

Research Article

The impact of melatonin and carbon ion irradiation on cancer stem cells

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Abstract

Aim: Cancer stem cells (CSCs) exhibited an excessive migratory and invasive potential. Melatonin may inhibit multiple crucial signals associated with tumor stem cell self-renewal, viability, invasiveness, tumor growth, and therapy resistance. Carbon ion irradiation may be a promising therapeutic modality in both non-stem-like and stem-like tumor cells in contrast to photon irradiation. A comprehensive understanding of the mechanisms and molecular pathways associated with invasive properties of CSCs is essential in developing novel treatment options for cancer therapy that target CSCs.

Materials and methods: A systematic review of the existing literature was conducted using the following search terms: 'melatonin', 'X-ray irradiation', 'charged particle irradiation', 'carbon ion irradiation', 'cancer stem cells', 'tumor-initiating cells' and 'cancer stem-like cells'. The search used PubMed and spanned the period from January 2000 to December 2016.

Results: The collected data included the impact of melatonin and carbon ion irradiation on tumor stem cells. The impact of melatonin on tumor stem cells consisted of 'Melatonin inhibits tumorigenicity of brain tumor stem cells via the AKT-EZH2-STAT3 signaling axis', 'Melatonin-induced methylation of the ABCG2/BCRP promoter overcomes multidrug resistance in brain tumor stem cells', 'Involvement of autophagy in melatonin-induced cytotoxicity in brain tumor stem cells', 'Melatonin inhibits estrogen receptor binding to estrogen response elements sites on the OCT4 gene in breast cancer stem cells' and 'Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells'. The impact of carbon ion irradiation on tumor stem cells consisted of 'Carbon ion irradiation effectively eradicates brain tumor stem cells', 'Carbon ion irradiation counteracts cancer stem cells' migration and invasion process in head and neck squamous cell carcinoma', 'Carbon ion beam combined with cisplatin effectively disrupts triple negative breast cancer stem cells', 'Effects of carbon ion beam on putative colon cancer stem cells and its comparison with X-rays' and 'Different effects of carbon ion beams and X-rays on clonogenic survival and DNA repair in human pancreatic cancer stem-like cells'.

Conclusion: Cancer stem cells possess the capacity of self-renewal and pluripotency, generating all cells within a tumor, and are responsible for tumor growth, therapy resistance and metastasis. Melatonin attenuated AKT activation, EZH2 S21 phosphorylation, EZH2-STAT3 interactions and altered histone modifications to reduce tumor initiation and propagation of brain tumor stem cells (BTSC). Melatonin increases the efficacy of chemotherapeutic agents, targeting both the tumor bulk and BTSCs through the regulation of the expression and function of the ABCG2/BCRP transporter by inducing the methylation of its promoter. Melatonin treatment induced cell death with ultrastructural characteristics of autophagy. Breast cancer stem cells (BCSCs) are responsive to melatonin treatment by way of reducing the viability and the invasiveness of breast cancer mammospheres as well as regulating the expression of OCT4, N-cadherin and vimentin proteins associated with epithelial mesenchymal transition in BCSCs. Carbon irradiation is effective in brain tumor stem cell (BTSC) elimination with relative biologic effectiveness (RBE) in the range of 1.87-3.44. Carbon ion irradiation may be a promising therapeutic modality because it reduces migration and invasion processes in both head and neck squamous cell carcinoma and cancer stem cells in contrast to photon irradiation. Low LET X-ray irradiation may essentially eradicate the non-stem-like tumor cells, consequently the radioresistant cancer stem-like cell population is obviously enriched. In contrast, carbon ion irradiation may eradicate both non-stem-like and stem-like tumor cells at the same time. Carbon ion irradiation is a promising tool to eradicate putative colon cancer stem-like cells. Further investigation to elucidate the mechanisms and molecular pathways involved in cancer stem cells particularly associated with melatonin and carbon ion irradiation certainly is warranted.

Introduction

Cancer stem cells (CSCs), also called tumor-initiating cells, comprise a distinct portion of the tumor mass that possess the capacity of self-renewal and pluripotency, generating all cells within a tumor; these specialized cells are responsible for tumor recurrence, resistance to chemotherapy and radiotherapy, and for metastasis [1-4]. Cancer stem cells are also responsible for the development of tumor cell heterogeneity which is often related to the failure of conventional therapies [5]. Therefore, targeting CSCs is critical for developing innovative therapies to counteract cancer relapse and emergence of drug resistance [6].

Melatonin (N-acetyl-5-methoxytryptamine), an indole compound synthesized by the pineal gland and many other tissues, is a critical

inhibitor of tumors [7-14]. This molecule executes its anticancer effects in various types of cancer due to its pro-apoptotic, anti-proliferative, anti-cell differentiation and anti-angiogenic actions [15,16]. Numerous studies indicate that melatonin inhibits multiple crucial signals associated with brain tumor stem cell self-renewal, viability,

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invasiveness, tumor growth, and therapy resistance [17-19]. Melatonin not only exhibits an antitumor effects on total tumor mass but also antitumor actions on subpopulations of CSCs [21]. Radiation therapy is an imperative modality of cancer treatment. X-rays consist of photons and are commonly used in radiation therapy. X-rays are defined as low LET (Linear Energy Transfer) radiation which produce only occasional ionizations along their trajectories [22]. Failure of radiation therapy using photon irradiation is usually related to metastasis [23]. There is an increasing application of high-LET charged particles such as protons and carbon ions in the treatment of cancer [24-26]. Carbon ions create numerous ionizations along their trajectories, and induce unreparable clustered DNA damage. Carbon ion irradiation is more potent in the induction of cytogenetic damage and cytotoxicity of irradiated cells than low-LET X-rays [26,27]. Several recent studies indicate that carbon ion irradiation may be a promising therapeutic modality because of complex DNA damage, increased apoptosis, and counteractive actions on migration and invasive processes in both parental cells of head and neck squamous cell carcinoma and CSCs that it displays. This distinguishes it from photon irradiation [28-32].

A comprehensive understanding of the mechanisms and molecular pathways associated with invasive properties of CSCs is essential in developing novel treatment options for cancer therapy that target CSCs.

Materials and methods

Literature search strategy

A systematic review of the existing literature was conducted using the following search terms: 'melatonin', 'X-ray irradiation', 'charged particle irradiation', 'carbon ion irradiation', 'cancer stem cells', 'tumor-initiating cells' and 'cancer stem-like cells'. The search used PubMed and spanned the period from January 2000 to December 2016.

Inclusion and exclusion criteria

We identified reports containing the impact of melatonin and carbon ion irradiation on tumor stem cells for inclusion. Reports which were published in languages other than English, only published in abstract form, not related to tumor stem cells, duplicate articles and those containing insufficient detail were excluded. All titles and abstracts were screened to assess whether they were eligible for inclusion. Then abstracts and full texts of all eligible studies were examined and data was evaluated.

Results

Literature search results

The search identified 109 potentially eligible articles. After application of the exclusion criteria, only 10 met the criteria and were therefore evaluated. The collected data included the impact of melatonin and carbon ion irradiation on tumor stem cells. The impact of melatonin on tumor stem cells consisted of 'Melatonin inhibits tumorigenicity of brain tumor stem cells via the AKT-EZH2-STAT3 signaling axis', 'Melatonin-induced methylation of the ABCG2/BCRP promoter overcomes multidrug resistance in brain tumor stem cells', 'Involvement of autophagy in melatonin-induced cytotoxicity in brain tumor stem cells', 'Melatonin inhibits estrogen receptor binding to estrogen response elements sites on the OCT4 gene in breast cancer stem cells' and 'Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells'. The impact of carbon ion irradiation on tumor stem cells consisted of 'Carbon ion irradiation effectively eradicates brain tumor stem cells',

'Carbon ion irradiation counteracts cancer stem cells' migration and invasion process in head and neck squamous cell carcinoma', 'Carbon ion beam combined with cisplatin effectively disrupts triple negative breast cancer stem cells', 'Effects of carbon ion beam on putative colon cancer stem cells and its comparison with X-rays' and 'Different effects of carbon ion beams and X-rays on clonogenic survival and DNA repair in human pancreatic cancer stem-like cells'.

The impact of melatonin on tumor stem cells

Melatonin inhibits tumorigenicity of brain tumor stem cells via the AKT-EZH2-STAT3 signaling axis: Several studies demonstrated that glioblastoma stem-like cells (GSCs) or brain tumor stem cells exhibiting self-renewing and tumor propagating capacity play an essential role in maintaining tumor growth, therapeutic resistance, and tumor recurrence [33-35]. Polycomb group proteins are related to the development of various types of cancer [36]. Epigenetic perturbations are the possible basis for transcriptional misregulation of tumor suppressor genes. The results of this misregulation relates to the formation of cancer stem cells, which generate tumors through a combination of increased self-renewal and incomplete cellular differentiation (36). Polycomb proteins comprise at least two different complexes, the Polycomb-repressive complexes 1 and 2 (PRC1 and PRC2) [36]. Polycomb repressive complex 2 (PRC2) plays a principal role in transcriptional silencing by placing methylation marks on lysine 27 of histone H3 [37,38]. Dysregulation of PRC2 function is associated with certain malignancies and poor prognosis [37,38]. Enhancer of zeste homologue 2 (EZH2) is the catalytic subunit of PRC2. Therefore, EZH2 is regarded as an essential target for pharmacological intervention [37,38]. EZH2 is involved in inhibiting gene expression through methylation of histone H3 on lysine 27. EZH2 overexpression is associated with tumorigenesis and related to poor prognosis in various tumor types [38-40]. AKT is an oncogenic protein kinase that plays a critical role in cell transformation, cancer cell proliferation, invasion, migration, and angiogenesis of tumor tissues [41-43]. Phosphoinositide 3-kinase (PI3K)/AKT signaling is frequently overexpressed in glioblastoma multiforme (GBM) specimens [44-48].

Chen and colleagues utilized glioblastoma stem-like cells (GSCs) isolated from surgical specimens of patients with GBM to analyze the role and underlying mechanisms associated with melatonin in GSC biology [17]. They noticed that treatment with 1 mol/L melatonin changed the shape of neurospheres. They evaluated the effect of melatonin treatment on *in vitro* cell proliferation, and found that GSCs presented moderate reduction in proliferation rate as illustrated by roughly a 25% reduction in BrdU incorporation. Utilizing a clonogenic assay to estimate the effect of melatonin treatment on GSC self-renewal, neurospheres were treated with 1 mol/L melatonin for 5 days; the spheres were then disrupted and plated as single-cell suspensions in CSC medium to evaluate the sphere-forming ability. The clonogenic index was reduced by >75% in all GBM samples due to melatonin treatment.

Western blotting showed that melatonin induced a decrease in expression of cancer stem cell markers. After pretreatment with luzindole, a melatonin membrane receptor antagonist, GSCs were treated with melatonin (1 mol/L) for 5 days. CD133 expression was subsequently detected by Western blot analysis. This analysis revealed that the downregulation of CD133 by melatonin was interrupted by blockade of the melatonin membrane receptors. The data further suggested an inhibitory effect of melatonin on GSC self-renewal and tumor-initiating capacity [17].

EZH2-STAT3 interaction is noticeable in GSCs and necessary for GSC clonogenic growth [49-51]. Co-immunoprecipitation experiments were carried out to define the effect of melatonin treatment on EZH2-STAT3 interaction in GSCs. EZH2-STAT3 co-precipitation was abundant in GSCs without melatonin treatment. In contrast, it was barely detectable in lysates isolated from GSCs following melatonin treatment [17].

Melatonin inhibited EZH2 S21 phosphorylation (pS21) in GSCs samples. Measurement of the level of H3K27me3 in GSCs treated with melatonin was carried out to ascertain the effect of melatonin-induced EZH2 pS21 on the methylation status of histone H3. Noticeable increases in H3K27me3 in response to melatonin treatment were detected [17].

STAT3 activation is mediated by phosphorylation of EZH2 S21 and the subsequent methylation of STAT3 by EZH2 [52,53]. The role of melatonin in STAT3 methylation and its effect on GSCs was assessed. Significant inhibition of STAT3 methylation with melatonin treatment was found in all three GSCs [17]. At the same time, tyrosine-phosphorylated STAT3 (pY-STAT3) concentration was reduced by 30% as compared with that detected in GSCs without melatonin treatment. A luciferase reporter assay indicated that STAT3 transcriptional activity was essentially inhibited by melatonin treatment [17].

Briefly, AKT overexpression obviously increased EZH2 S21 phosphorylation levels, EZH2-STAT3 interactions, and STAT3 activity, but reduced H3K27me3 levels [17]. The data revealed that melatonin directly targeted glioma tumor cells by modifying GSC biology and inhibiting GSC proliferation. AKT-STAT3-EZH2 signaling and EZH2 phosphorylation play essential roles in GSC growth. Melatonin treatment attenuated AKT activation, EZH2 S21 phosphorylation, EZH2-STAT3 interactions and altered histone modifications to repress tumor initiation and propagation. These results show that melatonin attenuates multiple crucial signals associated with GSC self-renewal and survival, and further suggest melatonin as an encouraging therapeutic agent for the treatment of GBM [17].

Melatonin-induced methylation of the ABCG2/BCRP promoter overcomes multidrug resistance in brain tumor stem cells: Recent studies demonstrate that brain tumor stem cells in malignant glioblastomas, which overexpress members of the adenosine triphosphate-binding cassette (ABC) family transporters, accounts for multidrug resistance and tumor recurrence [54-56]. Thus, a therapeutic strategy that targets both the tumor bulk and the brain tumor stem cell (BTSC) compartment is essential to bring about a stable and enduring remission [18].

Martin and colleagues observed that melatonin markedly increased the cancer killing activity of temozolomide, doxorubicin or mitoxantrone [18]. Their study indicated that co-incubation of melatonin plus a chemotherapeutic drug resulted in a synergistic inhibitory action in the three BTSC lines (NSC23, NSC7-2 and NSC11) tested. BTSCs revealed much higher mRNA levels for ABCG2/BCRP and ABCB1/MDR1 than in glioblastoma cell lines [18]. This finding was compatible with a higher resistance of BTSCs to temozolomide, doxorubicin and mitoxantrone. They found that melatonin induced a significant reduction in the mRNA levels of ABCG2/BCRP in BTSCs. These findings indicate that melatonin may inhibit multidrug resistance in BTSCs through the downregulation of ABCG2/BCRP expression, resulting in an increase in intracellular drug accumulation and the subsequent enhancement of cell death [18].

Martin and colleagues observed that glioblastoma samples isolated from patients had lower methylation levels in the ABCG2 promoter than the normal brain. Treatment of BTSCs with melatonin induced a significant reduction in the levels of unmethylated promoter and an accompanying increase in the levels of hypermethylation and partial methylation. Methylation-specific PCR (MSP) analysis verified the methylation of the ABCG2/BCRP promoter in BTSCs after treatment with melatonin [18]. Thus, melatonin may reduce transporter expression and BTSC resistance to chemotherapeutic drugs by inducing the methylation of the ABCG2/BCRP promoter.

These authors also reported that melatonin not only enhanced chemotherapy induced cell death in BTSCs but also in a human malignant glioblastoma cell line (A172). An elevation in promoter methylation by melatonin was inhibited by preincubation with 5-azacitidine (AZA). This finding suggests epigenetic regulation of ABCG2/BCRP expression and function by melatonin [18].

Briefly, melatonin increases the efficacy of chemotherapeutic agents, targeting both the bulk tumor and the BTSCs through the regulation of the expression and function of the ABCG2/BCRP transporter by inducing the methylation of its promoter [18]. The data indicate a probable correlation between the downregulation of ABCG2/BCRP function and the synergistic effect of melatonin and chemotherapeutic agents. Melatonin may be a worthy candidate to counteract multidrug resistance in the treatment of glioblastomas, and consequently improve the efficacy of contemporary therapies.

Involvement of autophagy in melatonin-induced cytotoxicity in brain tumor stem cells: Glioma-initiating cells (GIC) or brain tumor stem cells are resistant to contemporary therapeutic modalities, probably accounting for the frequent tumor recurrence [33,57]. Autophagy, the process of cellular self-eating, is recognized as a lysosomal degradation pathway that plays the role of a dynamic regulator of tumorigenesis [58,59].

In the study performed by of Martin and colleagues, neurospheroid cultures were established from cells dissociated from human glioblastoma postsurgical specimens [21]. Neurospheroid cultures display a GIC phenotype (self-renewal, proliferation, expression of stem cell markers, pluripotency, and ability to form tumors *in vivo*). To examine the effect of melatonin on cell proliferation in GIC derived from glioblastoma patients, three GIC lines (GIC-A, GIC-B, and GIC-C) were treated with different concentrations of melatonin (1-1000 μ M). Only the highest concentration (1 mM) inhibited the growth of the three GIC lines [21].

For the purpose of evaluating the effect of melatonin on self-renewal, limiting dilution assays were carried out [21]. Melatonin reduces formation of secondary spheres after dissociation. In the melatonin-treated groups, at least a doubling of cell number is required to generate a secondary neurosphere. Melatonin also reduces self-renewal capability in the clonogenic assay for the three GIC lines tested. Melatonin treatment reduces mRNA expression levels of stem cell markers such as the transcription factors Sox2, Oct3/4 and Nanog in the three GIC lines tested, implying the role of melatonin in the modulation of stem cell properties in GIC [21].

Martin and colleagues assessed the effect of melatonin on the viability of the GICs subpopulation. They found that melatonin induced a time-dependent increase in the lactate dehydrogenase (LDH) release starting at 48 hours after treatment which pointed out an induction of cell death. The effect of melatonin on the viability of a normal human

neural cell line (hNSC.100) was likewise assessed. No significant change in LDH release at any time point was found, indicating that the cancer killing effect of melatonin is limited to cancer-derived stem cells [21].

These authors further examined the ultrastructural characteristics of GIC after melatonin treatment to determine the nature of the effect of melatonin on these cells. Cells treated with melatonin revealed a progressive accumulation of autophagosome vacuoles starting at 24 hours after treatment. Vacuoles enclosed cytoplasmic content and were more numerous with increased treatment time. Consequently, at a later stage after melatonin treatment, vacuoles occupied a major part of cytoplasm and an immense disruption of the cellular membrane associated with an extremely vacuolated cytoplasm and disruption of structures in the cell were found. Melatonin induction of autophagy in GIC was also validated by Western blot. Treatment with melatonin enhances conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3 I) to LC3 II, which suggested initiation of autophagy cascade and formation of autophagosomes [21].

Briefly, the study of Martin and colleagues showed that melatonin treatment reduced GIC proliferation and caused a reduction in self-renewal and clonogenic capability which coexisted with a reduction in the expression of stem cell markers. Furthermore, the study also indicated that melatonin treatment induced cell death with ultrastructural characteristics of autophagy. Melatonin not only presents an antitumor effect on bulk tumor cells but also exhibits antitumor actions on the GIC subpopulation. Accordingly, the results indicate that melatonin could be a promising therapeutic agent in the treatment of glioblastoma.

Melatonin inhibits estrogen receptor binding to estrogen response elements sites on the OCT4 gene in human breast cancer stem cells: The transcription factor OCT4, encoded by the POU5F1 gene, is a crucial factor for self-renewal and maintenance of pluripotency of cancer stem cells [60-62]. Estradiol (E2) is the principal growth stimulant of estrogen receptor (ER) positive breast tumors [63,64]. Bisphenol A (BPA) is an environmental estrogen with analogous biological functions of E2. BPA plays a role in the initiation or progression of breast cancer and activates the transcription of genes which promote the proliferation of the breast cancer cells [65,66]. Lopes and colleagues assessed the effect of melatonin on the regulation of OCT4/POU5F1 in breast cancer stem cells (BCSC) after activation with tumor initiation chemical BPA and E2 in MCF-7 cells. Two individual techniques, cell suspension and anchorage independent growth, of three-dimensional growth of mammospheres were utilized [20]. The growth in a three-dimensional (3D) model provides an artificial tumor environment where the cells segregate appropriately to form components of adult tissues comparable to the situation found *in vivo* [67,68]. Both techniques were carried out to assess the effect of melatonin on mammospheres treated with BPA and E2. The results of both techniques were similar. The treatment with 10 nM E2 or 10 μ M BPA significantly increased the number and size of the mammospheres as compared with the control group. On the contrary, 1 mM of melatonin significantly reduced the number and size of mammospheres as compared with the control. Moreover, when the cells were stimulated by E2 or BPA and treated with melatonin simultaneously, there was an obvious reduction in the number and size of mammospheres [20].

The chromatin immunoprecipitation assay was carried out to determine the effect of melatonin on estrogen receptor binding to the POU5F1 gene. Mammospheres treated with 10 nM E2 or 10 μ M BPA markedly increased the binding of ER to the putative ERE (estrogen

response element) sequences in OCT4 transcription site OCT4-3544. On the contrary, the cells stimulated by 10 nM E2 or 10 μ M BPA and treated with 1 mM melatonin revealed a significant lowering in the binding of ER to the putative ERE sequences in OCT4 transcription site OCT4-3544. Moreover, mammospheres treated with E2 or BPA slightly enhanced the binding of ER to ERE sites at -1999 kb of OCT4 promoter region. This enhancement was reduced when mammospheres were treated with melatonin [20].

Quantitative real time PCR (qPCR) was conducted to examine the OCT4 and ER α gene expression levels after E2 and BPA treatment with or without melatonin. Cells treated with E2 or BPA exhibited increased levels of transcript for OCT4 and ER α genes. Levels of OCT4 and ER α mRNA were remarkably decreased in cells treated with melatonin alone, or simultaneous treatment with either E2 or BPA [20].

The effect of estrogen, BPA and melatonin on ER and OCT4 protein levels in these cells was evaluated. E2 and BPA markedly stimulated levels of ER α protein. OCT4 protein levels were also enhanced in cells treated with E2 or BPA. ER α protein levels were decreased in all cells treated with melatonin, but the extent of the reduction was not precisely related to the changes in mRNA levels, indicating that additional levels of regulation probably influenced ER α levels. In addition, melatonin reduced the levels of OCT4 protein. Only minimal changes in OCT4 transcript levels were noticed, implying that the regulation of protein levels of OCT4 may be modulated at a translational and/or post-translational level [20].

In summary, the study of Lopes and colleagues demonstrated that melatonin inhibited the effects of E2 and BPA treatment on mammosphere growth together with the expression of ER α and the stem cell marker OCT4. Melatonin treatment is effective in inhibiting the proliferation of BCSC and exerts an influence on the ER pathway, indicating it may be useful as a therapy in breast cancer.

Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells: The epithelial mesenchymal transition (EMT) is a process that allows cancer stem cells to become invasive and metastatic [69-71]. This process is mediated by the activity of growth and transcription factors, leading to loss of the intercellular junction structure of epithelial cells, obtaining a mesenchymal morphology, loss of apical-basal cell polarity and migration/invasion capability [72-74]. Various investigations have also indicated that EMT is involved in cell plasticity, the process by which non-stem cells acquire stem cell characteristics [75-77]. The principal EMT molecular features include loss of the epithelial marker E-cadherin, and overexpression of mesenchymal markers N-cadherin and vimentin [78,79]. Various studies also indicate that melatonin has anti-invasive and anti-metastatic effects, which involve multiple cellular processes including EMT [80-82].

A number of studies demonstrated the existence of stem cells in canine and human breast cancer cell lines [83-89]. The study of Dontu and colleagues demonstrated that the stemness of tumor cells is assessed *in vitro* by their capability to produce mammospheres [90]. Canine and human breast cancer cells readily produced mammospheres, and include CD44+/CD24low/- cells, which verify the cancer stem cell phenotype [90]. Breast cancer cells with CD44+/CD24low/- surface phenotype possessed tumor initiating features with pluripotency properties and invasive capability [19]. Ponti and colleagues observed that a breast cancer cell line that developed into spheroids also had CD44+/CD24low/- phenotype and expressed the transcription factor OCT4 [91]. The expression of OCT4 has an

essential role in carcinogenesis and offers a probable mechanism by which cancer cells obtain or sustain the therapy resistance phenotype [92,93]. Moreover, overexpression of this gene was related to metastasis and poor prognosis in various types of cancer, including colorectal, lung and gliomas [94-99].

In the study of Goncalves and colleagues, mammospheres were generated from the canine mammary cancer cell line CMT-U229 and human breast cancer cell line MCF-7 in MammoCultTM medium (StemCell Technologies). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to estimate the number of viable cells after the treatment with 1 mM melatonin for 24 hours. Cell viability from mammospheres of both cell lines was significantly reduced after treatment with 1mM melatonin for 24 hours as compared with control groups [19]. To assess the effects of melatonin on breast cancer stem cells from both cell lines, the protein expression of the cancer stem cell marker OCT4, the epithelial marker E-cadherin, the mesenchymal markers N-cadherin and vimentin, were estimated in cells from mammospheres. In CMT-U229 cells treated with 1 mM of melatonin, OCT4 protein expression was significantly reduced compared to the control group and E-cadherin protein expression was significantly enhanced after melatonin treatment compared to the control group. N-cadherin and vimentin protein expression were markedly reduced in the melatonin treated cells compared to the controls. Regarding MCF-7 cells, OCT4 protein expression was also lowered in melatonin treated cells compared to the controls ($p = 0.0001$) and E-cadherin protein expression increased after melatonin treatment ($p = 0.0001$). Low expression of N-cadherin and vimentin proteins was found in melatonin treated cells. The assessment of cell migration and invasion was performed in Boyden Chamber. CMT-U229 and MCF-7 cells were treated with 1 mM of melatonin for 24 hours. Melatonin reduced migration and invasion of CMT-U229 and MCF-7 mammospheres as compared with the control groups ($p = 0.0017$, $p = 0.0377$, respectively) [19]. The results collectively reveal that canine and human breast cancer stem cells are responsive to melatonin treatment with a reduction of viability and the invasiveness of breast cancer mammospheres as well as the expression of stem cell and EMT markers, indicating the potential therapeutic role of melatonin in the treatment of breast cancer.

The impact of carbon ion irradiation on tumor stem cells

Carbon ion irradiation effectively eradicates brain tumor stem cells: The capability of cells to survive radiation and generate colonies is associated with tumor radiosensitivity. Clonogenic survival assays have assessed radiosensitivity and establish predictive models for treatment result [100,101]. In the study of Chiblak and colleagues, human primary glioma stem cells (GSC or brain tumor stem cells) spheroid cultures were established from tumor specimens of six consenting glioblastoma patients [28]. Human U87MG was utilized as a conventional glioblastoma radioresistant cell line. NCH601, NCH620, NCH644, NCH441, NCH421k, and NCH636 primary cell lines were cultured as neurospheres employing serum-free cancer stem cell medium. The authors developed a setting by which tumor stem cells were grown under serum-free conditions in a semi-solid 3D matrix, offering an environment that maintained the stem cell-like characteristics. Using the XRAD320 X-ray device (Precision X-Ray, North Branford, CT), these cells were irradiated at 0-, 2-, and 4-Gy photon at 320 KeV. Particle irradiation with proton and carbon ion was performed using a pencil beam in a spread-out Bragg peak with 1.5 cm width equivalent to a depth of 14.0 cm in water. Beam energies were up to a maximum of 221 MeV/u for protons and 430 MeV/u for

carbon ions [102-104]. After irradiation of GSC with 0-, 2-, and 4-Gy photon, survival fraction (SF) was examined in a 2-step process. At first colonies were initially manually counted. Sphere-like colonies were counted using microscopy 2 weeks after irradiation. Two response patterns of GSC were observed after irradiation with increasing doses. NCH644 and NCH421k were most resistant to photon, with 40-50% SF2GyX and 20-25% SF4GyX. On the contrary, survival of NCH441, NCH663, NCH620, and NCH601 was reduced by increasing doses, with SF2GyX values ranging between 3% and 17%, and SF4GyX values ranging between 0.5% and 2%.

Subsequently, the four photon radioresistant GSCs (ie, NCH644, NCH421k, NCH441, NCH636) and human U87MG GBM cells were irradiated at 4-Gy photon, proton, or carbon. Cell survival was assessed by measuring fluorescence. The data revealed NCH644 (SF4GyX 87%, $P < 0.137$) and NCH421k (SF4GyX 81%, $P < 0.0001$) as the two most photon-resistant GSCs, with a radioresponsiveness similar to that of U87 (SF4GyX 80%, $P < 0.023$). Irradiation of GSCs and U87 with 4-Gy proton resulted in only a slight decrease in survival compared with photon irradiation. Carbon ion irradiation at an isodose of 4 Gy demonstrated conspicuous cell killing as revealed by the highest survival reduction. Clonogenic survival for proton irradiation demonstrated that relative biologic effectiveness (RBE) was in the range of 0.7-1.20. Nevertheless, carbon irradiation made the photon-resistant GSC cultures sensitive, with RBE in the range of 1.87-3.44.

Radioresistance of GSCs was partly due to DNA repair proficiency compared with non-GSCs (33, 105). As compared with photon irradiation, a higher percentage (2.4-fold) of GSCs stained positive for nuclear γ -H2AX 24 hours after carbon ion irradiation, demonstrating residual unrepaired double-strand breaks. Likewise, carbon ion irradiation increased persistent γ -H2AX foci as compared with photon irradiation in putative pancreatic stem cells (32, 106). These results indicate that the sensitivity of cancer stem cells to carbon ions might exhibit an impaired capability of GSCs to repair carbon ion-induced DNA double-strand breaks. Carbon irradiation is effective in GSC elimination with RBE in the range of 1.87-3.44. The study indicates that carbon ion radiation therapy may constitute a therapeutic approach for treatment of glioblastoma.

Carbon ion irradiation counteracts cancer stem cells' migration and invasion process in head and neck squamous cell carcinoma (HNSCC):

Approximately two-thirds of patients with head and neck squamous cell carcinoma (HNSCC) exhibit an advanced stage disease at diagnosis [107-109]. Combined modality therapy consists of surgery and radiotherapy with or without chemotherapy or molecular targeted therapy [110-112]. No matter what the therapeutic intervention, however, HNSCC is still associated with a high rate of recurrence [113,114]. In addition, metastatic disease remains the principal cause of death in cancer [115,116]. Cell migration and invasion are fundamental steps of the metastatic phenomenon [117,118]. Moncharmont and colleagues reported that migration and invasion were significantly increased by a 2 Gy photon irradiation in head and neck squamous cell carcinoma cells derived from a recurrent laryngeal cancer (SQ20B cells) [29]. Enhancement of migration by photon radiation had been previously reported in several studies [119,120]. After photon radiation, EGFR is activated by cellular stress induced by radiation [121,122]. EGFR enhancement could be related to activation of intracellular signaling pathways, resulting in the secretion of matrix metalloproteases (MMP) [121,123,124].

A subpopulation of cancer cells, the cancer stem cells (CSCs), exhibit excessive migratory and invasive potential [125,126]. These

cells are present in HNSCC, and overexpress CD44 and aldehyde dehydrogenase (ALDH) proteins, which are currently regarded as HNSCC CSCs' markers [127-129]. Moncharmont and colleagues reported that consecutive cell sorting was performed to isolate SQ20B/CSCs from SQ20B parental population utilizing Side Population (SP). SQ20B/CSCs migration and invasion capacities were greater than SQ20B parental cells in basal conditions. These capacities are related to their mesenchymal phenotype with presentation of a high N-cadherin expression and a low E-cadherin expression [29]. These phenotypic characteristics are similar to those reported in other studies where CSCs present a mesenchymal phenotype [130,131]. The aggressive characteristics (high migratory and invasive potential) of cancer stem cells (CSCs) in head and neck squamous cell carcinoma may explain their resistance to conventional chemotherapy and radiotherapy [29]. Various means to improve local control and long-term survival in advanced SCCHN have been implemented [132,133].

EGFR inhibition plays an essential role in decreasing tumor cell repopulation by modulation of cellular proliferation and enhancement of radiosensitivity of the tumor [134,135]. Cetuximab, a chimerized immunoglobulin G1 monoclonal antibody against the ligand-binding domain of EGFR, inhibits the tyrosine kinase activity of EGFR and increases the cytotoxic effects of radiation in squamous-cell carcinoma [136,137].

Beuve and colleagues reported that high linear energy transfer (LET) radiation induces a twofold increase in relative biological effectiveness (RBE) compared to that of photon radiation [138]. Mizoe and colleagues demonstrated that carbon ion irradiation was effective in the treatment of malignant melanoma and adenoid cystic carcinoma of the head and neck [139,140].

Moncharmont and colleagues observed that 2 Gy photon irradiation failed to inhibit cell proliferation of SQ20B cells and SQ20B/CSCs. Treatment with cetuximab or combined treatment with cetuximab and photon irradiation reduced SQ20B proliferation. No reduction of SQ20B/CSCs proliferation was noted. The calculated SF2 of SQ20B was significantly decreased with cetuximab (0.81 vs 0.62 without or cetuximab, respectively, $p = 0.007$) in contrast to SQ20B/CSCs (0.77 vs 0.73, with and without cetuximab, $p = 0.62$). Carbon ion radiation decreased the survival fraction of SQ20B and SQ20B/CSCs, with a relative biologic effectiveness (RBE) at 10% survival of 1.6 and 1.8, respectively. The combination of carbon ion radiation with cetuximab completely inhibited migration and invasion in SQ20B cells ($p < 0.01$ and $p < 0.005$, respectively). Cetuximab was without effect on the survival fraction of SQ20B/CSCs. Migration of SQ20B/CSCs was significantly reduced by carbon ion radiation ($p < 0.05$). A significant reduction of the invasion in SQ20B/CSCs subpopulation was observed after the combined therapy with carbon ion irradiation and cetuximab ($p < 0.005$) [29].

These results demonstrate the existence of a subpopulation of head and neck cancer stem cells characterized by high migratory and invasive capacities, low EGFR expression and resistance to cetuximab, which could account for the local recurrence and distant metastasis in HNSCC after conventional treatment. Carbon ion irradiation seems to be a promising therapeutic modality because it reduces the migration and invasion processes in both head and neck squamous cell carcinoma cells and cancer stem cells in contrast to photon irradiation.

Carbon ion beam combined with cisplatin effectively disrupts triple negative breast cancer stem-like cells *in vitro*: Triple-negative breast cancers (TNBC), why tumors that are negative for estrogen

receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) exhibit invasive behavior, poor prognosis and have few targeted therapies [141,142]. Breast cancer stem-like cell (BCSC) populations have recently been recognized on the basis of the cell membrane markers CD44+/CD24- ESA+ cells [143,144]. BCSCs present the capacity for self-renewal and multi-lineage differentiation, tumorigenicity, and chemotherapy and radiotherapy resistance, features that account for tumor progression, disease recurrence, and metastasis [145,146]. Thus, the development of innovative CSC targeting therapeutics is critical [147,148].

Heavy ion beams present a specific range and minor scatter in tissues with well-localized energy deposition at the end of the beam path; this is referred to as the "spread-out Bragg peak (SOBP)", a distinctive physical feature of charged particle beams. This results in the release of tremendous amount of energy at the end of their range. Accordingly, these ion beams bring about more cell cycle- and oxygenation-independent, irreparable DNA damage and eradicate more resistant cancer cells than conventional radiation [149,150]. Recently, a phase I clinical trial of early stage breast cancer treated with heavy ion irradiation noticed a limitations of dose escalation owing to adverse effects of skin, ribs, and lungs after carbon ion radiotherapy, particularly for some aggressive subtypes of breast cancer such as TNBC [30,151]. The authors considered that carbon ion beam combined with chemotherapy might decrease the dose of irradiation but preserve certain advantages to eliminate breast cancer [30,151]. The combination of chemotherapy with heavy ion radiotherapy might open new means to counteract this challenging breast cancer subgroup which has poor prognosis and highly limited treatment choices.

Sai and colleagues investigated the effects of a carbon ion beam alone or in combination with CDDP on triple negative (TN) BCSCs survival, DNA repair, and variations of in the expression of various genes compared to that of X-ray irradiation. Human TNBCSCs isolated from MDA-MB-231 and MDA-MB-453 cells were treated with carbon ion or X-ray irradiation alone or in combination with CDDP. Colony, spheroid and tumor formation assays, RT-PCR array analysis of gene expression, and immunofluorescence γ H2AX foci assay were carried out subsequently [30].

The colony, spheroid formation, and tumorigenicity assays verified that CD44+/CD24- and ESA+/CD24- cells presented CSC properties. The percentage of CD44+/CD24- cells increased dose dependently after X-ray irradiation, while no substantial changes were observed after carbon ion irradiation with equivalent dose of X-ray. The proportion of CD44+/CD24- cells increased substantially when X-ray combined with CDDP compared to that of carbon ion beam with CDDP. The RBE values estimated at the D10 level for CSCs were approximately 2.14, whereas those for non-CSCs were roughly 1.78. These data indicate that the carbon ion beam is more effective in eliminating CSCs [30].

The number of colonies formed of both CSCs and non-CSCs was remarkably decreased when carbon ion beam was combined with 25 μ M of CDDP compared to carbon ion beam alone or X-ray combined with CDDP. Likewise, tumor spheroid formation of cancer stem like CD44+/CD24- cells sorted from MDA-MB-231 cells was significantly reduced after carbon ion beam irradiation compared to that following X-ray irradiation; these were further reduced when the carbon ion beam was combined with CDDP. Spheroid formation ability of ESA+/CD24- cells sorted from MDA-MB-453 was substantially inhibited by carbon ion beam alone but not X-ray, and it was markedly suppressed by the combination of carbon ion beam irradiation with CDDP [30].

RT-PCR array analysis of multiple gene expression variations in radioresistant CSCs (CD44+/CD24- cells) sorted from MDA-MB-231 cells revealed that treatment with a carbon ion beam combined with 25 μ M of CDDP for 5 days significantly elevated the expression of apoptosis-related cytochrome c, and had substantially increased Bax and autophagy-related genes LC3 compared to X-ray, cisplatin alone or X-ray combined with cisplatin. These findings document that carbon ion beam treatment combined with CDDP may have a greater effect in inducing multiple cell death. Moreover, expression of CSC markers, CD44 and ESA, were nearly eliminated by carbon ion beam combined with CDDP, while X-ray, CDDP alone or X-ray combined with CDDP significantly increased the expression of ESA. Furthermore, expressions of angiogenesis- and metastasis-related genes such as HIF1 α and CD26 were remarkably inhibited by carbon ion beam combined with CDDP, while cisplatin alone or X-ray combined with CDDP significantly increased the expression of HIF1 α and CD26. Thus, carbon ion beam irradiation in combination with CDDP may be highly effective in inhibiting tumor angiogenesis and metastasis.

The immunofluorescence assay showed that a high number of γ H2AX foci formed at 1 hour after a carbon ion beam, X-ray alone, and in combination with CDDP further increased the number of γ H2AX foci in CD44+/CD24- cells sorted from MDAMB-231 cells. Nevertheless, at 24 h after carbon ion irradiation, the induced γ H2AX foci level persisted at a level significantly higher than that in X-ray irradiated cells with an equal dose. Carbon ion beam in combination with cisplatin markedly elevated the number of γ H2AX foci compared to carbon ion beam, X-ray, cisplatin alone or X-ray combined with cisplatin. Additionally, not only was there a noticeable rise in the number, but also in the size, of foci (clustered DSB) commonly found in carbon ion beam exposure combined with cisplatin-treated cells compared to carbon ion beam, X-ray, cisplatin alone or X-ray combined with cisplatin-treated cells. By means of the immunofluorescence assay, the number and size of nuclear γ H2AX foci formed in CSCs (ESA+/CD24- cells) sorted from MDA-MB-453 cells at 24 h after a carbon ion beam, X-ray alone or in combination with 25 μ M of CDDP revealed that a much larger abundance of γ H2AX foci persisted after carbon ion beam combined with CDDP [30].

These findings demonstrate that principal effects of carbon ion beam in combination with CDDP on TNBC cell killing primarily result from effective elimination of radioresistant TNBCSCs. The combined use of carbon ion irradiation with CDDP provides strong evidence for targeting TNBCSCs because of the complex DNA damage, increased apoptosis, autophagy, and subsequent cell death compared to conventional X-ray or carbon ion irradiation alone.

Effects of carbon ion beam on putative colon cancer stem cells and its comparison with X-rays: Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related deaths in developed countries [152,153]. Complete resection of the tumor is the exclusively curative treatment modality for localized colon cancer. Nevertheless, treatment outcome for locally advanced colon cancer remains discouraging despite recent advances in surgery with adjuvant therapy [154,155]. Accordingly, it is imperative to investigate innovative treatment strategies for the purpose of finding a solution to the problems of discouraging treatment consequences resulting from tumor invasion to contiguous tissues, widespread metastasis, and resistance to chemotherapy and radiotherapy.

Because heavy-ion radiotherapy possesses clear advantages in treating various human radioresistant tumors, Cui and colleagues hypothesized that heavy ion irradiation may efficaciously target cancer

stem cells. They investigated whether heavy-ion irradiation may have advantages over X-rays in targeting human colon cancer stem-like cells [31]. The colon adenocarcinoma cell lines HCT116 and SW480 were acquired from American Type Culture Collection. For assays of clonogenicity and ability to grow as "tumor spheres" in suspension, HCT116 and SW480 cells were isolated to obtain populations of CD133++ and CD133- , CD44+/ESA+ and CD44- /ESA- cells by BD FACSAria (Becton Dickinson). The initial energy of the carbon-ion beams was 290 MeV/n, 50KeV/mm, 6-cm SOBP. The energy of heavy-ion beams at the irradiation site was obtained by comparing the calculated and measured depth-dose distribution [31].

The HCT116 and SW480 cells were irradiated with carbon ion or X-rays up to 6 Gy, and their survival fraction was measured based on colony formation. The surviving fractions for the HCT116 and SW480 irradiated with X-rays and carbon ions caused exponentially reductions with increasing doses. Based on the survival curves, the RBE values calculated by the D10 is about 1.63 to 1.74 for carbon ion beams. After the isolation of CD133+, CD44+/ESA+ cells from the HCT116 and SW480 cells, respectively, CD133+, CD44+/ESA+ cells exhibited higher clonal and spheroid formation capacities *in vitro* and vigorous tumorigenicity in a xenograft model. The data implied that CD133+, CD44+/ESA+ cells exhibited the characteristics of cancer stem-like cells [31].

FACS (fluorescence-activated cell sorting) analyses revealed that the percentage of cancer stem-like cells with positive CD133, ESA, and CD44 was more enriched after X-ray irradiation compared with carbon ion irradiation. The percentage of CD133+/ CD44+ and ESA+/ CD44+ cells were enriched 2- to 3-fold after irradiation with 2 or 4 Gy X-rays. Conversely, the percentage of CD133+/ CD44+ and ESA+/ CD44+ cells reduced or remained stationary after 1 or 2 Gy carbon ion irradiation [31].

The surviving fractions for the cancer stem-like cells sorted from HCT116 or SW480 cell lines after irradiation with X-rays and carbon ions were reduced exponentially with increasing doses. Based on the survival curves, the RBE values calculated at the D10 level for cancer stem-like cells were about 2.05 to 2.28, while RBE values for non-cancer stem-like cells were about 1.22 to 1.44. Judging from the tumor growth delay, the RBE values of 50 keV/mm carbon ion at the middle of a 6-cm SOBP relative to X-ray were calculated as 3.05 to 3.25 [31].

Histopathologic alterations of xenograft tumors after irradiation with X-rays or carbon-ion beams for 4 weeks were examined by H&E staining. Histopathologic features revealed that majority of the tumor cells were not disrupted by 15 Gy X-rays or 5 Gy carbon ion irradiation. Fifteen Gy carbon ion irradiation primarily induced colon cancer cell cavitations, fibrosis and total disruption of the duct-like architecture. On the contrary, 30 Gy X-ray irradiation merely incompletely destroyed colon cancer cells while the duct-like architecture was preserved. These findings revealed that nearly all of the tumor cells were eliminated after 30 Gy carbon ion irradiation [31].

In vivo FACS analyses showed that the percentage of CD133+/ ESA+ cancer stem-like cells increased 1 month after 15 and/or 30 Gy X-ray irradiation. Nonetheless, these cancer stem-like cells significantly reduced 1 month after 60 Gy X-ray irradiation. In contrast, 15 Gy of carbon ion irradiation did not alter the percentage of the cancer stem-like cells, while 30 Gy of carbon ion irradiation markedly lowered the percentage of cancer stem-like cells [31].

The findings verify that low LET X-ray irradiation may essentially eradicate the non-stem-like tumor cells, consequently the radioresistant

cancer stem-like cell population is obviously enriched. By comparison, carbon ion irradiation eradicates both non-stem-like and stem-like tumor cells; subsequently, the proportion of cancer stem-like cells was slightly increased or remained stationary. Briefly, carbon ion irradiation is a promising therapeutic modality because of its improved targeting of putative colon cancer stem-like cells.

Different effects of carbon ion beams and X-rays on clonogenic survival and DNA repair in pancreatic cancer stem-like cells: Pancreatic cancer represents approximately 3% of newly diagnosed cancers annually worldwide and is the fourth leading cause of cancer-related deaths in the United States and Europe [156-158]. It is an aggressive disease. Despite recent advances in treatment, it remains a fatal malignancy and the 5-year overall survival (OS) rate does not exceed 5% [156,157,159]. The cell surface markers CD44, CD24, and epithelial-specific antigen (ESA) have been reported as markers for detecting pancreatic cancer stem cells. CD44+CD24+ESA+ pancreatic cancer cells display the stem cell characteristics of self-renewal, the capability to produce differentiated progeny, and enhanced expression of the molecules which are essential in developmental signaling pathways [160,161].

Radiotherapy is commonly used for cancer therapy and depends on ionizing radiation -induced DNA damage, especially the induction of DNA double strand breaks (DSBs). Phosphorylation of H2AX is a sensitive marker for detection of radiation-induced DSB [162,163]. The number of radiation induced γ H2AX foci is closely related to the number of DSB (164, 165). Slowly repaired or unrepaired DSB may account for cell death [32,166]. Carbon ion irradiation is regarded as a more effective therapy than X-ray irradiation owing to the high RBE, the lack of the oxygen effect, and less cell cycle-related radiosensitivity [32,167]. Furthermore, some studies have demonstrated that DSBs induced by heavy ion irradiation are repaired with slow kinetics compared to those induced by photon irradiation [168].

Cancer stem-like cells sorted from human pancreatic cancer cell lines, MIA PaCa-2 and BxPc-3 cells, were cultured with serum-free culture medium. These cells were treated with and without carbon ion or X-ray irradiation, and then colony, spheroid, tumor formation assays together with γ H2AX foci formation assay were carried out [32]. CD44+/CD24+ cells had significantly higher clonal formation capability than that of CD44-/CD24- cells. The ability to form spheroid bodies in CD44+/CD24+ cells was noticeably higher than that in CD44-/CD24- cells. The tumorigenicity of CD44+/CD24+ cells was also much higher than that of CD44-/CD24- cells. The data indicate that CD44+/CD24+ cells isolated from MIA PaCa-2 and BxPc-3 cells exhibited the characteristics of cancer stem-like cells [32].

The surviving fractions for the MIA PaCa-2 and BxPc-3 cells irradiated with X-rays and carbon-ion beams declined exponentially with increasing doses. The RBE values calculated at the D10 level, were 1.85-2.10 for carbon-ion beams. The surviving fractions for the cancer stem like cells isolated from the two cell lines after irradiation with X-rays and carbon-ion beams declined exponentially with increasing doses. The RBE values calculated at the D10 level for cancer stem-like cells were 2.0-2.19. The number of tumor spheroid formations was significantly lower in carbon ion irradiated cancer stem-like cells sorted from both cell lines compared to that of X-ray irradiated cells. A high number of γ H2AX foci formed at 1 or 6 hours after X-ray or carbon ion irradiation was observed both in CD44+/CD24+ and CD44-/CD24- cells. Nevertheless, at 24 hours after carbon ion irradiation, the γ H2AX foci level persisted at a significantly higher level than that of X-ray irradiated cells. A remarkable increase in the size of foci (clustered

DNA damage) was exclusively found in carbon-ion beam irradiated cells. Furthermore, the number of γ H2AX foci formed in CD44+/CD24+ cells declined more markedly than in CD44-/CD24- cells after X-ray irradiation, suggesting that CD44+/CD24+ cells possess an increased capability to repair X-ray-induced DSBs, which is probably a principal contributor to their greater degree of radioresistance [32].

The percentage of cancer stem-like CD44+/CD24+ cells was more enriched after X-ray irradiation compared to carbon ion irradiation. The percentage of cancer stem cell-like CD44+/CD24+ cells (in MIA PaCa-2 and BxPc-3 cells) rose markedly by 3- to 6-fold after X-ray irradiation, while the percentage of these cells merely doubled in MIA PaCa-2 cells or decreased in BxPc-3 cells after carbon ion irradiation. The data indicate that cancer stem cell-like CD44+/CD24+ cells may be resistant to X-ray causing selective killing of non-cancer stem cell-like CD44-/CD24- cells resulting in a rise in the relative proportion of cancer stem cell-like cells. However, carbon ion irradiation may eliminate both non-stem-like and stem-like tumor cells simultaneously, with minimal changes in the percentage of cancer stem cell-like cells in the population. The surviving fractions for the cancer stem-like cells after irradiation with X-rays or carbon ion beams declined exponentially with increasing doses, and the RBE values of carbon ion calculated at the D10 level for cancer stem-like cells isolated from MIA PaCa-2 and BxPc-3 were 2.0-2.19; this indicates that the carbon ion irradiation may be highly useful to eliminate cancer stem-like cells. On the contrary, RBE value at the D10 level for non-cancer stem-like cells sorted from MIA PaCa-2 was only 1.47, indicating that the difference in killing pancreatic cancer cells between carbon ion beam and X-rays might essentially result from the powerful effects of carbon ion irradiation on cancer stem-like cells [32]. In summary, these findings demonstrate the potential benefits of utilizing carbon ion irradiation for targeting pancreatic cancer stem-like cells which are resistant to conventional radiotherapy.

Discussion

The results of recent research indicate that tumors exhibit a cellular hierarchy, with a subpopulation of cancer cells possessing a tumorigenic potential much greater than that of other cancer cells. This extremely tumorigenic subpopulation of cells at the top of the hierarchy consists of cancer stem cells (CSCs) and generates progenitors and cells at different levels of differentiation along a variety of lineages [5].

CSCs possess the ability to self-renew and differentiate into various tumor components through stemness pathways, such as Wnt, TGF- β , STAT, and Hippo-YAP/TAZ. Stemness pathways in normal stem cells (NSCs) are stringently regulated and modulate many important biologic processes. On the contrary, stemness pathways in CSCs are highly dysregulated [1]. CSCs depend on distinct reprogrammed pathways to retain stemness and to play a part in the progression of cancers. The exact targeting of CSCs, along with chemotherapy or radiotherapy, may achieve steady remission or be an aid in curing cancer [2].

The study of Chen and colleagues demonstrated that melatonin precisely targeted glioma tumor cells by modifying glioblastoma stem-like cell (GSCs) biology and inhibiting GSC proliferation. AKT-STAT3-EZH2 signaling and EZH2 phosphorylation play essential roles in GSC growth. Melatonin attenuated AKT activation, EZH2 S21 phosphorylation, EZH2-STAT3 interactions and altered histone modifications to reduce tumor initiation and propagation. These results show that melatonin reduces multiple crucial signals associated with GSC self-renewal and survival [17].

Drug efflux by ATP-Binding Cassette (ABC) transporters is one of the mechanisms of CSC therapy resistance [169]. ABC-transporters are renowned for their involvement in multiple drug resistance in various human cancers [170]. Three proteins of the ABC transporter family were comprehensively examined as regulators of the multidrug resistance in tumors, including P-glycoprotein (P-gp, MDR1, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2) [171,172]. These three transporters have an extensive overlap in drug specificity offering tumor resistance to the principal classes of chemotherapeutic drugs and molecularly targeted therapies [32,33,173]. Inhibition of ABCG2 transporter activity with phosphodiesterase-5 inhibitors and fumitremorgin-type indolyldiketopiperazine, Ko143, might intensify the efficacy of the chemotherapeutic agents [174,175]. In addition, Martin and colleagues documented that melatonin increases the efficacy of chemotherapeutic agents, targeting both the tumor bulk and brain tumor stem cells (BTSCs) through the regulation of the expression and function of the ABCG2/BCRP transporter by inducing the methylation of its promoter [18]. This result implies a probable correlation between the downregulation of ABCG2/BCRP function and the synergistic toxic effect of melatonin and chemotherapeutic agents [18]. Based on this, melatonin is a likely candidate to counteract multidrug resistance in the treatment of glioblastomas.

OCT4, a member of POU family, is a transcription factor that is required for self-renewal and maintenance of pluripotency of cancer stem cells [60,176]. Mammospheres treated with E2 or BPA markedly increased the binding of ER to the putative ERE (estrogen response elements) sequences in OCT4 transcription site OCT4-3544. Conversely, mammospheres exposed to E2 or BPA and treated with melatonin revealed a significant reduction in the binding of ER to the putative ERE sequences in OCT4 transcription site OCT4-3544. Additionally, mammospheres exposed to E2 or BPA slightly enhanced the binding of ER to ERE sites at -1999 kb of OCT4 promoter region. This enhancement was reduced when mammospheres were treated with melatonin [20].

Autophagy captures, degrades, and recycles intracellular proteins and organelles in lysosomes. In most situations autophagy promotes tumorigenesis [177]. Cancers are able to upregulate autophagy to survive microenvironmental stress and to enhance growth and aggressiveness. Attempts to suppress autophagy to improve cancer therapy have attracted great attention [177]. The study of Martin and colleagues indicated that melatonin inhibited BTSCs proliferation and induced a reduction in self-renewal and clonogenic capability coexisted with a reduction in the expression of stem cell markers. In addition, the study also indicated that melatonin treatment induced cell death with ultrastructural characteristics of autophagy [21].

Recent studies have pointed out a connection between epithelial-to-mesenchymal transition (EMT) and cancer stem cell (CSC) formation. EMT is involved in the acquisition and maintenance of stem cell-like characteristics and is adequate to endow differentiated normal and cancer cells with stem cell properties. In addition, CSCs usually exhibit EMT properties. This mutual relationship between EMT and CSCs might be related to tumor progression [178]. The study of Goncalves and colleagues demonstrated that melatonin exhibited an inhibitory role in the viability and invasiveness of breast cancer mammospheres as well as in inhibiting the expression of OCT4, N-cadherin and vimentin proteins associated with EMT in breast CSCs [19].

Intratumoral hypoxia, resistance to radiation-induced apoptosis, a high capacity for the repair of DNA double-strand breaks (DSBs), and

mutations in certain oncogene and tumor suppressor genes are related to tumor resistance to X-ray radiotherapy [179]. Intratumoral hypoxia is a principal contributor to the X-ray resistance of cancer cells. Low pretreatment intratumoral pO₂ values is related to poor outcomes after X-ray radiotherapy. On the contrary, carbon ion radiotherapy revealed favorable antitumor effects in patients with locally advanced uterine cervical cancer, regardless of pretreatment intratumoral pO₂ levels. Cancer cell resistance to radiation-induced apoptosis is another crucial factor that plays a part in X-ray resistance. Carbon ions effectively eliminate cancer cells that are resistant to apoptosis induced by X-ray irradiation. The high capacity of cancer cells for double-strand break (DSB) repair gives rise to X-ray resistance. Carbon ion-induced complex DSBs are more arduous to repair than X-ray-induced DSBs; these sustained unrepaired DSBs then result in mitotic catastrophe [179].

Glioma stem cells (GSCs) are related to tumorigenesis, recurrence and treatment resistance. CD133-positive glioma cells indicate the subpopulation that presents glioma radioresistance and is regarded as the source of tumor recurrence after radiotherapy. Huynh and colleagues demonstrated that GRP78, an antistress protein, was highly expressed in GBM cells associated with the development of GSCs. When GRP78 was silenced, GSC properties were inhibited and radiosensitivity increased [180]. Four photon radioresistant GSCs and U87MG GBM cells were irradiated with 4-Gy photon, proton, or carbon (28). Cell survival data demonstrated that NCH644 and NCH421k were the two most photon-resistant GSCs. Carbon ion irradiation at an isodose of 4 Gy revealed noticeable cell killing. Carbon ion irradiation is effective in GSC eradication [28].

There is a considerable amount of evidence that crosstalk between cancer cells and cells of neoplastic stroma is essential in the acquired capability for invasion and metastasis [181]. Migration and invasion were substantially increased by a 2 Gy photon irradiation in head and neck squamous cell carcinoma cells (SQ20B cells) [29]. Migration of SQ20B/ CSCs was profoundly reduced by carbon ion radiation. Carbon ion irradiation may be a prospective treatment option because it counteracts migration and invasion processes in both head and neck squamous cell carcinoma cells and CSCs in contrast to photon irradiation [29].

The relationship between XRCC4 and radiosensitivity of human colon cancer stem-like cell to X-ray or carbon ion beam was investigated by Sai and colleagues. XRCC4, a member of NHEJ (non-homologous end-joining) for double strand breaks, may play an essential role in carcinogenesis. XRCC4 inactivation notably radiosensitized human colon cancer stem-like cells. The expression of cancer stem-like cell markers were significantly enhanced by X-ray in contrast to carbon ion irradiation [182]. Carbon ion irradiation may simultaneously eliminate both non-stem-like and stem-like tumor cells; accordingly, the proportion of cancer stem-like cells slightly increases or remains stationary. Fifteen Gy carbon ion irradiation induced a more noticeable xenograft tumor cell cavitation and fibrosis without obvious enhancement of cells with putative cancer stem cell markers compared with that induced by 30 Gy X-ray irradiation. Carbon ion irradiation is a promising therapeutic modality owing to improved targeting of putative colon cancer stem-like cells.

Most patients diagnosed with pancreatic cancer do not attain factual responses owing to the existence of intrinsic and acquired radioresistance. Distinguishing of molecular mechanisms that diminish the efficacy of radiation therapy and targeting these pathways is important for improving radiation response in patients with pancreatic cancer [183]. In the study of Oonishi and colleagues, the proportion of

cancer stem-like CD44+/CD24+ cells was more enriched after X-ray irradiation compared to carbon ion irradiation. The percentages of cancer stem cell-like CD44+/CD24+ cells increased remarkably by 3- to 6-fold after X-ray irradiation, whereas the proportion of these cells only doubled or decreased in pancreatic cancer stem-like cells after carbon ion irradiation [32]. The RBE values of carbon ion for pancreatic cancer stem-like cells ranged from 2.0 to 2.19, indicating that the carbon ion beam has significant capability to eliminate pancreatic cancer stem-like cells.

Conclusion

Cancer stem cells exhibit an excessive migratory and invasive potential. These cells possess the capacity of self-renewal and pluripotency, generating all cells within a tumor, and are responsible for tumor growth, therapy resistance and metastasis.

Melatonin attenuated AKT activation, EZH2 S21 phosphorylation, EZH2-STAT3 interactions and altered histone modifications to reduce tumor initiation and propagation of brain tumor stem cells (BTSC). Melatonin reduces multiple crucial signals associated with BTSC self-renewal and survival. Melatonin increases the efficacy of chemotherapeutic agents, targeting both the tumor bulk and BTSCs through the regulation of the expression and function of the ABCG2/BCRP transporter by inducing the methylation of its promoter. There is also a possible correlation between the downregulation of ABCG2/BCRP function and the synergistic toxic effect of melatonin and chemotherapeutic agents. Melatonin may be a worthy candidate to counteract multidrug resistance in the treatment of glioblastomas. Melatonin treatment induced cell death with ultrastructural characteristics of autophagy.

Melatonin inhibited the effects of E2 and BPA treatment on mammosphere growth together with the expression of ER α and the stem cell marker OCT4. Melatonin treatment is effective in inhibiting the proliferation of breast cancer stem cells (BCSCs) and influences the ER pathway. The BCSCs are responsive to melatonin treatment by way of reducing the viability and the invasiveness of breast cancer mammospheres as well as regulating the expression of OCT4, N-cadherin and vimentin proteins associated with EMT in BCSCs.

As compared with photon irradiation, a higher percentage (2.4-fold) of brain tumor stem cells (BTSCs) stained positive for nuclear γ -H2AX 24 hours after carbon ion irradiation, documenting residual unrepaired double-strand breaks. Sensitivity of cancer stem cells to carbon ions might be a result of the impaired capability of BTSCs to repair carbon ion-induced DNA double-strand breaks. Carbon irradiation is effective in BTSCs elimination with RBE in the range of 1.87-3.44.

A subpopulation of head and neck cancer stem cells characterized by high migratory and invasive capacities, a low EGFR expression and a resistance to cetuximab, which may promote local recurrence and distant metastasis in HNSCC after treatment. Carbon ion irradiation seems to be a promising therapeutic modality because it resists migration and invasion processes in both head and neck squamous cell carcinoma cells and cancer stem cells in contrast to photon irradiation.

Principal effects of carbon ion beam in combination with CDDP on TNBC cell killing primarily result from effective elimination of radioresistant TNBCSCs. Carbon ion irradiation combined with CDDP provides significant advantages for targeting TNBCSCs as a result of complex DNA damage, increased apoptosis, autophagy, and subsequent cell death compared to conventional X-ray or carbon ion irradiation alone.

In vivo FACS analyses revealed that the percentage of CD133+/ESA+ colon cancer stem-like cells increased 1 month after 15 and 30 Gy X-ray irradiation. In contrast, 30 Gy of carbon ion irradiation significantly reduced the percentage of colon cancer stem-like cells. Low LET X-ray irradiation may essentially eradicate the non-stem-like tumor cell, consequently the radioresistant cancer stem-like cell population is obviously enriched. In contrast, carbon ion irradiation may simultaneously eradicate both non-stem-like and stem-like tumor cells at the same time. Carbon ion irradiation is clearly a promising agent to eradicate putative colon cancer stem-like cells.

The number of tumor spheroid formations is significantly lower in carbon ion irradiated cancer stem-like cells sorted from human pancreatic cancer cell lines, MIA PaCa-2 and BxPc-3 cells, compared to that of X-ray irradiated ones. The surviving fractions for the cancer stem-like cells after irradiation with X-rays or carbon ions declined exponentially with increasing doses, and the RBE values of carbon ion for cancer stem-like cells isolated from MIA PaCa-2 and BxPc-3 were about 2.0-2.19, indicating that the carbon ion beam has significant potential to eliminate pancreatic cancer stem-like cells.

Further investigation to elucidate the mechanisms and molecular pathways involved in cancer stem cells particularly associated with melatonin and carbon ion irradiation certainly is warranted. Finally, considering the similar actions of melatonin and carbon ion irradiation in terms of altering the features of cancer stem cells in tumors, a combination therapy using these two agents may be highly worthwhile.

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