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Ring finger protein 126: a potential biomarker for colorectal cancer

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Summary. Background. Colorectal cancer (CRC) is the most common cancer of the digestive system. However, effective therapeutic targets against CRC have not been found yet. Further, the relationship between the expression of ring finger protein 126 (RNF126) and CRC is not clear.

Material and Methods. The expression level of RNF126 in CRC tissues and cell lines was detected by immunohistochemical staining and western blot. Subsequently, endogenous *RNF126* expression was inhibited in a CRC cell line using a short hairpin RNA. Next, the effect of RNF126 on the properties of CRC cells was studied through different experimental methods.

Results. We found that the RNF126 protein was mainly localized in the cytoplasm. High RNF126 expression was observed to be an independent risk factor for poor prognosis in CRC patients. *In vitro* studies showed that RNF126 was able to promote the proliferation, migration, and invasion ability of CRC cells.

Conclusion. RNF126 acts as an oncogene during CRC development, and may serve as a novel target for CRC treatment.

Key words: Colorectal Cancer, RNF126, Prognosis, Oncogene

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Introduction

Colorectal cancer (CRC) is the most common cancer of the digestive system, especially widespread in economically developed areas (Siegel et al., 2014; Arnold et al., 2017). According to statistics, there are approximately 1,200,000 new CRC cases and 700,000 CRC-related deaths, annually (Miller et al., 2016; Global Burden of Disease Cancer Collaboration., 2017). Despite the improved prognosis of CRC patients by the surgery-based comprehensive anti-tumor model and the application of new targeted drugs, the five-year survival rate of CRC patients remains unsatisfactory. Tumor metastasis is the main cause of death in CRC patients; therefore, it is critical to explore the mechanism of CRC metastasis and find effective therapeutic targets to improve the prognosis of CRC patients at the molecular level.

Ring finger protein 126 (RNF126), a ubiquitin ligase, is mainly involved in the quality control of cellular proteins (Rodrigo-Brenni et al., 2014). The molecular weight of RNF126 protein is about 34 kDa and contains 311 amino acids. It is widely accepted that failure to identify and degrade defective or redundant proteins can, in time, lead to protein accumulation, which can further lead to the occurrence of cancer, kidney disease, Alzheimer's disease, and other relevant diseases (Hipp et al., 2014; Trigo et al., 2019). RNF126 contains an N-terminal zinc finger domain and a C-terminal RING domain in structure which performs the ubiquitin ligase activity through interaction with an E2 (ubiquitin-conjugating) enzyme (Krysztofinska et al., 2016). Abnormal expression of RNF126 has been closely associated with poor prognosis in a variety of cancers (Wang et al., 2016b). However, to the best of our knowledge, there have not been any studies on RNF126 in CRC. In the present study, we examined the expression of RNF126 in CRC tissues and analyzed its

correlation with the prognosis of CRC patients. Moreover, we investigated the effects of RNF126 on CRC cell functions *in vitro*.

Materials and methods

Clinical specimens

A total of 156 cases of CRC cancer tissues and matched adjacent tissues (defined as at least 5 cm from the cancer tissues with no cancer cells detected under microscope) were collected from Zhongnan Hospital of Wuhan University. All the tissue samples were embedded in paraffin. CRC patients were diagnosed by pathology, with complete clinicopathological data. Of the 156 CRC patients, 113 were male and 43 were female, with a mean age of 56.7±12.5 years (range: 42-75 years). None of them received radiotherapy, chemotherapy, or immunotherapy before surgery. This study followed the Helsinki Declaration, and the collected tissue samples were approved by patients. The study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (No. 2015022).

Cell culture and transfection

The human colon epithelial cell line NCM460, and CRC cell lines (HT-29, HCT-116, SW480, and SW620) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Fisher Scientific UK Ltd., Loughborough, UK) and 1% penicillin/streptomycin at 37°C in an incubator containing 5% CO_2. Culture medium was changed every two days.

Endogenous *RNF126* expression was inhibited using a short hairpin RNA (shRNA). A shRNA targeting *RNF126* was designed and synthesized by GeneChem Technologies (GeneChem, Shanghai, China). In brief, CRC cells were seeded into the 6-well plates and incubated until the cell confluency reached about 50%, followed by the addition of lentivirus into each well in the appropriate amount. The target sequences of *RNF126* shRNA were as follows: sh-1, GCACTCAAACC CTATGGACTA; sh-2, GTCCTGCACTCAAACCCT ATG. In this study, cells treated with empty lentivirus were used as the control group (sh-NC). Finally, western blot was used to confirm the inhibition rate of shRNA.

Immunohistochemical staining (IHC)

The paraffin-embedded tissues were cut into 4 μm-thick sections, dewaxed in xylene, and subsequently hydrated in gradient ethanol. Sodium citrate buffer (pH=6.0) was used for antigen retrieval. Afterwards, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were incubated with Anti-RNF126 antibody (1:100; Abcam, Cambridge, UK) at 4°C overnight. After rinsing with phosphate buffered saline (PBS, pH=7.4) three times, the sections were incubated with anti-Rabbit IgG (Abcam, Cambridge, UK) at 37°C for 1 h. Finally, the sections were stained with hematoxylin for 5 min, fixed with neutral resins, and observed under a microscope (Olympus BX53, Japan). A semi-quantitative scoring method was used to evaluate the intensity of RNF126 expression (0: negative; 1: weak; 2: moderate; 3: strong). The percentage of stained tissue was also estimated and converted to a score (0: none; 1: 1% to 33%; 2: 34% to 66%; 3: 67% to 100%). Afterwards, the two scores were multiplied. A final score of 0-1 indicated low expression of RNF126, ≥2 indicated high expression.

Western blot

RIPA lysis buffer (Applygen, Beijing, China) was used to extract total protein from cells. 30 μg of total protein was separated by polyacrylamide gels, transferred to the PVDF membrane (Millipore, Billerica, MA, US). After blocking with 5% skim milk for 1 h, the PVDF membrane was incubated with the anti-RNF126 antibody (1:1000; Abcam) at 4 °C overnight. The next day, after washing with PBS three times, the membrane was incubated with the anti-Rabbit IgG (Abcam, Cambridge, UK) for 2 h. Finally, the protein bands were visualized by chemiluminescence (Millipore). GAPDH (Cell Signaling Technology, Shanghai, China) was used as the internal reference.

CCK-8

Cell proliferation ability was measured with a Cell Counting Kit-8 kit (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. In brief, cells were seeded in the 96-well plates at a density of 3×10^4 cells/well, followed by addition of CCK-8 reagent at 0, 24, 48, 72, and 96 h. After incubation for 1 h, absorbance was measured at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Measurements were repeated three times at each time point.

Transwell

A Transwell assay was used to detect cell migration and invasion capacities strictly according to the manufacturer's instructions. The Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) were used with or without Matrigel (BD Biosciences). Briefly, cells were seeded into the upper chamber in serum-free medium (1×10^5 cells/well), and 500 μL of complete medium was added to the lower chamber. The cells crossing through the lower membrane were fixed in 3.7% formaldehyde for 5 min, stained with 0.2% crystal violet, and subsequently counted under a microscope (Olympus Corp, Tokyo, Japan).

Wound healing

A wound healing assay was used to assess the migration capacity of the cells. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 cells/well. When cell confluency reached 100%, a 200 μL pipette tip was used to scratch the monolayer cells. Cell debris was rinsed with PBS solution and supplemented with fresh medium. Cells were photographed 0, 24, and 48 h after scratching. ImageJ software was used to quantify the area covered by the migrating cells (%).

Statistical analysis

Data are shown as mean ± standard deviation (SD). Categorical data were compared by Chi-square test, and measurement data were compared by t-test. The Kaplan-Meier method was used to plot the survival curve of CRC patients, followed by log-rank test to compare the differences of overall survival (OS). Univariate and multivariate Cox regression models were used to determine independent prognostic indicators for CRC patients. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Expression of RNF126 in CRC

IHC was used to detect the expression status of RNF126 protein in CRC tissue samples. As shown in Fig. 1, RNF126 protein was mainly localized in the cytoplasm. According to the aforementioned semi-quantitative scoring method, the high expression rate of RNF126 in CRC tissues was 66.7% (104/156), which was significantly higher than that in adjacent tissues (40.4%, 63/156) (P<0.001). Meanwhile, we analyzed the correlation between the expression status of RNF126 protein and clinicopathological characteristics of CRC patients. The results showed that high RNF126 expression was closely associated with lymph node metastasis, distant metastasis, TNM stage, venous invasion, perineural invasion, and preoperative CEA in patients with CRC (Table 1). For example, this part of CRC patients with lymph node metastasis, distant metastasis, nerve and blood vessel invasion has a higher expression rate of RNF126 (respectively 60.57%, 25.0%, 23.08% and 21.15%).

RNF126 and prognosis of patients with CRC

According to the evaluation criteria of IHC, 156 CRC patients were divided into RNF126 low expression group (n=52) and RNF126 high expression group (n=104). After a long-term follow-up of CRC patients, a Kaplan-Meier survival curve was plotted. Consequently, the OS was significantly better in CRC patients with low RNF126 expression than those with high RNF126 expression (P=0.002; Fig. 2A). In addition, we analyzed the correlation between clinicopathological features (including lymph node metastasis, distant metastasis, and TNM stage) and the prognosis of CRC patients. Meanwhile, the survival curve of subgroup of CRC patients was also plotted, as shown in Fig. 2B-D. In short, CRC patients with lymph node metastasis, distant metastasis, and later staging have a shorter survival time.

A Cox regression model was constructed to assess the risk factors associated with OS in CRC patients. Univariate analysis showed that age, lymph node metastasis, distant metastasis, TNM stage, venous invasion, and RNF126 expression status were associated with the OS of patients (Table 2). The Cox multivariate analysis suggested that, similar to the distant metastasis

Table 1. RNF126 expression and clinicopathological characteristics of patients with CRC.

Characteristics	n	RNF126 expression (%)		χ^2	P-value
		Low	High		
Total	156	52	104		
Gender				0.257	0.612
Male	113	39 (75.00)	74 (71.15)		
Female	43	13 (25.00)	30 (28.85)		
Age				0.055	0.815
≤60y	58	20 (38.46)	38 (36.54)		
>60y	98	32 (61.54)	66 (63.46)		
Location				0.212	0.645
Colon	64	20 (38.46)	44 (42.31)		
Rectum	92	32 (61.54)	60 (57.69)		
Differentiation				0.403	0.817
Well	43	16 (30.77)	27 (25.96)		
Moderate	60	19 (36.54)	41 (39.42)		
Poor	53	17 (32.69)	36 (34.62)		
T stage				2.048	0.562
T1	5	1 (1.92)	4 (3.85)		
T2	22	10 (19.23)	12 (11.54)		
T3	93	29 (55.77)	64 (61.54)		
T4	36	12 (23.08)	24 (23.08)		
Lymph node metastasis				6.625	0.036
N0	67	26 (50.00)	41 (39.42)		
N1	48	19 (36.54)	29 (27.88)		
N2	41	7 (13.46)	34 (32.69)		
Distant metastasis				5.153	0.023
M0	125	47 (90.38)	78 (75.00)		
M1	31	5 (9.62)	26 (25.00)		
TNM stage				5.308	0.021
I	37	16 (30.77)	18 (17.31)		
II	77	25 (48.08)	48 (46.15)		
III	29	7 (13.46)	22 (21.15)		
IV	13	4 (7.69)	16 (15.38)		
Venous invasion				4.151	0.042
Negative	135	47 (90.38)	80 (76.92)		
Positive	21	5 (9.62)	24 (23.08)		
Perineural invasion				10.199	0.001
Negative	133	51 (98.08)	82 (78.85)		
Positive	23	1 (1.92)	22 (21.15)		
Preoperative CEA				6.810	0.009
<5 ng/ml	73	32 (61.54)	41 (39.42)		
≥5 ng/ml	83	20 (38.46)	63 (60.58)		

and TNM stage, RNF126 was an independent risk factor of OS in CRC patients (Table 2).

Effect of RNF126 on CRC cells

We further explored the effects of RNF126 on CRC cells. First, we examined the expression level of RNF126 protein in CRC cell lines. Western blot analysis revealed that the expression level of RNF126 protein in CRC cell line is higher than that of normal human intestinal epithelial cells, and HCT-116 cells had the highest RNF126 protein level among the different CRC

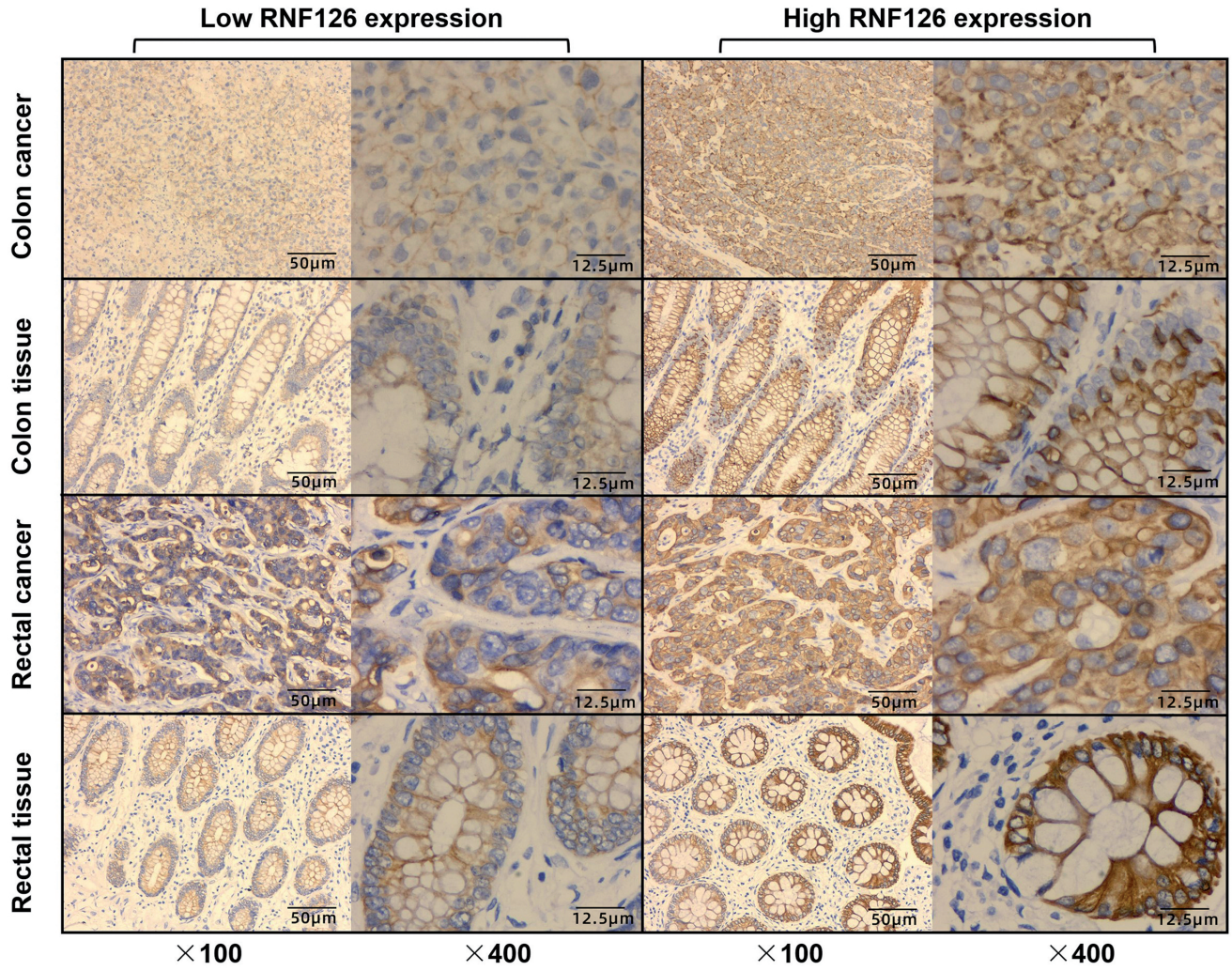

Fig. 1. Ring finger protein 126 was mainly localized in the cytoplasm which was detected by immunohistochemistry.

Table 2. Univariate and multivariable analysis of prognostic factors for OS in CRC.

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P-value
Age (year) (vs <60y)	1.627	1.041-2.542	0.033			
Gender (vs Male)	0.863	0.538-1.384	0.541			
Lymph node metastasis (vs N0)	1.082	0.844-1.387	0.036	1.673	1.152-2.457	0.032
Distant metastasis (vs M0)	2.108	1.318-3.369	0.002	2.840	1.366-5.904	0.005
TNM stage (vs I + II)	2.871	1.389-5.936	0.004	1.747	1.065-2.868	0.027
Venous invasion (vs Negative)	1.642	1.007-2.676	0.047	1.432	0.868-2.363	0.160
RNF126 expression (vs Low)	2.129	1.306-3.470	0.002	1.840	1.106-3.062	0.019

cell lines (Fig. 3A). Therefore, HCT-116 cells were selected for subsequent functional assays. The endogenous expression of *RNF126* in HCT-116 cells was suppressed through lentivirus-mediated shRNA knockdown. As shown in Fig. 3B, western blot analysis validated the inhibition rate of RNF126 (the knockdown rates of sh-1 and sh-2 are 40% and 46%, respectively). Afterwards, a CCK-8 kit was used to assess the effect of RNF126 on the proliferation of HCT-116 cells. As expected, depletion of RNF126 significantly inhibited the proliferation capacity of CRC cells (Fig. 3C). Transwell assays were used to analyze the effects of

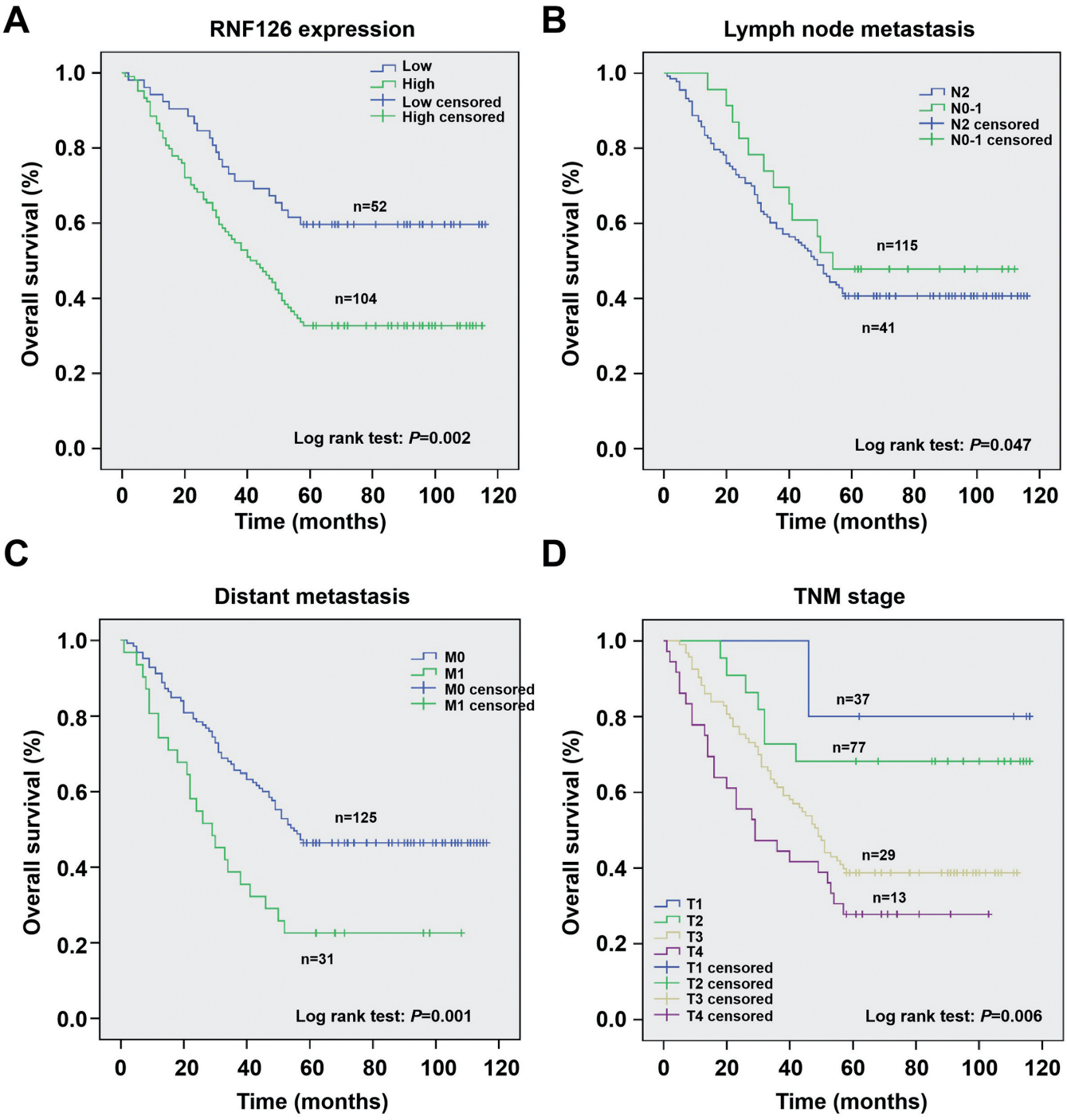

Fig. 2. Overall survival curves of patients with colorectal cancer were estimated with the Kaplan-Meier method by log-rank test.

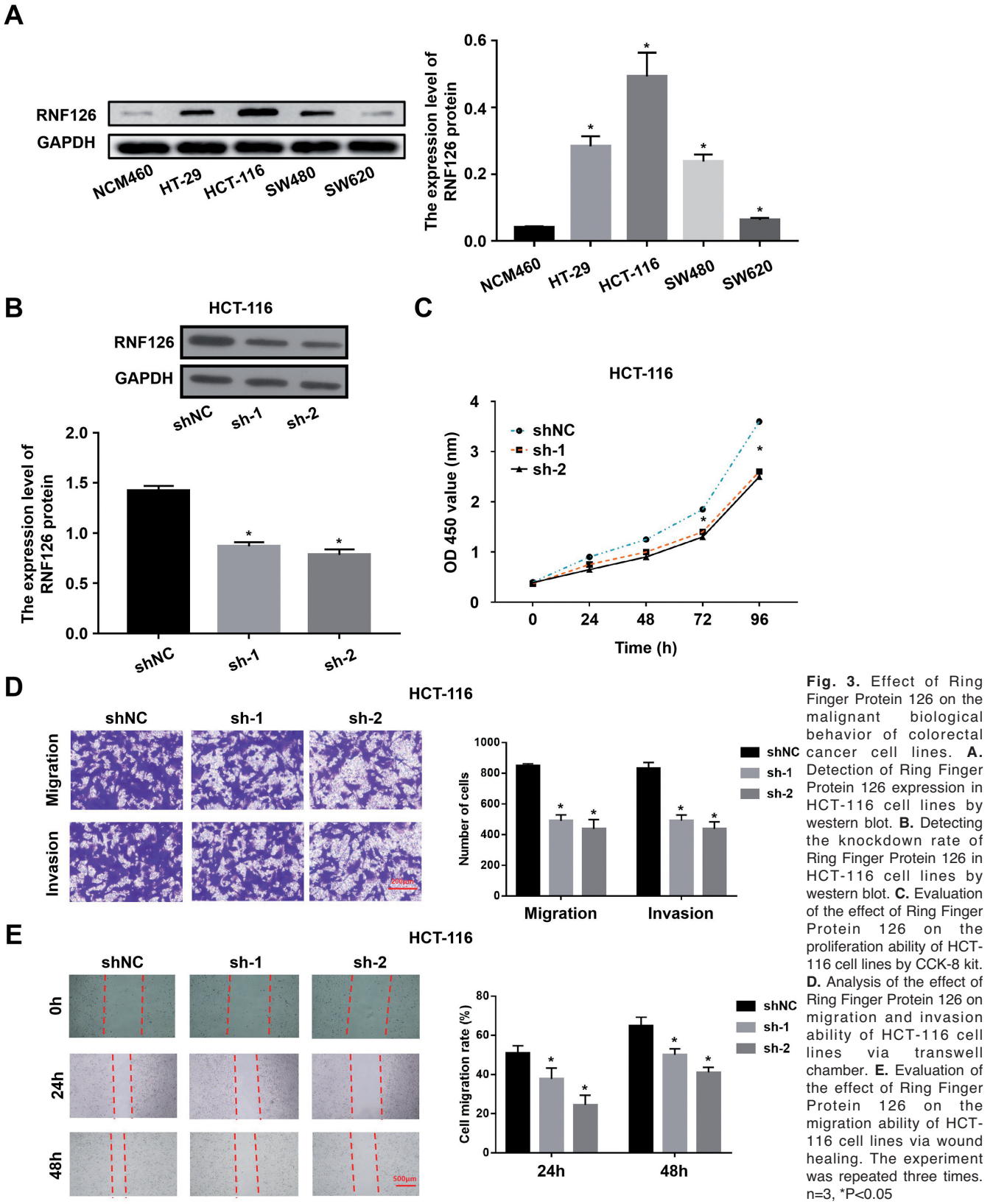

Fig. 3. Effect of Ring Finger Protein 126 on the malignant biological behavior of colorectal cancer cell lines. **A.** Detection of Ring Finger Protein 126 expression in HCT-116 cell lines by western blot. **B.** Detecting the knockdown rate of Ring Finger Protein 126 in HCT-116 cell lines by western blot. **C.** Evaluation of the effect of Ring Finger Protein 126 on the proliferation ability of HCT-116 cell lines by CCK-8 kit. **D.** Analysis of the effect of Ring Finger Protein 126 on migration and invasion ability of HCT-116 cell lines via transwell chamber. **E.** Evaluation of the effect of Ring Finger Protein 126 on the migration ability of HCT-116 cell lines via wound healing. The experiment was repeated three times. n=3, *P<0.05

RNF126 on the migration and invasion of CRC cells. As shown in Fig. 3D, knockdown of *RNF126* significantly inhibited the migration and invasion ability of HCT-116 cells. Finally, wound healing assay was used to detect the effects of RNF126 on the migration ability of HCT-116 cells, showing that knockdown of *RNF126* could significantly suppress the migration capacity of tumor cells (Fig. 3E).

Effect of RNF126 on apoptosis of CRC cells

In order to explore the potential mechanism of RNF126 to promote cancer in the process of CRC, we tested the expression changes of apoptosis-related molecules after RNF126 knockdown by western blot. The results showed that the expression of p21, Bax and activated caspase9 were up-regulated after RNF126 knockdown, while the expression of Bcl-2 was inhibited (Fig. 4).

Discussion

RNF126 is highly conserved throughout the evolution process. However, little is known about its biological role (Delker et al., 2013). RNF126 has three functional domains: an N-terminal zinc domain, a C-terminal RING finger domain and an abnormal C-terminal serine extension (Burger et al., 2006; Bacopulos et al., 2012; Delker et al., 2013). Upstream of the C-terminal serine sequence is a C3H2C3 type RING domain, which has been shown to have E3 ligase activity (Burger et al., 2006). The N-terminal zinc domain is homologous to the ubiquitin-binding domain of the related E3 ligase BCA2 (Bacopulos et al., 2012). The presence of two ubiquitin-related domains on RNF126 suggests their roles in post-translational modification. Moreover, RNF126 has been reported to play key roles in multiple types of cancer progression. However, there has been no study on RNF126 in CRC.

Zhi et al. found an abnormally high expression of RNF126 in breast cancer and prostate cancer cell lines. Meanwhile, the group constructed the siRNA library on E3 ubiquitin ligase, revealing that knockdown of *RNF126* could inhibit cell proliferation (Zhi et al., 2013). In addition, RNF126 promotes tumor cell proliferation and vitality by regulating the AKT signaling pathway in the progression of tongue cancer (Wang et al., 2016a). In a study on the correlation between RNF126 and anoikis in breast cancer and lung cancer cells, RNF126 acted as a ubiquitin ligase for pyruvate dehydrogenase kinase, leading to the proteasome degradation, thereby altering mitochondrial metabolism and ultimately regulating anoikis of cancer cells (Yoshino et al., 2016). Moreover, RNF126 has been found to be highly expressed in invasive breast cancer and promote the expression of CHEK1 (Yang et al., 2018). Similarly, RNF126 also plays a key role in gastric cancer. The abnormal expression of RNF126 in gastric cancer is closely associated with tumor invasion depth and venous invasion; and RNF126 also promotes tumor cell proliferation and cell cycle progression (Migita et al., 2020).

In this study, we preliminarily identified the abnormally high expression of RNF126 in CRC tissues. By using IHC, we determined that high RNF126 expression was closely associated with clinico-pathological characteristics, such as lymph node metastasis, distant metastasis, TNM stage, venous invasion, perineural invasion, and preoperative CEA in CRC patients. Based on the Cox regression model, we confirmed that high expression of RNF126 was a biomarker affecting the prognosis of CRC patients.

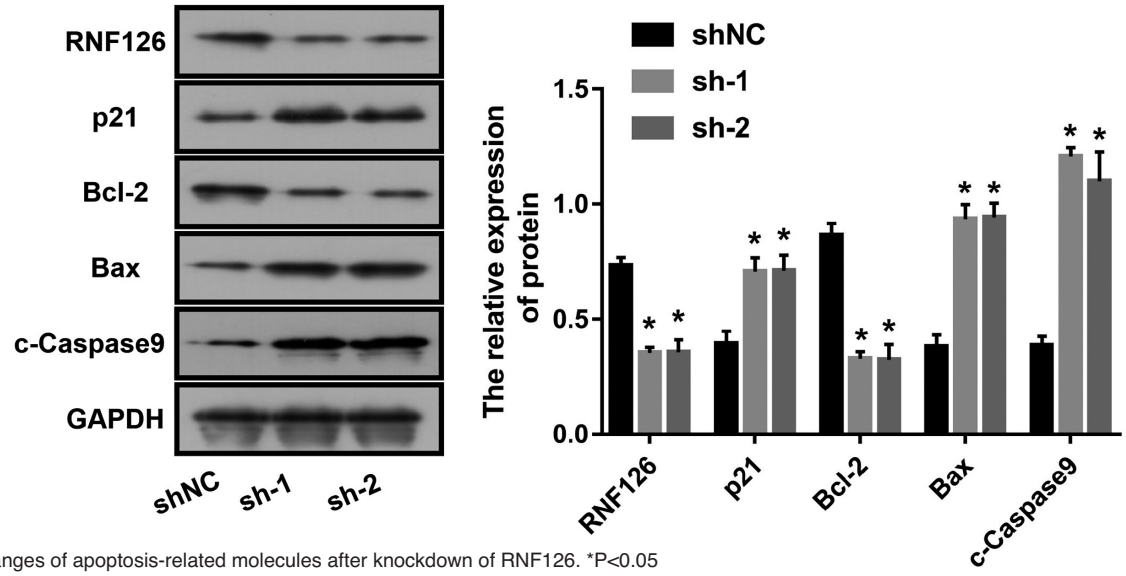

Fig. 4. Changes of apoptosis-related molecules after knockdown of RNF126. *P<0.05

Functionally, *in vitro* studies showed that RNF126 was able to promote malignant biological behaviors of CRC cells, such as cell proliferation, migration, and invasion. However, the underlying carcinogenic mechanism of RNF126 remains unknown. We speculate that RNF126 might act as an E3 ubiquitin ligase to promote the degradation of certain tumor suppressor genes, such as p21 (Zhi et al., 2013).

Conclusion

Collectively, by analyzing CRC tissues and cell lines we have clarified that RNF126 expression is upregulated in CRC, thereby promoting the malignant properties of tumor cells. In addition, we also demonstrated that high RNF126 expression is an independent risk factor for CRC patients, and thus this protein can become a potential therapeutic target for CRC.

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Conflicts of interest. The authors declare no conflict of interest.

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