Effects of Carbon Ion Beam on Putative Colon Cancer Stem Cells and Its Comparison with X-rays

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ABSTRACT

Although carbon ion therapy facilities are expensive, the biological effects of carbon ion beam treatment may be better against cancer (and cancer stem cells) than the effects of a photon beam. To investigate whether a carbon ion beam may have a biological advantage over X-rays by targeting cancer stem-like cells, human colon cancer cells were used in vitro and in vivo. The in vitro relative biological effectiveness (RBE) values of a carbon ion beam relative to X-rays at the D10 values were 1.63-1.74. Cancer stem-like CD133+, CD44+/ESA+ cells had a greater ability for colony and spheroid formation, as well as in vivo tumorigenicity compared to the CD133-, CD44-/ESA- cells. FACS data showed that cancer stem-like cells were more highly enriched after X-rays compared to carbon ion irradiation at doses that produced the same level of biological efficacy. A colony assay for cancer stem-like cells showed that RBE values calculated by the D10 levels were 2.05-2.28 for the carbon ion beam relative to X-rays. The in vivo xenotransplant assay showed an RBE of 3.05-3.25, calculated from the slope of the dose-response curve for tumor growth suppression. Carbon ion irradiation with 15 Gy induced more severe xenograft tumor cell cavitation and fibrosis without significant enhancement of cells with putative cancer stem cell markers, CD133, ESA and CD44, compared to 30 Gy X-rays, and marker positive cells were significantly decreased following 30 Gy carbon ion irradiation. Taken together, carbon ion beam therapy may have an advantage over photon beam therapy by improved targeting of putative colon cancer stem-like cells.

Key Words: carbon-ion radiation, cancer stem cell, xenograft tumor
1. Introduction

Colorectal cancer is currently the most common gastrointestinal malignancy and remains the third most common cancer and second leading cause of cancer-related deaths in developed countries.\textsuperscript{1} Although surgical resection has been the first choice for treatment of colorectal cancer, half of patients still suffer recurrence, presumably because of disseminated micrometastases present at the time of surgery.\textsuperscript{2} Radiation therapy is the most effective nonsurgical intervention for cancer treatment, but most cancers also invariably recur after radiation therapy. Therefore, determination of the mechanisms of recurrence and radioresistance in these tumors and development of powerful therapeutics could lead to advances in the treatment of cancer.

The heavy ion medical accelerator in Chiba (HIMAC), is the first heavy-ion accelerator specially dedicated to medicine in the world, and has now become the world’s leading heavy ion cancer treatment facility.\textsuperscript{3,4} Although heavy ion beam facilities are large scale and hugely expensive, several new heavy ion therapy facilities, such as CNAO (Italy), PTC Marburg (Germany), SAGA HIMAT (Japan), ETOILE (France), Shanghai Particle Therapy Hospital (China), and Mayo Clinic (USA) are under construction or in planning worldwide. A one-third smaller and cheaper carbon ion radiotherapy facility based on the highly advanced technology was designed and constructed in Gunma University in Japan, and clinical trial was successfully initiated in March 2010. High linear energy transfer (LET) particle therapy has various advantages because of the production of spread out Bragg’s peaks (SOBP), which cover tumors with biologically equivalent dose distributions. Therefore, high LET heavy-ion therapy has several potential advantages over low LET photon therapy, such as increased relative biological effect, reduced oxygen enhancement ratio, decreased cell cycle-dependent radiosensitivity, and induced complex DNA damage that is not easily repaired.\textsuperscript{5,6} Over the past decades, HIMAC has
been successful in treating more than 5000 cases of various human cancers and achieved promising clinical outcomes for many radioresistant tumor types, including recurrent colorectal cancer, hepatocellular carcinoma, chondroma, sarcoma etc. 7-10

Recently, cancer stem cells have been identified in a growing number of solid tumors, which are typically recognized by virtue of the expression of cell surface markers; most of them are transmembrane glycoproteins, such as CD133, CD44, and EpCAM (ESA). 11,12 Cancer stem cells have the ability to generate tumors that recapitulate the original tumor when xenotransplanted into animals, whereas the remaining non-cancer stem cell tumor bulk most often cannot. 13-18 The cancer stem cells that populated the original tumor may have resistance to the treatments to repopulate the recurrent tumor even after the bulk of the tumor has been removed by resection or chemoradiation therapy. 19,22 Therefore, the key point in curatively treating cancer is how to effectively eradicate those minor cancer stem cells in the bulk of the tumor. 23,24 Since heavy ion radiotherapy has potential advantages in treating many human radioresistant cancers, we hypothesize that heavy ion irradiation may effectively target these cancer stem-like cells. To the best of our knowledge, our study is the first to explore whether heavy ion irradiation may have advantages over X-rays in targeting human colon cancer stem-like cells.

2. Materials and Methods

2.1. Cell Lines

The colon adenocarcinoma cell lines HCT-116 and SW480 were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% with heat-inactivated 5% (v/v) fetal calf serum (Beit-Ha’Emek, Israel), 100 unit/mL penicillin and 100 μg/mL streptomycin (Invitrogen) in a 37°C with 5% CO2-in-air. The medium was changed
every other day.

2.2. Colony and Spheroid Formation Assay

A clonogenic survival assay was performed as described previously. In brief, the appropriate plating density was designed to produce 20–40 surviving colonies in each 6-cm dish or T-25 flask. After incubation for 14 days, the colonies were fixed and stained with 0.3% methylene blue in ethanol, and colonies containing more than 50 cells were counted as survivors. At least three parallel samples were scored in three to five repetitions performed for each irradiation condition.

For assays of clonogenicity and ability to grow as "tumor spheres" in suspension, HCT116 and SW480 cells were sorted to obtain populations of CD133+ and CD133−, CD44+/ESA+ and CD44-/ESA−-cells by BD FACSAria (Becton Dickinson, San Jose, CA, USA). For the clonogenicity assay and spheroid assay, the sorted CD133+ and CD133−, CD44+/ESA+ and CD44-/ESA−-cell subpopulations were then resuspended in a cell density of 500 cells/ml or 2000 cells/ml and were plated triplicate in a 6-cm dish or 96 well plates pre-coated with a layer of 1% agarose and left to grow for 1-2 weeks. To quantify the number of colonies and sphere formations as well as spheroid formation rates, each positive or negative stem-like cell was applied to 12 wells, and the rate of spheres per well calculated, and the data is presented as percentage of the wells that contained spheres.

2.3. In Vivo Tumorigenic Assays

Immediately after sorting, aliquots of the particular cell populations were counted and cell viability was determined using a conventional trypan blue test. The sorted cells were centrifuged at 300 xg for 5 min at 4°C, suspended in serum free medium (DMEM with 1% penicillin/streptomycin), then various numbers of CD133+/CD133−, CD44+/ESA+ and CD44-/ESA−- cells ranging from 1 x10³ to 2 x 10⁵ cells are injected subcutaneously into the hind legs of 6- to 8-week-old male NOD/SCID immunodeficient mice. The animals were sacrificed
at the indicated time intervals (4–10 weeks) when tumor nodules were identified on their body surfaces. Tissues were fixed in formaldehyde and examined histologically. All experiments involving the use of animals were performed in accordance with NIRS institutional animal welfare guidelines. NOD/SCID mice (Charles River Laboratories, Yokohama, Japan) were maintained under defined conditions at the NIRS animal facility.

2.4. Tumor Growth Delay Assays

BALB/cAJcl-nu/nu male mice (5-week-old) were purchased from CLEA Japan, Inc., Tokyo, Japan. Mice were provided with water and food ad libitum and were housed at 5 animals per cage. All surgical procedures and care administered to the animals were in accordance with the NIRS Animal Care and Use Committee. Tumors were established by subcutaneous inoculation of $8 \times 10^5$ HCT-116 cells into the mouse’ right leg. Tumor growth was monitored every 3 days by measuring two perpendicular diameters. Tumor volume was calculated according to the formula $0.52 \times a \times b^2$, where $a$ and $b$ are the largest and smallest diameters, respectively. The tumor growth delay (TGD) of xenograft tumors after treatment with X-rays or carbon-ion was estimated at the tumor volume of 500 mm$^3$. The relative biological effectiveness (RBE) of carbon-ions at the middle of a 6-cm SOBP relative to 200 KeV X-ray’ was calculated by Kaleidagraph software.

2.5. Irradiation

Cells or mice were irradiated with carbon-ion beams accelerated by the HIMAC at NIRS in Japan. The details concerning the beam characteristics of the carbon-ion beams, biological irradiation procedures, and dosimetry have been described elsewhere. Briefly, the initial energy of the carbon-ion beams was 290 MeV/n, 50KeV/μm, 6-cm, SOBP. The energy of heavy-ion beams at the irradiation site was obtained by comparing the calculated and measured depth-dose distribution. As a reference, mice were also irradiated with conventional 200 kVp X-rays (Pantac HF-320S, Shimadzu Co., Tokyo) at NIRS. Cells were irradiated with 2, 4, 6, 8
Gy of X-rays or 1, 2, 4, 6 Gy carbon ions. Transplanted xenograft tumors were irradiated with various doses of X-rays (15, 30, 60 Gy) or carbon-ions (5, 15, and 30 Gy).

2.6. **FACS Analysis**

FACS analysis for the cells or xenograft tumors irradiated with X-rays or carbon ion was performed with BD FACS Aria according to the manufacturer's protocol (Becton Dickinson, San Jose, CA, USA). For *in vitro* analysis, the cells were prepared and labeled with conjugated anti-human CD133-PE (Miltenyi Biotec), CD44-FITC (Miltenyi Biotec), ESA-PE (Miltenyi Biotec). Isotype matched immunoglobulin served as control. Cells were incubated for 20 min at each step and were washed with 2% FCS/PBS between steps. The percentage of CD133+, CD44+ and ESA+ present was assessed after correction for the percentage of cells reactive with an isotype control. For *in vivo* analysis, tumors were minced with scissors under sterile conditions, rinsed with Hank’s balanced salt solution (HBSS) and incubated for 2 hours at 37°C in serum-free DMEM medium supplemented with 200 units/ml Collagenases type II and type IV (Sigma-Aldrich, USA), 120 μg/l penicillin and 100 μg/mL streptomycin. Cells were further disaggregated by pipetting and serial filtration through cell dissociation sieves (size 40 and 80 meshes; Sigma-Aldrich). Contaminating erythrocytes were lysed by incubation in ammonium chloride. Single cell suspensions were assessed with BD FACS Aria in the same way as in *in vitro* cell analysis.

2.7. **Gross Morphology and Histopathology**

Gross morphological changes were followed up to 12 weeks after a single fraction of X-ray or carbon-ion radiation. At selected time points, tumors were excised and histopathological examinations were performed. Xenograft tumors from different groups were fixed in 10% neutral formalin and processed in paraffin-embedded followed by sectioning (4 μm) onto slides. Sections were stained with hematoxylin and eosin (HE) and assessed microscopically.
2.8. **Immunohistochemistry**

Immunohistochemical staining was performed with the Elite ABC Kit (Vector Laboratories; Burlingame, CA) according to the manufacturer's protocol.\(^{25,26}\) In brief, sections cut from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized and rehydrated through a graded series of ethanol, and incubated in 0.3% hydrogen peroxidase in methanol to block endogenous peroxidase action. For antigen retrieval, sections were placed in boiling 10 mM citrate buffer (pH 6.0). The slides were preincubated with normal horse serum (1:50 dilution; Vector Laboratories, Burlingame, CA) to diminish nonspecific binding of the secondary antibody and then incubated overnight at 4 °C with anti-CD133 (AC133, human monoclonal, Miltenyi Biotec, 1:200 dilution), anti-CD44 (mouse monoclonal, BD Transduction Labs, 1:100 dilution), and anti-ESA (human monoclonal, Miltenyi Biotec, 1:200 dilution). Slides were then rinsed and incubated with universal secondary antibody containing anti-mouse/anti-goat IgG (Vectastain ABC Elite kit; Vector Laboratories) for 30 min, developed with diaminobenzadine (Vectastain ABC Elite kit; Vector Laboratories) for 10 min, and counterstained with hematoxylin for 2 min. Ten fields were selected and expression was evaluated in 1000 tumor cells with high power (x200) microscopy. As a negative control, the sections were stained without primary antibodies to monitor the background staining level. Cytoplasmic and/or membrane staining were considered to indicate specific CD133, CD44 and ESA immunoreactivity. Each slide was assessed for the intensity of immunostaining, background, and percentage of cells expressing the target protein.

2.9. **Western Blotting**

Western blot analyses were performed as previously described (10). Cells or tumor tissues were lysed using the Pierce Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce). Samples were normalized for protein concentration using the Pierce BCA protein assay; 50 µg of each cytoplasmic or nuceara extract sample was analyzed by SDS/5% PAGE and transferred to a
polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 2% ECL Advance Blocking Agent in Tris Buffered Saline Tween 20 for 1 hour and probed overnight with monoclonal antibodies specific for CD133, ESA, and CD44. β-actin was used as a loading control. Subsequently, membranes were incubated with rabbit anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich). Blots were visualized by ECL Advanced™ Kit (GE Healthcare Life Sciences) and quantitated using ImageQuant™ LAS 4000 Mini Biomolecular Imager. Densitometric analysis was obtained with Fujifilm Multi Gauge software (version 3.0).

2.10. Statistical Analysis

One-way ANOVA and Bonferroni multiple comparison tests were used when mean differences between the groups were evaluated by StatView software (SAS Institute, Inc., Cary, NC). For all comparisons, \( p \) values less than 0.05 were defined as significant.

3. Results

3.1. Survival Fraction by Carbon-ion vs X-ray Irradiation

The HCT116 and SW480 cells were irradiated with carbon-ion or X-rays up to 6 Gy, and their survival fraction was measured from the colony formation. Figure 1 shows dose-response curves for cell killing effects on the 2 human colon cancer cell lines irradiated with X-rays or 50 keV/\( \mu \)m SOBP carbon ion beams. The surviving fractions for the HCT116 and SW480 irradiated with X-rays and carbon ions decreased exponentially with increasing doses. Based on these survival curves, the RBE values calculated by the D10, which is determined as the dose (Gy) required to reduce the surviving fraction to 10%, relative to X-rays, is about 1.63 to 1.74 for carbon-ion beams.

3.2. Determination of Cancer Stem-like Cell Properties of CD133+ and CD44+/ESA+
Cells

Having isolated the CD133+; CD44+/ESA+ cells from the HCT116 and SW480 cells, we next determined their cancer stem-like cell properties. CD133+, CD44+/ESA+ cells have higher clonal and spheroid formation capacities in vitro and robust tumorigenicity in xenograft model. When equal numbers of 500 cells were plated in a dish, CD133+, CD44+/ESA+ colorectal cancer cells from HCT116 formed 64 ± 10 and 87 ± 6 clones, whereas CD133-, or CD44-/ESA-cancer cells formed only 20 ± 6 and 22 ± 3 clones (p<0.01; Figure 2A). These data showed that CD133+, or CD44+/ESA+ colorectal cancer cells had much greater clonal formation capacities than that of CD133-, or CD44-/ESA- cancer cells. To investigate the ability to form spheroid bodies, isolated CD133+, CD44+/ESA+ and CD133-, CD44-/ESA- cells were cultured in 96 well plates pre-coated with a layer of 1% agarose. After being in culture for 1 week, CD133+, CD44+/ESA+ aggregated and formed spheroid bodies (Figure 2B). The ability to form spheroid bodies in CD44+/ESA+ was significantly higher than that in CD133-, CD44-/ESA- (p<0.01; Figure 2B). Figure 2C shows representative positive cells for CD133, CD44, ESA markers.

To determine the efficacy of tumor initiation from cells with or without CD133, CD44, ESA markers, we performed limiting dilution experiments. A variable number of human cells, ranging from 1000 to 2,000,000, were injected into NOD-SCID mice to test their xenotumor abilities. As few as 1000 CD133+, or CD44+/ESA+ cells were sufficient to generate a tumor within 6-10 weeks after implantation, whereas the number of CD133-, CD44-/ESA- cells that had a similar capacity was more than 20,000 (Table 1). These data showed that the tumorigenicity of CD133+, CD44+/ESA+ colorectal cancer cells was much higher than CD133-, or CD44-/ESA- cells (Figure 2D, Table 1). Combining these results, our data suggested that CD133+, CD44+/ESA+ cells isolated from HCT116 and SW480 cells present the characteristics of cancer stem-like cells.
3.3. Changes in Proportion of Cancer Stem-like Cells in vitro by Carbon-ion vs X-ray Irradiation

In vitro FACS analyses showed that the percentage of cancer stem-like cells which positive for CD133, CD44, and ESA were more significantly increased after 48 h or 72 h X-ray than carbon ion irradiation. The percentage of CD133+ cells in unirradiated HCT116 cells was about 3%, and it was increased more than 3-fold after irradiation with 2 Gy X-rays and further increased 5-fold after 4 Gy X-ray irradiation. In comparison, CD133+ cells were increased only 2-fold by 1Gy carbon ion and 3-fold by 2 Gy carbon ion irradiation which the doses induced equivalent effects by X-ray. The percentage of CD44+ cells was increased more than 1.5-fold after irradiated with 2 Gy X-rays and further increased 2.5-fold after 4 Gy X-ray irradiation. In contrast, CD44+ cells were unchanged by 1Gy carbon ion and decreased 0.5-fold by 2 Gy carbon ion irradiation. Interestingly, the percentage of ESA+ cells did not change either 2 or 4 Gy but increased 2-fold by 6 Gy X-rays irradiation. In contrast, ESA+ cells were unchanged after carbon ion irradiation even by the dose up to 4 Gy (Figure 3A). The proportion of CD133+/CD44+ and ESA+/CD44+ cells were enriched 2-3 fold by 2 or 4 Gy X-rays, whereas those of double positive cells were decreased or unchanged by 1 or 2 Gy carbon ion irradiation. (Figure 3A).

3.4. Clonogenic Assays of Cancer Stem-like Cells in vitro by Carbon-ion vs X-ray Irradiation

Clonogenic assays were performed to determine the different radiosensitivity of cancer stem-like cells between carbon ion and X-ray irradiation. Figure 3B shows dose-response curves for cell killing effects on cancer stem-like CD133+, CD44+/ESA+ cells and non-cancer stem-like cells sorted from HCT116 or SW480 cells. The results showed that the surviving fractions for cancer stem like CD133+, CD44+/ESA+ cells are significantly higher than non-cancer stem-like CD133-, CD44-/ESA- cells after exposure to either X-rays or carbon ion.
beams, suggesting that cancer stem-like cells show resistance to both X-rays and carbon ions.

(We have also determined the resistance of CD133+, CD44+/ESA+ cells to chemotherapy (data not shown). The surviving fractions for the cancer stem-like cells sorted from the 2 cell lines after irradiation with X-rays and carbon ions decreased exponentially with increasing doses. Based on these survival curves, the RBE values calculated at the D10 level for cancer stem-like cells were calculated to be about 2.05 to 2.28, whereas RBE values for non-cancer stem-like cells were about 1.22 to 1.44.

3.5. **Tumor Growth Delay and Relative Biological Effects of Carbon-ion Relative to X-ray Irradiation**

Transplanted xenograft tumors grow fast without any treatment and the tumor volume became more than 400 mm³ after being subcutaneously implanted into mice for 3 weeks. Treatment with X-rays (30 Gy) effectively suppressed tumor growth and reduced the tumor size and volume about 10%, but the tumor rapidly re-grew after 4 weeks and to double in volume after another 4 weeks (**Figure 4A**). In contrast, treatment with carbon-ion (30 Gy) radiation increased tumor size at the first week and then gradually decreased. The tumor was reduced to the same size as before radiation after 4 weeks and actually became less than half in volume after 8 weeks, and finally disappeared after 12 weeks without any regrowth and relapse (**Figure 4A**). To determine the tumor growth control possibility by carbon-ion radiation, the xenograft tumors were also treated with various doses. Carbon-ion irradiation with 15 Gy can suppress tumor growth without significant initial increase in tumor size and volume, but re-grew after 7-8 weeks. As expected, treatment with 5 Gy carbon-ion or 15 Gy X-ray failed to control tumor growth anymore, however the tumor was completely regressed without re-growth with 60 Gy X-ray irradiation in the 12-week follow-up (data not shown). Xenotransplanted tumor control possibility by carbon-ion and X-ray radiation at various doses is summarized in **Table 2a**.

To calculate in vivo RBE values, the TGD of xenotransplanted tumors after treatment...
with 15, 30 Gy of X-rays and 5, 15 Gy carbon-ions were estimated. Due to the tumor bed effect, the extent of TGD determined from tumor growth curves is highly dependent on the end volume chosen. To minimize the influence of the tumor bed effect on the growth delay, we calculated by choosing a smaller size and essentially an earlier time for regrowth. The TGD was obtained according to the endpoint of the tumor regrown to a volume of about 500 mm³. As shown in Table 2b, TGD was 4 and 28 days for 15 and 30 Gy of X-rays, but 10 and 76 days for 5 and 15 Gy of carbon ion beams, respectively (Figure 4B). Based on this TGD, the RBE values of 50 keV/μm carbon ion at the middle of a 6 cm SOBP relative to X-ray were calculated as 3.05 to 3.25 according to the formula analyzed by Kaleidagraph software (Figure 4B, Table 2c).

3.6. Gross Morphological and Histopathological Changes After Carbon-ion vs X-rays Irradiation

Figure 4C illustrates gross tumor morphology before and after treatment with carbon-ion (15, 30 Gy) or X-ray (30 Gy) irradiation. Tumor-supplying vessels were very clearly seen in the unirradiated mice as well as in the X-ray irradiated mice, but markedly reduced in carbon-ion irradiated mice. It is further confirmed by microscopy observation that the microvessel counts were significantly reduced by carbon ion compared to X-rays irradiation (Figure 4D). Histopathological changes of xenograft tumors after irradiation with X-rays or carbon-ion for 4 weeks were examined by H&E staining. Histopathological features showed that most of the tumor cells were not disrupted by 15 Gy X-rays or 5 Gy carbon ion irradiation (data not shown). At the same level of biological efficacy, 15 Gy carbon-ion irradiation predominantly induced colon cancer cell cavitations, fibrosis and the duct-like architecture was completely disrupted. In contrast, 30 Gy X-ray irradiation only partially destroyed colon cancer cells and the duct-like architecture still remained (Figure 4D). It is clearly shown that almost all of the tumor cells were destroyed after irradiated with 30 Gy carbon-ions (Figure 4D).

3.7. Expression of Cancer Stem-like Cell Marker CD133, CD44 and ESA after Carbon-ion
vs X-ray Irradiation

The expression changes of cancer stem cell marker CD133, CD44 and ESA in the xenograft tumors at 4 weeks after irradiation with 30 Gy X-rays or carbon-ion 15, 30 Gy were examined by immunohistochemical staining. It was shown that expression of both CD133 and CD44 was significantly suppressed by 15 Gy carbon-ion irradiation compared to unirradiated tumors, except ESA protein, but all three putative cancer stem cell proteins were remarkably inhibited after treatment with 30 Gy carbon ion irradiation. In contrast, 30 Gy X-rays significantly increased expression of CD133, CD44, and ESA compared to those of unirradiated tumors (Fig. 5A). It was further confirmed by western blotting analyses that expression levels of all CD133, CD44, and ESA proteins was significantly enhanced by 30 Gy X-rays compared to 15 Gy carbon-ion irradiation and all three proteins were predominantly reduced by 30 Gy carbon-ion irradiation (Fig. 5B).

3.8. Changes in Proportion of Cancer Stem-like Cells in vivo by Carbon-ion vs X-ray Irradiation

In vivo FACS analyses showed that the percentage of cancer stem-like cells which positive for CD133, ESA were increased with 15 and/or 30 Gy but significantly decreased with 60 Gy X-rays irradiation after 1 month. In comparison, carbon ion irradiation with 15 Gy did not change the percentage of these positive cancer stem-like cells, whereas irradiation with 30 Gy significantly reduced the percentage of positive cancer stem-like cells (Fig. 5C). It is not detectable any of cancer stem-like cells by in vivo FACS analysis after the tumor irradiated with 30 Gy carbon ion or 60 Gy X-rays for 2-3 months.

4. Discussion

We found that the in vitro RBE values, calculated by the D10 relative to the X-rays, are about 1.63 to 1.74 for the 50-keV/μm SOBP carbon ion beam on HCT116 or SW480 cells in this study. RBE values are known to be dependent on LET, and our results are almost consistent
with previous reports using HIMAC carbon ion beams on various human cancer cell lines such as lung, pancreas and brain tumors, which reported 1.06 to 1.33 for a 13-keV/μm-beam, 1.42-1.69 for a 50-keV/μm-beam and 2.00 to 3.01 for a 77-keV/μm-beam. Recent accumulating experimental evidence suggests that cancer stem cell content may differ among tumors, and that a higher proportion of cancer stem cells is correlated with higher radioresistance. To determine cancer stem properties of CD133+, CD44+/ESA+ cells in HCT116 and SW480, we have sorted cancer stem-like positive and negative cells and confirmed that CD133+, CD44+/ESA+ cells have a significantly higher possibility for colony and tumor sphere formation than CD133-, CD44-/ESA- cells. In vivo tumorigenicity study showed that the tumorigenicity of CD133+, CD44+/ESA+ colorectal cancer cells exactly much higher than CD133-, or CD44-/ESA- cells.

In the present study, FACS analyses showed that the proportion of cancer stem-like cells which were positive for CD133, ESA, and CD44 was more highly enriched after X-ray compared to carbon ion irradiation, particularly, the population of CD133+, CD44+ cells was increased more than 2-fold (2-4 Gy) by X-ray irradiation. In contrast, CD133+ and CD44+ cells were unchanged or decreased by carbon ion irradiation (1-2 Gy). Interestingly, the proportion of ESA+ cells was increased by 6 Gy X-ray irradiation, but without changes by carbon ion irradiation even the dose was up to 4 Gy. The proportion changes of double positive CD133+/CD44+ and ESA+/CD44+ cells by X-ray versus carbon ion irradiation were showed same responses. These finding suggests that low LET X-ray irradiation may mainly kill the non stem–like tumor cells, as a result the radioresistant cancer stem-like cell population was predominantly enriched. In contrast, carbon ion irradiation may concurrently kill both non stem-like and stem-like tumor cells, consequently, the population of cancer stem-like cells was only slightly increased or unchanged. To directly determine the radiosensitivity of cancer stem-like cells between carbon ion and X-ray irradiation, a colony assay was performed. Based
on the dose-response curves for cell-killing effect on cancer stem like cells and non-cancer stem like cells after irradiation with either X-rays or carbon ion beams, the cancer stem-like cells showed resistance to both X-rays and carbon ions. The surviving fractions for the cancer stem like cells after irradiation with X-rays or carbon ions decreased exponentially with increasing doses. The RBE values calculated at the D10 level for cancer stem-like cells were about 2.05 to 2.28, suggesting that carbon ion beam has a promising potential to destroy cancer stem-like cells. In contrast, RBE values at the D10 level for non-cancer stem-like cells were only 1.22 to 1.44, implying that there are no significant differences in the killing of non-cancer stem-like cells between the carbon ion beam and X-ray irradiation. All together, these results can also partially explain why the proportion of cancer stem-like cells after irradiation with X-rays is more enriched than those of carbon ion beams.

In vivo study demonstrated that 15 or 30 Gy carbon ion irradiation predominantly induced colon cancer cell cavitations, fibrosis and completely disrupted the duct-like structure, whereas 30 Gy X-ray irradiation only partially disrupted colon cancer cells and the duct-like structure still remained when the xenograft tumors were histopathologically examined after one month. The tumor-supplying vessels were exactly reduced in carbon-ion irradiated mice compared to those of X-rays irradiated mice. This finding is in agreement with a previous report that heavy-ion irradiation inhibits in vitro angiogenesis. Several previous studies reported that the in vivo RBE values for high LET carbon ion beams ranged from 2.0 to 3.1. In the present study, the in vivo RBE was calculated as 3.05 to 3.25 from the slope of the dose response curve for tumor growth suppression by carbon ions relative to X-rays, which is almost in line with previous reports. It is known that, RBE is a complex quantity, depending on many factors such as particle type, dose per fraction, and LET, as well as on biological factors like cell or tissue type and the selected biological endpoint. The extent of TGD determined from tumor growth curves is highly dependent on the end volume chosen because of tumor bed effects.
This may be the reason why the RBE values calculated in this study are a little higher than other reports. In addition, since a low number of dose levels was applied in this study, further studies are needed on in vivo RBE of carbon ions for local tumor control.

Recently, accumulating evidence showed that expression of cancer stem-like cell markers such as CD133, CD44 were closely related with patient’s clinical outcome, and prognosis as well as chemoresistance.\textsuperscript{33-36} In the present study, we surprisingly found that 15 Gy carbon ion radiation induced more severe tumor cell disruption without significant increment of putative cancer stem cell markers CD133, CD44, and ESA, whereas X-rays irradiation remarkably increased these protein levels. All these cancer stem-like cell markers were remarkably inhibited after treatment with 30 Gy carbon-ion irradiation. Furthermore, in vivo FACS analyses showed that the proportion of cancer stem-like cells which were positive for CD133, ESA were enriched by 15 and/or 30 Gy but significantly decreased by 60 Gy X-ray irradiation after 1 month. In comparison, carbon ion irradiation with 15 Gy did not change the percentage of these positive cancer stem-like cells, whereas irradiation with 30 Gy significantly reduced the percentage of positive cancer stem-like cells. However, none of cancer stem-like cells were detectable by FACS analysis after the tumors were irradiated with 30 Gy carbon ions or 60 Gy X-rays for 2-3 months. Presumably, the doses to completely destroy the cancer stem cells are dependent on the tumor cell type as well as cancer stem cell population in the tumor bulk.

All together, according to the cancer stem cell model, therapeutic approaches that are not capable of eradicating the cancer stem cell subset are unlikely to be successful because they might be able to kill the majority of tumor cells and induce regression of tumor lesions, but fail to prevent disease relapse and metastatic dissemination.\textsuperscript{23-27, 37, 38} Based on this understanding, although carbon ion beam facilities are very expensive, to achieve better outcomes as well as a better quality of life for patients with some types of cancer, such as unresectable sacral chordomas\textsuperscript{39}, or locally recurrent rectal cancer\textsuperscript{40}, carbon ion beam therapy may be worth the cost.
In conclusion, our findings presented here are the first to demonstrate that carbon ion beam therapy may have advantages over photon beam therapy in targeting putative colon cancer stem-like cells.

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Conflict of Interest Notification: none.
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Legends to Figures

**Figure 1.** Survival curves of HCT116 and SW480 cells plated immediately after X-ray or carbon ion irradiation. The graphs show the mean and standard error calculated from three independent experiments.

**Figure 2.** Colony, spheroid, and tumor formation of cancer stem-like and non-cancer stem-like cells delivered from HCT116 cells. Colony (A) and spheroid formation (B) of CD133+, CD133-, CD44+/ESA+ and CD44-/ESA- cells after being in culture for 1-2 week. C. Representative photos of positive cancer stem-like HCT116 cells. D. Tumorigenicity of CD133+, CD133-, CD44+/ESA+ and CD44-/ESA- cells after subcutaneously injection into the hind legs of NOD-SCID mice. *, p<0.01, compared to colony or sphere formed from CD133+ or CD44+/ESA+ cells.

**Figure 3.** A. Percentage changes of CD133+, CD44+, ESA+, CD133+/CD44+ and CD44+/ESA+ cells by FACS analysis 48 h or 72 h after X-ray or carbon ion irradiation in HCT116 cells. *, p<0.01, compared to non-IR cells. B. Survival curves of cancer stem-like cells and non cancer stem-like cells sorted from HCT116 and SW480 cells after irradiation with X-rays or carbon ion beams. Representative photos of colony formation from CD133+, CD133-, CD44+/ESA+, and CD44-/ESA- cells after X-ray or carbon ion irradiation are displayed. The graphs show the mean and standard error calculated from three independent experiments.

**Figure 4.** A. HCT116 xenograft tumor growth control by 30 Gy X-ray or 15, and 30 Gy carbon-ion irradiation. B. HCT116 xenograft tumor growth delay after treatment with X-rays or carbon-ion irradiation. Gross morphological changes (C) and histopathological features (D) of HCT116 xenograft tumors at 4 weeks after being treated with 30 Gy X-rays or 15, and 30 Gy
carbon-ion irradiation. Arrows indicate microvesseles in which red blood cells were seen. #, p<0.05; *, p<0.01 compared to that of control tumors.

**Figure 5.** A. Immunohistochemistry for the expression changes of CD133, CD44 and ESA in the HCT116 xenograft tumors at 4 weeks after treated with carbon-ion or X-ray irradiation. Original magnification x100. #, p<0.05, *, p<0.01, compared to non-IR tumors. B. Western blotting for the expression changes of CD133, CD44 and ESA in the HCT116 xenograft tumors at 4 weeks after being treated with carbon-ion or X-rays irradiation. β-actin was used as loading control. C. Percentage changes of CD133+/CD44+ and CD44+/ESA+ cancer stem-like cells by FACS analysis at 4 weeks after X-ray or carbon ion irradiation in HCT116 xenograft tumors. #, p<0.05, *, p<0.01, compared to non-IR tumors.
Table 1. Tumor formation ability of sorted HCT116 colorectal cancer cells using surface markers (number of tumors formed/number of injections)

<table>
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<tr>
<th>Groups</th>
<th>$2 \times 10^5$</th>
<th>$2 \times 10^4$</th>
<th>$1 \times 10^4$</th>
<th>$5 \times 10^3$</th>
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<td>1/6</td>
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<tr>
<td>CD133+</td>
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<td>6/6</td>
<td>5/6</td>
<td>3/5</td>
<td>3/5</td>
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<tr>
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<td>2/6</td>
<td>0/6</td>
<td>0/5</td>
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</tr>
<tr>
<td>$p$</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
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<tr>
<td>CD44+/ESA+</td>
<td>0/0</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
<td>3/5</td>
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<tr>
<td>CD44-/ESA-</td>
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<td>0/5</td>
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<td>$p$</td>
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$P<0.01$ compared with results from marker-negative cells.
Table 2. a. Therapeutic efficacy of X-ray and Carbon-ion irradiation in HCT116 colon cancer xenograft (12-week follow up).

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<td>-</td>
<td>-</td>
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<tr>
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<td>4</td>
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<td>60Gy</td>
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<tr>
<td>Carbon-ion</td>
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<td>0</td>
<td>5</td>
</tr>
<tr>
<td>15Gy</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>30Gy</td>
<td>5</td>
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b. Tumor growth delay (TGD) and RBE values estimated in this study

<table>
<thead>
<tr>
<th>TGD ± SE (95% CI) (Days)</th>
<th>X-ray</th>
<th>Carbon-ion</th>
<th>RBE ± SE (95% CI)</th>
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<tbody>
<tr>
<td></td>
<td>15 Gy</td>
<td>30 Gy</td>
<td>5 Gy</td>
</tr>
<tr>
<td>4 ± 1 (2-6)</td>
<td>28 ± 3 (24-33)</td>
<td>8 ± 2 (7-13)</td>
<td>76 ± 4 (71-82)</td>
</tr>
</tbody>
</table>
Fig. 1
Fig. 2
Fig. 3
Fig. 5
# Effects of Carbon Ion Beam on Putative Colon Cancer Stem Cells and Its Comparison with X-rays

Xing Cui, Kazuhiko Oonishi, Hirohiko Tsujii, et al.

_Cancer Res_ Published OnlineFirst March 31, 2011.

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