Comparative Study on the Regulation of PD-L1 Expression in Cancer Cells by Heavy Ion and X-ray Radiation

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Abstract

As one of the most promising advanced radiotherapy technologies, heavy-ion radiotherapy has developed rapidly in China; however, it still faces challenges regarding the escape and metastasis of malignant tumors. Recent studies have found that radiotherapy combined with immune checkpoint inhibitors can eliminate or control distant metastasis of malignant tumors. Nevertheless, research data on the regulation of immune checkpoint protein expression by heavy ions remain scarce.

In this study, 92-1 melanoma, A549 lung cancer, and HGC-27 gastric cancer cell lines were irradiated with carbon-ion beams and X-rays. The protein and transcriptional levels of programmed death-ligand 1 (PD-L1) were detected using Western Blot and RT-qPCR techniques. The study found that both types of radiation significantly upregulated the protein and mRNA expression levels of PD-L1.

Regarding dose-effect relationships, the carbon-ion beam exhibited a stronger dose-dependent effect on PD-L1 protein levels. Within the range of 0.5 Gy to 4 Gy, PD-L1 protein levels showed a two-stage linear increase with increasing dose. In contrast, X-rays caused significant upregulation of PD-L1 protein levels at 2 Gy, but the upward trend slowed as the dose increased.

Regarding time-effect relationships, PD-L1 protein levels showed a trend of initially decreasing and then increasing after carbon-ion beam irradiation, whereas this phenomenon was absent following X-ray irradiation. These results indicate that, similar to X-rays, carbon-ion beams can significantly promote PD-L1 expression in different cancer cells, though differences exist in terms of dose and time effects. This conclusion suggests a potential risk of immune escape by cancer cells during heavy-ion radiotherapy and provides an experimental basis

for the necessity and application strategies of combining heavy-ion radiotherapy with immunotherapy.

Full Text

Preamble

Comparative Study of Heavy Ion and X-ray Radiation Regulating PD-L1 Expression in Cancer Cells

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Abstract

Objective: To investigate the differences in the expression of Programmed Death-Ligand 1 (PD-L1) in cancer cells induced by heavy ion radiation compared to X-ray radiation, and to explore the underlying molecular mechanisms.

Methods: Human lung cancer cells (A549) and breast cancer cells (MCF-7) were irradiated with varying doses of Carbon-ion beams (heavy ions) and X-rays. PD-L1 mRNA and protein expression levels were quantified using quantitative Real-Time PCR (qRT-PCR) and Western Blotting at different time points post-irradiation. Flow cytometry was employed to detect the expression of PD-L1 on the cell surface. Furthermore, the activation of DNA damage response pathways and related signaling pathways, such as the JAK/STAT and cGAS-STING pathways, was analyzed to elucidate the mechanisms driving radiation-induced PD-L1 upregulation.

Results: Both heavy ion and X-ray radiation significantly upregulated PD-L1 expression in a dose- and time-dependent manner. However, heavy ion radiation induced a more pronounced and sustained increase in PD-L1 expression at lower physical doses compared to X-rays. Mechanistically, heavy ion radiation caused more complex DNA double-strand breaks (DSBs), leading to enhanced activation of the cGAS-STING pathway and subsequent interferon (IFN) production, which in turn mediated the upregulation of PD-L1.

Conclusion: Heavy ion radiation is more effective than X-rays in inducing PD-L1 expression. As one of the most promising advanced radiotherapy technologies, heavy ion therapy is developing rapidly in China. However, the escape and metastasis of malignant tumors remain significant clinical challenges. Recent studies have demonstrated that combining radiotherapy with immune checkpoint inhibitors can eliminate or control distant metastases. However,

research regarding the regulation of immune checkpoint protein expression by heavy ions remains scarce.

In this study, 92-1 melanoma, A549 lung cancer, and HGC-27 gastric cancer cell lines were irradiated with carbon ion beams and X-rays. The protein and transcriptional levels of Programmed Death-Ligand 1 (PD-L1) were subsequently detected using Western Blot and RT-qPCR. The results indicated that both types of radiation significantly upregulated the protein and mRNA expression levels of PD-L1. Regarding dose-effect relationships, the carbon ion beam exhibited a stronger dose-dependent effect on PD-L1 protein levels. Within the range of 0.5 Gy to 4 Gy, PD-L1 protein levels showed a two-stage linear increase with dose. In contrast, while X-rays induced significant PD-L1 upregulation at 2 Gy, the upward trend slowed as the dose increased. Regarding temporal effects, PD-L1 protein levels exhibited a trend of initially decreasing and then increasing following carbon ion beam irradiation, a phenomenon not observed with X-rays. These findings suggest that, similar to X-rays, carbon ion beams significantly promote PD-L1 expression across different cancer cells, though differences exist in their dose and time-dependent responses. This conclusion highlights the potential risk of immune escape by cancer cells during heavy-ion radiotherapy and provides an experimental basis for the necessity and strategic application of combining heavy-ion radiotherapy with immunotherapy.

Keywords: Carbon ion beam; X-ray; PD-L1; Immune escape **CLC number:** Q691 **Document code:** A

1 Introduction

With the aging trend of the domestic population, the number of new cancer cases and deaths in China has ranked first globally in recent years, accounting for approximately 25% of the global total [**?**]. Developing more advanced and precise cancer treatment technologies for human life and health has become a strategic requirement for national modernization. Compared to photon radiation (such as X-rays or γ-rays), heavy ions possess several distinct characteristics as radiotherapy particles: 1. They exhibit a unique depth-dose distribution known as the Bragg peak, which allows the beam energy to be precisely released within the tumor region by adjusting parameters, thereby reducing damage to surrounding normal tissues; 2. Their primary biological effect is the direct induction of DNA double-strand breaks (up to 70%), resulting in a higher Relative Biological Effectiveness (RBE) that is more difficult for cells to repair, thus reducing the probability of tumor recurrence [**?**, **?**]; 3. They have low oxygen dependence, providing a stronger killing effect on hypoxic tumors and tumor cells in different phases of the cell cycle; 4. The interaction between heavy ions and human tissue can excite positron beams, facilitating the evaluation of in vivo dose distribution using PET-CT (Positron Emission Tomography-Computed Tomography).

Consequently, heavy-ion radiation is considered the most ideal radiotherapy modality of the 21st century [**?**]. Since the release of the "2018 Catalogue for

the Management of Large-scale Medical Equipment Configuration Licenses" and related implementation rules by the National Health Commission in April 2018, proton and heavy-ion therapy systems have been explicitly managed as Class A medical equipment. These systems have developed rapidly during the "14th Five-Year Plan" period. According to the latest statistics from "Proton China—Proton Research," as of December 31, 2025, there are 96 proton and heavy-ion projects currently in operation, under construction, or planned in China.

Heavy ions offer significant advantages in treating single tumor lesions or achieving local tumor control; however, challenges remain regarding the escape and metastasis of malignant tumors [**?**, **?**]. Recent studies have found that conventional radiotherapy combined with immunotherapy can significantly eliminate or control distant metastases. One of the most promising immunotherapies currently in global clinical use involves immune checkpoint protein inhibitors. The body' s immune system maintains a delicate balance between tolerating its own normal cells and clearing foreign substances or mutated cells. If the immune system is strongly inhibited, low immunity may fail to suppress tumor growth; conversely, an overactive immune system may lead to autoimmune reactions and related diseases. Normal cells achieve immune tolerance through the secretion of inhibitory cytokines or the expression of immune cell inhibitory receptor proteins (immune checkpoints) for mutual recognition. Since the 1990s, increasing research has shown that tumor cells can express ligands that bind to these immune checkpoints to avoid clearance by immune cells. This mechanism, known as immune escape, is a primary reason why some malignant tumors are difficult to eradicate [**?**, **?**].

Following the discovery of immune escape and the elucidation of its underlying mechanisms, an increasing number of immune checkpoint proteins have been identified and used to develop immunotherapy drugs. The first identified immune checkpoint protein was Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). In animal models, antibodies blocking CTLA-4 significantly inhibited tumor occurrence and progression [**?**]. In 2011, the FDA approved the first immune checkpoint inhibitor, Ipilimumab, for the treatment of advanced melanoma. However, patients receiving anti-CTLA-4 drugs are prone to various adverse reactions, such as skin rashes, colitis, thyroiditis, and hepatitis, all of which resemble autoimmune diseases [**?**]. Subsequently, Tasuku Honjo and Lieping Chen discovered a more specific combination of immune checkpoint proteins: Programmed Cell Death Protein 1 (PD-1) and Programmed Death-Ligand 1 (PD-L1). PD-1 is an inhibitory receptor expressed on activated tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes, while its primary ligand, PD-L1, is highly expressed in many types of cancer. Unlike CTLA-4, which regulates the immune activation of most T lymphocytes, the PD-1 checkpoint primarily regulates the activity of infiltrating cytotoxic T lymphocytes. This makes drugs blocking the PD-1/PD-L1 pathway significantly less toxic than anti-CTLA-4 agents [**?**]. Since 2014, the US FDA has approved several therapeutic drugs targeting the PD-1/PD-L1 proteins, including Pembrolizumab, Nivolumab, and Atezolizumab [**?**]. Since 2017, 23 therapeutic drugs targeting PD-1/PD-L1 have been approved for marketing

in China.

Studies have found that ionizing radiation significantly upregulates PD-L1 protein expression in various cancer cells, suggesting that radiotherapy may enhance the immune escape capabilities of these cells [**?**]. Beginning in 2016, Joe Y. Chang' s team at the MD Anderson Cancer Center conducted research on the combination of Stereotactic Body Radiation Therapy (SBRT) and PD-1/PD-L1 monoclonal antibodies. Through extensive animal models and clinical trials, they found that SBRT combined with immunotherapy significantly improved tumor control rates compared to monotherapy [**?**]. Increasing clinical evidence also indicates that radiotherapy combined with PD-1/PD-L1 inhibitors can synergistically suppress both primary and distant tumors [**?**]. These studies established the necessity of combining radiotherapy with immunotherapy. However, most current research results are based on photon radiation (X-rays or γ-rays), and data related to heavy ions are scarce. Therefore, this study focuses on heavy-ion radiation, utilizing carbon ion beams and X-rays to irradiate various cancer cells. By detecting the effects on PD-L1 expression at both the protein and mRNA levels, we compare the differences in how heavy ions and X-rays regulate PD-L1. This work aims to provide a research foundation for clinical trials involving heavy-ion radiotherapy combined with PD-1/PD-L1 monoclonal antibodies to control malignant tumor metastasis.

2 Materials and Methods

2.1 Reagents and Instruments

The reagents required for the experiment included RPMI-1640 medium (Gibco, USA), DMEM medium (Gibco, USA), and Fetal bovine serum (FBS) purchased from Biological Industries (Israel). Penicillin-streptomycin solution was obtained from Solarbio (China), and the BCA protein assay kit was sourced from KangCheng Bio-tech (Shanghai, China). The following antibodies were used: PD-L1 (Proteintech, 28076-1-AP), α-Tubulin (Abcam, ab52866), HRP-conjugated goat anti-rabbit secondary antibody (Abcam, ab205718), and HRP-conjugated goat anti-mouse secondary antibody (Abcam, ab205719).

Heavy-ion irradiation was performed using the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics, Chinese Academy of Sciences. Other primary laboratory instruments included an X-ray irradiator (Precision, USA), a refrigerated high-speed centrifuge (Centrifuge 542R, Eppendorf, USA), a Uvitec imaging system (Alliance-LD4, UK), and an ultrasonic cell crusher (JY92-IIN, Ningbo Scientz Biotechnology, China).

2.2 Cell Culture

Human uveal melanoma cells (92-1), human non-small cell lung cancer cells (A549), and human gastric cancer cells (HGC-27) were provided by the Biological Radiation Effects Laboratory of the Institute of Modern Physics, Chinese Academy of Sciences. The 92-1 and HGC-27 cell lines were cultured in

RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The A549 cell line was cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. All three cell lines were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.3 Irradiation Protocol

Irradiation treatment was performed 24–36 hours after the cells had adhered to the surface. Prior to irradiation, the culture medium was replaced with fresh medium. To minimize the attenuation of the radiation beam by the liquid, the volume of the medium was adjusted during irradiation to just sufficiently cover the cell monolayer. Heavy-ion irradiation was conducted using carbon ion beams ($^{12}C^{6+}$) generated by the Heavy Ion Research Facility in Lanzhou (HIRFL). The linear energy transfer (LET) was maintained between 30 and 50 keV/μm, with a dose rate of approximately 2 Gy/min. X-ray irradiation was provided by an X-RAD 225 X-ray irradiator operating at a voltage of 225 kV and a dose rate of approximately 2 Gy/min.

2.4 Western Blot (WB)

Sample Preparation: The culture medium was discarded, and the cells were washed twice with pre-cooled PBS buffer. An appropriate volume of RIPA lysis buffer (Invitrogen, USA) was added, and the cells were lysed on ice for 10 min. The lysates were then collected into 1.5 mL centrifuge tubes using a cell scraper. The lysates were subjected to ultrasonication in an ice bath using an ultrasonic cell crusher. Subsequently, the samples were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was transferred to new centrifuge tubes. Protein concentrations were determined using a BCA protein assay kit and standardized accordingly. After adding loading buffer and vortexing, the samples were heated in a metal bath at 98 °C for 10 min to denature the proteins and then stored for subsequent use.

Electrophoresis: Resolving gels of appropriate concentrations were selected based on the molecular weights of the target proteins. A total of 20 μg of protein was loaded into each well. Electrophoresis conditions were set as follows: constant voltage of 80 V for 30 min during the stacking gel phase, followed by 120 V for 1 h during the resolving gel phase. Simultaneously, PVDF membranes were cut to the appropriate size, activated by immersion in methanol for 1 min, and equilibrated in transfer buffer.

Protein Transfer: The transfer sandwich was assembled from the anode to the cathode in the following order: sponge, filter paper, PVDF membrane, gel, filter paper, and sponge. Wet transfer was performed at a constant voltage of 120 V. The transfer duration was adjusted according to the protein molecular weight: 60 min for proteins <20 kDa, 90 min for proteins between 20–100 kDa, and 150 min for proteins >100 kDa. Following transfer, the PVDF membranes were blocked with 5% non-fat milk at room temperature for 2 h. The membranes

were then incubated with a PD-L1 primary antibody diluted in 5% non-fat milk for 2 h at room temperature. The membranes were washed three times with 0.1% PBST (PBS containing 0.1% Tween-20) for 10 min each. Subsequently, the membranes were incubated with secondary antibodies diluted in 5% non-fat milk for 2 h at room temperature, followed by three additional washes with 0.1% PBST (10 min each). Finally, protein bands were detected using an ECL chemiluminescence kit.

2.5 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA Extraction: The culture medium was discarded, and the cells were washed twice with PBS buffer. An appropriate volume of Trizol reagent (Qiagen, USA) was added, and the mixture was allowed to stand at room temperature for 1 min. The adherent cells were repeatedly pipetted to ensure complete lysis, and the lysate was transferred to an RNase-free 1.5 mL centrifuge tube. Chloroform was added at a ratio of 200 μL per 1 mL of Trizol, followed by vigorous shaking for 15 s and incubation at room temperature for 5 min. The mixture was then centrifuged at 12,000 rpm for 15 min at 4 °C. Approximately 350 μL of the upper colorless aqueous phase was carefully collected and transferred to a new RNase-free centrifuge tube. An equal volume (350 μL) of isopropanol was added, mixed by inversion, and incubated at room temperature for 30 min to precipitate the RNA. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatant was discarded. The pellet was washed with 1 mL of 75% ethanol. Following another centrifugation at 12,000 rpm for 15 min at 4 °C, the ethanol was discarded, and the pellet was air-dried. The RNA pellet was dissolved in RNase-free water. The absorbance values at A_{260}, A_{280}, and A_{320} were measured to calculate the concentration and purity (A_{260}/A_{280} ratio).

RT-qPCR: Real-time quantitative PCR amplification was performed using the TB Green® Premix Ex Taq™ II kit (Takara, Japan). The 20 μL reaction system was prepared as follows: 10 μL of TB Green Premix Ex Taq II (2×), 0.8 μL each of forward and reverse primers (10 μM) (Sangon Biotech, China), 0.4 μL of ROX Reference Dye (50×), 2 μL of cDNA template, and 6 μL of ddH_2O. The reaction conditions were: initial denaturation at 95 °C for 30 s; PCR amplification (40 cycles) consisting of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 31 s. Data analysis was conducted using GAPDH as the internal reference gene, and the relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6 Statistical Analysis

Experimental results are expressed as mean ± standard deviation. Comparisons of means between multiple groups were performed using one-way analysis of variance (ANOVA), and comparisons between two groups were performed using t-tests. Statistical significance was determined using Analysis of Variance (ANOVA). Significance levels were defined as follows: *, #, + indicate $p < 0.05$,

representing a statistically significant difference; **, ##, ++ indicate $p < 0.01$, representing a highly significant statistical difference. Data analysis was performed using GraphPad Prism 10 (GraphPad Software, USA), and all figures were generated using OriginPro 2018 (OriginLab Corporation, USA).

3 Results

3.1 Effects of Carbon-ion Radiation on PD-L1 Protein Levels in Cancer Cells

Research was conducted to investigate the effects of carbon ion beam irradiation on PD-L1 protein levels in cancer cells from two perspectives: time-dependent effects and dose-dependent effects.

Regarding the time-dependent effects, 92-1, A549, and HGC-27 cells were irradiated with a 2 Gy carbon ion beam. Protein samples were extracted at 8 h, 24 h, 48 h, and 72 h post-irradiation, and PD-L1 protein levels were detected using Western Blot (WB) technology. The results indicated that compared to the Ctrl group (unirradiated), PD-L1 protein levels in the three types of cancer cells decreased slightly within 8–24 h after irradiation. However, after 24 h, PD-L1 protein levels increased significantly, reaching a plateau at 48 h post-irradiation, with no further linear upward trend observed thereafter [Figure 1: see original paper] (a and b).

In terms of dose-dependent effects, the three types of cancer cells were irradiated with carbon ion beams at doses of 0.5, 2, and 4 Gy. Protein samples were extracted 48 h post-irradiation, and PD-L1 protein levels were measured via WB. The results showed that in 92-1 and HGC-27 cells, even a 0.5 Gy carbon ion beam dose led to a significant increase in PD-L1 protein levels (greater than 1.5-fold) compared to the Ctrl group, followed by a gradual increase as the dose intensified. In contrast, for A549 cells, PD-L1 protein levels increased only slightly after 0.5 Gy and 2 Gy carbon ion irradiation, while a significant increase was observed following the 4 Gy dose, reaching approximately 1.5 times the control level [Figure 1: see original paper] (c and d). These results indicate that carbon ion radiation can significantly upregulate PD-L1 protein levels in all three types of cancer cells. Furthermore, PD-L1 expression levels exhibited a sharp upward trend following specific time points and dosage thresholds. Notably, 92-1 and HGC-27 cells demonstrated greater sensitivity to carbon-ion radiation compared to A549 cells.

[Figure 1: see original paper] Effects of carbon-ion radiation on PD-L1 protein levels in cancer cells. (a) PD-L1 protein levels in 92-1, A549, and HGC-27 cells detected at 8 h, 24 h, 48 h, and 72 h following irradiation with a 2 Gy carbon-ion beam; (b) Quantitative analysis of PD-L1 protein levels from panel (a), *p < 0.05, **p < 0.01; (c) PD-L1 protein levels in 92-1, A549, and HGC-27 cells detected 48 h after irradiation with carbon-ion beam doses of 0.5, 2, and 4 Gy; (d) Quantitative analysis of PD-L1 protein levels from panel (c), *p < 0.05, **p < 0.01.

3.2 Effects of X-ray Irradiation on PD-L1 Protein Levels in Cancer Cells

Simultaneously, the effects of X-ray irradiation on PD-L1 protein levels in three types of cancer cells were examined to provide a comparison with the effects of carbon ion beam irradiation. Based on common single-fraction doses used in radiotherapy, 92-1, A549, and HGC-27 cells were irradiated with 2 Gy and 4 Gy of X-rays. Protein samples were collected at 24 h, 48 h, and 72 h post-irradiation, and PD-L1 protein levels were detected using Western blot (WB) analysis.

As shown in [Figure 2: see original paper], regarding temporal effects, PD-L1 levels in 92-1 cells increased significantly as early as 24 h post-irradiation and exhibited a linear upward trend over time. In A549 cells, PD-L1 protein levels began to rise at 24 h and reached a peak at 48 h. In HGC-27 cells, PD-L1 levels initially showed a downward trend at 24 h, followed by a significant linear increase over time. Regarding dose-response effects, compared to the control (Ctrl) group, both 2 Gy and 4 Gy X-ray irradiation significantly upregulated PD-L1 protein levels across all three cell lines; however, no clear dose-dependent effect was observed. These results indicate that while X-ray irradiation significantly upregulates PD-L1 protein levels in cancer cells, differences in dose and time-response patterns persist across different cell types.

[Figure 2: see original paper] Effects of X-ray irradiation on PD-L1 protein levels in cancer cells. (a) PD-L1 protein levels in 92-1, A549, and HGC-27 cells detected at 24 h, 48 h, and 72 h following 2 Gy and 4 Gy X-ray irradiation; (b) Quantitative analysis of PD-L1 protein levels from panel (a), $^{*}p < 0.05$, $^{**}p < 0.01$.

3.3 Effects of Carbon Ion and X-ray on PD-L1 mRNA Expression Levels in Cancer Cells

The results from the previous two sections demonstrate that both carbon ion and X-ray radiation can significantly increase PD-L1 protein levels in cancer cells. To determine whether PD-L1 mRNA expression levels exhibit a similar trend, we investigated the dose-response and time-course effects of carbon ion and X-ray irradiation on PD-L1 mRNA expression across three types of cancer cells.

First, we utilized doses of 0.5, 2, and 4 Gy of the carbon ion beam, and 2 and 4 Gy of X-ray radiation to irradiate 92-1, A549, and HGC-27 cells. At 24 h post-irradiation, RNA samples were extracted from the cells, and the relative changes in PD-L1 mRNA expression were measured using RT-qPCR. As shown in [Figure 3: see original paper], both carbon ion (Fig. 3a) and X-ray (Fig. 3c) irradiation significantly promoted PD-L1 mRNA expression in the three types of cancer cells compared to the Ctrl group (unirradiated), exhibiting a clear dose-dependent effect. Furthermore, the three types of cancer cells were irradiated with 2 Gy carbon ion beams and 4 Gy X-rays, and RNA samples were collected

at 8 h, 24 h, 48 h, and 72 h post-irradiation (for carbon ions) and 24 h, 48 h, and 72 h post-irradiation (for X-rays) to detect relative changes in PD-L1 mRNA expression. It was observed that following irradiation with carbon ion beams (Fig. 3b) and X-rays (Fig. 3d), the PD-L1 mRNA expression in all three cancer cell lines showed a trend of initially increasing and then declining, generally peaking between 24 and 48 h post-irradiation. These results indicate that both carbon ion beams and X-rays can significantly increase the expression levels of PD-L1 mRNA in cancer cells, demonstrating similar dose- and time-dependent effects.

[Figure 3: see original paper] Effects of carbon ion beam and X-ray irradiation on PD-L1 mRNA expression in cancer cells. (a) Relative expression levels of PD-L1 mRNA in 92-1, A549, and HGC-27 cells 48 h after irradiation with 0.5, 2, and 4 Gy carbon ion beams; (b) Relative expression levels of PD-L1 mRNA in the aforementioned three cell lines from 8 to 72 h after irradiation with a 2 Gy carbon ion beam; (c) Relative expression levels of PD-L1 mRNA in the three cell lines 48 h after irradiation with 2 and 4 Gy X-rays; (d) Relative expression levels of PD-L1 mRNA in the three cell lines from 24 to 72 h after irradiation with 4 Gy X-rays. *, #, +, $P < 0.05$; **, ##, ++, $P < 0.01$.

3.4 Comparison of Carbon Ion Beam and X-ray Regulation of PD-L1 Protein Levels

Through the detection of protein and mRNA levels, it was found that both carbon ion and X-ray radiation significantly promote the expression of the $PD-L1$ gene and increase PD-L1 protein levels in various cancer cells. However, differences exist in their dose-response and time-course effects.

Regarding the dose-response effect, carbon ions exert a more significant impact on PD-L1 protein levels compared to X-rays. Furthermore, the upward trend in protein levels slowed down after dose points of 0.5 Gy for carbon ions and 2 Gy for X-rays [Figure 4: see original paper] (a and b). In terms of the time-course effect, PD-L1 protein levels exhibited a trend of initially decreasing and then increasing following carbon ion irradiation, a phenomenon not observed with X-ray irradiation. Similarly, the upward trend in PD-L1 protein levels induced by both carbon ion and X-ray radiation appeared 24 hours post-irradiation [Figure 4: see original paper] (c and d). However, the maximum radiation dose and longest time point set in this study were 4 Gy and 72 h, respectively. Changes in PD-L1 protein levels following higher radiation doses and longer irradiation times warrant further investigation.

[Figure 4: see original paper] Comparison of the effects of carbon ion beam and X-ray radiation on PD-L1 protein levels in cancer cells. (a-b) Comparison of the dose-response effects of PD-L1 protein in cancer cells to carbon ion beam and X-ray radiation; (c-d) Comparison of the time-course effects of PD-L1 protein in cancer cells to carbon ion beam and X-ray radiation.

4 Discussion

Heavy-ion radiotherapy and PD-1/PD-L1 monoclonal antibodies represent the most promising advanced radiotherapy techniques and immunotherapy drugs for the future. The combined use of immunotherapy helps inhibit the immune escape of tumor cells and is expected to address the challenges of malignant tumor metastasis and recurrence faced by heavy-ion radiotherapy. However, many fundamental questions regarding the combination of heavy-ion radiotherapy and immunotherapy remain to be explored, such as the effects of heavy-ion radiation on PD-L1 expression in different tumor cells and its impact on the immune escape capacity of cancer cells, as well as the optimal timing for administering PD-1/PD-L1 monoclonal antibodies following clinical radiotherapy [**?**]. Currently, basic research data related to heavy-ion radiation remains scarce.

This study focuses on carbon ion radiation by comparing the effects of carbon ion beams and X-rays on PD-L1 expression in various cancer cells (92-1, A549, and HGC-27). It was found that both types of radiation significantly upregulate PD-L1 expression at both the transcriptional and protein levels within certain dose and time ranges. This result is consistent with existing research conclusions regarding the regulation of tumor immunity by ionizing radiation [**?**]. For example, X-rays lead to the upregulation of PD-L1 protein levels on the surface of tumor cells in various mouse cancer models [**?**]; meanwhile, studies have also found that carbon ion beams induce the upregulation of PD-L1 protein levels in human cervical cancer cell lines Hela and SiHa in a dose-dependent manner [**?**]. Therefore, radiation may induce immunosuppression by promoting PD-L1 expression in cancer cells, thereby limiting its therapeutic efficacy; this also provides a molecular-level explanation for the radiotherapy resistance observed clinically.

This study specifically compared the differences between carbon ion beams and X-rays in regulating PD-L1 protein levels. In terms of dose-response, the effect of carbon ion beams on PD-L1 protein levels showed a stronger dose-dependent relationship. Within the range of 0.5 Gy to 4 Gy, PD-L1 protein levels increased in a two-stage linear fashion with increasing dose. In contrast, X-rays induced a significant upregulation of PD-L1 protein levels at 2 Gy, but the upward trend slowed thereafter, reaching a plateau. This difference may be due to the distinct physical characteristics of the two types of radiation. Compared to X-rays, carbon ion beams have a higher Linear Energy Transfer (LET) and a higher energy deposition density. High-LET radiation triggers extensive, complex, and difficult-to-repair clustered DNA double-strand breaks (DSBs) in cancer cells [**?**]. This pattern of DNA damage may induce stronger molecular signaling for the regulation of PD-L1 expression [**?**].

Regarding the time-course effect, an interesting phenomenon was observed: in the early stage (0-24 h) after carbon ion beam irradiation, PD-L1 protein levels showed a significant downward trend before rising, whereas X-ray irradiation did not exhibit this trend. This early decline might be due to the impact of high-

LET radiation on the cellular protein synthesis process. After 24 h of radiation, the PD-L1 protein levels in the carbon ion group rose rapidly over time. Studies have shown that DNA double-strand breaks in tumor cells can upregulate PD-L1 expression by activating the ATM/ATR/Chk1 pathway. The level of DNA double-strand breaks induced by carbon ion beams is significantly higher than that of X-rays, which may explain why carbon ion beams induce a more significant and sustained upregulation of PD-L1 protein levels compared to X-ray irradiation [**?**]. Furthermore, research has found that the MYC transcription factor can bind to the PD-L1 promoter to enhance its expression. Radiation-induced stress responses are often accompanied by the activation of related transcription factors such as MYC, which may also be one of the potential mechanisms by which radiation promotes PD-L1 expression [**?**].

The aforementioned results provide an experimental basis for the necessity of combining radiotherapy with PD-1/PD-L1 immune checkpoint inhibitors to control the metastasis of malignant tumors. First, radiotherapy—especially carbon ion beam irradiation—significantly upregulates PD-L1 expression in cancer cells, an effect that may promote an immunosuppressive tumor microenvironment. Consequently, the combined use of PD-1/PD-L1 monoclonal antibodies can help relieve the inhibition of T-cell activity, achieving a synergistic "1+1 > 2" effect [**?**]. Furthermore, this study found that PD-L1 protein levels in cancer cells began to rise significantly 24 hours after irradiation, suggesting that PD-L1 antibody drugs should be administered at an early stage following radiotherapy. Existing clinical results have shown that in patients with non-small cell lung cancer, the prognosis for those receiving PD-L1 monoclonal antibodies within 14 days after combined chemoradiotherapy is significantly better than for those treated after 14 days [**?**]. For patients with melanoma brain metastases, when radiotherapy is combined with immune checkpoint inhibitors, the efficacy is significantly superior for patients who begin combination therapy within 4 weeks of radiotherapy compared to those who start more than 4 weeks after surgery [**?**].

Currently, there are very few studies on the combination of carbon-ion beams and PD-L1 monoclonal antibodies for cancer treatment. This study identified differences in the dose and time windows for PD-L1 protein upregulation induced by carbon-ion beams versus X-rays, providing a reference for future animal and clinical trials of heavy-ion radiotherapy combined with immunotherapy. Additionally, the study found that the rapid increase in PD-L1 protein levels induced by carbon-ion beams primarily occurred within the 0.5–2 Gy dose range, with the upward trend gradually slowing down after 2 Gy. This phenomenon suggests that employing hypofractionated irradiation plans during carbon-ion therapy may help reduce the risk of immune escape by tumor cells [**?**].

Although this study compares and preliminarily reveals the similarities and differences between carbon-ion and X-ray radiation regarding PD-L1 expression in cancer cells, certain limitations remain. First, cytokines within the tumor microenvironment are crucial for the regulation of PD-L1 expression, and it re-

mains difficult to simulate this complex cellular environment [**?**]. Second, while this study suggests that the ATM/ATR/Chk1 and MYC pathways play a role in radiation-induced PD-L1 upregulation, the specific mechanisms require further investigation. Furthermore, the maximum dose for carbon ions and X-rays in this study was set at 4 Gy, with a maximum detection time point of 72 hours; research data beyond these doses and time points remain unclear. Relevant expression changes in animal or clinical samples, as well as the optimal combination regimens, still need further exploration [**?**]. Recent research has discovered that ultra-high dose rate (FLASH) carbon-ion beams have the effect of inhibiting PD-L1 expression. Investigating the effects and mechanisms of conventional versus ultra-high dose rate carbon-ion beams on immune checkpoint proteins will also be a highly promising research direction [**?**].

5 Conclusion

In summary, the results of this study demonstrate that, similar to X-rays, carbon-ion beams can significantly promote PD-L1 expression in various cancer cells. However, differences exist between carbon ions and X-rays regarding the dose- and time-dependent effects of inducing elevated PD-L1 protein levels. These findings highlight the potential risk of cancer cell immune escape during heavy-ion radiotherapy and provide an experimental basis for the necessity of combining heavy-ion radiotherapy with immunotherapy. Furthermore, this work offers new insights and perspectives on the potential challenges faced in the future application of heavy-ion radiotherapy combined with PD-1/PD-L1 antibody drugs.

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A Comparative Study on the Regulation of PD-L1 Expression in Cancer Cells by Heavy Ion and X-ray

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Note: Figure translations are in progress. See original paper for figures.