SHP-2 Interacts with CD81 and Regulates the Malignant Evolution of Colorectal Cancer by Inhibiting Epithelial–Mesenchymal Transition

Huaqin Yuan1,*
Jun Zhao2,*
Yang Yang3
Rongfu Wei1
Liangxue Zhu1
Jie Wang1
MeiQing Ding1
Mingyun Wang1
Yanhong Gu1

1Department of Oncology, Nanjing Gaohun People's Hospital Affiliated to Yangzhou University, Nanjing, Jiangsu 211316, People's Republic of China; 2Department of Orthopedics, Nanjing Gaohun People's Hospital Affiliated to Yangzhou University, Nanjing, Jiangsu 211316, People's Republic of China; 3Department of Oncology, Gulou Hospital Affiliated to Medical College of Nanjing University, Nanjing, Jiangsu 210000, People's Republic of China; 4Department of Oncology, The First Affiliated Hospital with Nanjing Medical University, Nanjing, Jiangsu 210029, People's Republic of China

*These authors contributed equally to this work.

Correspondence: Mingyun Wang
Department of Oncology, Nanjing Gaohun People's Hospital Affiliated to Yangzhou University, 53 Maoshan Road, Nanjing, Jiangsu 211316, People's Republic of China
Tel: +86-18262636619
Fax: +86-25-57311232
Email: mingyun18748@126.com

Yanhong Gu
Department of Oncology, The First Affiliated Hospital with Nanjing Medical University, 300 Guangzhou Road, Nanjing, Jiangsu 210029, People's Republic of China
Tel: +86-18262643578
Fax: +86-25-57311232
Email: guhong183.com

Purpose: Colon cancer is a common malignant tumor of the digestive system. This project verified the negative role of protein tyrosine phosphatase (SHP-2) in the regulation of colon cancer and further clarified the key targets and molecular mechanisms in the regulation process.

Patients and Methods: The expression levels of SHP-2 in colon cancer tissues, adjacent tissues, normal colon cell lines, and cancer cell lines were detected via Quantitative Real-time PCR (qRT-PCR). The effect of SHP-2 on colon cancer cell function was verified using cell proliferation, Transwell, scratch, and apoptotic assays. CD81 was identified as the interaction protein of SHP-2 by immunoprecipitation.

Results: The expression of SHP-2 was decreased in colorectal cancer compared with that in adjacent tissues. This expression was also decreased in colon cancer cells compared with that in intestinal epithelial cells. In addition, the tumor tissues of patients with metastatic colon cancer exhibited downregulated expression of SHP-2 compared with those of patients with non-metastatic colon cancer. Cell proliferation, Transwell, scratch, and apoptotic assay showed that the overexpression of SHP-2 inhibited proliferation, adhesion, and metastasis of colon cancer cell lines and promoted apoptosis. CO-IP proved that SHP-2 could interact with CD81 and inhibit the function of CD81. Recovery experiments confirmed that the overexpression of CD81 reversed the anti-cancer effect of SHP-2.

Conclusion: Overexpression of SHP-2 inhibited malignant progression of colon cancer. Mechanism experiments showed that the anti-cancer effect of SHP-2 was realized through the interaction with CD81. This study elucidated the molecular mechanism of SHP-2 regulation in colon cancer and provided guidance for the diagnosis and prognosis assessment of colon cancer.

Keywords: SHP-2, CD81, EMT, colorectal cancer

Introduction
Colon cancer is one of the common malignant tumors of the digestive tract.1 It has a serious upward trend worldwide in recent years.2 The occurrence of colon cancer is believed to be a complex process involving multifactor, multistage, and multigene mutation accumulation and interaction.3 The treatment of colon cancer is mainly based on surgery, chemotherapy, biological targeted therapy, and other means. Colon cancer is usually diagnosed in the middle and advanced stages. The short survival time and poor prognosis have seriously threatened human life and health. Exploring relevant biomarkers, making early diagnosis, reasonably evaluating the prognosis,
and doing timely intervention are highly important for the clinical treatment of colon cancer.\textsuperscript{4,5}

Cell growth, mitosis, invasion, adhesion, and apoptosis are closely related to intracellular signal transduction.\textsuperscript{6} SHP-2 belongs to the protein tyrosine phosphatase family, and it is encoded by human \textit{PTPN11} gene. The molecular weight of the protein is 72 kDa. SHP-2 accepts the role of cytokines and extracellular stimuli, and it is widely expressed in various cells and tissues. It is a key regulatory factor in signal transduction. SHP-2 mutation could cause Noonan syndrome,\textsuperscript{7} LEOPARD syndrome,\textsuperscript{8} adolescent granular monocyte leukemia,\textsuperscript{9} and other human diseases. SHP-2 also plays an important role in various human malignancies. For example, SHP-2 plays a cancer-promoting function in breast cancer,\textsuperscript{10} lung cancer,\textsuperscript{11} and gastric cancer.\textsuperscript{12} However, SHP-2 inhibits the occurrence of cancer in liver cancer\textsuperscript{13,14} and colon cancer.\textsuperscript{15,16} Yu et al found that the increased expression of SHP-2 in the cancer cells of patients with colon cancer was associated with enhanced prognosis.\textsuperscript{15,16} A previous study also showed that the expression of SHP-2 protein in colon cancer tissues was significantly lower than that in normal colon tissues. The same results were shown in colon tumor cells and mouse colon tumors. Moreover, the expression of SHP-2 protein was relatively low in colon cancer with poor differentiation, late clinical stage, and high lymph node metastasis.\textsuperscript{16} However, the molecular mechanism of SHP-2 inhibition in colorectal cancer is unclear.

Tetraspanins are highly conserved Tetraspanins in evolution.\textsuperscript{17} They make use of their strong tendency to interact with some surrounding molecules to form a broad molecular interaction network structure, namely, the quaternary transmembrane protein network. CD81 is a four-fold transmembrane protein molecule with multiple biological activities.\textsuperscript{18} It is widely distributed in the organism, and it participates in a large number of physiological responses. CD81 plays an important role in multiple cellular processes; it is closely related to the pathogenesis of many human diseases.\textsuperscript{19} Tetraspanins could act directly or indirectly on cell signaling pathways, thereby affecting the adhesion, differentiation, migration, and invasion of tumor cells. In the present study, the interaction between SHP-2 and CD81 was predicted by protein interaction and verified by CO-IP. The effects of SHP-2 and CD81 on colorectal cancer were further studied.

This study aimed to further confirm the role of SHP-2 in the development of colon cancer at the molecular level. The key targets and main signaling pathways of SHP-2 regulation in colon cancer were explored. This study also aimed to provide updated guidance and effective predictors for the screening, diagnosis, and treatment of colon cancer.

### Patients and Methods

#### Methods

**Collection of Patient Specimens**

The colorectal cancer and adjacent normal intestinal mucosal tissue specimens used in the study were from patients admitted to Nanjing Gaochun People’s Hospital from January 2019 to December 2019. Thirty patients were pathologically diagnosed with colorectal cancer. The tumor tissues and adjacent tissues were rinsed with running water within 30 min. The specimen was cut into 0.5 cm\textsuperscript{3} and immersed in RNAlater (ThermoFisher, USA). The liquid volume was no less than five times the specimen volume. The specimen was transported on ice immediately after immersion and transferred to −80 °C refrigerator for storage within 30 min. The adjacent tissue obtained was a normal intestinal mucosal tissue of more than 5 cm from the tumor tissue. All patients signed informed consent forms. The study was approved by Nanjing Gaochun People’s Hospital.

**Cell Culture and Transfection**

The cell lines used in this study were purchased from American Germplasm Collection Center (ATCC) and cultured in accordance with ATCC’s recommended conditions and methods. SW620 cells were cultured in RPMI-1640 medium. The complete medium was made from each base medium mixed with 10% fetal bovine serum (Gibco, USA). One mL of 0.25% (w/v) trypsin solution was used for passage. The cells were digested at 37 °C for 2–4 min. Digestion was terminated with 10 mL complete medium. The cell suspension was centrifuged at 1500 g. After the supernatant was discarded, the cells were resuspended by adding a complete medium. The cells were inoculated after a blood count plate was used. A Lipofectamine2000 kit (ThermoFisher, USA) was used for culture and proliferation in six-well plates to occupy 60%–70% of the substrate area. SiRNA sequences (Ruibo, China) were dissolved in a basic medium in a 10 ng: 1 μL stem reagent proportion. After 10 min at room temperature, the cells were cultured at 50 μL/well. Plasmid transfection was performed using a Lipofectamine3000 kit (ThermoFisher, USA). In accordance with the instructions, 2 g/L of overexpressed plasmid DNA (Ruibo, China) and 4 μL of stem
reagent were dissolved in a basic medium for 15 min at room temperature. Then, 250 μL was added to each well, and cell culture was performed at 37 °C and 5% CO₂.

### Cell Proliferation Assay
Cells were inoculated in a 96-well cell culture plate at 5000/cm² (Corning, USA). Then, 10 μL of CCK-8 working fluid was added after 3 days of culture. After incubation for 4 hours at 37 °C, the absorbance OD value was measured using a microplate reader (BioTek, USA) at a wavelength of 450 nm.

### Transwell Experiment
Transfected cells were collected and resuspended in a serum-free RPMI-1640 medium. Matrigel was used to precoat the Transwell upper chamber. Then, 5 × 10⁴ cells were added to each well, and 600 μL of complete culture medium was added to the lower chamber. The upper chamber was removed from the incubator for 24 h. The cells were treated with 4% paraformaldehyde and stained with 0.01% crystal violet. The number of transmembrane cells was counted under a 200× optical microscope. Five fields were randomly selected, and the average value was obtained. The experiment was repeated three times.

### Colony Formation Assay
LOVO cells and SW620 cells were seeded into 12-well plates and transfected with si-NC or si-SHP-2, or vector-NC or vector-SHP-2. Then, they were stained with 0.5% crystal violet (Sigma–Aldrich, St. Louis, MO, USA). Snd colony numbers were counted directly.

### Flow Cytometry
The colorectal cancer cells at logarithmic growth stage in each group were digested with 0.25% trypsin. The cells were collected and centrifuged at 1000 r/min for 6 min at room temperature. The supernatant was discarded, and the cells were rinsed with precooled PBS. Then, 500 L of binding buffer was added. Five μL of Annexin V-FITC was also added, and the mixture was blended well. Subsequently, 5 μL of PI was added and the cells were incubated at room temperature in the dark for 10 min. Flow cytometry was used to detect the apoptotic rate.

### CO-IP Experiment
The cells were lysed with RIPA lysis buffer. Then, 50% proteinA/G-agarose working fluid was added with PBS. The horizontal shaker was shaken at 4 °C for 10 min. The cells were centrifuged at 14,000 g at 4 °C for 15 min. The supernatant was transferred to a new centrifuge tube to remove proteinA/G-agarose microspheres. The concentration of the total protein was determined using the BCA method. A primary antibody was then added. The mixture of antigens and antibodies was shaken slowly in a shaker and left overnight at 4 °C. The precipitation was collected via centrifugation at 14,000 g and washed three times with pre-cooled washing buffer. The supernatant was collected for further downstream Western blot analysis.

### Western Blot
Total protein was extracted using conventional methods. The total protein content was determined using the BCA method. The proteins via separated via SDS-PAGE electrophoresis at 30 μg protein per well. Then, they were electro-transferred to PVDF membrane. TBST was used to prepare 5% skimmed milk powder as the sealing solution. The proteins were sealed at room temperature for 1 h and rinsed with TBST three times, 5 min each. Primary antibodies (SHP-2, Abcam, 1:1000; CD81, Abcam, 1:1000) were added, and incubation was performed in a shaking table at 4 °C overnight. The TBST membrane was washed three times. Horseradish peroxidase-labeled antibody (1:1000) was incubated in PBST solution at 37 °C for 1 h. The membrane was washed with TBST solution three times. Finally, an appropriate amount of A and B luminescent substrates were mixed in the centrifuge tube at the same volume for reaction. Protein expression was measured using an exposure meter. The results were analyzed on ImageJ.

### Immunofluorescence
All treated cells were incubated on 18 mm-cover glass for 24 h. After the cells were completely attached to the wall, they were fixed with 4% paraformaldehyde. Nonspecific binding was blocked using 3% BSA. Primary antibodies were added, including SHP-2 (Abcam, 1:100 dilution) and CD81 (Abcam, 1:100 dilution). The cells were incubated at 4 °C overnight. After the cells were washed with PBS, they were incubated with AlexaFluor 488 or AlexaFluor 594 secondary antibody (1:500 dilution). The nuclei were stained with 4,6-diaminindole, and the slides were observed under a FV-1000 laser scanning confocal microscope.

### qRT-PCR
Cell and tissue RNA were collected using TRIZol (Invitrogen, USA), TriPurel Solution Reagent kit (Roche, USA), and GeneJETRNA (Invitrogen, USA). Fluorescence intensity was measured using KAPA SYBR FAST qPCR.
Master Mix (KAPA Biosystems, USA) and CFX96 (wave-guide, USA). The reaction conditions were as follows: 40 cycles of 95 °C for 5 min, 95 °C for 10 s, and 60 °C for 30 s. GAPDH was used as the internal reference: GAPDH forward, 5′-AAGTTCAACGGCACAGTCAA-3′ and reverse, 5′-TACTCAGCACCAGCATCACC-3′. All samples were provided with three multiple holes. The 2^{−ΔΔCT} method was used to calculate the gene expression.

**Statistical Analysis**

SPSS software (version 20.0, SPSS Inc., USA) was used to calculate the results, which were reported as mean ± standard deviation. Comparison between two groups was detected using T test. One-way ANOVA was used for multigroup comparisons. Pearson correlation analysis was used to detect the correlation between two groups of continuous variables. p < 0.05 was considered statistically significant.

**Results**

**Expression of SHP-2 Decreased in Colorectal Cancer**

The expression of SHP-2 in colon cancer and adjacent cancers was measured to investigate the role of SHP-2 in colorectal cancer. The experimental results showed that the SHP-2 in colon cancer was downregulated compared with that in the para-cancer control group (Figure 1A). The expression of SHP-2 in the tumor tissues of patients with metastatic and non-metastatic colon cancer was also analyzed. The results showed that SHP-2 was highly expressed in the tumor tissues of patients with non-metastatic colon cancer (Figure 1B), indicating that the expression of SHP-2 was positively correlated with the prognosis of colorectal cancer. Further, the expression of SHP-2 in intestinal epithelial cells and colon cancer cell lines was examined. The results showed that compared
with the intestinal epithelial cells, the colon cancer cell lines had decreased expression of SHP-2, which was lowest in LOVO and SW620 cells. Therefore, LOVO and SW620 cell lines were selected for subsequent experiments (Figure 1C).

**Overexpression of SHP-2 Inhibited Malignant Evolution of LOVO and SW620 Cells**

Cell proliferation, invasion, migration, and apoptosis were detected after overexpression and knockdown of SHP-2 to further investigate the role of SHP-2. The results showed that the overexpressed plasmid could significantly upregulate the expression of SHP-2, while siRNA could effectively inhibit it (Figure 2A and B). Cell proliferation was measured using cell proliferation assay. The experimental results showed that the overexpression of SHP-2 inhibited the proliferation of LOVO cells compared with the control group, whereas the knockdown of SHP-2 promoted it (Figure 2C). The detection results of SW620 cells were consistent with those of LOVO cells (Figure 2D). The ability of cell invasion was tested using Transwell assay. The experimental results showed that the overexpression of SHP-2 inhibited the invasion of LOVO cells compared with the control group, whereas the knockdown of SHP-2 promoted it. The detection results of SW620 cells were consistent with those of LOVO cells (Figure 2E). Furthermore, the growth of both cells was detected through clone formation experiments. The results showed that the overexpression of SHP-2 inhibited the clone formation of LOVO and SW620 cells compared with the control group, whereas the knockdown of SHP-2 promoted it (Figure 2F). Apoptosis was detected using flow cytometry. The apoptosis of LOVO and SW620 in colon cancer cells was induced by cisplatin (10 M). The experimental results showed that compared with the control group, the overexpression of SHP-2 promoted apoptosis of LOVO cells, whereas the knockdown of SHP-2 reduced it. The detection results of SW620 cells were consistent with those of LOVO cells (Figure 2G).

**SHP-2 Interacts with CD81**

A protein-protein interaction website was used to predict SHP-2 interaction proteins and investigate the molecular mechanism behind SHP-2 inhibiting the malignant progression of colon cancer. A Venn diagram was used to analyze the SHP-2 interaction protein and the poor prognostic gene of rectal cancer (Figure 3A). The experiment showed that 11 proteins may interact with SHP-2, among which CD81 ranked the highest. Further, the interaction between SHP-2 and CD81 was verified by CO-IP experiment. The results showed that the SHP-2 antibody could fish CD81 in LOVO and SW620 cells. SHP-2 similarly was fished from LOVO and SW620 cells by CD81 antibody (Figure 3B).

**Verification of Colocalization of SHP-2 and CD81**

The colocalization of SHP-2 and CD81 in colon cancer cells was verified via immunofluorescence. The experimental results showed that SHP-2 and CD81 had obvious colocalization (Figure 4A). Figure 4B shows the conformation of SHP-2 interacting with CD81 protein. The effect of SHP-2 on the prognosis of colorectal cancer was further verified. The expression of SHP-2 was decreased in colon cancer tissues. Survival analysis showed that patients with colorectal cancer with high expression of SHP-2 had a good prognosis (Figure 4C). The effect of CD81 on this prognosis was similarly analyzed. The expression of CD81 was increased in colon cancer tissues. Survival analysis also showed that patients with colorectal cancer with high expression of CD81 had poor prognosis (Figure 4D).

**SHP-2 Inhibited Expression of CD81 and EMT in Colorectal Cancer**

The effect of SHP-2 on the expression of CD81 was analyzed. The experiments were divided into four groups, namely, vector-NC, SHP-2, si-NC, and si-SHP-2. The experimental results showed that the overexpression of SHP-2 inhibited the expression of CD81 in LOVO cells, whereas the knockdown of SHP-2 upregulated this expression in LOVO cells (Figure 5A). The overexpression of SHP-2 inhibited the expression of CD81 in SW620 cells, while its knockdown upregulated it (Figure 5B). Next, changes in EMT markers were detected. The results showed that the overexpression of SHP-2 upregulated the expression of epithelial marker e-cadherin in LOVO and SW620 cells, whereas its knockdown inhibited it (Figure 5C and D). The detection results of the expression of mesenchymal marker protein Vimentin showed that the overexpression of SHP-2 inhibited the expression of Vimentin compared with that in the control group, whereas this knockdown upregulated it (Figure 5E and F). The expression levels of
EMT-related transcription factors (EMT-TFs), such as Snail1, ZEB1, and Twist1, in the SHP-2-activated/inactivated CRC cells were analyzed to further confirm the relationship between SHP-2 and EMT. Compared with the control group, the overexpression of SHP-2 inhibited the expression levels of Snail1, ZEB1, and Twist1, whereas its knockdown upregulated them (Figure 5G–L). These results showed that SHP-2 inhibited EMT in colorectal cancer by interacting with CD81 and inhibiting the expression of CD81.
Overexpression of CD81 Reverses the Anti-Cancer Effect of SHP-2

The following six groups of experiments were set up to further verify the relationship between SHP-2 and CD81: control, vector-NC-1, CD81, vector-NC-2, SHP-2, and CD81+SHP-2. The expression of CD81 was detected in LOVO cells. Compared with the control group, CD81 overexpressed plasmid could effectively upregulate the expression of CD81, whereas the overexpression of SHP-2 could inhibit this expression. Meanwhile, the overexpression of CD81 could reverse the inhibitory effect of SHP-2 (Figure 6A and B). The results of cell proliferation assay showed that compared with the control group, the overexpression of CD81 upregulated the proliferation of LOVO and SW620 cells, whereas the overexpression of SHP-2 inhibited it. Meanwhile, the overexpression of CD81 could reverse the inhibitory effect of SHP-2 (Figure 6C and D). The results of cell invasion experiments showed that compared with the control group, the overexpression of CD81 upregulated the invasion ability of LOVO and SW620 cells, whereas the overexpression of SHP-2 inhibited it. The overexpression of CD81 could also reverse the inhibitory effect of SHP-2 (Figure 6E and F). Detection of the expression of e-cadherin showed that the overexpression of CD81 inhibited this expression in LOVO and SW620 cells, whereas the overexpression of SHP-2 upregulated it. However, the overexpression of CD81 could reverse the effect of SHP-2 (Figure 6G and H). Compared with the control group, the overexpression of CD81 upregulated the expression of Vimentin in LOVO and SW620 cells, whereas the overexpression of SHP-2 inhibited it. However, the overexpression of CD81 could reverse the effect of SHP-2 (Figure 6I and J).
For further confirmation of the relationship between CD81 and EMT, the expression levels of EMT-TFs, such as Snail1, ZEB1, and Twist1, were analyzed in the CD81-activated and CD81/SHP-2-activated CRC cells. The test results showed that the overexpression of CD81 upregulated the expression levels of Snail1, ZEB1, and Twist1. By contrast, the overexpression of SHP-2 reversed the upregulation of CD81 (Figure S1).
Discussion

The progression of colorectal cancer is extremely complex. During the development of malignant tumors, cells may over-proliferate and apoptosis may be inhibited. This loss of balance may be a factor in tumor development. Therefore, inhibition of tumor cell proliferation and induction of apoptosis are two of the therapeutic approaches.\textsuperscript{20,21}
Figure 6 Overexpression of CD81 reverses the cancer suppressive effect of SHP-2. (A) Detection of CD81 expression in LOVO cells. (B) Detection of CD81 expression in SW620 cells. (C) Detection of LOVO cell proliferation rate. (D) Detection of SW620 cell proliferation rate. (E) Transwell detection of LOVO cells. (F) Transwell detection of SW620 cells. (G) Detection of E-cadherin expression in LOVO cells. (H) Detection of E-cadherin expression in SW620 cells. (I) Detection of Vimentin expression in LOVO cells. (J) Detection of Vimentin expression in SW620 cells. *represents p < 0.05 and ** represents p < 0.01.
SHP-2 is considered to be closely related to cancer; it plays an important role in various cancers. SHP-2 participates in multiple conduction pathways, such as RAS/MAPK pathway, PI3K/AKT pathway, JAK/STAT pathway, JNK pathway, NF- B pathway, RHO pathway, and NF pathay, and plays a key role in the process of information transmission. Lindsey et al. reported that intracellular SHP-2 dephosphorylates HoxA10, thereby inhibiting the transcriptional activity of CYBB and NCF2. Arachiche et al. revealed that SHP-2 is also present in the mitochondria, where it regulates respiratory chains and participates in cell apoptosis. As an oncogene, SHP-2 has been developed rapidly in cancer research. However, some researchers have demonstrated that SHP-2 is an inhibitor in liver cancer. Data have shown that SHP-2 is also an inhibitor in cartilage tissue, indicating that SHP-2 may have tissue specificity, and its multiple functions remain to be revealed. Through previous pioneering studies, this research group found that SHP-2 may play a negative regulatory role in the occurrence and development of colon cancer, but its specific molecular mechanism and mechanism of action are still unclear. The present study was an extension of previous studies conducted to further improve the correlation between SHP-2 and colon cancer by exploring the role and molecular mechanism of SHP-2 in regulating the occurrence and development of colon cancer at the molecular level.

In this study, the expression of SHP-2 in colorectal cancer tissues was detected via qRT-PCR. SHP-2 was found to be negatively correlated with metastasis and positively correlated with prognosis. This result seemed to be contrary to that of previous reports on the role of SHP-2 in promoting tumorigenesis and development in breast cancer and other tumors. However, some recent studies on liver cancer have found that SHP-2 was selectively knocked out in liver cells, and the inflammatory signal caused by SHP-2 deletion promoted the occurrence of liver cancer. The risk of hepatocarcinoma induced by diethylnitrosamine and spontaneous tumor in mice significantly increased. The RNA and protein expression levels of SHP-2 in human liver cancer samples were significantly higher than those in adjacent normal tissues. Survival analysis showed that patients with HCC with low expression of SHP-2 had a relatively short survival period. Therefore, the expression of SHP-2 in tumors may have complex regulatory mechanisms. It plays different roles in different tumors and has tissue specificity. However, more studies are needed to elucidate the mechanism. The decreased expression of SHP-2 in colon cancer may be related to the specific tumor microenvironment of the colon or intestinal bacteria.

CD81 is closely related to tumor invasion and metastasis. The expression of CD81 in esophageal carcinoma cells was significantly higher than that in normal esophageal epithelial cells. By interfering with the expression of CD81 in esophageal cancer cells, the invasion ability of cancer cells was downregulated and the expression of E-cadherin was upregulated. This finding suggested that high expression of CD81 may promote tumorigenesis. In the present study, the interaction between SHP-2 and CD81 was confirmed using protein interaction prediction and CO-IP experiment. Further studies have shown that CD81 could promote tumor EMT, while SHP-2 could inhibit the expression of CD81 through interaction, thereby reducing the malignant evolution of colorectal cancer.

**Conclusion**

On the basis of the finding that SHP-2 is negatively correlated with the occurrence, development, and prognosis of colon cancer, this study further verified the mechanism behind the negative regulation of colon cancer by SHP-2. The results showed that SHP-2 could interact with CD81, thereby reducing the function of CD81 and inhibiting tumor EMT. This study further elucidated the key targets and main signaling pathways of SHP-2 in regulating the malignant evolution of colorectal cancer and its biological significance for the treatment of colon cancer.

**Disclosure**

The authors report no conflicts of interest in this work.

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