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DNA-PKcs is important for Akt activation and gemcitabine resistance in PANC-1 pancreatic cancer cells

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ABSTRACT

Pancreatic cancer is one of the most aggressive human malignancies with extremely poor prognosis. The moderate activity of the current standard gemcitabine and gemcitabine-based regimens was due to pre-existing or acquired chemo-resistance of pancreatic cancer cells. In this study, we explored the potential role of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in gemcitabine resistance, and studied the underlying mechanisms. We found that NU-7026 and NU-7441, two DNA-PKcs inhibitors, enhanced gemcitabine-induced cytotoxicity and apoptosis in PANC-1 pancreatic cancer cells. Meanwhile, PANC-1 cells with siRNA-knockdown of DNA-PKcs were more sensitive to gemcitabine than control PANC-1 cells. Through the co-immunoprecipitation (Co-IP) assay, we found that DNA-PKcs formed a complex with SIN1, the latter is an indispensable component of mammalian target of rapamycin (mTOR) complex 2 (mTORC2). DNA-PKcs–SIN1 complexation was required for Akt activation in PANC-1 cells, while inhibition of this complex by siRNA knockdown of DNA-PKcs/SIN1, or by DNA-PKcs inhibitors, prevented Akt phosphorylation in PANC-1 cells. Further, SIN1 siRNA-knockdown also facilitated gemcitabine-induced apoptosis in PANC-1 cells. Finally, DNA-PKcs and p-Akt expression was significantly higher in human pancreatic cancer tissues than surrounding normal tissues. Together, these results show that DNA-PKcs is important for Akt activation and gemcitabine resistance in PANC-1 pancreatic cancer cells.

1. Introduction

Pancreatic cancer is one of the most aggressive human malignancies with a 5-year overall survival less than 5%. It is characterized by rapid disease progression without specific symptoms. Thus, early diagnosis and curative treatments are almost impossible, and the prognosis of pancreatic cancer is very low [1,2]. The fast majority of pancreatic cancer patients present in advanced stages, with only a minority (10–20%) being amenable to surgical intervention [3–5]. Chemotherapy has become an essential adjunct for pancreatic cancer treatment [6].

Currently, gemcitabine is only approved chemo-drug for pancreatic cancer [7]. However, pancreatic cancer is among the most intrinsically resistant tumors to almost all-known chemo-drugs including gemcitabine. The limitation of conventional chemotherapy appears mainly due to the profound chemo-resistance of pancreatic cancer cells towards all known anti-cancer drugs [8]. Recent pre-clinical and clinical studies have demonstrated that gemcitabine adjuvant therapy could significantly enhance median survival [9]. A number of factors contribute to the chemo-resistance, in this study, we explored the potential role of DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs) in it.

The serine/threonine protein kinase complex DNA-PK is composed of a 460-kDa catalytic subunit (DNA-PKcs) and the Ku hetero-dimer (Ku-70 and Ku-80) [10,11]. DNA-PKcs belongs to phosphatidylinositol-3-kinase (PI3K)-like protein kinase (PIKK) family and is one main kinase that is activated following DNA damages [12,13]. Its main function is to activate non-homologous end joining (NHEJ) pathway to repair DNA double-strand breaks [10,11]. Recent studies have implicated the important role of DNA-PKcs in cancer progression [14–20]. For example, DNA-PKcs protein expression correlates with resistance to etoposide in human chronic lymphocytic leukemia samples [19]. DNA-PKcs inhibitors were shown to enhance the cytotoxicity induced by radiation and a number of chemo-drugs (i.e., etoposide and

Abbreviations: MTT, 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; mTOR, mammalian target of rapamycin; mTORC2, mTOR complex 2; PI, propidium iodide; PI3K, phosphatidylinositol-3-kinase; SIN1, SAPK-interacting protein 1.

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doxorubicin) *in vivo* and *in vitro* [16,17,20,21]. However, the expression of DNA-PKcs in pancreatic cancer and its role in gemcitabine resistance have not been extensively studied.

2. Materials and methods

2.1. Chemicals and reagents

NU-7026 and NU-7441 were purchased from Calbiochem (San Diego, CA). Annexin V apoptosis kit was obtained from Promega (Madison, WI). 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) dye was purchased from Sigma (St. Louis, MO). Antibodies again Akt (1/2) and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho(p)-Akt (Ser 473), DNA-PKcs and SIN1 were purchased from Cell Signaling Technology (Denver, MA).

2.2. Cell culture

PANC-1 pancreatic cancer cell line was purchased from Shanghai Institute of Biological Science, Chinese Academy of Science (Shanghai, China). PANC-1 cells were cultured in RPMI medium (Invitrogen, Shanghai, China), supplemented with 10% fetal bovine serum (FBS, Invitrogen) with antibiotics in a CO_2 incubator at 37 °C.

2.3. Cell viability assay (MTT assay)

The cell viability was measured by the MTT assay. Twenty micro-liter (20 μl) of MTT tetrazolium salt (Sigma, St. Louis, MO) dissolved in PBS at a concentration of 5 mg/ml was added to PANC-1 cells after treatment, and incubated in CO_2 incubator for 2 h. The medium was then aspirated very carefully. Afterwards, 150 μl of DMSO (Sigma, St. Louis, MO) was then added to dissolve formazan crystals, the absorbance of each well was obtained using a plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm. The optical density (OD) value of treatment group was always normalized to that of control group.

2.4. Flow cytometry assay of cell apoptosis

After treatment, PANC-1 cells were washed and incubated in 500 μl binding buffer, 5 μl annexin V-FITC and 5 μl of propidium iodide (PI) (Invitrogen, Karlsruhe, Germany) at room temperature for 15 min under the dark. Cells were then detected through fluorescence-activated cell sorting (FACS) with a Becton-Dickinson machine (San Jose, CA). Annexin V positive cells were labeled as apoptotic cells, and its percentage was detected as apoptosis rate.

2.5. Caspase-3 activity assay

The cytosolic proteins from approximately 2×10^6 PANC-1 cells were extracted in hypotonic cell lysis buffer (25 mm HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 0.05% phenylmethylsulfonyl fluoride). The protein concentration of each sample was determined by the Bio-Rad Bradford protein assay kit (Bio-Rad, Shanghai, China). Ten micrograms of cytosolic extracts were added to caspase assay buffer (312.5 mM HEPES, pH 7.5, 31.25% sucrose, 0.3125% CHAPS) with benzyloxycarbonyl-DEVD-7-amido-4-(trifluoromethyl) coumarin as substrates (Calbiochem, Darmstadt, Germany). Release of 7-amido-4-(trifluoromethyl)-coumarin (AFC) was quantified, after 2 h of incubation at 37 °C, using a Fluoroskan system set to an excitation value of 355 nm and emission value of 525 nm. OD value was detected as an indicator of caspase-3 activity.

2.6. Western blots

After treatment, cell lysates were extracted from PANC-1 cells with a RIPA lysis buffer. Protein concentration was determined by Bio-Rad protein concentration assay (Sigma). Aliquots of 30 μg of lysates were electro-phoresed on 10% SDS–PAGE gel and transferred to PVDF membranes. The blots were then incubated with primary antibodies at 4 °C overnight. The appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotech, Santa Cruz, CA) were then added. Antigen-antibody complex was detected by enhanced chemiluminescence (ECL) reagent. Each condition was carried out in triplicate. The total gray of each indicated band was quantified using Image J software (NIH), which was then normalized to that of the corresponding loading control, the value was expressed as fold change vs. that of the untreated control group (labeled with "1.0").

2.7. Co-immunoprecipitation (Co-IP)

PANC-1 cells were lysed with lysis buffer containing 0.3% CHAPS and protease inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Aliquots of 1000 μg of proteins from each sample were pre-cleared by incubation with 25 μl of protein A/G Sepharose (Sigma, Shanghai, China) for 1 h at 4 °C. Pre-cleared samples were incubated with anti-SIN1 antibody (1 μg/sample) in lysis buffer (1000 μl) overnight at 4 °C. To this was added 350 μl of protein A/G beads and the samples were incubated for 4 h at 4 °C. The beads were then washed 5 times with cold PBS and once with lysis buffer, boiled, separated by 10% SDS–PAGE, and transferred onto a PVDF membrane followed by Western blot analysis.

2.8. SiRNA

DNA-PKcs-siRNA-#1 (Santa Cruz, sc-35200h) and DNA-PKcs-siRNA-#2 (leading strand: GAUCGCACCUUACUCUGUUdTdT Dharmacon Research Inc [18]) were gifts from Dr. He's Lab [14]. Scramble siRNA and SIN1 (MAPKAP1) siRNA (sc-60984) were also purchased from Santa Cruz Biotech. PANC-1 cells were seeded in a six-well plate with 50–60% confluence. For transfection, 3.0 μl PLUS™ Reagent (Invitrogen, Carlsbad, CA) was diluted in 90 μl of RNA dilution water (Santa Cruz, CA) for 5 min at room temperature. Then, 0.2 nmol of targeted siRNA or scramble siRNA was added and left for 5 min at room temperature. To this was added 3.0 μl of Lipofectamine (Invitrogen) and incubation for another 15 min at room temperature. Afterwards, the transfection complex was added to the cell well with 1.0 ml of medium (no antibiotics, no FBS). Cells were then cultured for additional 48 h. SiRNA efficiency was determined by Western blots.

2.9. Human pancreatic cancer tissues isolation

Human pancreatic cancer tissues and surrounding control tissues from patients with primary diseases were obtained at the time of surgery. Fresh tissues were thoroughly washed in PBS containing 100 units/mL penicillin–streptomycin and 2 mM DTT to remove debris, and then minced by scalpel into small pieces into DMEM plus 10% FBS containing 100 units/mL penicillin–streptomycin. Tissues were then lysed using tissue Western blot lysis buffer, and analyzed by Western blots. All patients enrolled provided individual informed consent with institutional review board approval of all protocols. The experiments conformed to the principles set out in the Declaration of Helsinki and the NIH Belmont Report.

2.10. Statistical analyses

The data were expressed as means ± standard deviation (SD). Data were collected using three set of independent experiments.

Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 16). Values of $p < 0.05$ were considered statistically significant. The significance of any differences between two groups was tested using paired-samples *t* test when appropriated.

3. Results

3.1. DNA-PKcs inhibitors sensitize gemcitabine-induced cytotoxicity against PANC-1 pancreatic cancer cells

In this study, we tested the potential role of DNA-PKcs in gemcitabine-induced cytotoxicity in pancreatic cancer cells. MTT [3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide] cell viability results in Fig. 1A showed that gemcitabine at concentration of 0.5–2.5 μM inhibited PANC-1 cell survival. Significantly, co-administration with DNA-PKcs inhibitors NU-7026 [22] and NU-7441 [23] enhanced gemcitabine-induced viability reduction in PANC-1 cells (Fig. 1A). Note that NU-7026 or NU-7441 alone had no significant effect on PANC-1 cell survival (Fig. 1A). We then examined the effect of DNA-PKcs inhibitors on gemcitabine-induced cell apoptosis. Annexin V FACS assay and Caspase-3 activity assay results showed that NU-7026 and NU-7441 sensitized cell apoptosis induction by gemcitabine in PANC-1 cells (Fig. 1B and C). Once again, NU-7026 and NU-7441 alone had almost no effect on PANC-1 cell apoptosis (Fig. 1B and C). These results show that DNA-PKcs inhibitors facilitate gemcitabine-induced cytotoxicity against PANC-1 cells, indicating a potential role of DNA-PKcs in gemcitabine resistance.

3.2. SiRNA-knockdown of DNA-PKcs facilitates gemcitabine-induced cytotoxicity in PANC-1 cells

Above results indicate a potential role of DNA-PKcs in gemcitabine resistance. To further support this hypothesis, siRNA strategy was applied. Two non-overlapping DNA-PKcs siRNAs, corresponding to different part of DNA-PKcs mRNA (see [14]), were utilized to selectively knockdown DNA-PKcs in PANC-1 cells. Western blot assay results in Fig. 2A demonstrated that these two siRNAs (#1 and #2) efficiently reduced DNA-PKcs expression in PANC-1 cells, with the knockdown efficiency around 70–80% (Fig. 2A, quantification). Importantly, gemcitabine-induced viability reduction was significantly enhanced in DNA-PKcs-depleted PANC-1 cells (Fig. 2B). Further, as shown in Fig. 2C, PANC-1 cell apoptosis by

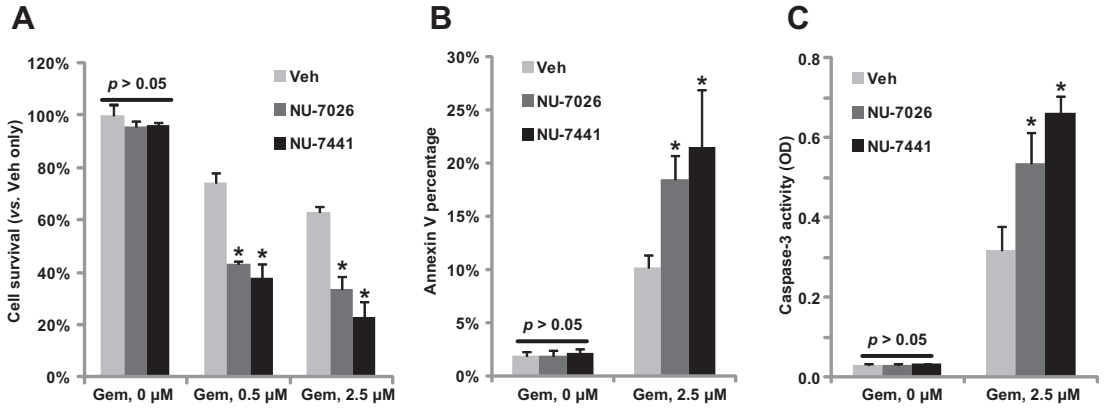

Fig. 1. DNA-PKcs inhibitors facilitate gemcitabine-induced cytotoxicity against PANC-1 pancreatic cancer cells. PANC-1 pancreatic cancer cells were treated with indicated dose of gemcitabine, or with NU-7026 (5 μM) and NU-7441 (5 μM), cells were cultured for 72 h, cell viability was tested by MTT assay (A), cell apoptosis was detected by Annexin V FACS assay (B) and caspase-3 activity assay (C). Experiments in this figure were repeated four times. Data were presented as mean ± SD. "Veh" stands for 0.1% DMSO. $^{*}p < 0.05$ vs. "Veh" group.

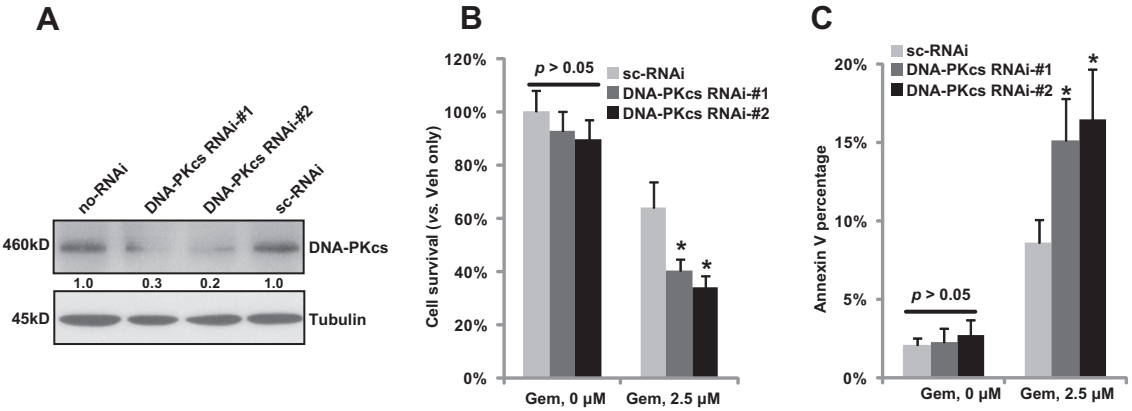

Fig. 2. SiRNA-knockdown of DNA-PKcs facilitates gemcitabine-induced cytotoxicity in PANC-1 cells. DNA-PKcs and tubulin expression in un-transfected control PANC-1 cells or PANC-1 cells transfected with indicated siRNA (200 nM each, 48 h) was tested by Western blots (A). PANC-1 cells transfected with scramble siRNA (sc-RNAi) or DNA-PKcs siRNA (1# or #2)(200 nM each, 48 h) were treated with gemcitabine (2.5 μM), cells were cultured for 72 h, cell viability and cell apoptosis was tested by MTT assay (B) and Annexin V FACS assay (C), respectively. DNA-PKcs expression was quantified. Experiments in this figure were repeated 4 times. Data were presented as mean ± SD. $^{*}p < 0.05$ vs. sc-RNAi group.

gemcitabine was also exacerbated with DNA-PKcs knockdown (Fig. 2C). These results further support the involvement of DNA-PKcs in gemcitabine resistance in PANC-1 cells.

3.3. DNA-PKcs–SIN1 association is important for Akt activation in PANC-1 cells

Above results suggest that DNA-PKcs might play a role in gemcitabine resistance. Elevated Akt activation is an important gemcitabine resistance factor [24–27], and Akt inhibition could enhance gemcitabine-induced apoptosis in human pancreatic cancer cells [27]. Interestingly, studies have demonstrated that DNA-PKcs could directly phosphorylate and activate Akt [14,17,28,29]. Tu et al., showed that ultraviolent (UV) radiation induces DNA-PKcs complexation with mTOR complex 2 (mTORC2) component SIN1 (SAPK-interacting protein 1), which is required for Akt Ser-473 phosphorylation, and subsequent cell survival/apoptosis-resistance [14]. Here we found that DNA-PKcs inhibitor NU-7441 or siRNA-knockdown largely suppressed Akt Ser 473 phosphorylation in PANC-1 cells (Fig. 3A). The results from the Co-immunoprecipitation (Co-IP) assay showed that DNA-PKcs formed a complex with SIN1 in PANC-1 cells, which was inhibited by DNA-PKcs inhibitors (NU-7441 and NU-7026), or by SIN1-siRNA knockdown (Fig. 3B, IP). Both DNA-PKcs inhibitors as well as SIN1 siRNA-knockdown prevented Akt Ser-473 phosphorylation (Fig. 3B, Inputs). Importantly, gemcitabine-induced viability decrease and apoptosis were enhanced by SIN-1 siRNA depletion (Fig. 3C and D). These results suggest that DNA-PKcs–SIN1 association is important for Akt activation and gemcitabine resistance in PANC-1 cells.

3.4. DNA-PKcs is over-expressed in human pancreatic cancer tissues

We then tested expression of DNA-PKcs in human pancreatic cancer tissues. As shown in Fig. 4A, in all three human pancreatic cancer tissues, expression of DNA-PKcs and p-Akt (Ser 473) was significantly higher than that in surrounding normal tissues. DNA-PKcs expression in cancer tissues was around 2–3 times higher than that in normal tissues (Fig. 4B). DNA-PKcs expression was closely correlated with p-Akt in cancer tissues (Fig. 4A), the latter was over 3 times higher than that in surrounding normal tissues (Fig. 4B). Thus, DNA-PKcs expression is elevated in human pancreatic cancer tissues, which might be a novel and important drug target.

4. Discussions

Pancreatic cancer is one of the most aggressive human malignancies with median overall survival of less than six months. The poor prognosis of this disease is due to a number of factors including late diagnosis, absence of diagnostic serum markers, resistance of conventional therapy, and the high metastatic potential of the cancer cells [30–34]. Gemcitabine is the only approved single agent for pancreatic cancer, which shows some survival benefit for patients with good conditions [35]. However, the efficiency of the current standard gemcitabine and gemcitabine-based regimens is moderate, and improved therapeutic options are greatly needed. The majority of phase III trials exploring gemcitabine-based combinations have been failed to improve overall survival. Our *in vitro* results showed that DNA-PKcs inhibition could significantly sensitize gemcitabine-induced cytotoxicity in PANC-1 cells.

Gemcitabine is a nucleoside analog in which the hydrogen atoms on the 2′ carbon of deoxycytidine are replaced by fluorine atoms, thus inhibiting DNA replication and arresting tumor cell growth [36,37]. Clinically, gemcitabine is being used in pancreatic cancer and various other carcinomas including non-small cell lung cancer, bladder cancer and breast cancer [34,36]. However, due to the pre-existing or acquired chemo-resistance, it fails to

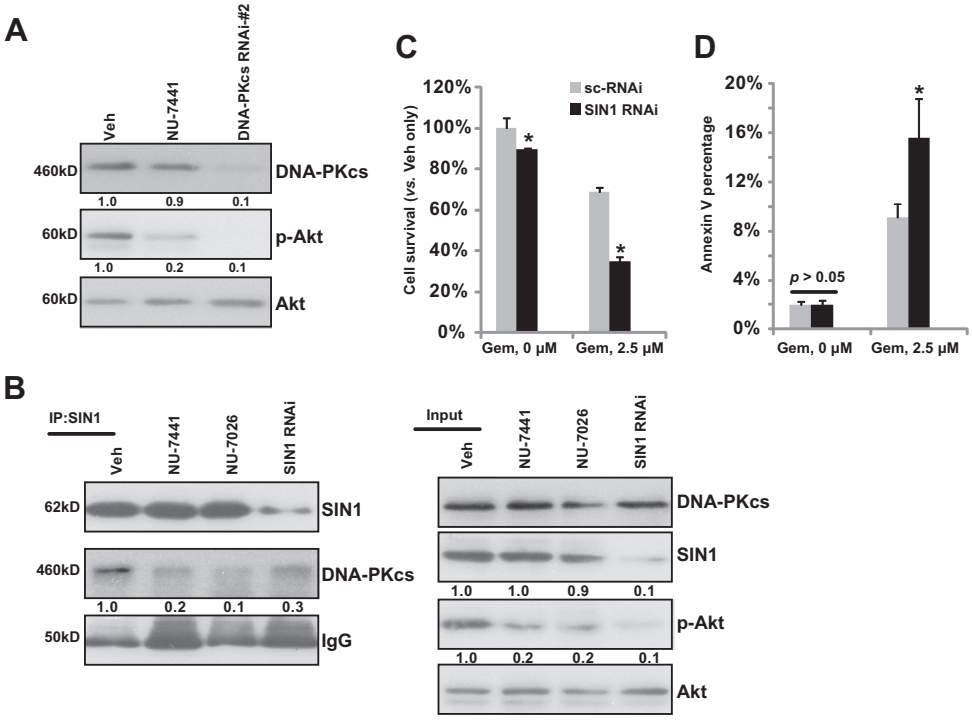

Fig. 3. DNA-PKcs–SIN1 association is important for Akt activation in PANC-1 cells. PANC-1 cells were either untreated, or treated with NU-7441 (5 μM, 12 h) or NA-PKcs siRNA (#2) (200 nM, 48 h), expression of indicated proteins was tested by Western blots (A). PANC-1 cells were either untreated, or treated with NU-7441 (5 μM, 12 h), NU-7026 (5 μM, 12 h) or SIN-1 siRNA (200 nM, 48 h), DNA-PKcs–SIN1 association (B, Co-IP) and expression (B, Inputs) were tested, p-Akt and regular Akt were also detected (B, inputs). PANC-1 cells transfected with scramble siRNA (sc-RNAi) or SIN1-siRNA (200 nM, 48 h) were treated with gemcitabine (2.5 μM), cells were cultured for 72 h, cell viability and cell apoptosis was tested by MTT assay (C) and Annexin V FACS assay (D), respectively. Experiments in this figure were repeated 4 times. Data were presented as mean ± SD. "Veh" stands for 0.1% DMSO. $^{*}p < 0.05$ vs. sc-RNAi group. Indicated proteins were quantified.

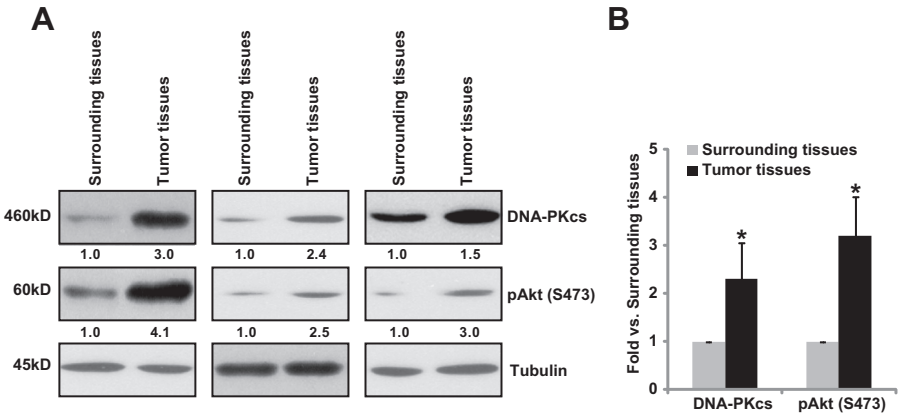

Fig. 4. DNA-PKcs is over-expressed in human pancreatic cancer tissues. DNA-PKcs, p-Akt Ser 473 and tubulin (loading control) expression in three human pancreatic cancer tissues and corresponding surrounded normal tissues was tested by Western blots (A), DNA-PKcs/p-Akt expression of three sets experiments was quantified (B). $^{*}p < 0.05$ vs. surrounding tissue group.

significantly improve the outcome of pancreatic cancer patients. Constitutive PI3K/Akt activation confers resistance against gemcitabine, and inhibition of Akt by pharmacological or genetic approaches could sensitize gemcitabine in the treatment of pancreatic cancer [24,25,27]. Thus, understanding the molecular mechanism of Akt activation in pancreatic cancer cells will be extremely importantly.

Akt Ser-473 could be phosphorylated by both mTORC2 [38,39] and DNA-PKcs [40,41] depending on type of stimuli. A recent study by Tu et al. has shown that after UVB radiation DNA-PKcs forms a complex with SIN1 at the cytosol, which is critical for Akt phosphorylation [14]. Similarly, in this study, we found that both DNA-PKcs and mTORC2 component SIN1 were required for Akt phosphorylation in PANC-1 cells. These two formed a complex. Disruption of this complex by DNA-PKcs inhibitors or by DNA-PKcs/SIN1 siRNA-knockdown not only prevented Akt phosphorylation, but also facilitated gemcitabine-induced apoptosis and cytotoxicity in PANC-1 cells. Thus, DNA-PKcs and SIN1 are both important gemcitabine resistance factors.

In summary, our data suggest that DNA-PKcs is important for Akt activation and gemcitabine resistance in PANC-1 cells, elevated DNA-PKcs in pancreatic cancer might be a novel target for treatment.

Author contributions

All authors carried out the experiments, participated in the design of the study, performed the statistical analysis, conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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