CDCA2 acts as an oncogene and induces proliferation of clear cell renal cell carcinoma cells

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Abstract. Cell division cycle-associated 2 (CDCA2) plays an important role in regulating chromosome structure during mitosis. It is highly expressed in oral squamous cell carcinoma, neuroblastoma and lung adenocarcinoma, and its upregulation is positively associated with tumor progression. However, the expression, biological function and underlying mechanisms of the role of CDCA2 in clear cell renal cell carcinoma (ccRCC) remain poorly understood. In the present study, CDCA2 was demonstrated to be upregulated in ccRCC tissues compared with normal kidney tissue, where higher expression was generally associated with the degree of malignancy. Small interfering RNA-mediated knockdown of CDCA2 expression inhibited the viability and proliferation of 786-O and CAKI-1 cells, as measured by an MTT assay, colony formation assay and flow cytometry. Furthermore, western blot analysis suggested that CDCA2 regulates cell proliferation through the cell cycle-associated proteins cyclin D1 and cyclin dependent kinase 4, and the apoptotic protein Bcl-2. In conclusion, the present study indicated that CDCA2 may be an important factor in ccRCC progression and could be a potential therapeutic target in this disease.

Introduction

Renal cell carcinoma (RCC) is one of the top 10 most common types of cancer worldwide, whereby its incidence increased by 8.1% from 1975-2016 (1,2). RCC is primarily composed of three subtypes: Clear cell RCC (ccRCC), papillary RCC and chromophobe RCC. ccRCC accounts for 75-80% of these tumors and is the most common RCC subtype with the highest degree of local invasion, metastasis and mortality (3,4). At present, the primary treatment of early stage, localized RCC is surgical resection. However, despite tumor removal, 20-40% of patients still experience tumor recurrence (5). It is generally well accepted that renal cell carcinogenesis is the result of multiple factors (6-8); however, a consensus in the field has not yet been reached and the precise underlying molecular mechanisms remain unclear.

Cell division cycle-associated protein 2 (CDCA2) belongs to a class of cyclin-associated proteins (9,10). Previous studies have demonstrated that CDCA2 can form a complex with protein phosphatase 1 (PP1) γ and control the PP1γ-dependent DNA damage response (DDR) (11,12). Furthermore, CDCA2 promotes major mitotic histone H3 dephosphorylation in a PP1-dependent manner (13). CDCA2 is highly expressed in a number of different types of tumors. Previous studies have demonstrated that CDCA2 protein expression is associated with tumor volume and Tumor-Node-Metastasis (TNM) stage of oral squamous cell carcinoma (14-16). Furthermore, silencing the CDCA2 gene can lead to cell cycle arrest, inhibition of cell proliferation and apoptosis (16,17) However, the expression of CDCA2 and its function in ccRCC remain unclear.

The present study demonstrated that CDCA2 expression in ccRCC tissue is upregulated compared with normal healthy tissue. Furthermore, silencing CDCA2 induced G_1 arrest and promoted apoptosis in 786-O and CAKI-1 cells. These results indicated that CDCA2 regulates ccRCC carcinogenesis and may serve as a potential therapeutic target for the disease.

Materials and methods

Bioinformatics analysis. The Cancer Genome Atlas (TCGA) dataset, TCGA kidney Clear Cell Carcinoma (KIRC) (18), was downloaded from the University of California Santa Cruz Xena website (xena.ucsc.edu) and includes 534 ccRCC cases and 72 normal controls (Table SI). The tumor samples were

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matched to TNM stage and G stage (19) in order to obtain data on CDCA2 expression and clinical progression.

Cell culture and RNA transfection. The two human ccRCC cell lines (786-O and CAKI-1) and a human tubular epithelial cell line (HK-2) were sourced from the Key Laboratory of Environment and Genes Related to Diseases at Xi'an Jiaotong University (Xi'an, China). 786-O and HK-2 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Biological Industries) and 1% penicillin/streptomycin (PS). CAKI-1 cells were cultured in McCoy's 5a Modified Medium (Thermo Fisher Scientific, Inc.) supplemented with 15% FBS and 1% PS. All cell lines were cultured in an incubator with 5% CO_2 at 37˚C, until they reached 80% confluence. Small interfering (si)RNA duplexes targeting human CDCA2 were synthesized and purified by Shanghai GenePharma Co., Ltd. Non-specific siRNA sequences, purchased from Shanghai GenePharma Co., Ltd., were used as a negative control. A total of two siRNAs were used, and the sequences were as follows: CDCA2 siRNA-1; Forward, 5'-CACCUGCCUUUCUAA AUAUTT-3' and reverse, 5'-AUAUUUAGAAAGGCAGGU GTT-3'; and CDCA2 siRNA-2; Forward, 5'GGGCAAAGG AUCAAGUGAUTT-3' and reverse, 5'-AUCACUUGAUCC UUUGCCCTT-3'. The non-specific siRNA sequence was as follows: Forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. A total of 20 μM of siRNA was used, and 3 μl was added to each well of the six-well plate. Transfection of siRNAs was performed using jetPRIME reagent (Polyplus-transfection SA), according to the manufacturer's protocol.

RNA extraction, cDNA synthesis and reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from transfected ccRCC cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol and expression levels were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed using the iQ5 Optical real-time PCR system (Bio-Rad Laboratories, Inc.) with SYBR Green Ex Taq™ II (Takara Bio, Inc.). The following primer sequences were used for the qPCR: CDCA2; Forward, 5'-ATGACCGGCTGTCTG GAAT-3' and reverse, 5'-GCTGAGACCTTCCTTTCTGGT-3' and GAPDH; Forward, 5'-TGAAGGTCGGAGTCAACGGAT T-3' and reverse, 5'-CCTGGAAGATGGTGATGGGATT-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 10 min; 40 cycles of 95˚C for 15 sec, annealing at 60˚C for 60 sec and extension at 72˚C for 30 sec. CDCA2 mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (20) and normalized to the internal reference gene GAPDH.

Cell proliferation assay. The effect of CDCA2 silencing on the proliferation of 786-O and CAKI-1 cells was assessed using an MTT assay. Cells were seeded into 96-well plates at 3,000 cells per 100 μl culture media per well. Transfections were performed the following day. A total of 10 μl MTT reagent (5 mg/ml) was added to every well at various time points following transfection (24, 48 and 72 h), and 150 μl dimethyl sulfoxide was added 4 h later. The absorbance of samples was measured at 490 nm using a high-throughput universal micro plate reader.

Colony forming assays. ccRCC cells were seeded into 12-well plates at a density of $5x10^4$ cells/well and transfection was performed the following day. 24 h post-transfection, cells were reseeded into 6-well plates at a density of 1,000 cells/well in triplicate and incubated at 37˚C with 5% CO_2 for 7-10 days until they reached 80% confluence. Cells were then fixed with 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma Aldrich; Merck KGaA) for 30 min at room temperature. Photos were captured and colonies were counted using the Quantity One® software (version 4.3.1; Bio-Rad Laboratories, Inc.).

Cell cycle assay. 786-O and CAKI-1 cells were seeded into 6-well plates at a density of $1.5x10^5$ cells/well in triplicate and transfected 24 h later. Cells were then trypsinized 24 h post-transfection, washed with cold PBS twice, and fixed in ice-cold 75% alcohol at 4˚C overnight. Fixed cells were washed with PBS and then resuspended in 150 μl RNase A (0.1 mg/ml) and 150 μl propidium iodide (PI; 0.05 mg/ml) for 30 min at room temperature. Cell cycle distributions were measured using a flow cytometer.

Cell apoptosis analysis. In order to determine the effects of CDCA2 on ccRCC cell apoptosis, Annexin V-FITC Apoptosis Detection kits (7Sea PharmTech Shanghai, China) were used according to the manufacturer's protocol. Cells were seeded into 12-well plates at a density of $5x10^4$ cells/well and transfected 24 h later. Cells were then trypsinized 48 h post-transfection and stained with 5 μl of FITC Annexin V for 15 min at room temperature in the dark, prior to incubating with 10 μl of PI on ice for 5 min in the dark. Cell apoptosis was measured using a flow cytometer and the percentage of apoptotic cells was calculated using ModFit software (version 3.3.11; Verity Software House, Inc.). Cells stained with Annexin V-FITC were considered to be early apoptotic cells, and Annexin V-FITC and PI double stained cells were considered to be late apoptotic cells.

Western blot analysis. Total protein was extracted from ccRCC cells 48 h post- transfection using radioimmunoprecipitation assay buffer (http://www.xfbio.com) supplemented with protease inhibitor cocktail (100X) and 1 mM phenylmethylsulfonyl protease inhibitor (both from MedChemExpress). Protein concentrations were determined using a BCA protein assay kit (Takara Bio, Inc.) and 20 μg protein/lane was separated via SDS-PAGE on a 7.5-12.5% gel. The separated proteins were subsequently transferred onto a methanol-activated polyvinylidene membrane (EMD Millipore) and blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween, for 1 h at room temperature. The membranes were incubated with primary antibodies against CDCA2 (cat. no. 14976), BAX (cat. no. 2774), Bcl-2 (cat. no. 15071), cyclin dependent kinase 4 (CDK4; cat. no. 12790) and cyclin D1 (cat. no. 2922) (all 1:1,000; all from Cell Signaling Technology, Inc.) overnight at 4˚C. Following the primary incubation, membranes were

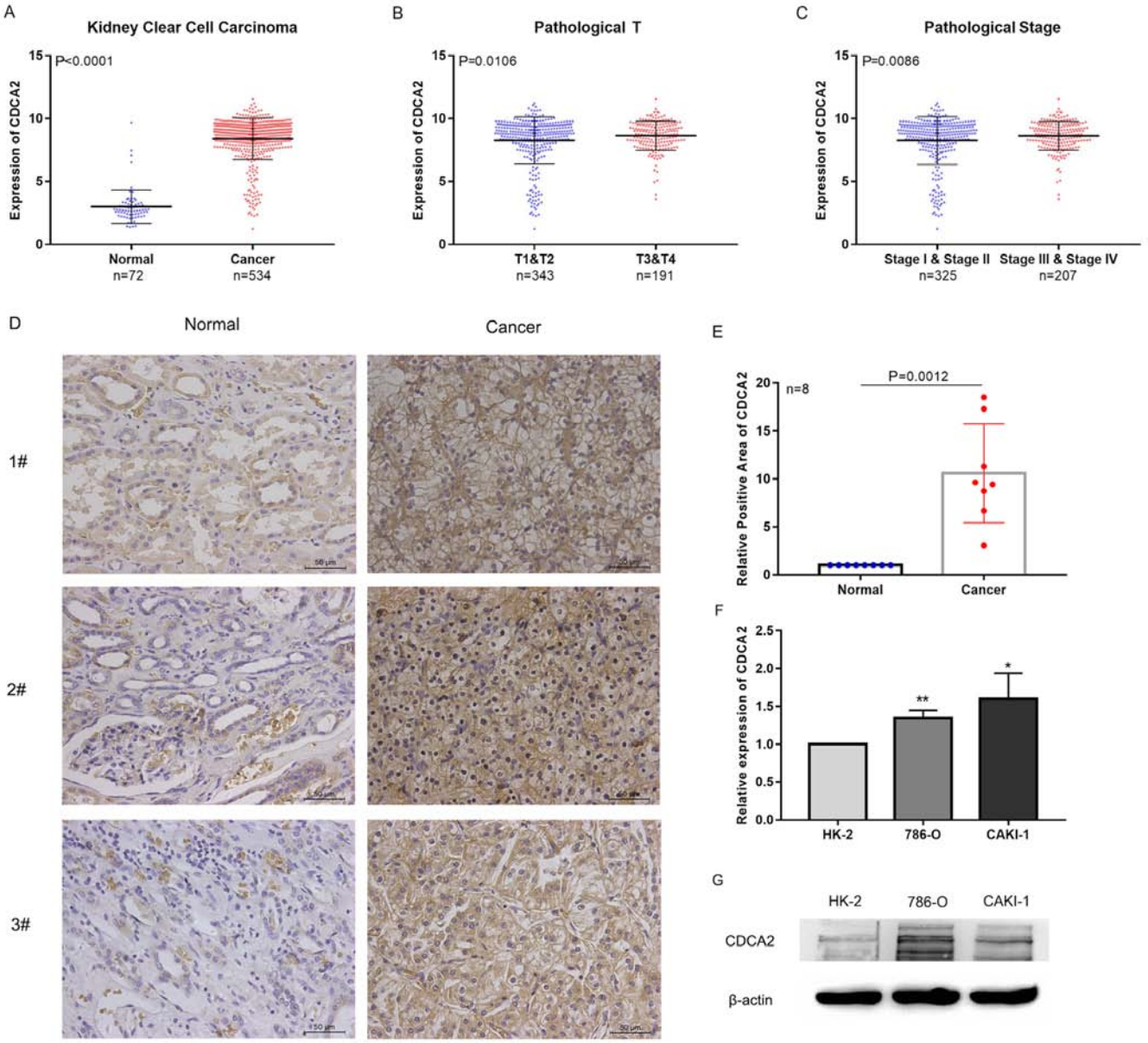

Figure 1. CDCA2 is upregulated in ccRCC and is associated with clinical stage in TCGA dataset. (A) Data from TCGA database demonstrated a significant upregulation of CDCA2 expression in ccRCC tumor samples (n=534) compared with normal tubular tissue (n=72). (B) Data from TCGA database demonstrated a significant upregulation of CDCA2 expression in T3 & T4 tumor samples (n=343) compared with samples from T1 & T2 (n=191). (C) Data from TCGA database demonstrated a significant upregulation of CDCA2 expression in tumor samples in Stages III & IV compared with samples in Stages I & II. (D) Representative images of CDCA2 immunohistochemistry in paired tumor and normal tissue samples. (E) The CDCA2 staining score in ccRCC tumor tissues was significantly increased compared with that in normal tissue (P=0.0012). The (F) reverse transcription-quantitative PCR and (G) western blotting results revealed that CDCA2 mRNA and protein levels were higher in the ccRCC cell lines 786-O and CAKI-1 compared with the normal renal epithelial cells HK-2. *P<0.05; **P<0.01 vs. control. CDCA2, cell division cycle-associated protein 2; ccRCC, clear cell renal cell carcinoma; TCGA, The Cancer Genome Atlas.

incubated with horseradish peroxidase-labeled secondary antibodies (cat. no. 7076 and cat. no. 7074; all 1:5,000; all from Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were visualized using the chemiluminescence detection Syngene GBox (Syngene Europe). The optical density of the image was analyzed using ImageJ software (version 1.4.3.67; National Institutes of Health) and protein levels were normalized to β-actin (1:5,000, cat. no. ab822, Abcam).

Immunohistochemistry. ccRCC tissues were fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin. Paraffin-embedded samples were cut into 4-μm thick sections. Sample information is presented in Table SII. Sections were deparaffinized in xylene and rehydrated in a descending ethanol series at room temperature. Deparaffinized sections were blocked with 10% goat serum working solution and incubated with 50 μl endogenous peroxidase inhibitor (both from OriGene Technologies, Inc.), according to the manufacturer's protocol, both at room temperature for 30 min. Antigen retrieval and blocking was subsequently performed. Tissue sections were incubated with primary antibody directed against CDCA2 (1:100; cat. no. 14976; Cell Signaling Technology, Inc.) overnight at 4˚C, followed by incubation with

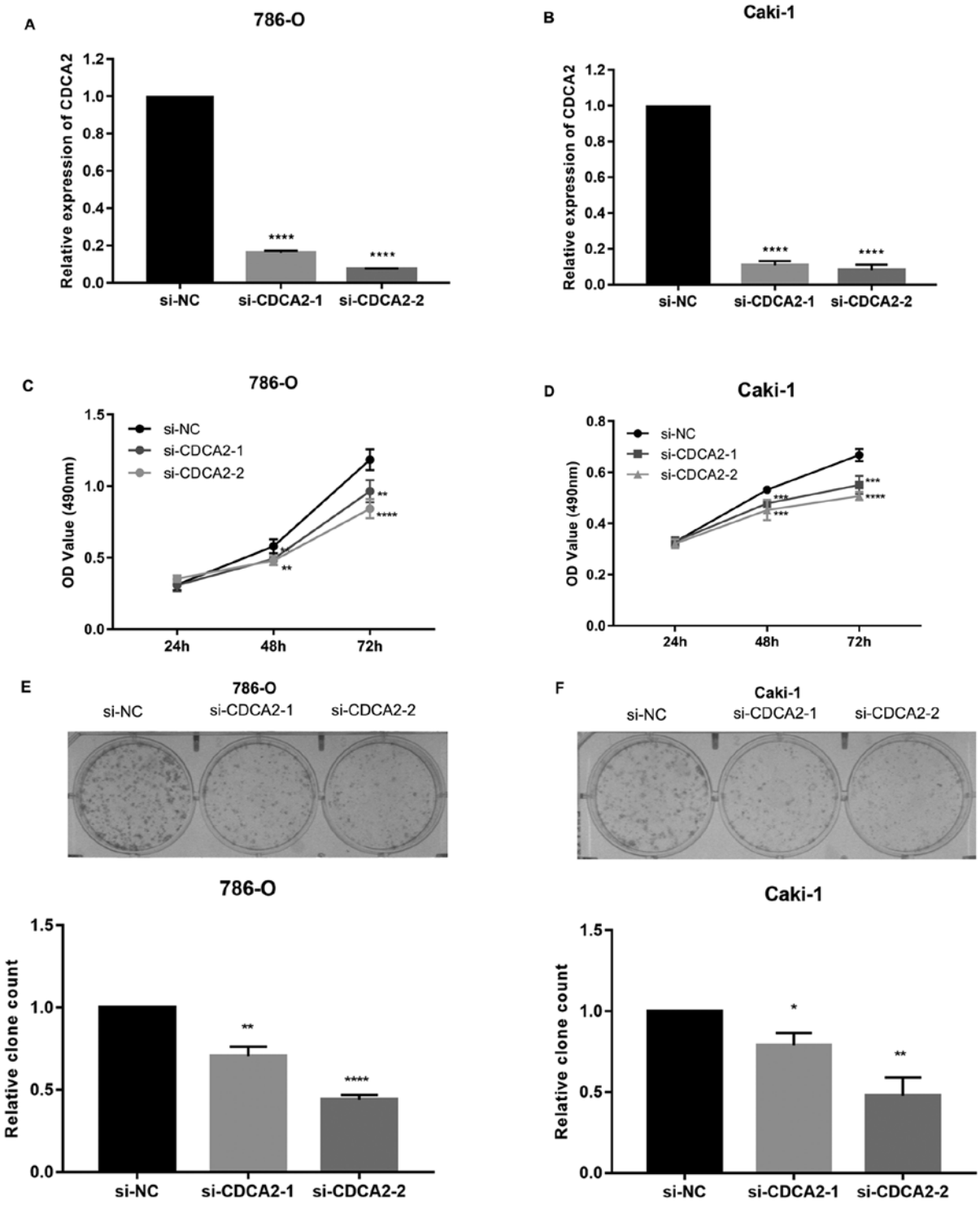

Figure 2. CDCA2 knockdown inhibits cell viability and proliferation of 786-O and CAKI-1 cells. The expression level of CDCA2 was detected via reverse transcription-quantitative PCR to confirm the knockdown efficiency of two siRNAs in (A) 786-O and (B) CAKI-1 cells. The MTT assay demonstrated that CDCA2 knockdown inhibited the viability of (C) 786-0 and (D) CAKI-1 cells. CDCA2 knockdown significantly decreased the number of (E) 786-O and (F) CAKI-1 cell colonies compared with the control group. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 vs. control. CDCA2, cell division cycle-associated protein 2; si, small interfering; NC, negative control.

100 μl of horseradish peroxidase-labeled secondary antibodies (cat. no. SP-9001; OriGene Technologies, Inc.) for 15 min at room temperature. Chromogenic development was performed using 3,3'-diaminobenzidine and hematoxylin staining for 15 sec at room temperature. Positive staining was analyzed by measuring the gray pixels using Image-pro Plus (version 6.0; Media Cybernetics, Inc.).

Statistical analysis. All statistical analyses were performed using SPSS software (version 22.0). All experiments were performed in triplicate. Unpaired Student's t-test and one-way ANOVA analysis followed by Dunnett's post-hoc test were performed for multiple comparison between the groups. Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

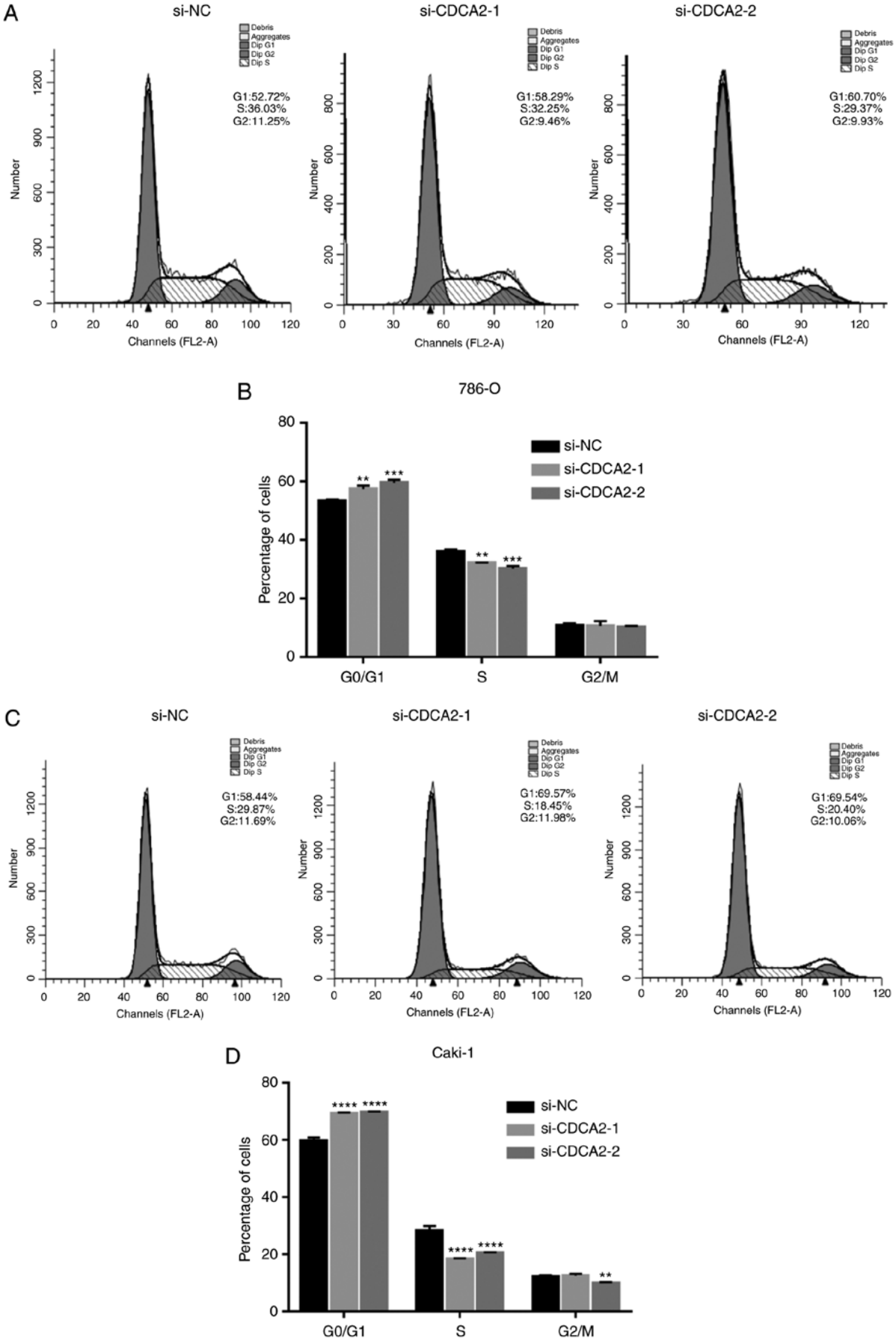

Figure 3. CDCA2 knockdown inhibits the expression of cell cycle proteins in clear cell renal cell carcinoma cells, causing G_1 arrest. Flow cytometry was performed in order to detect the effect of siCDCA2 on the cell cycle of 786-O and CAKI-1 cells. Flow cytometry results demonstrated that silencing of CDCA2 increased the proportion of cells in the G_1 phase and decreased the proportion of cells in the S phase in (A and B) 786-O and (C and D) CAKI-1 cells. **P<0.01, ***P<0.001, ****P<0.0001 vs. control group. CDCA2, cell division cycle-associated protein 2; si, small interfering; NC, negative control.

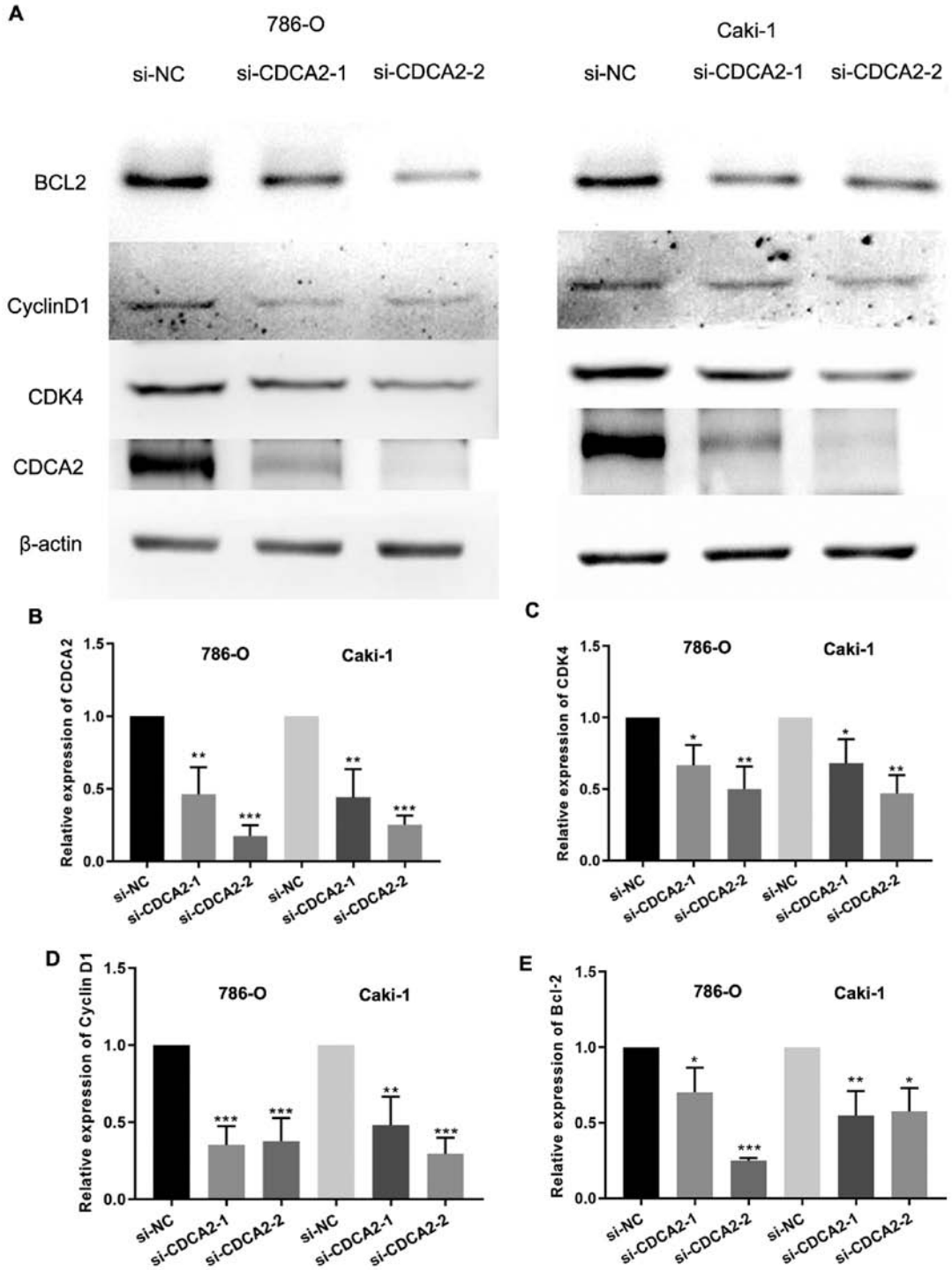

Figure 4. CDCA2 knockdown influences the expression of cyclin D1, CDK4 and Bcl-2. (A) Western blotting demonstrated that the expression of cyclin D1, CDK4 and Bcl-2 was decreased following CDCA2 knockdown in both 786-O and CAKI-1 cells. Histograms depicting quantitative analysis of the relative expression of (B) CDCA2, (C) CDK4, (D) cyclin D1 and (E) Bcl-2. *P<0.05; **P<0.01; ***P<0.001 vs. control. CDCA2, cell division cycle-associated protein 2; CDK4, cyclin dependent kinase 4; Bcl-2, B-cell lymphoma 2; si, small interfering; NC, negative control.

Results

CDCA2 is upregulated in ccRCC and is associated with clinical stage in TCGA dataset. Tumor samples had significantly higher CDCA2 expression compared with normal samples (P<0.0001) in the TCGA dataset (Fig. 1A). The present study then assessed CDCA2 protein levels in ccRCC paired tissue samples (n=8) using immunohistochemistry. As presented in Fig. 1D and E, positive staining of CDCA2 was higher in ccRCC tissue than normal, suggesting that CDCA2 protein expression is upregulated in ccRCC tissues compared with normal tissue controls (P=0.0012). In order to assess the expression of CDCA2 in the ccRCC cell lines 786-O and CAKI-1, the present study used RT-qPCR and western blotting. CDCA2 was highly expressed in 786-O and CAKI-1 cells compared with the normal renal epithelial cell line HK-2 (both P<0.05; Fig. 1F and G).

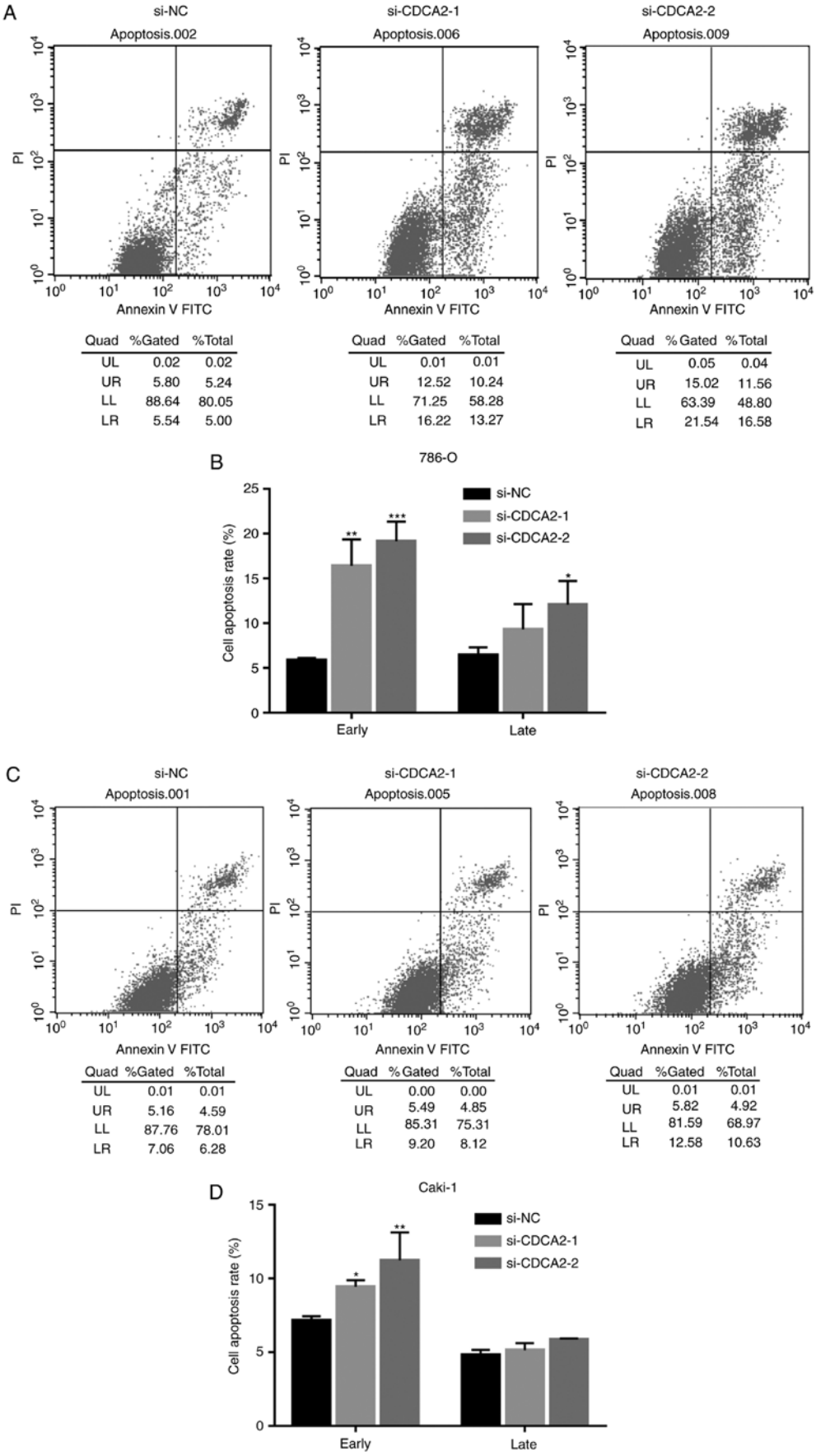

Figure 5. CDCA2 knockdown induces apoptosis in ccRCC cells. (A) Flow cytometry was performed to detect the effects of CDCA2 knockdown on apoptosis in 786-O cells. (B) Silencing of CDCA2 resulted in a significant increase in the number of apoptotic 786-O cells compared with controls. (C) Flow cytometry was performed to detect the effect of CDCA2 knockdown on apoptosis in CAKI-1 cells. (D) Silencing of CDCA2 resulted in a significant increase in the number of apoptotic CAKI-1 cells compared with controls. *P<0.05; **P<0.01; ***P<0.001 vs. control. CDCA2, cell division cycle-associated protein 2; si, small interfering; NC, negative control; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

The present study then assessed whether CDCA2 expression was associated with the clinical stage of ccRCC tumors. The statistical analysis indicated that CDCA2 expression was increased in tumors that had a high degree of malignancy compared with samples with a lower degree of malignancy (both P<0.05; Fig. 1B and C). These data suggest that CDCA2 may be associated with tumor cell proliferation.

CDCA2 knockdown inhibits cell viability and proliferation in 786-O and CAKI-1 cells. In order to investigate the effect of CDCA2 on ccRCC cell proliferation, the present study designed siRNAs that target human CDCA2. It was demonstrated that these CDCA2 siRNAs decreased CDCA2 mRNA levels in 786-O and CAKI-1 cells by >60% (both P<0.0001; Fig. 2A and B). MTT assays were used to measure the effect of CDCA2 on ccRCC cell viability. CDCA2 knockdown significantly inhibited ccRCC cell viability compared to the control group (both P<0.05; Fig. 2C and D). In order to further investigate the effect of CDCA2 on ccRCC cell proliferation, a colony formation assay was performed. Fewer and smaller colonies were observed in cells with CDCA2 knockdown compared with control cells (both P<0.05; Fig. 2E and F). These data demonstrated that silencing of CDCA2 inhibits the growth of ccRCC cells, and that CDCA2 promotes ccRCC cell viability and proliferation.

CDCA2 knockdown inhibits the expression of cell cycle proteins and promotes G_1 arrest in ccRCC cells. In order to determine whether cell cycle arrest drove the inhibition of cell proliferation that was observed with CDCA2 knockdown, flow cytometry was used to analyze cell cycle distribution 24 h post-transfection. Compared with controls, the percentage of siCDCA2 transfected 786-O and CAKI-1 cells in the G_1 phase increased, while the percentage of cells in S phase decreased (Fig. 3). Furthermore, CDK4 and cyclin D1 expression was significantly decreased in ccRCC cells transfected with siCDCA2 (both P<0.05; Fig. 4A, C and D), and the silencing efficiency of siCDCA2 was significant (P<0.05; Fig. 4B). These results demonstrated that silencing CDCA2 inhibits the expression of cell cycle proteins in ccRCC cells, causing G_1 arrest.

CDCA2 knockdown induces apoptosis of ccRCC cells. Dysfunction in apoptosis caused by the dysregulation of apoptosis-associated proteins plays an important role in the development of cancer. An apoptosis assay and western blot analysis were performed in order to determine whether CDCA2 affects apoptosis in ccRCC cells. CDCA2 knockdown increased the proportion of 786-O and CAKI-1 apoptotic cells (Fig. 5). Furthermore, B-cell lymphoma 2 (Bcl-2) expression significantly decreased in ccRCC cells transfected with siCDCA2 (Fig. 4A and E). These results revealed that silencing CDCA2 induces apoptosis in ccRCC cells.

Discussion

Trinkle-Mulcahy *et al* (21) first identified CDCA2 as a binding protein for PP1. Peng *et al* (12) reported that CDCA2 inhibits the activation of Ataxia-telangiectasia mutated-dependent signaling by promoting the binding of PP1c to chromatin. Peng *et al* (12) also demonstrated that CDCA2 upregulation during cancer progression enhances CDCA2-dependent DDR regulation, resulting in decreased DDR sensitivity. DNA damage delays cell cycle entry by affecting cell cycle checkpoints, causing cell cycle arrest at specific stages (22,23). Genomic stability is maintained by offsetting DNA damage through a series of pathways such as DNA repair, damage tolerance and checkpoint pathways. DDR defects can lead to apoptosis, genomic instability, dysregulation of cells and an increased risk of cancer (24,25). The aforementioned studies indicate that CDCA2 plays an important role in cell cycle progression and apoptosis. Studies have reported that CDCA2 is upregulated in neuroblastoma, melanoma and oral squamous cell carcinoma (15,16,18); however, to the best of our knowledge, the expression and function of CDCA2 in ccRCC has not been previously reported. The present study demonstrated that CDCA2 is widely upregulated in ccRCC, and the experiments in ccRCC cell lines revealed that CDCA2 knockdown can significantly inhibit cell proliferation by promoting G_1 phase arrest and apoptosis. This is consistent with previous findings in lung adenocarcinoma and oral squamous cell carcinoma (16,18). Since CDCA2 knockdown can cause G_1 arrest in ccRCC cells, the present study assessed changes in cyclin D1 and CDK4 protein levels, key downstream regulators of the G_1 to S transition. CDK4 and cyclin D1 expression levels were demonstrated to be decreased in 786-O and CAKI-1 cells with CDCA2 knockdown. Similarly, it was observed that silencing of CDCA2 significantly downregulated the apoptosis-associated protein Bcl-2 in 786-O and CAKI-1 cells, consistent with the results of the apoptosis assays.

Overall, the results of the present study demonstrated that CDCA2 is upregulated in ccRCC, and knockdown of CDCA2 promotes G_1 arrest by inhibiting the expression of CDK4 and cyclin D1. In addition, CDCA2 knockdown promoted apoptosis by inhibiting Bcl-2 expression. This indicates that CDCA2 is involved in the proliferation of human ccRCC cells and may play an important role in the progression of the disease. The present study investigated the role of CDCA2 in ccRCC development; however, its underlying molecular mechanisms remain unclear. Future studies are required on CDCA2 regulation of ccRCC and further research of its targeted drugs, in order to improve the treatment of ccRCC.

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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 upon reasonable request.

Authors' contributions

CH designed the present study. YW, ZW and XW collected the cancer tissues and interpreted the bioinformatics data. FL, HZ, QL and FW performed the experiments. CH and FL interpreted the data. FL and HZ drafted the initial manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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