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Original Article

Reticulocalbin 2 correlates with recurrence and prognosis in colorectal cancer

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Abstract: Reticulocalbin (RCN) family members could play oncogenic roles in human malignancies and facilitate tumor cell proliferation and metastasis. However, the expression pattern and potential function of Reticulocalbin 2 (RCN2) in colorectal cancer has not been addressed yet. In the present study, we investigated the protein expression of RCN2 by immunohistochemistry assay, and analyzed its association with tumor progression, recurrence and prognosis in 326 cases of patients. Results suggested that the expression of RCN2 was up-regulated in colorectal cancer compared with paired adjacent nontumor specimens. RCN2 expression was closely related to tumor size and the depth of invasion. Kaplan-Meier analysis proved that RCN2 was associated with both disease-free survival and overall survival of patients with colorectal cancer. Moreover, cox's proportional hazards analysis showed that high RCN2 expression was an independent prognostic marker of poor outcome. Consistently, overexpressing RCN2 promoted CRC cell proliferation both *in vitro* and *in vivo* and knockdown RCN2 showed the opposite results. These results provided the first evidence that RCN2 level was increased in colorectal cancer and significantly correlated with tumor growth and proliferation. It also indicated that RCN2 might serve as a potential marker of tumor recurrence and prognosis of colorectal cancer.

Keywords: RCN2, colorectal cancer, immunohistochemistry, recurrence, prognosis

Introduction

Reticulocalbin (RCN) is one member of the Ca^{2+}-binding proteins in the secretory pathway and is localized in the endoplasmic reticulum [1]. RCN was initially found in the mouse teratocarcinoma cell line by Ozawa *et al.* in 1993 [2]. Subsequently, a human RCN gene was cloned from the human transitional cell carcinoma cell line by Ozawa *et al.* in 1995 [3]. Recently, in addition to RCN, some RCN family proteins are identified in both vertebrates and invertebrates [4]. Although the exact functions of RCN remain unknown, it may play important roles in the maintenance of normal cell behavior and life. One of the potent evidence is that homozygous deletion of the RCN gene is lethal for mice [5]. Accumulating evidences suggest that RCN family members may also play a role in tumorigenesis, tumor invasion, and drug resistance. It was found that RCN1 level was significantly increased in a variety of solid tumors, such as renal cell carcinoma [6], breast [7], colorectal [8], lung [9] and liver cancer [10], and could facilitate tumor invasion and metastasis in these malignancies. In addition, ERC55, one of the RCN family proteins, was reported to play an important role in the oncogenesis of human cervical carcinomas infected by the papilloma virus [11]. Furthermore, Hou et al. [12] reported that RCN3, a new member of RCN family members, may be involved in the development of non-small cell lung cancer. These results indicated that RCN family members could play oncogenic roles in human malignancies and facilitate tumor cell invasion and metastasis.

Reticulocalbin-2 (RCN2, also named as ERC-55), is a 55-kDa Ca^{2+}-binding protein containing six EF-hands, which was identified to be localized in endoplasmic reticulum [13, 14]. Nevertheless, the reports on the role of RCN2 in various cancers are very limited. Ding et al. [15] found that RCN2 was upregulated in tumors compared with adjacent non-tumorous tissues in hepatocellular carcinoma patients.

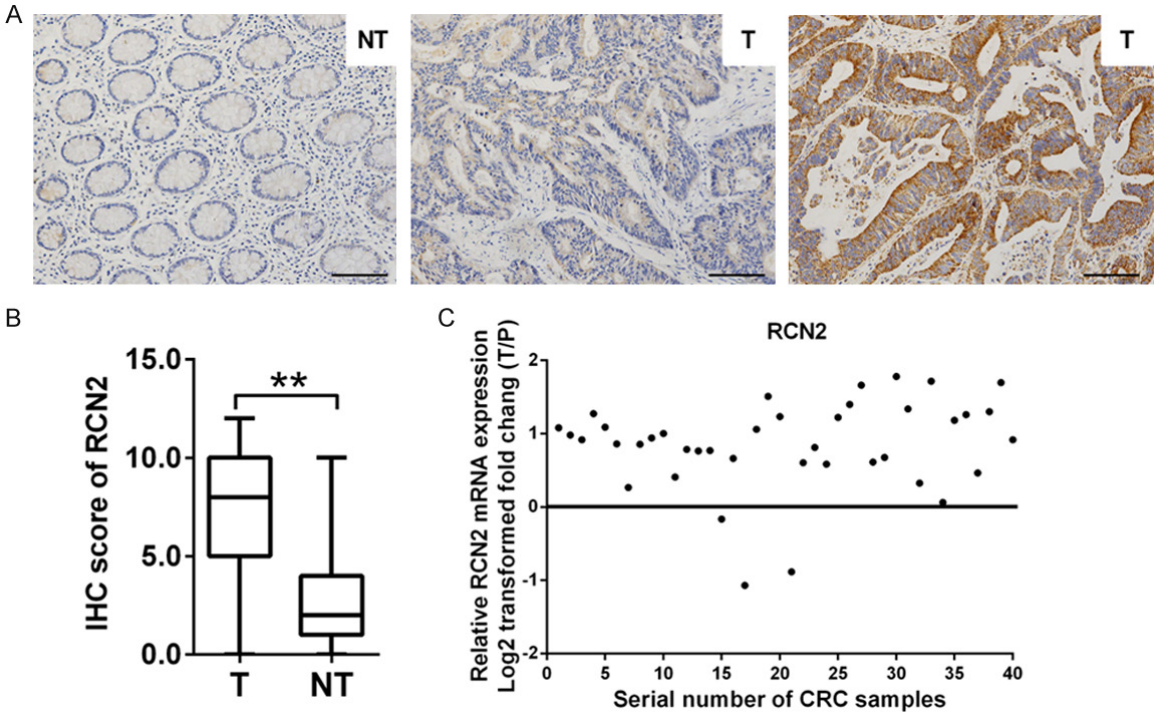

Figure 1. A significant over-expression of RCN2 in CRC tissues. A. IHC assays of RCN2 expression in 326 paired CRC samples and adjacent non-tumorous tissues. The left panel represents low RCN2 expression in adjacent non-tumorous tissues. The middle and right panel represents low and high RCN2 expression in CRC. Scale bar, 100 μm. B. RCN2 expression levels were compared with CRC and paired adjacent non-tumorous specimens. C. qRT-PCR analyses for expression levels of RCN2 in 40 paired tissues from CRC patients. The relative expression ratio of tumor to peritumor was log2 transformed. The data were displayed according to serial patient ID number. Statistical analysis was performed by paired-samples t-test. **P < 0.01. NT: non-tumorous tissues; T: tumorous tissues; CRC: colorectal cancer.

Furthermore, they proved that RCN2 plays a pivotal role in HCC cell proliferation and tumor growth. However, till now, the expression level and potential function of RCN2 remain unclear in colorectal cancer.

Therefore, in order to clarify the expression level and explore the potential function of RCN2 in colorectal cancer, we investigated the protein expression level of RCN2 in clinical specimens by immunohistochemistry assay, analyzed the association of RCN2 level with clinicopathological characteristics and postoperative survival of patients in the present study.

Results

RCN2 expression detected in clinical specimens

In the present study, we examined RCN2 expression in human colorectal cancer specimens by immunohistochemistry, including 236 cases of human colorectal cancer samples and 236 paired adjacent nontumor samples. According to the immunohistochemical staining evaluation protocol described in methods, 169 cases were defined as high RCN2 expression and 157 cases were defined as low RCN2 expression in human colorectal cancer samples. Among the 236 cases of paired adjacent nontumor specimens, high RCN2 expression was found in 59 cases of specimens and low expression was detected in 177 cases of specimens. With regard to its subcellular localization, RCN2 was found to be localized in cytoplasm (**Figure 1A**). Immunohistochemical results indicated that the positive rate of RCN2 in colorectal cancer specimens was significantly increased, compared with that in paired adjacent nontumor specimens ($P < 0.01$) (**Figure 1B**). Consistently, quantitative real-time reverse transcription (qRT-PCR) analysis showed that RCN2 was upregulated in CRC tissues at mRNA level (**Figure 1C**). These results suggested that

Table 1. Relationship between tumor RCN2 expression and clinic features

Variables	No. of cases	RCN2 expression		*P* value
		Low	High	
All	326	157	169	
Age				0.507
<60	170	85	85	
≥60	156	72	84	
Gender				0.824
Female	148	70	78	
Male	178	87	91	
Tumor size				0.003*
<5.0 cm	140	81	59	
≥5 cm	186	76	110	
Differentiation grade				0.311
Well	39	18	21	
Moderately	239	111	128	
Poor	48	28	20	
Depth of invasion				0.006*
T1+T2	120	70	50	
T3+T4	206	87	119	
Lymph node metastasis				0.819
Absent	122	60	62	
Present	204	97	107	
Distant metastasis				0.254
Absent	283	140	143	
Present	43	17	26	
TNM stage				0.015*
I + II	153	85	68	
III + IV	173	72	101	

Abbreviations: TNM, tumor-nodes-metastases; *P value < 0.05 was considered statistically significant.

the expression of RCN2 was upregulated in colorectal cancer and abnormal RCN2 expression might be related to the progression of colorectal cancer.

Association of RCN2 level with clinicopathologic characteristics

As results showed an increased expression pattern of RCN2 in colorectal cancer tissues, we next examined the relationship between RCN2 expression and selected clinicopathological parameters in colorectal cancer patients involved. The statistical analysis results showed that RCN2 was significantly associated with tumor size (P = 0.003). Moreover, a significant correlation was identified between RCN2 and depth of tumor invasion (P = 0.006) and TNM stage (P = 0.015). Nevertheless, no statistically significant associations were observed regarding gender (P = 0.824), age at diagnosis (P = 0.507), tumor site (P = 0.026), lymph node metastasis (P = 0.819) or distant metastasis (P = 0.254). The results are summarized in **Table 1**.

Association of RCN2 level with disease-free survival

Kaplan-Meier analysis was used to evaluate the disease-free survival of patients with colorectal cancer and RCN2 expression. The results showed that patients with tumors of RCN2 high expression had unfavorable disease-free survival compared with those with tumors of RCN2 low expression (**Figure 2**, log-rank test: P = 0.017). The median disease-free survival of patients with RCN2 positive tumors was 47.5 months (95% CI: 27.7-67.3). While the median disease-free survival time of patients with RCN2 low expression tumors cannot be estimated due to the fact that more than half of patients survived. This survival pattern indicated that patients with colorectal cancer of high RCN2 expression had a higher risk of tumor relapse compared with those with low RCN2 expression. In univariate survival analysis, tumor size (log-rank test: P = 0.003), depth of invasion (log-rank test: P = 0.002), and TNM stage (log-rank test: P < 0.001) were also proved to be associated with disease-free survival of these patients, which indicates that patients with colorectal cancer of large tumor size, deep depth of invasion or advanced TNM stage had shorter disease-free survival and higher risk of relapse than those without. However, no statistically significant associations were observed between disease-free survival and gender, age, or tumor site. The unadjusted hazard ratio (HR) was shown in **Table 2**.

To verify the independent effect of RCN2 level on disease-free survival of patients involved, cox proportional hazards model analysis was performed adjusting for gender, age at diagnosis, tumor depth of invasion and TNM stage, which aimed to control for confounding factors. The results showed that high RCN2 level was independently associated with unfavorable disease-free survival of patients after controlling for all these factors. The adjusted

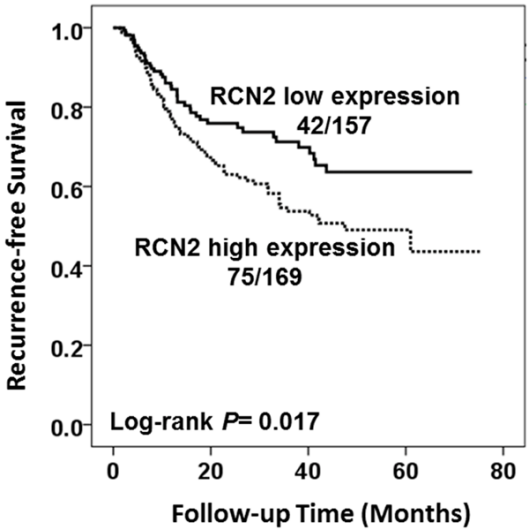

Figure 2. Kaplan-Meier curve analysis of disease-free survival in CRC patients by the expression of RCN2. Recurrence/total number of patients in each subgroup were presented. Log-rank test was used to calculate the difference significance.

HR of patients with colorectal cancer of high RCN2 expression was 0.82 (95% CI: 0.56-0.21, P = 0.034), compared with those with RCN2 low expression tumors (**Table 2**). Moreover, tumor size, tumor depth of invasion and TNM stage were also found to be independently correlated to disease-free survival of patients in multivariate analysis. These results suggested that patients with high RCN2 expression would have a higher risk to relapse than those with low level of RCN2.

Association of RCN2 expression with overall survival of patients

Similar to the results on disease-free survival, RCN2 expression level was found to be statistically related to overall survival of patients. Univariate survival analysis results showed that patients with tumors of high RCN2 expression had unfavorable overall survival, compared with those with tumors of low RCN2 expression (**Figure 3**, log-rank test: P = 0.024). The postoperative median overall survival of patients with RCN2 positive tumors was 52.4 months (95% CI: 31.6-69.4). However, the median overall survival time of patients with RCN2 negative tumors cannot be estimated due to more than half of patients survived. In addition, tumor size (log-rank test: P = 0.007), depth of invasion (log-rank test: P = 0.031) and TNM stage (log-rank test: P < 0.001) were also found to be associated with overall survival of these patients in univariate survival analysis, indicating that patients with large tumor size, deep depth of invasion or advanced TNM stage had unfavorable overall survival. Nevertheless, no statistically significant associations were found between overall survival and gender, age, or tumor site. The unadjusted hazard ratio (HR) was shown in **Table 3**.

Multivariate analysis indicated that RCN2 expression could be a prognostic factor for overall survival of patients with colorectal cancer after adjusting for gender, age at diagnosis, depth of invasion and TNM stage. In multivariate analysis, tumor size, depth of invasion and TNM stage were also found to be independently correlated to overall survival of patients. Nevertheless, no statistically significant associations were observed between overall survival and gender, age or tumor site (**Table 3**).

Overexpressing RCN2 promotes CRC cell proliferation both in vitro and in vivo

To determine the functional role of RCN2 in CRC cell, two CRC cells (SW480 and SW620) with moderate RCN2 expression were selected to establish cell models of RCN2 overexpression or knockdown (**Figure 4A** and **4B**). As shown in **Figure 4C**, CRC cells with RCN2 overexpression showed significantly elevated protein level. Given the previous results that RCN2 was significantly associated with tumor size, tumor invasion and TNM stage, we determined the effect of RCN2 on cell proliferation *in vitro*. Confirmedly, we found that forced expression of RCN2 showed significantly increased ability of cell proliferation (**Figure 4D**). Moreover, colony formation assay showed that overexpression of RCN2 could significantly elevate the ability of cell colony formation in CRC cells (**Figure 4E**). In addition, xenograft nude mice mode showed that elevated the level of RCN2 significantly enhanced tumor tumorigenesis capacity *in vivo* (**Figure 4F**). Furthermore, IHC staining of Ki-67, a proliferative cell marker, was performed in these tumors. As shown in **Figure 4G**, we found that the level of Ki-67 had a positive correlation with RCN2. In conclusion, the results demonstrated that highly expressed RCN2 could promote CRC cell proliferation both *in vitro* and *in vivo*.

Table 2. Association of RCN2 and clinical factors with disease-free survival

	Unadjusted HR* (95% CI)	*P*	Adjusted HR† (95% CI)	*P*
RCN2 high expression	0.633 (0.434-0.924)	0.018	0.820 (0.555-1.210)	0.034
Gender	1.118 (0.118-1.607)	0.547	-	-
Age at diagnosis	0.665 (0.459-0.962)	0.058	-	-
Tumor site	1.093 (0.759-1.574)	0.634	-	-
Tumor size	0.550 (0.372-0.813)	0.003	0.695 (0.466-1.036)	0.026
Depth of invasion	1.803 (1.237-2.628)	0.002	0.577 (0.385-0.866)	0.012
TNM stage	5.985 (3.708-9.660)	<0.001	2.209 (1.127-3.342)	0.008

*Hazard ratios in univariate models. †Hazard ratios in multivariable models. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

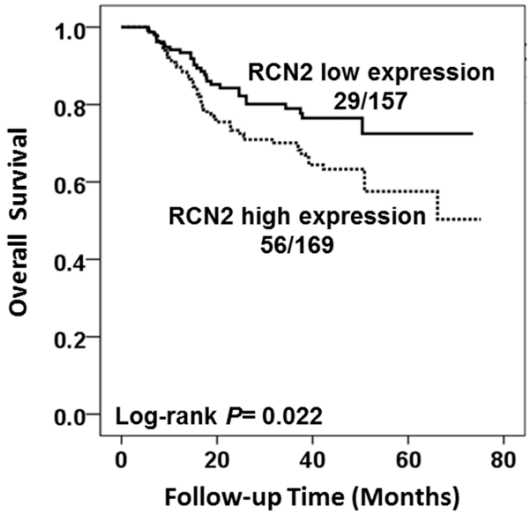

Figure 3. Kaplan-Meier curve analysis of overall survival in CRC patients by the expression of RCN2. Death/total of patients in each subgroup were presented. Log-rank test was used to calculate the difference significance.

Knockdown RCN2 inhibits CRC cell proliferation both in vitro and in vivo

We then established cell model with RCN2 knockdown using the same CRC cells. As shown in **Figure 5A**, SW480 and SW620 with RCN2 silencing showed RCN2 low expression. Next, the role of RCN2 *in vitro* CRC cell proliferation was determined by cell counting assay. Our results showed that CRC cells with RCN2 knockdown significantly decreased the ability of cell proliferation which was opposite to that of RCN2 overexpression (**Figure 5B**). In addition, colony formation assay showed the similar results (**Figure 5C**). Consistently, stable knockdown RCN2 significantly inhibited tumor growth *in vivo* (**Figure 5D** and **5E**). Taken together, these results suggest that knockdown RCN2 could inhibit CRC cell proliferation both *in vitro* and *in vivo*.

Discussion

Colorectal cancer (CRC) is one of the most prevalent malignancies throughout the world and the fourth most common cancer cause of death globally [16]. Several risk factors for CRC have been reported, including western dietary patterns [17], familial adenomatous polyposis [18], ulcerative colitis [19] and colorectal adenomas [20]. Although great progress has been made in the clinical diagnosis and treatment of CRC, the absence of well-defined molecular targets makes treatment of CRC challenging. Considering the high mortality rate of CRC and poor patient response to current therapies, the discovery of novel biomarkers involved in this pathophysiologic process will shed light on the understanding of the molecular mechanisms underlying cancer progression, providing more effective management strategies.

Recently, studies on RCN2 functions mainly focused on its role in differentiation and endocrine regulation in mouse [21, 22]. For example, RCN2 is involved in the regulation of extraembryonic endoderm differentiation of mouse embryonic stem cells. Manichaikul et al. [22] found that RCN2 can act as a novel regulator of cytokine expression in an atherosclerotic mouse model. Another study has suggested that RCN2 could be a potential tumor-associated antigen for mammary cancer immunological prevention [23]. Up to now, the experimental evidence uncovering the role of RCN2 in cancer is very limited. However, RCN1, a homology of RCN2, has been found aberrantly expressed in several cancerous cell lines. Furthermore, RCN1 has been identified as a potential tumor

Table 3. Association of RCN2 and clinical factors with overall survival

	Unadjusted HR* (95% CI)	*P*	Adjusted HR† (95% CI)	*P*
RCN2 high expression	0.596 (0.381-0.934)	0.024	0.806 (0.510-1.272)	0.041
Gender	1.116 (0.729-1.709)	0.612	-	-
Age at diagnosis	1.403 (0.911-2.161)	0.125	-	-
Tumor site	0.813 (0.531-1.245)	0.342	-	-
Tumor size	0.247 (0.139-0.438)	0.007	0.314 (0.176-0.561)	0.035
Depth of invasion	0.636 (0.411-0.986)	0.043	0.792 (0.508-1.233)	0.031
TNM stage	0.189 (0.108-0.333)	<0.001	0.24 (0.136-0.425)	0.012

*Hazard ratios in univariate models. †Hazard ratios in multivariable models. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

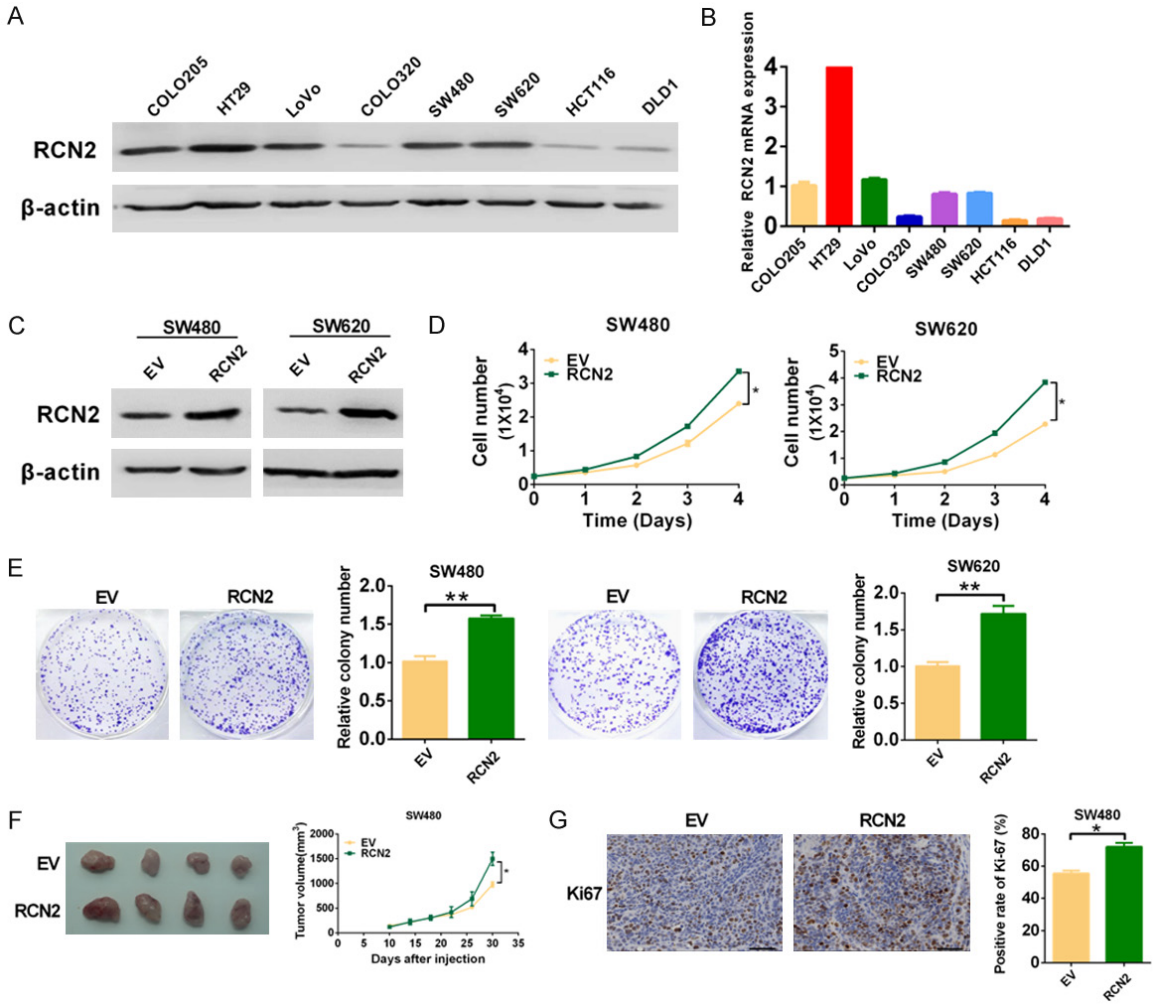

Figure 4. Overexpressing RCN2 promotes CRC cell proliferation both *in vitro* and *in vivo*. A, B. Protein and mRNA expression levels of RCN2 in different CRC cell lines. C. RCN2 expression in SW480 and SW620 cells, which were transiently transfected with expression vector as indicated. D. Cell counting of CRC cells with RCN2 overexpression. E. Colony formation assay of CRC cells with RCN2 overexpression. F, G. Tumor growth curves and comparison of Ki-67-positive cells in tumor tissues of subcutaneous xenograft tumor model developed from CRC cells which were stably transfected with forced-expression vector (n = 4). Scale bar, 50 μm. RCN2, expression vector encoding RCN2; EV, empty vector; Data shown are the mean ± S.E.M. from three independent experiments. *P<0.05; **P<0.01.

biomarker in renal cell carcinoma [6], breast [7], colorectal [8], lung [9] and liver cancer [10]. Moreover, a recent study indicates that RCN1 can confer non-small-cell lung cells resistance

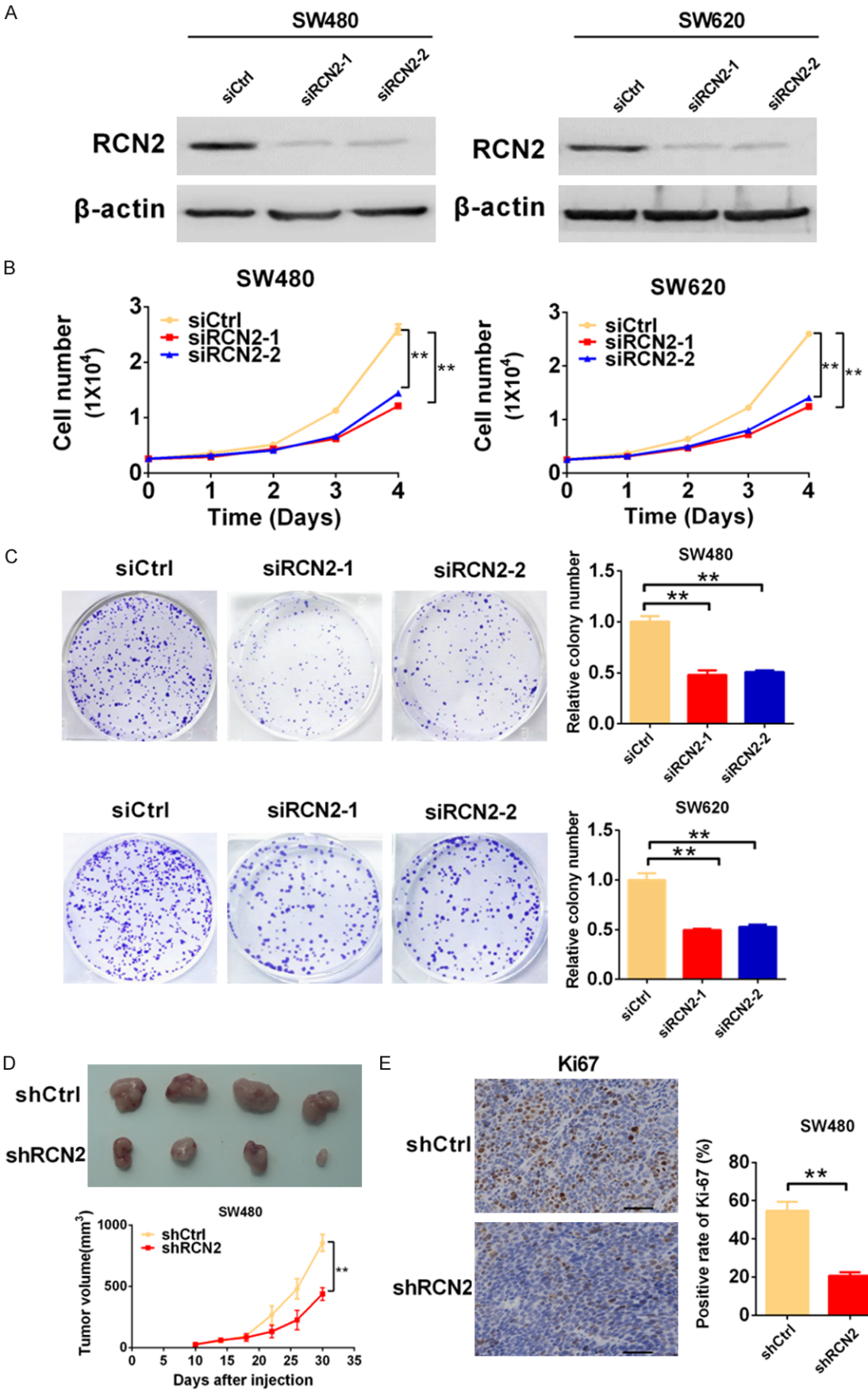
A
SW480
SW620
siCtrl
siRCN2-1
siRCN2-2
RCN2
β-actin
B
SW480
SW620
Cell number (1X10^4)
Time (Days)
C
siCtrl
siRCN2-1
siRCN2-2
Relative colony number
D
shCtrl
shRCN2
Tumor volume(mm^3)
Days after injection
E
Ki67
Positive rate of Ki-67 (%)

Figure 5. Knockdown RCN2 inhibits CRC cell proliferation both *in vitro* and *in vivo*. A. RCN2 expression in SW480 and SW620 cells, which were transiently transfected with siRNA as indicated. B. Cell counting of CRC cells with RCN2 knockdown. C. Colony formation assay of CRC cells with RCN2 knockdown. D, E. Tumor growth curves and comparison of Ki67-positive cells in tumor tissues of subcutaneous xenograft tumor model developed from CRC cells which were stably transfected with shRNA of RCN2 (n = 4). Scale bar, 50 μm. siRCN2-1 and siRCN2-2, siRNAs against RCN2; siCtrl, control siRNA. shRCN2, shRNA expression vector against RCN2; shCtrl, control shRNA. Data shown are the mean ± S.E.M. from three independent experiments. *P<0.05; **P<0.01.

to chemotherapy [9]. While RCN1 has been studied extensively, the role of RCN2 in oncogenesis and progression, especially CRC pathogenesis remains to be explored.

Therefore, we conducted the present study to determine RCN2 expression level and its association with clinical features, disease-free survival and overall survival of patients with colorectal cancer. RCN2 staining results indicated that RCN2 level in colorectal cancer was upregulated compared with that in paired adjacent nontumor tissues. Further statistical analysis revealed that high RCN2 expression level in colorectal cancer was significantly associated with tumor size, depth of invasion and advanced TNM stage. High RCN2 expression was more frequently to be detected in tumors with large tumor size, deep depth of invasion or advanced TNM stage in our investigation, indicating that RCN2 might promote tumor growth and progression in CRC. Next, we analyzed the association of RCN2 level with disease-free and overall survival. In our study cohort, high RCN2 expression was found to be correlated with unfavorable disease-free and overall survival. In both univariate and multivariate survival analysis, the prognostic impact of RCN2 for disease-free and overall survival was both statistically significant. Moreover, overexpressing RCN2 promoted CRC cell proliferation both *in vitro* and *in vivo* and knockdown RCN2 showed the opposite results. The results proved that RCN2 might be an independent molecular marker of tumor recurrence and survival for patients with CRC. Therefore, our findings have important clinical significance and applicable value.

It has been reported that RCN2 could affect dimerization and internalization of epidermal growth factor receptor (EGFR), and probably participated in the regulation of EGFR-ERK pathway and cell proliferation [15]. In hepatocellular carcinoma (HCC), RCN2 knockdown cells exhibited significant decrease in cell growth rate compared with control cells. Additionally, exogenous expression of RCN2 in knockout HCC cells significantly promoted cell growth, accelerated G1/S transition and increased cyclin D1 expression [15]. Overexpressed RCN2 not only correlates with disease progression in HCC but also plays a pivotal role in the developed resistance to tyrosine kinase inhibitors (TKIs) [15]. These findings may provide clues for the mechanism of the tumor promoting role of RCN2 in colorectal cancer demonstrated by our investigation. In this study, our data demonstrated that RCN2 promoted CRC cell proliferation both *in vitro* and *in vivo* which was consistent with clinical feature of patients with different RCN2 expression. However, further studies are still needed to explore the molecular mechanism of RCN2 in colorectal cancer.

In conclusion, we proved that RCN2 expression level in human CRC was up-regulated and significantly associated with tumor cell growth and proliferation. Our results also proved for the first time that RCN2 was independently associated with disease-free and overall survival of patients with CRC. In addition, our data also showed that RCN2 promoted CRC cell proliferation *in vitro* and *in vivo*. These results indicated that RCN2 may be a potential predictive marker of tumor recurrence and prognosis for patients with CRC.

Materials and methods

Cell culture and tissue collection

Human CRC cell lines SW480 and SW620 were routinely cultured in RPMI-1640 medium (Gibco) respectively, supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin (PAN Biotech, Adenbach, Germany). In accordance with the Helsinki declaration, all patients or family members involved have signed an informed consent form. The study cohort consisted of 236 cases clinical specimens from patients who were diagnosed as colorectal cancer between January 2011 and June 2013 in the Department

of General Surgery, Tangdu Hospital affiliated with the Fourth Military Medical University in Xi'an, China. The histomorphology of all tissue specimens were confirmed by the Department of Pathology, Tangdu Hospital. Patients with following criteria were subsequently excluded: refused consent; received treatment prior to surgery including neoadjuvant chemotherapy; diagnosed as colorectal stromal tumor; harvested insufficient specimens for protein expression evaluation; diagnosed with additional cancers. Clinicopathologic information and follow-up data of the remaining 236 patients were prospectively entered into a database, which was under a close follow-up scheme and updated with respect to survival status every three month by telephone visit and questionnaire letters. The present study has been approved by the Ethics Committee of Fourth Military Medical University.

Measurement of endpoints

Disease-free survival is defined as the time elapsed from surgery to the first occurrence of any of the following events: recurrence of colorectal cancer; distant metastasis of colorectal cancer; or death from any cause without documentation of a cancer-related event; development of second non-colorectal malignancy excluding basal cell carcinomas of the skin and carcinoma in situ of the cervix. The diagnosis of recurrence and distant metastasis was based on the imaging method such as computed tomography, endoscope, ultrasonography, magnetic resonance imaging and position emission tomography. Cytologic analysis or biopsy was performed if possible. Overall survival is defined as the time elapsed from surgery to death of patients with colorectal cancer. Death of participants was ascertained by reporting from the family and verified by review of public records. The disease-free and overall survival status was assigned by trained staff blinded to other clinicopathologic and RCN2 expression data.

Immunohistochemistry assay and staining evaluation

Immunohistochemistry assay on RCN2 was performed in all the 236 cases of colorectal cancer and 236 paired adjacent non-tumor samples. In addition, cell proliferation-associated nuclear antigen Ki67 was assessed in nude mice xenograft samples. Fresh tissues were fixed in 10% formalin and embedded in paraffin wax. One of the deepest sections from each tumor was selected for evaluation, and 4-μm sections were examined by immunohistochemistry. Tissue sections were deparaffinized in xylene, and then rehydrated in graded concentrations of ethyl alcohol. Antigen retrieval was performed by placing the tissues in sodium citrate buffer and applying a high voltage for 3 min (pH 6.0), followed by natural cooling. Then sections were placed in 3% H_2O_2 for 15 min to inhibit the endogenous peroxide activity, washed three times with phosphate-buffered saline (PBS) buffer for 5 min and placed in normal goat serum as blocking antibody at room temperature for 20 min. The primary antibody against RCN2 and Ki67 utilized was rabbit polyclonal antibody (1:200; Proteintech, Chicago, IL, USA), (1:200; Maixin Biotech, Fuzhou, China) respectively. After incubation at 4°C for 18 h, sections were washed three times with PBS buffer for 15 min. The samples were then incubated with the biotinylated goat anti-rabbit secondary antibodies and streptavidin peroxidase at 37°C for 20 min. Subsequently, the sections were washed with distilled water and PBS for 15 min. Prepared fresh 3,3'-diaminobenzidine (DAB) solution was dropped onto the slides, which were then incubated at 37°C for 5 min. The sections were then counterstained in hematoxylin, washed with distilled water, differentiated with 1% hydrochloric acid alcohol, washed with distilled water. The expression level of RCN2 was independently evaluated by 2 pathologists who were blind to the clinical data, according to the proportion and intensity of positive cells that were determined within 5 microscopic visual fields per slide. In brief, for quantitative scoring analysis, the percentage of positive stained cells was categorized as assigned to the one of five categories: score 0, 0-9%; score 1, 10%-25%; score 2, 26%-50%; score 3, 51%-75%; score 4, 76%-100%. The staining intensities was independently categorized as no staining (score 0), weak (score 1), moderate (score 2), or strong (score 3). The total score was calculated as the product of intensity and extent, ranging from 0 to 12.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Briefly, Total RNA was isolated from CRC tissues and cells and was used for reverse transcription. The cDNA was performed for qRT-

PCR analysis to evaluate the relative expression levels of RCN2. The sequences of primers were shown as followings: 5'-AGATGTATGA TCGTGTGATTGACT-3' and 5'-GGACCTGAATCC-TGGTTAGC-3' for RCN2. For β-actin, the sequences were 5'-TGACCCAGATCATGTTTGAG-3' and 5'-CGTACAGGGATAGCACAG-3'.

Western blot analysis

Protein lysates were separated using SDS-PAGE, transferred to polyvinylidenedifluoride (PVDF) membrane (Millipore, Bedford, MA), hybridized with primary antibodies against RCN2 (1:1000), and β-actin (1:3000, Sigma) overnight at 4°C, and then incubated with HRP-conjugated secondary antibodies. Immunoblots were detected using the enhanced chemiluminescence reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

Knockdown and forced expression of target genes

For the generation of shRNA expression vectors, a specific sequences targeting the human RCN2 mRNA sequence (5'-GACGGAAUU-UGUCAUUCAATT-3') was cloned into the pSilencer™ 3.1-H1 puro vector (Ambion). For overexpression, the coding sequence of mtSSB was amplified from cDNA derived from SW480 cells using primers (RCN2-sense: 5'-CCGACT-CGAGCGGATGCGGCTGGGCCCG-3'; RCN2-antisense: 5'-CGGGGTACCGTAAGCTCATCATGATA-3') and cloned into the pcDNA™ 3.1(+) vector (Invitrogen). Stable transfectants were generated after selection with G418 (Sigma-Aldrich, A1720) for 3 weeks. All siRNAs were synthesized by GenePharma (Shanghai, China). The sequences of siRNA for RCN2 were 5'-GA-CGGAAUUUGUCAUUCAATT-3' for siRCN2-1; 5'-CUUGGGUAGUACCUAAUAATT-3' for siRCN2-1 and 5'-UUCUCCGAACGUGUCACGUTT-3' for negative control siRNA. Both the vectors and siRNAs were transfected with Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's protocol.

Determination of in vitro cell proliferation

CRC cells were e transfected with vectors or siRNAs for 48 h. Then cells were seeded into 24-well plates at a density of 0.25×10^4 cells/well. Cells were trypsinized and counted at 0, 1, 2, 3 and 4 days. The number of cells was counted using a Countess® Automated Cell Counter (Invitrogen, Carlsbad, CA, USA). The assays were performed in triplicates.

Plate colony formation assay

Log phase cells with a density of 1000 cells/well were plated into 6-well plates. The colonies of CRC cells were stained with Giemsa after 2 weeks of culture, and the total number of colonies was counted. Each assay was performed in triplicates.

Nude mice xenograft model

Six-week-old BALB/c nude mice weighting 18-22 g were randomly divided into groups. Xenografts were initiated by injection of 1×10^7 CRC cells into the back of nude mice. Ten days later, the length (L) and width (W) of the tumors was measured every 4 days. Tumor volume (V) was calculated with the formula $V = 1/2 \times L \times W^2$. Thirty days later, the mice were sacrificed and tumor nodules were dissected. The study was approved by the ethics committee of the Fourth Military Medical University for animal research.

Statistical analysis

SPSS 17.0 software (SPSS, Chicago, IL) was used for all statistical analyses. Associations between RCN2 expression and categorical variables were analyzed by Pearson X^2 test. Correlation coefficients were analyzed by contingency or Spearman correlation analysis. Survival curves were estimated using the Kaplan-Meier method, and differences in survival distributions were evaluated by the log-rank test. Cox's proportional hazards modeling of factors potentially related to survival was performed in order to identify which factors might have a significantly independent influence on survival. Differences with a *P* value of 0.05 or less were considered to be statistically significant.

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