Pim-3 Contributes to Radioresistance through Regulation of the Cell Cycle and DNA Damage Repair in Pancreatic Cancer Cells

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Abstract

Resistance of cancer cells to chemoradiotherapy is a major clinical problem in pancreatic cancer treatment. Therefore, understanding the molecular basis of cellular resistance and identifying novel targets are essential for improving treatment efficacy for pancreatic cancer patients. Previous studies have demonstrated a significant role for Pim-3 in pancreatic cancer survival against gemcitabine-induced genotoxic stress. Here, we observed that radiation treatment enhanced Pim-3 expression in human pancreatic cancer cells *in vitro*. Stable overexpression of Pim-3 in pancreatic cancer cells significantly protected cells against radiation treatment by attenuating G2/M phase cell cycle arrest and DNA damage response. Silencing of Pim-3 expression significantly elevated the phosphorylation of histone variant H2AX, a marker of DNA double strand breaks, and decreased the activation of ataxia-telangiectasia-mutated (ATM) kinase, along with its downstream targets, eventually enhancing the radiosensitivity of human pancreatic cancer cells *in vitro* and *in vivo*. Hence, we demonstrated a novel function for Pim-3 in human pancreatic cancer cell survival against radiation. Targeting Pim-3 may be a promising way to improve treatment efficacy in combination with radiotherapy in human pancreatic cancer.

1. Introduction

Despite recent progress in early diagnosis, pancreatic cancer remains one of the most aggressive and lethal digestive system malignancies, with a 5-year relative survival rate of 7 % [1]. Although surgical resection and chemotherapy are the most effective treatments for pancreatic ductal adenocarcinoma (PDAC), locally-applicable external radiotherapy is becoming a major form of PDAC treatment [2]. However, because of the inherent ability of PDAC to become chemoradioresistant, combined modality therapy has failed to universally improve outcomes. Tumor resistance to radiotherapy remains an urgent problem, and a better understanding of the mechanisms involved in the induction of radioresistance may provide strategies for improving patient responses to therapy.

Radiation remains a mainstay in the treatment of cancer because of its clinical utility

in inducing DNA damage. Meanwhile, radiation is also known to induce DNA damage response (DDR), which is the central mechanism of radioresistance. In cancer treatment, DDR protects cells against genomic instability so that cells become resistant to ionizing radiation and chemotherapy by enhancing DNA damage repair [3,4,5]. ATM, ataxia telangiectasia mutated kinase, which belongs to the phosphatidylinositol-3-kinase family, is best known for its pivotal role in activating DDR[6,7]. Recent studies have indicated that loss-of-function ATM plays an important role in the poor prognoses of gastric and pancreatic cancers [8,9]. Depletion of ATM also accelerates the progression of pancreatic cancer [10].

Pim-3, a member of the Provirus integrating site for Moloney murine leukemia virus (Pim) family, exhibits serine/threonine kinase activity and is aberrantly expressed in many malignant tumors, including pancreatic cancer[11,12]. Pim-3 has the ability to regulate cell proliferation and apoptosis, contributing to tumorigenesis. It was reported that Pim-1 and Pim-3 play a crucial role in the control of self-renewal of ES cells [13]. Moreover, Pim-1 is overexpressed in radioresistant lung cancer cells via the phosphorylation of PRAS40. Pim-1 and AKT-activated pFOXO3a and 14-3-3 may be responsible for radioresistance [14]. Upon exposure to UV radiation, Pim-2 expression was increased and acted upstream of E2F-2 and ATM in UV-induced DNA damage response [15]. In addition, Xu et al. revealed that inhibition of Pim-3 sensitizes pancreatic cancer cells to gemcitabine [16]. These observations may indicate a potential role for Pim-3 in chemoradioresistance similar to that of other members of the Pim kinase family.

In this study, we showed that enhanced Pim-3 expression could induce resistance of pancreatic cancer cells to radiation treatment, whereas depletion of Pim-3 enhanced pancreatic cancer cell radiosensitivity. Radioresistance induced by Pim-3 is mainly attributed to the capacity of cells to prevent DNA-DSB (double-strand break) formation and to repair DNA damage. Our studies highlight a pivotal role for Pim-3 in DNA repair and cell survival in irradiation.

2. Methods and materials

2.1. Cell lines and reagents

Human pancreatic carcinoma cell lines PCI35 and PCI55 were a gift from Professor Mukaida Naofumi (Kanazawa University, Kanazawa, Japan). Human pancreatic cancer cell lines SW1990, MIAPaCa-2, PANC-1, and Capan-1 cells were purchased from ATCC and were cultured in RPMI 1640 medium supplemented with 10% FBS (Biosera, Inc. Loire Valley, France), and human embryonic kidney 293T cells were cultured in DMEM supplemented with 10% FBS (Biosera). The cell lines were authenticated by DNA profiling of short tandem repeats and amelogenin (Beijing Microread Genetics Co. Ltd.). The following antibodies were obtained commercially: Pim-3, CDC2, CylinB1, p-H2AX, H2AX, p-ATM, ATM, p-Chk1, Chk1, p-p53 and p53; all were purchased from Cell Signaling Technology, USA. β -Actin was purchased from Sigma-Aldrich (USA).

2.2. Ionizing radiation

Irradiation was performed using 225 kv X-rays generated by a linear accelerator at a dose rate of 3.845 Gy/min (SARRP, small animal radiation research platform). The radiation field was 10×20 cm. The source-skin distance was 350 mm.

2.3. Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation and immunoblotting were performed as described previously. [17,18]

2.4. Cell cycle and cell viability analyses

After the cells were treated with a single dose of radiation (0, 1, 4, 6 or 8 Gy), cell cycle analysis was performed using propidium iodine (Invitrogen) according to the manufacturer's protocol. Cell viability was determined at 0, 24, 48, 72, and 96 h using the Cell Counting Kit-8 reagent (Dojindo) according to the manufacturer's protocol. At least three independent experiments were performed.

2.5. Colony formation assay

Cells were seeded at a density of 1×10^6 cells per T25 flask in complete medium. Twenty-four hours later, the cells were treated with a single dose of radiation (0, 2, 4, 6 or 8 Gy), and then seeded into 6-well tissue culture plates at a density of 600 cells per well and allowed to incubate for 10-14 days. After colonies formed, the cells were fixed with 4 % formaldehyde and stained with 1 % crystal violet, and surviving colonies of > 50 cells were scored under a dissection microscope. The surviving fraction for a given treatment dose was calculated as the plating efficiency of the irradiated samples relative to that of the sham-irradiated ones. The Multi-target click model of GraphPad Prism 6.0 was used to fit cell survival curves.

2.6. Analysis of γ -H2AX foci

Cells were seeded onto slides in 60 mm dishes, irradiated with 4 Gy, and harvested at 24 h after irradiation. After washing twice with PBS, the cells were fixed with 4% paraformaldehyde and permeabilized using 0.3% Triton-X-100. The cells were then blocked, followed by incubation with primary antibodies overnight at 4°C. The following day, after three washes with PBS, the cells were incubated with fluorescently-labeled secondary antibodies in the dark and observed using confocal laser scanning microscopy.

2.7. Xenograft experiments

Twenty female BALB/c nude mice (5 weeks old; 18-23 g) were randomly divided into two groups. The right flank of each mouse was inoculated subcutaneously with 2×10^6 MIAPaCa-2, or MIAPaCa-2-Pim-3 cells. Seven days later, each group was randomly divided into two subgroups (five mice each). When the tumors reached a size of 70-100 mm³, two different subgroups were irradiated once with 15 Gy. Tumor volume was calculated using the following formula: $(\text{length} \times \text{width}^2)/2$. Two weeks after irradiation, the tumor tissues were removed and subjected to Western blot analysis. The study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Fudan University. The protocol was approved by the

Committee on the Ethics of Animal Experiments of Fudan University [permit number: SYXK (Hu) 2009-0082].

2.8. Statistical analysis

Group comparisons of normally-distributed data were performed using Student's t-test (two sample) or one-way ANOVA (multiple comparisons). Data were expressed as the mean $\pm$ standard deviation (SD). A p value < 0.05 was considered statistically significant.

3. Results

3.1 Pim-3 induces pancreatic cancer cell resistance to radiation *in vitro*

To define the role of Pim-3 in the radioresistance of pancreatic cancer cells, we first examined the radiosensitivities of PDAC cells. As shown in Fig. 1A, PANC-1 and Capan-1 cells were more resistant to radiation, whereas MIAPaCa-2 and SW1990 cells were more sensitive, which is consistent with the data for Pim-3 expression, as shown in Fig. 1B, demonstrating that PANC-1 and Capan-1 cells had relatively higher Pim-3 expression than MIAPaCa-2 and SW1990 cells. Moreover, ionizing radiation induced Pim-3 expression in both MIAPaCa-2 and PANC-1 cells (Fig. 1C). We next investigated MIAPaCa-2 cells that overexpressed Pim-3 (MIAPaCa-2-Pim-3) and PANC-1 cells with silenced Pim-3 expression (PANC-1-shPim-3) (Fig. 1D and E) to further evaluate the role of Pim-3 in radioresistance. As shown in Fig. 1F and 1G, the colony formation assay showed that Pim-3-overexpressing cultures increased the number of colonies and protected cells from irradiation compared with parental cells (Fig. 1F). In contrast, silencing of Pim-3 expression significantly decreased the number of colonies after irradiation (Fig. 1G). Consistent with the colony formation data, cell viability assays showed that Pim-3 overexpressing cultures promoted cell proliferation compared with parental cells after irradiation, whereas silencing of Pim-3 expression significantly inhibited cell proliferation following irradiation (Fig. 1H and 1I). Thus, these results suggest that Pim-3 is involved in the radioresistance of PDAC cells.

3.2 Pim-3 protects cells from radiation damage through ATM-mediated DNA damage repair

It is well documented that after irradiation treatment, cells activate DNA damage response (DDR), which comprises the initial sensing of DNA breaks and the subsequent DNA damage repair[3]. The phosphorylated form H2AX, γ -H2AX, leads to the formation of nuclear foci at sites of DNA damage and is necessary for the assembly of repair complexes[19]. We hypothesize that Pim-3 could reduce both the number of DNA damage lesions and the accumulation of DNA double-strand breaks (DSBs). Therefore, we first measured the formation of γ -H2AX foci in Pim-3-overexpressing cells and Pim-3-silenced cells after 4 Gy irradiation. As shown in Fig. 2A and 2B, fluorescence microscopy showed that Pim-3 overexpression decreased the number of radiation-induced γ -H2AX foci, whereas Pim-3 silencing increased the number of γ -H2AX foci after irradiation. Similar results were observed by Western blot analysis, suggesting increased DNA repair in Pim-3 overexpressing cells (Fig. 2C and D).

ATM, the major core molecular sensor, can be recruited in response to DNA damage, thereby triggering cell cycle arrest and DNA repair [20]. We further examined whether ATM was affected in Pim-3-overexpressing and Pim-3-silenced cells after 4 Gy irradiation. We found that overexpression of Pim-3 augmented phosphorylated ATM expression after irradiation, whereas silencing of Pim-3 decreased phosphorylated ATM expression but not total ATM protein expression (Fig. 2E and 2F). Levels of both phosphorylated Chk1 and p53, two substrates of ATM, were increased in Pim-3-overexpressing cells, whereas they were decreased in Pim-3-silenced cells after irradiation. Total Chk1 and p53 protein levels were unaffected (Fig. 2E and 2F). Moreover, immunoprecipitation experiments revealed that Pim-3 was bound to ATM, with elevated levels of phosphorylated ATM in Pim-3-overexpressing cells, whereas decreased levels of phosphorylated ATM were seen in Pim-3-silenced cells (Fig. 2G-2J), indicating that Pim-3 could bind to and phosphorylate ATM. To further ensure that Pim-3 accelerates DNA repair by phosphorylating ATM, we performed ATM knockdown using ATM siRNA in MIAPaCa-2-Pim-3-overexpressing cells. Immunostaining of γ -H2AX

showed that Pim-3 overexpression decreased the numbers of radiation-induced γ -H2AX foci, which was reversed by ATM knockdown (Fig. 2K). The phosphorylation of Chk1 and p53 was also decreased in ATM knockdown cells (Fig. 2L). Taken together, these results suggest that Pim-3 may be involved in DNA damage repair via phosphorylation of ATM.

3.3 Pim-3 protects cells from radiation damage through cell cycle arrest

To evaluate whether Pim-3 affected DNA damage response through cell cycle arrest, we examined the distribution of the cell cycle phases in Pim-3-overexpressed and Pim3-silenced cells after exposure to 4 Gy irradiation. Flow cytometry analysis revealed that the G2/M phase was longer after irradiation in both the MIAPaCa-2 and the PANC-1 cell lines compared with the non-irradiated cells (Fig. 3A and 3B, lanes 1 and 3). However, Pim-3 overexpression (Fig. 3A, lanes 2 and 4) weakened the arrest of cells in the G2/M phase and enhanced S phase accumulation, whereas Pim-3 silencing led to the opposite results (Fig. 3B, lanes 2 and 4). Consistent with these results, Pim-3 overexpression significantly increased the expression of Cdc2 and Cyclin B1, which attenuated radiation-induced G2/M phase arrest (Figure 3C). In contrast, Pim-3 silencing combined with radiation remarkably decreased the expression of Cdc2 and Cyclin B1, which facilitated G2/M phase arrest (Figure 3D). These results suggest that Pim-3 protected cells against radiation through attenuation of G2/M phase cell cycle arrest.

3.4 Pim-3 induces pancreatic cancer cell resistance to radiation *in vivo*

To determine whether Pim-3 induces tumor resistance to radiation *in vivo*, we irradiated tumor tissue using a single dose of 15 Gy administered on day 15 after subcutaneous injection of human pancreatic cancer cells (Fig. 4A). We found that the tumor volumes of xenografts derived from Pim-3-overexpressing cells were much larger than those of controls exposed to the same dose of radiation (Fig. 4B). Western blot analysis of tumor tissue suggested that Pim-3 expression was induced and that the expression of phosphorylated ATM was upregulated in xenografts treated as described above (Fig. 4C).

These results indicate that Pim-3 could induce radioresistance *in vivo*.

4. Discussion

Pancreatic ductal adenocarcinoma continues to pose a major therapeutic challenge because of its multifaceted resistance mechanisms to conventional cancer therapies [2,21]. Due to progress made in advanced techniques, radiotherapy is becoming a useful neoadjuvant treatment for pancreatic cancer [22]. However, both intrinsic and acquired radioresistance are major obstacles for pancreatic cancer treatment[2]. Thus, understanding the molecular mechanisms underlying radiation sensitivity and resistance remains an important pursuit.

We previously observed that Pim-3 was aberrantly expressed in human malignant lesions of the pancreas but not in normal pancreatic tissue [23]. Accumulating evidence also indicates that Pim-3 kinase plays key roles in the development of pancreatic cancer by regulating cell apoptosis [24], cell cycle progression [18], angiogenesis and gemcitabine resistance [16]. It has been shown that inhibition of Pim-3 increases chemosensitivity of pancreatic cancer cells to gemcitabine[16]. However, little is known about whether Pim-3 can modulate radioresistance in pancreatic cancer cells. Here, for the first time, we report that Pim-3 plays a role in the radioresistance of pancreatic cancer cells. We found that Pim-3 can be induced by irradiation. Pim-3 overexpression enhanced PDAC radioresistance, whereas reduced levels of Pim-3 increased radiosensitivity *in vitro* and *in vivo*.

Ionizing radiation-induced DNA double strand breaks (DSBs) induce important signal transduction processes [3,25]. DNA damage response (DDR), an evolutionarily-conserved signaling complex that involves initiation of DNA repair, activation of cell cycle checkpoints, and extensive modulation of gene expression, is the main reason for the resistance to radiation [26]. ATM plays a key role in the DDR complex via its autophosphorylation and recruitment to DNA damage sites to phosphorylate downstream substrates that trigger DNA repair[27]. It is known that the other Pim kinase family members, Pim-1 and Pim-2, are involved in genome instability

[15,28]. Interestingly, we found that Pim-3 could block formation of γ -H2AX foci and augment phosphorylated ATM expression upon irradiation. Moreover, ATM activation leads to Chk1 and p53 phosphorylation. Furthermore, co-immunoprecipitation verified that Pim-3 could interact with ATM and augment phosphorylation of ATM. ATM knockdown in Pim-3-overexpressing cells reversed the protective effect of Pim-3 against radiation. In addition, Thus, Pim-3 may contribute to DNA damage repair capacity by enhancing phosphorylated ATM levels.

Cell cycle checkpoints sense DNA damage and execute cell cycle arrest through modulation of the activity of cell cycle regulators[3]. ATM, the major protein kinase in DDR, is also known to trigger the activation of DNA damage checkpoints, which in turn phosphorylate their own substrate repertoires. Upon exposure to radiation, the G2/M checkpoint is initiated by phosphorylation of checkpoint kinase (Chk1) and phosphatases[29], which subsequently prevents dephosphorylation of CDK1-Cyclin B, which is required for progression into mitosis[29]. The G2/M checkpoint represents an opportunity for preventing cells with damaged DNA to enter mitosis, where unrepaired DSBs may result in mitotic catastrophe and cell death. Consistent with these reports, we observed that Pim-3 overexpression attenuated G2/M cell cycle arrest induced by irradiation. Therefore, Pim-3 contributed to radioresistance by attenuating G2/M cell cycle arrest in pancreatic cancer cells.

In summary, our data demonstrate that Pim-3 is endogenously induced by ionizing radiation in pancreatic cancer cells and that Pim-3 overexpression enhances radioresistance of pancreatic cancer cells both *in vitro* and *in vivo*. We propose a model in which Pim-3 induces activation of ATM, which subsequently activates checkpoint 1, leading to amplification of DNA repair through cell cycle arrest and DNA repair pathways (Fig. 4D). Thus, tumors expressing high levels of Pim-3 may be more resistant to radiotherapy. However, more research is needed to investigate and identify the complex mechanisms within this pathway. Based on our results and those of previous studies, targeting Pim-3 may be an effective strategy to overcome resistance to radiotherapy in human pancreatic cancer treatment.

Conflicts of interest

The authors have no financial conflicts of interest.

Acknowledgments

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Figure Legends

Figure 1. Pim-3 induced cell resistance to radiotherapy *in vitro*. (A) A clonogenic survival assay was performed using four human pancreatic cancer cell lines. The surviving fractions are shown here. Results are expressed as the mean $\pm$ SD (n = 3). (B) The expression of Pim-3 in four human pancreatic cancer cell lines as determined by Western blot analysis. β -actin was used as an internal control. (C) Cell lysates were obtained from MIAPaCa-2 and PANC-1 cells, which were irradiated at different doses for the indicated time intervals. The resultant lysates were subjected to Western blot analysis. β -actin was used as an internal control. (D and E) Cell lysates were obtained from MIAPaCa-2 or PANC-1 cells stably transfected with pMEI-5-Pim-3 expression vector (Pim-3) or Pim-3-specific shRNA (shPim-3). The resultant lysates were subjected to Western blot analysis. β -actin was used as an internal control. (F and G) A clonogenic survival assay was performed using MIAPaCa-2-Pim-3 and PANC-1-shPim-3 stable cells and their parental cells with or without 4 Gy irradiation just after the cells adhered to the microplate. The colonies were observed by microscopy 2 weeks after irradiation. (H and I) MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells and their parental cells were plated in 96-well microplates. After the cells had adhered to the microplates, they were treated with or without 4 Gy of ionizing radiation. This time was designated as Day 0. Cell numbers were determined at indicated time intervals using the Cell Counting Kit-8 reagent and were normalized to those on Day 0. Data are expressed as the mean $\pm$ SD of five replicates from three independent experiments. *, P < 0.05, **, P < 0.01, ***, P < 0.001, ****, P < 0.0001.

Figure 2. Pim-3 protected cells from radiation damage by binding to and phosphorylating ATM. (A and B) MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells and their parental cells were irradiated at 4 Gy. Immunostaining was performed with the γ -H2AX antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Fluorescent images were digitally merged. Quantification of γ -H2AX foci was performed and analyzed per cell. Over 50 cells were counted for each cell line. Scale bar = 10 μ m.

Values are expressed as the means $\pm$ SD from 3 independent experiments. **, $P < 0.01$.

(C and D) Cell lysates were obtained from MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells, and their parental cells were treated with or without 4 Gy of ionizing radiation. The resultant lysates were subjected to Western blot analyses with the indicated antibodies. β -actin was used as an internal control. **(E and F)** Cell lysates obtained from MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells and their parental cells were irradiated at 4 Gy. The resultant lysates were subjected to Western blot analyses with the indicated antibodies. β -actin was used as an internal control. **(G and H)** Co-immunoprecipitation of endogenous Pim-3 and ATM in MIAPaCa-2 and PANC-1 cells. Resultant lysates obtained from MIAPaCa-2 and PANC-1 cells were subjected to immunoprecipitation and immunoblotting. **(I and J)** Cell lysates obtained from MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells and their parental cells were irradiated at 4 Gy. The resultant lysates were subjected to immunoprecipitation with anti-Pim-3 antibody and immunoblotting with anti-phospho-ATM, anti-ATM, and anti-Pim-3 antibodies. **(K)** MIAPaCa-2-Pim-3 cells were transfected with ATM siRNA, and 48 hrs later, the cells were immunostained with γ -H2AX antibodies and compared with MIAPaCa-2 and MIAPaCa-2-Pim-3-overexpressing cells. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Fluorescent images were digitally merged. Scale bar = 10 μ m. **(L)** MIAPaCa-2-Pim-3 cells were transfected with ATM siRNA, and 48 hrs later, the cell lysates were subjected to Western blot analysis with the indicated antibodies and compared with MIAPaCa-2 and MIAPaCa-2-Pim-3-overexpressing cells. β -actin was used as an internal control.

Figure 3. Pim-3 protected cells from radiation damage through cell cycle arrest. **(A and B)** MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells and their parental cells were irradiated at 4 Gy. The proportion of cells in each cell cycle phase was determined. **(C and D)** Cell lysates obtained from MIAPaCa-2-Pim-3 or PANC-1-shPim-3 stable cells and their parental cells were treated with or without 4 Gy of ionizing radiation. The resultant lysates were subjected to Western blot analyses with the indicated antibodies.

β -actin was used as an internal control.

Figure 4. Pim-3 induced cell resistance to radiotherapy *in vivo*. (A) Schematic overview of the xenograft mouse model. (B) Size of tumors in mice that received subcutaneous implants of MIAPaCa-2-Pim-3 and their parental cells. Mice were treated with or without 15 Gy of ionizing radiation on day 15 after the tumors had grown to 70-100 mm³, and tumor growth was measured twice per week. The data are expressed as the mean $\pm$ SE (n = 5). (C) Cell lysates were obtained from tumor tissues. The resultant lysates were subjected to Western blot analyses with the indicated antibodies. β -actin was used as an internal control. (D) A proposed schematic model describing the ATM-mediated involvement of Pim-3 in radiation-induced DDR.

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Figure 2. Chen et al.

Figure 3. Chen et al.

Figure 4. Chen et al.