

# Secreted phosphoprotein-1 accelerates the progression of human colorectal cancer through activating $\beta$ -catenin signaling

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**Abstract.** Colorectal cancer (CRC) is a common malignant tumor of the digestive tract and one of the leading causes of cancer-associated mortality. Secreted phosphoprotein-1 (SPP-1) is overexpressed in CRC and promotes cancer progression, but the underlying mechanisms underlying SPP-1 function remain unclear. The present study aimed to explore the effects of Wnt/ $\beta$ -catenin signaling in SPP-1-induced CRC progression. The expression patterns of SPP-1 in CRC tissues were examined using reverse transcription-quantitative (RT-q)PCR, western blotting and immunohistochemistry. SPP-1 expression in cells was assessed using RT-qPCR and western blotting. Cell-Counting Kit-8, flow cytometry and tumor-burdened mice experiments were used to determine cell proliferation, apoptosis and *in vivo* tumor formation abilities. The results showed that SPP-1 expression was markedly elevated in CRC tissues and cells compared with that in normal colorectal tissues and cells. High expression of SPP-1 was associated with advanced clinical process and low overall survival rate in patients with CRC. Besides, SPP-1 could interact with  $\beta$ -catenin and positively regulated  $\beta$ -catenin protein expression, and enhanced its nuclear accumulation. Moreover, SPP-1-upregulation significantly enhanced cell proliferation and *in vivo* tumor formation ability, and reduced apoptosis, whereas these effects were all abolished when  $\beta$ -catenin was silenced. Overall, the present study revealed that SPP-1 promoted the progression of CRC in a  $\beta$ -catenin-dependent manner.

## Introduction

Human colorectal cancer (CRC) is the third most common malignant tumor and the fourth most common cause of

cancer-associated mortality in the world (1,2). Notably, the incidence rate of CRC in younger people has increased in past years in China (3). Although notable progress has been made in the therapy and diagnosis of CRC, the therapeutic response and prognosis are still unsatisfactory with a 5-year survival rate of ~50% (4). Therefore, exploration of the mechanism underlying CRC progression and identifying effective molecular targets are of important to improve the treatment of CRC.

It is well documented that the Wnt/ $\beta$ -catenin signaling pathway is frequently hyper-activated in cancer and is strongly implicated in carcinogenesis, including CRC (5,6). Inhibition of the Wnt/ $\beta$ -catenin signaling pathway is a potential antitumor target for CRC (7). In the absence of Wnt,  $\beta$ -catenin protein is retained in cytoplasm through forming a protein complex with axis inhibitor (Axin), adenomatous polyposis coil (APC), casein kinase 1 $\alpha$  and glycogen synthase kinase 3 $\beta$  (8). However, the free  $\beta$ -catenin level is elevated in cytoplasm and then translocates into the nucleus when Wnt signaling is activated, followed by the increased transcription of its target genes, such as c-myc and cyclin D1, through interacting with the T-cell factor/lymphoid enhancer factor family of transcription factors (9,10). Although the inactivating mutation of APC is considered an important factor for Wnt/ $\beta$ -catenin activation in CRC (11-13), other factors or genes that induce Wnt/ $\beta$ -catenin signaling activation in CRC still need to be clarified.

Secreted phosphoprotein-1 (SPP-1), also known as osteopontin, is located at 4q22.1 and a multifunctional member of the small integrin-binding ligand N-linked glycoprotein family (14). Evidence has demonstrated that SPP-1 is upregulated in various malignant tumors (15,16), including CRC (17). For example, Xu *et al* (17) used bioinformatics methods and found that SPP-1 was significantly overexpressed in CRC tissues, and its high expression is closely associated with the advanced clinical process and poor prognosis in patients with CRC. Further experiments showed that downregulation of SPP-1 significantly repressed CRC cell viability, migration and tumor growth and induced apoptosis. Moreover, Huang *et al* (18) demonstrated that SPP-1-upregulation resulted in significant enhancements in cell proliferation, migration and invasion, and inhibited apoptosis and autophagy through regulating MAPK signaling. These findings demonstrate that SPP-1 plays an oncogenic role in CRC progression, but the underlying mechanisms remain unclear.

The present study aimed to explore the role of the Wnt/ $\beta$ -catenin signaling pathway in SPP-1-induced CRC

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progression with *in vivo* and *in vitro* experiments. Our results demonstrated that  $\beta$ -catenin was an indispensable factor for SPP-1-mediated CRC progression.

## Materials and methods

**Colorectal tissue specimens.** Primary CRC tissues and adjacent normal colorectal tissues (>2 cm from the tumor tissues) were obtained from 200 patients with CRC who underwent colectomy from May 2009 to May 2017. All patients signed the informed consent and received colectomy as the first treatment method. Patients were excluded if they suffered from other malignant tumors or if they received radiotherapy and/or chemotherapy before surgery. Tumor-Node-Metastasis (TNM) stage was evaluated according to previously reported (19). The fresh tissues were then immediately immersed in liquid nitrogen (-196°C) until analysis. Experiments involving human samples were performed according to the Helsinki Declaration and approved by The Ethical Committee of Ganzhou People's Hospital (approval no. Ky2019015).

**Immunohistochemistry (IHC).** For IHC, the routine three-step procedure was performed as previously described (20) with the primary antibody against SPP-1 (cat. no. ab8448; Abcam). The staining of SPP-1 was determined by three pathologists independent from the present study in a blinded manner. The extent of positively stained cells was scored as: 0 For 0-5%, 1 for 6-25%, 2 for 26-50%, 3 for 51-75%, and 4 for 76-100%. The staining intensity was scored as: 0 for negative staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The extent score was multiplied with intensity score to obtain the total score of SPP-1 staining in colon tissues. Total score of SPP-1 staining  $\leq$  medium score (9 points) of the total samples was defined as low expression, and > medium score was defined as high expression.

**Cell culture conditions.** Human normal colon cell line CCD-18Co was purchased from The Cell Bank of the Chinese Academy of Sciences and cultured in RPMI-1640 medium with 10% fetal serum bovine (FBS) (both Thermo Fisher Scientific, Inc.). Three human CRC cell lines, including SW620, COLO 205 and SW480 were obtained from BeNa Culture Collection. SW620 and COLO 205 cells were cultured in RPMI-1640 medium and SW480 cells were grown in DMEM-H medium (Thermo Fisher Scientific, Inc.) with 10% FBS. All cells were maintained at 37°C with 5% CO<sub>2</sub>.

**Alteration of gene expression in cells.** To upregulate SPP-1 expression, SW480 cells were infected with the overexpression lentiviral vector (OE-SPP-1; Shanghai GenePharma Co., Ltd.) at a multiplicity of infection of 5 and incubated with G418 (100  $\mu$ g/ml) for 14 days at 37°C to select the stably overexpressed cells. To silence  $\beta$ -catenin expression, three lentiviral vectors (sh- $\beta$ -catenin-1/-2/-3; OriGene Technologies, Inc.) and pumomycin (7  $\mu$ g/ml, incubation for 14 days) were used to select the stably transfected cells. The 2nd generationally stable cell lines were used for further studies. Two small interfering (si)RNAs of SPP-1 (si-SPP-1-1/-2; 100 pmol/6-well plates) and the scrambled control (si-NC) purchased from OriGene Technologies, Inc. were used to downregulate SPP-1

expression using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37°C. After 48 h of transfection, the cells were harvested for further analysis. The si sequences were as follows: si-SPP-1-1, 5'-UCAUAUUCUGAAUCUCAUCCU-3'; si-SPP-1-2: 5'-AGUUUCAACCGUCUUAUAUCAG-3'.

**RNA extraction and reverse transcription-quantitative (RT-q) PCR.** Total RNA was extracted from snap-frozen tissues or cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The first-strand cDNA was synthesized with random primers (Beijing Solarbio Science & Technology Co., Ltd.) using TaqMan Reverse Transcription kit (Takara Biotechnology Co., Ltd.) at 42°C for 60 min. After that, the RT-qPCR was carried out with SYBR Green of The SuperScript® III One-Step RT-PCR system (Thermo Fisher Scientific, Inc.). Primers for SPP-1 were 5'-GAATCTCTAGC CCCACAGACC-3' (sense) and 5'-ACTCCTCGCTTTCCA TGTGTG-3' (antisense); primers for  $\beta$ -catenin were 5'-GCG CCATTTTAAGCCTCTCG-3' and 5'-GGCCATGTCCAA CTCCATCA-3'; primers for GAPDH were 5'-CCACTAGGC GCTCACTGTTCTC-3' (sense) and ACTCCGACCTTCACC TTCCC-3' (antisense). Reaction conditions were as follows: 94°C For 5 min, followed by amplification for 40 cycles (94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec), and a final step at 72°C for 5 min. The levels of mRNAs were calculated using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (21). GAPDH was used as endogenous control to normalize the mRNA levels of SPP-1 and  $\beta$ -catenin.

**Western blotting.** Protein was extracted using RIPA lysis buffer (Shanghai Yeasen Biotechnology Co., Ltd.) and protease and phosphatase inhibitors (Beijing Solarbio Science & Technology Co., Ltd.). After centrifugation at 4°C for 25 min at 12,000 x g, the protein samples were quantified by using the Bicinchoninic acid Protein Assay kit (Thermo Fisher Scientific, Inc.) and then 25  $\mu$ g protein from each group was loaded per lane and separated by 10% SDS-PAGE. After that, the proteins were transferred into the polyvinylidene difluoride membranes (Thermo Fisher Scientific), following by being blocked with 5% non-fat milk for 1 h at room temperature. Then, the membranes were probed with the indicated primary antibodies, SPP-1 (1:3,000 dilution; cat. no. ab844; Abcam), Axin (1:3,000 dilution; cat. no. ab32197; Abcam),  $\beta$ -catenin (1:2,000 dilution; cat. no. ab16051; Abcam), Frz (1:1,000 dilution; cat. no. AF1617; R&D Systems, Inc.), DVL1 (1:2,000 dilution; cat. no. TA329899; OriGene Technologies, Inc.), TCF (1:2,500 dilution; cat. no. #9383; Cell Signaling Technology, Inc.), c-myc (1:2,000 dilution; cat. no. M4439; Sigma-Aldrich; Merck KGaA), APC (1:2,000 dilution; cat. no. ab15270; Abcam) and GAPDH (1:6,000 dilution; cat. no. 2118; Cell Signaling Technology, Inc.) at 4°C overnight and the HRP-conjugated secondary antibodies (1:10,000 dilution; cat. no. SA00001-1 and SA00001-2; ProteinTech Group, Inc.) for 1 h at room temperature in succession. The protein signaling was visualized using electrochemiluminescence reagent (EMD Millipore) and quantified by ImageJ software (version 1.48; National Institutes of Health).

**Co-immunoprecipitation (Co-IP).** The interaction between different proteins was assessed using a Co-IP assay. SW480 cells were rinsed with cold PBS and lysed in IP lysis buffer

(Beijing Solarbio Science & Technology Co., Ltd.), followed by centrifugation at 4°C for 25 min at 12,000 x g. Then, the proteins (200 µg) were incubated with 10 µl of Dynabeads® protein G (Thermo Fisher Scientific, Inc.) for 1 h. Next, the proteins were incubated with 2 µg of SPP-1 antibody or IgG antibody (negative control) overnight at 4°C, and Dynabeads® protein G for 1 h. The immunocomplex was washed five times with IP lysis buffer and boiled at 100°C for 8 min, and then subjected to western blotting analysis.

**Immunofluorescence microscopy.** SW480 cells with and without stable SPP-1 stable overexpression were cultured in culture glass slides in 24-well plates and incubated at 37°C for 48 h. Then, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by incubation with 1% Triton-100 for 10 min for cell membrane permeabilizing. Next, the cells were rinsed with phosphate buffer, blocked with 5% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature and incubated with the primary antibody against  $\beta$ -catenin (cat. no. ab16051; Abcam) overnight at 4°C and the Alexa Fluor® 488 Conjugated secondary antibody (1:1,000 dilution; cat. no. 4412; Cell Signaling Technology, Inc.) for 1 h at room temperature. DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) was used for nuclear staining at a concentration of 1:5,000 for 5 min at room temperature. The subcellular location of  $\beta$ -catenin was analyzed on a laser scanning microscope (magnification, x60; TCSSP2-AOBS-MP; Leica Microsystems, Inc.).

**Cell proliferation detection.** After 6 h of cell transfection with OE-SPP-1, OE-NC, si-SPP-1, si-NC or OE-SPP-1, SW480 cells were seeded into 96-well plates at 3,000 cells/well density and cultured at 37°C for 1, 2, 3, 4 or 5 days. Cell proliferation was tested by using a Cell-Counting Kit 8 (CCK-8; Dojindo Molecular Technologies, Inc.) based on the manufacturer's description.

**Apoptosis detection.** The effect of SPP-1/ $\beta$ -catenin axis on SW480 apoptosis was assessed by using the Annexin V/propidium iodide (PI) kit (Roche Diagnostics). After incubation with the Annexin V-FITC and PI solution in the dark for 15 min, SW480 cells were collected for flow cytometry using CytoFLEX (Beckman Coulter, Inc.) and cell apoptotic rates were analyzed by FlowJo 7.6 software (FlowJo LLC). FITC<sup>+</sup>/PI<sup>-</sup> quadrant were viable cells, FITC<sup>+</sup>/PI<sup>+</sup> were necrosis cells, FITC<sup>+</sup>/PI<sup>-</sup> were early apoptotic cells and FITC<sup>+</sup>/PI<sup>+</sup> were late apoptotic cells.

**In vivo tumor formation assay.** SW480 cells were transfected with OE-NC+sh-NC, OE-SPP-1+sh-NC, OE-NC+sh-NC and OE-NC+sh- $\beta$ -catenin, and then incubated with 100 µg/ml G418 and 7 µg/ml puromycin for 14 days to establish the stable transfection cell lines. Then, 5x10<sup>6</sup> of the aforementioned cells were resuspended in PBS buffer and injected subcutaneously into the flanks of 4-6-week-old male nude mice (n=20; 20±2 g; Experimental Animal Center Of The Fourth Military Medical University). Mice were fed with common feed and sterile water *ad libitum*, and housed in 22±1°C with 55±1% humidity with a 12 h light/dark cycle. After 28 days of injection, mice were euthanized via cervical dislocation and the tumors were

removed to weigh and photograph. Mice are considered as dead if the chests did not rise or fall and the hearts do not beat. Animal health and behavior were monitored every 4 days. Mice were sacrificed if the tumor diameter was >1.8 cm. No mouse died prior to the end of the study. These animal experiments were carried out in accordance to the Institutional principles for the concern and use of animals and the protocol was approved by The Ethical Committee of Ganzhou People's Hospital (approval no. Ky2019015).

**Statistical analysis.** Data are obtained from at least three times of independent experiments and expressed as means ± standard deviation or median + interquartile range only for the IHC score. Comparison of the IHC scores between normal group and tumor group in Fig. 1A was carried out using the paired Wilcoxon signed rank test. Other comparisons between two groups were executed using two-tailed Student's t-tests and one-way ANOVA test followed by Tukey's post hoc test was used for multiple groups. A paired t-test was used for the analysis of tumor and adjacent non-tumor samples of the same individuals, and unpaired t-test was used for other comparisons between two groups. Kaplan-Meier analysis with log rank tests was used to evaluate the clinical significance of SPP-1 levels in predicting the prognosis of CRC patients.  $\chi^2$  tests were used for the data comparisons listed in Table I. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Expression of SPP-1 is increased in CRC tissues and cells.** To uncover the mechanism underlying SPP-1 in the progression of CRC, SPP-1 expression profiles were compared between CRC tissues and the adjacent normal colorectal tissues using IHC, RT-qPCR and western blotting assays. It was observed that the expression of SPP-1 was significantly increased at protein and mRNA levels in CRC tissues, compared with that in the normal tissues (P<0.01 and P<0.001, respectively; Fig. 1A-C). Additionally, SPP-1 expression patterns in CRC cells and normal colon cells were evaluated using western blotting and RT-qPCR. The results demonstrated that SPP-1 expression in CRC cell lines SW620, COLO 205 and SW480 was significantly increased when compared with that in CCD-18Co cells at the mRNA (P<0.01, Fig. 1D) and protein levels (P<0.01, Fig. 1E). These findings confirmed that the expression of SPP-1 was increased in CRC.

**Assessment of the clinical value of SPP-1 in CRC.** To determine the clinical value of SPP-1 in CRC, Kaplan-Meier with log-rank analysis and  $\chi^2$  tests test were used to analyze the effects of SPP-1 expression on the overall survival rate and the clinicopathological parameters of patients with CRC. As shown in Fig. 2, the overall survival rates for patients with SPP-1 low expression were significantly higher compared with that of patients with SPP-1 high expression. Moreover, SPP-1 expression level was associated with the differentiation of CRC tissues (P=0.017), and associated with TNM stage (P=0.001), incidence rates of tumor infiltration (P=0.001), lymph node metastasis (P=0.001), distant metastasis (P=0.001) and vascular invasion (P=0.001) (Table I). These results indicated that SPP-1 played an important role

Table I. Association between SPP-1 expression and the clinicopathological characteristics of 200 patients with colorectal cancer.

Parameters	Total	Low expression	High expression	P-value
Sex				0.689
Male	102	45	57	
Female	98	46	52	
Age, years				0.293
<60	48	25	23	
≥60	152	66	86	
Differentiation				0.017
High	52	31	21	
Poor/moderation	148	60	88	
TNM stage				0.001
I/II	46	40	6	
III/IV	154	51	103	
Tumor infiltration				0.001
T1/T2	32	26	6	
T3/T4	168	65	103	
Lymph node metastasis				0.001
N0	53	38	15	
N1-N3	147	53	94	
Distant metastasis				0.001
M0	121	72	49	
M1	79	19	60	
Vascular invasion				0.001
Absent	102	61	41	
Present	98	30	68	

TNM, Tumor-Node-Metastasis.

in predicting the clinical stage and prognosis in patients with CRC.

*SPP-1 promotes proliferation and inhibits the apoptosis of CRC cells.* SPP-1 expression was increased in SW620, COLO 205 and SW480 cells, and SW480 cell line was selected for further study as SPP-1-overexpression was most notable in these cells compared with SW620 and COLO 205. Compared with the control group, SPP-1 expression level was significantly decreased following transfection with si-SPP-1-1 and si-SPP-1-2, and OE-SPP-1 significantly enhanced SPP-1 expression at both the mRNA and protein levels ( $P<0.001$ , Fig. 3A-D). Compared with the control group, downregulation of SPP-1 significantly inhibited cell proliferation, while overexpression of SPP-1 enhanced cell proliferation ( $P<0.05$ , Fig. 3E). Besides, knockdown of SPP-1 significantly induced apoptosis, and overexpression of SPP-1 resulted in the opposite result ( $P<0.01$ , Fig. 3F-G) as compared with the control group. These results verified that SPP-1 served as an oncogene in CRC.

*SPP-1 promotes the expression of  $\beta$ -catenin and its nuclear transportation in CRC cells.* To investigate whether Wnt/ $\beta$ -catenin signaling was involved in SPP-1-mediated

CRC progression, the effects of SPP-1 on the activation of Wnt/ $\beta$ -catenin signaling were assessed. Western blotting showed that SPP-1-overexpression significantly increased the expression of  $\beta$ -catenin and c-myc, whereas no obvious influence on the expression levels of Frz, DVL1, APC, Axin and TCF in SW480 cells were observed ( $P<0.001$ , Fig. 4A). The Co-IP assay showed that SPP-1 could combine with  $\beta$ -catenin protein (Fig. 4B). Additionally, SPP-1-overexpression promoted the nuclear accumulation of  $\beta$ -catenin (Fig. 4C). These results suggested that SPP-1 triggered the activation of  $\beta$ -catenin signaling.

*SPP-1 facilitates CRC progression in a  $\beta$ -catenin-dependent manner.* Finally, it was assessed whether  $\beta$ -catenin was involved in SPP-1-mediated CRC progression. Three shRNAs of  $\beta$ -catenin were applied to downregulate  $\beta$ -catenin expression in CRC cells, among which shRNA-1 significantly decreased  $\beta$ -catenin mRNA and protein expression levels compared with the sh-NC group ( $P<0.001$ , Fig. 5A and B). Downregulation of  $\beta$ -catenin in SPP-1 overexpressed cells significantly rescued the enhancement in cell proliferation induced by SPP-1 in SW480 cells ( $P<0.01$ , Fig. 5C), and increased apoptosis rate ( $P<0.01$ , Fig. 5D) compared with the OE-SPP-1+sh-NC group. Furthermore, SPP-1 stable overexpression in SW480 cells



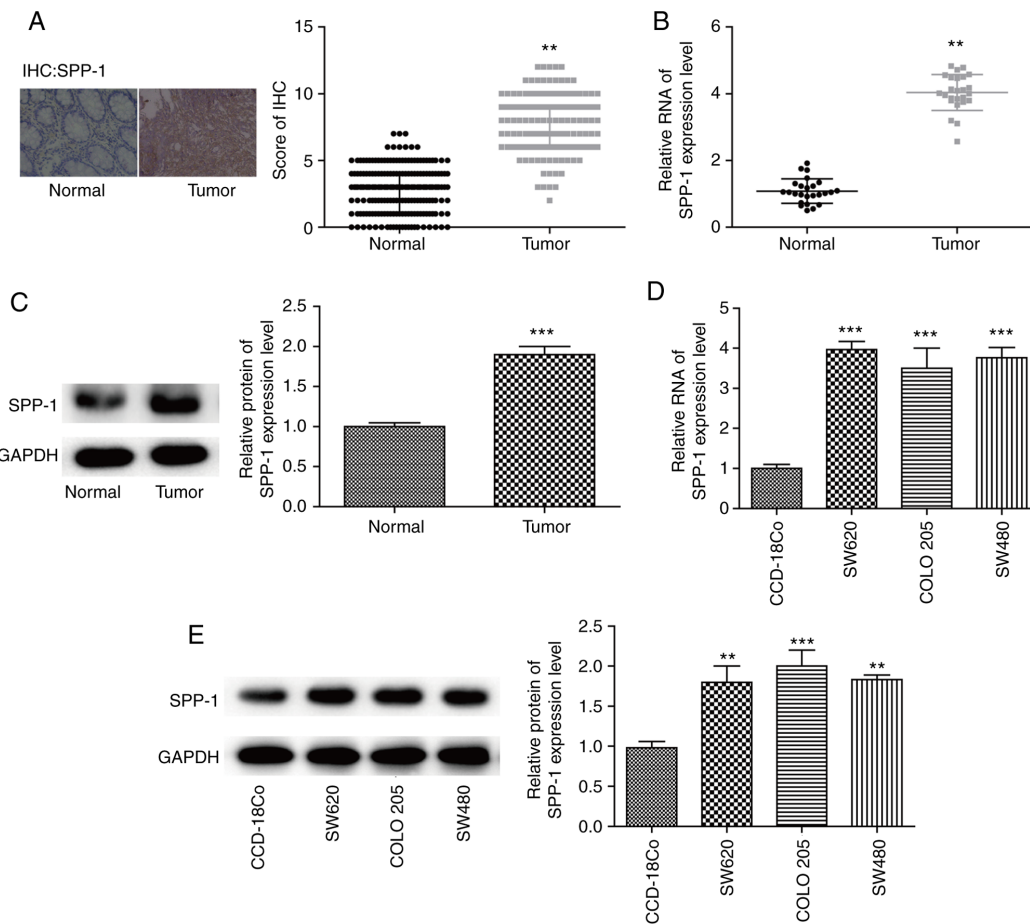


Figure 1. Increased expression of SPP-1 in CRC tissues and cells. (A) Representative images of protein expression of SPP-1 detected using immunohistochemical analysis of CRC and paired normal tissues (magnification, x20). SPP-1 mRNA and protein levels in 25 paired CRC tissues and the normal tissues were determined using (B) RT-qPCR and (C) and western blotting (n=25). SPP-1 expression patterns in normal colorectal cells and CRC cells were examined using (D) RT-PCR and (E) western blotting assays (n=3). \*\*P<0.01, \*\*\*P<0.001 vs. control. RT-q, reverse transcription-quantitative; SPP-1, secreted phosphoprotein-1.

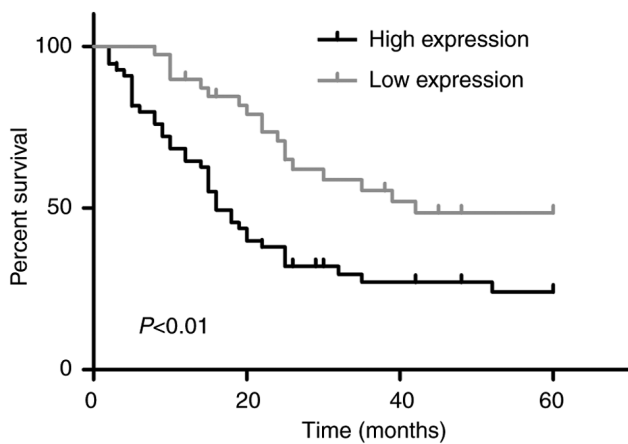


Figure 2. Evaluation of SPP-1 expression in predicting the prognosis of patients with CRC. Kaplan-Meier curve with log-rank analysis was conducted to assess the overall survival rates for patients with CRC with high and low SPP-1 expression. SPP-1, secreted phosphoprotein-1; CRC, colorectal cancer.

significantly promoted tumor formation *in vivo* as compared with the OE-NC+sh-NC group, which was abolished when  $\beta$ -catenin was stably downregulated (P<0.01, Fig. 5E and F). Additionally, the largest tumor size was ~0.18 cm<sup>3</sup> and the largest tumor diameter was ~1.0 cm (Fig. 5F). These results

suggested that SPP-1 facilitated CRC progression in a  $\beta$ -catenin-dependent manner.

## Discussion

SPP-1 has been previously demonstrated to be highly expressed in CRC cells and tissues (22), and overexpression of SPP-1 significantly enhances cell proliferation and motility *in vitro*, and tumorigenesis and angiogenesis *in vivo* (23). In addition, high SPP-1 expression predicts a poor prognosis in patients with CRC (24). These findings suggest that SPP-1 serves an important role in human CRC. The present study demonstrated, for the first time, that SPP-1 facilitated the progression of CRC through interacting with  $\beta$ -catenin and increasing its expression and nuclear accumulation.

SPP-1 is a secreted glycoprophosphoprotein, which is expressed in multiple cell types, and is strongly implicated in numerous biological functions, such as cell adhesion, migration, bone calcification, immune responses and carcinogenesis (25,26). Up to now, several studies have recognized that SPP-1 is overexpressed in multiple types of cancer and its high expression is associated with poor prognosis and advanced clinical process. For example, the plasma SPP-1 level is significantly elevated in patients with renal cell carcinoma with distant metastasis, and SPP-1 high

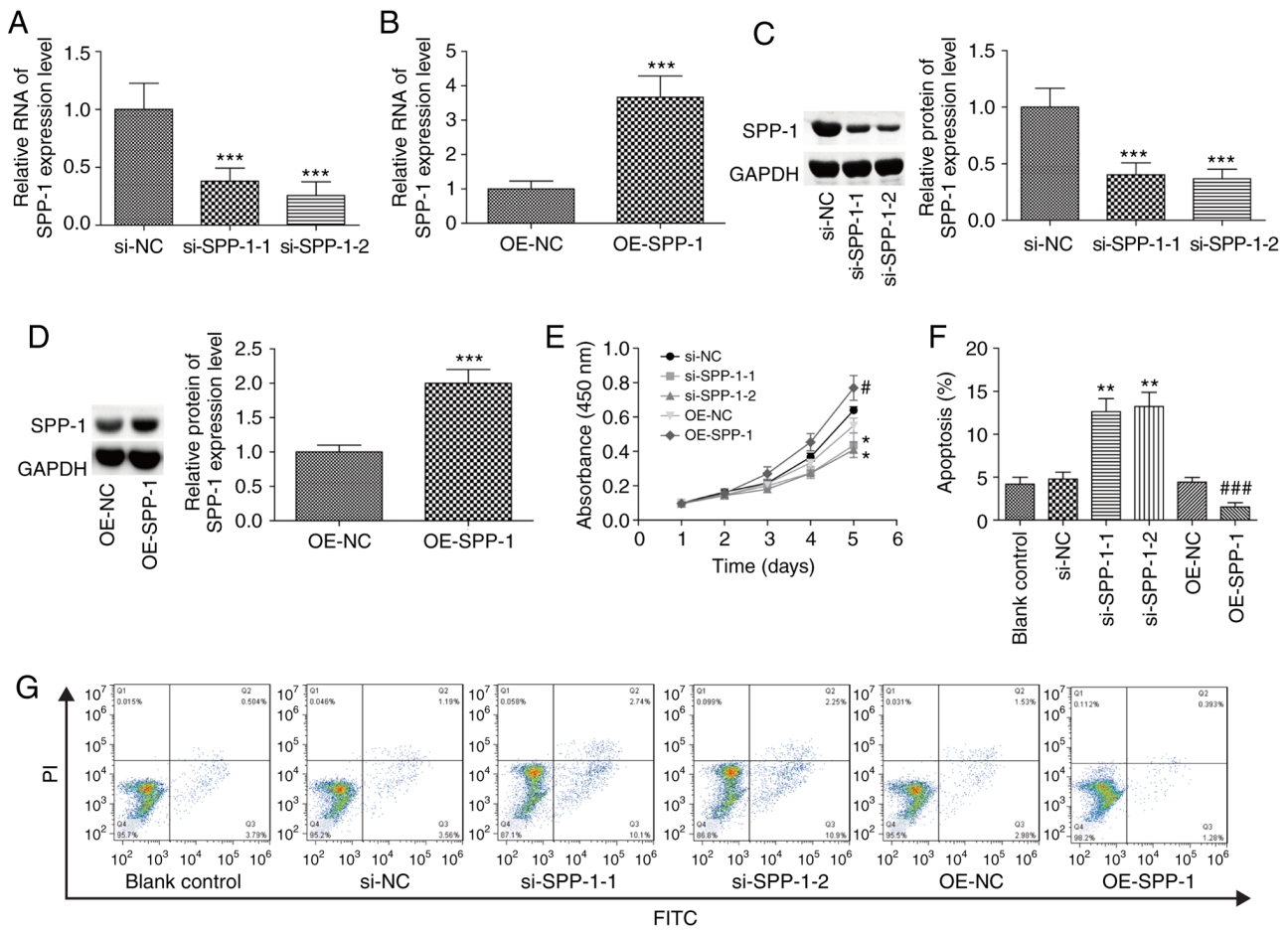


Figure 3. Increased expression of SPP-1 enhances cell proliferation and reduces apoptosis. SW480 cells were transiently transfected with OE-SPP-1, OE-NC, si-SPP-1 and si-NC, then (A-B) mRNA levels were determined using RT-PCR and (C-D) protein levels were measured by western blotting at 48 h post-treatments. (E) A Cell Counting Kit-8 assay was performed to assess the effects of altered expression of SPP-1 on the cell proliferation. (F and G) Flow cytometry was carried out to detect apoptosis (n=3). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. si-NC group; # $P < 0.05$ , ### $P < 0.001$  vs. OE-NC group. SPP-1, secreted phospho-protein 1; OE, overexpression, NC, negative control; si, small interfering.

expression levels predicts a lower survival rate (27). SPP-1 is upregulated in oral squamous cell carcinoma (OSCC) and stromal OSCC cells, which predicts a higher nodal stage, higher World Health Organization clinical stage and poor clinical prognosis in patients with OSCC (28). In addition, Loosen *et al* (29) reported that the increased expression of SPP-1 is associated with the poor survival in patients with postoperative cholangiocarcinoma. Similarly, in CRC, the high expression profile of SPP-1 is significantly correlated with the lymph node metastasis, lymphatic/venous invasion and TNM stage, as well as poor prognosis (22,30). Consistently, the current study demonstrated that the high expression level of SPP-1 was closely associated with a lower overall survival rate and advanced clinical process of patients with CRC, including TNM stage, tumor infiltration, lymph node metastasis, distant metastasis and vascular invasion. This suggested that SPP-1 may have value as a biomarker for CRC diagnosis and prognosis prediction.

SPP-1 has been identified to serve as an inducer of the aggressive behaviors in several types of tumor cell, including CRC. For example, Xu *et al* (17) reported that knockdown of SPP-1 significantly represses the proliferation, colony formation, migration and *in vivo* tumor growth and increased apoptosis in CRC cells. Likui *et al* (31) demonstrated that

SPP-1-downregulation significantly suppresses invasion and enhances the radiosensitivity of CRC cells. Huang *et al* (32) demonstrated that SPP-1-overexpression significantly promotes the hepatic metastasis of CRC. These findings indicate that SPP-1 plays an important role in promoting CRC progression. Except for the oncogenic role of SPP-1 in CRC, the present study also clarified that SPP-1 promoted the expression and nuclear accumulation of the  $\beta$ -catenin protein through protein-protein interactions. SPP-1 has been reported to induce the phosphorylation of GSK-3 $\beta$  at serine 9, which is the most well recognized means for GSK-3 $\beta$  inhibition (33), suggesting that SPP-1 may induce  $\beta$ -catenin expression through repressing GSK-3 $\beta$ . Moreover, Robertson *et al* (34) demonstrated that SPP-1-upregulation could significantly promote the expression and nuclear accumulation of  $\beta$ -catenin, potentially through repressing GSK-3 $\beta$  expression. The present study observed that SPP-1 could combine with  $\beta$ -catenin protein and promote its expression with no obvious influence in the expression of DVL1, Frz, TCF, Axin and APC. Evidence has shown that the increased expression of TCF4/lymphoid enhancer-binding factor induced by  $\beta$ -catenin activation can promote SPP-1 transcription (24,35). Taken together, it was speculated that there might be a positive feedback between SPP-1 and

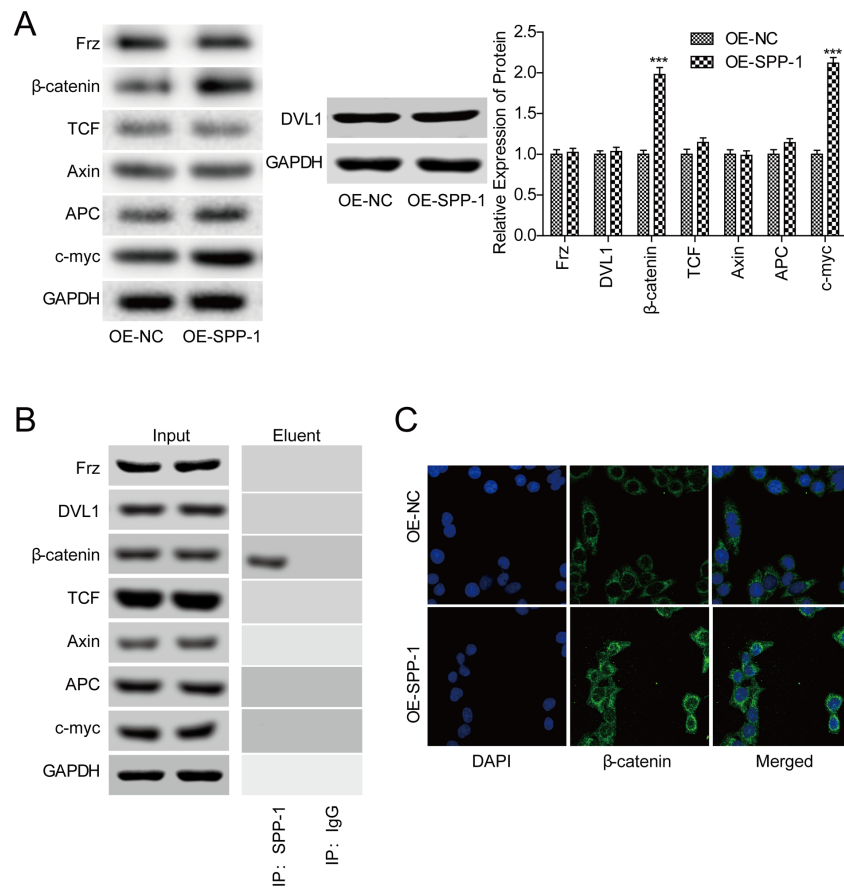


Figure 4. Increased expression of SPP-1 increases β-catenin expression and nuclear transportation. (A) Expression of Frz, DVL1, β-catenin, TCF, Axin, APC and c-myc were detected using western blotting after SW480 cells were infected OE-SPP-1 and OE-NC (n=3). (B) A co-immunoprecipitation assay was performed to assess the interactions between SPP-1 and Frz, DVL1, β-catenin, TCF, Axin, APC and c-myc. (C) Immunofluorescence microscopy was used to detect the effects of SPP-1 on the subcellular location of β-catenin. \*\*\*P<0.001 vs. OE-NC. SPP-1, secreted phosphoprotein-1; OE, overexpression; NC, negative control; APC, adenomatous polyposis coil; Axis, axin inhibitor.

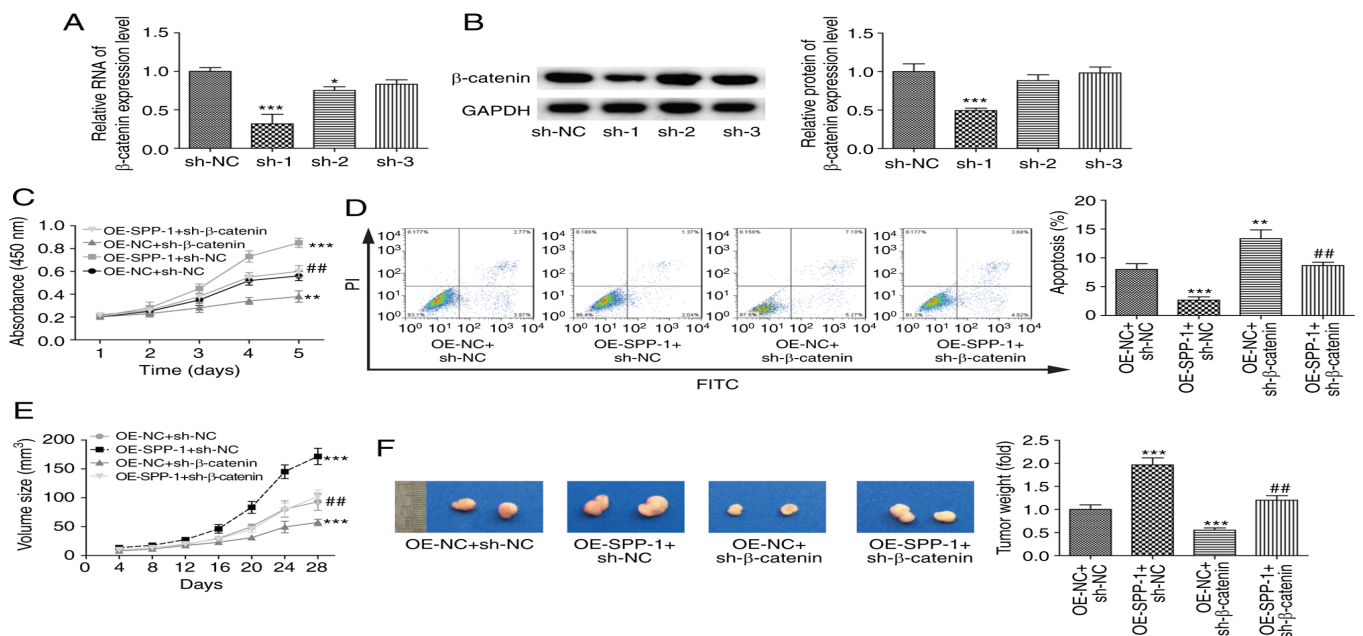


Figure 5. Effects of SPP-1/β-catenin signaling on cell proliferation, apoptosis and tumorigenesis. Knockdown efficiency of sh-β-catenin in SW480 cells was determined using (A) reverse transcription-quantitative PCR and (B) western blotting (n=3, \*P<0.05, \*\*\*P<0.001, vs. sh-NC group). SW480 cells were treated with OE-NC+sh-NC, OE-NC+sh-β-catenin, OE-SPP-1+sh-NC and OE-SPP-1+sh-β-catenin, then cell proliferation was determined using a (C) Cell Counting Kit-8 assay and (D) apoptosis was assessed using flow cytometry (n=3). (E and F) *In vivo* tumor formation ability was determined by nude mice xenotransplantation assays (n=5). (C-F) \*\*P<0.01, \*\*\*P<0.001 vs. OE-NC+sh-NC group; ##P<0.01 vs. OE-SPP-1+sh-NC group. SPP-1 secreted phosphoprotein 1; OE, overexpression; NC, negative control; sh, short hairpin.

$\beta$ -catenin, which needs to be verified in future studies using western blotting assays.

To further explore the role of  $\beta$ -catenin in SPP-1 induced CRC progression, flow cytometry, CCK-8 and *in vivo* tumor formation assays were performed. The results showed that the enhancements in cell proliferation, tumorigenesis and apoptosis inhibition induced by SPP-1-overexpression were abrogated when  $\beta$ -catenin was downregulated. These results demonstrated that  $\beta$ -catenin activation plays an important role in SPP-1-mediated CRC progression.

Drug resistance is a main cause for the dissatisfaction of CRC treatment (36), thus it is important to reveal the mechanisms of drug resistance in CRC. One main limitation of the present study is that the role of SPP-1 was not explored in response to anticancer drugs, such as cis-platin-mediated apoptosis of CRC cells. Further *in vitro* and *in vivo* experiments should be carried out to disclose SPP-1 role in the drug resistance of CRC.

In conclusion, the current study demonstrated that SPP-1 functions as an oncogene in CRC, which is highly expressed in CRC tissues and cells and is closely associated with the malignant clinical progression and poor outcome of patients with CRC. In addition, SPP-1 confers CRC cells with malignant phenotype in a  $\beta$ -catenin dependent manner. Overall, the present study provides evidence to support the value of SPP-1/ $\beta$ -catenin as a novel therapeutic target for human CRC.

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## Availability of data and materials

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

## Authors' contributions

CF conceived the project and revised the manuscript. JY and YL performed the experiments, collected and interpreted the data and wrote the manuscript. LZ analyzed the data. CF, JY, YK and LZ confirm the authenticity of all raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

This study was approved by The Ethical Committee of Ganzhou People's Hospital (approval no. Ky2019015). All patients 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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