CDC20 associated with cancer metastasis and novel mushroom-derived CDC20 inhibitors with antimetastatic activity

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Received November 14, 2018; Accepted March 26, 2019

DOI: 10.3892/ijo.2019.4791

Abstract. Aberrant expression of cell division cycle 20 (CDC20) is associated with malignant progression and poor prognosis in various types of cancer. The development of specific CDC20 inhibitors may be a novel strategy for the treatment of cancer with elevated expression of CDC20. The aim of the current study was to elucidate the role of CDC20 in cancer cell invasiveness and to identify novel natural inhibitors of CDC20. The authors found that *CDC20* knockdown inhibited the migration of chemoresistant PANC-1 pancreatic cancer cells and the metastatic MDA-MB-231 breast cancer cell line. By contrast, the overexpression of CDC20 by plasmid transfection promoted the metastasizing capacities of the PANC-1 cells and MCF-7 breast cancer cells. It was also identified that a triterpene mixture extracted from the mushroom *Poria cocos* (PTE), purified triterpenes dehydropachymic acid, and polyporenic acid C (PPAC) downregulated the expression of CDC20 in PANC-1 cells dose-dependently. Migration was also suppressed by PTE and PPAC in a dose-dependent manner, which was consistent with expectations. Taken together, the present study is the first, to the best of our knowledge, to demonstrate that CDC20 serves an important role in cancer metastasis and that triterpenes from *P. cocos* inhibit the migration of pancreatic cancer cells associated with CDC20. Further investigations are in progress to investigate the specific mechanism associated with CDC20 and these triterpenes, which may have future potential use as natural agents in the treatment of metastatic cancer.

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Key words: cell division cycle 20, cancer metastasis, triterpenes, *Poria cocos*

Introduction

Cell division cycle 20 (CDC20), which was first identified in yeast in 1973, is critical in cell cycle progression (1). It activates the anaphase-promoting complex/cyclosome, thus modulating mitotic exit through the proteasomal degradation of proteins (2-4). Aberrant expression of CDC20 is associated with malignant progression and poor prognosis in various types of cancer, including pancreatic ductal adenocarcinoma, gastric cancer, urothelial bladder cancer, astrocytoma, hepatocellular carcinoma, lung adenocarcinoma and oral squamous cell carcinoma (5-11). In addition, there is a significant correlation between a high expression of CDC20 and advanced tumor stage in carcinoma of the breast, colon, endometrium and prostate (12-14). Therefore, CDC20 may be a promising therapeutic target for combating human cancer.

The most widely investigated functions of CDC20 are associated with cell cycle, proliferation and apoptosis (15). For example, there is an increase in cell cycle arrest at the G2/M phase and a decrease in the proliferation of hepatocellular carcinoma cells transfected with *CDC20* small interfering (si)RNA (16). *CDC20* siRNA suppressed cell proliferation *in vitro* and growth of xenografted glioma cells in mice (17). Our previous study showed that a novel medicinal mushroom blend, ganodermanontriol, and a hydroxamic acid-derivative, 2-[benzyl-(2-nitro-benzenesulfonyl)-amino]-N-hydroxy-3-methyl-N-propyl-butyramide, suppressed the growth of breast cancer cells through the downregulation of CDC20 (18-20). Based on previous data, the focus of this study was on the novel function of CDC20 in cancer metastasis only.

Metastasis is the tendency of cancer cells to spread to distant organs, which is considered responsible for >90% of cancer-associated mortality (21-24). It involves a multi-step process including migration from primary tumors, invasion to

surrounding tissues and colonization in distant sites successfully (21,24). However, the functional roles of CDC20 involved in the process of cancer metastasis remain to be fully elucidated.

In the present study, it was demonstrated that *CDC20* knockdown inhibited the migration of chemoresistant PANC-1 pancreatic cancer cells and metastatic MDA-MB-231 breast cancer cells (25). By contrast, the overexpression of CDC20 by plasmid transfection promoted the metastasizing capacities of PANC-1 and non-metastatic MCF-7 breast cancer cells (26). An identified triterpene mixture extracted from the edible and medicinal mushroom *Poria cocos* (PTE), purified triterpenes dehydropachymic acid (DPA) and polyporenic acid C (PPAC), downregulated the expression of CDC20 in PANC-1 cells dose-dependently. Migration was also suppressed by PTE in a dose-dependent manner, which was consistent with expectations. Taken together, it was demonstrated for the first time, to the best of our knowledge, that CDC20 serves an important role in cancer metastasis and that triterpenes from *P. cocos* inhibit the migration of pancreatic cancer associated with CDC20.

Materials and methods

Cell culture and reagents. The PANC-1 human pancreatic cancer cell line and MDA-MB-231 and MCF-7 breast cancer cell lines, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml; all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 10% fetal bovine serum (FBS) from ATCC. DMSO was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Anti-CDC20 (cat. no. sc-13162) and anti-β-actin (cat. no. sc-47778) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-FLAG M2 antibodies were from Sigma-Aldrich (Merck KGaA; cat. no. F3165).

Extraction and purification. Triterpenes were prepared from the pulverized sclerotium of *P. cocos* (Fujian, China). The method of preparation and identification follows as reported in our previous study (27). The quantification of HPLC analysis demonstrated that PTE contained 55.7% pachymic acid (PA), 31.7% DPA and 4.1% PPAC. The PTE, PA, DPA and PPAC were dissolved in DMSO at a concentration of 50 mg/ml and 50 mM, respectively and then stored at -20˚C.

siRNA transfection. The PANC-1 or MDA-MB-231 cells were transfected with human CDC20 siRNA (cat. no. sc-156154) or control siRNA-A (cat. no. sc-37007) using siRNA Transfection Reagent (cat. no. sc-29528) from Santa Cruz Biotechnology, Inc. as previously described (27). *CDC20* siRNA was used at 0.2 μM and control siRNA-A was used at 0.9 μM. After 48 h at 37˚C of transfection, the cells were harvested and *CDC20* knockdown was verified by western blot analysis.

DNA transfection. The FLAG-tagged plasmid DNA with CDC20 and FLAG-tagged plasmid DNA were provided by Professor Michele Pagano (NYU School of Medicine, New York, NY, USA). The PANC-1 or MCF-7 cells were transiently transfected with FLAG-tagged plasmid DNA with *CDC20* or control FLAG-tagged plasmid DNA using Lipofectamine™ LTX Reagent according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Inc.). After 24 h of transfection, the cells were harvested and the overexpression of CDC20 was verified by western blot analysis.

Western blot analysis. The sub-confluent (80-90%) PANC-1 cells were treated with PTE (30 and 60 μg/ml), PA (30 and 60 μM), DPA (30 and 60 μM), or PPAC (30 and 60 μM) for 24 h at 37˚C. Whole protein extracts isolated from cells were prepared using radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA). A total of 25 μg protein per lane was separated on gels and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% bovine serum albumin (cat. no. A7906; Sigma-Aldrich; Merck KGaA) in Tris-buffered saline buffer with Tween-20 (TBTS) buffer for 1 h at room temperature, followed by the incubation of the anti-CDC20, anti-FLAG and anti-β-actin antibodies (diluted 1:100) overnight at 4˚C as previously described (28). The PVDF membrane was then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies (cat. no. NA-931; diluted 1:5,000; Amersham Biosciences, Buckinghamshire, UK) for 1 h at room temperature and protein expression was visualized by the ECL Plus Western Blotting Detection system (Amersham Biosciences). The western blots were scanned with HP Scanjet 5470c scanner (Hewlett Packard, Palo Alto, CA, USA) and the optical densities of proteins were quantified with UN-SCAN-IT software (version 7.0; Silk Scientific, Inc., Orem, UT, USA).

Cell migration assay. Cell migration of the PANC-1 cells treated with PTE (30 and 60 μg/ml) or PPAC (30 and 60 μM) was assessed in Transwell chambers according to an established method (29). The PANC-1 cells ($0.2x10^6$) suspended in serum-free medium were added to the upper chamber of an insert, and the insert was placed in a 24-well plate containing medium with 10% FBS. The migration assays were performed for 24 h at 37˚C. Data points represent the mean ± standard deviation (SD) of three individual filters within one representative experiment repeated at least twice. The changes in cell migration in the different cell lines were examined. For the metastatic MDA-MB-231 breast cancer cell line, migration can be evaluated following 3 h of incubation. For the migration of the non-metastatic MCF-7 human breast cancer cell line, incubation for 24 h is necessary (29).

Cell viability. Cell viability was determined following incubation with PTE (30 and 60 μg/ml) or PPAC (30 and 60 μM) for 24 h by staining with Trypan blue (0.4%) at 22˚C for 5 min. The cells were then viewed using an inverted light microscope at a magnification of x40, as previously described (30). This method is used to assess cytotoxicity in a variety of cell lines. Data are presented as the mean ± SD from three independent experiments.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± SD. Statistical

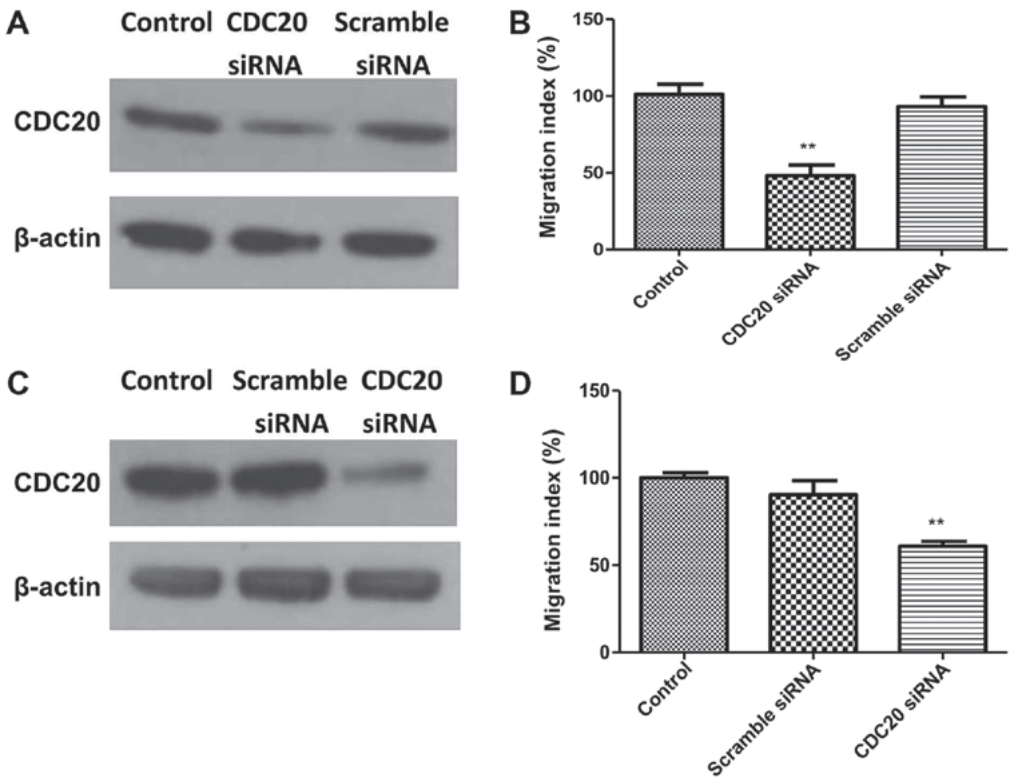

Figure 1. *CDC20* knockdown inhibits the migration of chemoresistant pancreatic cancer and metastatic breast cancer cells. PANC-1 cells and MDA-MB-231 cells were transfected with scrambled siRNA or *CDC20* siRNA. (A) After 48 h of transfection, western blot analysis of CDC20 in PANC-1 cells was evaluated. (B) Migration of PANC-1 cells in Transwell chambers. (C) After 48 h of transfection, western blot analysis of CDC20 in MDA-MB-231 cells was evaluated. (D) Migration of MDA-MB-231 cells in Transwell chambers. Each bar represents the mean ± standard deviation of three individual filters within one representative experiment repeated at least twice. **P<0.05 vs. Control. CDC20, cell division cycle 20; siRNA, small interfering RNA.

comparisons were performed using one-way analysis of variance with the significance level adjusted to P<0.05 using repeated t-tests with the Bonferroni correction.

Results

CDC20 knockdown inhibits the migration of chemoresistant pancreatic cancer and metastatic breast cancer. To evaluate whether CDC20 was involved in the process of cancer metastasis, *CDC20* was silenced with siRNA as described above. As shown in Fig. 1A and B, the knockdown of *CDC20* effectively suppressed the migration of chemoresistant PANC-1 pancreatic cancer cells by >40%. In the metastatic MDA-MB-231 breast cancer cell line, ~40% of cell migration was significantly inhibited by *CDC20* knockdown (Fig. 1C and D). These results indicate that CDC20 is an important target of cancer metastasis. However, the use of additional siRNAs is planned in future investigations to assess whether the inhibitory effects of siRNA CDC20 are not caused by the off-target effect.

Overexpression of CDC20 promotes the metastasizing capacities of pancreatic and breast cancer. In order to gain further insight into the functional role of CDC20 associated with cancer metastasis, FLAG-tagged plasmid DNA with *CDC20* was transfected into the PANC-1 cell line and MCF-7 breast cancer cell line, respectively, to induce the overexpression of CDC20. Induction of the expression of CDC20 markedly promoted the migration of PANC-1 and MCF-7 cells compared with the control (Fig. 2A-D). By contrast, transfection with FLAG-tagged plasmid DNA had no effect on cell migration, showing that promotion of the metastasizing capacities of pancreatic and breast cancer was closely associated with CDC20. The use of a mutant CDC20 expression vector is planned in future investigations to further confirm the crucial role of CDC20 in cell migration.

Triterpenes from P. cocos downregulate the expression of CDC20 in chemoresistant pancreatic cancer dose-dependently. As it is suggested that CDC20 serves an important role in cancer metastasis, the present study aimed to identify novel CDC20 inhibitors from natural compounds. The PANC-1 cells were treated with PTE (30 and 60 μg/ml), a triterpene mixture extracted from *P. cocos*, or three triterpenes PA, DPA and PPAC (30 and 60 μM), which were purified from PTE, for 24 h. Western blot analysis was then performed. As shown in Fig. 3, PTE suppressed the expression of CDC20 in PANC-1 cells dose-dependently. Among the purified triterpenes, PPAC was the most effective compound in downregulating the expression of CDC20, and DPA exerted moderate inhibition at a high dose. However, PA had no effect on the expression of CDC20. Different experimental methods resulted in different expression levels of CDC20 in the control PANC-1 cells. In Fig. 1, the endogenous expression of CDC20 was determined by CDC20 antibody. In Fig. 2, the overexpression of CDC20 was determined by FLAG antibody in cells which were transiently transfected with FLAG-CDC20 plasmid DNA.

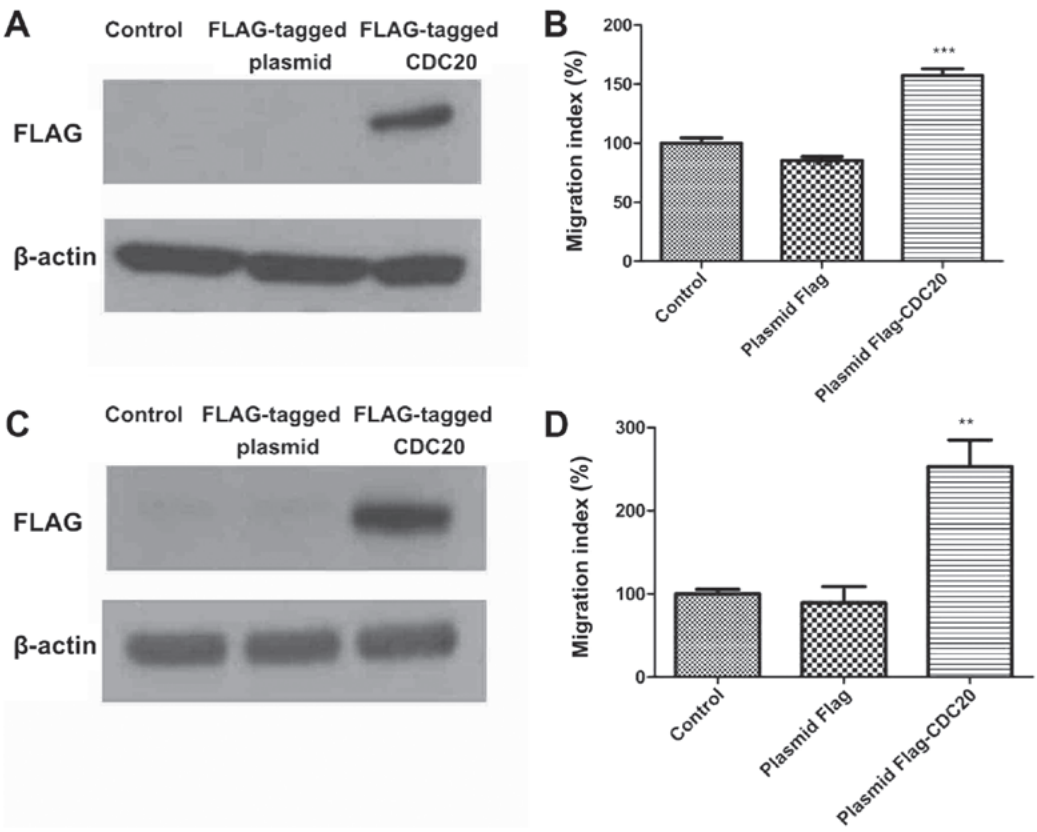

Figure 2. Overexpression of CDC20 promotes the migration of chemoresistant pancreatic cancer and breast cancer. PANC-1 cells and MCF-7 cells were transfected with FLAG-tagged plasmid DNA with *CDC20* or FLAG-tagged plasmid DNA. (A) After 24 h of transfection, western blot analysis of CDC20 in PANC-1 cells was performed. (B) Migration of PANC-1 cells in Transwell chambers. (C) After 24 h of transfection, western blot analysis of CDC20 in MCF-7 cells was performed. (D) Migration of MCF-7 cells in Transwell chambers was determined. Each bar represents the mean ± standard deviation of three individual filters within one representative experiment repeated at least twice. **P<0.05 and ***P<0.001 vs. Control. CDC20, cell division cycle 20.

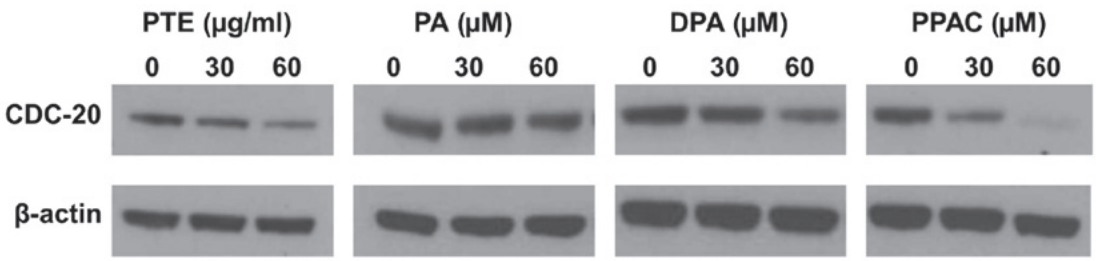

Figure 3. Effect of triterpenes from *P. cocos* on the expression of CDC20 in chemoresistant pancreatic cancer. PANC-1 cells were treated with PTE (30 and 60 μg/ml), or PA (30 and 60 μM), DPA (30 and 60 μM) and PPAC (30 and 60 μM), three triterpenes purified from PTE for 24 h, respectively. Whole protein extracts isolated from cells were prepared and western blot analysis with anti-CDC20 and anti-β-actin antibodies was performed. β-actin was used as a loading control. Representative images are shown. Similar results were obtained in at least two additional experiments. *P. cocos*, *Poria cocos*; CDC20, cell division cycle 20; PTE, triterpene mixture extracted from *P. cocos*; PA, pachymic acid; DPA, purified triterpenes dehydropachymic acid; PPAC, polyporenic acid C.

Therefore, the same cell line can exhibit different expression levels of CDC20.

Triterpenes from P. cocos suppresses the migration of chemoresistant pancreatic cancer in a dose-dependent manner. To determine whether the suppression of CDC20 by triterpenes from *P. cocos* is associated with the metastasizing capacities in chemoresistant pancreatic cancer, the PANC-1 cells were treated with PTE (30 and 60 μg/ml) or PPAC (30 and 60 μM) for 24 h and cell migration was evaluated, as described above. In accordance with expectations, PTE (Fig. 4A and B) and PPAC (Fig. 5A and B) significantly inhibited the migration of the chemoresistant PANC-1 pancreatic cancer cells in a dose-dependent manner. The inhibition of cell migration was not caused by the toxic effects of the tested triterpenes, since PTE or PPAC did not affect the viability of PANC-1 cells (Figs. 4C and 5C). These results further demonstrated that triterpenes from *P. cocos* inhibited the migration of pancreatic cancer associated with CDC20. Although cell motility analysis in Transwell chambers is an established method to asses cell migration, a scratch assay and animal experiments will be performed in future experiments to verify these results *in vitro* and *in vivo* and to reduce the limitation of the present study.

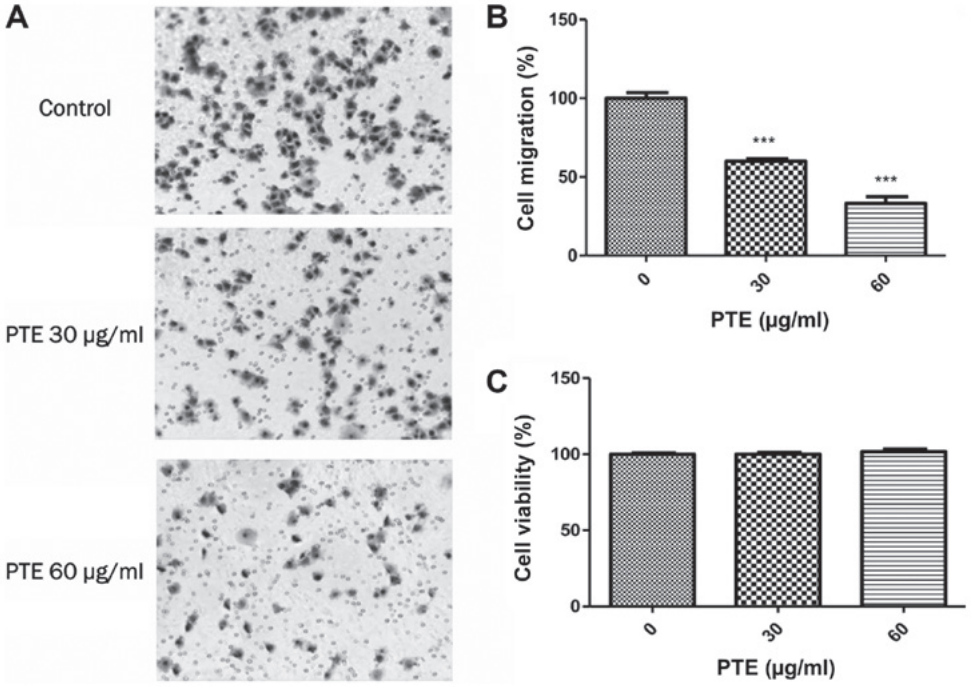

Figure 4. Effect of PTE on the migration of chemoresistant pancreatic cancer cells. PANC-1 cells were treated with PTE (30 and 60 μg/ml) and cell migration in Transwell chambers was assessed. (A) Representative images of cell migration. Magnification, x40. (B) Quantification of migration; each bar represents the mean ± SD of three individual filters within one representative experiment repeated at least twice. (C) Cell viability was determined; each bar represents the mean ± SD of three experiments. Similar results were obtained in three independent experiments. ***P<0.001 vs. 0 PTE. PTE, triterpene mixture extracted from *Poria cocos*; SD, standard deviation.

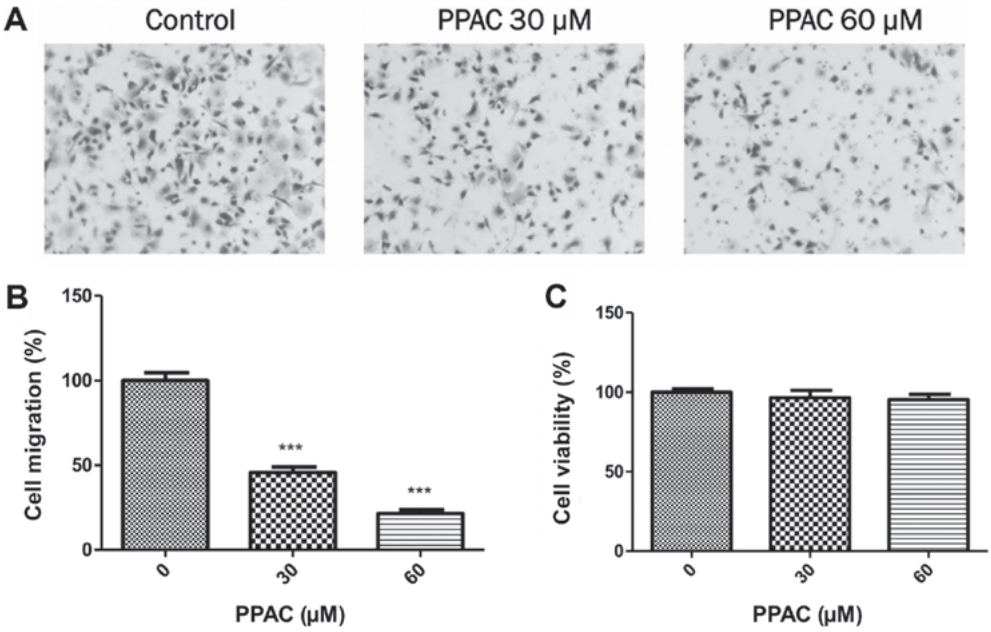

Figure 5. Effect of PPAC on the migration of chemoresistant pancreatic cancer. PANC-1 cells were treated with PPAC (30 and 60 μM) and cell migration in Transwell chambers was determined. (A) Representative images of cell migration are shown. Magnification, x40. (B) Quantification of migration; each bar represents the mean ± SD of three individual filters within one representative experiment repeated at least twice. (C) Cell viability was determined; each bar represents the mean ± SD of three experiments. Similar results were obtained in three independent experiments. ***P<0.001 vs. 0 PPAC. PPAC, polyporenicacid C; SD, standard deviation.

Discussion

A high expression of CDC20, a key component of the spindle assembly checkpoint, has been reported in various malignancies and serves a vital role in tumorigenesis and progression (13). It is reported that the silencing of *CDC20* suppresses metastatic castration-resistant prostate cancer growth and enhances chemosensitivity to docetaxel (31). Furthermore, the overexpression of CDC20 enhances cell proliferation and invasion in pancreatic cancer cells (32). The

present study indicates for the first time, to the best of our knowledge, that *CDC20* knockdown inhibited migration, a key component of the tumor metastatic process, in chemoresistant pancreatic cancer cells and metastatic breast cancer cells. By contrast, the overexpression of CDC20 by plasmid transfection promoted the metastasizing capacities of these cells. These results suggest that CDC20 is a critical regulator of cancer metastasis. The underlying molecular mechanism of CDC20 may be associated with the tumor suppressor scaffold matrix attachment region binding protein 1 (SMAR1). Paul *et al* indicated that CDC20 is responsible for maintaining the cellular levels of SMAR1 in higher grades of cancer and that the CDC20-mediated proteasomal degradation of SMAR1 promotes cell migration and invasion (12).

Based on a number of studies, the development of specific CDC20 inhibitors may be a novel strategy for the treatment of cancer with elevated expression of CDC20 (33). Curcumin, a polyphenol derived from the *Curcuma longa* plant, exhibits its anticancer function through the inhibition of CDC20 in pancreatic cancer cells (32). *P. cocos* is an edible and medicinal mushroom of the Polyporaceae family, which is widely used as nutritious food, dietary supplements and traditional medicine in Asia (34,35). Increasing experimental evidence suggests that triterpenes isolated from *P. cocos* exert direct anticancer effects through a variety of mechanisms, including the inhibition of cell proliferation, induction of apoptosis and suppression of invasive behavior (36-40). In our previous study, PTE, PA, DPA and PPAC were found to inhibit the growth of pancreatic cancer cells in a dose-dependent manner. In addition, PTE and PA significantly suppressed the invasive behavior of the BxPc-3 pancreatic cancer cell line by inhibiting the expression of matrix metalloproteinase-7 (27). PA also suppresses growth and induces the apoptosis of chemotherapy-resistant pancreatic cancer cells *in vitro* and *in vivo* by targeting endoplasmic reticulum stress (41). In the present study, it was observed that PTE, DPA and PPAC downregulated the expression of CDC20 in chemoresistant pancreatic cancer cells dose-dependently. Migration was suppressed by PTE in a dose-dependent manner, which was in accordance with expectations.

In conclusion, the results of the present study indicate that CDC20 serves an important role in cancer metastasis and that triterpenes from *P. cocos* inhibit the migration of pancreatic cancer associated with CDC20. Further investigations are in progress to investigate the specific mechanism associated with CDC20 and these triterpenes, which may have potential for use as natural agents in the treatment of metastatic cancer.

Acknowledgements

The authors thank Professor Michele Pagano (NYU School of Medicine) for providing FLAG-tagged plasmid DNA with CDC20 and FLAG-tagged plasmid DNA, Dr Hui Xu (China Pharmaceutical University) for formatting figures and Mr. Petr Sliva (IU School of Medicine) for English editing.

Funding

This study was supported by grants from the National Key R&D Program of China (grant no. 2017YFD0400203), the National Natural Science Foundation of China (grant no. 31701595) and the Natural Science Foundation of Jiangsu Province (grant no. BK20160750).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SC performed and analyzed experiments, and wrote the manuscript. VC performed experiments. DS designed experiments, analyzed data and edited final version of the 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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