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REVIEW

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Carbon ion irradiation mobilizes antitumor immunity: from concept to the clinic

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Abstract

Carbon ion radiotherapy (CIRT), a type of particle therapy, is at the forefront of clinical oncology treatments due to its superior physical properties and biological performance. Although CIRT has demonstrated outstanding therapeutic outcomes in clinical settings, the biological mechanisms underpinning its effects, particularly its immunogenic potential and the superiority of its induced antitumor immune response compared to photon radiotherapy, remain areas of active investigation. This review summarizes the latest research progress on the mechanisms of antitumor immune responses triggered by CIRT and discusses preclinical and clinical studies related to combined CIRT and immunotherapy (CCIT). Against the backdrop of extensive research and significant clinical efficacy achieved by combining radiotherapy with immunotherapy, this review provides a theoretical foundation for a better understanding of the superior tumor cell-killing effects of CIRT and the underlying immunological mechanisms. Further insights into the factors affecting the efficacy, toxic effects, and developmental limitations of this combination therapy mode will be instrumental in guiding the conduction of CCIT studies.

Keywords Carbon ion radiotherapy, Immunotherapy, Tumor immunogenicity, Antitumor immune response

Introduction

Photon radiotherapy (PhRT) remains the most commonly used form of radiotherapy today. Limited by its physical and biological characteristics, despite the assistance of computer technology and imaging methods to optimize radiotherapy plans, traditional radiotherapy still faces unresolved issues such as poor therapeutic effects and severe side effects [1, 2]. The American physicist Robert Rathbun Wilson first proposed the use of heavy charged particles for cancer treatment in 1946 [3]. Subsequently, researchers at the Lawrence Berkeley Laboratory (LBL) in the United States initiated studies from 1975 to 1992 on treating cancer patients with heavy ions, including carbon ions [3–5]. Since 1994, Japan has established the world's first medical facility dedicated to heavy ion radiotherapy at the National Institute of Radiological Sciences (NIRS) in Chiba, starting the use of carbon ions for treatment and scientific research and gradually becoming the largest carbon ion radiotherapy (CIRT) center in the world [6]. Currently, institutions with CIRT capabilities are mainly located in Asia and Europe, with the Mayo Clinic announcing the establishment of the first carbon ion radiation facility in the United States at Florida in 2020 [7]. CIRT plays an irreplaceable role in malignant tumors traditionally resistant to radiotherapy, such as bone and soft-tissue sarcomas, locally recurrent rectal cancer and pancreatic cancer [6, 8, 9].

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Tumor immune escape is a severe issue that plays a significant role in tumor development and resistance to immunotherapy. The immune system is involved in every stage of tumor occurrence and progression, which can be divided into the elimination, equilibrium, and escape phases, known as the three "E" stages [10, 11]. The immune escape mechanism evolved by tumors enables tumors to survive despite the host immune system's attempts to combat them [11]. Although immunotherapy has been successful in treating many advanced cancers, a portion of patients still do not respond or develop resistance after treatment, leading to therapeutic failure [12–14]. Consequently, a primary focus of current research has been to identify partners that enhance the efficacy of immunotherapy while maintaining acceptable side effects.

Notably, the combination of immunotherapy and radiotherapy has garnered considerable attention in recent years. Immunotherapy tends to be less effective against solid tumors with high tumor burdens in advanced stages, while radiotherapy, in addition to its debulking effect, can also stimulate the release of tumor antigens, induce immunogenic cell death (ICD), and reshape the tumor immune microenvironment (TIME), thereby reactivating the anti-tumor immune response [15–17]. Conversely, immunotherapy can amplify the immune response, significantly increase the occurrence of abscopal effects, and prolong patient survival. The immunotherapy and radiotherapy is synergistic, commonly referred to as radioimmunotherapy [15, 18]. However, the results of clinical trials for combined PhRT and immunotherapy (CPIT) have not always supported its superior effectiveness to either therapy alone [19–21]. This limitation may be due to the suboptimal dose delivery of PhRT, its substantial toxicity, and the insufficient specificity or intensity of the immune response it generates, making PhRT less compatible with immunotherapy. Preliminary evidence suggests that CIRT may enhance antitumor immunity more effectively than PhRT, potentially improving radioimmunotherapy outcomes [22–24]. Based on the limited data available, without strictly limiting the target patient population and clinical characteristics, this paper aims to provide a comprehensive review of the antitumor immune responses triggered by CIRT, revisit the related research on combined CIRT and immunotherapy (CCIT), and discuss the factors affecting the efficacy, toxic reactions, and developmental limitations of CCIT to offer a broader reference for the conduction of CCIT clinical research and its clinical application.

CIRT enhances the antitumor immune response

Radiation-mediated antitumor immune responses include adaptive and innate immune mechanisms. Initially, within an immunopermissive tumor microenvironment, ionizing radiation facilitates the generation or exposure of tumor antigens and the release of damage-associated molecular patterns (DAMPs). These tumor antigens are captured, processed, and presented to T cells by antigen-presenting cells (APCs), subsequently activating T cells to proliferate, infiltrate the tumor, and kill tumor cells, accompanied by further release of tumor antigens from the tumor [15, 25, 26]. Additionally, innate immune cells such as natural killer (NK) cells and related immune molecules in the microenvironment also play a critical role in this process [25, 26]. Although in CPIT, PhRT acts as an ignition, shifting the tumor from "cold" to "hot" and enhancing the likelihood of an abscopal effect, achieving a complete cure remains challenging [27]. Although not systematically summarized, abscopal effects have occurred in clinical cases where CIRT was used to treat advanced tumors with metastasis or recurrent tumors with multiple lymph node metastases [26, 28–31]. In preclinical experimental models, CIRT also seems to induce abscopal effects [32]. Notably, despite similarities in immune responses following exposure to low and high linear energy transfer (LET) radiations, CIRT, which has superior physical and biological characteristics compared to PhRT, may elicit even stronger antitumor immunity [22, 33]. Here, we summarize the CIRT-mobilized antitumor immune response and its related molecular mechanisms from three levels: immunogenicity, tumor immune microenvironment and systemic tumor immune environment.

Increased immunogenicity of tumor cells caused by CIRT

Complex DNA damage and repair

While radiotherapy promotes the generation and exposure of tumor antigens, recent clinical studies on CPIT have revealed that the immune response elicited by photon-induced tumor antigens often falls short of eradicating the primary tumor (let alone effective abscopal effects), with many patients showing either no response or short-lived responses [34, 35]. Compared to photon radiation, carbon ions induce a greater frequency and complexity of DNA double-strand breaks (DSBs), with clustered DSBs or multiple DSBs representing further increases in overall damage complexity and mutagenicity, as clustered DSBs carry a greater risk of misrepair. Unrepaired DNA damage molecules during mitosis lead to various chromosomal-level distortions and genomic instability [36–39]. Studies have explored the impact of isoeffective doses of 295 keV/μm carbon ions versus 1.3

MeV or 0.2 keV/μm photons on chromosomes, demonstrating that carbon ions increase the yield of chromosome shattering and chromothripsis [40]. Moreover, lost DNA or chromosome fragments (manifested as micronuclei, etc.) migrate from the nucleus to the cytoplasm during interphase after carbon ion irradiation [36]. This process is more pronounced with carbon ions than with photon irradiation, as these ions more effectively induce cytoplasmic double-stranded DNA (dsDNA) or micronuclei [41–43]. Recently, we observed a more pronounced decrease in LNC EBLN3P expression in tumor cells following carbon ion irradiation compared to X-ray irradiation [44]. Further investigation indicated that the downregulation of LNC EBLN3P in response to carbon ions enhances radiation-induced mitochondrial damage via the Keap1/Nrf2/HO-1 pathway [45], which leads to changes in membrane permeability and facilitates mitochondrial DNA (mtDNA) release into the cytoplasm [46, 47]. Aberrantly exposed dsDNA or mtDNA in the cytoplasm can be recognized by the DNA receptor cyclic GMP-AMP synthase (cGAS), triggering the cGAS/STING pathway and inducing a type I interferon (IFN-1) response [48]. Through transcriptome analysis of cancer cells and in vitro and in vivo experiments, it has been demonstrated that compared to low-LET radiation, carbon ions with high LET significantly induced stronger cGAS-STING-IFN signals, which also indirectly demonstrated the enhancement of the above process [43, 49–51].

To ensure genomic stability, cells (including tumor cells) undergo complex DNA damage repair (DDR) processes after DNA damage, and the DDR after carbon ion irradiation involves proteins different from those after photon irradiation [52, 53]. Compared to the classical nonhomologous end-joining pathway used after low-LET irradiation, these error-prone alternative DNA repair pathways become more prevalent after CIRT, increasing the likelihood of gene mutation or repair failure [54]. Therefore, it can be speculated that carbon ion radiation has greater potential to induce the generation of antigenic neoepitopes than photon radiation [36, 55].

Non-lethal effects

During the process of DNA repair following irradiation, cells undergo cell cycle checkpoints, such as G_1/S, intra-S, and G_2/M checkpoints. Due to the unique nature of DNA damage under high-LET carbon ion action, studies have shown that carbon ion radiation is more effective at reducing the cell proliferation index and increasing the expression of cell cycle inhibitors than X-rays [56]. Furthermore, carbon ion irradiation induces more prolonged G_2/M arrest than does photon irradiation at the same physical dose (isodose) [57–59]. Cell cycle arrest induced by ionizing radiation, including carbon ions, seems to be related to tumor immunity. For example, ionizing radiation activates the ATM/p53/p21 pathway, inhibiting CDK4/6 and causing G1/S arrest [60, 61]; however, effective inhibition of CDK4/6 can promote tumor immunogenicity and enhance T-cell activation through various mechanisms [62, 63]. Particularly, the effectiveness of carbon ions is less dependent on the cell cycle compared to photons [64].

The DNA damage inflicted by carbon ion radiation is complex and challenging to repair, resulting in lower repair efficiency and longer-lasting damage compared to the DNA damage induced by X-rays, which is predominantly repaired within 24 h with most residual damage associated with telomeric DNA [65]. A study with human uveal melanoma 92–1 cells demonstrated that heavy ions more effectively induced senescence than X-rays, as shown by measurements of senescence-associated β-galactosidase and cell proliferation [66]. Cell senescence, a stress-induced process, can participate in antitumor immune responses or radiation resistance processes by activating the senescence-associated secretory phenotype (SASP) and triggering the secretion of related cytokines [67].

Mitotic catastrophe is considered a cytostatic mechanism preceding cytotoxic processes such as apoptosis and necrosis or as a consequence of aberrant mitotic progression [68]. Daijiro and colleagues reported that carbon ions induce mitotic catastrophe more effectively than the isodose of X-rays by assessing clonogenic cell death with 4′,6-diamidino-2-phenylindole dihydrochloride staining [69]. This may be related to the inhibition of NBS1/BRCA1-mediated γ-tubulin protein monoubiquitination by high-LET carbon ions, thereby enhancing excessive replication of centrioles in tumor cells [70]. Mitotic catastrophe leads to the formation of giant nuclei, multinucleation, and micronuclei and triggers the cGAS/STING pathway, contributing to genomic instability of tumor cells and triggering antitumor immunity after recognition by the immune system [57, 69, 70].

Immunogenic cell death

Ionizing radiation causes DSBs through direct ionization of target molecules or indirect action by generating free radicals. Nucleases, proteases, and other enzymes are activated, leading to chromatin degradation, histone efflux, nuclear contraction and fragmentation, and ultimately cell death [71]. Additionally, factors such as membrane structural damage and disruption of cellular energy metabolism are also important causes of cell death induced by ionizing radiation [72, 73]. Radiation damage caused by low-LET radiation, such as photons, is mainly mediated

by the formation of radical oxygen species [74]. After carbon ion irradiation, due to the severity of DNA damage and the inefficiency of DNA repair, tumor cells (even cancer stem cells) that are too late or unable to repair the damage will undergo multiple types of cell death (p53-independent apoptosis, necrosis, necroptosis, ferroptosis, etc.). The above processes after carbon ion irradiation are more significant than those after the same dose of photon radiation [75–78]. These ICD modes allow tumor cells to produce or release antigens, becoming antigen sources; thus, these mechanisms are also known as in situ autovaccination [79–85].

The occurrence of ICD by tumor cells releases meaningful tumor antigens and also effectively coordinates the spatial–temporal release of DAMPs, thereby triggering another characteristic of ICD, including exposure to calreticulin (CRT), extracellular release of high mobility group box 1 (HMGB1) and adenosine triphosphate (ATP). These events promote APC recruitment, phagocytic activity, and maturation, enabling them to engulf antigenic material, migrate to lymph nodes, and initiate cytotoxic T lymphocyte (CTL)-dependent immune responses, namely, adjuvanticity [25, 86]. The mechanisms of DAMPs in immune cell activation are not further elaborated here but can be found in the relevant literature [35, 87]. Compared to isodose photon irradiation, carbon ion irradiation leads to a more significant increase in HMGB1 in a dose-independent manner [88]. However, under isoeffective dose irradiation, both groups exhibited comparable increases in HMGB1 [89]. Additionally, under isoeffective dose conditions, exposure to HMGB1 increases with increasing LET of carbon ions [90]. As one of the most critical markers of ICD, the translocation of calreticulin to the cell surface (ecto-CRT) promotes APC phagocytosis and activates antitumor immunity. In lung adenocarcinoma, tongue squamous carcinoma, and nasopharyngeal carcinoma cells, the level of ecto-CRT induced by 2 Gy and 4 Gy carbon ion irradiation is greater than that induced by isodose protons and photons [91]. Furthermore, this advantage exists in normoxic tumor cells, whereas under hypoxic conditions, the expression level of CRT is significantly upregulated at baseline (0 Gy), and radiation cannot further increase CRT expression. This situation may be due to a competitive relationship between hypoxia-induced endoplasmic reticulum stress (ER stress) and radiation-induced ER stress, leading to the upregulation of CRT [92, 93]. Although the above studies were conducted in vitro, obvious induction of ICD characteristics by carbon ions was also observed through in vivo detection using sera from tumor-bearing mice or tumor samples [50, 94, 95].

Immunorecognition-related molecules

Alternatively, the formation of immunogenic niches is crucial for immune responses. After photon irradiation, the expression of intercellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex class I antigen (MHC-I) on the cell membrane surface increases, enhancing the ability of T cells to recognize and kill tumor cells and leading to the aggregation of effector T cells at the tumor site [96, 97]. It has been reported that C3H/HeSlc mice bearing NR-S1 squamous cell carcinoma cells were irradiated with carbon ions. Immunohistochemistry showed that ICAM1 expression increased in a time-dependent manner after irradiation [98]. After carbon ion irradiation in MC38 colorectal tumor cells, the expression of MHC class I genes was upregulated and was significantly greater than that in response to the same dose of photons [99], which is conducive to the formation of effective immune synapses between T cells, APCs and tumor cells.

CIRT reshapes the tumor immune microenvironment

Even in the presence of sufficient antigenicity and adjuvanticity, the immune microenvironment of tumor cells is the primary determinant to initiate adaptive immune responses, referred to as the TIME [81, 100]. Tumor cells induce the infiltration of immunosuppressive cells into the TIME by secreting inhibitory cytokines, and immunosuppressive cells can also recruit more of the same type of cells by secreting these factors, leading to an immunosuppressive vicious cycle [101–103]. Once the tumor progresses to the immune escape stage (according to the immunoediting theory), an TIME that promotes malignant tumor progression, drug resistance, and metastasis is eventually established under the action of various immunosuppressive cells (such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and tumor-associated neutrophils (TANs)) and immunosuppressive molecules (such as transforming growth factor-β (TGF-β), IL-10, Fas ligand (FasL), and immune checkpoint molecules) through multiple complex mechanisms that inhibit antitumor immunity, maintain or promote self-immunosuppressive phenotypes, and facilitate malignant tumor progression [104, 105]. Here, we investigated the immunological effects of CIRT on the composition and function of TIME.

The distribution and function of immune cells such as T cells, Tregs, and mononuclear phagocytes and the levels of immunostimulatory or inhibitory molecules in the TIME are important indicators for evaluating the effect of radiation on the TIME [106, 107]. PhRT has been reported to reduce the infiltration of Tregs and MDSCs

into tumors and increase the infiltration and activation of CD4+ T and CD8+ T cells [15]. In contrast, compared with PhRT, CIRT elicits a limited induction of immunosuppressive cells such as MDSCs, and inhibits M2-like polarization [108, 109]. Additionally, carbon ions can increase the infiltration of CD8+ T cells, CD4+ T cells, macrophages, and natural killer cells into the TIME and increase the abundance of immunostimulatory molecules such as interferon-gamma (IFN-γ), IL-2, and IL-1β [43, 50, 51, 108–110]. Compared with X-rays, carbon ions relatively reduced the exposure levels of immunosuppressive factors IL-10 and TGF-β [88].

In terms of function, immunosuppressive cell subgroups play a crucial role in inhibiting the TIME [107]. MDSCs reduce the formation and function of NK cells by producing inhibitory cytokines, thus downregulating NKG2D expression and IFN-γ secretion [111]. Simultaneously, Tregs can downregulate NKG2D expression on NK cells via membrane-bound TGF-β or directly suppress NKG2D activity upon contact with NK cells [112], which are crucial cytotoxic cells in tumor innate immunity that directly kill tumor cells in a non-MHC-restricted manner using perforin and granzymes, making their functional status necessary for antitumor immune responses [113]. Carbon ion irradiation significantly upregulates *Klrk1* gene expression to activate the NKG2D/NKG2D-Ls pathway and enhance the cytotoxic pathway mediated by NK cells [114]. This finding suggested that inhibiting Tregs or MDSCs combined with CIRT can strengthen the immune response by increasing NK cell cytotoxicity. TAMs, which can function as specialized APCs to induce antitumor immunity by presenting tumor antigens, are also involved in the formation of a suppressive TIME [115, 116]. Conrad et al. compared the effects of 250 kV X-rays and 9.8 MeV/u carbon ions on RAW 264.7 macrophages at isodose and reported that carbon ions induce only a slight decrease in macrophage viability but exhibit greater enhancement of phagocytic activity than X-rays [117]. Carbon ion irradiation enhances CTL sensitivity to tumor cell recognition and killing, similar to the modulatory effect of photons at isoeffective doses [118]. A study simulating the effects of space radiation (including carbon ions) on Jurkat cells, a T-cell model widely used in space radiation studies, revealed that 1 Gy of carbon ion irradiation causes a smaller decrease in IL-2 secretion by T cells than photons at isodose, suggesting that CIRT may have less immunosuppressive effects on T cells [119]. Studies have reported that carbon ion irradiation does not significantly increase the number of dendritic cells (DCs) or promote functions such as phagocytosis, migration, and IL-12 secretion [120]. However, carbon ions trigger IFN-γ and IL-12 in DCs treated with lipopolysaccharide (LPS), suggesting the potential long-lasting induction of adaptive immune responses after carbon ion irradiation [121].

Tumors can induce a decrease in oxygen pressure (even below 2%, while the physiological range of normal tissues is 3–7.4%) in the TIME through a series of mechanisms, leading to the generation of hypoxic microenvironments [122]. Conversely, hypoxia, as a cellular stress factor, extensively affects multiple cellular metabolic pathways, thereby correlating with tumor invasive phenotypes and treatment resistance and serving as a critical factor in tumor microenvironment (TME) immune suppression [123, 124]. Hypoxia induces multiple effects by upregulating hypoxia-inducible factor-1α (HIF-1α), such as promoting angiogenesis, tumor invasion, and metastasis, as well as increasing Treg numbers and upregulating programmed cell death-ligand 1 (PD-L1) on tumor cells [125]. Interestingly, although PhRT can increase tumor immunogenicity to some extent, this effect is greatly influenced by oxygen pressure in the TIME (oxygen enhancement ratio, OER is 2–3). In contrast, carbon ion beams at the Bragg peak with high LET (up to > 200 keV/μm) primarily exert direct radiation effects, and their immunostimulatory effects are almost unaffected by oxygen pressure [93]. Since the DNA damage caused by carbon ion irradiation is mainly due to direct radiation damage, subsequent micronucleus formation and cGAS-STING activation, carbon ions are more effective than X-rays in inducing DDR defects, regardless of normoxia or hypoxia. Additionally, HIF is involved in the postirradiation DDR process in tumor cells, promoting the generation of hypoxic radiation resistance [126, 127]. Compared to photons, carbon ions significantly reduce HIF expression, thereby inhibiting the DDR to maintain a damaged DNA status, which also optimizes the tumor microenvironment via the cGAS-STING pathway to promote immunogenicity [51, 128, 129]. Furthermore, studies have shown that under hypoxic conditions, high-LET carbon ion beams at the SOBP further increase the radiation sensitivity of DNA repair-deficient Chinese hamster ovary cells [130].

Ionizing radiation also has a certain degree of immunosuppressive effect [131]. Local PhRT can recruit immunosuppressive bone marrow cells, directly promoting tumor growth, and can also upregulate tumor cell PD-L1 expression through the ATM/ATR/Chk1 pathway, activating the programmed cell death protein 1 (PD-1) receptor on inhibitory T cells [132, 133]. Studies have shown that the upregulation of PD-L1 on tumor cells and PD-1 expression on T cells after carbon ion irradiation is greater than that caused by PhRT [51, 99, 134]. However, the development of immune checkpoint inhibitors (ICIs) targeting PD-L1 or PD-1 is relatively mature, which further suggests the necessity of CIRT combined with ICI

drugs to further improve the efficacy of treatments for tumors with high immune checkpoint expression.

CIRT improves the systemic tumor immune environment to maintain the foundation of antitumor immunity

Measuring the antitumor immune response should extend beyond the local immune activity within the TIME to encompass the broader systemic tumor immune environment (STIE). The antitumor immune response is primarily mediated by immune effector cells and molecules from both the innate and adaptive immune systems. These immune-active substances circulate between the TIME, immune organs, and other bodily organs, playing a crucial role in the development of the STIE [106, 135]. The STIE serves as the foundation for establishing antitumor immune responses and affects patient prognosis after radiotherapy [136–138].

CIRT improves STIE by modulating immune cells and cytokines in the circulatory system (blood, spleen, etc.). Studies have confirmed that compared to PhRT, carbon-ion irradiation results in a greater increase in CD3+, CD4+, and CD8+ T cells; macrophages; and NK cells in the peripheral blood of tumor-bearing mice at an isoeffective dose, while MDSCs decrease more in the bone marrow, peripheral blood, spleen, and tumor tissue [109]. Similarly, research has shown that CIRT expands the populations of CD8+ T cells and effector memory T cells while reducing T-cell exhaustion in the spleens of tumor-bearing mice [43]. Recent studies have shown that low-dose radiation has significant immunostimulatory effects on the TIME [139, 140]. Low-dose irradiation can alleviate tumor-induced immune suppression, resulting in increases in the proportions of CD3+ T cells, CD3+CD4+ T cells, and CD3+CD8+ T cells, as well as in the levels of IFN-γ and IL-2 in the peripheral blood after irradiation [141]. Peripheral blood samples were collected from 32 prostate cancer patients before and after receiving CIRT. Analyses of these samples revealed changes in the frequency, proliferation, and cytokine expression of immune cells. Notably, CIRT preserved lymphocyte viability, augmented lymphocyte proliferation, improved T-cell functionality, curtailed the induction of immunosuppressive cells, and decreased the expression of immunosuppressive cytokines [142]. Although the evidence available is limited, this retrospective clinical study, to some extent, demonstrated that carbon-ion immune modulation is beneficial for improving STIE in patients.

The state of STIE affects the occurrence and development of tumors, and the progression of tumors also leads to immune damage in the body [106, 143, 144]. Ionizing radiation, including photons and charged particles, directly enhances antitumor immunity by rapidly and extensively inactivating tumor cells through DNA damage [145]. As noted, carbon ions achieve greater tumor cell inactivation than photons at equivalent doses, exhibiting a higher relative biological effectiveness (RBE), even in hypoxic tumor stem cells. Consequently, they exert a more potent suppressive effect on tumor-induced immune damage [108, 146–150]. Overall, as most cancers are chronic debilitating diseases, and many cancer patients suffer from leukopenia and diminished leukocyte function due to treatment-related toxicities associated with radiotherapy and chemotherapy, the functional state of the immune system declines. This reduction is detrimental to establishing an effective antitumor immune response [151]. Compared to PhRT, CIRT can inhibit the above processes with a superior tumoricidal effect.

The improvement in STIE is partly due to the more precise and limited dose delivery of CIRT compared to PhRT [152]. Lymphocytes are extremely sensitive to ionizing radiation, and in the process of three-dimensional PhRT, circulating lymphocytes are inevitably irradiated, leading to lymphopenia, a common side effect of radiotherapy [35]. CIRT has the characteristic of differential dose distribution, with Bragg peak effect and less lateral scattering, which preserves more normal tissues from being irradiated and delivers a lower dose to normal tissues in the entrance channel, further reducing the dose deposited in circulating lymphocytes and reducing the proportion of chromosomal aberrations in lymphocytes, thus reducing the common complications of radiation-induced lymphopenia [110, 153–156].

In vivo study of combined CIRT and immunotherapy

Relying solely on tumor ablation rarely results in effective antitumor immunity. In the therapeutic setting, the TIME of most tumors can greatly limit immune-driven ICD [15, 34]. Therefore, unless additional immune-stimulating factors are provided, merely irradiating tumor lesions typically fail to generate a sufficiently strong immune response [157], which is typically evaluated using indicators such as inhibition of tumor metastasis, tumor suppression or rejection, abscopal effects, and survival in tumor-bearing mice [158]. Compared to other low-LET radiation, CIRT has advantages in terms of immunomodulation [23, 35]. Therefore, we propose that the combination of immunotherapy with CIRT, termed CCIT, may elicit a comparable or more effective antitumor immunity compared to its combination with PhRT, known as CPIT. Here, we compiled preclinical studies on the efficacy of CCIT, with or without comparisons to CPIT, detailed in Sects. "CIRT combined with ICIs" to "CIRT combined with DCs" below (Table 1).

Table 1 Preclinical studies demonstrating the antitumor immune response to carbon ion radiotherapy combined with immunotherapy

Tumor model	Immunotherapy type; dosage; administration route (intravenous injection-iv, intratumoral injection-it, intraperitoneal injection-ip); timing	CIRT regimen	PhRT regimen	Relationship of dose	Combined CIRT and immunotherapy (CCIT) boosts antitumor response	References
C3H/HeSlc mice bearing NR-S1 squamous cell carcinoma	DC: a-GalCer–pulsed DCs; 1.5 × 10^6/mouse; it; after RT	290 MeV/u, 6 Gy/1 f	NA	NA	Inhibition of tumor metastasis was stronger than CIRT alone	[98]
Mouse models bearing a poorly immunogenic SCCVII squamous cell carcinoma	DC: iDCs; 1 × 10^6/mouse; it; after RT	290 MeV/u, 77 keV/μm	NA	NA	Rejection of secondary tumor challenge was stronger than CIRT alone	[159]
C3H/He mice bearing NR-S1 squamous cell carcinoma	DC: iDCs; 1 × 10^6/mouse; it or iv; after RT	290 MeV/u, 70–80 keV/μm, 2 Gy/1 f	Cs137 γ-rays, 0.6617 MeV, 4 Gy/1 f	Isoeffective dose	Inhibition of tumor metastasis was stronger than CIRT alone or CPIT	[94]
C3H mice bearing LM8 osteosarcoma	ICIs: anti PD-L1 antibody and anti CTLA-4 antibody; 150 ug/mouse; ip; before, concurrent with and after RT	290 MeV/u, 50 keV/μm, 5.3 Gy/1 f	NA	NA	Suppression of primary tumor was stronger than CIRT alone; abscopal effect and survival were stronger than CIRT or ICIs alone	[95]
C57BL/6 mice bearing B16-OVA melanoma	ICIs: anti-PD-L1 and anti-CTLA-4 antibodies; 200 ug/mouse; ip; after RT	118.41–140.01 MeV/u, 96.94 keV/μm, 4 Gy/1 f	NA	NA	Suppression of primary tumor, abscopal effect and survival were better than CIRT or ICIs alone	[76]
C3H/He female mice bearing LM8 osteosarcoma	ICIs: anti-PD-1; 600 ug/mouse; ip; before RT and anti-CTLA-4; 200 ug/mouse; ip; concurrent with and after RT	290 MeV/u, 50 keV/μm, 10 Gy/1 f	X-rays, 200 kVp, 20 mA, 10 Gy/1 f	Isodose	Inhibition of tumor metastasis was stronger than CPIT, CIRT or ICIs alone. Abscopal effect was stronger than CIRT or ICIs alone, and similar to CPIT	[160]
C57BL/6 mice bearing Lewis lung cancer	ICI: anti PD-1; 25 mg/kg/time; ip; after RT	80 MeV/u; peak LET, 50 keV/μm, 10 Gy/1 f	X-rays, 225 kV, 13.3 mA, 10 Gy/1 f	Isodose	Suppression of primary tumor was stronger than CIRT alone or CPIT; abscopal effect was stronger than CIRT alone, and similar to CPIT	[161]
C57BL/6 J mice bearing Her2+ EO771 breast cancer	ICI: anti-CTLA-4 antibody; 100 μg/mouse; after RT	103 keV/μm, 9.24 Gy/3 f	X-rays, 15 Gy/3 f	Isoeffective dose	Survival was stronger than CIRT alone, and similar to CPIT	[162]
C57BL/6 mice bearing B16 and S91 melanoma	ICI: anti-PD-1; 10 mg/kg; ip; after RT	80 MeV/u, 50 keV/μm, 2.94 Gy/1 f	X-rays, 225 kV, 13.3 mA, 5 Gy/1 f	Isoeffective dose	Survival and suppression of primary tumor were stronger than CIRT or ICI alone	[50]
C57BL/6 J male mice bearing B16 F10 melanoma	ICI: anti-PD-L1; 1 μmol/mouse; ip; before RT	2 Gy/1 f	X-rays, 6 Gy/1 f	Isoeffective dose	Suppression of primary tumor was stronger than CPIT or CIRT alone	[51]

Table 1 (continued)

Tumor model	Immunotherapy type; dosage; administration route (intravenous injection-iv, intratumoral injection-it, intraperitoneal injection-ip); timing	CIRT regimen	PhRT regimen	Relationship of dose	Combined CIRT and immunotherapy (CCIT) boosts antitumor response	References
C57BL/6 mice bearing Lewis lung cancer	Treg inhibitors: anti-CD25; 20 mg/kg; ip; before, concurrent with and after RT	80.55 MeV/u, 50 keV/μm, 0 Gy, 2 Gy, 5 Gy, and 10 Gy/1 f	NA	NA	Survival and suppression of primary tumor were stronger than CIRT alone	[114]
C57BL/6 mice bearing MC38 colorectal cancer	NeoAg RNA-LPX vaccine: NeoAg RNA-LPX (Rpl18 mRNA and MHC class II – restricted decatope mRNA, 1:1); 20 mg/mouse; iv; before, concurrent with and after RT	240 MeV/u, 55–60 keV/μm, 10 Gy/1 f	X-rays, 12 Gy/1 f	Isoeffective dose	Survival and suppression of primary tumor were stronger than CIRT alone, and similar to CPIT	[99]

CIRT, carbon ion radiotherapy; CCIT, combined CIRT and IT therapy; CPIT, Combined PhRT and IT Therapy; ICI (s), immune checkpoint inhibitor (s); DCs, Dendritic cells; PhRT, Photon radiotherapy; RT, radiotherapy; f, fraction

CIRT combined with ICIs

In the adaptive immune system, with the assistance of CD4+ T cells and DCs, CD8+ T cells are activated to become CTLs, which ultimately exert tumor-killing effects [136]. However, the presence of inhibitory costimulatory molecules (immune checkpoints), such as PD-1/PD-L1 and CTLA-4, renders CTL cells dysfunctional [163]. ICIs are important targeted drugs for various tumor immunotherapies [164]. Notably, both photons and carbon ions increase the expression of tumor PD-L1, with carbon ions showing a more significant increasing trend [51, 134]. Therefore, the combination of CIRT with ICI drugs may lead to more effective synergistic therapeutic strategies. Studies have shown that combining CIRT with dual immune checkpoint blockade therapy (anti-PD-L1 and anti-CTLA-4 antibodies) results in better control of irradiated tumors than CIRT alone ($p < 0.01$) [95]. Importantly, the complete remission rate of mice with nonirradiated tumors in the combination group was significantly greater (64%) than that in the ICI alone group (20%) ($p = 0.0392$); the overall survival rate in the combination group was greater than that in the CIRT or ICIs alone groups (vs CIRT, $p = 0.0014$; vs ICIs, $p = 0.0009$) [95]. Similarly, Huang et al. reported that combination therapy with ICIs and CIRT significantly inhibited the growth of locally irradiated and distant melanoma tumors compared to CIRT or ICIs alone, improving the survival rate of mice [76]. There are numerous studies on the combination of PhRT with ICIs, with Zhang et al.'s review detailing positive outcomes [157]. In recent years, studies have preliminarily confirmed to varying degrees that CCIT demonstrates similar or superior antitumor immunity compared to CPIT, where tumors were irradiated with isodose or isoeffective doses of carbon ions or photons combined with ICIs, with the detailed experimental designs shown in Table 1.[50, 51, 160–162].

The above studies demonstrated that effective immune responses, including abscopal effects, are induced by the combination of CIRT with ICIs and are closely related to the immunomodulatory processes triggered by irradiation. Bioinformatics analysis of infiltrating immune cells, molecular components, and tumor cell transcriptomes revealed that changes in tumor cells and the TIME in the combination group were consistent with that in CIRT alone, as discussed in Sect. "CIRT reshapes the tumor immune microenvironment". Furthermore, ICI drugs further enhance this advantage. For example, more complex and persistent DNA damage, along with higher levels of cGAS-STING and more significant activation of cell death pathways, was observed in tumor cells. In the TIME at the irradiated site, there was increased infiltration and activation of immune-active cells such as CD45+ and CD8+ cells, NK cells, and macrophages, and the levels of innate immune signaling markers such as IL-2, INF-γ, inflammatory factors, and chemokines were significantly increased. Increased CD8+/GzmB+ and CD4+ tumor-infiltrating lymphocytes (TILs) were also observed in nonirradiated tumor tissues, with a greater frequency of activated naïve T cells in the combination group [50, 51, 76, 95, 160–162]. In a recent clinical case, a 24-year-old woman with alveolar soft part sarcoma underwent pelvic tumor irradiation with carbon ions at 67.2 GyE in 16 fractions, followed by pembrolizumab (ICI) administration 20 days after radiotherapy. The results showed significant shrinkage of both irradiated pelvic tumors and nonirradiated leg tumors, with MRI 10 months post-CIRT indicating an 80% reduction in both tumors compared to baseline [165].

CIRT combined with Treg inhibitors

As mentioned earlier, Treg cells can downregulate NK cell surface NKG2D expression via membrane-bound TGF-β or inhibit NK cell activity through cell contact [112]. Recent studies have shown that Treg inhibitors can effectively inhibit Treg activity by specifically blocking the interleukin-2 receptor alpha subunit (IL-2Rα, CD25) on Tregs without affecting the IL-2 signaling pathway on effector T cells [166]. Additionally, CIRT significantly induces *Klrk1* gene expression and activates the NKG2D/NKG2D-ls pathway to enhance NK cell-mediated cytotoxic pathways. Therefore, the combination of CIRT with Treg inhibitors (anti-CD25 and niclosamide) significantly increases NK cell infiltration and function in the lung cancer tumor microenvironment, prolonging the survival of Lewis lung cancer-bearing C57BL/6 mice, with superior efficacy compared to either single-agent Treg inhibitor immunotherapy or CIRT ($p < 0.001$) [114].

CIRT combined with NeoAg RNA-LPX vaccines

In terms of combining active immunotherapy, studies have demonstrated that the combination of RNA-LPX vaccines with conventional PhRT can achieve potent tumor suppression [167]. Salomon et al. further investigated whether the inhibitory effect of the neoAg RNA-LPX vaccine combined with CIRT on murine colorectal tumors was superior to that of the combination with photon radiation. Compared with photon radiation, CIRT at isodose significantly upregulated the expression of MHC class I and extracellular calcium-binding protein in MC38 cells and increased HMGB1 secretion and PD-L1 expression [99], consistent with the trends we summarized earlier. Moreover, immune cell and molecular component analyses revealed greater infiltration of total CD8+ T cells, CD4+ T cells (including neoantigen-specific CD4+ T cells), and NK cells in the combination of

CIRT with neoAg RNA-LPX-treated mouse tumors than in all other treatment groups. These T cells showed reduced expression of the inhibitory markers PD-1 and Tim-3, along with high secretion of the cytokines IFN-γ and TNF-α. In terms of tumor control and survival, the neoAg RNA-LPX vaccine showed similar overall therapeutic efficacy in mediating tumor growth inhibition and survival when combined with CIRT or PhRT, even at a lower physical radiation dose for carbon ions than for X-rays [99].

CIRT combined with DCs

DCs, as potent professional APCs in vivo, play a crucial role in presenting and activating naïve T cells and helper T cells after processing tumor antigens released under radiation [168]. Pretreated DCs administered intravenously or intratumorally in combination with CIRT have shown promising results [94, 98, 159]. Significant upregulation of tumor cell DAMPs (such as ecto-CRT), costimulatory molecules (ICAM-1), and antigens, which promote DC maturation, activation, and antigen presentation, thereby enhancing systemic antitumor immunity, was observed after carbon ion irradiation. In a mouse squamous cell carcinoma model, the administration of DCs after carbon ion treatment significantly improved the rate of tumor rejection after secondary tumor inoculation compared to that in the CIRT alone group (70.4% vs 88.5%; $p < 0.01$) and improved the rate of SCCVII-induced tumor-specific CTLs compared to that in the CIRT alone group [159]. In another study, the early lung metastasis marker S100A8 and the number of lung metastases in the CIRT combined with DC treatment group were significantly lower than those in the CIRT alone group [98]. Ando et al. compared the effects of 290 MeV/u, 70–80 keV/μm carbon ions and 137 Cs γ-rays combined with DCs on tumor immune responses. They first prepared tumor-bearing mice and irradiated them with CIRT at 2 Gy or PhRT at 4 Gy on the seventh day, followed by DC administration 1.5 days after irradiation, and observed the number of lung metastases 2 weeks postirradiation. The results showed that intravenous injection of DCs combined with CIRT significantly suppressed lung metastasis compared to CIRT or DCs combined with PhRT. Compared with those in response to photon irradiation, the levels of ecto-CRT in tumor cells significantly increased after CIRT, and subsequent coculture of these irradiated tumor cells with DCs significantly upregulated the expression levels of CD40 and IL-12 in DCs, indicating improved functional levels. Intravenously injected cocultured DCs into nonirradiated NR-S1 mice exerted antimetastatic effects [94].

Clinical study of CIRT combined with immunotherapy

In summary, as we have come to understand, CCIT provides a potent synergistic approach for tumor treatment, offering new avenues in this continuously evolving field. Currently, clinical trials investigating CCIT are actively underway, mostly targeting recurrent, metastatic, and advanced malignant tumors that are resistant to standard treatments. We identified 4 relevant studies related to CCIT on the ClinicalTrials.gov website. These trials cover various pathologies and immunotherapy characteristics, including CIRT combined with ICI (camrelizumab) for locally recurrent nasopharyngeal carcinoma (NCT04143984), CIRT combined with ICI (pembrolizumab) for stable disease in non-small cell lung cancer, head and neck squamous cell carcinoma, melanoma, and urothelial carcinoma (NCT05229614), SABR CIRT combined with the immunocytokine L19-IL2 for stage IV metastatic non-small cell lung cancer (NCT03705403) [169], and CIRT combined with granulocyte–macrophage colony-stimulating factor (GM-CSF) for hepatocellular carcinoma (NCT02946138) (withdrawn due to enrollment issues). Additionally, we found two studies on the jrct.niph.go.jp website: a phase Ib study registered in 2021 evaluating the safety and efficacy of durvalumab (an anti-PD-L1 antibody) combined with CIRT and weekly cisplatin for locally advanced cervical cancer (jRCT2031210083), which has completed patient recruitment. In the same year, another phase Ib study explored the safety and efficacy of durvalumab (MEDI4736) or tremelimumab combined with CIRT for treating advanced hepatocellular carcinoma patients with macrovascular invasion (jRCT2031210046), but this study is currently being suspended.

Summary and prospects

In summary, the superior immunomodulatory effects of CIRT can be attributed to several factors: first, the enhanced tumor immunogenicity induced by CIRT efficiently activates tumor immune responses; second, CIRT leads to the formation of an immunopermissive TME and suppresses the negative immune regulatory process, which not only promotes the successful construction of an adaptive immune response but also effectively recruits and activates nonspecific innate immune cells (NK, macrophages, NKT, etc.)-mediated antitumor effects; third, tumor shrinkage mediated by the high RBE characteristics of CIRT and precise dose distribution improve the STIE to maintain the basis of antitumor immunity. These theoretical foundations hope to promote the development of research related to CCIT.

In this era of immunotherapy, radiation therapy has ushered in new opportunities. By adjusting the protocols

of radiotherapy and immunotherapy to make combinations more compatible with antitumor immune activation and to reduce side effects, the efficacy of radioimmunotherapy can be maximized, which is known as adaptive radioimmunotherapy (ART) [15, 170]. Currently, large fractionation is often recommended for CIRT [171]. Using single high-dose irradiation (such as 8 or 10 Gy), carbon ion irradiation dose-dependently upregulated the expression of the DNA exonuclease three-prime repair exonuclease 1 (TREX1), which degrades cytoplasmic dsDNA, in tumors, thereby inhibiting IFN-1 responses [161, 172]. However, by silencing TREX1 (siRNA), the activation of IFN-1 responses is enhanced under carbon ion radiation, IFN-β levels are significantly increased, and tumor cell apoptosis is enhanced [161]. The method of DC administration may affect the synergistic effect of CIRT combined with DCs. Compared with intratumoral injection of DCs, intravenous injection of DCs combined with CIRT has a greater antimetastatic effect, which may be related to the influence of the TME on intratumorally injected DCs [94]. However, it must be acknowledged that there is currently no standard protocol for determining the optimal dose, administration method, or sequence of CCIT, and further investigations are needed within the context of clinical trials. In terms of safety, it is worth noting that while ICIs combined with CIRT exhibit synergistic efficacy, the occurrence of adverse events does not seem to have a similar synergistic effect. A retrospective analysis revealed that among advanced melanoma patients treated with sequential CIRT combined with ICI, 21% experienced G3 + adverse events, similar to ICI monotherapy, while the frequency of local adverse events was similar to that of CIRT monotherapy [173].

The development and clinical use of CCIT still have a long way to go. Firstly, the rarity of CIRT facilities limits the progress of research and further clinicalization. Currently, the number of institutions globally with corresponding equipment and relatively mature carbon ion treatment planning systems is far less than that for PhRT, greatly limiting the acquisition of experimental samples and clinical data [174]. Clinical data on the combination of carbon ions and immunotherapy are lacking, especially for large-scale RCT trials comparing the efficacy and safety of CCIT with CPIT or combined therapy with protons. Currently, numerous plans for carbon ion facilities are underway or under construction [7]. The immune response induced by carbon ion radiation and its combination with immunotherapy provide evidence of excellent efficacy and safety for further approval and construction. Secondly, while comprehensive statistical data are lacking, as mentioned above, most carbon ion radiobiology research primarily targets tumor cells, with limited studies on immune and stromal components, which are equally crucial for the efficacy of radioimmunotherapy [106]. There is also a pressing need for translational research and development of clinically relevant biomarkers. Additionally, clearer understanding of CIRT's effects and mechanisms may suggest potential benefits when combined with targeted therapies or chemotherapy. Finally, the overall quantity and quality of CCIT research are lower than those of CPIT-related studies, however, the latter can provide some methodological experience. Moreover, the cost of CIRT is relatively high, and combination therapy with immunotherapy will inevitably incur substantial expenses. However, the radiobiology of carbon ions provides many targets for sensitizing PhRT, such as differential metabolic factors, epigenetic, and posttranslational modification targets. Based on the differential molecular signaling pathways underlying the radiobiological effects, targeted drugs for sensitizing PhRT are expected to be developed, providing a new approach for radioimmunotherapy. Can we replace CCIT with a combination of PhRT, targeted drugs, and immunotherapy in parallel? Only further research can provide answers to these questions.

Abbreviations

APC	Antigen-presenting cell
ART	Adaptive radioimmunotherapy
CAF	Cancer-associated fibroblast
CCIT	Combined carbon ion radiotherapy and immunotherapy
CPIT	Combined photon radiotherapy and immunotherapy
CIRT	Carbon ion radiotherapy
CTL	Cytotoxic T lymphocyte
cGAS	Cyclic GMP-AMP synthase
DC	Dendritic cell
DDR	DNA damage repair
DAMPs	Damage-associated molecular patterns
DSBs	DNA double-strand breaks
ER stress	Endoplasmic reticulum stress
FasL	Fas ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High mobility group box 1
HIF-1α	Hypoxia-inducible factor-1α
ICAM-1	Intercellular adhesion molecule-1
ICI	Immune checkpoint inhibitor
ICD	Immunogenic cell death
IFN-1	Type I interferon
IFN-γ	Interferon-γ
LET	Linear energy transfer
LPS	Lipopolysaccharide
MDSCs	Myeloid-derived suppressor cells
mtDNA	Mitochondrial DNA
NIRS	National Institute of Radiological Sciences
NK cells	Natural killer cells
OER	Oxygen enhancement ratio
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death-ligand 1
PhRT	Photon radiotherapy
RBE	Relative biological effectiveness
SASP	Senescence-associated secretory phenotype
STIE	Systemic tumor immune environment
TAMs	Tumor-associated macrophages
TANs	Tumor-associated neutrophils
TGF-β	Transforming growth factor-β

TIME	Tumor immune microenvironment
TILs	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
Tregs	Regulatory T cells
TREX1	Three-prime repair exonuclease 1

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval and consent to participate
Not applicable.

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