellular carcinoma cells (Huh7 and HCCLM3). The mean fold change of SPAG5 mRNA expression was revealed to be significantly lower in MIHA compared with Huh7 and HCCLM3, with a 2.8-fold increase in Huh7 compared with MIHA cells, and a 2-fold increase in HCCLM3 compared with MIHA cells (Fig. 2D). Western blot analysis was conducted to ascertain SPAG5 protein expression in these cell lines, which also indicated higher SPAG5 expression in HCC cells compared with MIHA (Fig. 2E and F).

*Curcumin inhibits the proliferation, migration and SPAG5 expression of HCC cells.* CCK-8 results confirmed that treatment of Huh7 and HCCLM3 cells with curcumin for 24 h led to statistically significant differences in cell viability ($P<0.05$), with optical density (OD) decreasing as curcumin concentration increased. (Fig. 3A and B) The optimal curcumin concentrations for HCC cells, Huh7 and HCCLM3, were determined to be 80 and 100 $\mu$M respectively, and these were selected for further experimentation. A scratch test was then performed, with a control group treated with DMSO and an experimental group treated with the optimal concentration of curcumin. The results indicated

that after 24 h, the migration ability of curcumin-treated HCC cells was significantly reduced, with the difference being statistically significant (P<0.01 Fig. 3C and D). Western blot results revealed that the expression of SPAG5 protein in Huh7 cells co-cultured with varying concentrations of curcumin was curcumin concentration-dependently inhibited (Fig. 3E and F).

*Curcumin inhibits SPAG5-induced cyclin D1 expression, and knockdown of SPAG5 decreases the expression of β-catenin*. The expression of cyclin D1 was examined using western blot analysis, and the results demonstrated that the expression of cyclin D1 protein in both cell types was significantly weakened in the curcumin-treated group (Fig. 4A and B). In Huh7 cells overexpressing SPAG5, curcumin could significantly inhibit the expression of SPAG5 and cyclin D1 (Fig. 4C and D). On the other hand, the expression of SPAG5 was significantly reduced in siSPAG5 HCCLM3 cells, and curcumin could further reduce the expression of SPAG5 and cyclin D1, (Fig. 4E and F). In the SPAG5-knockdown cell line, the expression of β-catenin was also significantly decreased (Fig. 4G and H). Although the overexpression of SPAG5 promoted proliferation and migration, curcumin inhibited this promoting effect, but not by inhibiting cyclin D1.

Further investigation was conducted to better understand the mechanism by which SPAG5 contributes to HCC cell growth, specifically examining the effects of SPAG5 knockdown on apoptosis. The findings, as presented in Fig. 5, revealed that the percentage of apoptotic cells was significantly increased in the SPAG5-knockdown group compared with the control group. The application of curcumin resulted in a further increase in the rate of apoptosis. Collectively, these data indicated that the knockdown of SPAG5 exerted a tumor-suppressive effect in human HCC cells, an effect which was amplified by the action of curcumin (Fig. 6).

## Discussion

The present study investigated the potential carcinogenic role of SPAG5 in HCC and demonstrated via experimental evidence that the expression of SPAG5 was significantly elevated in HCC at both the tissue and cellular levels. Not only was the high expression of SPAG5 in HCC validated, but also its mechanism and association with Wnt, and the intervention of curcumin were revealed. The present research, exploring uncharted territories, has provided some preliminary conclusions.

Initially, the study revealed that in HCC cell lines, the most suitable concentration of curcumin, which effectively inhibited their proliferation and migration, ranged between 80-100 $\mu$M. This inhibition was further confirmed at the gene level by RT-qPCR and at the protein level by western blot analysis. Next, the potential effect of curcumin on SPAG5 was investigated. It was revealed that the expression of SPAG5 in HCC cell lines exhibited a negative association with curcumin concentration. Finally, HCC cell lines overexpressing SPAG5 were co-cultured under the optimal concentration of curcumin, revealing that curcumin could still significantly inhibit the expression of SPAG5 and the Wnt/β-catenin pathway-related oncogene cyclin D1. Moreover, experimental results indicated that curcumin could directly inhibit the expression of β-catenin in HCC cells. Cyclin D1 and β-catenin were inhibited when SPAG5 was knocked down. In addition, when SPAG5 was knocked down, curcumin further inhibited cyclin D1, which indicated that the inhibition of cyclin D1 by curcumin was associated with the inhibition of SPAG5.

The present study elucidated the effect of curcumin on SPAG5 targets in HCC from multiple angles and provided a theoretical basis for the treatment of clinical liver cancer. However, whether curcumin influences normal liver cells, was not addressed during the experimental process of the present study. In addition, these findings are only preliminary conclusions, as additional pathway proteins have not yet been used to corroborate these findings. Whether curcumin directly inhibits Wnt or inhibits Wnt by inhibiting SPAG5 still necessitates further investigation. These questions will be the focus of a future follow-up study.

In summary, the findings of the present study indicated that SPAG5 is a highly significant cancer-promoting molecule and therapeutic target, especially its association with the Wnt pathway, which warrants further investigation. This research could potentially address the issues of drug side effects targeting Wnt. The inhibition of SPAG5 by curcumin also presents a valuable approach for the early intervention and adjuvant treatment of HCC, thus warranting the execution of more clinical trials. While the effect of curcumin has been experimentally confirmed again, the correct application of curcumin in clinical practice requires resolution in the following areas: i) Further elucidation of the mechanism and target of curcumin; ii) addressing the bioavailability of curcumin; and iii) conducting clinical research.

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## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Authors' contributions

BR conceived and designed the study. HL performed the experiments and drafted the manuscript. YQ undertook the responsibility of data collection and performed data analysis. The task of gathering and verifying the references was performed by JW, while YH participated in discussing the results. HL and YQ confirm the authenticity of all the raw data. All authors have critically reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

The present study was approved (approval no. DK2018002) by the Ethics Committee of the Second People's Hospital of Hunan Province (Changsha, China) and each patient provided written informed consent.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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