

Original Article

Carbon-ion beam irradiation combined with paclitaxel/nab-paclitaxel effectively induces cell death in pancreatic cancer cells

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Abstract: This study investigated the molecular mechanisms by which carbon-ion beam irradiation (IR), either alone or in combination with paclitaxel (PTX) or nab-PTX, induces cell death in pancreatic cancer (PC) cells *in vitro*. Human PC cell lines, PANC-1 and MiaPaCa-2, were treated with carbon-ion beam IR alone or combined with PTX/nab-PTX. Cell viability, caspase-3/7 activity, and the expression of apoptosis-, autophagy-, and angiogenesis-related genes and proteins were analyzed using quantitative real-time RT-PCR (RT-qPCR) and western blotting. Additionally, cell invasion assays were performed. We found that PANC-1 cells were more resistant to carbon-ion beam IR than MiaPaCa-2 cells; however, combination treatment with PTX/nab-PTX enhanced the cytotoxic effects of carbon-ion beam IR (1 Gy). Caspase-3/7 activity was increased by carbon-ion beam IR or PTX alone and was further elevated by the combination treatment. RT-qPCR analysis showed that combined carbon-ion beam IR and PTX decreased the expression of the pro-apoptotic gene *Bax* while increasing *Bcl-2* expression in PANC-1 cells. Furthermore, carbon-ion beam IR, both alone and in combination with PTX, significantly increased the expression of the autophagy-related genes *ATG7* and *Beclin1* in PANC-1 cells. PTX/nab-PTX alone, or combined with carbon-ion beam IR, enhanced the expression of the apoptosis-associated proteins cleaved PARP and cleaved caspase-3 in both PC cell lines. Combination treatment with carbon-ion beam IR and PTX/nab-PTX effectively suppressed the expression of p-ERK and p-EGFR, but not p-AKT, in PANC-1 cells. In contrast, carbon-ion beam IR (1 Gy) alone, PTX/nab-PTX alone, or the combination treatment effectively inhibited p-ERK, p-EGFR, and p-AKT expression in MiaPaCa-2 cells. Additionally, carbon-ion beam IR combined with PTX significantly inhibited VEGF expression and cell invasion. In conclusion, carbon-ion beam IR in combination with PTX/nab-PTX effectively enhances PC cell death via apoptosis induction and reduces its invasive potential.

Keywords: Carbon-ion beam, paclitaxel, pancreatic cancer

Introduction

Pancreatic cancer (PC) remains an extremely aggressive malignancy characterized by a poor prognosis [1]. Despite advances in treatment strategies, locally advanced PC continues to be a clinically challenging disease [2]. While surgical resection is the definitive treatment for PC, the 5-year survival rate remains below 10% due to high rates of systemic recurrence and metastasis [3]. Multimodality approaches,

such as neoadjuvant therapy combining chemotherapy (e.g., FOLFIRINOX or gemcitabine/nab-paclitaxel) with surgery, or combining chemotherapy with advanced radiation techniques - such as stereotactic body radiotherapy (SBRT), intensity-modulated radiotherapy (IMRT), and proton beam therapy (PBT) - have demonstrated improved local control rates. Nevertheless, the survival rate for unresectable cases remains limited to approximately 20%-30% [4-9].

Carbon-ion radiotherapy (CIRT) offers higher biological effectiveness for pancreatic cancer (PC) compared to photon and proton beam therapies, as evidenced by its superior relative biological effectiveness (RBE) values [10, 11]. The efficacy of CIRT is relatively independent of tumor oxygenation [12] and cell cycle phase [13], potentially enhancing therapeutic outcomes beyond those of traditional approaches. To date, QST Hospital has treated more than 1,000 patients with LAPC using CIRT in combination with gemcitabine (GEM), achieving promising outcomes [14-17]. Our previous studies have demonstrated that high-linear energy transfer (LET) carbon-ion beam irradiation (IR), either alone or combined with chemotherapy and molecularly targeted drugs, significantly eradicates cancer stem cells (CSCs) in various malignancies [18-25]. This includes the finding that combining CIRT with GEM effectively targets pancreatic CSCs, providing a mechanistic basis for these clinical results [25]. However, the 2-year overall survival rate remains suboptimal at approximately 50% [15, 16]. Meanwhile, paclitaxel (PTX), a microtubule-stabilizing agent that induces cell cycle arrest and apoptosis, is an established treatment for advanced lung and pancreatic cancers [26, 27]. Nab-paclitaxel (Abraxane), an albumin-bound nanoparticle formulation of paclitaxel, delivers higher doses of the drug directly to tumor cells without the use of toxic solvents; its combination with GEM has been shown to improve PC survival [28, 29]. Despite these advancements, the potential synergistic effects of combining PTX with CIRT specifically for PC remain largely unexplored. Therefore, we hypothesized that the addition of PTX or nab-PTX may further enhance the sensitivity of PC cells to carbon-ion beams, potentially improving therapeutic efficacy. This study investigates the effects of carbon-ion beam IR, alone or in combination with PTX/nab-PTX, on human PC cells *in vitro*.

Materials and methods

Cell culture

PANC-1 and MiaPaCa-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum

(Biological Industries, Beit-Haemek, Israel), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The medium was replaced every three days.

Reagents

The following antibodies were used in this study: mouse anti-human β-actin (BD Biosciences, San Jose, CA, USA); mouse anti-human PARP (Miltenyi Biotec, Bergisch Gladbach, Germany); and caspase-3, cleaved caspase-3, EGFR, phospho-EGFR, AKT, phospho-AKT, ERK, and phospho-ERK (Cell Signaling Technology, Danvers, MA, USA). Paclitaxel (PTX) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and nab-PTX (Abraxane) was obtained from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan). Gemcitabine (GEM) was purchased from Eli Lilly Japan K.K. (Kobe, Japan). A stock solution of gemcitabine (1 mM in PBS) was diluted to the appropriate concentrations immediately before use.

Irradiation

Cells were irradiated with a 290 MeV/n carbon-ion beam with a 6-cm spread-out Bragg peak (SOBP), generated by the Heavy Ion Medical Accelerator in Chiba (HIMAC) at QST, Japan [18]. The carbon-ion beam IR was delivered at doses of 1, 2, or 3 Gy.

Cell viability assay

Cell viability was assessed using the trypan blue exclusion assay. Cells were stained with trypan blue dye, which is excluded by viable cells but taken up by dead or dying cells. The number of viable cells was then quantified using a Countess 3 FL automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA).

Caspase-Glo 3/7 assay

PC cells were treated with carbon-ion beam IR alone, or in combination with PTX or GEM, and incubated for 96 h. Caspase-3/7 activity was measured using the Caspase-Glo 3/7 Assay kit (G8091; Promega, Madison, WI, USA) according to the manufacturer's instructions.

Table 1. The primer sequences for quantitative real-time RT-PCR

Gene	Primer sequence
GAPDH	
Forward	5'-TGAACGGGAAGCTCACTGG-3'
Reverse	5'-TCCACCACCCTGTTGCTGTA-3'
Bax	
Forward	5'-CAAACCTGGTGCTCAAGGCC-3'
Reverse	5'-GCACCTCCGCCACAAAGAT-3'
Bcl-2	
Forward	5'-ATGTGTGTGGAGAGCGTCAACC-3'
Reverse	5'-TGAGCAGAGTCTTCAGAGACAGCC-3'
Beclin-1	
Forward	5'-AGCTGCCGTTTACTGTTCTG-3'
Reverse	5'-ACTGCCTCCTGTGTCTCAATCTT-3'
HIF1 α	
Forward	5'-CTATGGAGGCCAGAAGAGGGTAT-3'
Reverse	5'-CCCACATCAGGTGGCTCATAA-3'
VEGF	
Forward	5'-CTTGTTTCAAGCGGAGAAAGCT-3'
Reverse	5'-ACATCTGCAAGTACGTTCTGTT-3'

Cell invasion assays

The invasive potential of PC cells was evaluated using Transwell chambers (Millipore, Billerica, MA, USA) as previously described [30]. Cells were seeded into the upper chambers in 150 μ L of medium and subjected to carbon-ion beam IR alone or in combination with PTX/nab-PTX for 24 h. Cells that invaded through the Matrigel/gelatin-coated membrane were fixed and stained using the cell staining solution provided in the Transwell Chamber Assay Kit (Chemicon, Millipore).

Quantitative real-time RT-PCR (RT-qPCR) analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the RT² First Strand Kit (SABiosciences, Frederick, MD, USA). The expression levels of apoptosis- and autophagy-related genes were analyzed using a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). Data were analyzed using the Ct method with the system's software, and statistical analysis was performed as previously described [22]. The primer sequences used in this study are listed in **Table 1**. GAPDH was used as the internal housekeeping control.

Western blotting

PC cells were treated with carbon-ion beam IR alone or in combination with PTX/nab-PTX and incubated for 96 h. The cells were then lysed in RIPA buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% (v/v) skim milk in PBS containing 0.1% Tween 20 and then incubated with primary antibodies (1:1,000), followed by appropriate secondary antibodies (1:1,000). The protein bands were visualized using Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific) and scanned.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test using StatView software (SAS Institute, Inc., Cary, NC, USA). A *p*-value of less than 0.05 was considered statistically significant for all comparisons.

Results

Carbon-ion beam irradiation (IR) combined with PTX/Nab-PTX decreases PC cell viability

A cell viability assay using trypan blue staining was performed to examine the inhibitory effects of combining PTX/nab-PTX with carbon-ion beam IR on PC cells. As shown in **Figure 1**, treatment with 1 Gy of carbon-ion beam IR decreased the viability of MiaPaCa-2 cells but not PANC-1 cells. In contrast, PTX/nab-PTX alone significantly inhibited the viability of both PANC-1 and MiaPaCa-2 cells. Furthermore, the combination of carbon-ion beam IR and PTX/nab-PTX further suppressed the viability of MiaPaCa-2 cells but did not have a significant additional effect on PANC-1 cells. These results indicate that PANC-1 cells are more resistant to carbon-ion IR than MiaPaCa-2 cells.

Effects of carbon-ion beam IR alone or in combination with PTX on the expression of apoptosis- and autophagy-related genes in PC cells

qPCR was performed to investigate the effects of carbon-ion beam IR, alone or in combination

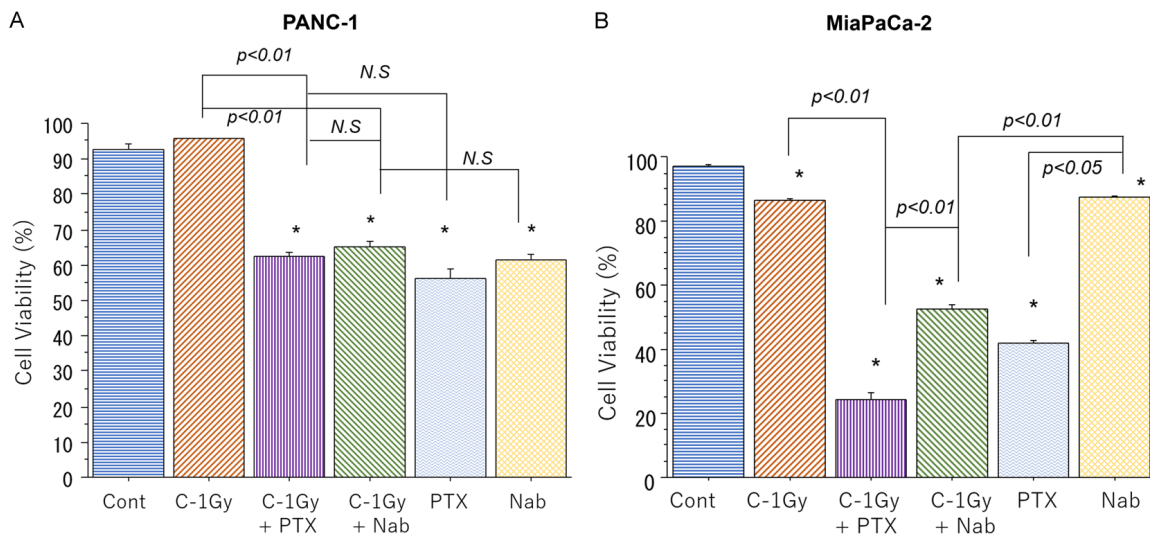


Figure 1. Effects of carbon-ion beam IR and chemotherapy on cell viability. A. Cell viability of PANC-1 cells following carbon-ion beam IR alone or in combination with PTX (100 nM) or nab-PTX (100 nM). B. Cell viability of MiaPaCa-2 cells following carbon-ion beam IR alone or in combination with PTX or nab-PTX (Nab). **P < 0.01 compared to the control.

with PTX, on the expression of apoptosis-related genes. As shown in **Figure 2A**, the expression of the pro-apoptotic gene *Bax* remained unchanged after treatment with carbon-ion beam IR alone, but decreased after PTX alone or in combination with carbon-ion beam IR. Conversely, the expression of the anti-apoptotic gene *Bcl-2* was increased by PTX alone or its combination with carbon-ion beam IR in PANC-1 cells. Regarding autophagy-related genes, the expression of *ATG7* and *Beclin1* significantly increased following treatment with carbon-ion beam IR alone, PTX alone, and the combination treatment in PANC-1 cells (**Figure 2B**).

Effects of carbon-ion beam IR alone or in combination with PTX on the expression of angiogenesis-related genes in PC cells

To investigate the impact of carbon-ion beam IR alone or in combination with PTX on the expression of angiogenesis-related genes, the levels of *VEGF* and *HIF1α* were determined by qPCR. As shown in **Figure 3**, the expression of *VEGF* was not significantly altered by carbon-ion IR or PTX alone; however, the combination of carbon-ion beam IR and PTX significantly inhibited *VEGF* expression. *HIF1α* expression showed no significant changes across all treatment groups.

Effects of carbon-ion beam IR alone or in combination with PTX on apoptosis induction

To elucidate apoptosis induction at the protein level, we performed caspase-3/7 activity assays and western blot analysis of cleaved caspase-3 and cleaved PARP expression. As shown in **Figure 4A** and **4B**, caspase-3/7 activity was increased by carbon-ion beam IR alone and PTX alone and was further enhanced by the combination treatment in both PANC-1 and MiaPaCa-2 cells. In PANC-1 cells, 1 Gy of carbon-ion beam IR alone did not alter the expression of cleaved caspase-3 or cleaved PARP; however, PTX/nab-PTX alone or in combination with carbon-ion beam IR significantly induced the expression of both proteins (**Figure 4C**). In contrast, 1 Gy of carbon-ion beam IR alone, nab-PTX alone, and their combination all induced cleaved caspase-3 and cleaved PARP expression in MiaPaCa-2 cells (**Figure 4D**). The uncropped, full-length scan of the original blot data are available in [Supplementary Data](#).

Effects of carbon-ion beam IR alone or in combination with PTX/nab-PTX on cell survival signaling pathways

To determine whether cell proliferation signaling pathways were affected by carbon-ion beam IR alone or in combination with PTX/nab-

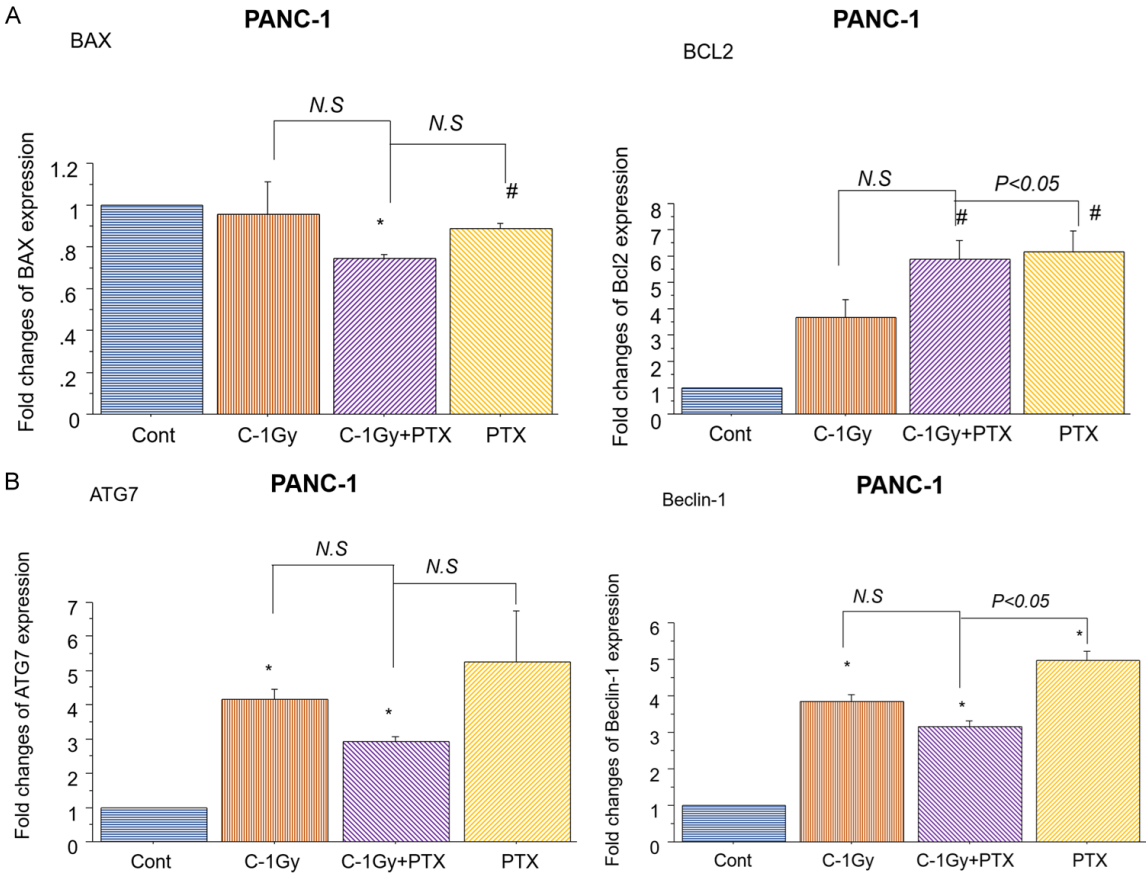


Figure 2. mRNA expression of apoptosis- and autophagy-related genes. A. Changes in mRNA expression of apoptosis-related genes after carbon-ion beam IR with or without PTX. B. Changes in mRNA expression of autophagy-related genes after carbon-ion beam IR with or without PTX. #P < 0.05, *P < 0.01 compared to the control.

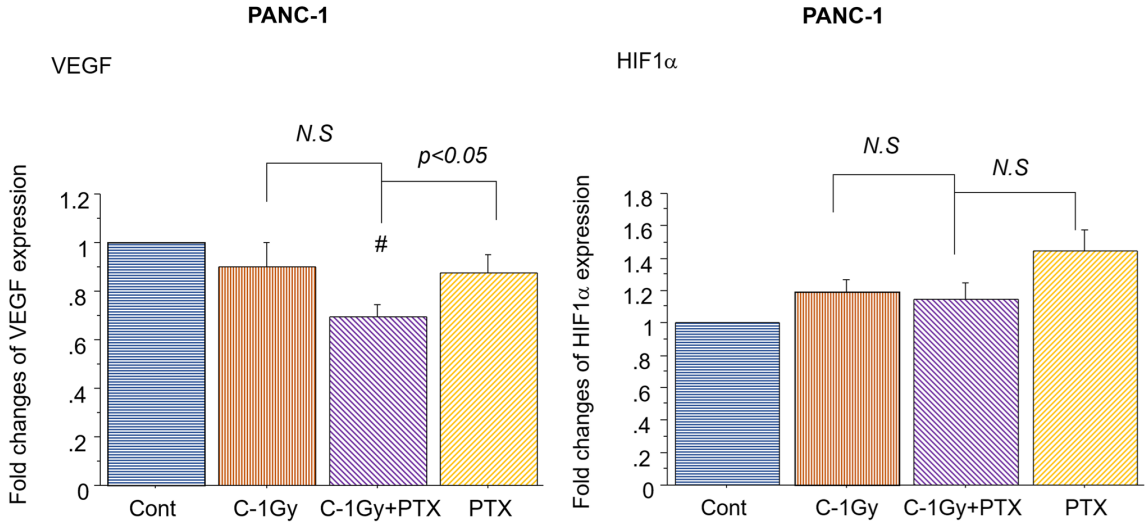


Figure 3. mRNA expression of angiogenesis-related genes. mRNA expression changes of VEGF and HIF-1 α after carbon-ion beam IR with or without PTX. #P < 0.05, *P < 0.01 compared to the control.

PTX, the expression levels of phosphorylated (p)-EGFR, p-AKT, and p-ERK were examined by

western blotting. As shown in **Figure 5**, carbon-ion beam IR alone or in combination with PTX/

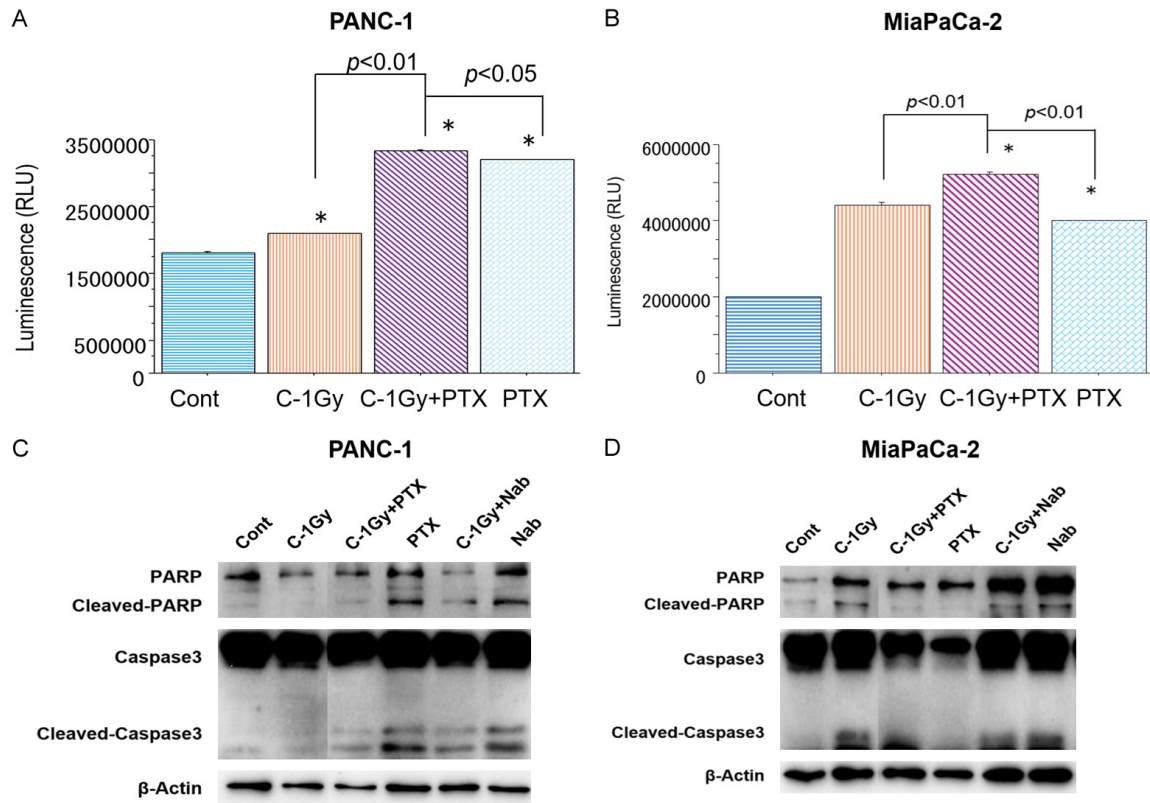


Figure 4. Apoptosis induction by carbon-ion beam IR and PTX. Caspase-3/7 activity in PANC-1 (A) and MiaPaCa-2 (B) cells after carbon-ion beam IR with or without PTX. Protein expression of cleaved PARP and cleaved caspase-3 after carbon-ion beam IR alone or in combination with PTX/nab-PTX (Nab) in PANC-1 (C) and MiaPaCa-2 (D) cells.

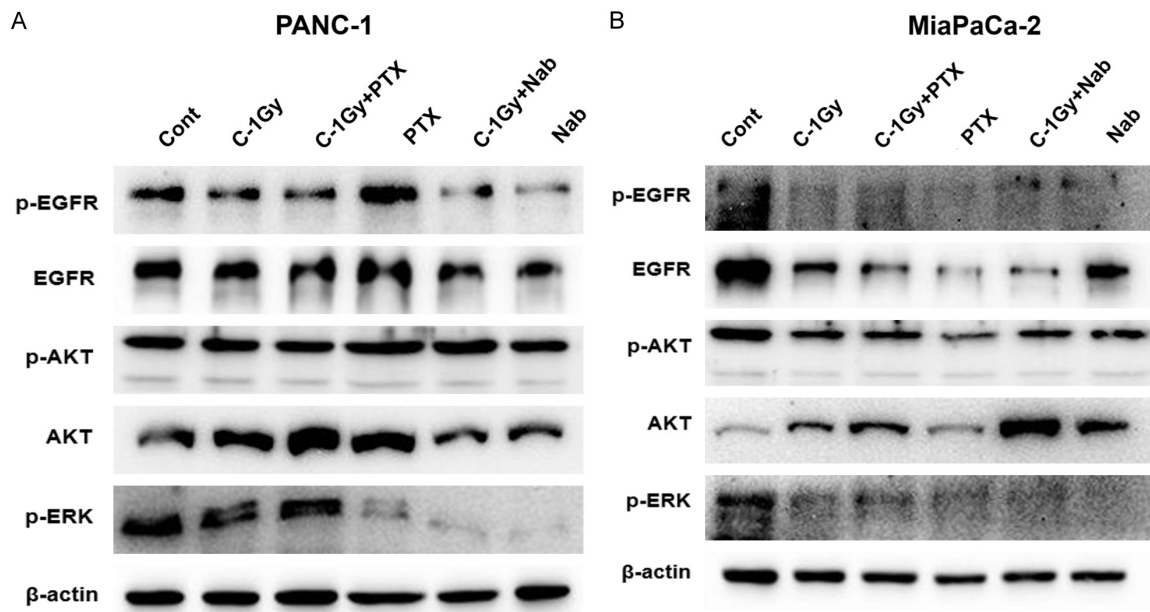


Figure 5. Changes in cell signaling pathways. Expression levels of EGFR, ERK/MAPK, and AKT signaling pathways after carbon-ion beam IR alone or in combination with PTX/nab-PTX (Nab) in PANC-1 (A) and MiaPaCa-2 (B) cells.

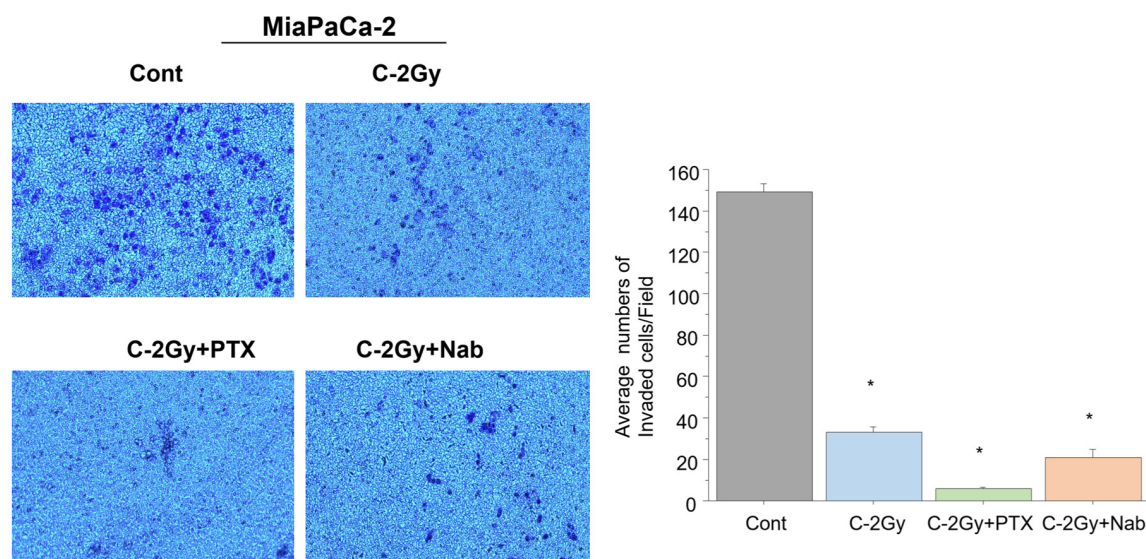


Figure 6. Changes in cell invasion ability. Cell invasion ability after carbon-ion beam IR alone or in combination with PTX or Nab-PTX (Nab) in MiaPaCa-2 cells.

nab-PTX predominantly suppressed p-EGFR and p-ERK, but not p-AKT, in PANC-1 cells. In contrast, the expression of p-AKT, p-EGFR, and p-ERK were all inhibited by carbon-ion beam IR alone, PTX/nab-PTX alone, or the combination treatment in MiaPaCa-2 cells.

Effects of carbon-ion beam IR alone or in combination with PTX/nab-PTX on cell invasion

To investigate the role of PTX/nab-PTX in cell invasion, the effects of PTX/nab-PTX alone or in combination with 2 Gy of carbon-ion beam IR were evaluated using a Transwell invasion assay. The results showed that PTX/nab-PTX alone significantly suppressed cell invasion compared to the control group. This inhibitory effect was further pronounced when combined with carbon-ion beam IR (**Figure 6**).

Discussion

In the present study, we observed that carbon-ion beam IR decreased cell viability in MiaPaCa-2 cells but not in PANC-1 cells, confirming that PANC-1 is inherently more radioresistant. However, PANC-1 remained susceptible to PTX/nab-PTX. This finding suggests that the biological pathways targeted by PTX - specifically microtubule stabilization and G2/M cell cycle arrest [31, 32] - remain functional and effectively act as radiosensitizers, enhancing the effects of CIRT in PC cells. This is con-

sistent with previous studies demonstrating that PTX/nab-PTX increases the radiosensitivity of various human cancer cells [33-35]. Regarding the molecular mechanisms, combining carbon-ion beam IR with PTX/nab-PTX reduced Bax (pro-apoptotic) mRNA levels while simultaneously elevating Bcl-2 (anti-apoptotic) mRNA levels. These results further underscore the high level of resistance in PANC-1 cells to both radiotherapy and chemotherapy.

Subsequent analysis showed that caspase-3/7 activity was significantly enhanced by 1 Gy of carbon-ion beam IR alone, PTX alone, and the combination treatment. In PANC-1 cells, 1 Gy of carbon-ion beam IR failed to induce cleaved caspase-3 and cleaved PARP; however, PTX/nab-PTX alone or in combination with carbon-ion beam IR induced these markers. This suggests that the cell death observed in the combination group for PANC-1 is primarily driven by the chemotherapy component rather than the radiation. Conversely, the induction of cleaved caspase-3 and PARP by 1 Gy of carbon-ion beam IR alone suggests that even a low dose is sufficient to activate the caspase cascade in MiaPaCa-2 cells. This finding aligns with previous reports that carbon-ion beam IR induces apoptosis, an effect that is further enhanced when combined with chemotherapy [20, 22, 25].

Interestingly, the expression of autophagy-related genes *ATG7* and *Beclin1* significantly increased after carbon-ion beam IR, PTX, and combination therapy in PANC-1 cells. This upregulation suggests that PANC-1 responds to radiation by activating autophagy as a survival mechanism rather than undergoing apoptosis. While PTX/nab-PTX does induce apoptosis in PANC-1, the simultaneous activation of autophagy in the combination group might antagonize the treatment. PTX promotes apoptotic cell death, but the cell likely responds by increasing autophagy to repair damage and maintain viability [36]. This “countervailing mechanism” may explain why no further suppression of viability was observed in PANC-1 compared to MiaPaCa-2 cells.

Furthermore, we found that while *VEGF* expression remained unchanged with either carbon-ion beam IR or PTX alone, it was significantly suppressed by the combination therapy. *HIF-1α* expression was unaffected across all groups. The fact that *VEGF* decreased only during combination therapy indicates a synergistic inhibitory effect, suggesting that the combination not only promotes direct cytotoxicity but may also inhibit tumor angiogenesis. Indeed, PTX/nab-PTX alone or in combination with 2 Gy of carbon-ion beam IR significantly suppressed cell invasion, suggesting a potential role in preventing metastasis. This is supported by previous data indicating that carbon-ion beam IR, especially in combination with chemotherapy, can suppress migration and invasion in various cancers [30, 31, 37, 38].

Mechanistically, carbon-ion beam IR, alone or combined with PTX/nab-PTX, primarily suppressed the expression of p-EGFR and p-ERK, while p-AKT expression remained unaffected in PANC-1 cells. This suggests that PANC-1 cells are effectively “stunned” - ceasing proliferation and invasion due to ERK inhibition - but do not undergo cell death because the AKT survival pathway remains active. In contrast, in MiaPaCa-2 cells, p-AKT, p-EGFR, and p-ERK were all significantly suppressed, suggesting that MiaPaCa-2 cells are killed by the simultaneous inhibition of the “growth engine” (ERK) and the “survival insurance” (AKT). Consequently, these cells are forced to undergo apoptosis, as evidenced by increased cleaved caspase-3/PARP. These findings explain the superior responsive-

ness of MiaPaCa-2 to combination therapy and are consistent with reports that MiaPaCa-2 cells are more sensitive to chemotherapy while PANC-1 cells exhibit higher resistance [39, 40].

In summary, PANC-1 cells resist low-dose carbon-ion IR through the activation of autophagy (increased *ATG7* and *Beclin1*). While PTX induces some cell death, the combination does not provide a synergistic effect on viability in this resistant line. In comparison, even low-dose (1 Gy) carbon-ion IR, especially when combined with PTX, significantly reduces MiaPaCa-2 cell viability by triggering apoptosis. However, treatment with 2 Gy IR and PTX strongly inhibits the invasive potential of both cell lines, suggesting a powerful effect in preventing cancer dissemination. The combination (but not single agents) significantly suppresses *VEGF* expression through a *HIF-1α* independent pathway. In conclusion, the combination of carbon-ion beam IR and PTX/nab-PTX is a potent multi-pronged therapy. It eliminates sensitive cells (MiaPaCa-2) and, although it may not eliminate highly resistant cells (PANC-1) at low doses, it successfully inhibits their invasion and angiogenic potential. The resistance in PANC-1 is specifically driven by the persistence of AKT signaling and the induction of autophagy.

Disclosure of conflict of interest

None.

Abbreviations

CIRT, carbon-ion radiotherapy; HIMAC, heavy ion medical accelerator in Chiba; QST, National Institutes for Quantum Science and Technology; PTX, paclitaxel; LET, linear energy transfer.

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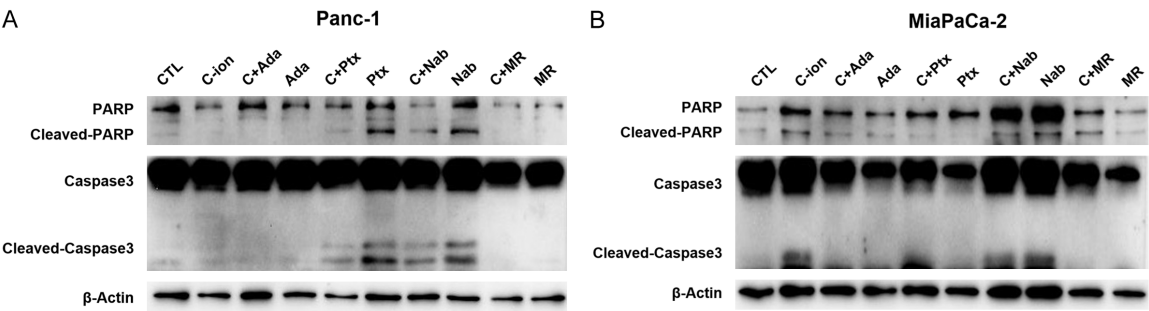
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Paclitaxel sensitizes pancreatic cancer cells to carbon-ion beam irradiation



Supplementary Data. Original data showing the protein expression of cleaved PARP and cleaved caspase-3 following carbon-ion beam IR alone or in combination with PTX, nab-PTX, or other reagents in PANC-1 (A) and MiaPaCa-2 (B) cells (Ada, Adagrasib; MR, MRTX113).