RESEARCH ARTICLE

CRKL protein overexpression enhances cell proliferation and invasion in pancreatic cancer

Lin Fu · Qianze Dong · Chengyao Xie · Yan Wang · Qingchang Li

Abstract CRKL is an adapter protein which is overexpressed in many malignant tumors and plays crucial roles in tumor progression. However, expression pattern and biological roles of CRKL in pancreatic cancer have not been examined. In the present study, we found that CRKL expression in pancreatic cancer specimens was higher than that in normal pancreatic tissues. Colony formation assay and Matrigel invasion assay showed that the overexpression of CRKL in Bxpc3 and Capan2 cell lines with low endogenous expression increased cell proliferation and invasion. Flow cytometry showed that CRKL promoted cell proliferation by facilitating cell cycle. Further analysis of cell cycle- and invasion-related molecules showed that CRKL upregulated cyclin D1, cyclin A, matrix metalloproteinase 2 (MMP2) expression, and phosphorylated extracellular signal (ERK)-regulated kinase. In conclusion, our study demonstrated that CRKL was overexpressed in human pancreatic cancers and contributed to pancreatic cancer cell proliferation and invasion through ERK signaling.

Keywords CRKL · Proliferation · Invasion · Pancreatic cancer

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the USA [1]. The prognosis of pancreatic cancer is poor with a 5-year survival rate of about 5 % [2–4]. The mechanism of its carcinogenesis and progression is still a blind spot. Thus, it is necessary to find reliable biomarkers predicting its biological aggressiveness.

CRKL (v-crk sarcoma virus CT10 oncogene homologue (avian)-like) is a member of the CRK family of adapter proteins, which plays a role in multiple biological processes, including cell proliferation, adhesion, and migration, as well as signal transduction [5]. CRKL was once thought to be as a key substrate and effective downstream molecule of BCR-ABL fusion protein, which mediates the inhibition of BCR-ABL gene-associated apoptosis in chronic myeloid leukemia [6–8]. Recently, CRKL protein had been shown to overexpress in a subset of malignant cancers [9]. CRKL is located at the center of the 22q11.21 amplicon, which was identified as one of the most commonly amplified regions in many cancers such as lung cancer, pancreatic cancer, and colorectal cancer [10–12]. The above findings suggest that CRKL may act as an oncogene in human cancers. However, little is known about the role of CRKL in pancreatic cancer progression. So it is necessary to investigate its expression pattern and functional significance in pancreatic cancer tissues and cell lines.

In our study, we demonstrated that CRKL expression was higher in pancreatic cancer tissues than in normal tissues. We also explored the role of CRKL on the proliferation and invasion of pancreatic cancer cells. In addition, we found that CRKL overexpression upregulated cyclin A, cyclin D1, p-extracellular signal (ERK), and matrix metalloproteinase 2 (MMP2), implicating the potential mechanism of CRKL on pancreatic cancer progression.

Materials and methods

Patients and specimens

This study was conducted with the approval of the Institutional Review Board at China Medical University. Primary tumor

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specimens were obtained from 116 patients who were diagnosed with pancreatic cancer and subsequently underwent complete surgical resections in the First Affiliated Hospital of China Medical University between 2004 and 2010. Follow-up information was obtained by reviewing patients' medical records. None of the patients received radiotherapy or chemotherapy before surgical resection, and all of the patients were treated with routine chemotherapy after the operation.

Immunohistochemistry

Surgically excised tumor specimens were fixed with 10 % neutralized formalin and embedded in paraffin, and 4-µm-thick sections were prepared. Normal bronchial epithelium present in the tumor slides was used as a control. Immunostaining was performed using the avidin-biotin-peroxidase complex method (Ultrasensitive TM, MaiXin, Fuzhou, China). The sections were deparaffinized in xylene, rehydrated with graded alcohol, and then boiled in 0.01 M citrate buffer (pH 6.0) for 2 min with an autoclave. Hydrogen peroxide (0.3 %) was applied to block endogenous peroxidase activity, and the sections were incubated with normal goat serum to reduce non-specific binding. Tissue sections were incubated with CRKL rabbit polyclonal antibody (1:150 dilution) (Millipore, USA). Mouse immunoglobulin (at the same concentration of the antigen-specific antibody) was used as a negative control. Staining for either antigen-specific or non-specific antibodies was performed at room temperature for 2 h. Biotinylated goat anti-mouse serum IgG was used as a secondary antibody. After washing, the sections were incubated with streptavidin-biotin complex conjugated with horseradish peroxidase, and then the peroxidase reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride. Counterstaining with hematoxylin was performed, and the sections were dehydrated in ethanol before mounting.

As indicated above, all the stained sections were evaluated independently by two pathologists. Five random fields of tumor were examined per slide, and 100 cells were evaluated per field under ×400 magnification. Immunostaining of CRKL was scored following a semiquantitative scale by evaluating representative tumor areas for staining intensity and percentage of positive cells. Nuclear and cytoplasmic immunostaining or staining in either location in tumor cells was considered to be positive. The staining intensity was categorized as follows: 0, negative; 1, weak; and 2, strong. The percentage of stained tumor cells was scored as follows: 0, 0 %; 1, 1–25 %; 2, 26–50 %; 3, 51–75 %; and 4, 76–100 %. The scores of each tumor sample were multiplied to give a final score of 0 to 8, and the tumor samples with a final score <4 were regarded as negative or weak staining; tumor samples with a final score of 4–8 were finally determined as CRKL overexpression. The median value of this series (35 % of positive cells) was used as a threshold value to distinguish tumors with low (<35 %) versus high (≥35 %) index of cell proliferation.

Cell culture and transfection

Capan2 and Bxpc3 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10 % fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 µg/ml streptomycin (Sigma-Aldrich). Cells were grown on sterile tissue culture dishes and were passaged every 2 days using 0.25 % trypsin (Invitrogen).

The plasmid of CRKL was purchased from OriGene. Plasmid was transfected into cells using Attractene Transfection Reagent (Qiagen, Hilden, Germany). Empty vector was used as a negative control. Cells were harvested 48 h later, and the protein levels of CRKL, cyclin D1, cyclin A, and others were assessed by Western blot analysis.

Western blot analysis

Total protein from cultured cells was extracted in cell lysis buffer (Pierce) and quantified using the Bradford method. Fifty micrograms of protein was loaded and separated on SDS-PAGE (12 %). After transferring to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), the membrane was incubated overnight at 4 °C with antibody against CRKL (1:500, Millipore), cyclin D1, cyclin A, cyclin B1, cyclin-dependent kinase (CDK)2, CDK4, CDK6 (1:1000, Cell Signaling, USA), or mouse monoclonal antibody against GAPDH (1:500, Santa Cruz Biotechnology, USA). Mouse monoclonal antibodies to MMP2 (1:1000, San Diego, CA) were purchased from Calbiochem (San Diego, CA). Antibodies against ERK1/2 (1:1000, Cell Signaling, Boston, MA, USA), phospho-ERK1/2 (1:1000, Cell Signaling, Boston, MA, USA), p38 MAPK (1:1000, Cell Signaling, Boston, MA, USA), phospho-p38 MAPK (1:1000, Cell Signaling, Boston, MA, USA), c-Jun N-terminal kinase (JNK; 1:1000, Cell Signaling, Boston, MA, USA), phospho-JNK (1:1000, Cell Signaling, Boston, MA, USA), and β-actin (1:1000, Santa Cruz) were obtained from Cell Signaling Technology. After incubation with peroxidase-conjugated antimouse IgG (Santa Cruz Biotechnology) at 37 °C for 2 h, bound proteins were visualized using ECL (Pierce) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). The relative protein levels were calculated by normalizing to GAPDH protein as a loading reference.

Quantitative real-time PCR (SYBR Green method)

Total RNA was extracted from cells using TRIzol Reagent (Qiagen). Reverse transcription of 1 μg of RNA was done using the high-capacity cDNA RT Kit (Applied Biosystems) following the manufacturer's instructions. Quantitative real-time PCR was done using SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 20 μl on 7900HT Fast Real-Time PCR System (Applied Biosystems) as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. The sequences of the primer pairs are as follows:

> CRKL forward, 5′ CCTTTGCCATCCACACAGAAT 3′,
> CRKL reverse, 5′ TTTCACGATGTCACCAACCTCTA 3′.
> β-actin forward, 5′ ATAGCACAGCCTGGATAGCAAC GTAC 3′,
> β-actin reverse, 5′ CACCTTCTACAATGAGCTGCGT GTG 3′.
> MMP2 forward, 5′-TGTGTTCTTTGCAGGGAATGAA T-3′,
> MMP2 reverse, 5′-TGTCTTCTTGTTTTTGCTCCAG TTA-3′.
> cyclin A forward, 5′GCAGAGGCCGAAGACGAGA 3′,
> cyclin A reverse, 5′ TCCAAGGAGGAACGGTGACA 3′.
> cyclin D1 forward, 5′ GCTGGAGGTCTGCGAGGA 3′,
> cyclin D1 reverse, 5′ ACAGGAAGCGGTCCAGGT AGT 3′.

A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. β-Actin was used as the reference gene. The relative levels of gene expression were represented as $\Delta Ct = Ct$ gene$-Ct$ reference, and the fold change of gene expression was calculated by the $2-\Delta\Delta Ct$ method. Experiments were repeated in triplicate.

Flow cytometry for cell cycle analysis

Cells (500,000) were seeded into 6-cm tissue culture dishes. Twelve hours later, cells were transfected with CRKL plasmid or empty vector. Forty-eight hours after transfection, cells were harvested, fixed in 1 % *para*-formaldehyde, washed with phosphate-buffered saline (PBS), and stained with 5 mg/ml propidium iodide in PBS supplemented with RNase A (Roche, Indianapolis, IN) for 30 min at room temperature. Data were collected using BD systems. One-parameter histogram was plotted according to the distribution of nuclear DNA content in each cell detected by a flow cytometer. Cells in each individual phase of the cell cycle were determined based on their DNA ploidy profile.

Colony formation assay

For the evaluation of colony formation, Capan2 and Bxpc3 cell lines were transfected with CRKL plasmid for 48 h before being seeded into three 6-cm cell culture dishes (1000 per dish) and incubated for 12 days. The plates were washed with PBS and then stained with Giemsa stain. The number of colonies with more than 50 cells was manually counted under the microscope.

Matrigel invasion assay

Thereafter, cells were planted into three 6-cm cell culture dishes (1000 per dish f) and incubated for 12 days. Plates were washed with PBS and stained with Giemsa stain. The number of colonies with more than 50 cells was counted.

Cell invasion assay was performed using a 24-well Transwell chamber with a pore size of 8 μm (Costar), and the inserts were coated with 20 μl Matrigel (1:3 dilution, BD Bioscience). Forty-eight hours after transfection, Bxpc3 and Capan2 cells were trypsinized and transferred to the upper Matrigel chamber in 100 μl of serum-free medium containing 3×105 cells and incubated for 18 h. Medium supplemented with 10 % FBS was added to the lower chamber as the chemoattractant. Negative controls without chemoattractant were poerformed. Then the non-invading cells on the upper membrane surface were removed with a cotton tip, and the cells passed through the filter were fixed with 4 % *para*formaldehyde and stained with hematoxylin. The experiments were performed in triplicate.

Statistical analysis

SPSS version 11.5 for Windows was used for all analyses. All *p* values were based on the two-sided statistical analysis and $p<0.05$ was considered to be statistically significant in difference.

Results

Expression and localization of CRKL in the cancer of pancreas

In order to investigate CRKL protein levels in pancreatic carcinoma, we used immunohistochemistry to examine a panel of 116 primary pancreatic cancer samples and

Fig. 1 Expression pattern of CRKL in pancreatic cancers. **a** Negative staining of CRKL in normal pancreatic tissue. **b** Negative staining of CRKL in pancreatic ductal carcinoma. **c** Moderate cytoplasmic staining of CRKL in pancreatic carcinoma. **d** Strong cytoplasmic and nuclear staining in pancreatic ductal carcinoma
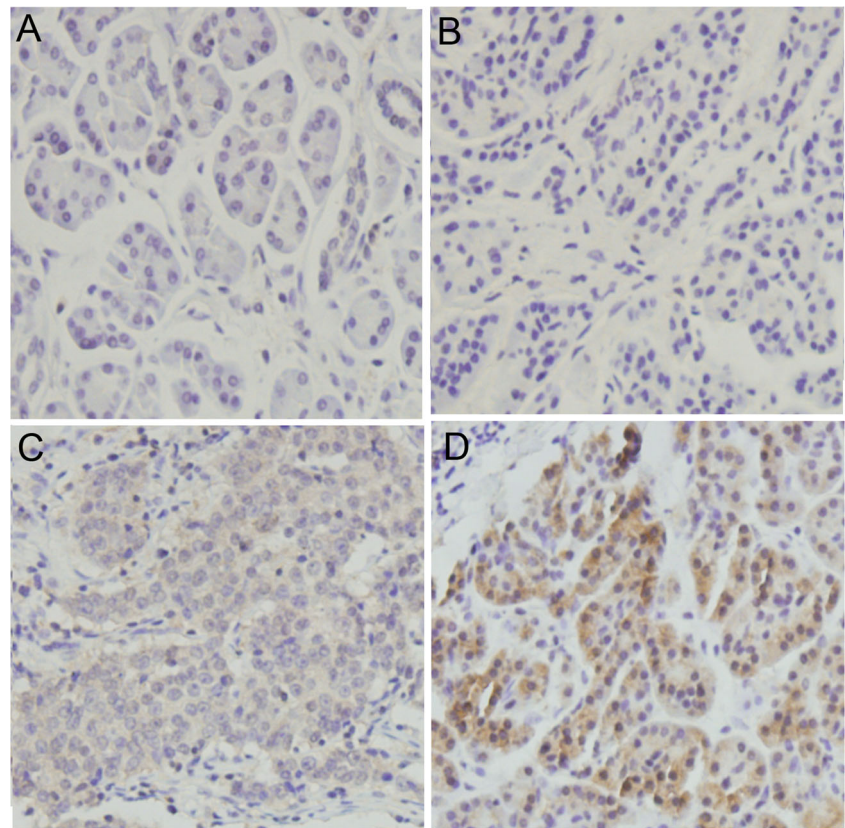
corresponding normal pancreatic tissues. The expression level of CRKL in pancreatic ductal carcinoma was higher than that in normal pancreatic tissues. Of 116 pancreatic patients, 45 (38.8 %) were scored as CRKL positive, which was localized in cytoplasmic and nuclear compartments of tumor cells (Fig. 1d). According to previous studies, strong or moderate cytoplasmic staining was considered as CRKL positive. Negative CRKL staining was detected in normal pancreatic tissue (Fig. 1).

Overexpression of CRKL induces cell proliferation and invasion in pancreatic cancer cell lines

We examined the expression pattern of CRKL protein in pancreatic cancer cell lines by Western blot. We found lower expression in Capan2 and Bxpc3 pancreatic cancer cell lines (Fig. 2a). To determine the role of CRKL in biological behavior of pancreatic cancer cells, transfection of CRKL plasmid was performed in these two cell lines. CRKL plasmid

Fig. 2 Expression pattern of CRKL in pancreatic cancer cell lines. Experiments were performed in triplicate. **a** Western blot and real-time PCR analysis of CRKL expression in pancreatic cancer cell lines. **b** Western blot and real-time PCR analysis showed that CRKL plasmid transfection markedly increased CRKL levels in Bxpc3 and Capan2 cells in comparison with empty vector
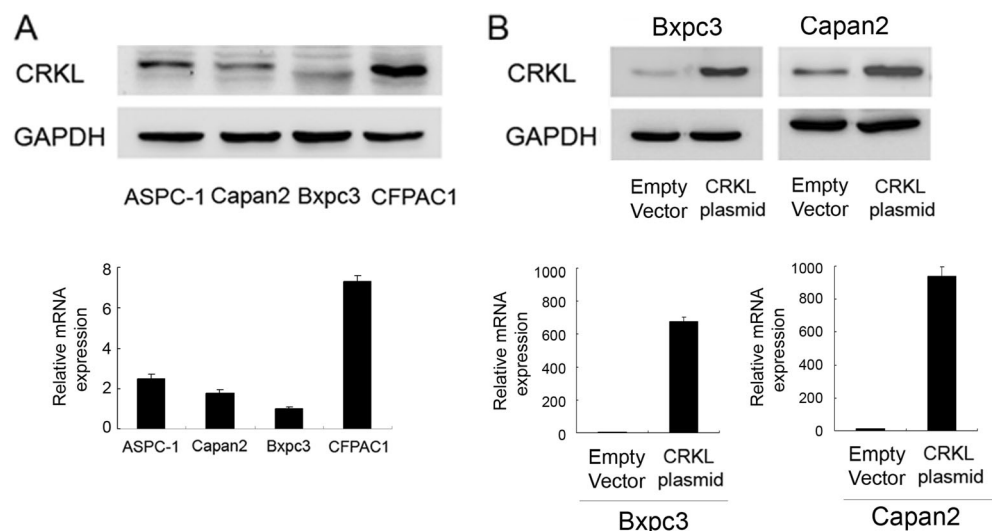
Fig. 3 CRKL overexpression enhanced pancreatic cancer cell proliferation and invasion. **a** Colony formation assay was performed in cells transfected with CRKL plasmid and negative control. Note that a significant increase was observed in the groups with CRKL plasmid compared with the control in colony formation. **b** Matrigel invasion assay showed a marked increase of invading ability in cells transfected with CRKL plasmid. Experiments were performed in triplicate and Student's *t* test was used for comparison
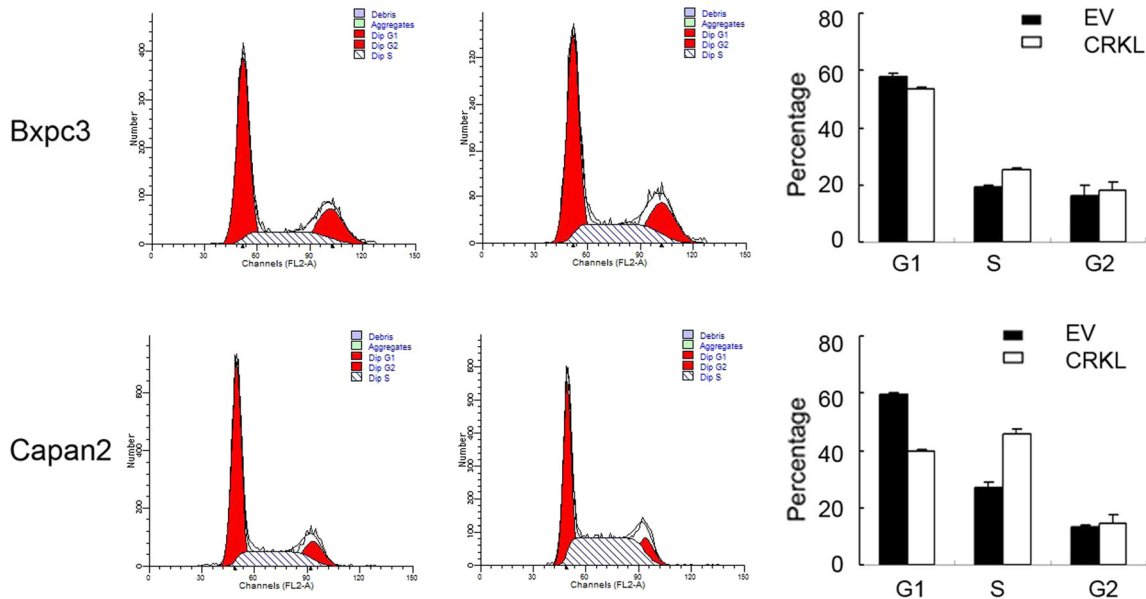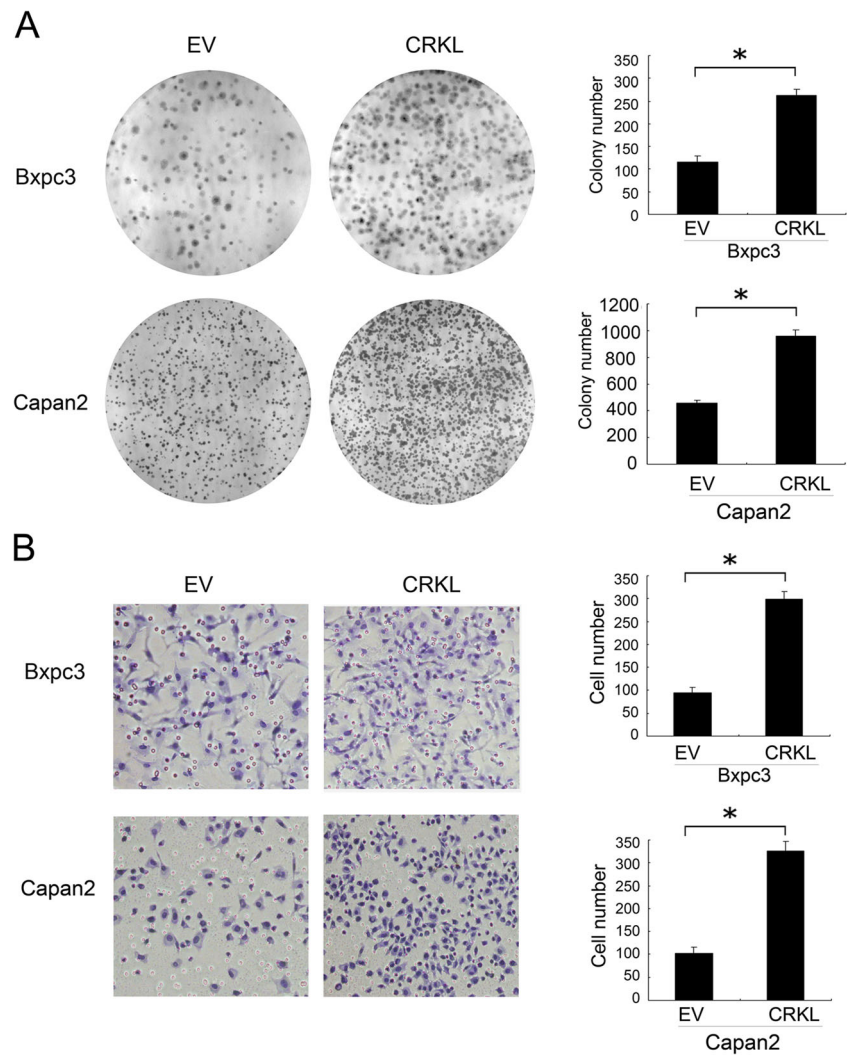

Fig. 4 Flow cytometry for cell cycle analysis after CRKL transfection. Cell cycle analysis was performed in triplicate and showed that CRKL transfection increased the percentage of S phase cells and decreased G1 percentage in both Bxpc3 and Capan2 cell lines

transfection considerably increased its protein and mRNA expression levels (Fig. 2b).

Colony formation assay was employed to characterize the role of CRKL on cell proliferation. We found that CRKL transfection caused an obvious increase in colony formation ability compared with empty vector in both cell lines (Bxpc3 EV vs CRKL: 116±12 vs 262±13; Capan2 EV vs CRKL: 456±23 vs 960±43) ($p<0.05$, Fig. 3a). Cell cycle analysis was employed to characterize the cell cycle status of Capan2 and Bxpc3 with CRKL overexpression. We found that the percentage of G1 phase in CRKL-transfected cells was decreased compared with that for the control groups (Bxpc3 EV vs CRKL: 57.5±1.5 vs 53.4±0.5; Capan2 EV vs CRKL: 59.8±0.6 vs 39.4±0.9), whereas the percentage of S phase was increased (Bxpc3 EV vs CRKL: 19.3±0.8 vs 25.2±0.8; Capan2 EV vs CRKL: 27±0.7 vs 45.7±0.5) (Fig. 4). These findings suggest that the upregulation of CRKL can promote cell proliferation by facilitating cell cycle progression in

pancreatic cancer cells. We further examined whether cell invasion capacity was altered. As shown in Fig. 3b, CRKL overexpression enhanced pancreatic cancer cell invasion in both cell lines using Matrigel invasion assay (Bxpc3 EV vs CRKL: 95±12 vs 299±16; Capan2 EV vs CRKL: 102±12 vs 326±20).

Overexpression of CRKL upregulates cyclin D1, cyclin A, MMP2 expression, and ERK phosphorylation in pancreatic cancer cells

To investigate the mechanism by which CRKL affected the pancreatic cancer proliferation and invasion, we examined the effect of CRKL transfection on the expression of several cell cycle- and invasion-related factors by Western blot. We revealed that the overexpression of CRKL increased the mRNA and protein expression level of cyclin D1, cyclin A, and MMP2 in both cell lines. We also examined the level of
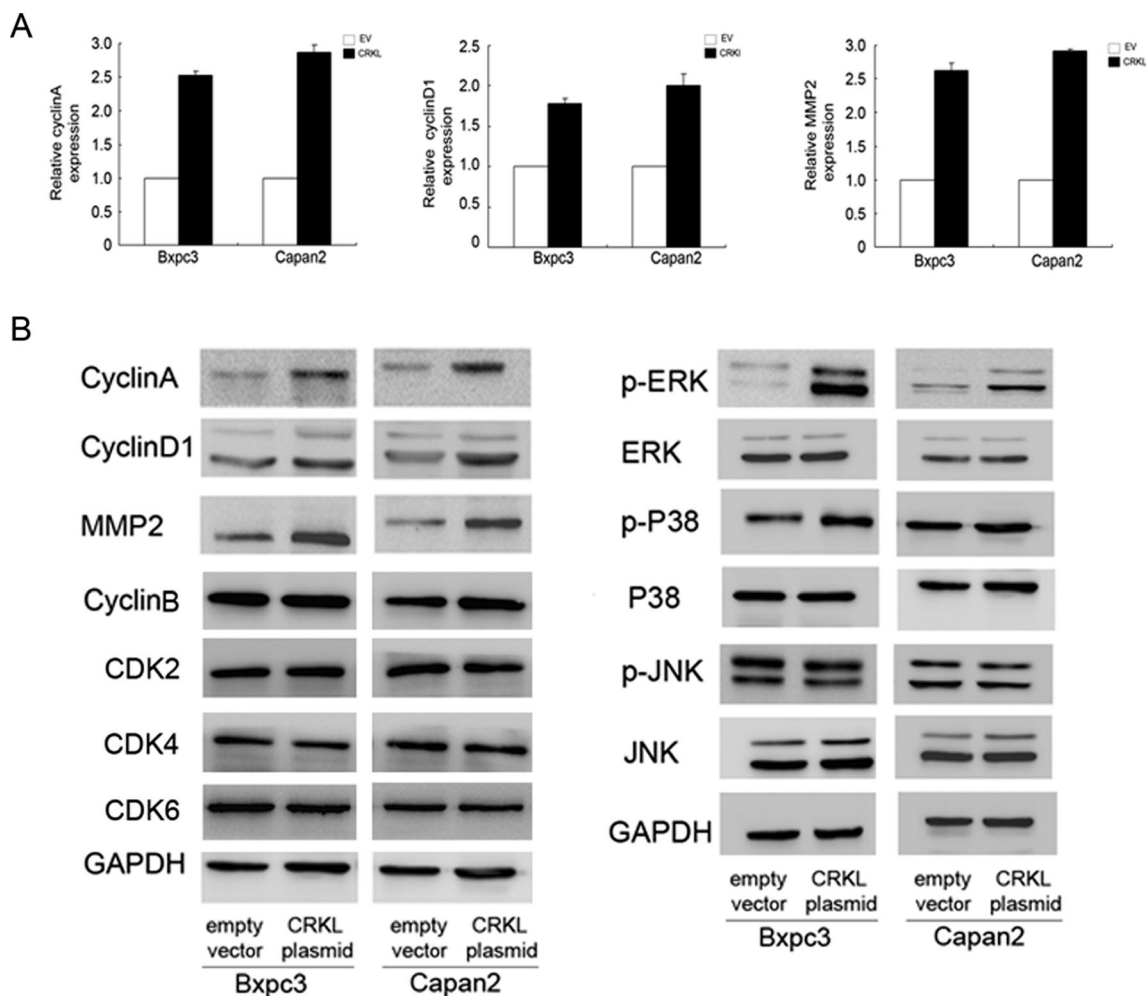

Fig. 5 CRKL overexpression upregulated the expression of cyclin D1, cyclin A, MMP2, and phosphorylated ERK. Experiments were performed in triplicate. **a** Real-time PCR analysis revealed that CRKL transfection increased the expression of cyclin D1, cyclin A, and MMP2. **b** Western blot analysis revealed that CRKL transfection increased the expression of cyclin D1, cyclin A, and MMP2. Activities of ERK1/2, p38 MAPK, and JNK were determined by Western blotting using antibodies specific for total and phosphorylated forms of ERK1/2, p38 MAPK, and JNK. The result showed that CRKL promoted ERK phosphorylation levels

ERK phosphorylation. We found that CRKL transfection led to the increase of ERK phosphorylation level, without a significant change of total ERK. These results suggest that overexpression of CRKL promotes pancreatic cancer cell proliferation and invasion, likely through the regulation of cyclin D1, cyclin A, MMP2, and ERK phosphorylation (Fig. 5).

Discussion

Pancreatic cancer is still one of the most common cancers in many areas of the world. Like other malignant tumors, mechanism for carcinogenesis and progression of pancreatic cancer still remains unclear. Previous studies provided important clues suggesting CRKL as an oncogene. High levels of CRKL gene amplification and protein overexpression were reported [13]. Furthermore, CRKL was also proved to be involved in the construction of tumor microenvironments and finally affected the origin and progression of tumor [14, 15]. However, until now, there was no report concerning its effect on pancreatic cancer. In this study, we performed experiments to identify whether CRKL is involved in pancreatic cancer and to explore its potential mechanism.

We performed immunohistochemistry to quantitatively detect the staining of CRKL protein in pancreatic cancer tissue. We found that IHC staining of CRKL was mainly localized in the cytoplasmic and nuclear compartments of cancer cells, which was in accord with previous reports [9]. The expression level of CRKL in pancreatic ductal carcinoma was higher than that in normal pancreatic tissues. There was a growing body of evidences suggesting that CRKL was involved in the carcinogenesis of solid tumors by regulating biological behavior, such as proliferation, differentiation, invasion, and apoptosis [16–18].

In order to assess the function of CRKL in pancreatic tumor progression, we established two CRKL overexpressed cell line models by a transient transfection of CRKL plasmid. Increased CRKL protein level indicated effective transfection. Our experiments showed that CRKL overexpression caused an obvious increase in the proliferation rate and invading ability. In addition, cell cycle analysis showed that the percentage of cells in S phase was increased in CRKL upregulated cells, suggesting that CRKL promotes cell growth by facilitating G1–S phase transition. We thereafter examined a series of cell cycle-related factors in the CRKL overexpressed cells and noted apparently upregulation of cyclin A and cyclin D1. It has been well documented that cyclin D1 was overexpressed in a variety of cancers, which regulates cell proliferation by controlling progression through the restriction point at the G1 phase of the cell cycle [23–26]. Cyclin A is expressed in S phase, low expression of which has been shown to be associated with cell cycle arrest [19]. These results correlated well with the cell cycle progression after CRKL overexpression. After examining the change of MMP family members, which was closely correlated with cell invasion, we found that the protein and RNA levels of MMP2 was increased. MMP2, which could degrade collagen IV, the major extracellular matrix (ECM) component of the basement membrane, is implicated in non-physiological tissue invasion. MMP2 facilitates tumor cell invasion and metastasis in pancreatic, lymph, ovarian, and colorectal cancers [20–23]. While the data in the present study suggested that CRKL influenced cell proliferation and migration via the regulation of cyclin A, cyclin D1, and MMP2 expression levels, the exact machinery by which CRKL modulates cyclin and MMPs needs to be elucidated in future studies. Like many other malignant tumors, abnormal cell signal transduction pathways are involved in pancreatic cancer development [24, 25]. CRKL was proved as a "switch" and adaptor of several signaling pathways [5, 6, 11, 26]. The MEK/ERK signaling pathway is one of the most commonly activated pathways in malignant human cancers and has been highlighted in a number of studies of invasiveness and metastasis [27, 28]. The role of CRKL activating MEK/ERK signaling was investigated in hematologic neoplasms, such as chronic myelogenous leukemia (CML), as well as solid tumors such as lung cancer [29, 30]. Furthermore, MMP family regulated pancreatic cancer cell invasion dependent on ERK signal pathway activation [31, 32]. In support of this, we demonstrated that CRKL overexpression promoted ERK phosphorylation level, without a significant change of total ERK.

In conclusion, this study demonstrated that CRKL is overexpressed in pancreatic cancer tissues. CRKL promotes pancreatic cancer cell proliferation, by facilitating cyclin D1- and cyclin A-mediated cell cycle progression and enhances cell invasion through MMP2 upregulation. Importantly, CRKL could induce ERK phosphorylation, suggesting that CRKL contributes malignant phenotype through ERK pathway. Given these findings, our study confirmed CRKL as a potential therapeutic target in human pancreatic cancer development.

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