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Functional characterisation of cell cycle-related kinase (CCRK) in colorectal cancer carcinogenesis

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ABSTRACT

Cell cycle-related kinase (CCRK) is a newly identified protein kinase homologous to Cdk7. We have previously shown that *CCRK* is a candidate oncogene in human glioblastoma. However, whether *CCRK* is a bona fide oncogene remains to be tested. The aim of this study was to investigate the role of CCRK in human colorectal cancer carcinogenesis. By Western blotting, we analysed the expression profile of CCRK protein in 10 colorectal cancer tissue samples and their adjacent normal colon tissues and in seven colorectal cancer cell lines. CCRK protein expression was also investigated by immunohistochemistry in a colorectal tissue microarray, which contained 120 cases of primary colorectal cancer and adjacent normal colorectal mucosa. The effects of CCRK knock-down on cell cycle profile and proliferation of colorectal cancer cells were examined by transfecting LoVo and DLD1 human colorectal cancer cell lines by either short-hairpin RNA (shCCRK) or small interfering RNA targeting *CCRK* (siCCRK). We found that CCRK protein levels were elevated by more than 1.5-fold in 70% of colorectal cancer patient samples examined and CCRK was detectable in all seven colorectal cancer cell lines tested. Colorectal tissue microarray indicated that overexpression of CCRK was detected in 62/109 (56.9%) of informative colorectal cancer cases and was significantly associated with the tumour pT and pN status ($p < 0.05$). Suppression of CCRK by siCCRK led to G1 phase cell cycle arrest and reduced cell growth. Consistently, stable clones of LoVo and DLD1 cells expressing shCCRK exhibited decreased cell proliferation rates. Furthermore, we showed that CCRK is required for the phosphorylation of Cdk2 (on Thr-160) and Rb (on Ser-795) and the expression of cyclin E. These results suggest for the first time that CCRK is involved in colorectal cancer carcinogenesis and G1/S cell cycle transition by regulating Cdk2, cyclin E and Rb.

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1. Introduction

Colorectal cancer, or commonly referred to as colorectal cancer, is the third most common cancer worldwide and it ranks only after breast and lung cancers in females and prostate and lung cancers in males. The American Cancer Society estimated that approximately 146,970 new cases and 49,920 deaths from colorectal cancer were expected to occur in the United States in 2009.[1] In Hong Kong, the incidence of colorectal cancer is increasing rapidly and it has become the second most common cancer with more than 4000 new patients diagnosed every year.[2] Compared with other parts of the world, Hong Kong has more colorectal cancer patients under 40-years-old.[3] Therefore, understanding the molecular pathology of colorectal cancer is critical for establishing novel therapeutic and diagnostic strategies against this potentially fatal disease.

Cell cycle-related kinase (CCRK), also known as p42 or cyclin-dependent kinase (Cdk)-related kinase PNQALRE, is a newly identified 42 kDa protein which contains all 11 conserved subdomains characteristic of serine/threonine protein kinase. CCRK is homologous to Cdk1, 2 and 7 and, to a lesser extent, Csk.[4,5] It has been reported to activate Cdk2 and support cell proliferation and was found to phosphorylate male germ cell-associated kinase (MAK)-related kinase at Thr-157 in mammalian cell.[4,6]

We have previously demonstrated that *CCRK* transcript was expressed predominantly in the brain and it identified *CCRK* as a candidate oncogene in human glioblastoma.[7] We showed that *CCRK* is overexpressed in a majority of the glioma patient samples and cell lines. Suppression of CCRK by small interfering RNA (siRNA) inhibits glioblastoma cell growth, whereas overexpression of CCRK is able to confer tumourigenicity to a non-tumourigenic glioblastoma cell line, indicating that *CCRK* expression level may associate with the tumourigenicity of the glioblastoma cells. Consistently, we also found that CCRK enhances the proliferation of ovarian carcinoma cells.[8] However, the function of CCRK in other cancer types and how it regulates cell cycle remains unclear. Although CCRK is expressed at virtually undetectable level in the normal colon,[7] it may play a role in colorectal cancer if it is a bona fide oncogene. Therefore, we investigated the potential role of CCRK in colorectal cancer carcinogenesis in this study.

In the present study, we determined CCRK protein levels in the colorectal cancer tissues by Western blotting and immunohistochemistry. We also investigated the function of CCRK in colorectal cancer by suppressing CCRK expression by small interfering RNA (siCCRK) or short-hairpin RNA (shCCRK). Finally, the effects of CCRK knock-down on the expression and phosphorylation of proteins important for cell cycle regulation were analysed.

2. Materials and methods

2.1. Cell culture

Seven human colorectal cancer cell lines (Colo205, DLD1, HCT116, HT29, LoVo, SW1116 and SW480) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cell lines were cultured in RPMI 1640 medium containing 10% FBS (Invitrogen, Rockville, MD).

2.2. Protein and RNA sample extraction

A total of 10 pairs of tissue samples were obtained from individual tumour foci and adjacent normal colon tissues from patients with colorectal cancer. The use of these archival tissues from the current study was approved by the Ethics Committee of The University of Hong Kong. The samples were snap-frozen in liquid nitrogen and stored at −80 °C. Part of these samples (10 pairs) were homogenised in RIPA buffer (Sigma, St. Louis, MO) containing 1 mM PMSF and protease inhibitor cocktail (Sigma) and centrifuged. Supernatants were stored at −80 °C until analysis.

2.3. Antibodies

A 16-amino acid peptide corresponding to a sequence near the N-terminus of human CCRK (RIGEGAHGIVFKAKHV) was used for rabbit immunisation (Boster, China). Crude antisera were affinity-purified with the same peptide affinity chromatography. Other antibodies were obtained commercially: anti-Cdk2 (D-12), anti-cyclin D1 (M-20), anti-cyclin E (HE12) and anti-cyclin B1 (H20) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-GAPDH, anti-pho-Cdk2 (Thr-160), anti-Pho-Rb (Ser-795), anti-Rb and anti-α/β-tubulin from Cell Signalling Technologies (Danvers, MA).

2.4. RT-PCR

RNA was reverse-transcribed using SuperScript First Strand cDNA System (Invitrogen, Carlsbad, CA). The first strand cDNA products were then amplified with *GAPDH*-specific (NM002046; 5′-TGCCTCCTGCACCACCAACT-3′ and 5′-CCCGT TCAGCTCAGGGCTGA-3′) and *CCRK*-specific (NM178432; 5′-TCATCCTGGAGGGGTGAGAAGT-3′ and 5′-CCACCTTAGCCATT TCCCTTGA-3′) primers by PCR. The PCR conditions were 94 °C for 45 s, 59 °C for 45 s and 72 °C for 1 min; 27 cycles for GAPDH and 35 cycles for CCRK. The PCR products were analysed by 1% agarose gel electrophoresis and the band intensity was quantitated by ImageQuant (Molecular Dynamics).

2.5. Western blotting

Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extract (PIERCE, Rockford, IL). For the whole-cell lysates, the cells were lysed with RIPA buffer containing 1 mM PMSF and protease inhibitor cocktail for 30 min at 4 °C. After centrifugation for 15 min at 13,000 rpm, the supernatants were recovered and the protein concentration was measured by BCA Protein Assay Kit (PIERCE). Equal amounts of cell lysates were resolved in 10% SDS–PAGE and transferred onto nitrocellulose membranes (Sigma). After blocking, the membranes were incubated sequentially with the appropriate diluted primary and secondary antibodies. Proteins were then detected by the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). To ensure equal loading of the samples, the membranes were

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reprobed with an anti-GAPDH antibody (Cell Signalling Technologies).

2.6. Patients and tissue specimens

For the preparation of the colorectal tissue microarray, 120 colorectal carcinoma patients who underwent partial coloproctectomy were selected consecutively from the surgical pathology archives of the Department of Pathology, Cancer Centre and the First Affiliated Hospital, Sun Yat-Sen University (Guangzhou, China) between 1997 and 2001. All colorectal cancer cases selected were conventional adenocarcinoma. Patients with mucinous carcinoma and/or familial CRC were excluded from this study. The age of these colorectal cancer patients selected ranged from 33 to 82-years-old at the time of surgery (mean age, 56.1 years) and the male/female ratio was 1.3:1. Tumour grade was defined according to the criteria of the World Health Organisation. The pTNM status of all colorectal cancer tissues was assessed according to the criteria of the 6th edition of the TNM classification of the International Union against Cancer (UICC, 2002). The clinicopathological features of these colorectal cancer cohorts are outlined in Table 1. The specimens were recruited from paraffin blocks of 120 primary carcinomas and adjacent normal colorectal mucosa. This study was approved by the Medical Ethics Committee of Sun Yat-Sen University.

2.7. Tissue microarray (TMA)

The colorectal TMA was constructed as described previously.[9] Briefly, haematoxylin and eosin (H&E)-stained slides were examined under the microscope. The individual donor tissue block and the corresponding histological H&E-stained slides were overlaid for TMA sampling. A TMA instrument (Beecher Instruments, Silver Spring, MD) was used to create holes in a recipient paraffin block to obtain cylindrical core tissue biopsies with a diameter of 0.6 mm. These biopsy samples were then transferred to a recipient block at defined array positions. TMA tissue specimens for each patient were composed of three sample from adjacent normal mucosa and three samples from primary carcinoma at different loci. Thus, a total of 720 (120×6) colorectal tissue samples were constructed. Multiple sections (5 μm thick) were cut from the TMA block and mounted on microscope slides.

2.8. Immunohistochemistry (IHC)

IHC studies were performed using a standard streptavidin–biotin–peroxidase complex method.[10] Non-specific binding was blocked with 10% normal rabbit serum for 20 min. In brief, TMA sections were deparaffinised and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. For antigen retrieval, TMA slides were microwave-treated in 10 mM citrate buffer (pH 6.0) for 10 min. The slides were incubated with rabbit anti-CCRK polyclonal antibody (ABGENT, San Diego, CA, 1:500 dilution) overnight at 4 °C. The slides were then incubated with a biotinylated goat anti-rabbit serum for 30 min and subsequently reacted with a streptavidin–peroxidase conjugate and 3′-3′ diaminobenzidine. The nucleus was counterstained using Meyer's haematoxylin. Negative controls were

Table 1 – Association between CCRK expression and clinico-pathological characteristics in colorectal cancers.

	CCRK expression			
	Total cases	Informative cases	Overexpression (%)	*p* Value[a]
Age				0.661
⩽56.1 years	55	49	29 (59.2%)	
>56.1 years	65	60	33 (55.0%)	
Gender				0.286
Male	68	62	38 (61.3%)	
Female	52	47	24 (51.1%)	
Tumour location				0.956
Right colon	18	16	9 (56.3%)	
Left colon/rectum	102	93	53 (57.0%)	
WHO Grade				0.190
G1/G2	103	93	50 (53.8%)	
G3	17	16	12 (75.0%)	
pT status				0.013
pT1–2	19	17	5 (29.4%)	
pT3–4	101	92	57 (62.0%)	
pN status				0.0076
pN0	53	49	21 (42.9%)	
pN1–2	67	60	41 (68.3%)	
pM status				0.363
pMX	95	86	47 (54.7%)	
pM1	25	23	15 (65.2%)	

[a] Fisher's exact test.

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performed by replacing the primary antibody with rabbit IgG. The slides with known CCRK immunoreactivity were used as positive controls.

To evaluate the IHC staining of CCRK in colorectal TMA, a semi-quantitative scoring criterion for IHC of CCRK was used, in which both staining intensity and positive areas were recorded. A staining index (values 0–12) was obtained by multiplying the intensity of CCRK-positive staining (negative = 0, weak = 1, moderate = 2 or strong = 3) by the staining proportion (<25% = 1, 25–50% = 2, >50% to <75% = 3, ⩾75% = 4). For cell counting, the whole area of each TMA sample was evaluated and a minimum of 300 epithelial cells was counted in each case. The association of CCRK protein expression with CRC patient's clinico-pathological features was assessed by the Fisher's exact test.

2.9. Immunofluorescence

Cells grown on coverslips were fixed with cold methanol for 10 min at −20 °C and permeabilised with permeabilisation buffer (1% BSA, 0.25% Triton X-100 and 0.05% NaN_3 in PBS) for 10 min at 37 °C. The cells were first incubated with a 1:50 diluted anti-CCRK antibody for 1 h and then with a fluorescence-conjugated secondary antibody (Santa Cruz) (1:50) for 1 h. Nuclei were stained with DAPI at concentration of 1 μg/ml. Sample was examined with a fluorescent microscope and images were acquired with a SPOT CCD camera and analysed by SPOT software.

2.10. Construction of short-hairpin RNA-expressing plasmids

Short-hairpin RNAs targeting *CCRK* (shCCRK and shCCRK2) were generated using the GeneEraser shRNA Mammalian Expression Vector System (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Briefly, the oligonucleotides containing the shRNA sequences targeting *CCRK* mRNA (shCCRK and shCCRK2) and firefly luciferase (shLuc) were separately cloned into pGE-1 vector (Stratagene), generating pGE-1-shCCRK, pGE-1-shCCRK2 and pGE-1-shLuc expression plasmids respectively. The sequences of the shCCRK, shCCRK2 and shLuc oligonucleotides were as follows: shCCRK; 5′-GAAGGTGGCCCTAAGGCGGTTGGAAGACG-3' shCCRK2; 5′-GGCGGTTGGAGGACGGCTTCCCTAACCAG-3′ shLuc; 5′-GTGAACATC ACGTACGCGGAATACTTCGA-3′.

2.11. Gene transfection and establishment of stable cell lines

Cells were grown in six-well plates to 70–80% confluence. The cells were transfected with 4 μg/well of the expression plasmids (pGE-1-shCCRK, pGE-1-shCCRK2 or pGE-1-shLuc) using Lipofectamine 2000 (Invitrogen). After 6 h, the transfection mixture was replaced with fresh growth medium and the cells were allowed to recover for 48 h. The cells were then trypsinised and split into 1:10 in growth medium containing 0.8 mg/mL G418. Resistant clones were either selected separately using cloning cylinders or pooled together and maintained in growth medium containing 0.6 mg/mL G418 until further analysis.

2.12. Synthetic siRNA oligonucleotides

siRNA duplex oligonucleotide targeting human *CCRK* mRNA (siCCRK; 5′-GAAGGUGGCCCUAAGGCGG-3′) or firefly (*Photinus pyralis*) luciferase mRNA (siLuc; 5′-CGUACGCGGAAUACUUCGA-3′) was synthesised and purified by Proligo (Boulder, CO).

2.13. MTT assay

Cells were plated at an initial density of 2×10^4 cells/well in 96-well plates, and transfected with siCCRK, siCCRK2 or siLuc. For the MTT assay, the cells were incubated with 0.5 mg/mL 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma) for 4 h. The medium was then discarded and formazan was dissolved by the addition of 200 μl of dimethylsulphoxide (DMSO). The absorbance was measured at 570 nm.

2.14. Cell cycle analysis

LoVo or DLD1 MG cells (7.5×10^5) were seeded onto a 100-mm dish and cultured for 24 h. The cells were then transfected by incubation with siCCRK or siLuc at a final concentration of 200 nM and Oligofectamine transfection reagent and incubated for 48 h at 37 °C. The transfected cells were then fixed in ice-cold 70% ethanol and stained with the use of a Coulter DNA-Prep Reagents kit (Beckman Coulter, Fullerton, CA). Cellular DNA content of 1×10^4 cells from each sample was determined with the use of an EPICS ALTRA flow cytometer (Beckman Coulter). Cell cycle phase distribution was analysed with the use of ModFit LT 2.0 software (Verity Software House, Topsham, ME) using data obtained from two separate experiments in which each transfection was performed in triplicate.

2.15. Statistical analysis

The data were obtained from at least three different experiments and expressed as mean ± SD. Statistical analysis was performed by Student's *t* test and differences were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. CCRK expression profiles in colorectal cancer patient tissues and cell lines

Western blotting analysis of CCRK protein levels in 10 colorectal cancer patient samples showed that 70% (7 of 10) of the tumour tissues exhibited elevated CCRK protein level as compared to the adjacent normal tissues (Fig. 1A and B). We also determined the expression levels of CCRK transcript and protein in seven tumourigenic colorectal cancer cell lines (colo205, DLD1, HCT116, HT29, LoVo, SW1116 and SW480) (Fig. 1C and D). We found that CCRK mRNA and protein were expressed at variable levels in these cell lines and no association between *CCRK* expression and *p53* status of these colorectal cancer cells was observed. In the subsequent studies, LoVo and DLD1, which express CCRK at high and low levels, were chosen for further analysis.

CCRK protein expression was also investigated by IHC in a colorectal TMA, which contained 120 cases of primary

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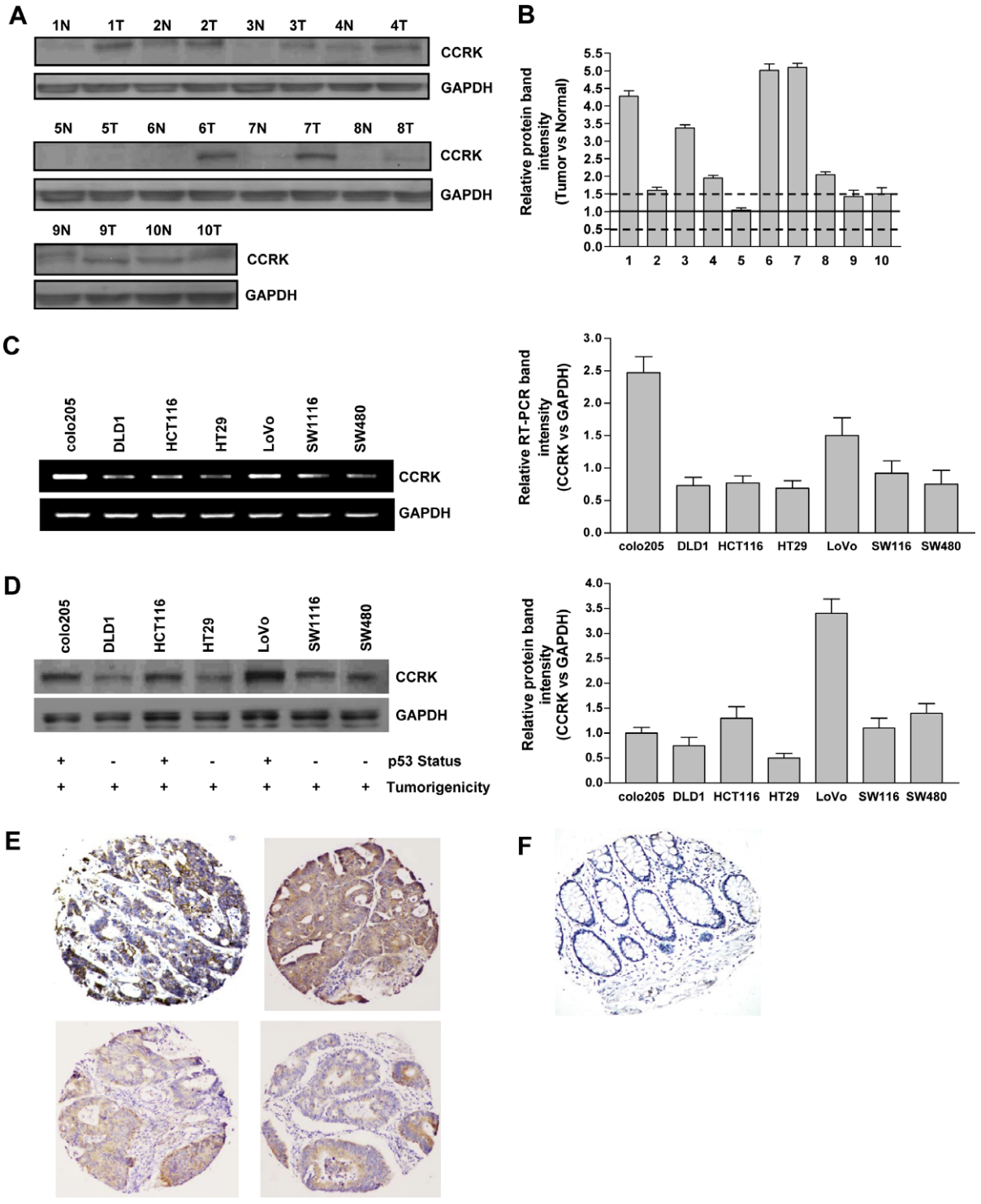

Fig. 1 – CCRK protein expression in human colorectal cancer tissues. (A) Immunoblotting showing the expression of CCRK and GAPDH in paired normal (N) and tumour (T) samples. (B) The intensity of the protein bands for CCRK and GAPDH was quantified by the ImageQuant software. The bar charts shown represent the GAPDH-normalised CCRK expression levels in tumour samples (T) relative to those in the normal colon tissues (N). Levels above 1.5-fold were regarded as up-regulation. (C and D) CCRK mRNA expression and protein levels in seven colorectal cancer cell lines were analysed by semi-quantitative RT-PCR and immunoblotting, respectively. The bar charts on the right represent the relative band intensities showing the GAPDH-normalised CCRK mRNA or protein levels in various colorectal cancer cell lines. (E) Immunohistochemical staining of CCRK in human colorectal tissues. Four representative cases of colorectal cancer with different degrees of CCRK overexpression are shown. (F) A representative image showing a normal level of CCRK expression in the adjacent normal colorectal mucosa, with a negative staining of CCRK in all of the epithelial cells.

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colorectal cancer and adjacent normal colorectal mucosa (Fig. 1E and F). CCRK expression could be evaluated informatively in 112/120 (93.3%) of the normal colorectal mucosa and 109/120 (90.8%) cases of the primary colorectal cancer. The non-informative samples included lost samples, unrepresentative samples and samples with too few tumour cells (<300 cells). These samples were not used for data analysis. Because the staining index of CCRK expression in each of the 112 informative normal colorectal mucosas was ⩽4, we designated the staining index 0–4 as the normal level of CCRK expression (Fig. 1F). Overexpression of CCRK was designated when the staining index was >4 (Fig. 1E). Using these criteria, overexpression of CCRK was detected in 62/109 (56.9%) cases of informative colorectal cancer.

The potential association between CCRK expression and tumour clinicopathologic features in a cohort of colorectal cancer patients was also evaluated (Table 1). Our results showed that CCRK expression was significantly associated with the tumour pT and pN status in colorectal cancer ($p < 0.05$), in which the frequency of CCRK overexpression in CRCs in pT3–4 stage (62.0%) was significantly larger than that in CRCs in pT1–2 stage (29.4%). Overexpression of CCRK was more frequently observed in colorectal cancers with lymph node metastasis (pN1/2, 68.3%) than in those without lymph node metastasis (pN0, 42.9%). No significant association was found between CCRK expression and other clinico-pathological features, such as patients age, gender, tumour grade, location and pM status ($p > 0.05$).

3.2. *Subcellular localisation of CCRK in colorectal cancer cells*

Next, we examined the subcellular localisation of CCRK in LoVo and DLD1 cells by indirect immunofluorescence staining using an affinity-purified CCRK antibody. We found that endogenous CCRK was mainly expressed in the nuclear and perinuclear regions in both cell lines (Fig. 2A and B). In some of the CCRK-positive cells, fluorescent signals were also detected in the cytoplasm, implying that the localisation of CCRK may change with different stages of the cell cycle.

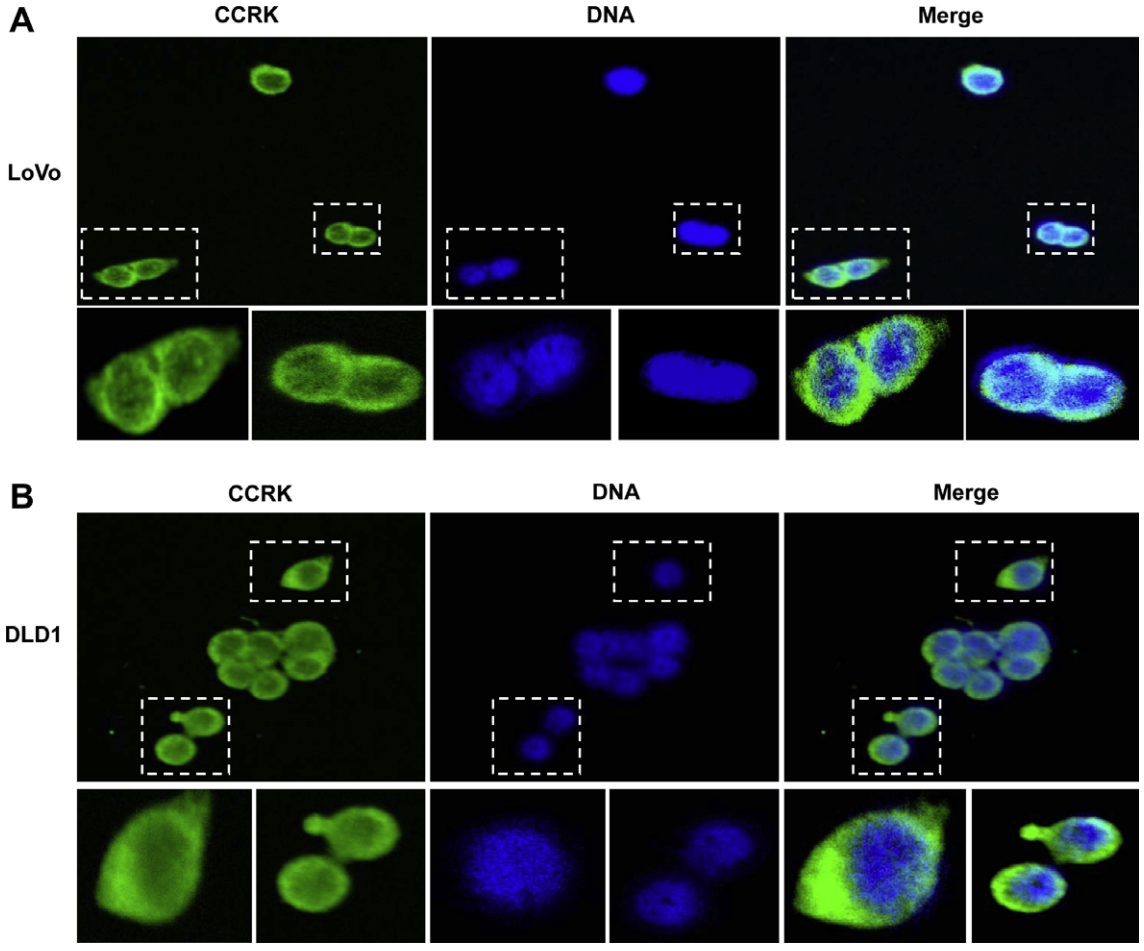

Fig. 2 – Subcellular localisation of CCRK in colon cancer cells. Indirect immunofluorescence was performed on methanol-fixed LoVo and DLD1 cells, respectively, using an anti-CCRK antibody. The bound anti-CCRK was visualised with a fluorescein-conjugated secondary antibody (in green), whereas 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) counterstaining was used to visualise the cell nuclei (in blue). (A) LoVo cells treated consecutively with anti-CCRK (left) and DAPI (middle). (B) DLD1 cells treated consecutively with anti-CCRK (left) and DAPI (middle). The merged images are shown on the right panels and insets below show the enlargement of the cells highlighted in the dotted rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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3.3. *CCRK knock-down inhibited colorectal cancer cell proliferation*

To test whether CCRK affects cell proliferation, we first established LoVo and DLD1 cell lines which stably expressed short-hairpin RNA (shCCRK). Stable cell lines expressing another shRNA which targets CCRK at a different region (shCCRK2) was also generated to ensure that the designed shCCRKs are specific in action. The pGE-1-Luc-transfected LoVo or DLD1 cells served as the control in this experiment. After G418 selection, pGE-1-shCCRK-, pGE-1-shCCRK2- or pGE-1-shLuc-transfected cell clones were isolated and the expression of CCRK in these cell clones was screened by Western blotting. Both shCCRK and shCCRK2 significantly suppressed CCRK protein expression by more than 70% in LoVo and DLD1 cells (Fig. 3A and B). In addition, shCCRK or shCCRK2 expression was able to significantly reduce the cell proliferation rates of both LoVo and DLD1 cells. At day 7 post-transfection, the cell

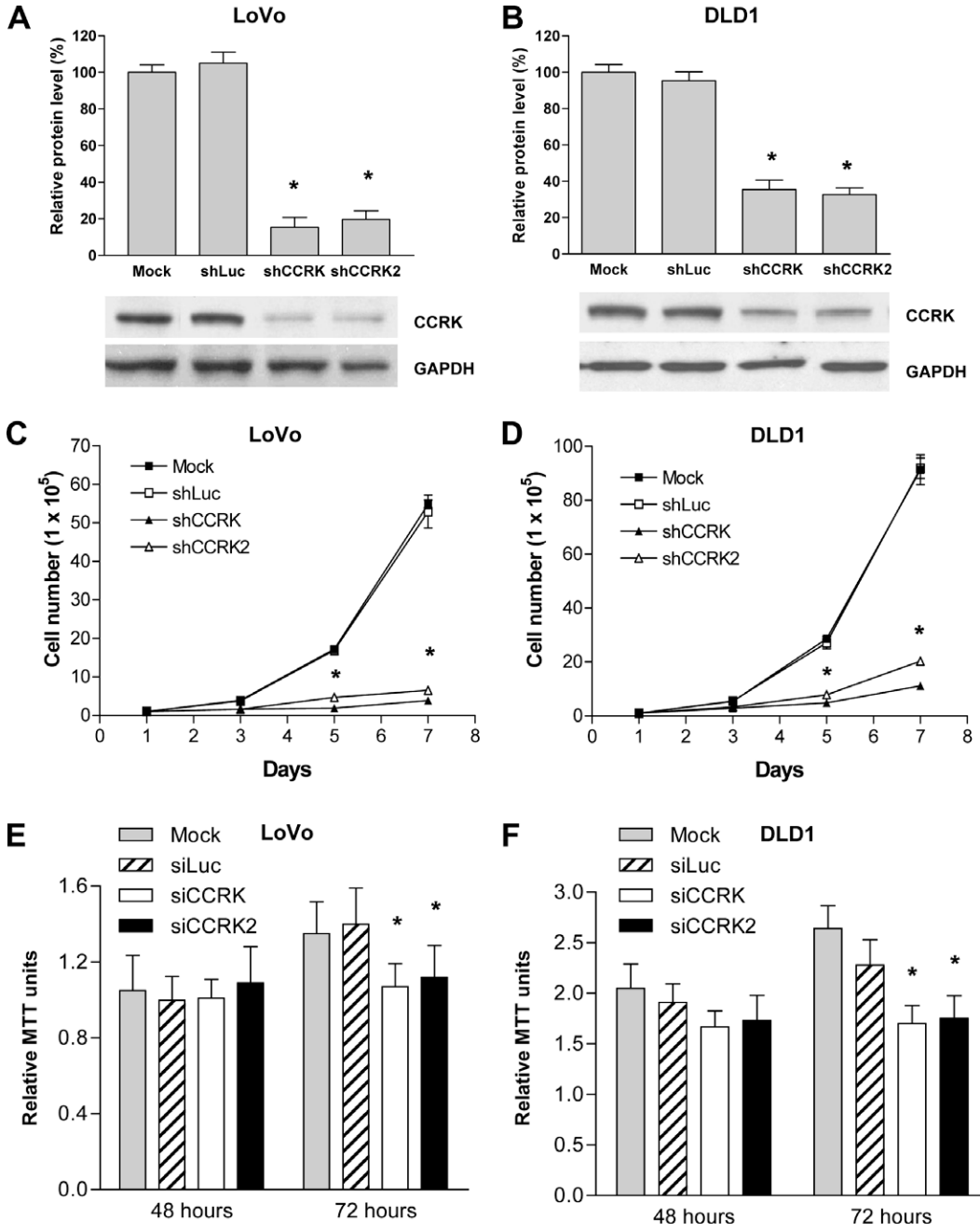

Fig. 3 – CCRK knock-down inhibited cell proliferation. Pooled stable clones of mock-, shLuc-, shCCRK- and shCCRK2-transfected LoVo (A) or DLD1 cells (B) were constructed and the protein expression levels of CCRK were determined by Western blotting and densitometry. The viable cell numbers of the mock-, shLuc-, shCCRK- or shCCRK2-transfected LoVo (C) or DLD1 (D) cells were determined by trypan blue exclusion method. MTT assays on mock-, siLuc-, siCCRK- and siCCRK2-transfected LoVo (E) and DLD1 (F) cells were also performed for comparison. *$p < 0.01$ as compared with siLuc-transfected cells. The results represent the means ± SD of three experiments with each performed in triplicate.

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Table 2 – Cell cycle distribution of mock-, siLuc- and siCCRK-transfected LoVo and DLD1 cells.

Sample	G0–G1 phase (%)	S phase (%)	G2–M phase (%)	Apoptosis (%)
LoVo	55.85 ± 1.48	29.82 ± 0.94	14.36 ± 0.71	0.09 ± 0.01
Mock	57.16 ± 1.67	28.10 ± 0.86	14.61 ± 1.27	0.13 ± 0.01
siLuc siCCRK	74.15 ± 1.75*	11.53 ± 1.28*	18.21 ± 1.23*	0.11 ± 0.01
DLD1	58.93 ± 1.32	25.24 ± 1.17	15.83 ± 1.24	0.02 ± 0.01
Mock	58.65 ± 2.23	25.07 ± 1.21	16.28 ± 1.48	0.04 ± 0.01
siLuc siCCRK	64.09 ± 2.48*	19.96 ± 1.62*	15.95 ± 1.48	0.01 ± 0.01

* $p < 0.05$ compared with siLuc control ($n = 5$).

number of shCCRK- or shCCRK2-transfected cells was only 10–20% of the control mock- or shLuc-transfected lines (Fig. 3C and D). Similar results were obtained when LoVo and DLD1 cells were transfected with siRNAs targeting CCRK (siCCRK and siCCRK2). We found that the viability of both LoVo and DLD1 cells was markedly reduced at 72 h after transfection with siCCRK or siCCRK2 as measured by MTT assays (Fig. 3E and F).

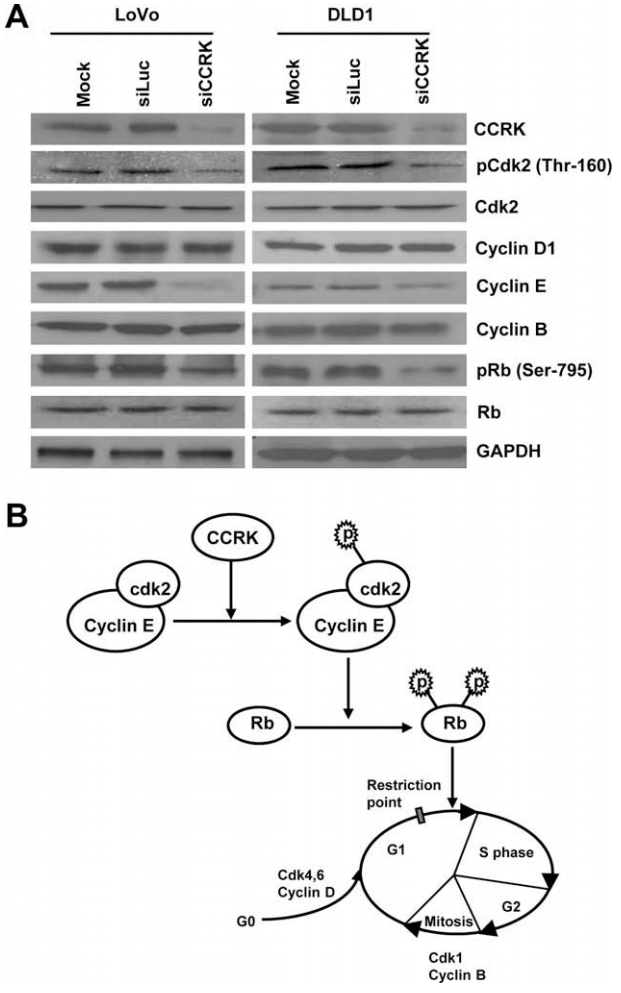

Fig. 4 – CCRK knock-down inhibited cyclin E expression and the phosphorylation of Cdk2 and Rb. (A) Cdk2, phosphorylated Cdk2 (pCdk2), cyclin D1, cyclin E, cyclin B, Rb and phosphorylated Rb (pRb) protein levels were determined in mock-, siLuc- or siCCRK-transfected LoVo cells (left) or DLD1 cells (right). Western blotting for GAPDH was done in parallel as a loading control. (B) A hypothesised model illustrating how CCRK controls cell cycle progression in colorectal cancer cells.

3.4. *Suppression of CCRK by siRNA caused cell cycle arrest at the G1 phase*

To understand how CCRK induces growth inhibition in colorectal cancer cells, we analysed the cell cycle profile of siCCRK-transfected LoVo or DLD1 cells by flow cytometry. As shown in Table 2, no statistical significant differences in cell cycle profile were found between the mock- and siLuc-transfected cells. For LoVo cells, the ratio of population at G0–G1 and G2–M phases increased from 57.16% to 74.15% and 14.61% to 18.21% in siCCRK-transfected cells, respectively. Concomitantly, cell population at S phase was reduced from 28.10% to 11.53%. Similar results were observed in DLD1 cells, but there was no significant change in the cell population at G2–M phase. In both cell lines, no notable increase in the number of apoptotic cells was detected.

3.5. *CCRK knock-down decreased the phosphorylation of Cdk2/Rb and the expression of cyclin E but not cyclin D1/cyclin B*

Our cell cycle profile data showed that CCRK suppression would cause cell cycle arrest at the G0–G1 phase in both LoVo and DLD1 cells. Therefore, we investigated the expression of some important proteins involved in G1/S transition, such as Cdk2, cyclin E and Rb, in siCCRK-transfected LoVo and DLD1 cells. Western blotting showed that CCRK knock-down reduced Cdk2 phosphorylation without affecting the expression level of total Cdk2 in both cell lines (Fig. 4A). Moreover, siCCRK-transfection markedly reduced the levels of cyclin E expression and Rb protein phosphorylation. By contrast, the expression levels of cyclin D1 and cyclin B1 remained unchanged in both cell lines.

4. Discussion

CCRK is a newly discovered protein kinase expressed mainly in brain and kidney. We have shown previously that *CCRK* acts as a candidate oncogene in the most aggressive form of brain tumour, glioblastoma.[7] However, it is not known whether *CCRK* is a bona fide oncogene in other cancers. Previously, it has been reported that *CCRK* is expressed in many other cancer cell lines derived from tissues with low *CCRK* expression, including cervical adenocarcinoma (HeLa), osteogenic

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sarcoma (U20), breast adenocarcinoma (MCF-7), lung fibroblast (WI-38), myoblast (C2C12), lymphocyte (GM08336) and embryonic kidney (HEK 293).[4,5] This finding has raised the possibility that *CCRK* also has oncogenic activities in these cancers.

In this study, we investigated the function of CCRK in colorectal cancer carcinogenesis. We found that CCRK protein levels are elevated in the 70% of colorectal cancer tissues examined. Furthermore, our colorectal TMA study showed that overexpression of CCRK was detected in 62/109 (56.9%) of informative colorectal cancer cases and was significantly associated with the tumour pT and pN status ($p < 0.05$), suggesting that CCRK may be associated with colorectal cancer development. Supporting this notion, CCRK knock-down by siCCRK or shCCRK significantly reduces cell growth in both LoVo and DLD1 human colorectal cancer cell lines. There are two possible mechanisms responsible for the growth inhibition. First, suppression of CCRK may induce apoptosis. Second, CCRK knock-down may inhibit cell cycle progression. The later was supported by flow cytometric analysis whereby siCCRK-transfection could induce cell cycle arrest at G1 phase in both LoVo and DLD1 cells. Because no noticeable increase in the number of apoptotic cells was observed, it is likely that the growth inhibition induced by CCRK knock-down is mainly due to cell cycle arrest rather than cell apoptosis. It is worth noting that suppression of CCRK by siCCRK also resulted in a G2–M phase arrest in the LoVo cells, but not the DLD1 cells. The reason for such discrepancy in these two cell lines is currently unknown; nevertheless, this observation suggests that siCCRK may have additional function in LoVo cells by inducing G2–M phase arrest.[11,12]

Cell cycle progression is tightly regulated by Cdks and cyclins.[13] Different cyclin/Cdk complexes are activated in specific stages of the cell cycle.[14–16] As cell cycle progresses through G1 to S phase, cyclin–Cdk complexes, such as cyclin D1–Cdk4/6 and cyclin E–Cdk2 complexes,[17] are sequentially activated and hyperphosphorylate retinoblastoma protein (pRB) on serine and threonine residues.[18] The hyperphosphorylated pRB releases active E2F transcription factors, which in turn stimulate the transcription of numerous genes required for G1 to S transition and S phase progression.[19] Western blot analyses of various cyclins demonstrated that there was no apparent change in the levels of cyclin D1 or cyclin B in siCCRK-transfected LoVo and DLD1 cells compared with the control siLuc-transfected cells. By contrast, the expression level of cyclin E, phosphorylated Cdk2 (pCdk2) and phosphorylated Rb (pRb) significantly declined. Based on our data and the previous findings that CCRK was able to interact with Cdk2,[7] we proposed that CCRK phosphorylates the cyclin E/Cdk2 complex, which subsequently promotes the transition from G1 to S phase through the phosphorylation of Rb (Fig. 4B). Nevertheless, the possibility that CCRK can directly phosphorylate Rb should not be excluded.

In conclusion, we have demonstrated for the first time that CCRK expression is associated with colorectal cancer development and CCRK is responsible for the regulation of G1/S cell cycle transition, the expression of cyclin E and the phosphorylation of Cdk2 and Rb. Further studies on how the dysregulation of CCRK expression in colorectal cancer cells causes these cellular defects may provide important insights into the carcinogenesis of colorectal cancer.

Role of the funding source

The authors declared that the study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

Conflict of interest statement

None declared.

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