CKS2 Predicts A Poor Prognosis and Contributes to Malignant Progression of Pancreatic Cancer

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Abstract

Background: Cyclin-dependent kinase subunit 2 (CKS2) has been reported to promote various malignancies. This study aims to investigate the prognostic significance and functional role of CKS2 in pancreatic cancer.

Methods: The analysis of abnormal expression genes and prognosis value on pancreatic cancer by Gene Expression Profiling Interactive Analysis (GEPIA) database and immunohistochemical staining of 64 samples of tumors. CCK-8 assay, EdU staining, colony formation, flow cytometry, wound healing assay, transwell assay, and a xenograft tumor model were used to analyze the biological function of CKS2 in pancreatic cancer. Western blotting was performed to explore the mechanisms underlying the effect of CKS2 on cell cycle progression and apoptosis.

Results: A significantly higher expression of CKS2 was found in pancreatic cancer compared with adjacent normal tissues and high CKS2 expression indicated poor prognosis in patients with pancreatic cancer. Moreover, functional assays revealed that CKS2 knockdown suppressed cell proliferation, migration and invasion ability, induced cell cycle G2/M phase arrest and apoptosis *in vitro* and reduced tumor growth *in vivo*. In addition, CKS2 knockdown increased the expression of Bax, caspase-3, P53, P21 and GADD45α, but decreased the expression of Bcl-2, Cyclin B1, CDK1, Cyclin A, and Cdc25C. CKS2 overexpression obtained the opposite results to CKS2 knockdown.

Conclusions: Our findings suggested that CKS2 may act as a promising prognostic indicator and therapeutic target for the treatment of pancreatic cancer.

Background

Pancreatic cancer is the third leading cause of tumor-related death with an estimated 432,000 death cases in the world [1], whose main tumor type is pancreatic ductal adenocarcinoma accounting for 85% of all cases [2]. Currently, the five-year survival rate of pancreatic cancer patients is less than 5%, even though great progress has been made on available therapies, including surgical resection and adjuvant medical therapy [3, 4]. Therefore, exploring the mechanisms and identifying diagnostic targets are of great significance for improving the prognosis of pancreatic cancer.

Cyclin-dependent kinase subunit (CKS) proteins are highly conserved small cyclin-dependent kinase-interacting proteins, which play an important role in regulating cell cycle progression [5]. The mammalian CKS family consists of two paralogs, including CKS1 located at chromosome 8q21 and CKS2 located at chromosome 9q22 [6]. CKS1 and CKS2 share more than 80% sequence identity [7], which have been shown to be associated with cell growth and proliferation [8]. CKS1 is essential for the G1/S transition in cell cycle progression by acting as a specific cofactor required for Skp1-Cullin-F-box (SCF)$^{S-phase\ kinase–associated\ protein\ 2\ (SKP2)}$ in the degradation and ubiquitination of p27 [9, 10]. CKS2 is essential for the first metaphase/anaphase transition of mammalian meiosis [11]. Compared with CKS1, CKS2 lacks the residues in the N terminus that are required for interacting with SKP2 [12].

To our best knowledge, CKS2 has been reported to contribute to tumor progression [13]. For example, CKS2 was significantly elevated in hepatocellular carcinoma (HCC) tissues and identified as an independent prognostic biomarker of HCC [14]. In uterine leiomyosarcoma (ULMS), Deng et al [15] showed that CKS2 predicts poor prognosis and promotes tumor progression. High CKS2 expression was markedly associated with poor overall survival, relapse-free survival, and distant metastasis-free survival in patients with breast cancer [16]. In addition, CKS2 has been also demonstrated to function as an oncogene in esophageal carcinoma [17], gastric cancer [18] and cholangiocarcinoma [19]. Functionally, suppression of CKS2 expression resulted in decreased cell viability, increased cell apoptosis, cell cycle arrest and impaired cell invasion in colorectal cancer [20]. Knockdown of CKS2 expression inhibited proliferation and induced programmed cell death in prostatic cancer [21]. Nevertheless, there is currently lack of direct evidence on the clinical significance and functional role of human CKS2 in pancreatic cancer, although a recent study by Li et al [22] identified that CKS2 was pancreatic duct adenocarcinoma-associated gene through integrative analysis of gene expression profiles in GEO database.

In current study, we first investigate the clinical relevance of CKS2 in pancreatic cancer by the Gene Expression Profiling Interactive Analysis (GEPIA: http://gepia.cancer-pku.cn/) web tool and a large scale of immunohistochemical analysis. Moreover, the function role of CKS2 on cell proliferation and tumor growth was assessed *in vitro* and *in vivo* by performing loss-of-function and gain-of-function assays. Our finding indicated that inhibition of the CKS2 activity might provide new hints for pancreatic cancer therapeutic strategies.

Materials And Methods

Data analysis of public databases

To get the expression pattern of CKS2 in pancreatic cancer, the data sets were downloaded from a newly developed interactive web database Gene Expression Profiling Interactive Analysis (GEPIA: http://gepia.cancer-pku.cn/) based on the TCGA and GTEx projects for RNA expression analyses. Total 350 samples, including 171 normal cases and 179 tumor cases were obtained for CKS2 expression and the prognostic significance of CKS2 expression for the overall survival and disease-free survival in pancreatic cancer patients by Kaplan-Meier plotter. Disease-free survival was defined as the length of time following resection during which a patient survived without signs of recurrence or metastasis. Low and high expression of CKS2 in the pancreatic cancer was defined according to the median, and those above the median were defined as high expression and those under the median were defined as low expression. Meanwhile, the expression pattern of MKi67 was also obtained in the 179 tumor cases and Spearman's correlation analysis was performed to assess the relationship between CKS2 and MKi67 in pancreatic cancer patient samples.

Clinical sample information

A total of 64 of paraffin-embedded tumor tissues and matched adjacent non-cancerous tissues (2 cm away from the tumor border) were obtained from our Hospital, with all pancreatic cancer patients signed

written informed consent. Radiotherapy or chemotherapy was not received by any of these patients before the operation and follow-up continued up to November 2018. The basic clinical characteristics are summarized in **Table S1**. The overall survival (OS) is taken to indicate the time from surgery to death or final contact for any reason. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Nanjing Medical University.

Immunohistochemistry

Immunohistochemical staining for CKS2 was performed using an ultrasensitive kit (MXB; Fuzhou, China) according to the manufacturer's instructions. In brief, paraffin-embedded specimens were fixed with 10% buffered-formalin and made into 4 μm-thick sections. The sections were subsequently dewaxed in xylene and rehydrated in decreasing gradient ethanol. After rinsed in PBS, antigen retrieval was done with high-pressure steam treatment at 100 °C in 10 mM citrate buffer (pH, 6.0). Next, the endogenous peroxidase activity was blocked with peroxidase blocking solution for 10 min. Then, the sections were incubated with rabbit polyclonal anti-human CKS2 antibody (1:100; Santa Cruz Biotechnology) overnight at 4 °C, followed by probed for 2 h with horse-radish peroxidase (HRP)-conjugated secondary antibodies at 37 °C. Reaction product was developed with 3, 3'-diaminobenzidine (DAB) substrate kit. Two independent pathologists evaluated the immunohistochemical staining based on the proportion of staining area (0–100 scale) and the intensity of staining (0 = negative; 1 = weak; 2 = moderate; 3 = strong). Finally, these two scores were then multiplied together to yield an immunohistochemistry score between 0 and 300. All patients were divided into high- and low-expression groups with the mean immunohistochemistry score as a cutoff value.

Cell culture

Human pancreatic cancer cell lines, including BXPC-3, Mia PaCa-2, PANC-1, SW1990, COLO 357, CFPAC-1, and in immortalized non-neopastic pancreatic dual cell line hTERT-HPNE were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in RPMI1640 medium (Wako, Tokyo, Japan) with 10% heat-inactivated FBS (Gibco, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 incubator.

Lentivirus transfection

Lentiviruses carrying short hairpin RNA targeting CKS2 (Lv-sh-CKS2), negative control (NC), CKS2 (Lv-CKS2) or empty vector were produced by RiboBio (Guangzhou, China). Media containing lentivirus particles carrying Lv-sh-CKS2 or NC were used to transfect Mia PaCa-2 and SW1990 cells, while those carrying Lv-CKS2 or vector were used to transfect PANC-1 cells using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA), followed by puromycin selection.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and 2 μg RNA from each specimen was used for complementary DNA (cDNA) synthesis with PrimeScript™ Reverse Transcription kit (Takara

Bio, Inc., Otsu, Japan) according to the manufacturer's protocols. RT-qPCR was conducted with SYBR® Green Master Mix (Takara Bio, Inc., Otsu, Japan) following the reaction parameters (denaturation at 94 °C for 30 sec, then 30 cycles of 56 °C for 30 sec and 72 °C for 90 sec, and final extension at 72 °C for 5 min). Primer sequences used in this study were listed as follows: CKS2: forward, 5'-CTTCGCGCTCTCGTTTCATT-3' and reverse, 5'-CACCAAGTCTCCTCCACTCC-3'; GAPDH: forward, 5'-GTCGATGGCTAGTCGTAGCATCGAT-3' and reverse, 5'-TGCTAGCTGGCATGCCCGATCGATC-3'. Relative expression level of CKS2 was calculated using the 2-ΔΔCt method with GAPDH as an endogenous control.

CCK-8 assay

After 48 h transfection, cells were seeded at a density of 3,000 per well in a 96-well plate and incubated for consecutive five days. At each day, cells in each well were incubated with 10 μl CCK-8 reagent (Dojindo Laboratories) for 2 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad, Berkeley, CA, USA). Triplicates were prepared for each sample and means were calculated.

5-Ethynyl-2′-Deoxyuridine (EdU) assay

After 48 h transfection, cells were placed at a density of 1×10^5 cells per well into 96-well plates and incubated with 100 μL of 50 μM EdU reagent (RiboBio, Guangzhou, China). Then, cells were washed with PBS and fixed with 4% polyformaldehyde for 30 min at room temperature, which were treated with Apollo staining solution and Hoechst 33342 for 30 min in dark. The stained cells were examined under a fluorescent microscope (magnification 20×) and the percentage of Edu-positive cells was calculated using Image J software. Triplicates were prepared for each sample and means were calculated.

Colony formation

After 48 h transfection, cells (500 cells per well) were seeded in six-well plates and cultured for two weeks until forming colonies. Visible colonies were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet for 30 min. Subsequently, colonies with more than 50 cells were photographed and counted in five randomly selected fields. Triplicates were prepared for each sample and means were calculated.

Flow cytometry

Cells were harvested after transfection for 48 h and reseeded on 6-cm dishes at a density of 3×10^5 cells per well. For cell cycle analysis, cells were fixed with 70% ethanol and washed with PBS twice. Then, cells were re-suspended and stained with 5 μL of 25 μg/mL propidium iodide (PI, Keygentec, Nanjing, China) for 30 min in the dark. The percentage of cell population at different phases was determined by flow cytometry (BD Biosciences, San Jose, CA, USA). For cell apoptosis analysis, cells were stained with 5 μL Annexin V-FITC and 5 μL PI solution in 1× binding buffer (Keygentec) for 15 min at room temperature in

the dark. Apoptotic cells were quantified by flow cytometry (BD Biosciences). Triplicates were prepared for each sample and means were calculated.

Wound healing assay

Cell migratory ability was assessed by performing wound healing assay. Briefly, transfected cells were plated in 12-well plates and cultured until 80% confluence. Then, an artificial scratch wound was created on cell plate with a 200 μl pipette tip. The wound closure was recorded at 0, 24 and 48 h, respectively under a light microscope. Relative wound healing percentage was calculated at 24 h and 48 h relative to 0 h in different groups. Triplicates were prepared for each sample and means were calculated.

Transwell assay

Cell migration and invasion was assessed using 24-well Transwell chamber (Corning, Corning, NY, USA) coated without or with diluted Matrigel (BD Biosciences). In brief, the upper chamber was supplemented with 600 μL of serum-free medium containing 3×10^4 cells, while the lower chamber was filled with medium containing 10% FBS as a chemoattractant. Following 24 h of incubation, cells that migrated or invaded to the lower chamber were fixed in 4% paraformaldehyde for 15 min and stained with crystal violet for 10 min. Stained cells were photographed (magnification 200×) and counted using a light microscope. Triplicates were prepared for each sample and means were calculated.

Xenograft tumor in vivo

Six-week-old male BALB/c nude mice (18-21 g) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and maintained in a specific pathogen-free (SPF) animal facility. Four groups of PDAC cells were prepared prior to inoculation as follows: empty vector-transfected PANC-1 cells, Lv-CKS2-transfected PANC-1 cells, NC-transfected Mia PaCa-2 cells and Lv-sh-CKS2-transfected Mia PaCa-2 cells. Approximately 1×10^6 transfected above cells were subcutaneously injected into the left dorsal flank of the mice and these mice were accordingly divided into four groups with six mice in each group. After inoculation, the tumor length (L) and width (W) were measured at 7, 14, 21, 28 and 35 days with calipers and tumor volume were calculated using the equation $(L \times W^2)/2$. On day 35, the mice were sacrificed by cervical dislocation and the tumors were excised and weighed. Excised tumors were paraffin-embedded for histologically examination by immunohistochemistry using anti-human Ki-67 (1:100; Santa Cruz Biotechnology). All the animal experimental procedures were approved by the Ethics Committee of Nanjing Medical University.

Western blotting

Total protein from cells was extracted using RIPA lysis buffer (Pierce, Rockford, IL, USA) and protein concentration was measured by the BCA protein assay kit (Beyotime, Jiangsu, China). Equal amount protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes. After blocked with 5% skimmed milk for 2 h, the membranes were

incubated overnight at 4 °C with primary antibodies against CKS2, Bax, Bcl-2, Caspase-3, Cyclin B1, CDK1, Cyclin A, P53, P21, GADD45α, Cdc25c, Cyclin D, CDK2, CDK4, β-actin and GAPDH. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. Protein signals were visualized using enhanced chemiluminescence (Keygentec) with β-actin or GAPDH as the internal control.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). The Kaplan-Meier method was conducted for the effect of CKS2 on survival analyses and the log rank test was used to examine the differences in survival. The correlation of CKS2 expression levels with clinicopathological features was evaluated using chi-square test. Independent risk factors among pancreatic cancer patients were identified using the univariate and multivariate Cox proportional hazards model. All data are presented as the means ± SD. For continuous data from in vitro and in vivo experiments, significant differences were determined using Student's t-test for two groups and one-way ANOVA for more than two groups. P values less than 0.05 were regarded as statistically significant.

Results

Elevated CKS2 expression in pancreatic cancer indicates poor prognosis

To investigate the expression pattern of CKS2 in pancreatic cancer, we acquired 171 normal cases and 179 tumor cases from GEPIA dataset. As shown in **Figure 1A**, the expression level of CKS2 was significantly elevated in pancreatic cancer tissues compared with normal tissues. In the same tumor tissues, we found CKS2 expression was inversely correlated with MKi67 expression by Spearman's correlation analysis (**Figure 1B**). Then we performed Kaplan–Meier analysis using GEPIA dataset and observed that high level of CKS2 in pancreatic cancer patients predicted shorter overall survival (**Figure 1C**) and disease-free survival (**Figure 1D**).

Subsequently, we collected pancreatic cancer and matched adjacent non-cancerous tissues to examine the expression of CKS2. Immunohistochemical staining showed negative, weak, moderate and strong staining intensity of CKS2 in the cytoplasm of tumor and adjacent tissues in adjacent tissues, highly differentiated, moderately differentiated, poorly differentiated pancreatic cancer tissues, respectively (**Figure 2A**). By comparison of immunohistochemistry score, we found CKS2 expression score in tumor tissues is significantly higher than that in adjacent normal tissues (**Figure 2B**). Spearman's correlation analysis indicated that CKS2 expression was positively correlated with T stage, tumor size and histology stage, but negatively correlated with survival time (**Table S2**). According to the mean immunohistochemistry score, 64 patients were divided into high- (n = 33) and low-CKS2 (n = 31) expression groups. Chi-square test (**Table 1**) further demonstrated that CKS2 expression was significantly associated with T stage ($P = 0.005$), tumor size ($P = 0.047$) and histology stage ($P = 0.042$). Kaplan-Meier analysis found the overall rates for these patients with low CKS2 expression were significantly higher than the high CKS2 expression group (**Figure 2C**). Furthermore, a Cox proportional hazard model was

applied to identify the independent factors affecting overall survival. As shown in **Table S3**, CKS2, histological grade, blood vessel invasion and nerve invasion were recognized as independent prognostic factors of survival. Thus, CKS2 might have significant correlation with poor prognosis of pancreatic cancer patients.

CKS2 is abnormally expressed in pancreatic cancer cells

Consistent with the expression levels of CKS2 in pancreatic cancer tissues, we showed that CKS2 mRNA and protein (**Figure 3A**) expression was significantly higher in six pancreatic cancer cell lines than that in normal pancreatic dual cell line hTERT-HPNE. Among these pancreatic cancer cell lines, Mia PaCa-2 and SW1990 cells exhibited relatively higher CKS2 expression, while PANC-1 showed relatively lower CKS2 expression, as compared with the other pancreatic cancer cells. To investigate the biological function of CKS2 in pancreatic cancer cells in vitro, we suppressed the expression levels of CKS2 in Mia PaCa-2 and SW1990 cells by Lv-sh-CKS2 transfection compared with NC transfection, but elevated the expression levels of CKS2 in PANC-1 cells by Lv-CKS2 transfection compared with vector transfection. RT-qPCR and western blot further demonstrated that Lv-sh-CKS2 transfection remarkably down-regulated CKS2 expression in Mia PaCa-2 (**Figure 3B**) and SW1990 (**Figure 3C**) cells, while Lv-CKS2 transfection obviously up-regulated CKS2 expression in PANC-1 cells (**Figure 3D**).

CKS2 promoted pancreatic cancer cell growth and proliferation *in vitro* and *in vivo*

Given the clinical importance of CKS2 in pancreatic cancer patients, we further explored the effects of CKS2 on the biological behavior of pancreatic cancer cells. CCK-8 assay showed that CKS2 knockdown significantly suppressed cell viability in Mia PaCa-2 and SW1990 cells, while CKS2 overexpression increased cell viability in PANC-1 cells (**Figure 3E-3G**). Edu staining and colony formation assays were used to evaluate the role of CKS2 on the proliferation of pancreatic cancer cells. The percentage of Edu-positive cells was obviously reduced in Mia PaCa-2 and SW1990 cells after Lv-sh-CKS2 transfection, but elevated in PANC-1 cells after Lv-CKS2 transfection, compared with NC transfection (**Figure 3H, 3I**). Colony formation consistently showed that CKS2 knockdown significantly decreased the number of colonies in Mia PaCa-2 and SW1990 cells, while overexpression increased the number of colonies in PANC-1 cells (**Figure 3J, 3K**). Next, we employed a xenograft tumor assay to evaluate cancer cell proliferation ability *in vivo*. A shown in **Figure 4A**, the size of the tumors was obviously increased after CKS2 overexpression and decreased after CKS2 knockdown. The weight of the tumors was remarkably increased in CKS2-overexpressed mice and decreased in CKS2-silenced mice (**Figure 4B**). Moreover, the time-dependent analysis showed the volume of the tumors was significantly suppressed in mice inoculated with CKS2-silenced Mia PaCa-2 cells (**Figure 4C**), but enhanced in mice inoculated with CKS2-overexpressed PANC-1 cells compared with control groups (**Figure 4D**). Additionally, the Ki67 staining results showed that the expression of Ki67 was down-regulated in mice inoculated with CKS2-silenced Mia PaCa-2 cells (**Figure 4E**), but up-regulated mice inoculated with CKS2-overexpressed PANC-1 cells (**Figure 4F**), in comparison with control groups. This finding indicated that CKS2 could contribute to pancreatic tumorigenesis.

CKS2 promoted cell migration and invasion in pancreatic cancer cells

In addition to proliferation, we analyze the effects of CKS2 on the ability of cell migration and invasion in pancreatic cancer cells. Wound healing assay (**Figure 5A-5D**) showed that the migratory distance of Mia PaCa-2 and SW1990 cells was significantly impaired after CKS2 knockdown, while that of PANC-1 cells was notably increased after CKS2 overexpression at 24 h and 48 h. Transwell assay (**Figure 5E-5G**) further confirmed that the number of migrated and invasive cells was remarkably reduced in Lv-sh-CKS2-transfected Mia PaCa-2 and SW1990 cells compared with NC-transfected cells, but notably increased in Lv-CKS2-transfected PANC-1 cells compared with vector-transfected cells. The above results indicated that CKS2 was associated with the metastasis of pancreatic cancer cells.

Mechanism study of CKS2 in promoting pancreatic cancer cell proliferation

To investigate the mechanism underlying CKS2 promoting pancreatic cancer cell proliferation, we performed flow cytometry to analyze the effects of CKS2 on cell apoptosis and cell cycle distribution in pancreatic cancer cells. As depicted in **Figure 6A**, CKS2 knockdown significantly increased the percentage of apoptotic cells in both Mia PaCa-2 and SW1990 cells. Conversely, CKS2 overexpression obviously decreased the percentage of apoptotic cells in PANC-1 cells. We then observed that the percentage of cells at G2/M phase was increased, that at G0/G1 and S phase was accordingly decreased in Lv-sh-CKS2-transfected Mia PaCa-2 and SW1990 cells compared with NC-transfected cells. However, Lv-CKS2 transfection obtained the opposite results in PANC-1 cells, as shown by decreased percentage of cells at G2/M phase and increased percentage of cells at S phase (**Figure 6B**). Next, we determined the protein markers associated with apoptosis and cell cycle progression using western blotting. As shown in **Figure 6C**, pro-apoptotic Bax and caspase-3 protein expression was up-regulated and anti-apoptotic Bcl-2 was down-regulated in CKS2-silenced Mia PaCa-2 and SW1990 cells, while CKS2 overexpression caused the opposite effects on Bax, Bcl-2 and Caspase-3 expression. As shown in **Figure 6D**, we observed that the expression of Cyclin B1, CDK1 and Cyclin A, associated with G2/M transition was decreased in CKS2-silenced Mia PaCa-2 and SW1990 cells, but increased in CKS-overexpressed PANC-1 cells. Meanwhile, there were no significant differences in the expression of G0/G1 phase markers (CDK4 and Cyclin D) and S phase marker CDK2 between Lv-sh-CKS2 group and NC group in Mia PaCa-2 and SW1990 cells, as well as between Lv-CKS2 group and vector group in PANC-1 cells. These data indicated that CKS2 knockdown caused G2/M phase arrest in pancreatic cancer cells. Considering the crucial role of Cyclin B-Cdc2 (CDK1) binding in G2/M transition, we further detected its upstream molecules, including Cdc25C, P53, P21 and GADD45α. The results showed that Cdc25C expression was reduced, while P53, P21 and GADD45α were elevated in CKS2-silenced Mia PaCa-2 and SW1990 cells. Inversely, CKS2 overexpression increased the expression of Cdc25C, but decreased the expression of P53, P21 and GADD45α. Collectively, these findings suggested that CKS2 promoted pancreatic cancer cell proliferation might by escaping the G2/M DNA damage checkpoint.

Discussion

Here, we showed that the expression of CKS2 was significantly elevated in pancreatic cancer tissues compared with normal tissues. Based on the available clinical samples and follow-ups of patients, we further demonstrated that CKS2 predicted poor prognosis in pancreatic cancer, which was correlated with poor overall survival and disease-free survival. Consistently, previous studies focused on colorectal cancer [20], breast cancer [23] and esophageal squamous cell carcinoma [24]. Notably, a recent study by Li et al [22] showed that high CKS2 expression is significantly associated with shorter survival time in patients with pancreatic ductal adenocarcinoma by integrative analysis of six GEO datasets. Different from this, our bioinformatics analysis was performed on GEPIA database, including 171 normal cases and 179 pancreatic cancer cases. The similar results from different data source will be more powerful for demonstrating that CKS2 was a poor prognostic factor affecting pancreatic cancer patients' overall survival rate.

Next, we investigated the biological function of CKS2 in pancreatic cancer tumorigenesis. The *in vitro* functional assays revealed that CKS2 significantly increased pancreatic cancer cell proliferation, migration and invasion capacity. Moreover, the *in vivo* assay revealed that CKS2 overexpression increased, while knockdown decreased the average weights and volumes of the tumors. Similar reports have been also presented on the role of CKS2 on the aggressive behaviors of cancer cells. Hua et al [25] showed that overexpression of CKS2 contributed to thyroid cancer cell proliferation, migration and invasion. Deng et al [15] indicated that CKS2 promotes cell proliferation and the clonogenic survival of ULMS cells. In addition, knockdown of CKS2 suppressed proliferation, invasion, and migration of epithelial ovarian cancer cells and CKS2 could promote EMT progress by modulating EMT-related molecules [26]. Li et al [22] consistently showed that CKS2 knockdown substantially inhibited cell proliferation in pancreatic ductal adenocarcinoma in vitro. Compared with the study from Li et al [22], our in vitro data used three pancreatic cancer cell lines (Mia PaCa-2, SW1990 and PANC-1) and conducted loss-of-function and gain-of-function assays to analyze the function role of CKS2 on cell proliferation, which is more convincing for exerting the oncogenic role of CKS2 in pancreatic cancer.

Flow cytometry analysis indicated that CKS2 decreased cell apoptosis and promoted G2/M transition in pancreatic cancer cells. Western blotting analysis further demonstrated that CKS2 down-regulated the protein expression of pro-apoptotic Bax and caspase-3, but down-regulated anti-apoptotic Bcl-2. Furthermore, CKS2 increased the expression of Cyclin B1, CDK1, Cyclin A, and Cdc25C, associated with G2/M transition, but decreased the expression of P53, P21 and GADD45α. As the downstream genes of CKS2, Cyclin B1, CDK1 and Cyclin A are important players in cell cycle G2/M transition involved in tumor cell growth and proliferation [21, 27]. Lv et al [28] showed that down-regulated CKS2 expression caused G2/M phase-arrest in thyroid cancer cells and Shen et al [19] also found that silencing of CKS2 caused cell cycle G2/M phase arrest in cholangiocarcinoma, while G0/G1 phase arrest in colorectal cancer after CKS2 knockdown [20]. In molecular level, P21 is a universal inhibitor of cyclin-dependent kinases and its phosphorylation could precede the activation of the cyclin B1-Cdc2 kinase and the appearance of hyperphosphorylated Cdc25C [29], which indicated that the disruption of cyclin B-Cdc2 binding may be responsible for the G2/M arrest. GADD45α, encoded by DNA damage 45 (GADD45), could inhibit the activation of cyclin B-Cdc2 kinases to cause the inhibition of cell cycle G2-M transition [30]. Additionally,

GADD45α, acts as a downstream mediator of P53, and is capable of deactivating P53, binding with cyclin-dependent kinases and proliferating nucleus antigen, thereby contributing to cell cycle G2-M transition [31, 32]. Related study shows that tumor suppressor P53 could repress CKS2 [33] and P53 was down-regulated, accompanied with increased cell growth in CKS2-overexpressing gastric cancer cells [34]. In line with our data, CKS2 knockdown induced cholangiocarcinoma cell cycle arrest in G2/M phase through down-regulation of Cyclin A and Cyclin B1 and Bax up-regulation and caspase-3 activation, which resulted in facilitating cholangiocarcinoma apoptosis [19]. These findings further confirmed that the suppressive effects of CKS2 knockdown on tumor cell proliferation were closely associated with induced cell cycle G2/M phase arrest and apoptosis in pancreatic cancer.

Conclusions

In summary, we obtained comparative data that supported that CKS2 was a poor prognostic factor in pancreatic cancer patients. Most importantly, CKS2 overexpression promoted tumorigenicity by elevating cell proliferation, migration and invasion, as well as escaping the G2/M DNA damage checkpoint. These findings suggest that targeting CKS2 might provide a novel strategy for pancreatic cancer treatment.

Abbreviations

CKS2: Cyclin-dependent kinase subunit 2; BAX: BCL2 associated X, apoptosis regulator; P53: transformation related protein 53; P21: cyclin dependent kinase inhibitor 1A; GADD45α: growth arrest and DNA damage inducible alpha; Bcl-2: BCL2 apoptosis regulator; CDK1: cyclin dependent kinase 1; CDC25C: cell division cycle 25C.

Declarations

Ethics approval and consent to participate

This study was approved by ethical committees of the Nanjing Medical University. Written informed consent was obtained from all patients and data was analyzed anonymously. All Animal experiments complied with the national guidelines for the care and use of laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors confirm that there is no conflict of interest.

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Availability of data and materials

Can be provided upon a reasonable request.

Authors Contribution

QYC, YF and JLW Conceived and designed the work that led to the submission. QYC, XCL, PW, SND and XLZ acquired data. FZ, TTL and WBX played an important role in interpreting the results. JLW and YW drafted and revised the manuscript. All authors approved the final version.

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Table

Table 1 Correlation of CKS2 expression with clinicopathological features of patients with pancreatic cancer

Variable	Group	CKS2 expression		*P* value
		Low (n = 31)	High (n = 33)	
Gender	Male	20	22	0.856
	Female	11	11	
Age (year)	≤ 63	15	17	0.802
	< 63	16	16	
T stage	T1 or T2	17	7	0.005*
	T3 or T4	14	26	
N stage	Absent	16	19	0.632
	Present	15	14	
Metastasis	Absent	31	31	0.164
	Present	0	2	
TNM	IA-IIA	14	15	0.981
	IIB-IV	17	18	
Tumor size	< 3.5	18	11	0.047*
	≥ 3.5	13	22	
Histology stage	I/I-II/II	21	14	0.042*
	II-III/III	10	19	
Blood vessel invasion	Absent	30	29	0.185
	Present	1	4	
Nerve invasion	Absent	14	9	0.136

Present	17	24	

Figures

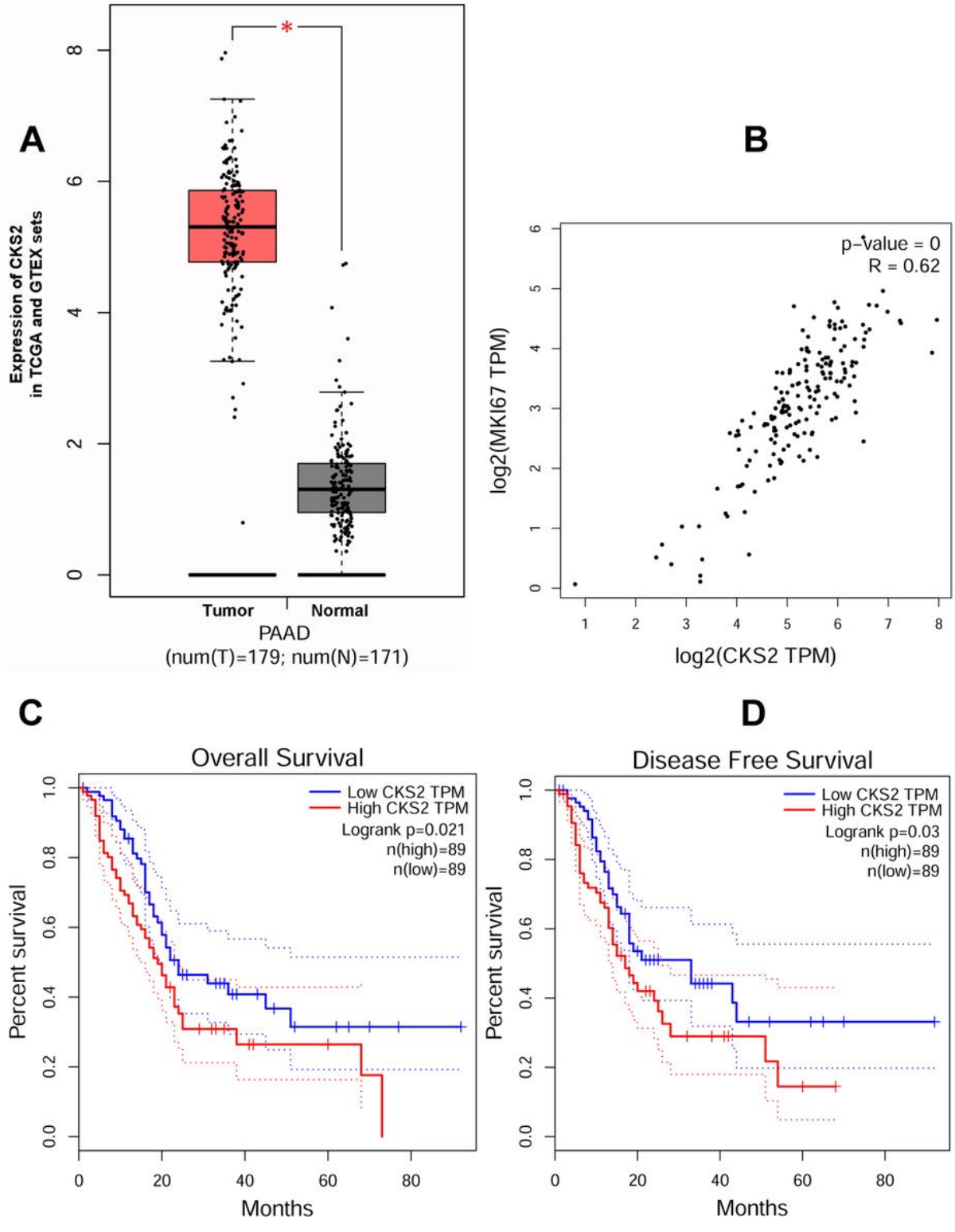

Figure 1

Overexpressed SKS2 was associated with poor prognosis in pancreatic cancer by bioinformatics analysis. (A) The mRNA expression level analysis of CKS2 in pancreatic cancer patient specimens (n = 179) and pancreatic non-tumor tissues (n = 171) by using the GEPIA web tool. *P < 0.05; (B) CKS2 expression was inversely correlated with MKi67 expression by Spearman's correlation analysis. (C, D) The overall survival time disease and free survival time of pancreatic cancer patients with different expression level of CKS2 was determined by GEPIA, P values as indicated.

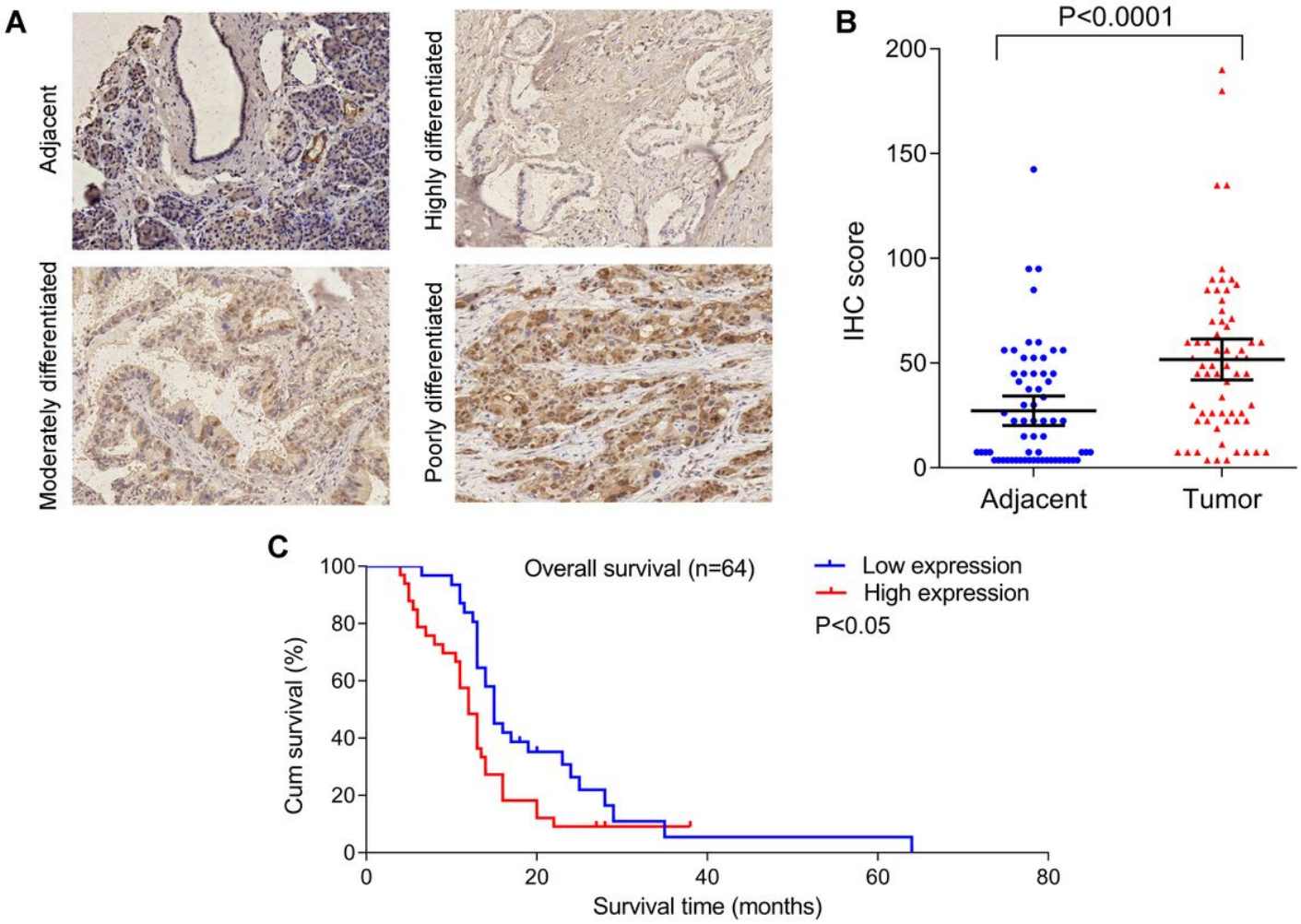

Figure 2

Increased CKS2 expression predicted poor prognosis in clinical pancreatic cancer samples. (A) Representative images of CKS2 expression in adjacent tissues, highly differentiated, moderately differentiated, poorly differentiated pancreatic cancer tissues detected by Immunohistochemistry. (B) Immunohistochemistry scores of CKS2 in pancreatic tissue microarray (Adjacent n = 64, Tumor n = 64). (C) Patients with pancreatic cancer were divided into high-expression and low-expression groups, and then overall survival of the two groups was compared by Kaplan-Meier analysis.

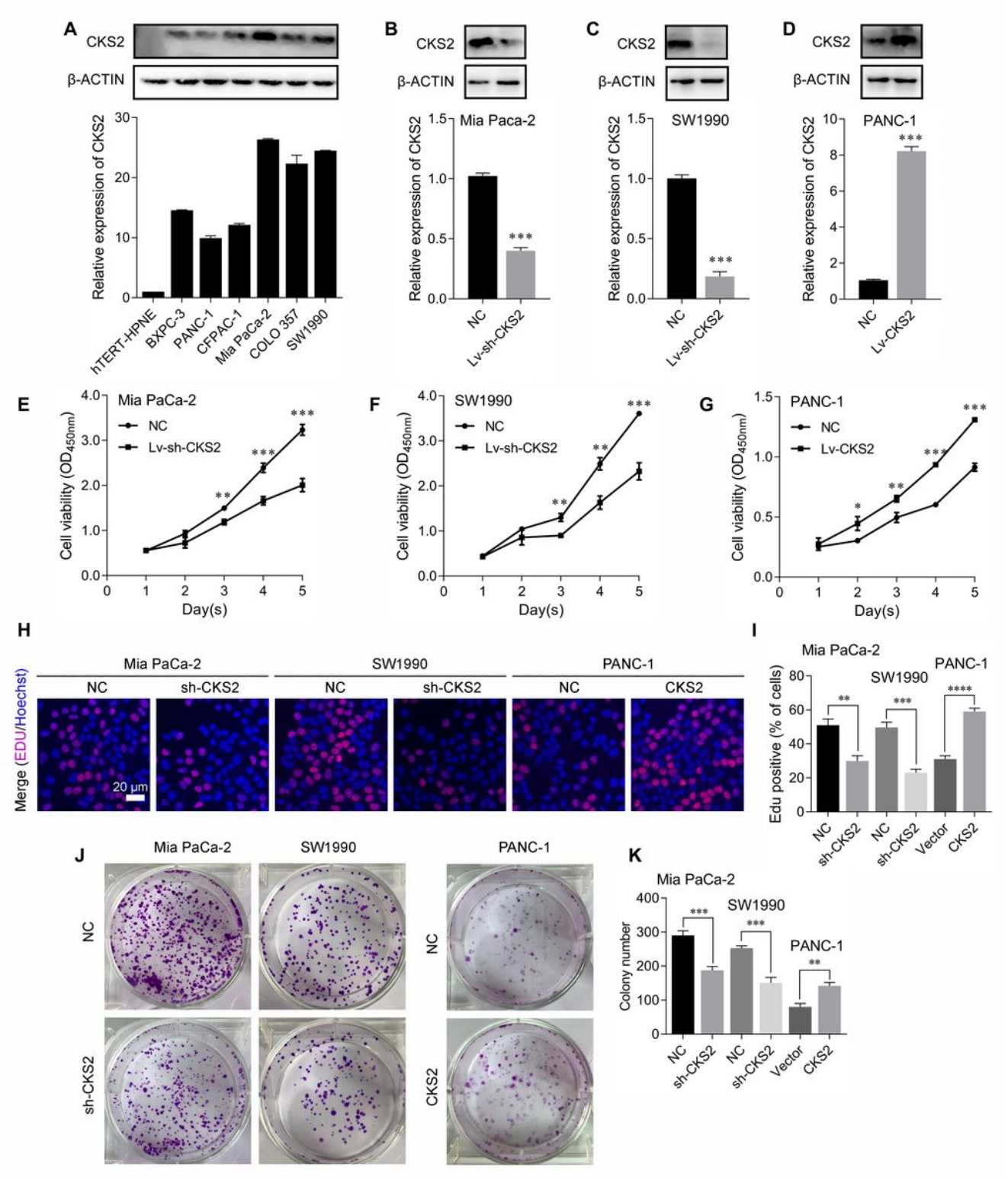

Figure 3

CKS2 promoted pancreatic cancer cell growth and proliferation in vitro. (A) RT- qPCR (lower) and western blotting (upper) were used to determine the expression of CKS2 in six pancreatic cancer cell lines and immortalized non-neopastic pancreatic dual cell line hTERT-HPNE. RT-qPCR (lower) and western blotting (upper) were conducted to analyze CKS2 expression levels in Lv-sh-CKS2-transfected Mia PaCa-2 (B) and SW1990 (C) cells, as well as Lv-CKS2-transfected PANC-1 cells (D). (E-F) CCK-8 assay was performed to

determine cell viability in Lv-sh-CKS2-transfected Mia PaCa-2 and SW1990 cells, as well as Lv-CKS2-transfected PANC-1 cells. (H, I) Edu staining and (J, K) colony formation was carried out to assess the cell proliferation ability in Lv-sh-CKS2-transfected Mia PaCa-2 and SW1990 cells, as well as Lv-CKS2-transfected PANC-1 cells. Data are presented as the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, compared with NC or Vector.

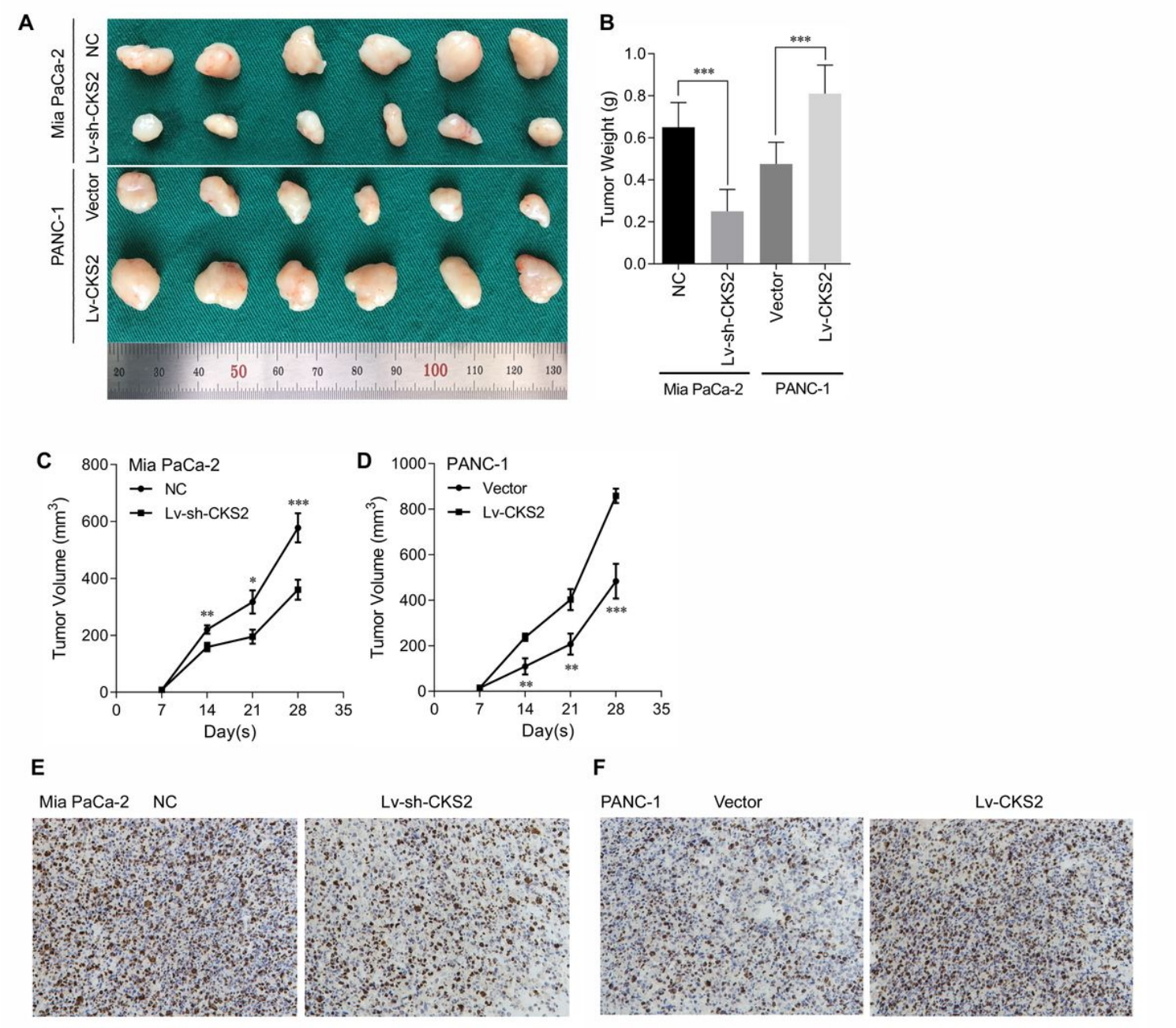

Figure 4

CKS2 promoted pancreatic cancer cell growth and proliferation in vivo. (A) Representative images of tumors formed in the mice in which Lv-CKS2- or vector-transfected PANC-1 cells and Lv-sh-CKS2- or NC-transfected Mia PaCa-2 cells were implanted. (B) Changes in the tumor weight in mice after CKS2 silencing or CKS2 overexpression. (C-D) Changes in the tumor volume on the 7th, 14th, 21st, 28th and 35th days after transfected-Mia PaCa-2 or PANC-1 cells were implanted subcutaneously. Data are

presented as the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, compared with NC or vector; (E-F) Representative images of Ki67 staining in tumors formed by transfected-Mia PaCa-2 or PANC-1 cells were presented.

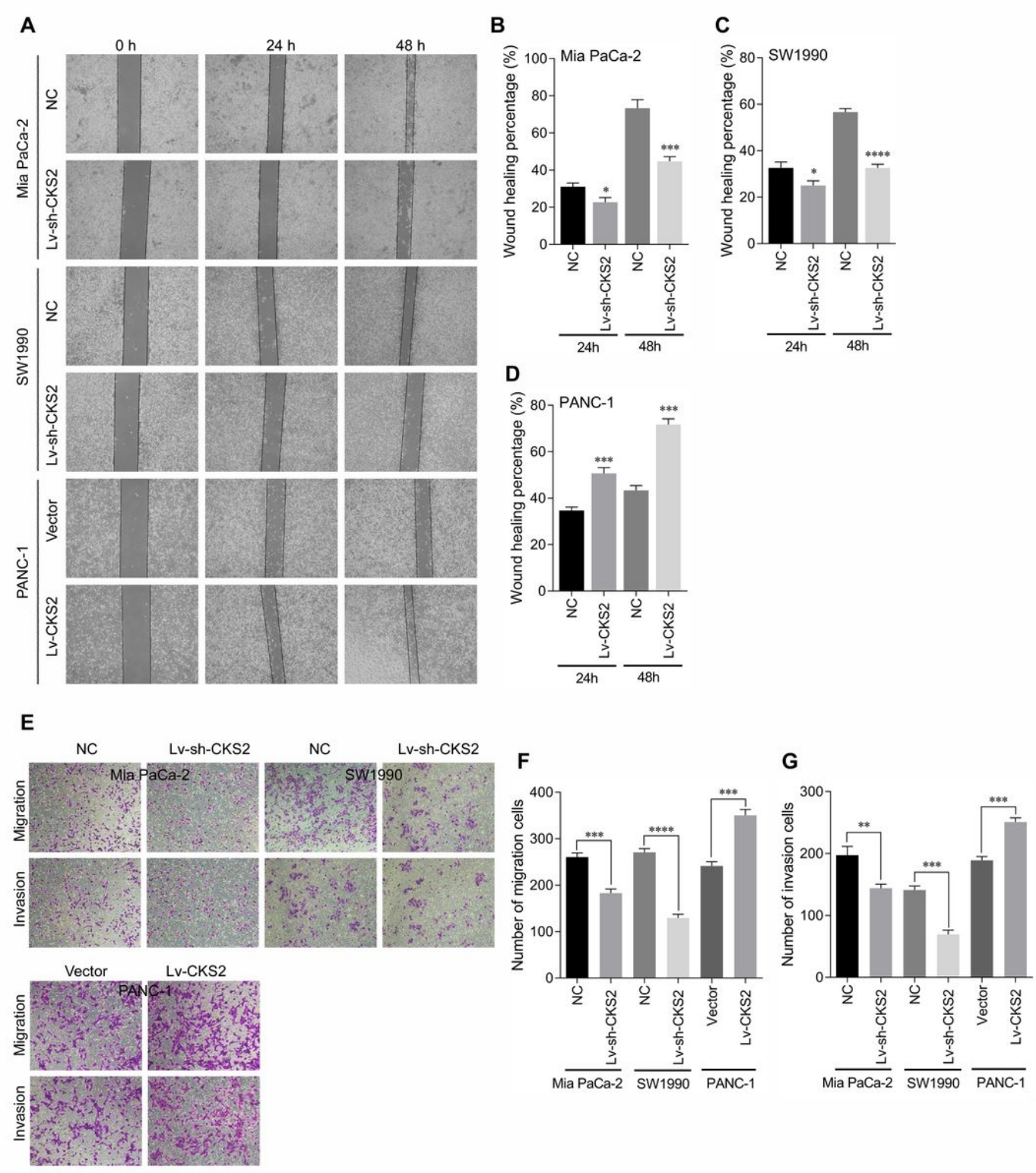

Figure 5

CKS2 promoted cell migration and invasion in pancreatic cancer cells. Mia PaCa-2 and SW1990 cells were transfected with lentivirus particles carrying Lv-sh-CKS2 or NC, PANC-1 cells were transfected with

lentivirus particles carrying Lv-CKS2 or vector. (A-D) Wound healing assay was performed to assess cell migration ability in Mia PaCa-2, SW1990 cells and PANC-1 cells as above transfected at 24 h and 48 h, respectively. (E-G) Transwell assay was conducted to analyze cell migration and invasion in Mia PaCa-2, SW1990 cells and PANC-1 cells as above transfected. Data are presented as the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, compared with NC or Vector.

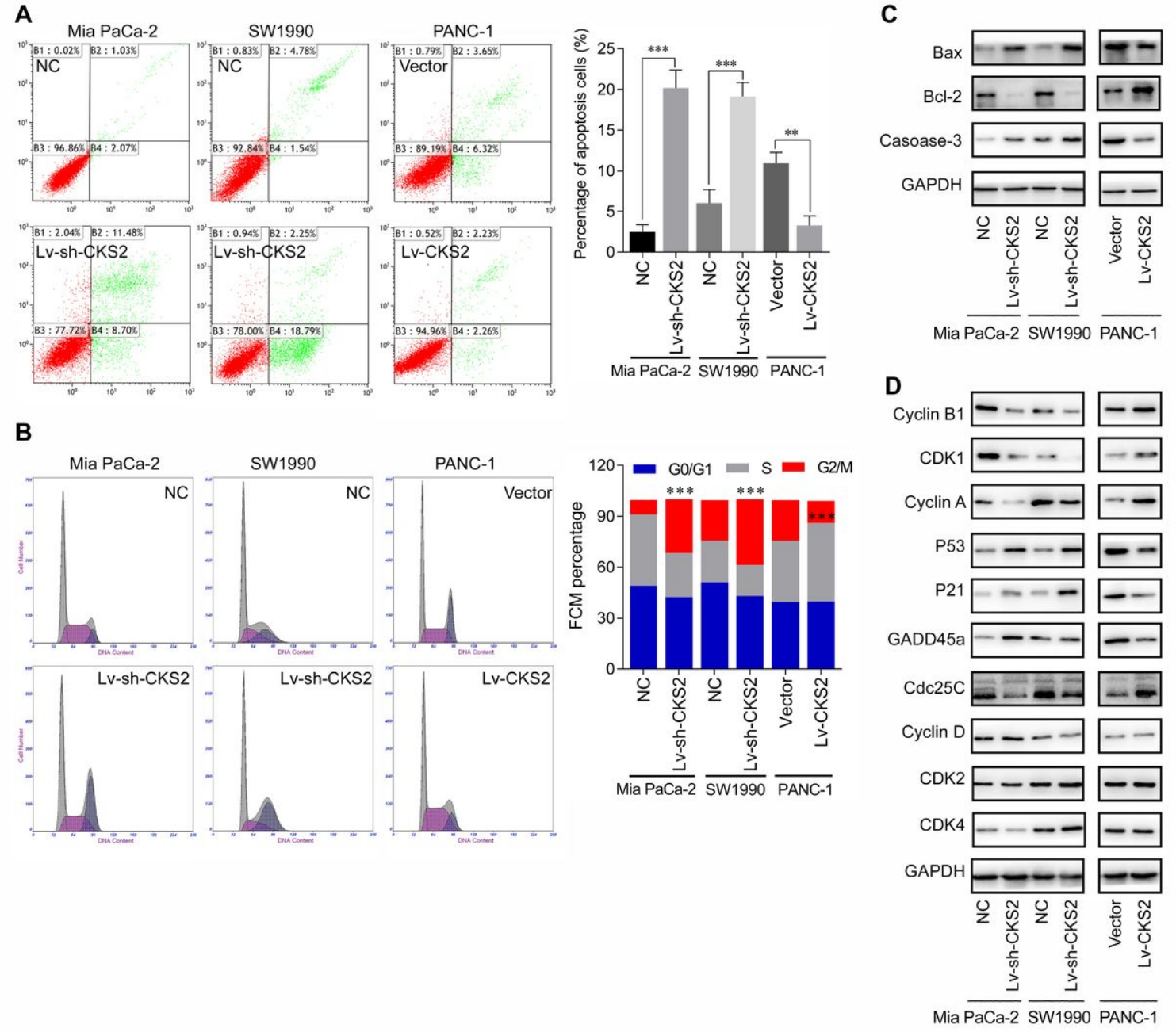

Figure 6

The effects of CKS2 on cell apoptosis and cell cycle distribution in pancreatic cancer cells. Mia PaCa-2 and SW1990 cells were transfected with lentivirus particles carrying Lv-sh-CKS2 or NC, PANC-1 cells were transfected with lentivirus particles carrying Lv-CKS2 or vector. (A) The percentage of apoptotic cells was determined in the above transfected cells. (B) Cell cycle distribution was measured in the above transfected cells. Data are presented as the means ± SD. **P < 0.01, ***P < 0.001, ****P < 0.0001,

compared with NC or Vector. (C) The protein expression of Bax, Bcl-2 and Caspase-3 was measured in the above transfected cells. (D) The protein expression of Bax, Bcl-2 and Caspase-3 was measured Cyclin B1, CDK1, Cyclin A, Cdc25C, P53, P21, GADD45α, Cyclin D, CDK2 and CDK4 was detected in the above transfected cells.

Supplementary Files

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