

# hcrn81 promotes cell proliferation through Wnt signaling pathway in colorectal cancer

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**Abstract** The objective of the study was to investigate the role of hcrn81 gene in Wnt/ $\beta$ -catenin signaling pathway related to human colorectal cancer. A total of 30 pairs of human colorectal cancer tissues with control normal tissues were analyzed by qRT-PCR. The proliferation, apoptosis, cell cycle, cell colony and metastasis of LS174T<sup>-hcrn81</sup>, HCT116<sup>-hcrn81</sup>, LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells were tested, of which hcrn81 was knockdown in LS174T, HCT116 cells and hcrn81 was overexpressed in LoVo, SMMC-7721 cells. Besides, the mRNA and protein levels of hcrn81,  $\beta$ -catenin, c-Myc, cyclinD1, GSK-3 $\beta$  and survivin in colon cancer cell lines were evaluated by qRT-PCR and western blot. The mRNA levels of  $\beta$ -catenin and Survivin were up-regulated in 76.7 % (23/30) and 63.3 % (19/30) of the tumor samples, respectively. hcrn81 and GSK-3 $\beta$  mRNA expression levels were down-regulated in 20/30 (66.7 %) and 21/30 (70.0 %) of the tumor samples as compared to the adjacent normal tissues, respectively. Furthermore, in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells, the mRNA and protein levels of  $\beta$ -catenin, c-Myc, cyclinD1 and Survivin were up-regulated, whereas those of GSK-3 were down-regulated. In LS174T<sup>-hcrn81</sup> and HCT116<sup>-hcrn81</sup> cells, the mRNA levels of  $\beta$ -catenin, c-Myc, cyclinD1 and Survivin were down-regulated, whereas GSK-3 $\beta$ mRNA was up-regulated. Cell proliferation in LoVo<sup>+hcrn81</sup> and SMMC-

7721<sup>+hcrn81</sup> groups was significantly enhanced ( $P < 0.05$ ). Proliferation index in both LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> groups was significantly higher than that in the control groups ( $P < 0.05$ ). The number of colony in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells were significantly higher than that in the control groups ( $P < 0.05$ ). In addition, the percentage of apoptotic cells in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> groups were significantly lower than that in the control groups ( $P < 0.01$ ,  $P < 0.01$ ). Finally, the number of migrating cells was significantly higher in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> groups than that in the control group ( $P < 0.05$ ). hcrn81 might promote carcinogenesis and progression through regulation of the Wnt/ $\beta$ -catenin signaling pathway and plays an important role in the carcinogenesis of colorectal cancer.

**Keywords** hcrn81 · Proliferation · Wnt signaling pathway · Colorectal cancer

## Introduction

Colorectal adenocarcinoma is one of the second most common malignancies, and its prevalence has increased recently. With the third highest incidence among all cancers, it is also the common cause of cancer-related mortality worldwide [1, 2]. The 5-year survival for poorly differentiated colorectal adenocarcinoma is only 29 % [3]. Currently, at least 100 genes have been reported to be correlated with the invasion and metastasis of colorectal cancer, and the foundation for colorectal cancer prevention and treatment have been established based on these findings [4, 5]. Current studies have revealed the association between tumorigenesis and inactivation of tumor suppressor genes, and the deletion or mutation of oncogenes.

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Knockdown or overexpression of some genes closely related to cancer might be an alternative approach to decrease cancer occurrence rates [6, 7]. The carcinogenesis of colorectal adenocarcinoma is a multistep process that involves a great number of genomic alterations and multiple signal pathways. Therefore, investigating the molecular mechanisms might lay the foundation for cancer prevention, early diagnosis and effective treatments.

In our previous studies, we have identified 86 differentially expressed sequences from human colorectal adenocarcinoma tissues by cDNA subtractive library and cDNA microarray. Among these newly identified differentially expressed taqs, *hcrn81* mRNA level was down-regulated in 83 % (25/30) of the patients with colorectal adenocarcinoma [8]. Rapamycin treatment induced *hcrn81* up-regulation in SW480 and LoVo cells on both the mRNA and protein levels [9]. The full-length cDNA of *hcrn81* is composed of 4283-bp nucleotides and codes HCRCN81 protein, which belongs to the UPF0561 family. Until now, the function of the UPF0561 family has not been characterized. But potential NLS and NES from the protein sequence of HCRCN81 and the domain of EF-hand indicate that HCRCN81 could function as a cell cycle regulator in the nucleus, which might explain its possible role in colorectal adenocarcinoma pathogenesis [10, 11].

The Wnt signaling pathway is an ancient system highly conserved during evolution. Change in the Wnt pathway might induce diseases or cause most malignant tumors. The Wnt/ $\beta$ -catenin pathway plays a vital role in the regulation of intestinal epithelial cells and is aberrantly activated in colon neoplasms. However, the role of HCRCN81 in the development of colorectal cancer and the underlying mechanisms remained unknown. To verify and elaborate the function of *hcrn81* in Wnt signaling pathway and mechanism for human colorectal adenocarcinoma, human cancer tissue samples and cancer cells were examined.

## Materials and methods

### Cell culture

LoVo, SMCC7721, HCT116 and LS174T cell lines (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) containing 10 % fetal bovine serum (FBS; HyClone), penicillin (100 U/mL), and streptomycin (0.1 mg/mL) in a humidity controlled 37 °C incubator with 5 % CO<sub>2</sub> atmosphere. Culture medium was replaced to keep cells in monolayer. Cells were passaged using 0.25 % trypsin at 70–80 % confluency. Cells at the logarithmic growth phase were used for experiments.

### Collection of tissue samples

Colorectal adenocarcinoma tissues and corresponding adjacent normal tissues (>5 cm away from the edge of the tumor) were obtained from 90 patients at the West China Hospital of Sichuan University, Chengdu, China. The sample acquisition was approved by the Medical Research Ethics Committee of Sichuan University. Written informed consent was obtained from all patients. The collected normal tissue was confirmed by pathological examination. Samples were snap-frozen and stored in liquid nitrogen until subsequent analysis.

### Construction of pcDNA3.1-*hcrn81* vector and cell transfection

*hcrn81* gene was synthesized and inserted into the XhoI/EcoRI site of pcDNA3.1 eukaryotic expression vector (GenePharma Company, Shanghai, China). The engineered pcDNA3.1-*hcrn81* eukaryotic expression vector was verified by restriction digestion followed by sequencing (Beijing Genomic Institute, Beijing, China). LoVo and SMMC-7721 cell lines at  $3 \times 10^5$  cells/well in a six-well plate were transfected with 1  $\mu$ g of pcDNA3.1-*hcrn81* vector or negative control (NC) vector using Lipofectamine 2000<sup>TM</sup> (Invitrogen Life Technologies, Carlsbad, CA, USA). Transfection medium was removed after 6 h and replaced with fresh growth medium. After 48 h, transfected LoVo and SMMC-7721 cells were treated with 600  $\mu$ g/mL G418 (Sigma, St. Louis, MO, USA). After 14 days, monoclonal cells were cultured in the presence of 300  $\mu$ g/mL G418.

### Establishment of HCT116<sup>-*hcrn81*</sup> and LS174T<sup>-*hcrn81*</sup> cell lines by RNAi

siRNA for *hcrn81* was designed and synthesized by RiboBio (Guangzhou, China). Cells were transfected with 100 nM siRNA to induce the knockdown of *hcrn81* expression. Cells were transiently transfected with a pool of six siRNA duplexes targeting *hcrn81*. HCT116 and LS174T cells were seeded at a density of  $2.3 \times 10^6$  cells/well in a six-well plate and transfected with *hcrn81* siRNA or control oligonucleotides using Lipofectamine<sup>TM</sup> 2000. Transfection medium was removed 6 h later and replaced with fresh growth medium. The transfected cells were collected at 48 h for qRT-PCR to detect mRNA expression level.

### RNA extraction and qRT-PCR

Total RNA from the tumor and corresponding adjacent normal tissues was isolated using the TRIzol RNA isolation

reagent (Invitrogen Life Technology, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA yield was determined by absorbance at 260 nm using a spectrophotometer. The quality of RNA products was confirmed by the sharp bands representing 28S and 18S rRNA molecules and the intensity ratio of 2:1 of these two bands (28S:18S) on 1 % agarose gel. cDNA was synthesized using the M-MuLV Reverse Transcriptase kit (Fermentas, Waltham, MA, USA) and stored at  $-20\text{ }^{\circ}\text{C}$ , and qPCR was performed using SYBR Premix Ex Taq (Takara Bio Inc, Shiga, Japan) according to the manufacturer’s instructions and associated international standards [7, 8]. qPCR primers used to amplify *hcrn81*,  $\beta$ -catenin, GSK-3 $\beta$  and Survivin genes are shown in Table 1. The mean threshold cycle ( $C_t$ ) and standard error were calculated from individual  $C_t$  values obtained from three replicates per specimen. The normalized mean  $C_t$  was calculated as  $\Delta C_t$  by subtracting the mean  $C_t$  of GAPDH from the target genes.  $\Delta\Delta C_t$  was calculated as the difference between the control  $\Delta C_t$  and the values obtained for each sample. The  $n$ -fold change in gene expression relative to the untreated controls was calculated using the  $2^{-\Delta\Delta C_t}$  method.

**Western blot analysis**

Cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer’s instructions. The total protein concentration was determined using an Enhanced BCA Protein assay kit (Beyotime Institute of Biotechnology, USA). Thirty micrograms of the denatured protein was separated on 12 % SDS-PAGE and then transferred to PVDF membrane (Bio-Rad, USA). After blocking for 3 h in Tris-buffered saline containing 0.1 % Tween-20 and 3 % BSA, the membrane was incubated overnight at

$4\text{ }^{\circ}\text{C}$  with primary antibodies for HCRCN81,  $\beta$ -catenin, GSK-3 $\beta$  and Survivin. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies at  $37\text{ }^{\circ}\text{C}$  for 2 h, followed by ECL western blotting reagent (Thermo Scientific, USA) to visualize the proteins. All antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).  $\beta$ -actin was used as the loading control.

**MTT assay**

MTT assay was performed on LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells. Cells were seeded into 96-well culture plates at a density of  $2 \times 10^3$  cells/well. Each group contained six replicates. 20  $\mu\text{L}$  of MTT reagent (Sigma, St. Louis, MO, USA) was added at 0, 12, 24, 48 and 60 h after cell seeding. Cells were cultured for an additional 4 h, the medium was removed, and 150  $\mu\text{L}$  of DMSO was added into each well. Cells were incubated for another 15 min. The plate was read at the absorbance wavelength of 490 nm on a microplate reader (Bio-Rad, USA). The experiment was repeated three times.

**Flow cytometry**

LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells were analyzed by flow cytometry on BD FACSAria II cell sorter to determine cell apoptosis and cell cycle. Cells were cultured in six-well culture plates at a cell density of  $3 \times 10^5$ /well. To determine cell apoptosis, cells were cultured at  $-20\text{ }^{\circ}\text{C}$  for 48 h, washed twice with PBS and centrifuged at 2000 rpm,  $4\text{ }^{\circ}\text{C}$  for 5 min. Cells were suspended in 400  $\mu\text{L}$  of binding buffer (KeyGEN Biotech, Nanjing, Jiangsu, China), and 5  $\mu\text{L}$  of Annexin V-FITC was added into cells followed by gentle agitation. The mixture was incubated at room temperature for another 10 min. A total of 10  $\mu\text{L}$  of

**Table 1** qRT-PCR primers

Name	Primer sequences	Product location	Product length (bp)
hcrn81	Forward: 5'-acgcaaccagactatgaagag-3'	315–336	169
	Reverse: 3'-tccttcactcactctccac-5'	462–483	
gapdh	Forward: 5'-ggaaggtgaaggcggagt-3'	107–125	117
	Reverse: 3'-ctgggaaagtaactggagt-5'	223–205	
$\beta$ -catenin	Forward: 5'-gctggtgacaggaagacatc-3'	1628–1648	116
	Reverse: 3'-ggtagtcctatggaaggcgaac-5'	1743–1721	
c-Myc	Forward: 5'-tcaagaggcgaacacacaac-3'	1631–1550	110
	Reverse: 3'-ggcctttcattgtttcca-5'	1740–1721	
cyclinD1	Forward: 5'-gtggcctctaagatgaaggaga-3'	534–555	169
	Reverse: 3'-ggaagtgtcaatgaaatcgtg-5'	702–681	
GSK-3 $\beta$	Forward: 5'-cacagaaccttgctggat-3'	1536–1553	391
	Reverse: 3'-agagccatgatgccctgg-5'	1638–1657	
Survivin	Forward: 5'-tcaagaccaccgatctcta-3'	78–98	156
	Reverse: 3'-aagaagacgaagttcctcgacc-5'	209–230	

PI (20 ug/ml) was added into the mixture, and the cells were incubated at 4 °C for 30 min. Cell apoptosis was then measured by flow cytometry. The experiment was repeated three times. For the cell cycle assay, cells were collected 48 h after culture, washed twice using ice-cold PBS and then fixed in 75 % ice-cold ethanol at −20 °C for 48 h. Cell cycle assay was performed using the Cell Cycle Detection kit (KeyGEN Biotech, Nanjing, Jiangsu, China). The experiment was repeated three times.

### Cell colony formation assay

LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells were cultured in six-well plates until they reached  $2 \times 10^2$  cells/well. Medium was removed and replaced with medium containing FBS for 15 days. Cells were washed three times with PBS and dehumidificated in the air. Cells were then fixed with methanol for 15 min and stained with Giemsa for 15 min. Colony numbers were counted under the microscope (200×). Five random fields were selected for each well to determine total number of colonies. The experiment was repeated three times.

### Cell migration assay

LoVo and SMMC-7721 cells were seeded into the upper chamber of a transwell (Millipore, Billerica, MA, USA) at a cell density of  $2 \times 10^5$ /well in DMEM containing no FBS. Medium containing 10 % FBS was placed in the lower chamber to act as a chemoattractant. After 48 h, non-migratory cells remained on the upper chambers were removed by scraping with a cotton swab. Cells migrating through the 8.0 μm polycarbonate membrane to the lower surface were stained with 0.1 % crystal violet. Migrated cells were quantified by counting stained cells under a microscope (200×). Five random fields were selected for each well to determine total number of migrated cells. The assay was performed in triplicate and repeated three times.

### Statistical analysis

Data were presented as the mean ± SD of three or more replicates. Statistical analysis was performed using Student's *t* test, Fisher's exact probability test, Chi-square test or ANOVA with SPSS19.0 software (SPSS Inc., Chicago, IL, USA). The tumor volume was analyzed with one-way ANOVA and independent sample *t* test. The association between hcrn81 high expression and clinicopathological feature was assessed using either Fisher's exact test or Chi-square test. A backward stepwise multivariate logistic regression analysis was carried out to investigate the most significant variables related to hcrn81 overexpression after adjustment.  $P < 0.05$  was considered statistically significant.

## Results

### mRNA expression levels of hcrn81, β-catenin, GSK-3β and Survivin in human colorectal adenocarcinoma tissues

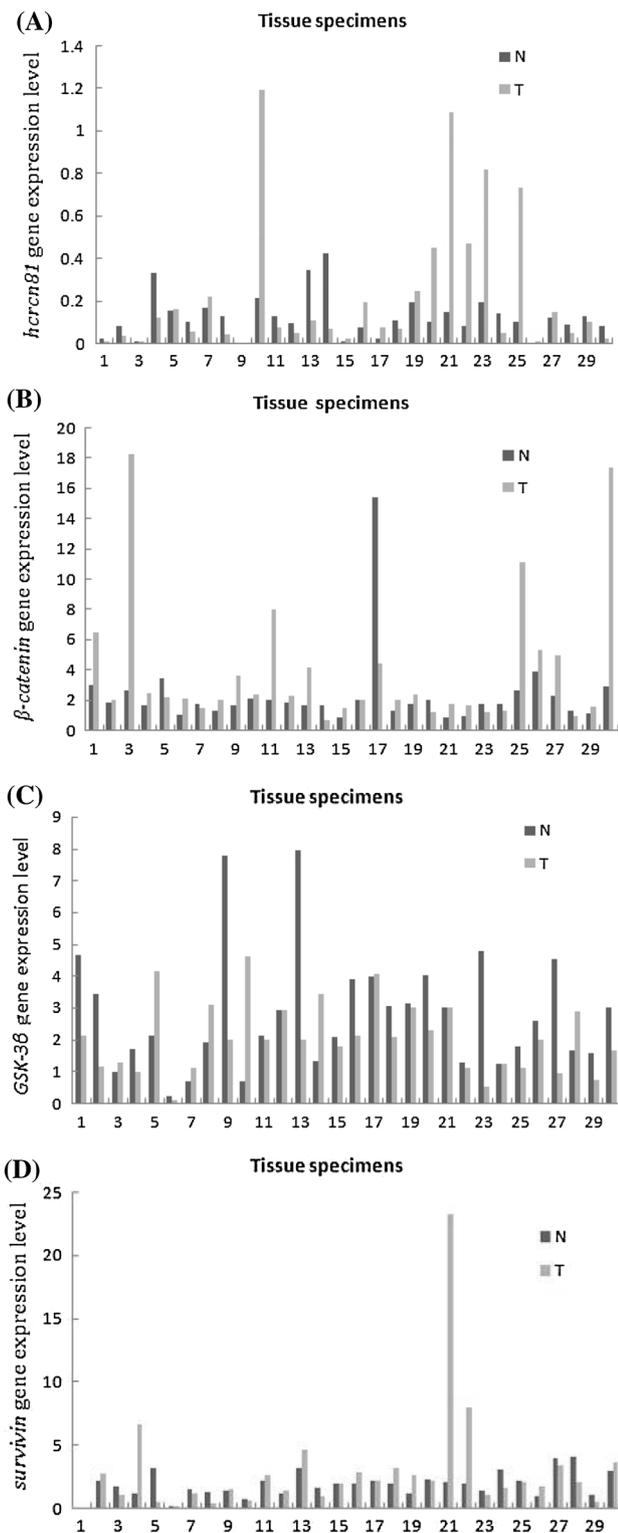
Compared to paired adjacent normal tissues, mRNA level of β-catenin was significantly up-regulated by 76.7 % (23/30) of tumor samples (Fig. 1a,  $P < 0.005$ ). Meanwhile, Survivin was found to be markedly increased by 63.3 % (19/30) of the tumor samples (Fig. 1b). However, hcrn81 and GSK-3β mRNA levels were down-regulated by 66.7 % (19/30) and 63.3 % (20/30), respectively, of the tumor samples (Fig. 1c, d). No significant correlation was found between hcrn81 mRNA expression and gender, Dukes' stage or differentiation degree (all  $P > 0.05$ , Table 2).

### mRNA expression levels of hcrn81, β-catenin, GSK-3β, c-Myc, cyclinD1 and Survivin in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells

The mRNA expression level of hcrn81 in LoVo<sup>+hcrn81</sup> cells was significantly higher than that in LoVo or LoVo<sup>-NC</sup> cells ( $P < 0.05$ ), i.e., the increase by 3.15-fold. hcrn81 mRNA expression level in SMMC-7721<sup>+hcrn81</sup> cells was significantly higher than that in SMMC-7721 or SMMC-7721<sup>-NC</sup> cells ( $P < 0.05$ , Fig. 2). The mRNA expression levels of β-catenin, c-Myc, cyclinD1 and Survivin were significantly up-regulated in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells compared to those in the corresponding control cells (all  $P < 0.05$ ). There was a 1.259-, 1.186-, 1.674- and 1.440-fold increase in β-catenin, c-Myc, cyclinD1 and Survivin, respectively, for LoVo<sup>+hcrn81</sup> cells, and a 1.725-, 2.64-, 1.615- and 1.687-fold increase for SMMC-7721<sup>+hcrn81</sup> cells. In contrast, the mRNA expression level of GSK-3β was decreased in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells by 1.344- and 1.577-fold, respectively.

### mRNA expression levels of hcrn81, β-catenin, GSK-3β, c-Myc, cyclinD1 and Survivin in HCT116<sup>-hcrn81</sup> and LS174T<sup>-hcrn81</sup> cells

The mRNA expression levels of hcrn81 in HCT 116<sup>-hcrn81</sup> and LS174T<sup>-hcrn81</sup> cells were significantly lower than those in control cells ( $P < 0.05$ ). The decrease was 70.29 and 70.81 %, respectively. Compared with control cells, the mRNA expression levels of β-catenin, c-Myc, cyclinD1 and Survivin were significantly reduced in HCT116 and LS174T hcrn81 knockdown cell lines ( $P < 0.05$ , Fig. 3). In HCT116 cells, the decrease was 66.5, 43.32, 54.5 and 17.9 %, respectively, for β-catenin, c-Myc,



**Fig. 1** Quantitative real-time polymerase chain reaction analysis of hcrn81 (a),  $\beta$ -catenin (b), GSK-3 $\beta$  (c) and Survivin (d) mRNA expression in human colorectal adenocarcinoma samples (T) and corresponding adjacent normal samples (N). GAPDH was used as a loading control

cyclinD1 and Survivin. The mRNA expression levels of GSK-3 $\beta$  increased to 161.8 %. In LS174T cells, the decrease was 50.4, 39.6, 32.8 and 26.4 %, respectively, for  $\beta$ -catenin, c-Myc, cyclinD1 and Survivin. The mRNA expression levels of GSK-3 $\beta$  increased to 156.8 %. Spearman correlation analysis showed that hcrn81 was negatively correlated with GSK-3 $\beta$  with a correlation coefficient of  $-0.413$  ( $P = 0.018$ ). hcrn81 was positively correlated with  $\beta$ -catenin and c-Myc with the correlation coefficients of  $0.860$  ( $P = 0.000$ ) and  $0.643$  ( $P = 0.024$ ), respectively. Survivin was positively correlated with c-Myc and cyclinD1, and the correlation coefficients were  $0.643$  and  $0.706$  ( $P = 0.0248$ ,  $P = 0.010$ ) (Table 3).

### Protein expression levels of HCRCN81, $\beta$ -catenin, GSK-3 $\beta$ and Survivin in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells

As shown in Fig. 4, overexpression of HCRCN81 induced a significant increase in the expression of Survivin and  $\beta$ -catenin in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells, compared with that in LoVo and SMMC-7721 cells (all  $P < 0.05$ , Fig. 4). The increase of HCRCN81, Survivin and  $\beta$ -catenin proteins were 80.17, 55.6 and 33.27 % in LoVo cells and 105.82, 42.21 and 41.68 % in SMMC-7721 cells, respectively. In contrast, overexpression of HCRCN81 induced a significant decrease in the expression of GSK-3 $\beta$  (24.47 % for LoVo cells and 34.36 % for SMMC-7721 cells,  $P < 0.05$ ).

### Cell proliferation

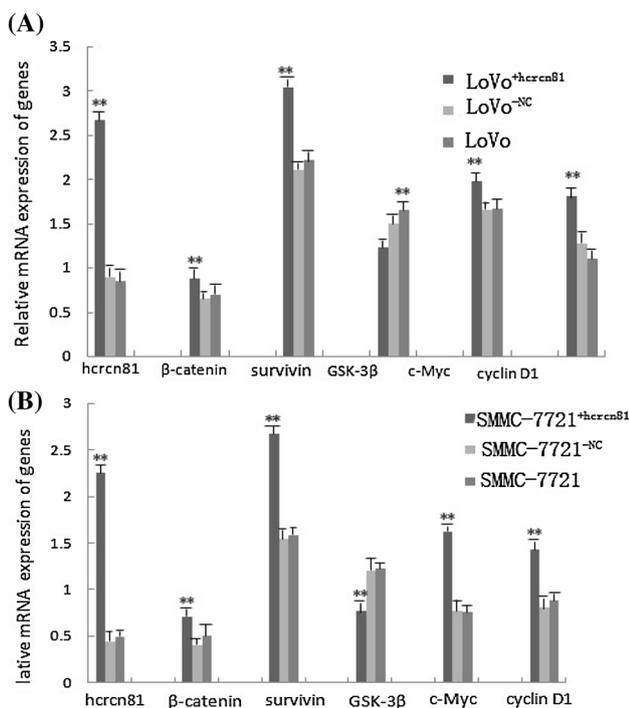
As shown in Fig. 5, cell proliferation was significantly increased in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells compared to their corresponding controls ( $P < 0.05$ ). There was an increase of 83.4 and 35.1 % at 24 h, respectively, in the proliferation rate of LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells.

### Cell apoptosis

As shown in Fig. 6, the percentage of apoptotic cells was significantly higher in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells than in their corresponding controls ( $P < 0.01$ ). The percentage of apoptotic cells were  $6.3 \pm 0.42$ ,  $13.1 \pm 0.22$  and  $12.7 \pm 0.32$  %, respectively, in LoVo<sup>+hcrn81</sup>, LoVo<sup>-NC</sup> and LoVo cells; and  $7.5 \pm 0.43$ ,  $12.9 \pm 0.19$  and  $11.3 \pm 0.64$  %, respectively, in SMMC-7721<sup>+hcrn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells.

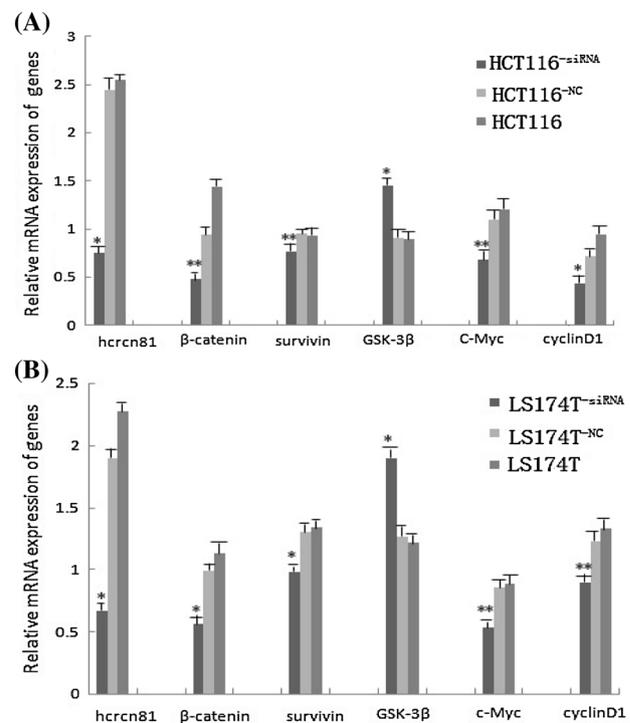
**Table 2** Expression of hcrn81,  $\beta$ -catenin, GSK-3 $\beta$  and Survivin and patient characteristics

	Expression		Gender		Age Dukes' stage				Differentiation degree		
	Male	Female	$\geq 50$	$< 50$	A	B	C	D	Well	Moderately	Poor
Up-regulation											
hcrn81	8	2	4	1	0	2	3	0	0	3	1
$\beta$ -catenin	14	8	18	2	0	9	9	0	0	19	1
GSK-3 $\beta$	15	6	20	1	0	10	7	0	1	17	2
Survivin	13	6	19	2	0	11	8	0	0	18	3
Down-regulation											
hcrn81	13	7	24	1	1	15	9	0	1	22	3
$\beta$ -catenin	7	1	10	0	1	6	3	0	1	6	3
GSK-3 $\beta$	6	3	8	1	1	7	5	0	0	8	2
Survivin	8	3	9	0	1	6	4	0	1	7	1

**Fig. 2** Overexpression of hcrn81 affected the expression of other genes in colorectal cells. hcrn81 overexpression caused an increase in  $\beta$ -catenin, c-Myc, cyclinD1 and Survivin mRNA expression and a decrease in GSK-3 $\beta$  mRNA expression in LoVo cells (a) and SMMC-7721 cells (b). \* $P < 0.05$  and \*\* $P < 0.01$ 

### Cell cycle assays

As shown in Fig. 7a, the proliferation index [calculated as  $(G2M + S)/(G0G1 + S + G2M) \%$ ] in LoVo<sup>+hcrn81</sup> cells was 56.4 %, which was significantly higher than that in the control groups (49.1 % for LoVo cells and 45.6 % for LoVo<sup>-NC</sup> cells). The difference between LoVo<sup>+hcrn81</sup> and LoVo cells was statistically significant ( $P < 0.01$ ). The proliferation index of SMMC-7721<sup>+hcrn81</sup>, SMMC-

**Fig. 3** Knockdown of hcrn81 affected the expression of indicated genes in colorectal cells. hcrn81 knockdown reduced  $\beta$ -catenin, c-Myc, cyclinD1 and Survivin expression but increased GSK-3 $\beta$  expression in HCT116 cells (a) and LS174T cells (b). \* $P < 0.05$  and \*\* $P < 0.01$ 

7721<sup>-NC</sup> and SMMC-7721 cells was 53.3, 43.2 and 39.9 %, respectively (Fig. 7b). The difference between SMMC7721<sup>+hcrn81</sup> and SMMC-7721 cells was statistically significant ( $P < 0.05$ , Fig. 7c).

### Cell colony assay

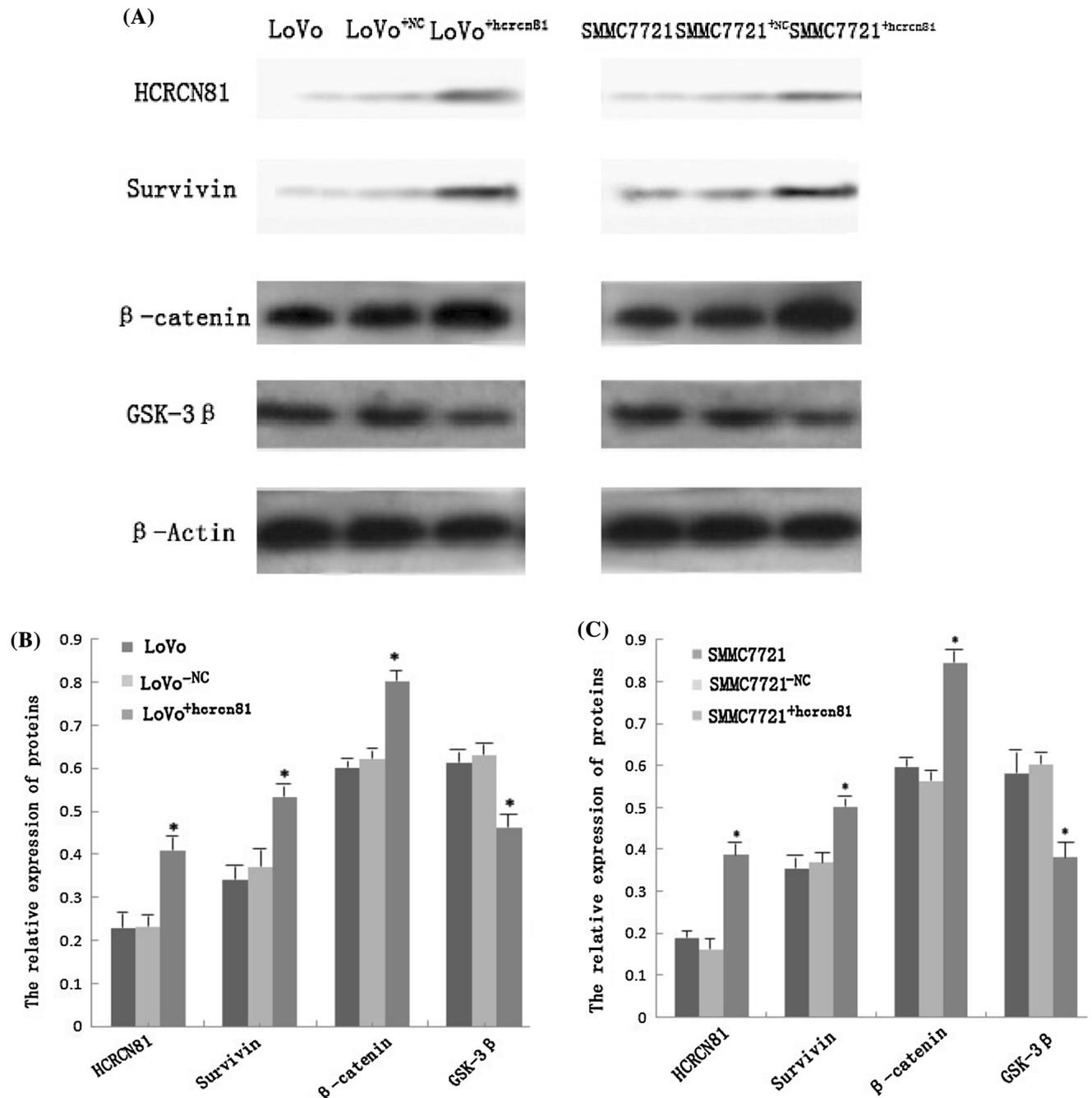
As shown in Fig. 8, the number of colonies was significantly higher in LoVo<sup>+hcrn81</sup> cells than that in the control

**Table 3** Spearman correlation analysis of hcrn81,  $\beta$ -catenin, GSK-3 $\beta$ , Survivin, c-Myc and cyclinD1

	$\beta$ -catenin	Survivin	GSK-3 $\beta$	c-Myc	cyclinD1
Correlation coefficient	0.860**	0.63	-0.413*	0.643*	0.513
<i>P</i>	0.000	0.846	0.018	0.024	0.075

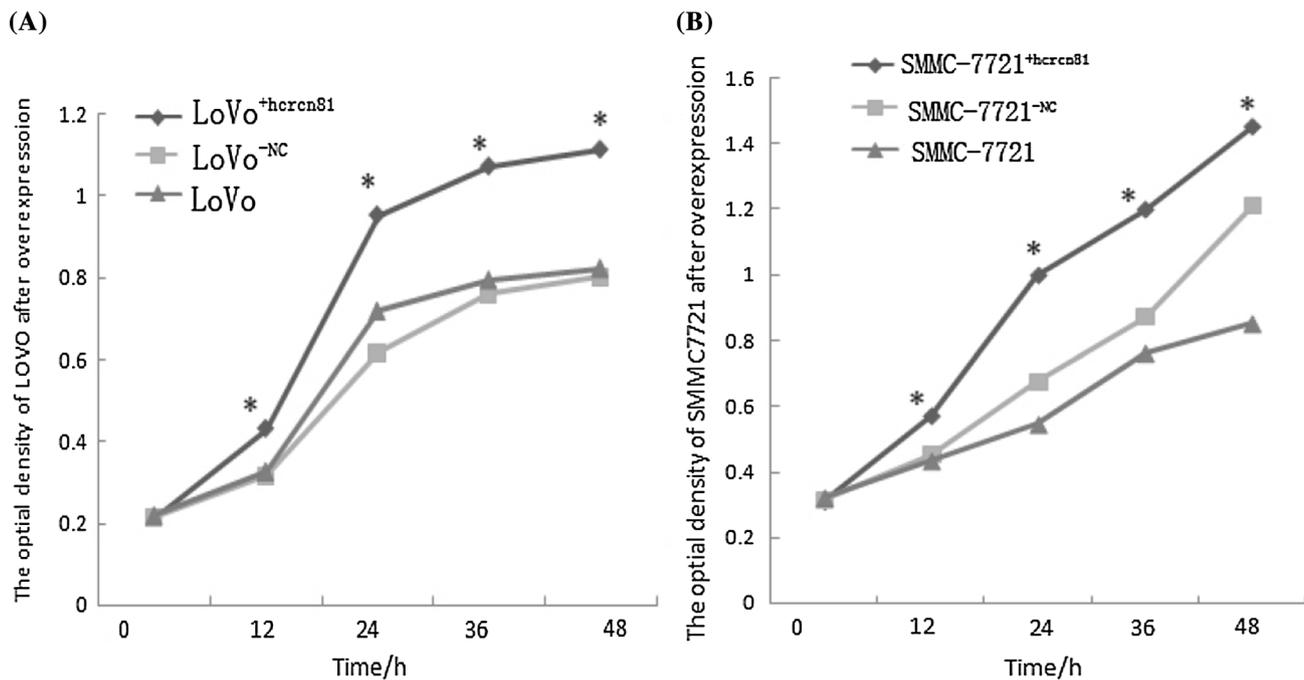
\* *P* < 0.05 was considered statistically significant

\*\* *P* < 0.01 was considered statistically highly significant



**Fig. 4** Overexpression of hcrn81 affected the expression of indicated proteins in LoVo and SMMC-7721 cells. Representative western blot results showing that hcrn81 overexpression increased

the protein expression levels of  $\beta$ -catenin and Survivin but reduced GSK-3 $\beta$  protein expression (a). Quantification of western blot results (b, c). \**P* < 0.05 and \*\**P* < 0.01



**Fig. 5** Growth increase in human colorectal cell lines overexpressing hcrn81. Cell proliferation curves of LoVo<sup>+hcrn81</sup>, LoVo<sup>-NC</sup> and LoVo cells (a). Cell proliferation curves of SMMC-7721<sup>+hcrn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells (b). \* $P < 0.05$  and \*\* $P < 0.01$

cells ( $P < 0.05$ ). The number of colonies in LoVo<sup>-hcrn81</sup>, LoVo<sup>-NC</sup> and LoVo cells was  $131 \pm 9$ ,  $105 \pm 4$  and  $96 \pm 2$ , respectively. The difference between LoVo<sup>+hcrn81</sup> and LoVo cells was statistically significant ( $P < 0.01$ , Fig. 8a). The number of colonies in SMMC-7721<sup>+hcrn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells was  $124 \pm 3$ ,  $90 \pm 7$  and  $97 \pm 4$ , respectively (Fig. 8b). The difference between SMMC7721<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells was statistically significant ( $P < 0.05$ , Fig. 8c).

### Cell migration assay

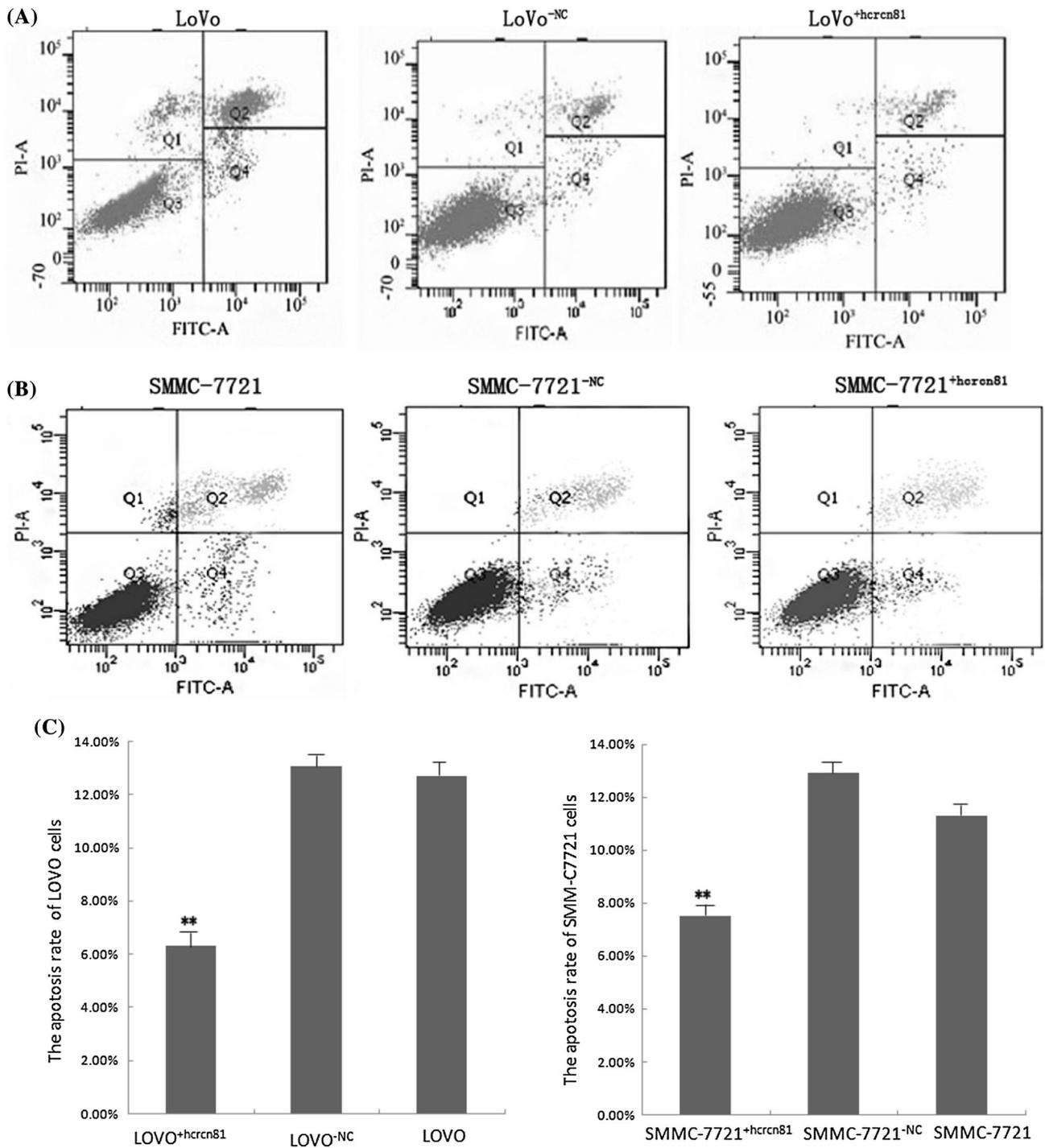
The invasive ability was evaluated by counting the number of migrating cells through the polycarbonate membrane of the Transwell invasion chamber, and migrating cells number was  $66 \pm 8$ ,  $65 \pm 7$  and  $95 \pm 1$  in LoVo groups and  $54 \pm 3$ ,  $57 \pm 6$  and  $86 \pm 2$  in SMMC-7721 groups (Fig. 7). The number of migrating cells was significantly higher in cells overexpressing hcrn81 than that in control cells (Fig. 9).

### Discussion

In this study, recombinant plasmid pcDNA3.1-hcrn81 was established and transfected into LoVo and SMMC-7721 cells. siRNA targeting hcrn81 gene was transfected into LS174T and HCT116 cells [12]. We found that cell proliferation rate was increased in LoVo<sup>+hcrn81</sup> and SMMC-

7721<sup>+hcrn81</sup> cells, but decreased in LS174T<sup>-hcrn81</sup> and HCT116<sup>-hcrn81</sup> cells. Cell apoptosis was inhibited in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells, but was enhanced in LS174T<sup>-hcrn81</sup> and HCT116<sup>-hcrn81</sup> cells. In addition, there were more cell colonies in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> groups as compared to controls. These results suggested that hcrn81 might stimulate the proliferation of cancer cells and block their apoptosis. Previous studies have shown that hcrn81 expression could be up-regulated by rapamycin treatment via mTOR signaling pathway to block cell cycle progression and inhibit metastasis in cancer cells [9]. Our data indicated that hcrn81 was involved in promoting the growth of LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells by regulating cell entry into G1/G0 phase, consistent with other studies [12]. Therefore, hcrn81 might play a vital role in colon cancer onset and progression. The molecular mechanism by which HCRCN81 regulates cancer development is yet to be investigated.

The Wnt signal transduction pathway plays an important role in controlling embryo development and cancer genesis. Previous studies have shown that over 90 % of colon cancers and a high percentage of other cancers originate from activating mutations in the Wnt signaling pathway [13]. Inhibition of the Wnt pathway can inhibit proliferation and induce differentiation [14, 15]. In our study, the increase of hcrn81 expression led to the increase of  $\beta$ -catenin, Survivin, cyclinD1 and c-Myc, but the decrease of GSK-3 $\beta$  in cancer cells.  $\beta$ -catenin mRNA and protein

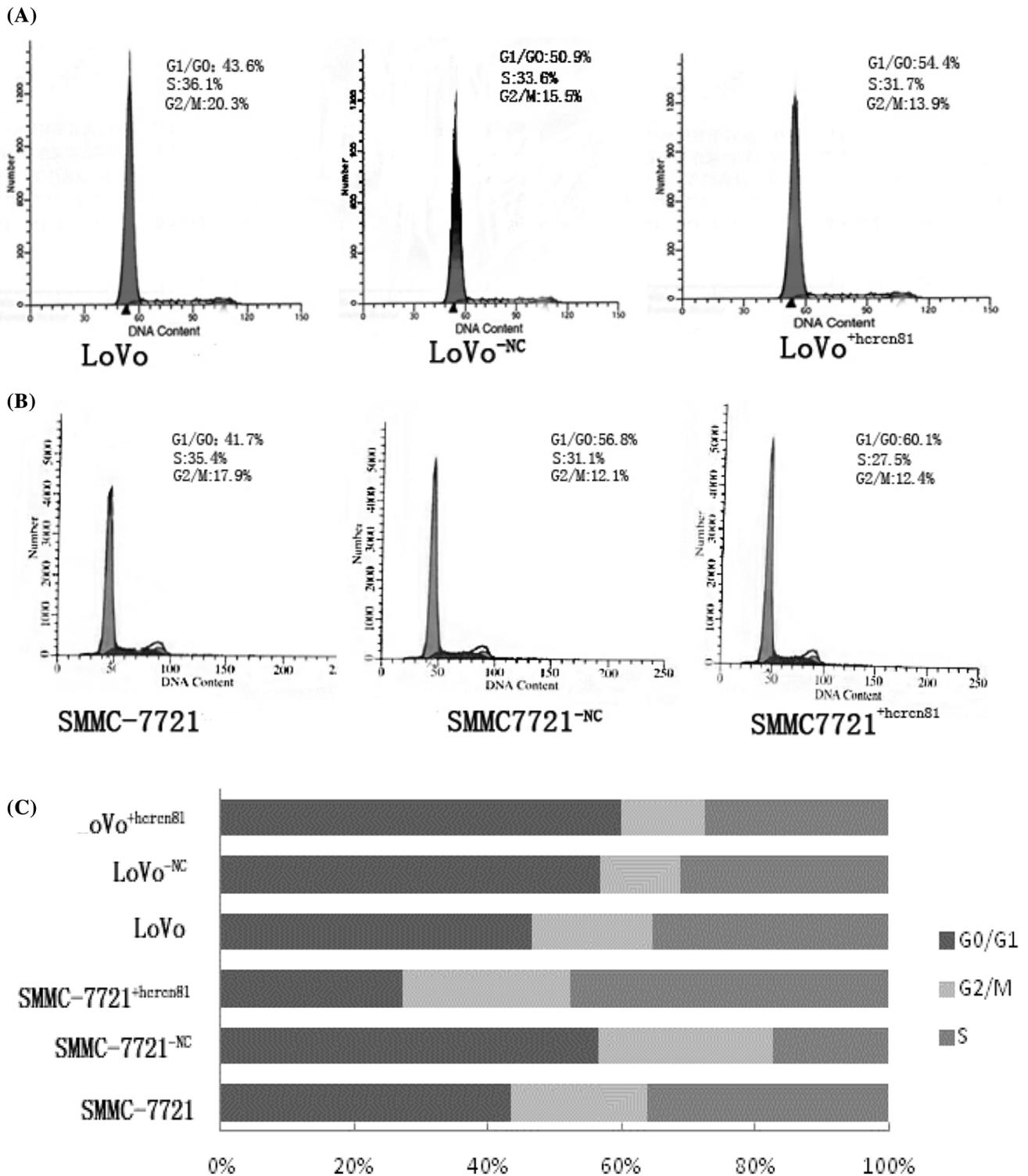


**Fig. 6** Cell apoptosis analysis of human colorectal cancer cells. Representative flow cytometry results showing the percentage of apoptotic cells in LoVo<sup>+hrcn81</sup>, LoVo<sup>-NC</sup> and LoVo cells (a); and SMMC-7721<sup>+hrcn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells (b).

Average apoptotic rate of LoVo<sup>+hrcn81</sup>, LoVo<sup>-NC</sup> and LoVo cells (c); and SMMC-7721<sup>+hrcn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells (d). \**P* < 0.05 and \*\**P* < 0.01

expression was significantly increased in LoVo<sup>+hrcn81</sup> and SMMC-7721<sup>+hrcn81</sup> cells. Most of the genes involved in cell cycle and cell adhesion arrest are repressed by c-Myc [16]. Based on the bioinformatics analysis, hrcn81 might

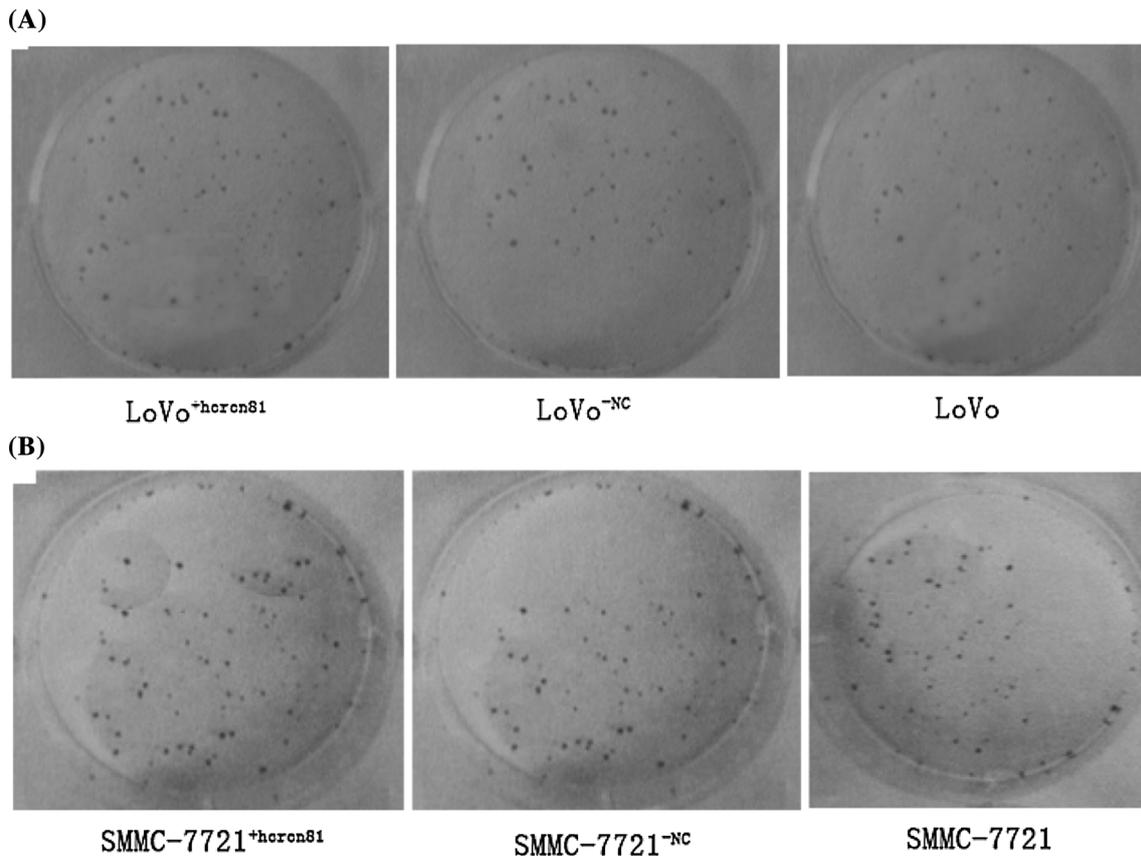
act as a serine/threonine kinase to directly or indirectly phosphorylate c-Myc at Thr358 [5]. c-Myc<sup>pThr358</sup> was reduced after hrcn81 interference, which then inhibited the proliferation of SW480<sup>-hrcn81</sup> and SW620<sup>-hrcn81</sup> cells



**Fig. 7** Cell cycle analysis of human colorectal cells by flow cytometry. DNA frequency distribution in LoVo<sup>+hcrn81</sup>, LoVo<sup>-NC</sup> and LoVo cells (a); and SMMC-7721<sup>+hcrn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells (b). Average percentage of cells in different cell cycles (c)

[9]. It is well known that cyclinD1 is involved in cell cycle regulation, and cyclinD1-CDK complex promotes cell cycle progression into the S phase. Here we found that the

percentage of cells in G1/G0 phase was higher in LoVo<sup>+hcrn81</sup> and SMMC-7721<sup>+hcrn81</sup> cells than in control groups. Therefore, we hypothesized that hcrn81 might



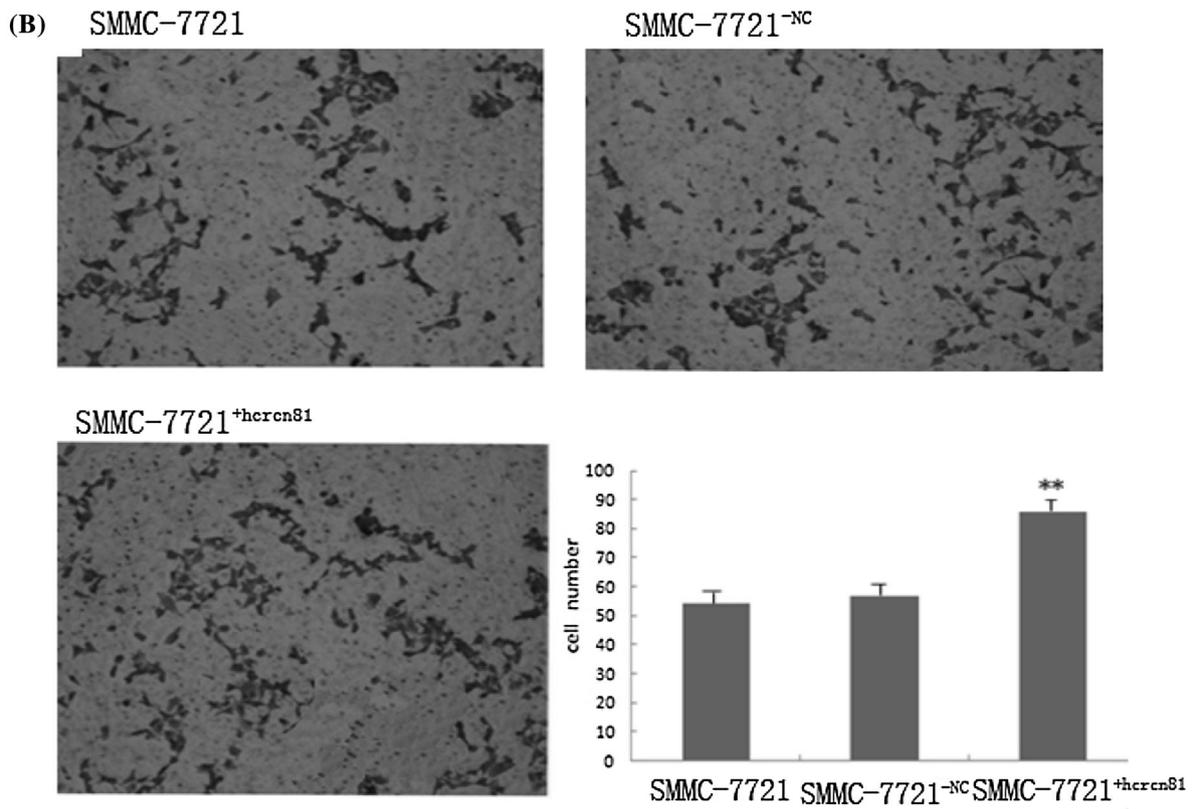
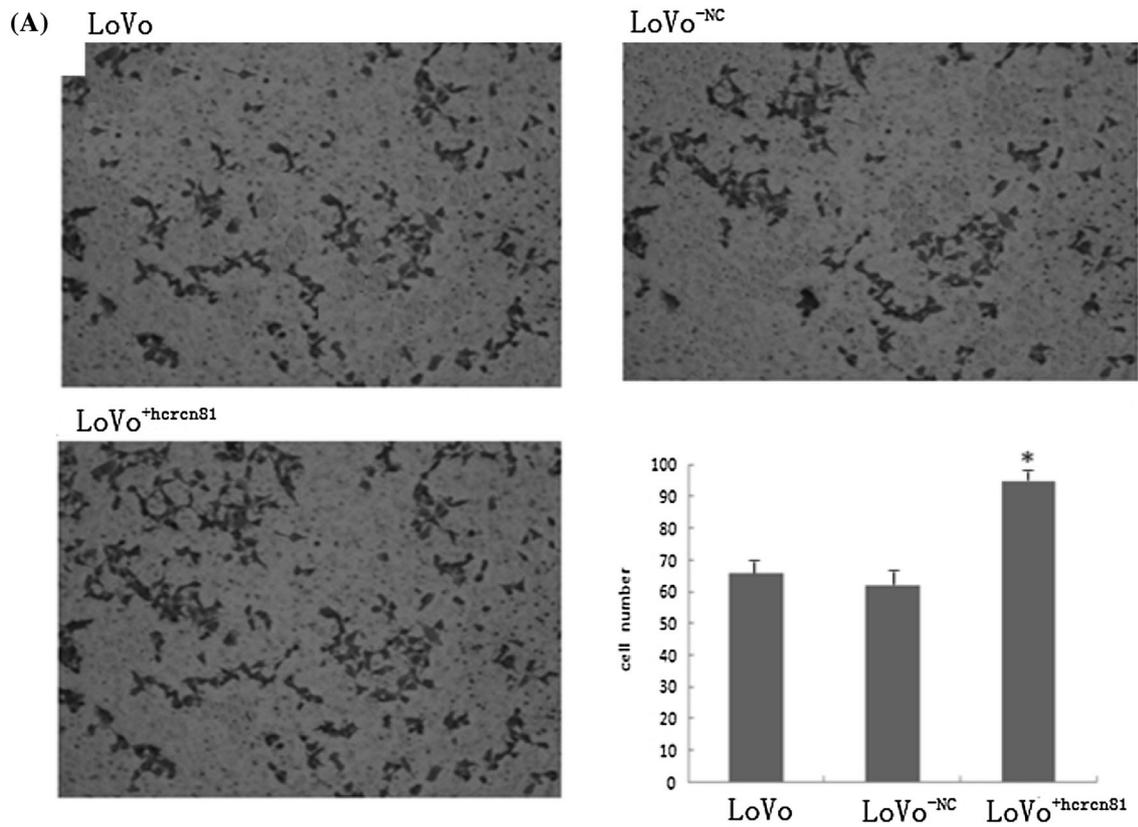
**Fig. 8** Increase in cell colonies by overexpressing hcrn81 in human colorectal cell lines. Representative cell colony images for LoVo<sup>+hcrn81</sup>, LoVo<sup>-NC</sup> and LoVo cells (a); and SMMC-7721<sup>+hcrn81</sup>, SMMC-7721<sup>-NC</sup> and SMMC-7721 cells (b)

regulate cell cycle by regulating cyclinD1. In our study, the mRNA and protein expression levels of Survivin were up-regulated when hcrn81 was overexpressed. Several oncogenic transcription factors stimulate expression of the Survivin gene [17]. In mammalian cells, Survivin participates in at least three homeostatic networks: the control of mitosis, the regulation of apoptosis and the cellular stress response [18]. Our data suggested that hcrn81 overexpression might cause the differential expression of c-Myc, cyclinD1 and Survivin, which might result in promoting proliferation of cancer cells.

The Wnt/ $\beta$ -catenin signaling pathway regulates cellular processes, such as proliferation, differentiation, motility, survival and apoptosis. It also plays key roles in embryonic development and in tumor formation, migration, metastasis and chemoresistance [19]. In the absence of Wnt-activating signals,  $\beta$ -catenin is sequestered in the cytoplasm by a multi-protein complex that includes GSK-3 $\beta$ .  $\beta$ -catenin is subsequently phosphorylated by GSK-3 $\beta$ , and phosphorylated  $\beta$ -catenin is degraded by ubiquitination. Activation of Wnt/ $\beta$ -catenin signaling inhibits formation of the multi-protein complex and phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$ . GSK-3 $\beta$ , a multi-functional serine-threonine protein

kinase, regulates numerous signaling pathways involved in diverse cellular processes, including metabolism, cell cycle control, proliferation, differentiation and apoptosis [20]. A large number of studies have suggested that sustained GSK-3 $\beta$  serine 9 phosphorylation could contribute to cancer genesis. GSK-3 $\beta$  was found to be hyperphosphorylated on serine 9 in human hepatoma cell lines as well as in human and murine liver tumors [21]. In this study, we found that down-regulation of GSK-3 $\beta$  might be caused by hcrn81 overexpression. Therefore, hcrn81 might be involved in Wnt/ $\beta$ -catenin signaling pathways via regulating GSK-3 $\beta$ . The possible mechanism might be that hcrn81 up-regulation leads to sustained phosphorylation of GSK-3 $\beta$  Ser9 and causes  $\beta$ -catenin to be accumulated and translocated to the nucleus. Hypophosphorylated  $\beta$ -catenin then associates with transcription factors of the TCF/LEF family to initiate the expression of a broad range of genes, such as c-Myc, cyclinD1, MMP-7, CD44 and Survivin [22, 23].

On the tissue level, hcrn81 was significantly down-regulated in human colorectal adenocarcinoma tissues, compared to the adjacent normal colorectal tissues. It is worth noting that we did not observe any significant



**Fig. 9** Cell migration assay of LoVo and SMMC-7721 groups. The number of migrating cells in LoVo groups (a) and SMMC-7721 groups (b). \* $P < 0.05$  and \*\* $P < 0.01$

correlation between hcrn81 expression level and patient characteristics, such as gender and Dukes' stage. The up-regulation rate in older patients (>50 years old) was higher than that in younger ones (<50 years old), suggesting the possible role of hcrn81 as a pathological factor in colorectal carcinoma. There were more moderately differentiated samples than poorly differentiated samples, in accordance with our previous studies. We also found that the mRNA expression levels of  $\beta$ -catenin and Survivin in human colorectal adenocarcinoma tissues were markedly up-regulated compared to those in the adjacent normal colorectal tissues, and were closely correlated with the age and differentiation of the patients. Spearman correlation analysis showed that there was a positive correlation between hcrn81 and  $\beta$ -catenin in tissues, different from the findings in cell culture, possibly due to small clinical sample size.

To summarize, expression of hcrn81 was significantly reduced in colorectal carcinoma tissues and cells, and overexpression of hcrn81 increased the proliferation and invasion and decreased the apoptosis of cancer cells. The promotion of hcrn81 expression might be responsible for the increase of  $\beta$ -catenin, Survivin, cyclinD1 and c-Myc, and the decrease of GSK-3 $\beta$  in cells. Therefore, hcrn81 might promote the carcinogenesis of colorectal adenocarcinoma and play a vital role in the pathogenesis of colorectal cancer through the activation of Wnt/ $\beta$ -catenin signaling pathway. hcrn81 might be a potential cancer gene and can provide a new perspective for biological therapy of colorectal carcinoma in the future. However, further studies are required to validate this correlation and to elucidate the underlying molecular mechanisms.

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