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Livin serves as a prognostic marker for mid-distal rectal cancer and a target of mid-distal rectal cancer treatment

QI-BIAO SU[1], LAI-YOU WANG[2], GUI-NING WEI[3], LI-ZHEN LIAO[1], JIE ZHAO[1], HONG-JUN LIU[3], YU-LONG SHI[4], LE-PING LI[4] and CHEN-SHENG LI[4]

[1]College of Health Science; [2]College of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, Guangdong 510006; [3]Department of Pharmacology, Guangxi Institute of Chinese Medicine and Pharmaceutical Science, Nanning, Guangxi 530022; [4]Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, P.R. China

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Abstract. Livin is a novel member of the inhibitor of apoptosis protein family, which has been identified to be expressed in various malignancies and is suggested to be associated with poor prognostic significance. However, no data are available concerning the significance of livin in mid-distal rectal cancer. In the present study, livin expression, and its association with clinicopathological characteristics and prognosis was examined in patients with mid-distal rectal cancer. Apoptotic susceptibility, invasion capacity and chemosensitivity of LoVo cells were investigated using small interfering RNA (siRNA)-mediated knockdown of livin. It was revealed that livin was highly expressed in mid-distal rectal cancer tissues compared with the normal rectal mucosal tissues. Livin expression was associated with pathological grade, extent of invasion (T stage) and extent of lymph node metastasis (N stage) of tumor, contributing to poor prognosis of mid-distal rectal cancer following surgery. The data suggest that aggressive surgery should be applied in patients with mid-distal rectal cancer with high expression of livin. It was also revealed that knockdown of livin by siRNA increased the apoptotic rate, suppressed invasion of LoVo cells, and decreased the half-maximal inhibitory concentration of oxaliplatin and 5-fluorouracil by ~50% in LoVo cells significantly compared with control groups. The data suggested that a combination of downregulation of livin and anticancer drugs may significantly decrease the toxicity of anticancer drugs. Taken together, the present study indicated that livin may be a promising target in clinical therapy of mid-distal rectal cancer.

Correspondence to: Dr Chen-Sheng Li, Department of Gastrointestinal Surgery, Shandong Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan, Shandong 250021, P.R. China
E-mail: chensheng76@163.com

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer in males, the second in females worldwide, and the fourth most common cause of cancer-associated mortality following lung, stomach and liver cancer (1).

High incidence rates are usually observed in the Western world, but during the past few decades, China has experienced an increase in the incidence of rectal cancer (2). CRC is a heterogeneous disease, particularly with respect to the ethnicity of patients, tumor stage and genetic alterations that contribute to its progression (3). Surgery, chemotherapy and radiotherapy are the traditional main treatments for rectal cancer (4). The majority of patients with rectal cancer are already at the advanced stage when they are diagnosed, which often leads to poor outcomes following surgery, thus, identification of key regulators governing the progression and malignant behavior of rectal cancer may aid in evaluating the patient prognosis, and provide targets for cancer prevention or therapy (5,6).

As is known, inhibition of apoptosis performs an important role in oncogenesis, which is mediated by a group of inhibitors of apoptosis proteins (IAPs) (7,8). Various studies demonstrated that overexpression of IAP facilitates the accumulation and survival of tumor cells, and improves tumor drug resistance to antitumor drugs (9,10). To date, identified IAPs comprise eight members in human cells, including insulin-like peptide (ILP)-1, cellular (c)-IAP1 (HIAP-2), c-IAP2 (HIAP-1), neuronal apoptosis inhibitory protein, Survivin, BRUCE (apollon), livin (ML-IAP, KIAP) and ILP-2 (11). Livin was originally identified in melanoma and is expressed in several types of malignant tumors, including bladder cancer, lung cancer, breast carcinoma, acute lymphoblastic leukemia and gastric cancer (12-16).

Although Korean (17) and Chinese (18) scientists reported the association of livin with colorectal cancer progression, in regard to the fact that mid-distal rectal cancer comprise 70-80% of rectal cancer in China (19), and to a certain extent, the effect of surgery for mid-distal cancer is attenuated by the anatomy constraint of narrow pelvis. Furthermore, thus far, there are no studies that have evaluated the role of livin in mid-distal rectal cancer. To the best of our knowledge,

the present study examined for the first time livin expression in human mid-distal rectal cancer tissues, evaluated the association between its expression and clinicopathological characteristics and prognosis. In addition, this study investigated the effects of livin on apoptotic susceptibility, invasion capability and the chemosensitivity of LoVo cells. The present data indicated that livin contributes to the aggressive behavior of human mid-distal rectal cancer and may serve as a potential target for mid-distal rectal cancer therapy.

Materials and methods

Patients. A total of 180 patients with mid-distal rectal cancer (median age, 58 years old; age range, 40-76 years old), including 117 tubular adenocarcinomas and 63 mucinous adenocarcinomas receiving total mesorectal excision were enrolled in the present study. All tumor specimens were obtained from patients operated on at Shandong Provincial Hospital Affiliated to Shandong University (Shandong, China) between May 2001 and October 2005. Written informed consent was obtained from all 180 patients. Each tumor was assigned a histological type according to the Chinese Classification of Rectal Carcinoma, which abides by the World Health Organization classification of malignant tumors (20), and infiltration was graded according to the International Union against Cancer TNM Classification of Malignant Tumors (21). The study obtained ethical approval from the Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University and observed the Helsinki Declaration. In total, 72 tumors were stage I-II, 108 were stage III-IV and 76 were at the lymph node metastasis stage. The corresponding normal rectal mucosal tissues (5 cm away from the tumor edge) were evaluated as normal controls. Diagnosis was performed by two independent pathologists, and those cases with questionable diagnosis were omitted from the present study. All the patients enrolled were followed up post-surgery by telephone, letter or re-examination in the outpatient department, with a median follow-up time of 71 months. No cases treated by radiotherapy and/or chemotherapy were included.

Immunohistochemistry. Samples sections were fixed with 10% neutral buffered formalin for 24 h at 4˚C, dehydrated in graded ethanol (50, 70, 80, 90, 95 and 100%) and embedded in paraffin. Paraffin sections (4 μm) were deparaffinized and rehydrated in graded ethanol (100, 95, 80, 70 and 50%). Heat-mediated antigen retrieval was performed using a pressure cooker treatment for 10 min in the citrate buffer (pH 6.0) at 100˚C. Endogenous peroxide activity was quenched by hydrogen peroxide (3%) for 10 min at room temperature. The slides were incubated overnight at 4˚C with a primary antibody for livin (rabbit polyclonal livin antibody; catalog no. ab97350; dilution, 1:100; Abcam, Cambridge, MA, USA) and with a horseradish peroxidase-labeled dextran polymer-conjugated goat anti-rabbit antibody (catalog no. K4003; dilution, 1:100; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at 37˚C subsequent to washing with TBS (pH 7.4). Negative controls were processed in the absence of primary antibody. The reaction products were viewed using diaminobenzidine and hematoxylin staining at room temperature for 5 min. Livin expression levels were classified into two categories depending on the percentage of cells stained. The mean percentage of positive tumor cells for the expression of livin was determined in at least five areas at 200-fold magnification under a light microscope, and cases with <10% positively stained tumor cells were defined as negative, while the remainder were defined as positive.

Cell culture and small interfering RNA (siRNA) transfection. CRC LoVo cells were obtained from the Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere at 37˚C with 5% CO_2. Cells were periodically detached with 0.25% trypsin-ethylene diamine tetraacetic acid solution and subsequently replated. Cells in the logarithmic phase were used for the experiments. Livin was knocked down with siRNA against livin (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Duplex sequences of siRNA-livin were as follows: a-GGAGUUGCGUCUGGGCUCCUCUAU and b-AUAGAAGGAAGGCCAGACGCAACUCC. The transfection mixture consisted of antibiotic-free culture medium and 100 nM siRNA. The non-targeting scrambled siRNA (Invitrogen; Thermo Fisher Scientific, Inc.) was used as a negative control. Following siRNA transfection according to the manufacturer's protocol, the cells were incubated and then used for various analyses.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells 48 h post-transfection using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using a one-step RT-polymerase chain reaction (PCR) kit (Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 37˚C for 15 min and 85˚C for 5 sec and then the mix was stored at 4˚C. PCR reactions were undertaken using the Real-time PCR Master Mix (SYBR-Green) reagent kit (Toyobo Life Science, Osaka, Japan), according to the manufacturer's protocol. PCR was run in 20 μl total volume. Primer sequences were: β-actin forward, 5'-AGTGTGACGTTGACATCCGT-3' and reverse, 5'-TGCTAGGAGCCAGAGCAGTA-3'; livin forward, 5'-CGGCCCCTGACAGAGGAGGA-3' and reverse, 5'-AGGCCAGACGCAACTCCTCA-3' (22). The PCR was performed by initial incubation at 95˚C for 5 min followed by 30 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 15 sec and extension at 72˚C for 45 sec. Livin mRNA quantification was performed by the $2^{-\Delta\Delta Cq}$ method (23).

Western blot analysis. At 48 h post-transfection, cells were collected, lysed in an ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Nonidet P-40 and a protease inhibitor cocktail (Boehringer, Mannheim, Germany). Lysates were centrifuged at 100,000 x g for 30 min at 4˚C and supernatants were collected. Protein concentrations were determined using the Bio-Rad protein determination method (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

An aliquot of each sample equivalent to 100 μg protein was boiled in sample buffer, separated on a 8-12% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1 h, washed with TBST (50 mM Tris, 300 mM NaCl pH 7.6, 0.1% Tween) and incubated with the primary antibody (livin antibody; catalog no. 2505; dilution, 1:500; housekeeping gene β-actin monoclonal antibody; catalog no. 3777; dilution, 1:5,000; ProSCI, Inc., Poway, CA, USA) at 4˚C overnight. Blots were then washed with TBST (50 mM Tris, 300 mM NaCl pH 7.6, 0.1% Tween) and incubated with peroxidase-linked secondary antibodies (catalog No. 97100, dilution 1:5000, Abcam, Inc., Cambridge, MA, USA) at 37˚C for 1 h. Bound antibodies were developed using the Super Enhanced Chemiluminescence plus detection reagent (Applygen Technologies, Inc., Beijing, China). Densitometric analysis was performed using GDS-8000 UVP photo scanner (Bio-Rad Laoratories, Inc.) and Quantity one 4.2.2 software (Bio-Rad Laboratories, Inc.). Cells transfected with siRNAs that had the highest downregulation rates of livin were selected for the subsequent assays.

Analysis of cell apoptosis. At 48 h post-transfection, LoVo cells on plates were harvested by trypsin-EDTA (Invitrogen; Thermo Fisher Scientific, Inc.); cells were washed with ice-cold PBS and incubated with 500 μl of the annexin V-binding buffer at room temperature for 5 min. Annexin V-fluorescein isothiocyanate (5 μl) and 10 μl of PI were added to each cell suspension, and incubated at room temperature for 10 min in the dark prior to analysis by flow cytometry (BD Biosciences, San Jose, CA, USA). The apoptotic rate was determined using CellQuest software (version 5.1; BD Biosciences).

Cell invasion assay. Invasion of LoVo cells was measured using Transwell cell culture chambers (8 μm pore size) containing a Matrigel-coated polycarbonate membrane filter. Cells (1x10^4 cells/well) in 500 μl of serum-free RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) were placed in the upper chamber of the Transwell insert, and the lower chamber was filled with 750 μl culture medium with 10% FBS. Following incubation at 37˚C for 24 h, non-invasive cells on the upper surface of the filter were removed using a cotton swab, and the invasive cells on the bottom side of the membrane were stained with hematoxylin and eosin at room temperature for 10 min. The number of cells was counted under a light microscope from six random fields (magnification, x200). The experiment was repeated three times.

Chemosensitivity assay. LoVo cells were treated with a concentration gradient of anticancer drugs prior to and following livin gene knockdown. The anticancer drugs, oxaliplatin (L-OHP) and 5-flourouracil (5-FU), both purchased from Bristol-Myers Squibb (Princeton, NJ, USA), were tested in six dilutions corresponding to 200, 100, 50, 25, 12.5, and 6.25% of each drug standard test drug concentration (TDC; 100% TDC used as follows: L-OHP 16 μM, 5-FU 8 μM). The cells were seeded at a density of 3x10^4 cells/well on 96-well plates in RPMI-1640 medium, each well was repeated in triplicate. Following incubation overnight at 37˚C, the medium was replaced by fresh medium containing the test drug. All the cells were treated for 48 h. At the end of the treatment, cell viability was determined colorimetrically using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Following incubation for 2 h, the 96-well plate was scanned by an ELISA reader at 450 nm for optical density values to calculate the percentage of cell inhibition and half-maximal inhibitory concentration (IC_{50}) (inhibitory concentration to achieve 50% cell death).

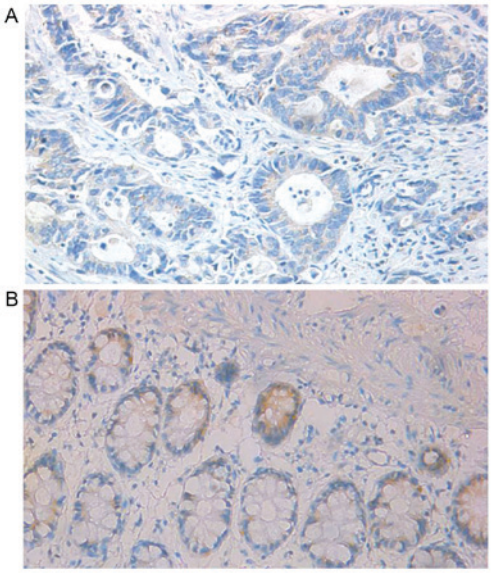

Figure 1. Livin protein expression in mid-distal rectal cancer tissues and normal rectal mucosal tissues by immunohistochemistry. (A) Livin protein was strongly immunostained in mid-distal rectal cancer tissues (magnification, x200). (B) Livin protein was weakly immunostained in normal rectal mucosal tissues (magnification, x200).

Statistical analysis. All data were analyzed by SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). Each assay was performed at least 3 times. The data were expressed as mean ± standard deviation. The expression of livin was analyzed by the χ^2 test. Recurrence-free survival curves were constructed using the Kaplan-Meier method and life tables, and were compared using the log-lank test. The prognostic indicators of livin expression for recurrence were assessed using the Cox proportional hazards model. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of livin in mid-to-distal rectal cancer tissues. Among 180 cases of cancer, 96 cases (53.33%) exhibited positive staining for livin, whereas out of 30 control cases, only 2 cases (6.67%) exhibited positive staining (Fig. 1). There was a significant difference in the positive staining rate between the mid-to-distal rectal cancer and the control group (χ^2=22.50; $P<0.01$). Livin expression was associated with the known prognostic variables, including pathology stage, T stage and lymph node metastasis (all $P<0.05$; Table I), but not with age, sexuality, blood transfusion, course of disease, and tumor size (all $P>0.05$; Table I). A significant difference in the 5-year survival rate between patients with positive and negative

Table I. Association between livin expression and the clinicopathological features of patients with mid-distal rectal cancer.

Characteristic	Number of cases	Livin			χ^2	P-value
		-	+	Positive ratio		
Gender						
Male	105	51	54	0.51	0.37	0.54
Female	75	33	42	0.54		
Age, years						
≤50	66	30	36	0.55	0.06	0.80
>50	104	54	60	0.58		
Blood transfusion						
Yes	116	55	61	0.53	0.07	0.79
No	64	29	35	0.55		
Course, months						
≤6	116	49	67	0.58	2.57	0.11
>6	64	35	29	0.45		
Tumor size, cm						
≤5	93	48	45	0.48	1.89	0.17
>5	87	36	51	0.59		
Pathology stage						
Adenocarcinoma	117	62	55	0.47	5.37	0.02
Mucinous adenocarcinoma						
Signet ring cell carcinoma	63	22	41	0.65		
Depth of invasion						
T_1+T_2	72	58	14	0.19	55.37	<0.01
T_3+T_4	108	26	82	0.76		
Lymph node metastasis						
Yes	114	56	48	0.42	5.10	0.02
No	76	28	48	0.63		

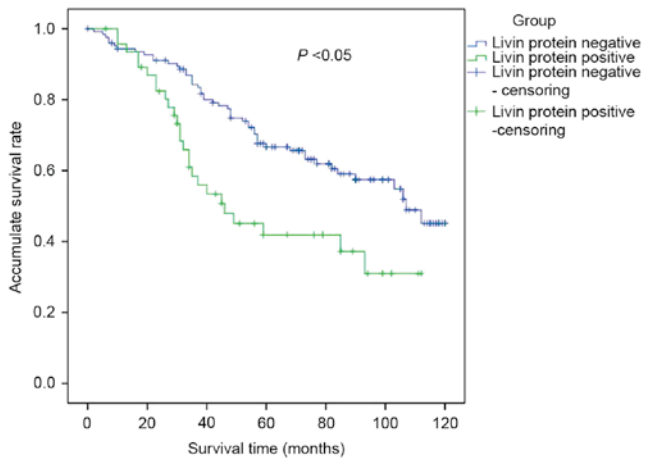

Figure 2. Kaplan-Meier analysis. The 5-year survival rate of patients with positive livin expression was lower compared with that of patients with negative livin expression (45 vs. 68%; P<0.05).

expression of livin was observed (45 vs. 68%; P<0.05, Fig. 2). Univariate analysis in the Cox regression model revealed that 5-year survival rate was associated with pathology stage, T stage, lymph node metastasis and livin expression (P<0.05; Table II), but not with age, sexuality, blood transfusion, course of disease, and tumor size (P>0.05; Table II). Multivariate Cox regression analysis confirmed an independent and significant effect of livin expression, and lymph node metastasis on the prognosis of patients (P<0.05; Table III).

Stable knockdown of livin by siRNA in LoVo cells. Expression of livin protein in LoVo cells was examined by western blot analysis (data not shown). To characterize the biological role of livin in mid-to-distal rectal cancer, a specific siRNA was employed for livin to knockdown livin expression in LoVo cells expressing the highest level of endogenous livin. Stable transformants were identified using RT-qPCR and western blot analysis. The results showed that the livin mRNA and protein levels in cells transfected with specific siRNA for livin were significantly reduced compared with those transfected with control siRNA (both P<0.05; Fig. 3).

Knockdown of livin promotes LoVo cell apoptosis. To detect whether livin siRNA was able to induce apoptosis of LoVo cells, at 48 h after infection of livin siRNA, control siRNA or PBS, the cell apoptotic rate was detected by flow cytometry. Apoptotic cells were determined by annexin V-positive and PI-negative cells. The results demonstrated that the apoptotic

Table II. Univariate Cox proportional regression analysis on the 5-year overall survival rate in patients with lower and middle rectal cancer.

Characteristic	Number of cases	5-year overall survival, %	P-value
Gender			
Male	105	68.8	0.762
Female	75	62.5	
Age, years			
≤50	66	70.0	0.824
>50	104	65.0	
Blood transfusion			
Yes	116	67.5	0.793
No	64	61.9	
Course, months			
≤6	116	65.8	0.985
>6	64	63.9	
Tumor size, cm			
≤5	93	66.3	0.173
>5	87	58.8	
Pathology stage			
Adenocarcinoma	117	69.1	0.041
Mucinous adenocarcinoma			
Signet ring cell carcinoma	63	59.0	
Depth of invasion			
T_1+T_2	72	65.4	0.032
T_3+T_4	108	53.9	
Lymph node metastasis			
Yes	114	66.5	0.016
No	76	49.2	
Livin expression			
Negative	96	45.0	0.002
Positive	84	68.0	

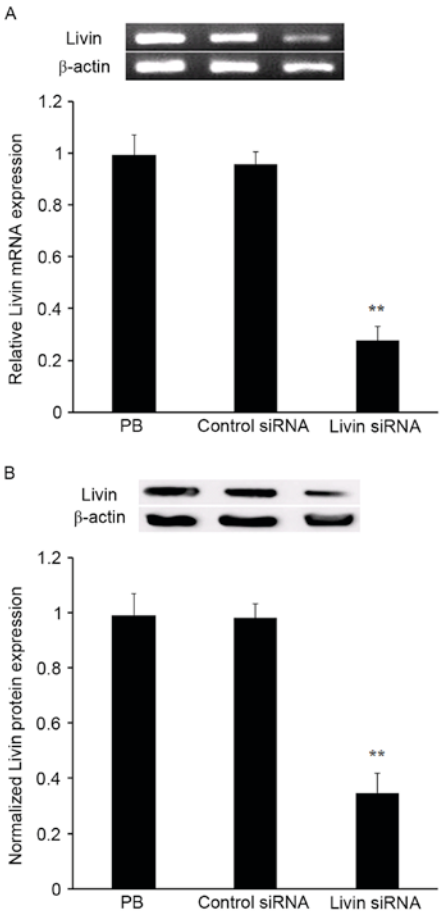

Figure 3. Specific downregulation of livin mRNA and protein expression by livin siRNA. LoVo cells were transfected with 100 nM livin siRNA or scramble siRNA (control siRNA) for 48 h. (A) The relative mRNA levels of livin were quantified by quantitative polymerase chain reaction analysis and (B) the relative protein levels of livin were determined by western blotting. Data were normalized by using β-actin as an internal standard. **P<0.05 vs. control siRNA. siRNA, small interfering RNA.

rate of LoVo cells significantly increased compared with control groups (P<0.05; Fig. 4).

Stable knockdown of livin expression decreases invasion capacity of LoVo cells. The Transwell invasion assay demonstrated that the invasion capacity was significantly reduced in stable livin knockdown cells as compared with control cells (P<0.05; Fig. 5).

Effect of knockdown of livin on LoVo cell chemosensitivity. The growth inhibition curves of L-OHP and 5-FU at six different concentrations were plotted in Fig. 6. It was demonstrated that knockdown of Livin by siRNA in LoVo cells resulted in much higher cell growth inhibition at different drug concentrations. The IC_{50} of L-OHP and 5-FU for the untransfected LoVo cells were 2.5, and 1.1 μM, respectively. However, the corresponding IC_{50} values dropped to 1.2 and 0.45 μM, respectively (both P<0.05) when livin expression was knockdown by siRNA (Fig. 6), suggesting that a significant enhancement in chemosensitivity of L-OHP, and 5-FU LoVo cells compared with the untransfected cells or control siRNA-transfected cells.

Discussion

Mid-to-distal rectal cancer is prone to invade into adjacent tissues due to no peritoneum coat covering the mid-to-distal part of the rectum, and furthermore, the outcome of radical resection is affected by narrow pelvis or obesity in certain patients, therefore leading to poor prognosis (24,25). Saito *et al* (26) reported that the 5-year survival rate following curative resection for local recurrence of rectal cancer in the asymptomatic group was significantly higher compared with that in the symptomatic group (62 vs. 23%; P<0.05). These findings suggested that timely treatment for local recurrence is associated with the prognosis of patients with rectal cancer. Local recurrence of rectal cancer occurs predominantly in the cavity and rarely in the anastomotic stoma, and anus examination, enteroscopy or barium enema are not applied following abdominoperineal excision. Despite the emergence of computed tomography, positron emission tomography and

Table III. Multivariate Cox proportional regression analysis on the 5-year overall survival in patients with lower and middle rectal cancer.

Characteristic	Partial regression coefficient	SE	OR	P-value
Livin	2.034	0.373	7.644	0.000
Lymph node metastasis	1.649	0.342	5.203	0.000

SE, standard error of the mean; OR, odds ratio.

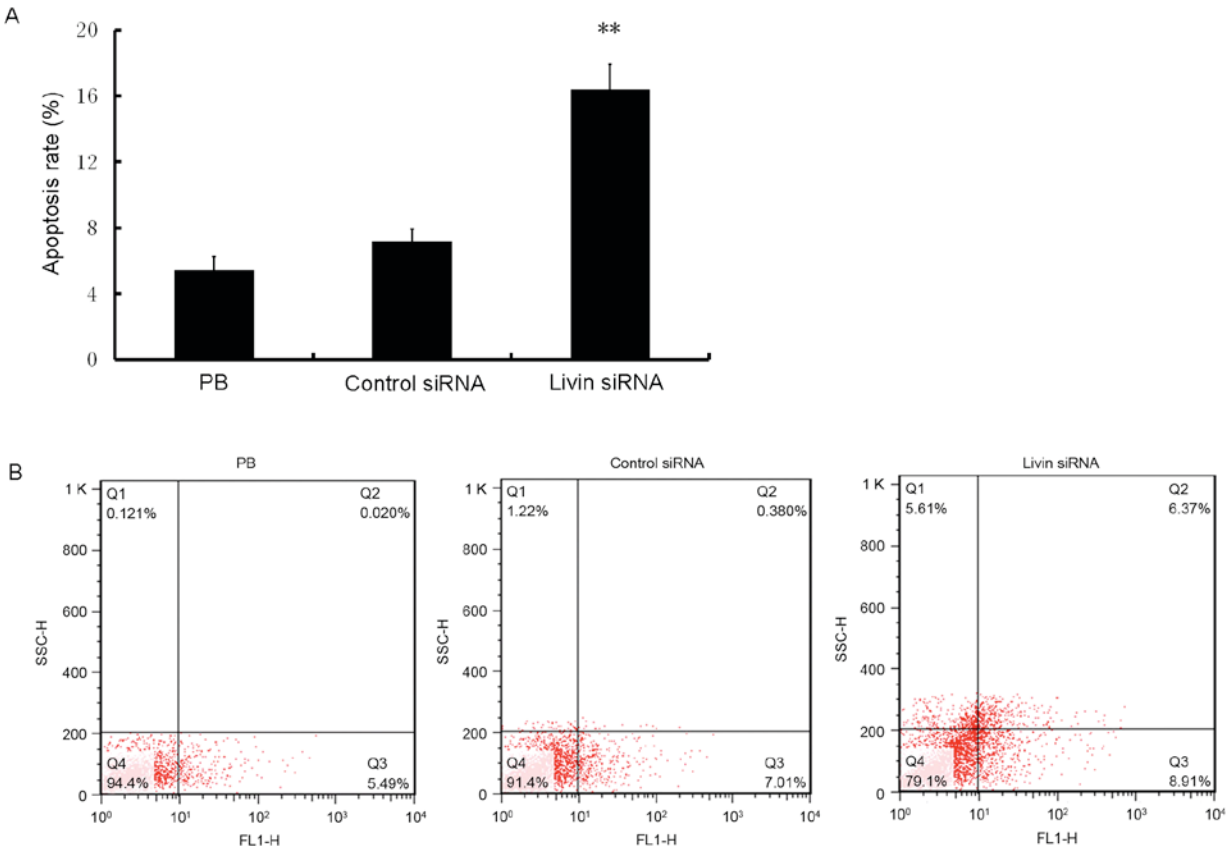

Figure 4. Analysis of cell apoptosis by flow cytometry assay. At 48 h post-transfection, apoptotic cells were determined by annexin V-positive and propidium iodide-negative cells. Livin siRNA induced apoptosis of LoVo cells. (A) The apoptosis rate of cells under the indicated conditions. Data are presented as the mean from three independent triplicate experiments. (B) Representative images of flow cytometry analysis of cell apoptosis. **P<0.05 vs. control siRNA. siRNA, small interfering RNA.

magnetic resonance imaging scans, the symptoms of pain, constipation, and bleeding are still considered the best predictors of signs of recurrence (27). However, for the majority of patients with rectal cancer, local recurrence is often diagnosed at late stage due to the high rate of asymptomatic patients (28). Therefore, diagnosis of recurrent rectal cancer is among the formidable challenges.

Various studies revealed that inhibited apoptosis contributes to the development and progression of cancer (7,29-31). Livin, an important member of the IAP family, was revealed to be expressed in multiple malignant tumors, and has been demonstrated to be essential for tumor progression and poor prognosis for several types of malignancies (12-16).

In the present study, it was revealed that the positive expression rate of livin was significantly higher in mid-to distal rectal cancer tissue compared with that in normal rectal mucosal tissues (53.3 vs. 6.67%; P<0.05), indicating that the positive expression of livin is significantly associated with the oncogenesis of mid-distal rectal cancer. Pathological grade, extent of invasion (T stage), extent of lymph node metastasis (N stage) and livin expression (P<0.05; Table II) were associated with 5-year survival rate.

Kanemitsu *et al* (32) reported that mucinous adenocarcinoma itself was an independent prognostic factor in the curative resected patients. The extent of invasion (T stage), extent of lymph node metastasis (N stage) are included in the Dukes

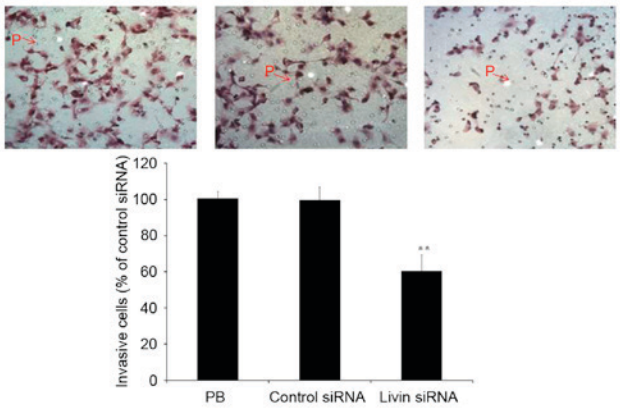

Figure 5. Stable-knockdown of livin suppressed LoVo cell invasion *in vitro*. The number of cells that had invaded the lower chamber was counted 24 h after seeding cells on the Transwells. **P<0.05 vs. control siRNA. siRNA, small interfering RNA.

classification, and are considered the independent prognostic factors in the majority of studies (33,34). In the present study, the pathological grade and extent of invasion (T stage) were excluded from the multivariate analysis model, suggesting that the other alternative factors in the model exhibit effects on the prognosis. As expected, multivariate Cox regression analysis revealed that livin expression and lymph node metastasis are two independent prognostic factors (both P<0.05), of which livin expression is more important. It was also revealed that livin-knockdown by specific siRNA caused an increased apoptotic rate and decreased invasion capability of LoVo cells compared with the control group, indicating that livin depletion may lead to decreased recurrence of mid-to-distal rectal cancer.

It was proposed that aggressive surgery treatments should be applied for patients with mid-distal rectal cancer with positive livin expression. Holm *et al* (35) reported that cylindrical abdominoperineal resection was applied to treat patients with rectal cancer in T3-T4 stage. This technique creates a cylindrical specimen with more tissue covering and surrounding the tumor, providing the benefits of a low risk bowel perforation, and tumor involvement of the circumferential resection margin, thus leading to a potentially lower risk of local recurrence. The technique may be a novel surgery treatment for patients with mid-distal rectal cancer with positive livin expression, additional studies regarding its effect and benefits are warranted.

As is known, surgery is the basis of therapy for colorectal cancer (36), and the majority of patients with colorectal cancer receive chemotherapy to palliate advanced unresectable disease or to reduce the risk of recurrence following radical surgery, but efficacy must be carefully balanced against toxicity (4). In the present study, it was revealed that the IC_{50} of L-OHP and 5-FU dropped by ~50% (both P<0.05) in LoVo cells when livin expression was knocked down by siRNA, indicating a significant decrease in toxicity of L-OHP and 5-FU by siRNA.

In summary, in the present study, livin was identified to be highly expressed in mid-distal rectal cancer tissues compared with normal rectal mucosal tissues. Livin expression was associated with pathological grade, extent of invasion (T stage) and

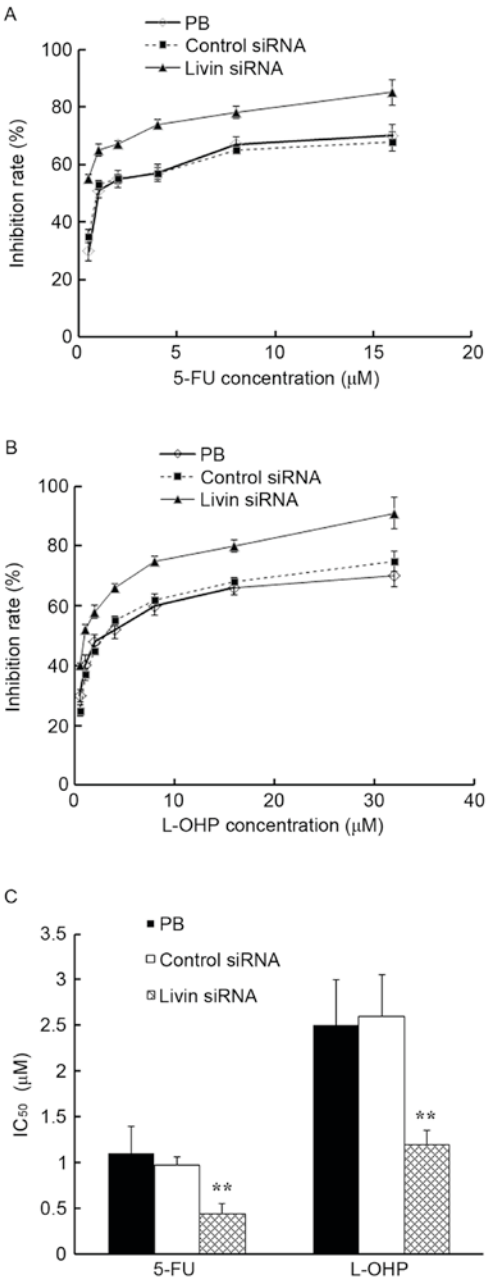

Figure 6. Live expression inhibition by siRNA potentiates effect of the 5-FU and L-OHP *in vitro*. Inhibition curves of (A) 5-FU and (B) L-OHP at different concentrations. (C) IC_{50}s of 5-FU and L-OHP to LoVo cells prior to, and following silencing livin inhibition. **P<0.05 vs. control siRNA. siRNA, small interfering RNA; 5-FU, 5- fluorouracil; L-OHP, oxaliplatin; IC_{50}, half-maximal inhibitory concentration; PB, phosphate buffered saline.

extent of lymph node metastasis (N stage) of tumor, contributing to poor prognosis of mid-distal rectal cancer following surgery. The data suggested that aggressive surgery techniques should be applied in patients with mid-distal rectal cancer with high expression levels of livin. It was also revealed that IC_{50} of L-OHP and 5-FU dropped by ~50% (both P<0.05) in LoVo cells when livin expression was knocked down by siRNA. The data suggest that a combination of livin downregulation and anticancer drugs may significantly decrease the toxicity of anticancer drugs. The present study suggests that livin is a promising target for further study in the clinical therapy of mid-distal rectal cancer.

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