

# Expression of the apoptosis inhibitor livin in colorectal adenoma-carcinoma sequence: correlations with pathology and outcome

Y. Wang · Y. Li · B. Zhou · W. Y. Zhang · J. T. Guan ·  
R. Wang · L. Yang · Q. J. Xia · Z. G. Zhou · X. F. Sun

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**Abstract** The inhibitor of apoptosis family member livin is expressed in several types of cancer but not in most benign tissues, and it has been considered to be a poor prognostic mark in various malignancies. However, livin expression and its prognostic relevance have not been evaluated in colorectal adenoma-

carcinoma sequence. In this study, we analyzed the difference of livin expression among normal mucosa, adenoma, and adenocarcinoma and investigated the relationship of livin expression in carcinomas with clinicopathological variables using immunohistochemistry and real-time reverse transcription-PCR. We observed that the expression of livin protein was mainly present on base of colorectal crypts in adenoma and throughout the epithelium in carcinoma, whereas did not present in accompanying normal mucosa, and the expression of livin messenger RNA (mRNA) in adenocarcinomas was significantly higher than in adenomas and in normal mucosa ( $P=0.001$ , respectively), whereas, compared with normal mucosa, the expression level of livin mRNA was up-regulated in adenomas but no significant difference ( $P=0.196$ ). We also found that the expression levels of livin mRNA in rectal cancer was significantly higher than those in colonic cancer, and livin mRNA expression was strongly related to colorectal cancer invasive depth but not to clinical tumor stage, differentiation, lymph node metastasis, tumor morphological category and pathological type, and patient's age and gender. These findings support the possibility that the livin gene may play a role in colorectal tumorigenesis, and increased expression of livin mRNA may serve as a new target for colorectal cancer treatment.

Y. Wang

Department of Colorectal Surgery, Institute of Digestive Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China  
e-mail: wydoctor1974@aliyun.com

Y. Li · B. Zhou · J. T. Guan · R. Wang · L. Yang · Z. G. Zhou (✉)  
State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China  
e-mail: zhou767@aliyun.com

Y. Li

e-mail: liyuanletters@163.com

B. Zhou

e-mail: 10444157@qq.com

J. T. Guan

e-mail: 670793370@qq.com

R. Wang

e-mail: 1728101707@qq.com

L. Yang

e-mail: lie\_222@163.com

W. Y. Zhang

Department of Pathology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China  
e-mail: zhangwylu@hotmail.com

Q. J. Xia

Ocular Surgery Laboratory, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China  
e-mail: 519082056@qq.com

X. F. Sun

Division of Oncology, and Department of Clinical and Experimental Medicine, Linköping University, Linköping S-58185, Sweden  
e-mail: xiao-feng.sun@liu.se

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## Introduction

During epithelial homeostasis, proliferation, migration, differentiation, and apoptosis occur; disturbance of these processes leads to neoplastic transformation and progression [1, 2]. Some studies reported that the ability of cells to evade apoptosis plays a crucial role during development and progression of cancer and is considered to be a major cause of therapeutic resistance to cytotoxic therapies [3–5]. Several gene families are involved

in the negative regulation of apoptosis, including the inhibitor of apoptosis protein (IAP) family [6–8]. The IAP family consists of a group of structurally related proteins that protect against various endogenous and exogenous apoptotic triggers [9], and the basal level of apoptosis is tightly controlled by endogenous IAPs in mammalian cells [10, 11]. Although inhibition of caspases is an important mechanism of IAPs to exert their antiapoptotic effects, they may also affect protein degradation, signal transduction pathways, and mitosis [11–13]. Livin, also known as ML-IAP and KIAP which initially identified computational analysis of the nucleotide sequence databases, is the most recently discovered member of the IAP family [14–17]. It displays a rather restricted expression pattern, as it has been detected to be predominantly expressed in various cancer cell lines and in a subset of human malignancies, including melanoma, neuroblastoma, lung, bladder, and renal cell cancer, but at substantially lower levels or almost nondetectable in most normal adult tissues [14, 15, 17, 18]. Two splicing variants of livin protein have been identified, termed livin  $\alpha$  and livin  $\beta$ , which are almost identical, except for 54-bp that is truncated from the 5'-part of exon 6 in livin  $\beta$  [17, 19]. Although some reports showed that these two isoforms have different antiapoptotic properties [19], both variants have been shown to potently block death receptor- and chemotherapy-induced apoptosis [17]. This may be obtained through either direct inhibition of various caspases or indirectly by acting as E3 ubiquitin ligases to target the degradation of Smac/DIABLO [20]. These characteristics render livin an attractive target for cancer treatment. In fact, specific inhibition of endogenous livin expression in tumor cell lines has been reported to strongly increase their response to various apoptotic stimuli [19, 21, 22], and its expression has recently been proven to relate to an increased chemotherapeutic resistance. Moreover, in the majority of tumors investigated for livin expression, high levels of the livin expression were predictive signs of tumor progression and it could provide prognostically relevant information [23, 24]. Gazzaniga et al. [18] had found that livin might be involved in the progression of superficial bladder cancer and used as a marker of early recurrence. Meanwhile, in several neoplasms, the association with tumor progression has been also corroborated in the context of comprehensive analysis of gene expression profiling by DNA microarray or PCR-based assays [24]. However, livin could not serve as a predictive sign in all kinds of cancers. Takeuchi et al. [25] have demonstrated that livin expression in metastatic melanomas could not influence disease outcome and had no significant prognostic utility. Kemphensteffen et al. [5] also reported that livin expression levels in renal cell carcinomas did not correlate with pathological or clinical parameters and were not predictive of patient outcome.

Colorectal cancer is the second leading cause of cancer-related death in the Western world [26], and in recent years, the adenoma-carcinoma sequence is the one of the most important fundamental concepts in colorectal cancer to emerge and it

gives us a wonderful interface to study the sequence of gene mutation and the diversification of gene expression [27], including livin. Furthermore, like renal cell carcinomas, colorectal carcinomas also easily endure chemotherapy and radiotherapy [26], and up to now, the expression of livin in the sequence of colorectal adenoma-carcinoma has not been investigated. The objectives of this study were to determine the expression levels of livin in the colorectal adenoma-carcinoma progression sequence and also investigate the gene expression data with clinicopathological parameters.

## Materials and methods

### Patients and specimens

All tissue specimens were collected from patients underwent operation or colonoscopy excision at the West China Hospital of Sichuan University from June 2006 to December 2006, included 64 patients with colorectal adenocarcinomas, 36 with colorectal adenomas, and 12 accompanying normal colorectal mucosa for comparison (taken from uninvolved morphologically normal colorectal tissue distant from colorectal cancer above 10 cm). None of the patients had received preoperative chemotherapy and/or radiotherapy. The specimens were flash-frozen in liquid nitrogen and stored at  $-150^{\circ}\text{C}$ . Each specimen was divided into two parts, one for immunohistochemistry and the other for real-time reverse transcription (RT)-PCR. Histological diagnosis, confirmed by pathologist at the Department of Pathology in the West China Hospital of Sichuan University according to the histological diagnostic criteria of the World Health Organization, was determined in formalin-fixed tissue immediately adjacent to the frozen sample used for real-time RT-PCR. Each tumor sample was considered suitable for RNA extraction if the proportion of tumor cells was  $>80\%$ . The clinical tumor stage of these patients with colorectal cancer was assessed according to the tumor, node, and metastasis (TNM) Classification of Malignant Tumors of the International Union Against Cancer (2003).

### Immunohistochemistry

For immunohistochemical staining, from each sample block, five serial 4- $\mu\text{m}$  sections were cut and place on glass slides. Sections 1–4 were used for immunostaining, and section 5 was stained with hematoxylin and eosin; this latter section was reviewed and compared with the original slide of the Department of Pathology used for diagnostic purposes.

Sections were stained with polyclonal rabbit antibody against livin (BA1743, Boster Biotechnology, Wuhan city, China). Briefly, the sections were deparaffinized and those to be stained with livin were subjected to microwave antigen retrieval in citrate buffer for 15 min twice. The sections were incubated with a polyclonal livin antibody of rabbit at 1/200

dilution over night at 4 °C. The avidin-biotin detection method was employed on a DAKO autostainer universal system (DAKO, Ely, UK). A negative control was performed by omission of the primary antibody of rabbit.

#### RNA extraction and cDNA preparation

Total RNA was extracted from tissue specimens using TRIzol LS reagent (Jinmei Biotech Co., Ltd, Shenzhen, China) as described previously [28]. The quality of the RNA was determined by electrophoresis through agarose gels and staining with ethidium bromide and the 18 s and 28 s RNA bands were visualized under UV light. To generate complementary DNA (cDNA), 5 µl RNA was first denatured at 70 °C with 2.5 µmol/l random hexamers (TaKaRa Biotechnology Co. Ltd, Dalian, China) for 5 min before quenching on ice; then, a 10 mmol/l final of each of the four deoxynucleotide triphosphates, 20 U ribonuclease inhibitor, 100 U M-MLV, and 5× M-MLV buffer (TaKaRa Biotechnology Co. Ltd) were added together to make up a final volume of 20-µl reaction mix. The reaction mix was incubated for 10 min at 20 °C and 1 h at 42 °C. The reverse transcriptase was inactivated at 95 °C for 10 min and cooling at 5 °C for 5 min.

#### Real-time PCR

Theoretical basis real-time PCR was performed using relative quantification protocol on an iCycler iQ System (Bio-Rad, CA, USA). Equation (1) (Michael 2001) was applied to calculate the relative expression ratio of the target gene (livin) in a sample versus a control in comparison with a reference gene (β-actin):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{reference}})^{\Delta CT_{\text{ref}}(\text{control} - \text{sample})}}$$

$E_{\text{target}}$  and  $E_{\text{reference}}$ , respectively, represent the real-time PCR efficiency of target gene and reference gene transcript.  $\Delta CT_{\text{target}}(\text{control} - \text{sample})$  and  $\Delta CT_{\text{ref}}(\text{control} - \text{sample})$  are the Ct (threshold cycle) deviations of control minus sample of the target or reference gene transcript, respectively. Real-time PCR efficiency ( $E$ ) was 2, approximately, by calculation.

#### Primers and probes

Specific primers and probes for livin and β-actin were designed based on sequence data from the ensemble database (<http://www.ensembl.org>). They were purchased from TaKaRa Biotechnology Co. Ltd, Dalian, China (Table 1).

#### PCR amplification

All PCR reactions were performed using an iCycler iQ System (Bio-Rad, CA, USA). For each PCR run, a mastermix of the following reaction components was prepared on ice as follows: 1× PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.3 mM dNTP, 0.16-µM TaqMan probe, 0.33 µM each primer, 1.25 U of AmpliTaq Gold DNA polymerase (TakaRa Biotechnology Co. Ltd), and 1 µl of cDNA add as a PCR template. The following iCycler iQ run protocol was used: denaturation program (94 °C, 3 min) and amplification and quantification programs repeated 45 times (94 °C for 15 s, annealing temperature for 45 s, 60 °C for 45 s); in addition, a nontemplate control (ddH<sub>2</sub>O control) was analyzed for each mastermix. All of the samples were amplified simultaneously in triplicate in a one assay run.

#### Statistical analysis

In our experiment, the relative expression analysis of target gene was performed using a software, named REST-XL© [29] (relative expression software tool, available at <http://www.wzw.tum.de/gene-quantification/>), which compares the expression of target gene in sample relative to control on the basis of Eq. (1) and tests the group differences for significance with a newly developed pairwise fixed reallocation randomization test (<http://www.biostat.ac.uk/smart/unix/mrandt/slides/frames.htm>).  $P$  values less than 0.05 were regarded as statistically significant.

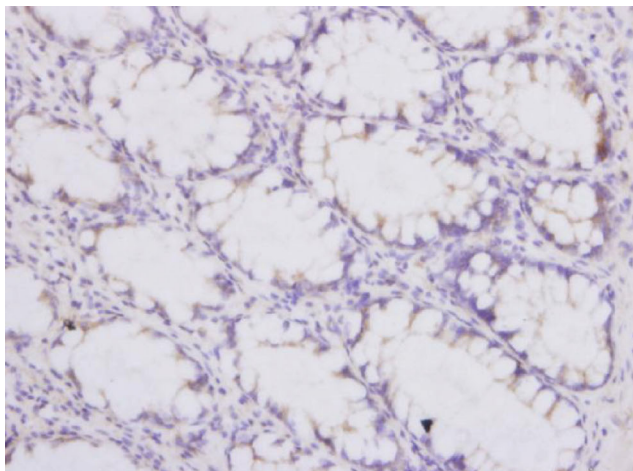
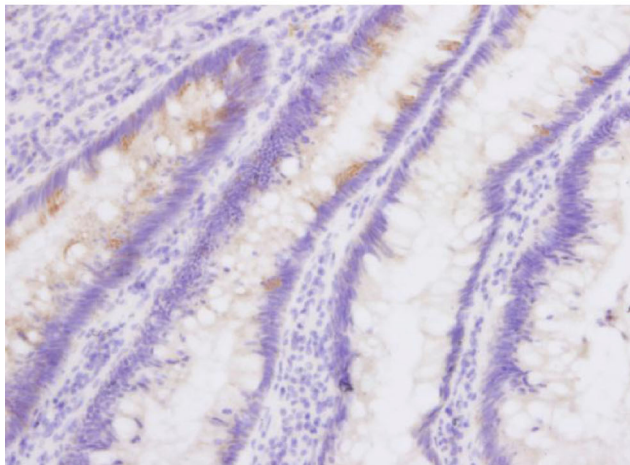
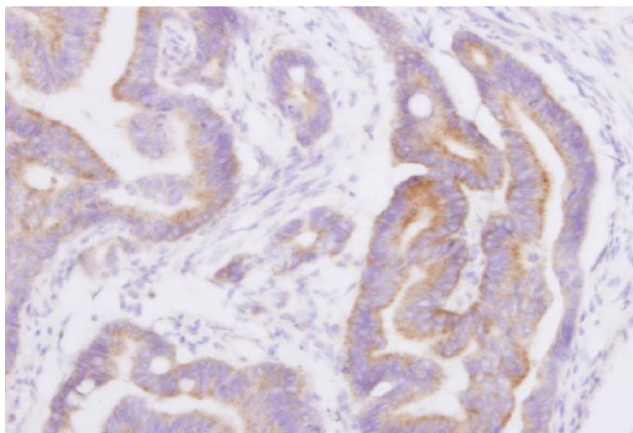
## Results

#### Immunohistochemistry

In colorectal adenoma, the expression of livin protein was expressed in the cytoplasm of cell and mainly present in the base of crypts, and no expression was on surface mucosa

**Table 1** Specific primers and probes for livin and β-actin gene

	Livin	β-Actin
Forward primer	5'-GGCGTGGTGGGTTCTGA-3'	5'-AAGGCCAACCGCGAGAA-3'
Reverse primer	5'-CGCACGGCACAAGACGA-3'	5'-CCTCGTAGATGGGCACA-3'
Probe	5'-FAM-TCCAGGCACACCTTGCACGTCT-TAMRA-3'	5'-FAM-TCAACACCCCAGCCATGTACGT-TAMRA-3'

**A** normal colorectal mucosa livin (20×10)**B** Villous adenoma livin (40×10)**C** rectal adenocarcinoma livin (20×10)

**Fig. 1** **a** Normal colorectal mucosa livin (20×10). **b** Villous adenoma livin (40×10). **c** Rectal adenocarcinoma livin (20×10)

(Fig. 1b). However, livin protein was expressed in all epithelia throughout the crypts of adenocarcinoma (Fig. 1c). There was none of accompanying normal mucosa in which livin protein was expressed (Fig. 1a).

#### Real-time RT-PCR

##### *Comparison of livin mRNA levels between adenocarcinoma and normal mucosa*

To compare the general expression level of livin in adenocarcinoma relative to normal mucosa and test the group difference for significance, we put the Ct values for reference and target genes both in sample group (adenocarcinomas) and control group (normal mucosa) into the REST-XL© software to run it and chose 2000 as the randomization number. The Ct values for reference and target gene were jointly reallocated to the control and sample groups (pairwise fixed reallocation), and the expression ratios were calculated on the basis of the Eq. (1); then, the randomization test was performed to test the group difference for significance, and the numeric results of the randomization test were given in the randomization data output box. The relative amounts of livin messenger RNA (mRNA) to  $\beta$ -actin mRNA in the adenocarcinoma group were significantly higher than those in the normal mucosa group ( $P=0.001$ ), and livin was up-regulated in adenocarcinoma group (in comparison to normal mucosa group) by the factor of 44.08 (Table 2).

##### *Comparison of livin mRNA levels between adenocarcinomas and adenomas*

Briefly, to adopt the previous way described in the test, we put the Ct values of reference and target genes both in adenomas (control group) and adenocarcinomas (sample group) into the REST-XL© software and also chose 2000 as the randomization number. To run the software, we obtain that expression of livin mRNA was up-regulated in sample group, in comparison to control group, by the factor of 12.47, and the difference has a statistical significance ( $P=0.001$ , Table 3).

##### *Comparison of livin mRNA levels between adenomas and normal mucosa*

We also compared the livin mRNA expression between adenomas and normal mucosa and stipulated the group of adenomas as sample group and the group of normal mucosa as control group. To employ the same way described previously, we found that the difference of livin mRNA expression between adenomas and normal mucosa did not reach a statistical significance, although the expression of livin mRNA was up-

**Table 2** Randomization data output in comparison of livin mRNA expression between colonic normal mucosa and adenocarcinoma

Type of specimen	Number of specimens	Means of livin gene	Means of reference gene	Expression ratios	<i>P</i> value
Normal mucosa	12	34.15	24.59	–	
Adenocarcinoma	64	28.22	24.12	44.08 <sup>a</sup>	0.001

<sup>a</sup> The ratio of livin mRNA expression, presented as the fold change in adenocarcinoma normalized to an endogenous reference gene and relative to the group of normal mucosa

regulated in adenomas group, in comparison to normal mucosa group, by the factor of 3.66 ( $P=0.196$ , Table 4).

#### *Livin mRNA expression in adenocarcinomas in relation to clinicopathological parameters*

We examined the relationship of livin mRNA expression in adenocarcinomas with patients' age, gender, and tumor location, tumor invasive depth and degree of tumor differentiation, clinical tumor stage or lymph node metastasis, morphological category, and histopathological type. As shown in Table 5, the expression levels of livin mRNA in rectal cancer were significantly higher than those in colonic cancer ( $P=0.001$ ), and livin mRNA expression was strongly related to colorectal cancer invasive depth ( $P=0.009$ ), but not to clinical tumor stage, degree of tumor differentiation, lymph node metastasis, tumor morphological category and pathological type, and patient's age and gender ( $P>0.05$ ).

## Discussions

Adenomas are the precursors of most sporadic colorectal cancers. The adenoma-to-carcinoma sequence is characterized by recognizable histological changes [2, 27, 30]. Lesions, starting with dysplastic aberrant crypt foci and benign tubular adenomas, have the potential to progress to advanced adenomas, which have a significant potential to transform into invasive adenocarcinomas [29, 31, 32]. Livin, as one of the novel human IAP family members, has been studied in some types of cancers, including melanoma, lung cancer, and renal cell cancer [4, 8, 9, 12–14], but little is known about the diversification of livin expression during the colorectal

adenoma-carcinoma sequence and the livin expression in colorectal cancer related to the clinicopathological parameters. In this study, we first found the expression of livin protein during the colorectal adenoma-carcinoma sequence using immunohistochemical assay and quantitatively examined the expression of livin gene by means of real-time RT-PCR in a series of 12 normal mucosa samples, 36 colorectal adenomas, and 64 adenocarcinomas. In accompanying colorectal normal mucosa, there was none of the samples that had livin protein expression, and similar result was reported by Vucic and Yan et al. [3, 11, 12]. Interestingly, we found that the livin protein was expressed in the cytoplasm of cell and mainly expressed in the base of crypts in adenoma, whereas throughout the epithelium in carcinoma. In 12 paired samples of normal mucosa and colorectal cancer, we also found that livin mRNA was significantly up-regulated in adenocarcinomas in comparison with normal mucosa. Our data further showed that livin mRNA expressed in adenoma and its expression were significantly down-regulated in comparison with adenocarcinoma, whereas the difference of livin mRNA expression between adenoma and normal mucosa did not reach statistical significance, although the expression of livin mRNA was up-regulated in the adenoma in comparison with the normal mucosa samples by the factor of 3.66. Taken together, considering the biological function of livin gene, the findings suggest that, compared with the sequence of normal mucosa-adenoma, livin, as one of important factor, could play a role to affect cell apoptosis in the phase of adenoma malignant to adenocarcinoma and possibly imply that the status of livin is likely to be one of main reasons for the reduction of apoptosis in colorectal carcinogenesis, although at present purely speculative. Xi et al. [33] also speculated that livin play important roles in the development and progression of colorectal cancer through the study of correlation between livin

**Table 3** Randomization data output in comparison of livin mRNA expression between adenomas and adenocarcinomas

Type of specimen	Number of specimens	Means of livin gene	Means of reference gene	Expression ratios	<i>P</i> value
Adenoma	36	31.63	23.90	–	
Adenocarcinoma	64	28.22	24.12	12.47 <sup>a</sup>	0.001

<sup>a</sup> The ratio of livin mRNA expression, presented as the fold change in adenocarcinomas normalized to an endogenous reference gene and relative to the group of adenoma

**Table 4** Randomization data output in comparison of livin mRNA expression between colonic normal mucosa and adenoma

Type of specimen	Number of specimens	Means of livin gene	Means of reference gene	Expression ratios	P values
Normal mucosa	12	34.15	24.59	–	
Adenoma	36	31.63	23.90	3.66 <sup>a</sup>	0.196

<sup>a</sup> The ratio of livin mRNA expression, presented as the fold change in adenomas normalized to an endogenous reference gene and relative to the group of normal mucosa

expression and overall survival in human colorectal cancer. This hypothesis was primarily based on consistent epidemiological evidence and strong biological support for the critical roles of livin (as antiapoptosis factors) in the regulation of cell

**Table 5** Relationship between livin mRNA and clinicopathologic variables of colorectal adenocarcinomas randomization test by REST-XL©

Variables	Colonic adenoma N=64	Randomization test by REST-XL©	
		FURS	P value
<b>Age</b>			
Median age(range)	57.2 (21–80)		
≥60 years	33	–	0.164
<60 years	31	1.61	
<b>Sex</b>			
Male	34	–	0.057
Female	30	1.81	
<b>Location of tumor</b>			
Colon	17	–	0.001*
Rectum	47	3.025	
<b>Degree of differentiation</b>			
Well-moderate 54	–	0.419	
Poor	10	1.41	
<b>Lymph node metastasis</b>			
Negative	37	–	0.878
Positive	27	1.05	
<b>Tumor type</b>			
Mucinous carcinoma 13	–		
Adenocarcinoma 51	1.97	0.061	
<b>Pathologic category</b>			
Ulcerative type 30	–		
Proliferative type 34	1.28	0.422	
<b>Invasive depth</b>			
Submucosa or proper muscle	23	–	0.009*
Serosa or penetrate the serosa	41	2.24	
<b>TNM stage</b>			
I+II	37	–	0.889
III+IV	27	1.05	

FURS factor of up-regulated in sample group

\* are statistically significant values

growth and differentiation. Similar results are reported by Kempkensteffen et al. [5], who showed that livin expression was present at early tumor stages and well-differentiated renal cell carcinomas and that up-regulation of livin expression may occur early during tumor development of renal cell carcinoma. Tanabe et al. [34] also demonstrated that livin was expressed in early-stage nonsmall cell lung cancer. Furthermore, Kawasaki et al. [2] studied survivin expression, another human IAP family member, and showed that survivin expression, like livin expression in the phase of adenoma malignant to adenocarcinoma, was markedly increased at the transition from adenomas to adenocarcinomas, whereas apoptosis index was significantly decrease in this sequence. Therefore, those findings may partly explain why the cell apoptosis was significantly decreased, may be because of the up-regulation of some IAP family member in the phase of adenoma malignant to adenocarcinoma.

Up to now, data on livin expression have been controversial in normal tissue. Although most studies did not detect livin protein in normal tissue [12, 16, 17, 20], Vucic et al. [11] found livin expression in a normal testicular sample using Northern blot analysis. Wagener et al. [35] also found livin protein expression in nontumorous adult kidney and that livin protein expression was only detectable in specific cell types and restricted to the cytoplasm in nontumorous kidney tissue. In our study, we used real-time RT-PCR to quantify the expression of livin mRNA among normal mucosa, adenoma, and adenocarcinoma. Although livin gene expression was in substantially lower levels in the corresponding colorectal normal mucosa compared to adenoma and adenocarcinoma, the expression of livin mRNA was present in colorectal normal mucosa, but no expression of livin protein was detected in all of normal colorectal mucosa. Considering with the application of this highly sensitive technique [29, 34] and livin protein, which was easily cleaved by many protein, including caspase3/7 and Omi/HtrA2 protease [36], our results showed that the expression of livin mRNA was present in colorectal normal mucosa, but livin protein was not present in colorectal normal mucosa. Similar findings were observed by Myung et al. [37] who showed that the livin protein did not or only weakly immunostained in normal colorectal mucosa, and they also confirmed up-regulation of livin expression in cancer tissues compared to that in paired normal mucosa at the RNA and protein levels. Xi and Liu et al. [16, 33] also showed

that no expression of survivin and livin was detected in normal colorectal mucosa.

An increasing resistance toward apoptosis may enhance the metastatic potential of tumor cells [12–14]. In our study, according to the differences in tumor location, tumor invasive depth, degree of tumor differentiation, clinical tumor stage or lymph node metastasis, morphological category, histopathological type, and patient's age and gender, we divided all samples of adenocarcinomas into different groups and further compared the difference of livin mRNA expression in each group, and we observed that livin overexpression was significantly associated with invasion depth, but not with lymph node metastasis, clinical tumor stage and tumor differentiation, tumor morphological category and pathological type, and patient's age and gender. The result was partly similar to the findings by Xi et al. [33] who reported that the livin protein expression was not correlated with patient's age and gender, degrees of differentiation, and TNM stage in colorectal cancer. We also observed that the expression level of livin mRNA in patients with lymph node metastasis was up-regulated in comparison with those in patients without lymph node metastasis, but the difference has no statistical significance. One of the reasons why the difference had no statistical significance may be because of the small number of the samples. Interestingly, considering with the recent epidemiological studies that have reported a proximal migration of colorectal cancers evidenced by increased incidence of right-sided colon cancers and a decrease in incidence of rectosigmoid tumors, suggestive of different risk factors associated with carcinogenesis in proximal and distal colon and with these data support that cancers originating from these two different anatomical locations should have distinct molecular pathogenesis, we first observed that livin expression in rectal cancers was significantly higher than in colonic cancers. Similar report was observed by Minoo et al. [38] who showed that MSI-H status is more common in proximal colon cancers as compared to distal colon and rectum.

These data suggest that livin overexpression plays an important role in tumor invasiveness by mediating resistance to apoptosis. Although at present purely speculative, one could envision that the aberrant expression of livin maybe one of the several factors contributing to tumorigenesis in the sequence of adenoma-carcinoma and may thus constitute to play an important role in tumor invasiveness. Our results are consistent with the other reports and suggest that targeting livin has the potential for the treatment of colorectal cancer.

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**Conflicts of interest** None.

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