Prognostic and clinical significance of subcellular CDC27 for patients with rectal adenocarcinoma treated with adjuvant chemotherapy

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Abstract. Rectal adenocarcinoma (READ) constitutes one-third of newly diagnosed colorectal cancer cases. Surgery, chemotherapy and concurrent chemoradiotherapy are the main treatments to improve patient outcomes for READ. However, patients with READ receiving these treatments eventually relapse, leading to a poor survival outcome. The present study collected surgical specimens from patients with READ and determined that cytoplasmic cell division cycle 27 (CDC27) expression was associated with the risk of lymph node metastasis and distant metastasis. Nuclear CDC27 expression was negatively associated with 5-year disease-free survival (DFS) and 5-year overall survival (OS) rates. Multivariate Cox proportional regression analysis showed that nuclear CDC27 was an independent prognostic factor in the patients with READ, especially in those treated with adjuvant chemotherapy. High nuclear CDC27 expression was significantly associated with poorer 5-year DFS (HR, 2.106; 95% CI, 1.275-3.570; P=0.003) and 5-year OS (HR, 2.369; 95% CI, 1.270-4.6810; P=0.005) rates. The data indicated that cytoplasmic CDC27 expression could affect tumor progression and that it plays an important role in metastasis. Nuclear CDC27 expression was markedly associated with poorer survival outcomes and was an independent prognostic factor in patients with postoperative adjuvant chemotherapy-treated READ. Thus, CDC27 expression serves as a potential prognostic marker for rectal tumor progression and chemotherapy treatment.

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Introduction

Colorectal cancer is one of the leading causes of cancer-related death globally, and ~30% of these cases are rectal adenocarcinoma (READ). Although surgery is a principal approach for READ treatment, preoperative concurrent chemoradiotherapy, post-operative adjuvant chemotherapy and radiotherapy are often used as a combined therapeutic regimen for high-risk patients and those with advanced READ (1). These multidisciplinary approaches not only decrease local recurrence and distant metastasis rates, but also improve survival outcome for patients with locally advanced rectal cancer (1,2). Fluoropyrimidine-based postoperative adjuvant chemotherapy followed by surgery is usually the standard treatment regimen for patients with READ (3). However, drug resistance quickly occurs within months, eventually followed by distant metastasis, and is the major cause of treatment failure and cancer-associated

death in patients with advanced READ (4). Therefore, suitable prognostic factors to stratify advanced READ for personalized therapeutic strategies are urgently required.

Cell division cycle 27 (CDC27) is a crucial subunit of the anaphase-promoting complex/cyclosome (APC/C) and regulates the cell cycle by interacting with different coactivators through targeting protein ubiquitination and degradation. APC/C is reported to play a role in genome integrity, apoptosis, autophagy, energy metabolism and tumorigenesis (5). CDC27 also participates in the control of the mitotic checkpoint and surveys the mitotic spindle to maintain chromosomal integrity. CDC27 has been reported to be involved in apoptosis, stemness, epithelial-mesenchymal transition (EMT) and efferocytosis regulation (6,7). Previous studies have revealed that overexpression of CDC27 promotes tumor cell proliferation, invasion and metastasis in patients with colorectal cancer, breast cancer, gastric cancer and lymphoma (8-11). Downregulation of CDC27 has been reported to be associated with clinical outcomes of patients with breast cancer and sensitivity to radiotherapy (12-14). The alteration or mutation of CDC27 is also associated with tumorigenesis, tumor progression, drug resistance and survival (6,15). However, postoperative adjuvant chemotherapy is mostly used for first-line treatment of patients with colorectal cancer, and these chemotherapeutic drugs can interrupt DNA replication and cell cycle processes. There is no direct clinical evidence to explain the correlation between CDC27 expression and chemotherapeutic treatment response, which is still controversial and uncertain.

Furthermore, the amount of tumor-infiltrating lymphocytes (TILs) is an important prognostic factor for colorectal cancer and in previous studies (16,17). In the present study, in the TIMER database (http://timer.cistrome.org/), CDC27 expression was associated with immune cell infiltration. Hence, the present study aimed to investigate the expression profiles of CDC27 and intratumor-infiltrating $CD3^+$ lymphocytes by immunohistochemical staining, and examine their clinical significance and effect on survival outcome in patients with READ. These findings provide new insights into the potential of CDC27 as a prognostic factor in patients with READ.

Materials and methods

Patients, clinical staging and pathological evaluation. Between January 2011 and December 2016, 290 patients with rectal cancer were recruited from China Medical University Hospital (CMUH) (Taichung, Taiwan). Among these patients, due to insufficient tumor tissue, only 255 patients who received surgery with or without postoperative chemotherapy were enrolled in this study cohort. Resected specimens were reviewed by pathologists, and pathological staging was based on the 7th American Joint Committee on Cancer (AJCC) staging system (18). A postoperative chemotherapeutic regimen was recommended for patients with high-risk stage II disease and lymph node metastasis stage III identified in surgical specimens, according to the status of the patients. The clinicopathological characteristics were extracted from the electronic medical records. This study was approved by the Institutional Review Board (IRB) of CMUH (approval no. CMUH105-REC2-072) and written informed consent was obtained from all patients.

Tissue microarray (TMA) construction. A TMA was constructed from the surgically resected primary tumor tissues of patients with READ as previously described (17,19), as approved by the IRB of CMUH (approval no. CMUH105-REC2-073). Briefly, the tissues were fixed in 10% neutral buffered formalin (Leica Microsystems, Inc.) for 18-24 h at RT, dehydrated with ethanol, infiltrated with paraffin wax, embedded into paraffin at 60˚C and then cooled to become formalin-fixed, paraffin-embedded (FFPE) blocks. Next, the sections of FFPE blocks were cut by a microtome (Leica Microsystems, Inc.) onto the slides. The slides were deparaffinization with xylene, stained with hematoxylin for 3 min and eosin for 1 min, and then mounted in resin. Next, the tumor areas were evaluated and marked on hematoxylin and eosin-stained (H&E) slides by a pathologist using light microscopy (Leica Microsystems, Inc.). Finally, the corresponding area was identified, marked, and punched on the matching FFPE block, and then transferred into a paraffin-embedded recipient block for TMA construction using an AutoTiss 10C system (EverBio Technology, Inc.). A single TMA block comprised a maximum of 60 cores, 2 mm in diameter, and the sections were cut by a microtome (Leica biosystems, Inc.) and mounted on capillary-gap slides (Dako, Inc.).

Immunohistochemical (IHC) staining and evaluation. IHC staining was performed using 3-μm sections of TMAs as previously described (19). The antibodies used in this study were anti-human CDC27 (1:100; cat. no. ab10538; Abcam) and anti-human CD3 (1:100; cat. no. ab16669; Abcam). A HRP-conjugated Vectastain Elite ABC Kit (Vector Laboratories, Inc.) and DAB chromogen (Vector Laboratories, Inc.) were used for detection according to the manufacturer's protocol. Briefly, the slides were retrieved with sodium citrate buffer (pH 6.0) for 20 min at 80˚C and then incubated with 3% H_2O_2 in 1X PBS at room temperature (RT) for 10 min to block endogenous peroxidase activity. Next, the slides were incubated with primary antibodies at RT for 2 h after blocking. According to the manufacturer's protocol, blocking solution, biotinylated antibody and Vectastain Elite ABC reagent were used and incubated in the slides at RT for 20, 30 and 30 min, respectively. The slides were incubated in a peroxidase substrate solution with DAB chromogen for 10 min at RT according to the manufacturer's protocol. Finally, the slides were counterstained with hematoxylin for 1 min at RT, washed and mounted in resin mount.

Evaluation of CDC27 expression in the cytoplasm and nucleus was assessed through the histoscore (H-score) (20). The immunostaining was performed using a semi-quantitative H-score. The intensity of the staining was categorized as follows: Negative, weak, moderate and strong. The H-score was determined as the intensity and the percentage of the staining area of tumor cells at its intensity grade using the following formula: H-score=[1 x (% of weak staining) + 2 x (% of moderate staining) + 3 x (% of strong staining)]. The range of the H-score was from 0 to 300. The CDC27 expression status was categorized as low or high according to the mean value of the H-score. Evaluation of $CD3^+$ TILs was performed, with counting at x400 magnification [number of positively stained TILs/high-power field (HPF)], as previously described (17). The

mean count of $CD3^+$ TILs in five HPFs was scored and graded as follows: 0, no positively stained TILs/HPF; 1, 1-3 positively stained TILs/HPF; 2, 4-10 positively stained TILs/HPF; and 3, >10 positively stained TILs/HPF. Finally, specimens with grade 2 or 3 were regarded as the high group and those with grade 0 or 1 were regarded as the low group.

TIMER database analysis. The Tumor Immune Estimation Resource (TIMER) database (http://timer.cistrome.org/) is used for the systematic analysis of immune infiltrates in the tumor microenvironment of various cancer types (21). The association between CDC27 expression and $CD4^+$/$CD8^+$ T cells with purity adjustment was analyzed via gene modules in patients with READ.

Statistical analysis. MP Pro statistical software version 12 (SAS Institute, Inc.) was used to perform the statistical analysis. The H-score of nuclear and cytoplasmic CDC27 expression was analyzed with the Wilcoxon matched-pairs test, and the correlation was examined with Spearman's analysis. Associations between CDC27 expression and clinicopathological variables were analyzed by a χ^2 test. The 5-year DFS and OS rates were estimated using the Kaplan-Meier method, and survival differences were analyzed with the log-rank test. The Cox proportional hazards model was used for multivariate analysis to estimate the HR, 95% CI and prognostic factors. $P<0.05$ (two-sided) was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics in patients with READ. Tissue specimens from 255 patients with READ who underwent surgery were collected and the clinicopathological characteristics are shown in Table I. The majority of patients were male (67%), and the mean age at diagnosis was 63.2±13.7 years (range, 25-97 years). The patients were staged according to the 7th edition of the AJCC staging system. There were 135 patients (53%) who had nodal metastasis, and 148 patients (58%) received fluorouracil-based postoperative chemotherapy. KRAS mutations were assessed in 121 of the 255 patients (47%), and of these, 64 patients (53%) were determined to have wild-type KRAS.

Association between clinicopathological characteristics and subcellular distribution of CDC27 expression in patients with READ. To determine the expression profiles of CDC27 in tumor tissues of patients with READ, CDC27 expression was examined by IHC staining according to the H-scoring system. CDC27 protein was present in the cytoplasm and/or nucleus with different expression patterns (Fig. 1A). The expression pattern of CDC27 was patchy or focally intense in the nucleus, but was diffusely in the cytoplasm. There were 93 patients (36%) with no cytoplasmic expression of CDC27, and 32 patients (13%) with no nuclear expression of CDC27. Among these patients, 30 patients (12%) lacked CDC27 expression in the cytoplasm as well as the nucleus. The distribution of CDC27 H-score in the cytoplasm and nucleus is shown in Fig. 1B; the mean H-scores of CDC27 expression in the cytoplasm and nucleus were 95.8 and 168.5, respectively. The H-score of nuclear CDC27 expression was significantly higher than that of cytoplasmic CDC27 (Wilcoxon matched-pairs test, $P<0.0001$). Spearman's correlation analysis showed that the H-score of CDC27 in the nucleus was positively correlated with that of the cytoplasm [correlation coefficient (rs) = 0.752; $P<0.0001$] (Fig. 1C).

To evaluate whether the subcellular distribution of CDC27 had different prognostic significance, the relationship between the subcellular distribution of CDC27 and the

Table I. Clinicopathological characteristics of patients with READ (n=255).

Clinicopathological parameters	Value
Sex, n (%)	
Male	159 (62)
Female	96 (38)
Age, years	
Mean (range)	63.2 (25-97)
<65, n (%)	142 (56)
≥65, n (%)	113 (44)
Histological grading, n (%)	
Well to moderate	233 (94)
Poor	14 (6)
NA	8
pTMN stage (7th AJCC), n (%)	
I	61 (24)
II	56 (22)
III	104 (41)
IV	34 (13)
pN, n (%)	
Negative	117 (46)
Positive	138 (54)
Lymphovascular invasion, n (%)	
Absent	151 (61)
Present	98 (39)
NA	6
Perineural invasion, n (%)	
Absent	151 (61)
Present	98 (39)
NA	6
Preoperative CEA, n (%)	
<5 ng/ml	138 (60)
≥5 ng/ml	92 (40)
NA	25
Postoperative chemotherapy, n (%)	
No	107 (42)
Yes	148 (58)
Kras mutation, n (%)	
Wild-type	64 (53)
Mutant	57 (47)
NA	134

NA, not available; CEA, carcinoembronic antigen; AJCC, American Joint Committee on Cancer.

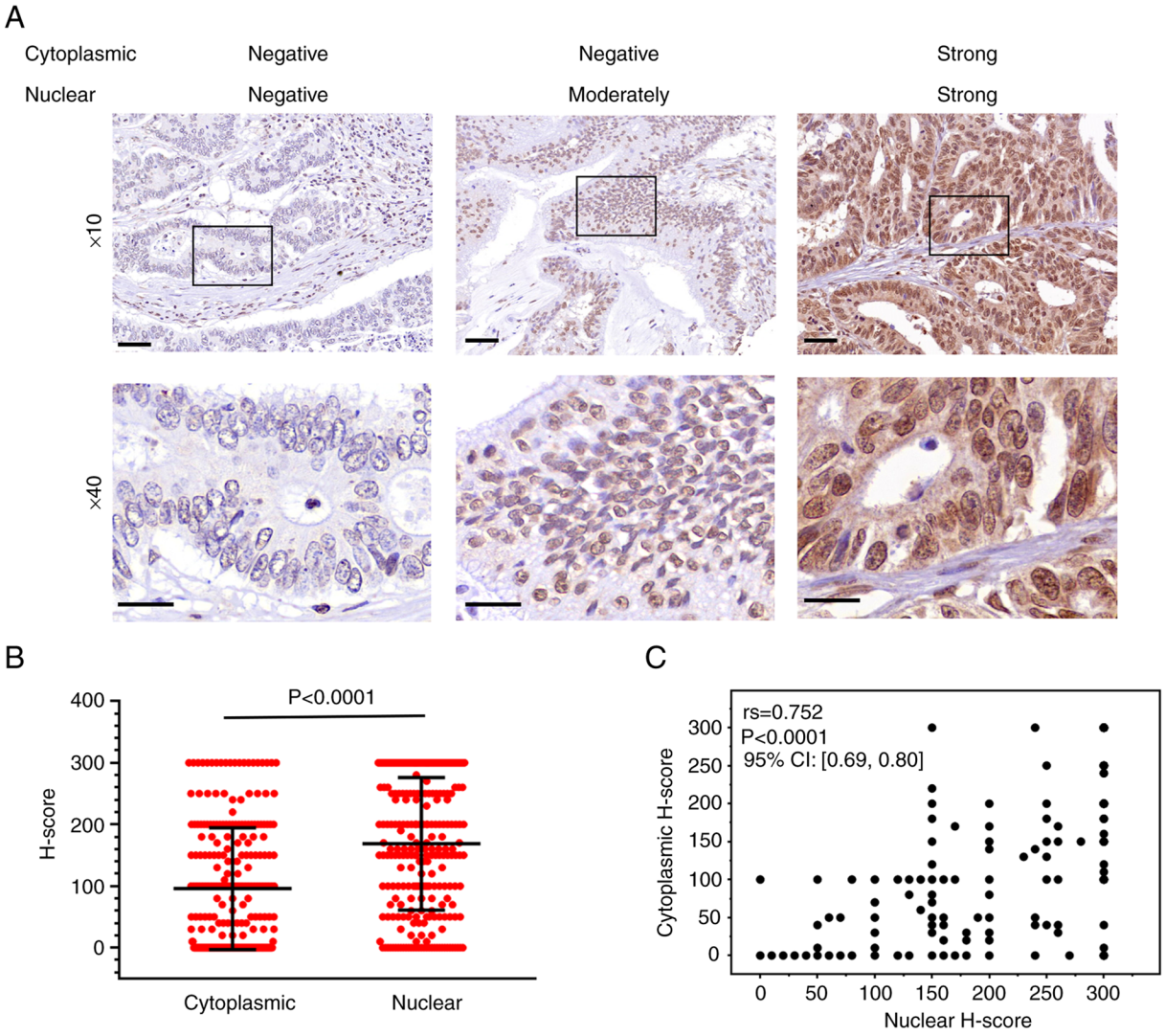

Figure 1. Representative images and analysis of CDC27 immunohistochemistry staining from the tissue microarray of specimens from patients with READ. (A) Expression of CDC27 in the nucleus and/or cytoplasm of tumor cells. Scale bars, 50 μm (magnification, x40) and 20 μm (magnification, x10). (B) Distribution of CDC27 expression in the cytoplasm and nucleus by the H-index. Data are expressed as the mean ± SD. (C) The correlation of CDC27 expression between the cytoplasm and nucleus as determined using Spearman's correlation analysis. CDC27, cell division cycle 27.

clinicopathological characteristics in patients with READ was analyzed. READ patients were stratified into high and low CDC27 expression according to the mean H-score, and their relationship with clinicopathological parameters was analyzed. As shown in Table II, it was found that cytoplasmic CDC27 expression was significantly associated with TNM stage (P=0.021), pN stage (P=0.003), lymphovascular invasion (LVI) (P<0.001), perineural invasion (PNI) (P=0.012) and distant metastasis (P=0.043). Patients with high cytoplasmic CDC27 expression exhibited advanced stage, lymph node metastasis, presence of LVI and PNI, and distant metastasis. Nuclear CDC27 expression was significantly associated with the presence of LVI (P=0.002). There was no association between nuclear CDC27 and other clinicopathological parameters. These findings suggest that cytoplasmic CDC27 expression may be associated with tumor progression and metastasis.

Association between CDC27 expression and CD3⁺ TILs in patients with READ. From the TIMER database, it was found that CDC27 expression was associated with immune cells infiltration in colorectal cancer (http://timer.cistrome.org/). To further explore these results, the association between the subcellular distribution of CDC27 and intratumor CD3⁺ TILs was analyzed in READ tumor tissues. First, the association between CD3⁺ TILs and clinicopathological parameters was analyzed, which found that CD3⁺ TILs was markedly associated with TNM stage (P<0.001), pN stage (P<0.001) and PNI (P<0.001) (Table SI). High-grade CD3⁺ TILs in tumor cells were associated with advanced stage, lymph node metastasis and presence of PNI. However, it was also found that intratumor CD3⁺ TILs had no association with cytoplasmic or nuclear CDC27 expression (Table II).

Nuclear CDC27 expression is associated with survival outcomes in patients with READ. Next, the present study evaluated whether the subcellular expression of CDC27 was associated with the clinical outcomes of patients with READ using the Kaplan-Meier survival analysis. As shown in Table III, several clinicopathological parameters, including age (P=0.011 and P<0.0001), TNM stage (P<0.0001 and

Table II. Association between clinicopathological parameters and cell division cycle 27 expression in patients with READ.

Clinicopathological parameters	Cytoplasmic expression, n (%)			Nuclear expression, n (%)		
	Low	High	P-value	Low	High	P-value
Total patients	129 (100)	126 (100)		127 (100)	128 (100)	
Sex						
Male	77 (60)	82 (65)	0.374	78 (61)	81 (63)	0.758
Female	52 (40)	44 (35)		49 (39)	47 (37)	
Age, years						
<65	75 (58)	67 (53)	0.424	71 (56)	71 (55)	0.944
≥65	54 (42)	59 (47)		56 (44)	57 (45)	
Histological grading						
Well to moderate	118 (95)	115 (93)	0.571	117 (94)	116 (95)	0.613
Poor	6 (5)	8 (7)		8 (6)	6 (5)	
NA	5	3		2	6	
pTNM stage (7th AJCC)						
Early	71 (55)	46 (37)	0.003[a]	63 (50)	54 (42)	0.234
Late	58 (45)	80 (63)		64 (50)	74 (58)	
pN						
Negative	71 (55)	46 (37)	0.003[a]	63 (50)	54 (42)	0.234
Positive	58 (45)	80 (63)		64 (50)	74 (58)	
Lymphovascular invasion						
Absent	90 (71)	61 (50)	<0.001[a]	88 (70)	63 (51)	0.002[a]
Present	36 (29)	62 (50)		38 (30)	60 (49)	
NA	3	3		1	5	
Perineural invasion						
Absent	86 (68)	65 (53)	0.012[a]	82 (65)	69 (56)	0.146
Present	40 (32)	58 (47)		44 (35)	54 (44)	
NA	3	3		1	5	
Preoperative CEA, ng/ml						
<5	71 (60)	67 (60)	0.957	67 (58)	61 (62)	0.59
≥5	47 (40)	45 (40)		48 (42)	44 (38)	
NA	11	14		12	13	
Kras mutation						
Wild-type	28 (46)	33 (49)	0.796	32 (54)	31 (49)	0.707
Mutant	24 (54)	35 (51)		27 (46)	32 (51)	
NA	77	58		68	65	
$CD3^+$ TILs						
High	59 (46)	66 (52)	0.288	55 (43)	70 (55)	0.068
Low	70 (54)	60 (48)		72 (57)	58 (45)	
Distant metastasis						
No	111 (86)	96 (76)	0.043[a]	106 (83)	101 (79)	0.351
Yes	18 (14)	30 (24)		21 (17)	27 (21)	

[a]P<0.05. NA, not available; CEA, carcinoembronic antigen; TIL, tumor-infiltrating lymphocyte; AJCC, American Joint Committee on Cancer.

P<0.0001), pN stage (P<0.0001 and P<0.0001), PNI (P<0.0001 and P=0.0002), preoperative CEA level (P<0.0001 and P<0.0001), $CD3^+$ TILs (P=0.002 and P=0.0043) and nuclear CDC27 expression (P=0.028 and P=0.028), were significantly associated with 5-year disease-free survival (DFS) and 5-year overall survival (OS), respectively. It is worth noting that cytoplasmic CDC27 expression had a non-significant tendency towards association with 5-year DFS (P=0.081, log-rank test; Fig. 2A) and 5-year OS (P=0.129, log-rank test; Fig. 2B). Nuclear CDC27 expression was significantly associated with

Table III. Association between clinicopathological parameters and 5-year survival outcome in patients with READ (n=255).

Clinicopathological parameters	Cases, n	5-year DFS rate, %	P-value	5-year OS rate, %	P-value
Total patients		50.3		62.2	
Sex					
Male	159	48.4	0.433	61.3	0.548
Female	96	53.7		63.8	
Age, years					
<65	142	57.7	0.011[a]	75.1	<0.0001[a]
≥65	113	40.2		44.9	
Histological grading					
Well to moderate	14	50.8	0.310	55.0	0.449
Poor	233	29.7		61.7	
NA	8				
pTNM stage (7th AJCC)					
Early	117	65.3	<0.0001[a]	77.2	<0.0001[a]
Late	138	37.8		49.6	
pN					
Negative	117	65.3	<0.0001[a]	77.2	<0.0001[a]
Positive	138	37.8		49.6	
Lymphovascular invasion					
Absent	151	53.4	0.213	66.2	0.121
Present	98	43.9		55.0	
NA	6				
Perineural invasion					
Absent	151	61.5	<0.0001[a]	71.2	0.0002[a]
Present	98	31.5		47.6	
NA	6				
Preoperative CEA, ng/ml					
<5	138	63.2	<0.0001[a]	72.8	<0.0001[a]
≥5	92	32.6		45.5	
NA	25				
$CD3^+$ TILs					
High	120	61.7	0.002[a]	71.0	0.0043[a]
Low	135	41.0		53.3	
Cytoplasmic CDC27 expression					
Low	129	55.9	0.08	67.4	0.121
High	126	44.3		56.8	
Nuclear CDC27 expression					
Low	127	56.8	0.028[a]	69.6	0.028[a]
High	128	43.8		55.0	

[a]P<0.05. NA, not available; CEA, carcinoembronic antigen; TIL, tumor-infiltrating lymphocyte; AJCC, American Joint Committee on Cancer.

5-year DFS and OS (P=0.029 and P=0.028, respectively, log-rank test) in patients with READ (Fig. 2C and D).

To further validate the prognostic values of nuclear CDC27, Cox proportional hazards model and multivariate linear regression analysis were used. As shown in Table IV, in the total patient group, nuclear CDC27 expression was an independent risk factor for 5-year DFS. Patients carrying high nuclear CDC27 expression had an increased risk for a poorer 5-year DFS rate (HR, 1.590; 95% CI, 1.075-2.369; P=0.020). Taken together, these results indicated that nuclear CDC27 expression acted as an independent prognostic factor for patients with READ.

Nuclear CDC27 expression as a predictive biomarker for adjuvant chemotherapy treatment in patients with READ. Furthermore, the present study examined whether the subcellular expression of CDC27 was associated with the response to postoperative adjuvant chemotherapy using

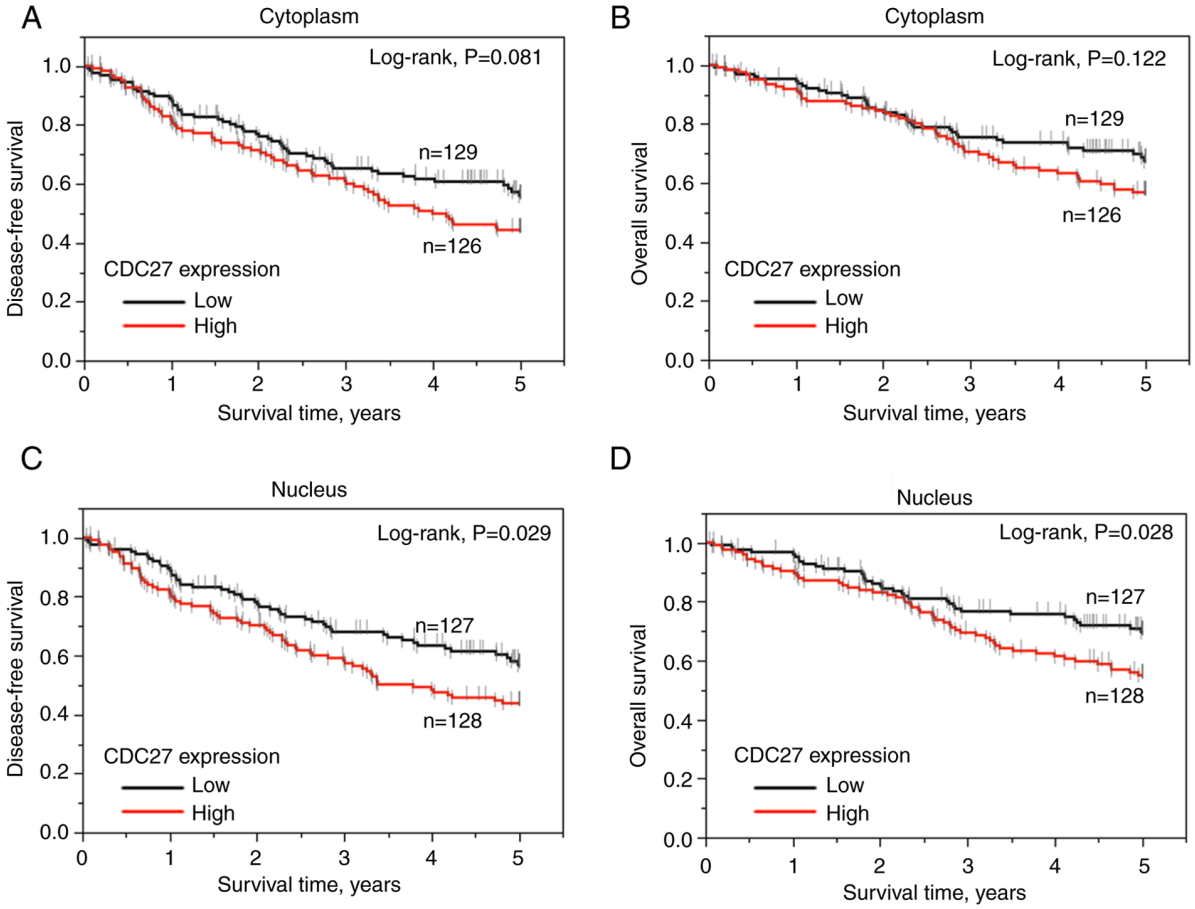

Figure 2. Association between survival outcomes and CDC27 expression in patients with READ. Survival curves were created by Kaplan-Meier survival analysis. (A) 5-year DFS and (B) 5-year OS based on the expression of CDC27 status in the cytoplasm. (C) 5-year DFS and (D) 5-year OS based on the expression of CDC27 status in the nucleus. CDC27, cell division cycle 27; OS, overall survival; DFS, disease-free survival.

Kaplan-Meier survival analysis. It was found that cytoplasmic CDC27 expression was not associated with 5-year DFS or 5-year OS rates in the adjuvant chemotherapy or surgery only groups (Fig. S1A-D). Nuclear CDC27 expression was significantly associated with 5-year DFS (P=0.004, log-rank test) and 5-year OS (P=0.002, log-rank test) in the adjuvant chemotherapy-treated group (Fig. 3C and D). According to the Cox proportional hazards model and multivariate linear regression analysis, patients with high nuclear CDC27 expression remarkably exhibited an increased risk for a poorer 5-year DFS (HR, 2.106; 95% CI, 1.275-3.570; P=0.003) and OS (HR, 2.369; 95% CI, 1.272-4.681; P=0.005) in the adjuvant chemotherapy-treated group (Table IV). These results indicated that nuclear CDC27 expression is an independent prognostic factor for patients with READ, especially those treated with postoperative adjuvant chemotherapy. The data revealed that nuclear CDC27 expression may influence the response to adjuvant chemotherapy.

Discussion

The present study evaluated the subcellular pattern of CDC27 expression and intratumor-infiltrating $CD3^+$ lymphocytes in resected tumors of a cohort of patients with READ using IHC. It was found that cytoplasmic and nuclear CDC27 expression had different influences on clinicopathological characteristics and survival outcomes. Cytoplasmic CDC27 expression was associated with TNM stage, nodal metastasis and distant metastasis. Nuclear CDC27 protein expression was significantly associated with survival outcomes and acted as an independent prognostic factor for patients with READ, especially in those receiving post-operative adjuvant chemotherapy. In addition, intratumor $CD3^+$ TILs were associated with TNM stage, nodal metastasis, PNI presence and survival outcomes in patients with READ. However, cytoplasmic or nuclear CDC27 expression was not associated with the density of intratumor $CD3^+$ TILs. Therefore, the data implied that cytoplasmic and nuclear CDC27 expression had different influences on tumor progression and the response to chemotherapy, respectively.

CDC27 is an important subunit of APC/C that regulates mitosis and chromosome segregation processes at the G_2/M and M/G_1 transitions. Accumulating studies revealed that the expression of CDC27 is associated with tumorigenesis, but is still ambiguous in different cancer types (6). For example, high CDC27 expression triggered cell proliferation, tumor growth, epithelial-mesenchymal transition and metastasis in colorectal and gastric cancer *in vitro* and *in vivo* (9,10,22). High CDC27 expression caused chromosome instability and recurrence in patients with breast cancer (8). However, downregulation of CDC27 was also associated with a poor response to radiotherapy in cervical and breast cancer (12,13). There are a number of isoforms of CDC27, the main one having 824 amino acids, and

Table IV. Multivariate analysis of clinicopathological parameters and nuclear CDC27 expression in 5-year DFS and OS.

A, All patients (n=225)[a]

	DFS			OS		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥65 years vs. <65 years)	1.499	1.015-2.216	0.042[b]	2.735	1.733-4.394	<0.0001[b]
pTNM stage (late vs. early)	-	-	-	-	-	-
pN stage (positive vs. negative)	1.610	0.988-2.669	0.055	2.151	1.242-3.843	0.005[b]
Perineural invasion (present vs. absent)	1.675	1.074-2.369	0.022[b]	1.470	0.898-2.444	0.125
CEA (abnormal vs. normal)	1.940	1.269-2.975	0.002[b]	1.856	1.143-3.036	0.012[b]
$CD3^+$ TILs (low vs. high)	1.599	1.058-2.440	0.025[b]	1.549	0.969-2.508	0.065
Nuclear CDC27 expression (high vs. low)	1.590	1.075-2.369	0.020[b]	1.567	0.998-2.492	0.051

B, No chemotherapy (n=95)

	DFS			OS		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥65 years vs. <65 years)	2.644	1.209-6.413	0.013[b]	6.214	2.332-21.550	<0.0001[b]
pTNM stage (late vs. early)	-	-	-	-	-	-
pN stage (positive vs. negative)	2.005	0.795-4.842	0.137	2.557	1.039-6.331	0.041[b]
Perineural invasion (present vs. absent)	2.484	1.024-5.841	0.044[b]	2.063	0.830-5.058	0.117
CEA (abnormal vs. normal)	0.690	0.283-1.613	0.396	0.664	0.270-1.584	0.359
$CD3^+$ TILs (low vs. high)	1.884	0.929-3.836	0.078	2.072	0.957-4.597	0.064
Nuclear CDC27 expression (high vs. low)	1.022	0.501-2.040	0.949	0.874	0.405-1.829	0.724

C, Chemotherapy (n=130)

	DFS			OS		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age (≥65 years vs. <65 years)	1.045	0.626-1.708	0.862	1.64	0.904-2.947	0.102
pTNM stage (late vs. early)	-	-	-	-	-	-
pN stage (positive vs. negative)	1.734	0.862-3.885	0.127	3.638	1.284-15.267	0.012[b]
Perineural invasion (present vs. absent)	1.662	0.980-2.889	0.059	1.611	0.866-3.134	0.133
CEA (abnormal vs. normal)	2.813	1.670-4.833	<0.0001[b]	2.995	1.573-5.964	0.0007[b]
$CD3^+$ TILs (low vs. high)	1.545	0.921-2.646	0.099	1.433	0.774-2.708	0.254
Nuclear CDC27 expression (high vs. low)	2.106	1.275-3.570	0.003[b]	2.369	1.272-4.681	0.005[b]

A multivariate linear regression was used, and the P-value was obtained from a likelihood ratio test. [a]The data of 30 patients were not available (NA) for the multivariate analysis. [b]P<0.05. CEA, carcinoembronic antigen; TIL, tumor-infiltrating lymphocyte; CDC27, cell division cycle 27; DFS, disease-free survival; OS, overall survival.

the somatic mutations of CDC27 have been found to disrupt APC/C activity in various cancer types (6,15). The vast majority of CDC27 protein is associated with APC/C and expressed in the nucleus, especially in the nuclear envelope membrane and chromosome (23,24). However, Huang *et al* (23) showed that the mutated Cdc27 on *Drosophila* was expressed in both nuclear and cytoplasmic locations. In the present study, an antibody that recognized amino acids 814-823 at the extreme C-terminus of CDC27 was selected for IHC detection. It was found that CDC27 could be expressed in the nucleus and/or cytoplasm. Hence, CDC27 was separated into cytoplasmic and nuclear expression for exploring the roles of CDC27 in patients with READ. It was found that cytoplasmic CDC27 was significantly associated with tumor progression and distant metastasis, but not with survival outcomes, while nuclear CDC27 was strongly associated with survival outcomes and was an independent prognostic factor for this. The results indicated that the location of CDC27 expression may have different functions in tumor development. However,

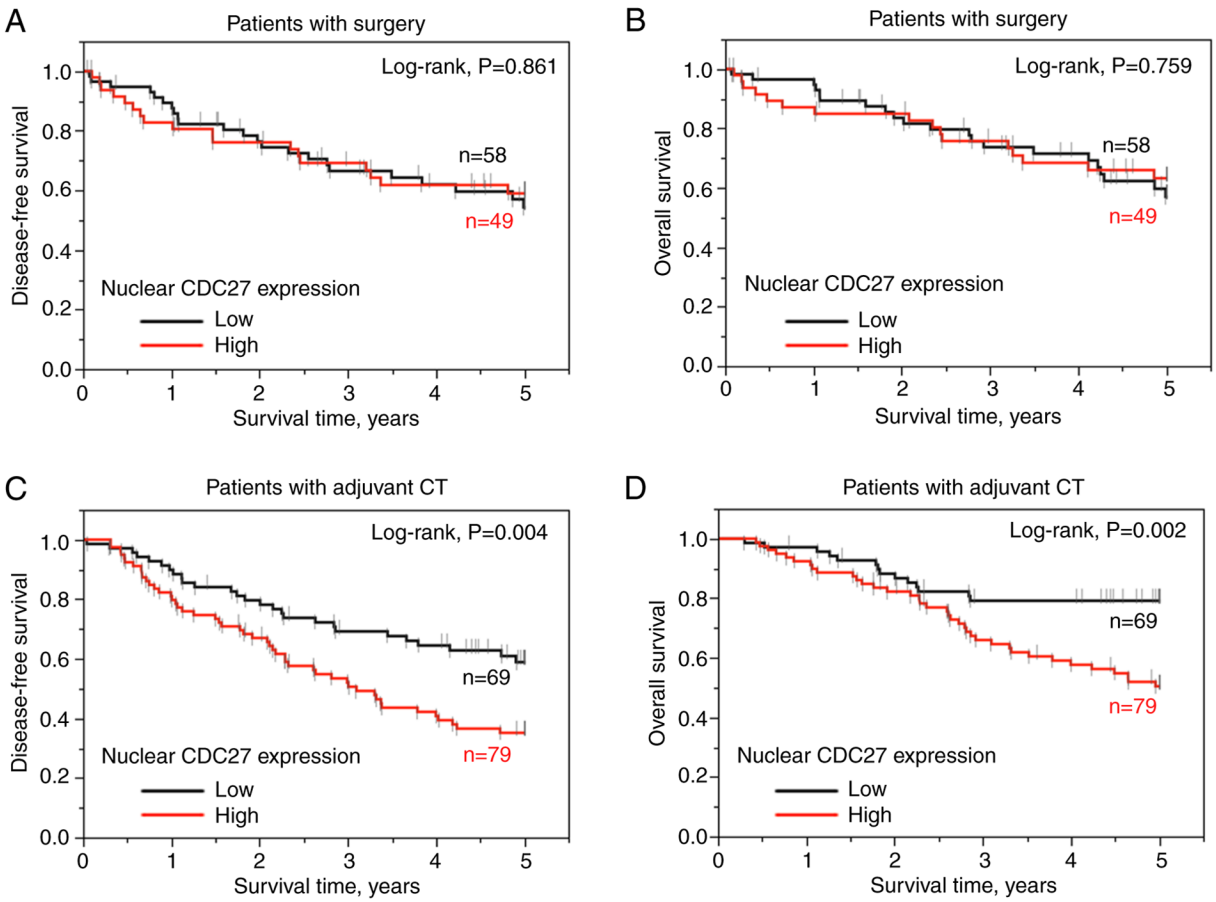

Figure 3. Nuclear CDC27 expression is associated with survival outcome in patients with chemotherapy-treated READ. Kaplan-Meier survival analysis showed that nuclear CDC27 expression was not associated with (A) 5-year DFS or (B) 5-year OS in patients with READ who received surgery only. The Kaplan-Meier curves showed that nuclear CDC27 expression was associated with (C) 5-year DFS or (D) 5-year OS in patients with READ who received adjuvant chemotherapy after surgery. CT, chemotherapy; CDC27, cell division cycle 27; OS, overall survival; DFS, disease-free survival.

considering the isoforms and somatic variants of CDC27 proteins, additional studies are necessary to survey and evaluate the results in terms of CDC27 isoforms and mutations.

Chemotherapy is the most common treatment for cancer, and 5-fluorouracil-based chemotherapeutic drugs combined with oxaliplatin and/or irinotecan are commonly used to treat patients with colorectal cancer to improve patient survival and tumor response. Post-operative adjuvant chemotherapy is often used after surgery to reduce the risk of distant metastasis and provide additional survival benefits in high-risk colorectal cancer patients (25,26). However, numerous deaths in patients with cancer are due to the failure and resistance to chemotherapy. Chemotherapeutic drugs often cause innate and acquired chemoresistance by intrinsic and extrinsic factors (27-29). Several studies have proven that dysfunction of the APC/C complex is associated with chemoresistance and poor clinical outcomes, as APC/C activity is reactivated and triggers cell cycle arrest for repair under chemotherapeutic agent exposure (15,30). Hu *et al* (31) demonstrated that the stability of bromodomain-containing 7 protein was regulated by APC/C and correlated with clinical outcomes in patients with osteosarcoma. Inhibition of APC/C function improved paclitaxel efficacy in breast cancer cells and increased the sensitivity of osteosarcoma cells to cisplatin and doxorubicin (31,32). Activation of APC/C led to temozolomide resistance in glioma cells, docetaxel resistance in the castration-resistant prostate cancer cell line and chemoresistance to anti-microtubule drugs (33-35). Moreover, altered expression or somatic mutation of CDC27 also reduced chromosomal instability, dysfunction of APC/C and spindle assembly checkpoint target therapy resistance (15). CDC27 also is a target of miRNA that can predict therapy efficacy and modulate radiosensitivity (36,37). In the present study, it was discovered that nuclear CDC27 expression was an independent prognostic factor in adjuvant chemotherapy-treated patients, but not in patients only treated with surgery. These results revealed that high nuclear CDC27 expression could attenuate the response to chemotherapy treatment. This phenomenon may be as the overexpressed CDC27 in the nucleus causes APC/C dysfunction and chemoresistance in cancer cells. Further studies are important in order to determine the roles of CDC27 in chemoresistance. Recently, the subunits of APC/C have become attractive innovative therapies for cancer treatment (5,15). Hence, CDC27 may act as a potential therapeutic target in patients with READ.

The subset and amount of TILs, especially the density of $CD3^+$ TILs, have been reported to correlate with antitumor immune response and survival outcome in patients with colorectal cancer (16,38). The present results revealed that the density of intratumor $CD3^+$ TILs was associated with TNM stage, pN stage and PNI ($P<0.001$). The density of $CD3^+$ TILs was also associated

with the 5-year DFS and OS rates in patients with READ. In the present study, $CD3^+$ TILs were a prognostic factor for patients with READ in terms of 5-year DFS, but not for adjuvant chemotherapy-treated patients in terms of either 5-year DFS or OS. Furthermore, using TIMER database analysis, the expression of CDC27 was positively associated with $CD4^+$ and $CD8^+$ TILs in patients with READ in the present study. However, there was no association between cytoplasmic/nuclear CDC27 expression and intratumor $CD3^+$ TILs in patients with READ. These inconsistent results may be due to the difference in the study population. Overall, there was no association between CDC27 expression and intratumor $CD3^+$ TIL density, and $CD3^+$ TILs were associated with survival outcome in patients with READ.

There are a number of different treatments for patients with READ, such as surgery, preoperative concurrent chemoradiotherapy, post-operative adjuvant chemotherapy, radiotherapy and targeted therapy. In the present study cohort, the resected specimens were only collected from patients with READ who received surgery with or without postoperative chemotherapy, as this group is more straight forward for exploring the effects of CDC27 expression. However, the variants of CDC27 at the RNA, DNA and protein levels are complex and may affect the functions of CDC27 on tumorigenesis, prognosis, survival outcome and response to treatment in patients with cancer. Also, only IHC staining was used to detect CDC27 expression in this study. Further studies are therefore needed to validate the present results using different methods and retrospective cohorts, and to investigate the functions of these CDC27 variants and their clinical significance. The present study just indicates an easy method to evaluate the effects of CDC27 in the clinic, and provides strong evidence that the subcellular location of CDC27 protein expression in cancer cells may have an impact on tumor progression, survival outcome and chemotherapy efficacy in patients with READ. Thus, the expression of CDC27 in the cytoplasm or nucleus may be used to predict postoperative distant metastasis after surgery and the benefits of adjuvant chemotherapy.

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Availability of data and materials

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

Authors' contributions

SFC, KCYH, YLL and CHK conducted and performed the experiments. CLC, WTLC, TWC, HHH, KSCC and TWK enrolled the patients with READ and performed the IHC evaluation. CLC and SFC performed the statistical analysis. TWK and SFC supervised this study. CLC, KCYH and SFC analyzed the data and wrote the manuscript. KSCC and SFC provided the funds. All authors have read and approved the manuscript. CLC, KCYH, TWK and SFC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was reviewed and approved by the Internal Review Board of China Medical University Hospital (Taichung, Taiwan; approval no. CMUH105-REC2-072). Informed consent was obtained from all participants in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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