Overexpression of calpain-1 predicts poor outcome in patients with colorectal cancer and promotes tumor cell progression associated with downregulation of FLNA

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Abstract. Several studies have demonstrated that calpain-1 is involved in a variety of pathophysiological processes, including tumorigenesis. However, the clinical relevance and role of calpain-1 in colorectal cancer (CRC) are unclear. Filamin A (FLNA) is an actin-binding protein that participates in cancer progression and can be cleaved by calpain-1. In the present study, the protein expression levels of calpain-1 and FLNA were detected by immunohistochemistry in 467 matched cancerous and paracancerous tissues from patients with CRC. The staining results and the clinicopathological characteristics of the patients were comprehensively analyzed. A high expression level of calpain-1 was strongly associated with age, metastasis, Dukes stage and survival time but not with sex, histologic grade, tumour location or tumor size. By contrast, a low expression level of FLNA was significantly associated with tumor size, histological grade, metastasis, Dukes stage and survival time, but not with age, sex, or tumor location. Kaplan-Meier survival analysis demonstrated that patients with calpain-1 overexpression had a shorter mean overall survival (OS) than patients with lower levels of calpain-1 expression. Unlike high levels of calpain-1, high levels of FLNA were associated with longer OS than lower levels of FLNA expression. Furthermore, calpain-1 expression was inversely correlated with FLNA expression. The relationship between calpain-1 and FLNA was further confirmed using CRC cell lines *in vitro*. When calpain-1 expression decreased in CRC cells, FLNA expression increased. Furthermore, calpain-1 knockdown in CRC cells resulted in decreased proliferation, colony formation, migration and invasion. The present findings suggest that calpain-1 overexpression predicted a poor outcome in patients with CRC and promoted tumor progression, possibly via FLNA downregulation.

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Introduction

In the past forty years, the incidence of colorectal cancer (CRC) has increased by 75%. Currently, CRC ranks fourth worldwide in terms of the number of cancer-related deaths and accounts for 8% of all cancer-related deaths (1). The current understanding of the genetic mechanisms underlying the development of CRC is not yet complete. Exploring new mechanisms involved in CRC is important for the comprehensive diagnosis and treatment of patients with CRC.

In unpublished observations from our group, sequence analysis of gene expression profiles of CRC tissues and adjacent normal tissues has revealed that calpain-1 gene expression was significantly upregulated in CRC tissues. Calpains are a family of calcium-dependent cysteine proteases that are widely expressed in many tissues and cells (2). Currently, 15 calpain genes have been discovered. Decreased calpain activity is related to various pathologies: Calpain-1 and calpain-2 are associated with Alzheimer's disease and neuronal degeneration, calpain-3 is associated with muscular dystrophy and cataracts, calpain-8 and calpain-10 are associated with type 2 diabetes mellitus and hyperglycemia, and calpain-9 is associated with gastric cancer (3-7). Among these genes, calpain-1 is the best characterized. In general, it exists in the form of an inactive zymogen in the cytoplasm and cell membrane

and is activated when the intracellular Ca^{2+} concentration increases. Calpain-1 consists of two subunits, a regulatory subunit and a catalytic subunit, which are the products of genes on chromosomes 1 and 11, respectively (8). Calpain-1 has an important effect on carcinogenesis by participating in multiple vital cellular processes, such as cell apoptosis and proliferation (9-14). The expression of calpastatin in endometrial carcinoma is higher compared with benign endometrial tissue, and calpain-1 influences the survival, apoptosis and migration of breast cancer cells. However, the specific role and mechanism of action of calpain-1 in the development of CRC remains unclear. Filamin A (FLNA) is an actin-binding protein that participates in cancer progression, and can be cleaved by calpains (15). In our unpublished genomic data, we found that the expression of calpain-1 and FLNA in CRC was inversely correlated, therefore, their interaction in CRC warrants further study.

In the present study, the expression levels of the calpain-1 and FLNA genes were examined in CRC tissues and the relationship between calpain-1 and FLNA expression and the clinicopathological features of CRC was analyzed. In addition, the effects of calpain-1 on the biological functions of CRC cells and the underlying mechanisms were investigated.

Materials and methods

Patients and specimens. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University, and informed consent was obtained from each patient. In total, 467 CRC and corresponding paracancerous tissue samples were collected at the Second Affiliated Hospital of Wenzhou Medical University, between June 2011 and December 2017. Tissue specimens were surgically resected from the patients, none of whom received neoadjuvant chemotherapy prior to the operation. The age of the patients ranged from 32 to 90 years old (61.77±12.7). The patients' clinical data and tumor characteristics are summarized in Table I. All patients were followed for 6-79 months following surgery.

Immunohistochemistry assay. Calpain-1 and FLNA expression in CRC tissue samples was detected by immunohistochemistry. Paraffin-embedded CRC tissue sections (4-μm thick) as well as the corresponding paracancerous samples were obtained. Sections were deparaffinized by submerging in xylene for 10 min three times and then hydrated in a graded series of ethanol. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) at 120˚C and 103 kPa for 2 min. Then, endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide (20 min, ambient temperature), after which the samples were blocked with 10% normal goat serum (cat. no. ab138478; Abcam, Cambridge, MA, USA; 30 min, ambient temperature). Next, the samples were sequentially incubated with anti-calpain-1 (rabbit monoclonal; cat. no. ab108400; Abcam; dilution 1:150) or anti-FLNA (rabbit monoclonal; cat. no. ab76289; Abcam; dilution 1:800) antibodies overnight at 4˚C in a humid environment followed by streptavidin-peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG; cat. no. zb2306; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., OriGene Technologies, Inc., Rockville, MD, USA; dilution 1:5,000) for 18 min at ambient temperature. Antibody detection was performed using a 3,5-diaminobenzidine (DAB) substrate kit (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., OriGene Technologies, Inc.), and the slides were counterstained with haematoxylin. Data were independently analyzed by two experienced pathologists. A double-headed microscope was utilized to assess discrepancies. Calpain-1 or FLNA protein expression levels were evaluated based on staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) and extent (0, <5% positive CRC cells; 1, ≥5-25%; 2, ≥26-50%; 3, ≥51-75%; 4, ≥76%). The product of both subscores represented the final score (0-12). A score ≤4 reflected low expression levels for both calpain-1 and FLNA, and was used as the cutoff for diving the patients to low-expression and high-expression groups for survival and correlation analyses.

Western blot assay. First, the calpain-1 protein expression levels in six CRC cell lines were tested. In brief, the human CRC cell lines HCT116, HT29, LS174T, RKO, SW480, and SW620 were prepared, and the normal colon cell line NCM460 (purchased from the Shanghai Fudan Zhongke Biomedical Research and Development Service Center) was used as control. All CRC cell lines were acquired from the Shanghai Cell Bank of Chinese Academy of Sciences. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; MediaTech, Herndon, VA, USA) at 37.5˚C in a humidified incubator containing 5% CO_2. CRC cells were harvested, washed with cold phosphate-buffered saline (PBS) and lysed with radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Lysates were centrifuged for 10 min at 11,000 x g at 4˚C, and the supernatant was discarded. Protein samples were prepared by mixing the cell lysates with 200 μl of BCA mixture and incubating them for 30 min at 37˚C. Then, we measured the absorbance value at 562 nm and detected the protein concentration of the samples. Proteins (30 μg) were separated by 12% SDS-PAGE, and then transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% non-fat milk, shaken gently for 2 h, and washed in Tris-buffered saline with Tween-20 (TBST) buffer on a shaking table for 10 min; this process was repeated three times before the addition of antibodies targeting GAPDH (cat. no. ab8245; Abcam; dilution 1:5,000) calpain-1 (rabbit monoclonal; cat. no. ab108400; Abcam; dilution 1:1,000) or FLNA (rabbit polyclonal; cat. no. ab51217; Abcam; dilution 1:2,000) at 4˚C overnight. Next, the membrane was washed in TBST buffer three times, secondary antibody (goat anti-rabbit IgG; cat. no. zb2306; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., OriGene Technologies, Inc.; dilution 1:5,000) was added, and the membrane was incubated on a shaking table for 2 h. The membrane was washed in TBST buffer three times. An ECL chemiluminescence assay (Clarity Western ECL Substrate; cat. no. 170-5060; Bio-Rad Laboratories, Inc.) was used to visualize the signals. The results revealed that HT29 and SW480 cells expressed the highest levels of calpain-1 protein, and therefore these cell lines were selected for the subsequent experiments. Second,

Table I. Association between Calpain-1 or FLNA expression and clinicopathological features.

	Calpain-1			FLNA		
Clinicopathological feature	Low	High	P-value	Low	High	P-value
Age (years)			0.041			0.566
≥60	158	163		185	136	
<60	57	89		80	66	
Sex			0.974			0.085
Male	130	152		151	131	
Female	85	100		114	71	
Tumor Site			0.280			0.367
Colon	126	160		167	119	
Rectum	89	92		98	83	
Size (cm)			0.618			0.000
>5	83	103		126	60	
≤5	132	149		139	142	
Histologic grade			0.069			0.000
High to moderate	143	147		95	195	
Low	72	105		170	7	
Metastasis			0.024			0.000
Yes[a]	103	147		165	85	
No	112	105		100	117	
Dukes stage			0.024			0.000
A-B	112	105		100	117	
C-D	103	147		165	85	
Mean survival time (months)	59.7	51	0.003	50.7	60.7	0.000

[a]Includes both distant organ and lymph node metastasis. FLNA, filamin A.

after calpain-1 was knocked down by liposome-mediated RNA interference, the calpain-1 and FLNA protein expression levels in HT29 and SW480 cells were detected by western blot assay. Third, different concentrations (0, 5, 20 and 40 μg) of MDL28170 (cat. no. ab145601; Abcam), a calpain-1 inhibitor that binds with active sulfhydryl and influences the Ca^{2+} binding site, were added to HT29 and SW480 cells. The calpain-1 and FLNA protein expression levels in HT29 and SW480 cells were detected by western blot assay at 48 h following MDL28170 treatment.

Calpain-1 knockdown by liposome-mediated RNA interference. HT29 and SW480 cells were incubated in DMEM containing 10% FBS at 37.5˚C in a humidified incubator containing 5% CO_2. When cells reached 90% confluence, the medium was removed, the cells were washed twice with PBS and 1 ml of 0.25% trypsin was added. When the cells had retracted, 4 ml of normal medium was added to stop the activity. The cells were centrifuged at 230 x g for 5 min, the supernatant was discarded, and normal medium was added. The cells were counted and seeded into 10 cm culture dishes at $1x10^6$ cells/ml. Then, three small interfering RNAs (siRNAs) against calpain-1 were transfected [capn1-1983 (siRNA1), 5'-CAUGGAUCGUGAUGGCAAUTT-3'; capn1-2287 (siRNA2), 5'-GGAGUUGUGACCUUUGACUTT-3'; and capn1-1293 (siRNA3), 5'-GAACACCACACUCUACGA ATT-3'; 20 pM each; Invitrogen; Thermo Fisher Scientific, Inc.] into the treatment group using the Lipofectamine RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The negative control (NC) group was transfected with a scrambled siRNA (5'-GCAGAA UCACAGCACUUUAUC-3'). To examine the efficiency of RNA interference, western blot analysis was performed. Cell lysates were mixed with 200 μl of BCA mixture and incubated for 30 min at 37˚C. Then, the absorbance value was measured at 562 nm, and the protein concentration of the samples was calculated. SDS-PAGE was performed as aforementioned. PVDF membranes were blocked with 0.5 mg/ml standard bovine serum protein before adding primary antibody (rabbit monoclonal; cat. no. ab108400; Abcam; dilution 1:1,000) at 4˚C overnight. The membrane was washed for 10 min three times on a shaking table. The secondary antibody (goat anti-rabbit IgG; cat. no. zb2306; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., OriGene Technologies, Inc.; dilution 1:5,000) was added to the membrane and incubated on a shaking table for 2 h at room temperature. The membrane was washed three times in TBST buffer, and the protein bands were visualized on a gel imaging analyser.

Colony formation assay. SW480 and HT29 cells were incubated in serum-free DMEM for 24 h. Five groups of cells at the logarithmic growth stage were used, digested with trypsin and centrifuged for 5 min at 64 x g, at room temperature. The cell density was adjusted to $4x10^4$ cells/ml, and 2 ml of cell suspension was added into each well of a 6-well plate. The cells were placed in an incubator overnight. The medium was changed to serum-free DMEM, and the cells were incubated for another 24 h before they were transfected with siRNA (Lipofectamine RNAiMAX; Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, cell samples were collected for colony formation detection. The monolayer of cells in the logarithmic growth phase was digested with 0.25% trypsin and suspended in culture medium with 10% FBS. The cell suspension underwent a gradient dilution and was seeded into culture dishes at the appropriate cell density (according to the proliferative capacity). Generally, a suspension containing 100 cells was seeded in a dish containing 10 ml of pre-heated culture solution at 37˚C and gently rotated to evenly disperse the cells. The cells were incubated at 37˚C in 5% CO_2 and saturated humidity for 2-3 weeks. An inverted light microscope (ECLIPSE Ti-S; Nikon Corporation, Tokyo, Japan) was used to count the number of colonies.

Detection of cell proliferation with the Cell Counting Kit-8 (CCK-8) assay. SW480 and HT29 cells were incubated in serum-free DMEM for 24 h and cells in the logarithmic growth stage were obtained and centrifuged for 5 min at 64 x g, at room temperature. The cell density was adjusted to $2x10^4$ cells/ml, and the suspensions were seeded into 96-well plates at 100 μl/well. After the SW480 and HT29 cells were incubated overnight, they were divided into experimental and negative control groups. In the experimental group, cells were transfected with siRNA for 0, 24, 48 and 72 h. The control group was transfected with the scrambled siRNA. Then, 10 μl of CCK-8 detection solution (cat. no. ab228554; Abcam) was added to each well, and a blank control well was established. After incubation at 37˚C for 1.5 h, the optical density (OD) value was read at 450 nm, and the cell survival rate was calculated as follows: (OD450 experimental group/OD450 control group) x 100%.

Detection of apoptosis by flow cytometry. SW480 and HT29 cells in the logarithmic growth phase were cultured in serum-free medium for 24 h to synchronize them, after which the cells were digested, counted and seeded into culture dishes at $2.5x10^5$ cells/dish. The next day, the cells were transfected with siRNA or NC for 72 h before they were collected for flow cytometry analysis of apoptosis. The cells were centrifuged for 5 min at 64 x g, at room temperature, washed twice with PBS, and then centrifuged again for 5 min at 100 x g. A total of 300 μl binding buffer was added to the cells; the cells were then mixed with 5 μl of Annexin V-fluorescein isothiocyanate (cat. no. a13199; Invitrogen; Thermo Fisher Scientific, Inc.) and incubated at room temperature for 15 min. Then, 5 μl of propidium iodide (cat. no. v13245; Invitrogen; Thermo Fisher Scientific, Inc.) was added to the samples, which were incubated in the dark for another 5 min. Finally, 200 μl binding buffer was added, and the cells were analyzed by flow cytometry (NovoCyte; ACEA Biosciences, Inc., San Diego, CA, USA.) using the Kaluza analysis software (Beckman Coulter, Inc., Brea, CA, USA).

Detection of cell migration by the scratch assay. SW480 and HT29 cells were cultured in DMEM medium containing 10% FBS and incubated at 37˚C in 5% CO_2 and saturated humidity. When SW40 and HT29 cells reached the logarithmic growth phase, they were transfected with NC or siRNA. Then, the cells were centrifuged for 5 min at 64 x g at room temperature and counted. Cells were seeded in 6-well plates, at $1x10^6$ cells/well (2 ml), and incubated overnight. When the cells grew to 90% confluence, the supernatant was discarded, and serum-free medium was added. After the cells were scratched with a 10 μl Microlance needle, the supernatant was discarded, the floating cells were washed off with PBS, and serum-free medium was added to the culture. Then, Leica Application Suite software was used to capture photos (magnification, x100) and record the scratch distance at 0, 24 and 48 h. Cells in each group were observed under an inverted microscope, and the cell mobility of each group was calculated. The formula was as follows: Cell migration rate % = (0 h scratch distance-scratch distance at a certain time)/0 h scratch distance x 100%.

Migration and invasion assays. SW480 and HT29 cells were incubated in serum-free DMEM for 24 h, transfected with NC or siRNA and collected 48 h later. The cells were treated with 0.25% trypsin and 0.02% EDTA, stained with trypan blue, and washed twice with PBS. A total of $5x10^4$ cells in 200 μl of medium was added to the upper chamber of migration and invasion Transwells, and 600 μl of medium with 10% FBS was added to the lower chambers. The cells were incubated for 24 h in 5% CO_2 at 37˚C, after which the Transwell chamber was removed, the non-migratory cells in the upper Transwell chamber were wiped off with cotton swabs, and the insert was inverted and dried. Next, 500 μl of 0.1% crystal violet per well was added to a 24-well plate, and the cells were stained at 37˚C for 30 min. The cells were washed with PBS, three random views were photographed per filter, and the average number of cells was calculated.

Statistical analysis. SPSS v22.0 (IBM Corp., Armonk, NY, USA) was employed for the statistical analyses. Data are presented as the mean ± standard error of the mean from three independent experiments and were assessed by one-way analysis of variance followed by SNK post hoc test. Chi-square test and the Kaplan-Meier method with the log-rank test were used to assess the associations between calpain-1 or FLNA expression and pathological indices. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Calpain-1 and FLNA expression levels are associated with the clinicopathological features of CRC patients. The immunohistochemistry results revealed that calpain-1 protein was highly expressed in 252 (252/467, 53.9%) CRC patients (Fig. 1A) but only in 42 (42/467, 9.0%) adjacent non-cancerous tissues (P=0.000). In addition, the expression levels of calpain-1 in most of the adjacent non-cancerous colorectal tissues was low (Fig. 1B). High expression levels

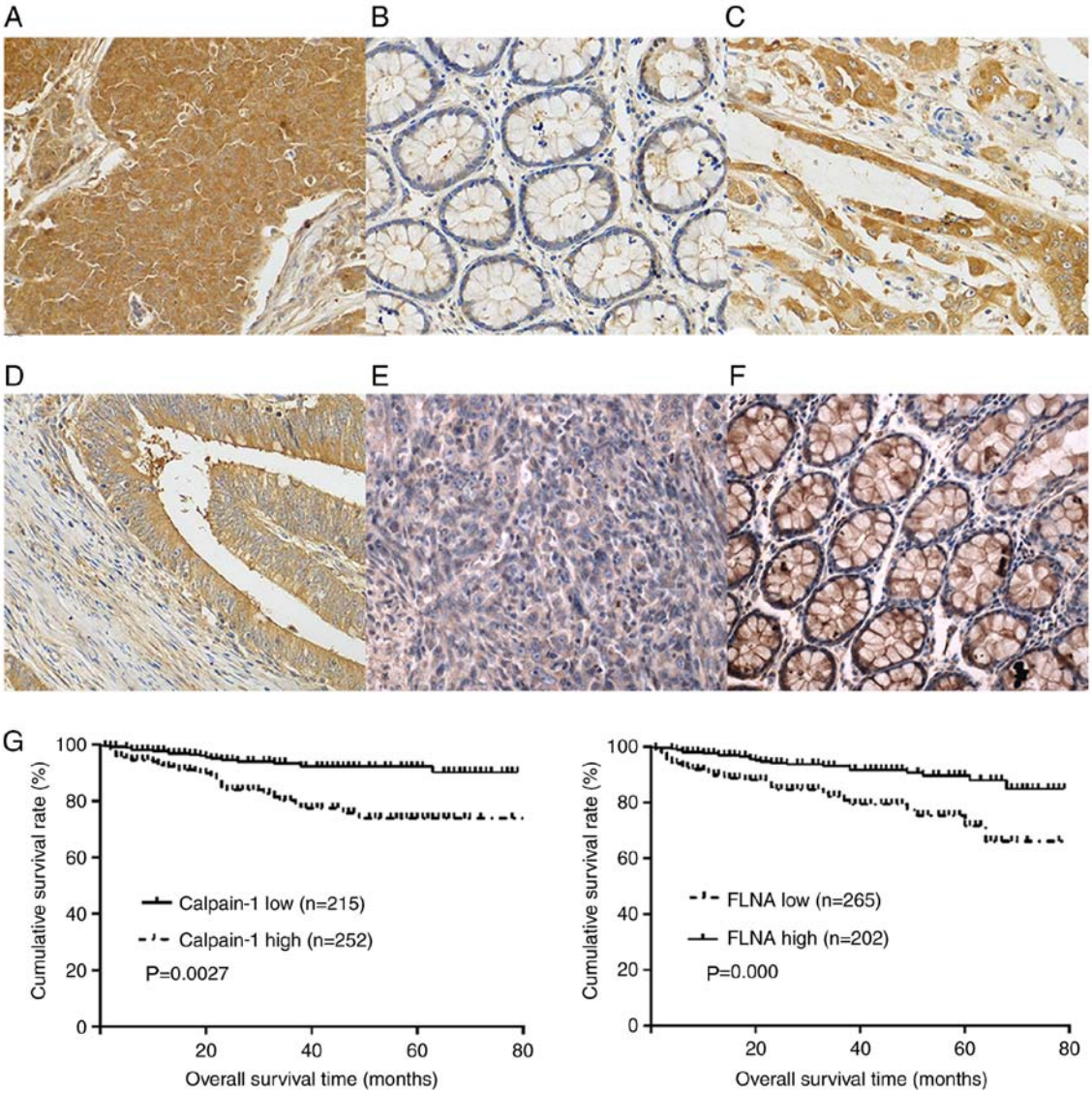

Figure 1. Calpain-1 and FLNA expression in CRC and paracancerous tissues detected by immunohistochemistry and its clinical relevance. (A) CRC tissue with a high level of calpain-1 expression. (B) Adjacent non-cancerous tissue with a low level of calpain-1 expression. (C) Poorly differentiated CRC tissue with a high level of calpain-1 expression. (D) Well-differentiated CRC tissue with a low level of calpain-1 expression. (E) FLNA protein was expressed at low levels in CRC tissue. (F) FLNA protein was highly expressed in adjacent non-cancerous tissues (magnification, x400). (G) Kaplan-Meier analysis of calpain-1 and FLNA expression in CRC patients and their correlation with overall survival. FLNA, filamin A; CRC, colorectal cancer.

of calpain-1 were more often found in the less differentiated CRC tissues (105/177, 59.3%; Fig. 1C) than in the moderately to well-differentiated CRC tissues (147/290, 50.7%; Fig. 1D; P=0.02). High expression levels of calpain-1 were significantly associated with age, metastasis, Dukes stage and survival time, but not with sex, histologic grade, tumor location or tumor size (Table I).

By contrast, FLNA protein was expressed at low levels in 265 (265/467, 56.7%) CRC tissues (Fig. 1E) and was highly expressed in 382 (382/467, 81.8%) adjacent non-cancerous tissues (Fig. 1F; P=0.000). A low expression level of FLNA was significantly associated with tumor size, histological grade, metastasis, Dukes stage and survival time, but not with age, sex, or tumor location (Table I).

Relationship between calpain-1 or FLNA expression and the prognosis of CRC patients. In total, 467 CRC patients were divided into two groups according to their immunohistochemical results. Group one consisted of 252 patients with high levels of calpain-1 expression and had a mean overall survival (OS) of 59.7 months [95% confidence interval (CI), 54.8-64.7]. Group two consisted of 215 patients with low levels of calpain-1 expression and had a mean OS of 51 months (95% CI, 45.7-56.2). The results indicated that patients with high levels of calpain-1 expression had shorter OS compared with patients with low levels of calpain-1 expression (P=0.0027; Fig. 1G). The patients were also grouped according to their level of FLNA expression, and the mean OS of the FLNA overexpression group was 60.7 months (95% CI, 52.9-62.6), while the mean OS of the group with low levels of FLNA expression was 50.7 months (95% CI, 45.7-56.2). In contrast to the calpain-1 results, patients with high levels of FLNA expression had longer OS than patients with low levels of FLNA expression (P=0.000; Fig. 1G).

Table II. Relationship between calpain-1 and FLNA expression.

	Calpain-1				
FLNA	High	Low	Total	χ^2-value	P-value
High	94	108	202	7.385	0.007
Low	158	107	265		
Total	252	215	467		

FLNA, filamin A.

Relationship between calpain-1 and FLNA expression. In all 467 patients, there were 94 cases with high expression of calpain-1 and FLNA, 108 cases with high expression of FLNA and low expression of calpain-1, 158 cases with low expression of FLNA and high expression of calpain-1, and 107 cases with low expression of both. In ~57% (268/467) of patients, the expression level of calpain-1 was inversely correlated to that of FLNA. A significant inverse relationship was found between the expression levels of calpain-1 and FLNA (P=0.007; Table II).

Calpain-1 expression and siRNA-mediated knockdown in CRC cells. Calpain-1 expression levels were examined by western blotting in 6 CRC cell lines (HCT116, HT29, LS174T, RKO, SW480 and SW620) and one normal colon cell line, NCM460. Five of the colon cancer cell lines expressed high levels of calpain-1 protein compared with the normal colon NCM460 cells, among which HT29 and SW480 cells had the highest levels (Fig. 2A and B). Therefore, HT29 and SW480 cells were used for all subsequent experiments. Three siRNAs were designed against calpain-1 and transfected into the CRC cells. In HT29 cells, calpain-1 levels of NC and three siRNA groups were 0.91±0.11, 0.58±0.05, 0.47±0.03 and 0.56±0.04, respectively. In SW480 cells, the levels of NC and three siRNA groups were 0.79±0.07, 0.80±0.08, 0.21±0.02 and 0.49±0.04, respectively. Calpain-1 protein was downregulated to different degrees in HT29 and SW480 cells following knockdown by the three siRNAs. siRNA2 was the most efficient in silencing calpain-1 in both cell lines (Fig. 2C and D), and therefore, siRNA2 was selected for use in subsequent experiments.

Calpain-1 knockdown inhibits colony formation in CRC cells. As presented in Fig. 3, the number of colonies in the SW480 NC, SW480 siRNA, HT29 NC, and HT29 siRNA groups was 151±13, 66±5 (P<0.05), 76±8, and 26±3 (P<0.05), respectively. Calpain-1 knockdown significantly inhibited the colony forming ability of both HT29 and SW480 cells compared with control (Fig. 3A and B).

Calpain-1 knockdown inhibits cell proliferation as measured by the CCK-8 assay. As presented in Fig. 3C, in the 0 h group, the viability of cells in the HT29 NC, HT29 siRNA, SW480 NC, and SW480 siRNA groups was 100.0±2.8%, 104.7±4.1% (P=0.174), 104.7±4.8%, and 102.5±5.0% (P=0.606), respectively. At 24 h, the viability of cells in the same groups was 121.3±1.8%, 114.4±2.2% (P=0.013), 125.7±4.9%, and 118.2±3.5% (P=0.097), respectively. At 48 h, the viability was 213.1±3.7%, 128.6±2.6% (P=0.000), 234.5±1.9%, and 170.7±4.4% (P=0.000), respectively. Finally, at 72 h, the viability was 294.0±3.5%, 184.3±7.6% (P=0.000), 310.1±18.2%, and 231.9±7.6% (P=0.002), respectively. Thus, calpain-1 knockdown significantly reduced the numbers of viable cells compared with control (Fig. 3C).

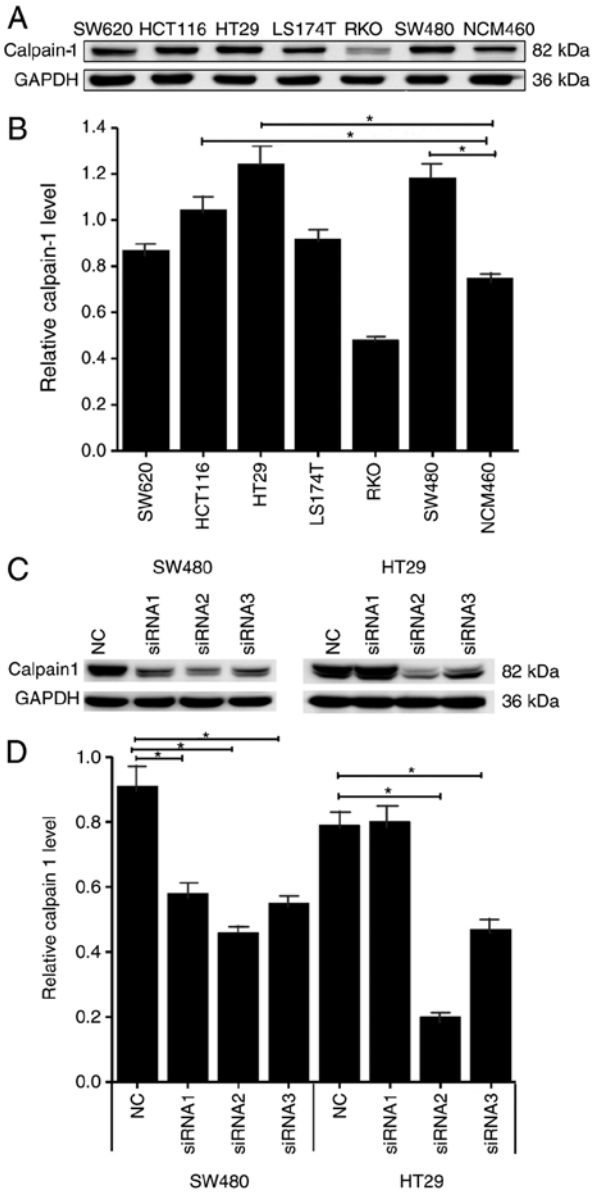

Figure 2. Expression of calpain-1 in CRC cell lines and following siRNA transfection. (A) Representative blots and (B) quantification of calpain-1 protein expression levels in CRC cell lines compared with the normal colon cell line NCM460, as detected by western blotting. *P<0.05 compared with NCM460. (C) Representative blots and (D) quantification of the relative expression levels of calpain-1 in SW480 and HT29 cells following transfection with either NC or calpain-1-targeting siRNAs. *P<0.05 compared with NC. CRC, colorectal cancer; si, small interfering; NC, negative control.

Calpain-1 knockdown inhibits the migration ability of CRC cells as measured by the scratch assay. After transfecting HT29 and SW480 cells with siRNA, scratch assays were performed to compare the migration ability of the cells. As presented in Fig. 3D and E, in the 24 h group, the relative migration rate

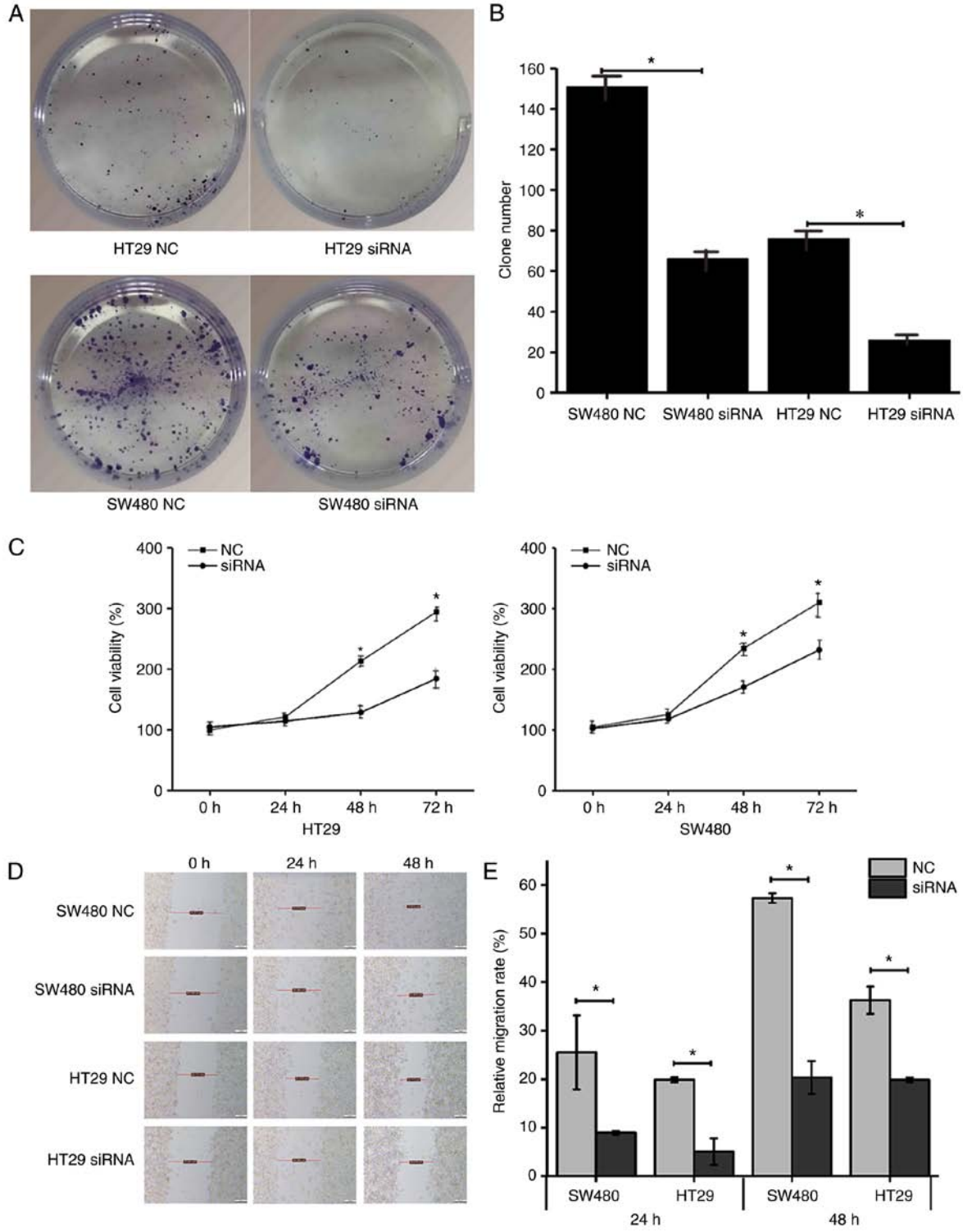

Figure 3. Calpain-1 knockdown inhibits colony formation, proliferation and migration ability of CRC cells. HT29 and SW480 cells were transfected with either NC or calpain-1-targeting siRNA. (A) Representative images and (B) quantification of results from colony formation assay. (C) Cell viability by Cell Counting Kit-8 assay. (D) Representative imaged and (E) quantification of scratch migration assay. In the SW480 NC group, distance at 0 h was 44.311 μm, at 24 h 34.919 μm, at 48 h 17.614 μm; in the SW480 siRNA group, distance at 0 h was 40.358 μm, at 24 h 36.380 μm, at 48 h 31.870 μm; in the HT29 NC group, distance at 0 h was 32.711 μm, at 24 h 27.679 μm, at 48 h 22.713 μm; and in HT29 siRNA group, distance at 0 h was 35.941 μm, at 24 h 36.380 μm, at 48 h 28.459 μm. *P<0.05 compared with NC. CRC, colorectal cancer; si, small interfering; NC, negative control.

of SW480 NC, SW480 siRNA, HT29 NC, and HT29 siRNA cells was 25.51±7.64, 8.97±0.39, 19.87±0.52 and 5.08±2.74%, respectively. In the 48 h group, the relative migration rate was 57.32±0.99, 20.34±3.38, 36.26±2.83 and 19.85±0.49%, respectively. The migration ability was decreased following calpain-1 knockdown and was higher in SW480 cells than in HT29 cells.

Calpain-1 knockdown inhibited the migration and invasion of CRC cells. As presented in Fig. 4A-D, calpain-1

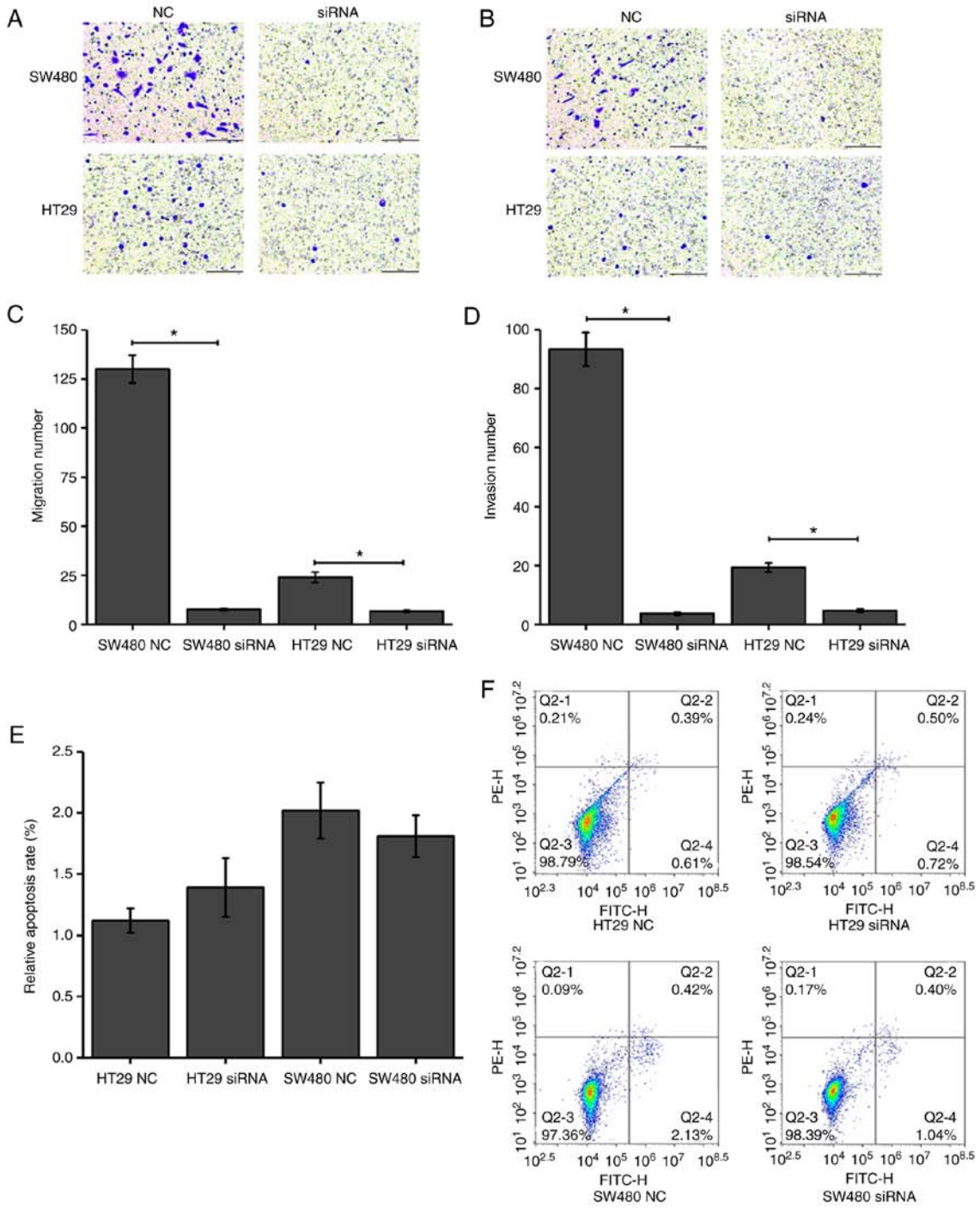

Figure 4. Effect of calpain-1 knockdown on CRC cell migration, invasion and apoptosis. HT29 and SW480 cells were transfected with either NC or calpain-1-targeting siRNA. (A) Representative images of Transwell migration assays (magnification, x200; Scale bar, 10 μm). (B) Representative images of Transwell invasion assays (magnification, x200; Scale bar, 10 μm). (C) Quantification of migration from panel A. (D) Quantification of invasion from panel B. (E) Quantification and (F) representative plots of flow cytometry analysis of apoptosis. Q1, necrotic cells; Q2, late apoptotic cells; Q3, normal cells; Q4, early apoptotic cells. *P<0.05 compared with NC. CRC, colorectal cancer; si, small interfering; NC, negative control.

knockdown resulted in fewer migrating [SW480 NC (93.33±5.69) vs. SW480 siRNA (7.67±0.58), P=0.0014; HT29 NC (24.00±2.65) vs. HT29 siRNA (6.65±0.48), P=0.0115] and invading [SW480 NC (93.33±5.69) vs. SW480 siRNA (3.67±0.58), P=0.0011; HT29 NC (19.33±1.53) vs. HT29 siRNA (4.67±0.58), P=0.0036] cells compared with the control groups. These results revealed that calpain-1 knockdown significantly inhibited the migration and invasion abilities of CRC cells.

Calpain-1 knockdown has no significant effect on the apoptosis of CRC cells. Following calpain-1 knockdown in HT29 and SW480 cells, flow cytometry was used to detect the apoptosis rate and compare it to control cells. As presented in Fig. 4E and F, the apoptosis rates of the HT29 NC and HT29 siRNA groups were 1.12±0.10 and 1.39±0.24, respectively, and the apoptosis rates of the SW480 NC and SW480 siRNA groups were 2.02±0.23 and 1.81±0.17, respectively. The results revealed that the apoptosis rates of both cell lines following

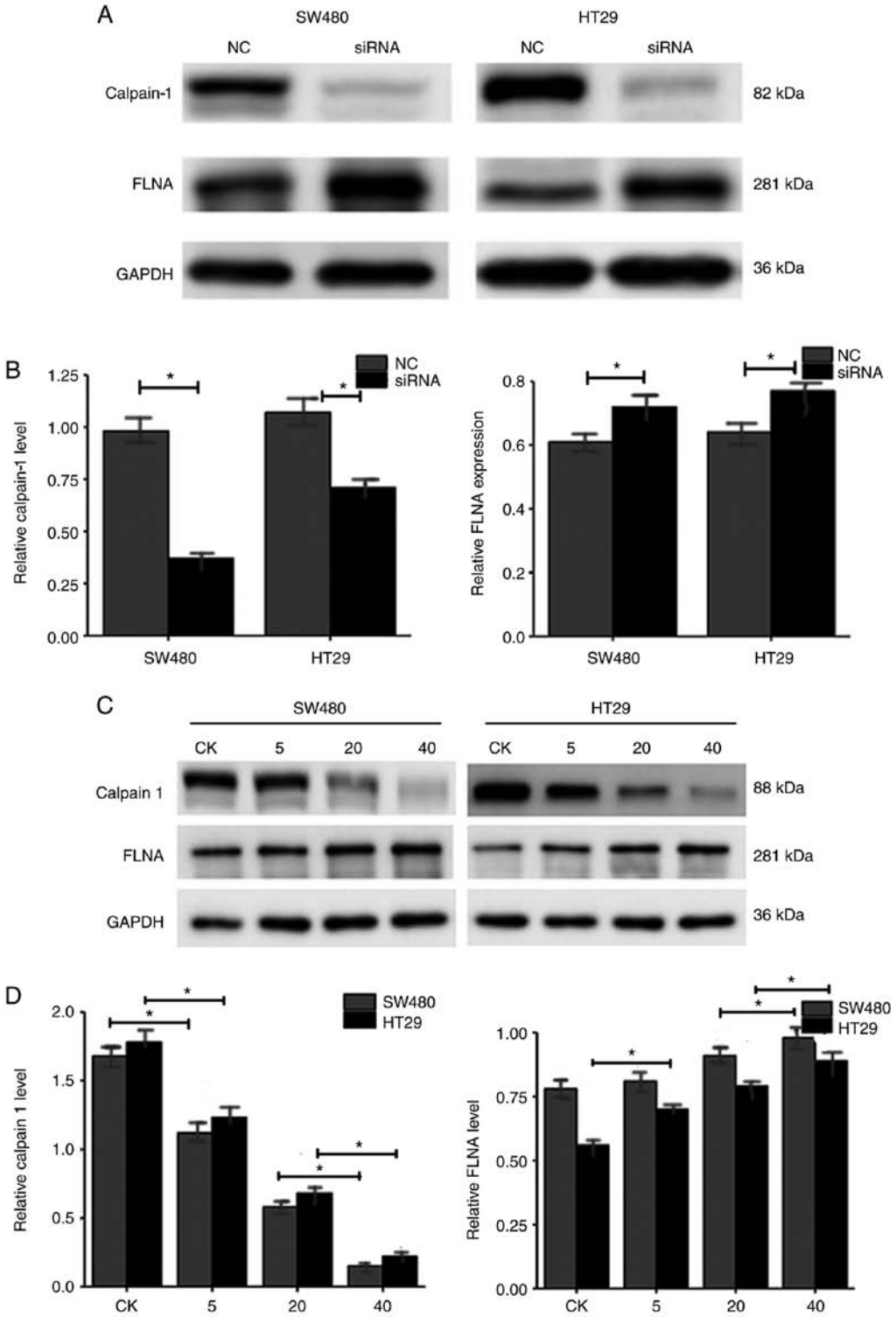

Figure 5. Calpain-1 and FLNA protein expression levels in colorectal cancer cells detected by western blotting. (A) Representative blots and (B) quantification of calpain-1 and FLNA levels following calpain-1 knockdown. (C) Representative blots and (D) quantification of calpain-1 and FLNA levels following treatment with the indicated concentrations of MDL28170. *P<0.05, with comparisons indicated by brackets. FLNA, filamin A; si, small interfering; NC, negative control; CK, control check.

calpain-1 knockdown were not altered compared with the control groups (P>0.05).

Calpain-1 knockdown in CRC cells is associated with the upregulation of FLNA. As presented in Fig. 5A and B, the relative protein expression levels of calpain-1 in SW480 NC, SW480 siRNA, HT29 NC, and HT29 siRNA groups were 0.98±0.09, 0.38±0.04, 1.08±0.10, 0.69±0.06, respectively. The relative protein expression levels of FLNA in SW480 NC, SW480 siRNA, HT29 NC, and HT29 siRNA groups were 0.61±0.03, 0.73±0.06, 0.65±0.04, 0.77±0.06, respectively. The western blot results revealed that calpain-1 knockdown decreased calpain-1 expression, while it increased FLNA expression in both CRC cell lines.

FLNA expression is upregulated when calpain-1 expression is decreased. MDL28170, a calpain-1 inhibitor, was used to test if FLNA expression is directly influenced by the expression of calpain-1. As the MDL28170 treatment amount increased from 0 to 40 μg, the levels of calpain-1 in SW480 control, SW480 5 μg, SW480 20 μg and SW480 40 μg groups were 1.65±0.13, 1.11±0.09, 0.61±0.05, 0.19±0.02, respectively (Fig. 5C and D). The levels of calpain-1 in the corresponding HT29 groups were 1.75±0.15, 1.22±0.11, 0.69±0.05, 0.22±0.03, respectively (Fig. 5C and D). The levels of FLNA in the corresponding SW480 groups were 0.77±0.06, 0.79±0.06, 0.87±0.08 and 0.95±0.10, and in the HT29 groups 0.57±0.03, 0.69±0.04, 0.76±0.05 and 0.84±0.08, respectively (Fig. 5C and D). The results revealed that as calpain-1 expression decreased in a dose-dependent manner, the FLNA expression levels increased, in both CRC cells lines.

Discussion

Calpain-1 was first recognized as a Ca^{2+}-dependent neutral protease in rat brain (16). Deficiency of the calpain-1 gene in mice results in abnormal growth, decreased platelet aggregation and poor blood clotting (17). Calpain is involved in target protein hydrolysis, thus has a role in the protein modification pathway (18). Calpain is also involved in cell mobility, proliferation, cell cycle progression, migration, necrosis, apoptosis (2,19), platelet activation, differentiation and membrane fusion (3,20,21). Calpain-1 is so versatile that it is easy to speculate that it may have a role in tumorigenesis and cancer progression.

Indeed, several studies have demonstrated that calpains are associated with certain types of cancer. In renal cell cancer, a higher expression level of calpain-1 was correlated with more advanced disease and worse prognosis (22). In melanoma, calpains are involved in mitogen-activated protein kinase (MAPK)-dependent cyclin-dependent kinase inhibitor 1B (p27Kip1) regulation, and calpain inhibitors impair MAPK-dependent p27Kip1 downregulation in melanoma cells (23). Based on these studies, calpain may act as a tumor promoter. However, the role of the calpain-1 gene in CRC has not been reported.

Immunohistochemical staining of 467 paired specimens of CRC and adjacent paracancerous tissues was performed to examine the relationship between calpain protein expression and the clinicopathological features of CRC. First, the expression level of calpain-1 protein in CRC tissues was demonstrated to be significantly higher compared with the adjacent normal tissues by immunohistochemistry. Overexpression of calpain-1 was associated with age, metastasis, and Dukes stage, and Kaplan-Meier analysis revealed that patients in the calpain-1 high expression group had a shorter OS compared with the low expression group. These results indicated that overexpression of calpain-1 may be associated with a worse prognosis in CRC patients.

To investigate the biological function of calpain-1 in CRC, calpain-1 expression levels in six CRC cell lines were detected by western blotting. Calpain-1 protein was highly expressed in five of the CRC cell lines compared with a healthy intestinal epithelial cell line (NCM460). Next, an *in vitro* model of calpain-1 knockdown was established by siRNA transfection in HT29 and SW480 CRC cells, and successful knockdown was confirmed by western blotting. Using this model, the effects of calpain-1 on the proliferation of HT29 and SW480 cells were examined and the results demonstrated that calpain-1 knockdown inhibited the proliferative ability of CRC cells. Similarly, Ma *et al* (24) reported that downregulation of calpain-1 in oral squamous cell carcinoma cells resulted in cell cycle arrest and inhibited proliferation. Colony formation assays in the present study revealed that the colony forming ability of CRC cells was significantly inhibited following calpain-1 knockdown. A previous study has also demonstrated that calpain inhibitors impair colony formation of mouse tumor cells (25). These results suggested that calpain-1 promoted cell proliferation in CRC cell lines. By contrast, the present results revealed that calpain-1 had no effect on apoptosis in HT29 and SW480 cells, which was in agreement with previously reported results. Calpain-1 knockdown did not increase the CRC cell apoptosis rate compared with the negative control group. Additionally, calpain-1 knockdown inhibited the migration and invasion abilities of CRC cells. This is consistent with the experimental results of Ma *et al* (24) in oral squamous cell carcinoma cells. The present results suggested that a high expression level of calpain-1 may promote the malignant biological behavior of CRC cells. The underlying mechanism requires further investigation.

FLNA is an actin filament cross-linking protein involved in cancer progression; it is the most abundant and widely expressed isoform of filamin in human tissues and can be cleaved by calpain. Research has demonstrated that FLNA is a novel cancer suppressor gene in colorectal adenocarcinoma (15). The relationship between calpain and FLNA in the development of CRC is of interest. The immunohistochemistry results revealed that FLNA protein expression levels in adjacent normal colorectal tissues were significantly higher than those in CRC tissues. Low expression of FLNA was associated with tumor size, histologic grade, metastasis and Dukes stage. Kaplan-Meier analysis revealed that the FLNA low expression group had a shorter OS compared with the high expression group. These results suggested that FLNA may act as a tumor suppressor in CRC. Notably, calpain expression is correlated with FLNA expression. One study reported that activation of calpain followed by the cleavage of FLNA resulted in enhanced motility of melanoma cells (26). To further confirm the relationship between calpain-1 and FLNA, the expression levels of FLNA and calpain-1 in HT29 and SW480 cells were examined. The results demonstrated that when calpain-1 expression was downregulated, FLNA expression was increased. Similarly, after adding different concentrations of a calpain inhibitor, the FLNA expression levels were increased in a dose-dependent manner. Therefore, it can be speculated that calpain-1 may promote CRC progression via cleavage of FLNA, which is consistent with the findings of Salimi *et al* (25); by proliferation, migration, invasion and colony formation assays, they concluded that a calpain inhibitor decreased human and mouse tumor cell growth by blocking the cleavage of FLNA.

In conclusion, the present findings suggested that high levels of calpain-1 predicted a poor prognosis and contributed to tumor progression in CRC. These tumor promoting functions of calpain-1 may occur through calpain-mediated cleavage of FLNA. In the future, calpain-1 might serve as a novel target gene for CRC therapy.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and ZX conceived, designed, reviewed and edited the manuscript. LY, YL, HY and XY performed the experiments. CX and YZ wrote the paper. CX wrote the manuscript, performed the data analysis and interpretation. YZ collected the pathological features and clinical data of colorectal cancer patients and approved the publication of the final version. ZC reviewed and edited the manuscript and also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University, and informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
2. Cotton SW, Kornegay JN, Bogan DJ, Wadosky KM, Patterson C and Willis MS: Genetic myostatin decrease in the golden retriever muscular dystrophy model does not significantly affect the ubiquitin proteasome system despite enhancing the severity of disease. Am J Transl Res 6: 43-53, 2013.
3. Arthur JS, Elce JS, Hegadorn C, Williams K and Greer PA: Disruption of the murine calpain small subunit gene, Capn4: Calpain is essential for embryonic development but not for cell growth and division. Mol Cell Biol 20: 4474-4481, 2000.
4. Suzuki K, Hata S, Kawabata Y and Sorimachi H: Structure, activation, and biology of calpain. Diabetes 53: (Suppl 1): S12-S18, 2004.
5. Huang Y and Wang KK: The calpain family and human disease. Trends Mol Med 7: 355-362, 2001.
6. Gonzalez A, Abril E, Roca A, Aragón MJ, Figueroa MJ, Velarde P, Royo JL, Real LM and Ruiz A: CAPN10 alleles are associated with polycystic ovary syndrome. J Clin Endocrinol Metab 87: 3971-3976, 2002.
7. Ehrmann DA, Schwarz PE, Hara M, Tang X, Horikawa Y, Imperial J, Bell GI and Cox NJ: Relationship of calpain-10 genotype to phenotypic features of polycysticovary syndrome. J Clin Endocrinol Metab 87: 1669-1673, 2002.
8. Ohno S, Minoshima S, Kudoh J, Fukuyama R, Shimizu Y, Ohmi-Imajoh S, Shimizu N and Suzuki K: Four genes for the calpain family locate on four distinct human chromosomes. Cytogenet Cell Genet 53: 225-229, 1990.
9. Storr SJ, Thompson N, Pu X, Zhang Y and Martin SG: Calpain in breast cancer: Role in disease progression and treatment response. Pathobiology 82: 133-141, 2015.
10. Storr SJ, Carragher NO, Frame MC, Parr T and Martin SG: The calpain system and cancer. Nat Rev Cancer 11: 364-374, 2011.
11. Storr SJ, Woolston CM, Barros FF, Green AR, Shehata M, Chan SY, Ellis IO and Martin SG: Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy. Int J Cancer 129: 1773-1780, 2011.
12. Luo W, Ren Z, Gao S, Jin H, Zhang G, Zhou L and Zheng S: Clinical correlation of calpain-1 and glypican-3 expression with gallbladder carcinoma. Oncol Lett 11: 1345-1352, 2016.
13. Salehin D, Fromberg I, Haugk C, Dohmen B, Georg T, Bohle RM, Bauerschlag D, Maass N and Friedrich M: Immunhistochemical analysis for expression of calpain 1, calpain 2 and calpastatin in endometrial cancer. Anticancer Res 30: 2837-2843, 2010.
14. Kovacs L and Su Y: The critical role of calpain in cell proliferation. Biomol Res Ther 3: 112, 2014.
15. Tian ZQ, Shi JW, Wang XR, Li Z and Wang GY: New cancer suppressor gene for colorectal adenocarcinoma: Filamin A. World J Gastroenterol 21: 2199-2205, 2015.
16. Guroff G: A neutral, calcium-activated proteinase from the soluble fraction of rat brain. J Biol Chem 239: 149-155, 1964.
17. Azam M, Andrabi SS, Sahr KE, Kamath L, Kuliopulos A and Chishti AH: Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. Mol Cell Biol 21: 2213-2220, 2001.
18. Sorimachi H, Hata S and Ono Y: Impact of genetic insights into calpain biology. J Biochem 150: 23-37, 2011.
19. Van Ba H and Inho H: Significant role of μ-calpain (CANP1) in proliferation/survival of bovine skeletal muscle satellite cells. In vitro Cell Dev Biol Anim 49: 785-797, 2013.
20. Santella L, Kyozuka K, De Riso L and Carafoli E: Calcium, protease action, and the regulation of the cell cycle. Cell Calcium 23: 123-130, 1998.
21. Wang KK: Calpain and caspase: Can you tell the difference? Trends Neurosci 23: 20-26, 2000.
22. Braun C, Engel M, Seifert M, Theisinger B, Seitz G, Zang KD and Welter C: Expression of calpain I messenger RNA in human renal cell carcinoma: Correlation with lymph node metastasis and histological type. Int J Cancer 84: 6-9, 1999.
23. Delmas C, Aragou N, Poussard S, Cottin P, Darbon JM and Manenti S: MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export. J Biol Chem 278: 12443-12451, 2003.
24. Ma D, Fang J, Liu Y, Song JJ, Wang YQ, Xia J, Cheng B and Wang Z: High level of calpain1 promotes cancer cell invasion and migration in oral squamous cell carcinoma. Oncol Lett 13: 4017-4026, 2017.
25. Salimi R, Bandaru S, Devarakonda S, Gökalp S, Ala C, Alvandian A, Yener N and Akyürek LM: Blocking the cleavage of filamin A by calpain inhibitor decreases tumor cell growth. Anticancer Res 38: 2079-2085, 2018.
26. O'Connell MP, Fiori JL, Baugher KM, Indig FE, French AD, Camilli TC, Frank BP, Earley R, Hoek KS, Hasskamp JH, *et al*: Wnt5A activates the calpain-mediated cleavage of filamin A. J Invest Dermatol 129: 1782-1789, 2009.