

Induction of cancer stem-like cells in A549 cells after exposure to carbon ions and X-rays

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ABSTRACT

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Background: Cancer stem-like cells (CSCs) play a crucial role in the initiation, progression, and recurrence of cancer. Evidence indicates that the high linear energy transfer (LET) carbon ion beam is more effective against CSCs than the conventional X-ray beam. Carbon ion radiotherapy is considered as a promising cancer strategy, however, information about whether, or not, new CSCs are induced after carcinoma cells exposed to carbon ions is limited and incomplete.

Materials and Methods: The selected A549 cells, derived from a single colony with low CD133 expression, were irradiated by high LET carbon ion beam or low LET X-ray, and then the cell clonal survival fraction, CD133⁺ expression subpopulations and soft agar colony formation potential were detected. **Results:** The cell-killing ability of carbon ions was higher than that of X-rays. The fractions of CD133⁺ or Oct4⁺ high expression cells increased in a dose-dependent manner at 12 h after both X-ray and carbon ion exposure. Compared to that of carbon ion irradiation, the efficiency of transformation in a cell colony was higher in the X-ray irradiation group after 30 d; however, the amount of colonies with higher fractions of CD133⁺ expression cell subpopulation in the carbon ion irradiation group was more than that in X-ray irradiation treatment group after 30 and 50 d. **Conclusion:** Our results indicate that, although high-LET radiation kill more cells including cancer stem-like A549 cells, it may induce more stable stem cell-like colonies than low LET radiation.

Keywords: Cancer stem-like cells (CSCs), CD133, low and high LET radiation, carcinogenesis.

INTRODUCTION

Low linear energy transfer (LET) g- or X-ray radiation have been commonly used for radiotherapy due to their effectiveness in tumour cell killing. It has been used to treat more than half of all patients who have malignant tumours ⁽¹⁾, however, there are issues associated with tumour recurrence and the lack of specific, targeted, delivery resulting in normal tissue damage and side effects, because the absorbed dose delivered by these types of radiation usually undergoes an exponential attenuation with penetration depth in patient bodies. A high-LET heavy ion beam is generally

characterised by an energy deposition peak (a Bragg peak) at the end of its range and an increased relative biological effectiveness (RBE) within the peak, which is benefit for the deep seated tumour radiotherapy in the body. These characteristics make it more popular in cancer radiotherapy than conventional radiation sources such as low-LET g- and X-rays ⁽¹⁻³⁾. The advantages of charged particle beams for medical applications were recognised as early as the 1950s and the first patient treatments were performed with proton beams. Thereafter, at several other accelerator facilities medical treatments were initiated, however, all of these therapies were used only in experimental

treatments. A major breakthrough was thus achieved by establishing dedicated clinical accelerators, *e.g.*, the proton treatment facility in Loma Linda in 1990 and the Heavy Ion Medical Accelerator in Chiba (HIMAC) in 1994, which used carbon ions for tumour treatment. Up to now, the clinical treatment, or trials, using accelerated particles have been started in the USA, Germany, Japan, and China ⁽⁴⁾. Although, it is the trend to treat cancer therapy with particle accelerators, the side effects induced by heavy ions during radiotherapy remain a concern.

For most tumour therapies, all of the cancer cells are treated as though they have similar proliferative potential and can acquire the ability to metastasise ^(5,6); however, the cancer stem-like cells (CSCs) hypothesis indicates that only a rare subpopulation of cancer cells has the ability to control tumour growth and shares similar characteristics with embryonic stem cell including self-renewal and differentiation ⁽⁷⁻⁹⁾. The majority of cancer cells within the tumour have only limited proliferative potential, and are known as non-stem cancer cells (NSCCs) ^(6,10,11). The rare subpopulation of CSCs can be discriminated by the surface makers through flow cytometry. For instance, lung CSCs are enriched in markers such as CD133 and Oct4 ⁽¹²⁻¹⁸⁾. Kurth *et al.* found that CSCs can be accumulated in the fraction of CD133⁺ cells after irradiation and reduce sensitivity in radiation-induced apoptosis ⁽¹⁹⁾. The CSC model and the stochastic model suggest different clinical strategies of tumour therapy ⁽²⁰⁾. Presently, the need lies in how to gain a better understanding of tumour heterogeneity and the dynamic variations of different subpopulations, specifically the CSCs and NSCCs in tumours ⁽²¹⁾.

High-LET carbon ion beam radiotherapy is reported to have higher radio-curability and a lower recurrence ratio than photon irradiation, and particle irradiation may effectively kill CSCs ⁽²²⁻²⁵⁾. Non-invasive control of tumours by use of charged particle therapy offers advantages over conventional radiotherapy ⁽²⁶⁾. Despite the use of ionising radiation (IR) is being widely used as a standard treatment for most solid cancers ^(27,28). Much evidence suggests that IR paradoxically promotes cancer malignancy ^(10,29-32), however,

information about whether, or not, the new CSCs are induced in cancer cells after exposure to high-LET heavy ions is limited and incomplete.

In the current study, we investigated the induction of CD133⁺ expression subpopulations and soft agar colony formation in human lung adenocarcinoma epithelial cells (A549) after being exposed to high-LET carbon ions and low-LET X-rays. Our aim was to ascertain whether, or not, the new CSCs are induced by heavy ions and to provide more comprehensive basic data for the application of heavy ions in clinical radiotherapy. Our results indicated that there is an intrinsic inter-conversion and dynamic equilibrium between CSCs and NSCCs of A549 after irradiation. In addition, subsequent experiments showed that the efficiency of oncogenic transformation induced by high-LET carbon ions was lower than that induced by low-LET X-rays, however, the transformed colonies induced by carbon ions were more stable and more stem-like than those induced by low-LET X-rays.

MATERIALS AND METHODS

Cell culture and low expression of CD133 cell subclone selection

The A549 cells (adenocarcinomic human alveolar basal epithelial cell) were purchased from the Centre for Cell Resources, Institute for Biological Sciences of Shanghai, Chinese Academy of Sciences (CAS). Cells were cultured in RPMI-1640 (Gibco, USA) medium supplemented with 10% FBS (Hyclone, USA) and 1% penicillin/streptomycin (Amresco, USA). Cell culture was incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

To avoid the higher background noise, and confirm that the CSCs were induced by irradiation rather than the surviving parental cells with a higher expression marker of CSCs, the A549 cell line was selected as follows: two hundred cells were seeded into a 100-mm-diameter dish and cultured to form single-cell colonies. The colonies were picked up and the biomarker of CD133 was analysed by flow cytometry. One subclone with a low expression

of CD133 was then used in the following experiments.

Radiation

Carbon ions beam was supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics, Chinese Academy of Sciences (IMP-CAS). Cell exposures were conducted at the therapy terminal of the HIRFL, which has a horizontal beam line. Due to the energy degradation caused by the vacuum window, air gap, T-25 flask wall, and medium, the energy of the ion beam on cell samples was calculated to be 165 MeV/u, corresponding to an LET of 30 keV/ μ m and the dose rate was

adjusted to be about 0.25 Gy/min. Low-LET irradiation was performed using Faxitron Cabinet X-ray System (RX-650, 5mA, USA) operated at 50 keV. The dose rate was 0.60 Gy/min.

The single colony with low expression of CD133 was expanded and then trypsinised, counted, and seeded in T-25 flasks at a density of 5×10^5 cells/flask. After incubation for 24 h, the cells were irradiated with different doses of the high-LET carbon ion beams, or X-rays, at room temperature. All experiments were carried out in accordance with the following protocol (figure 1).

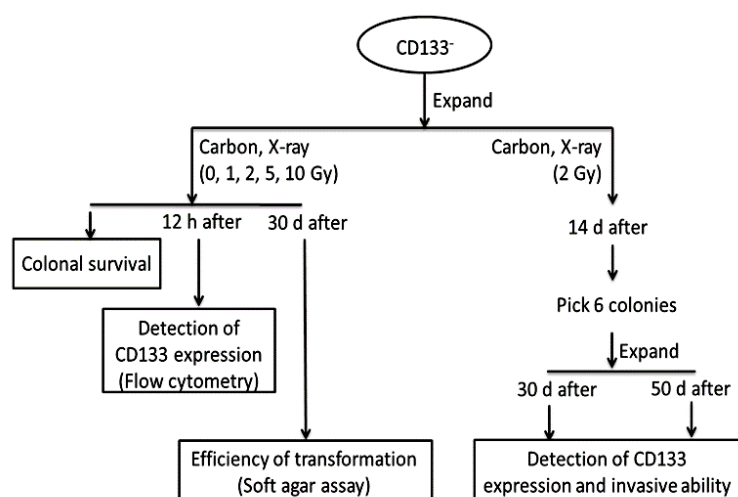


Figure 1. Schematic diagram of the protocol used to investigate the induction of CSCs in A549 cells after ionising irradiation.

Colony formation assay

Cells were trypsinised after irradiation and then resuspended in RPMI-1640 medium with 10% FBS and 1% penicillin/streptomycin. An appropriate number of cells were plated into each 100-mm-diameter dish to produce colonies. After incubation for 10 d, cells were stained with 0.5% crystal violet for 20 min. Colonies containing more than 50 cells were counted as survivors. Plating efficiencies (PE) were calculated as follows: numbers of colonies formed / numbers of cells plated. Surviving fractions were calculated as follows: PE (irradiated) / PE (unirradiated).

Flow cytometry

For the detection of CD133 expression, cells

were resuspended at up to 1×10^7 nucleated cells per 100 μ L of buffer (Miltenyi, Bergisch Gladbach, Germany). Then the cells were mixed with 10 μ L monoclonal antibodies (mouse anti-human CD133/1-PE (Miltenyi, Germany) and incubated for 10 min in the dark in the refrigerator (2 to 8 $^{\circ}$ C). The cells were washed by adding 1-2 mL of buffer and centrifuged at 300g for 54 min. The supernatant was completely aspirated. Cell pellets were re-suspended in a suitable amount of buffer for analysis by flow cytometry (FACScan flow cytometer, Becton Dickinson, USA).

For the detection of Oct4 expression, cells were re-suspended at up to 10^6 nucleated cells in 1 mL of cold, freshly prepared, Fixation/Permeabilisation Solution (Miltenyi) and

incubated for 30 min in the dark in the refrigerator (2 to 8 °C). Cells were washed by adding 1–2 mL of cold buffer and centrifuged at 300g for 9 min at 8 °C. Aspirate supernatant completely. Cells were re-suspended again up to 10^6 nucleated cells in 100 μ L of cold 1 $\times$ Permeabilisation Buffer. Then the cells were mixed with 10 μ L monoclonal antibodies (goat anti-human Oct4-FITC, Miltenyi, Germany) and incubated for 30 min in the dark in the refrigerator (2 to 8 °C). The cells were washed by adding 1–2 mL of cold 1 $\times$ Permeabilisation Buffer and centrifuged at 300g for 5 min at 4 °C. The supernatant was completely aspirated. Cell pellets were re-suspended in a suitable amount of buffer for analysis by flow cytometry.

Soft agar colony assay

Soft agar assays were performed as described previously (33). Briefly, cells were irradiated and then cultured for one month. Survival cells after irradiation were seeded in complete media at a density of 5000 cells in 60-mm dishes containing a top layer of 0.35% agarose and a base layer of 0.5% agar. They are incubated at 37 °C for 4 weeks and fed twice a week.

Cell invasion assays.

Cell invasion assay was performed using 24-well Transwell chambers with a pore size of 8- μ m (Corning Inc., Corning, NY, USA). Briefly, the inserts were coated with 80 μ L Matrigel (1:8 dilution; Corning Inc., Corning, NY, USA). Approximately 2×10^4 cells/well were re-suspended in 100 μ L serum-free medium and plated onto 8- μ m trans-well inserts. The lower chambers contained 600 μ L medium containing 10% FBS as a chemoattractant. After culturing for 48 h, the cells on the upper membrane surface were removed by scraping with a cotton swab, and the cells that passed through the filter were fixed and stained using the hematoxylin-eosin reagent. All the experiments were performed in triplicate with three replicates and the mean was calculated.

Statistical analysis

The statistical significance (p values) in mean values of two-sample comparison was

determined with the Students' t -test, and two-variance comparison was detected by F -test. A value of $p < 0.05$ was considered statistically significant and is represented by an asterisk on the bars in the figures. A value of $p < 0.01$ was considered extremely significant and is represented by two asterisks on the bars in the figures. Values shown on graphs represent the means $\pm$ S.D of at least three independent repeated experiments.

RESULTS

Carbon ions induce more cellular killing

The selected A549 cells were irradiated with different doses of low-LET X-rays or high-LET carbon ions and then plated for the clonogenic assay. Figure 2 shows that the clonal survival fraction of A549 cells after exposure to high-LET carbon ions induced more cellular killing, compared to the low-LET X-rays. The significance increased with increasing dose.

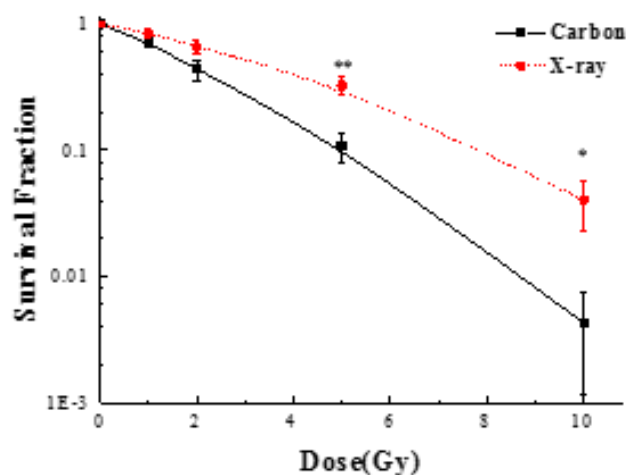


Figure 2. The survival curve of A549 cells after exposure to the low-LET X-rays and high-LET carbon ions. Each experiment was conducted at least three times (** $P < 0.01$, * $P < 0.05$). Compared to the low-LET X-rays, high-LET carbon ions induced more cellular killing.

The Subpopulation of CD133⁺ and Oct4⁺ Expression Induced by Ionising Radiation

It was reported that the expression of CD133 is higher in CSCs cells of human lung tumour (34–36). In addition, Oct4 was also reported to be more highly expressed in CSCs cells (37,38).

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Therefore, the biomarkers of both CD133 and Oct4 were used to identify the accumulation of CSCs in A549 cells after exposure to ionizing irradiation in the current study. The A549 cells with low expression of CD133 were irradiated with different doses of carbon ions or X-rays. The irradiated cells were stained with CD133 and Oct4 antibody 12 h after irradiation and the

subpopulation of CD133⁺ and Oct4⁺ expression were detected by flow cytometry (figures 3 and 4). The fraction of CD133⁺ and Oct4⁺ expression cells increased after exposure to carbon ions at 2 Gy or more. Similarly, X-rays also induced an increase in the numbers of CD133⁺ and Oct4⁺ expression cells (figures 3 and 4).

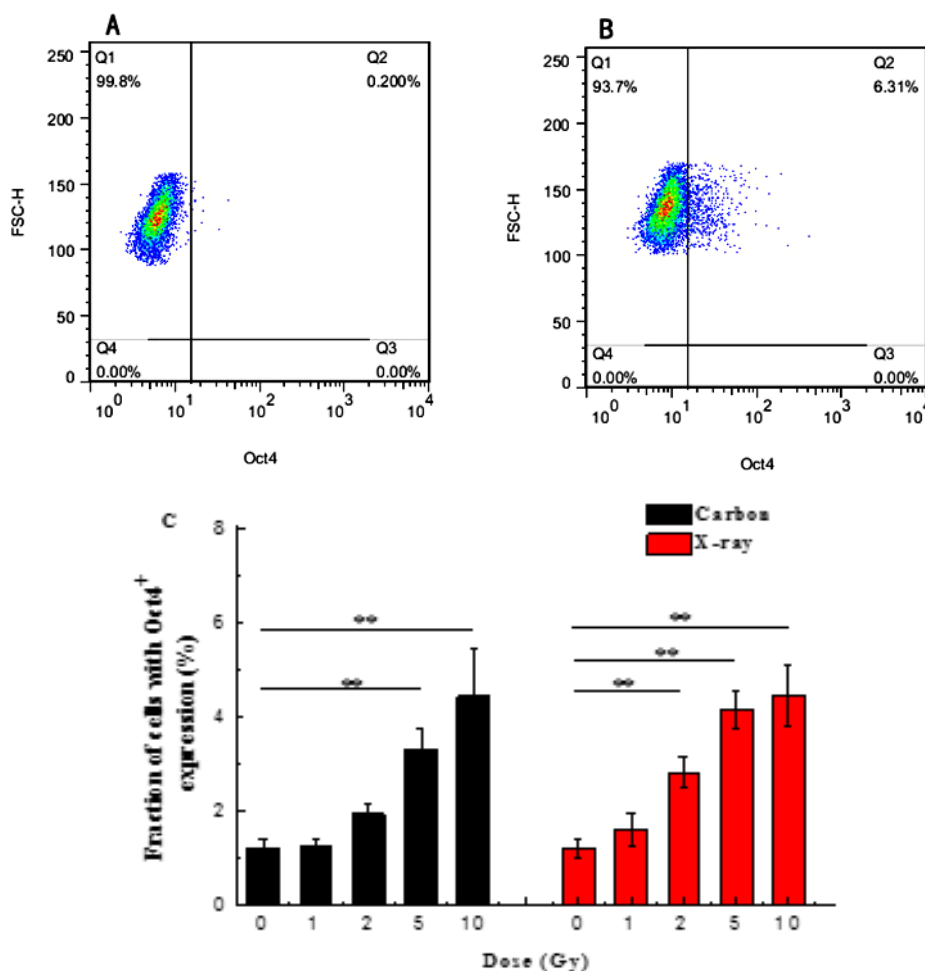


Figure 4. Flow-cytometry analysis of Oct4⁺ expression induced by irradiation. **(A):** Unirradiated control cell group. **(B):** Representative dot image of irradiated cell group 12 h after exposure to carbon ions at 10 Gy. **(C):** Accumulation of Oct4⁺ expression subpopulation within the A549 cells after exposure to the high-LET carbon ions and low-LET X-rays. The Oct4⁺ expressions were analysed by flow cytometry in cells 12 h after irradiation. Each experiment was conducted at least three times (* P < 0.05, ** P < 0.01).

Furthermore, we detected the enrichment of CD133⁺ expression of A549 cells as a function of the time after exposure to the carbon, or X-ray, irradiation at 2 Gy, a dose mostly used in clinical radiotherapy. The irradiated cells were continuously cultured and then harvested at

different times (0, 0.5, 1, 2, 6, 12, 24, 48, and 72 h, and 5, 10, 20, and 30 d). Cells were stained with CD133 antibody and then detected by flow cytometry. Figure 5 shows that the fraction of CD133⁺ cells gradually increased to a peak at 12 h for X-ray irradiation and 24 h for carbon

irradiation (figure 5B), and then gradually returned to a normal level thereafter until 30 d (figure 5A).

Carbon ions induces fewer transformation colonies compared to X-rays

The soft agar assay is an anchorage independent growth assay in soft agar, which is considered as the most stringent assay available for measuring malignant transformation of a

single cells *in vitro* ⁽³⁹⁾. The A549 cells with low expression of CD133 were irradiated by carbon ions or X-rays at doses of 0, 1, 2, 5, and 10 Gy, respectively. The irradiated cells were cultured for 30 d and then subjected to the soft agar colony formation test. Figure 6D shows that the efficiency of transformation after all doses of high-LET carbon ion irradiation were significantly lower than those induced by the low-LET X-rays.

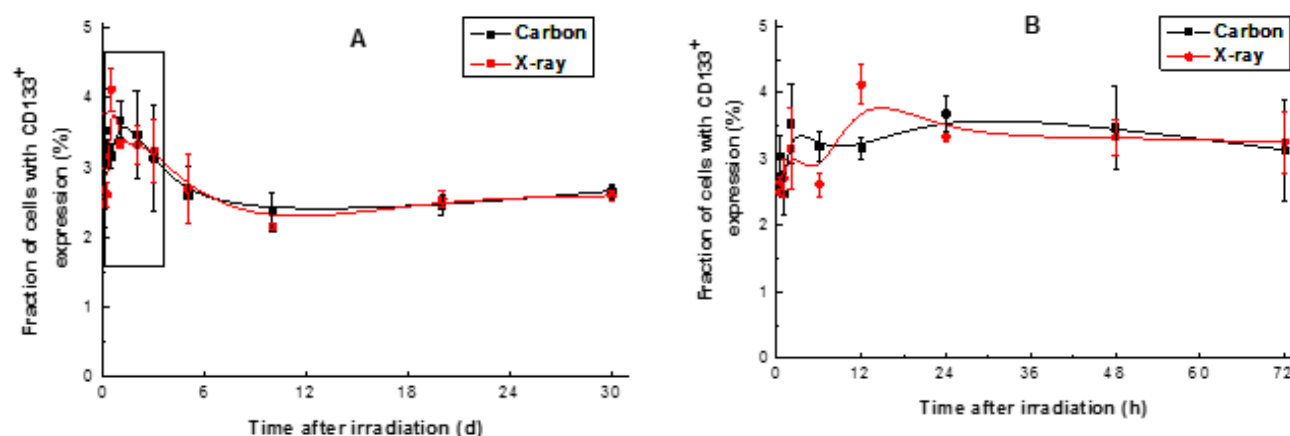


Figure 5. The accumulation of CD133⁺ expression cell as a function of the time after exposure to 2 Gy of X-rays or carbon ions. (A): Detected data from each whole time point after irradiation. (B): The data obtained during the short-term (0-72 h) after irradiation which were included in Figure 5A. Each experiment was conducted at least three times.

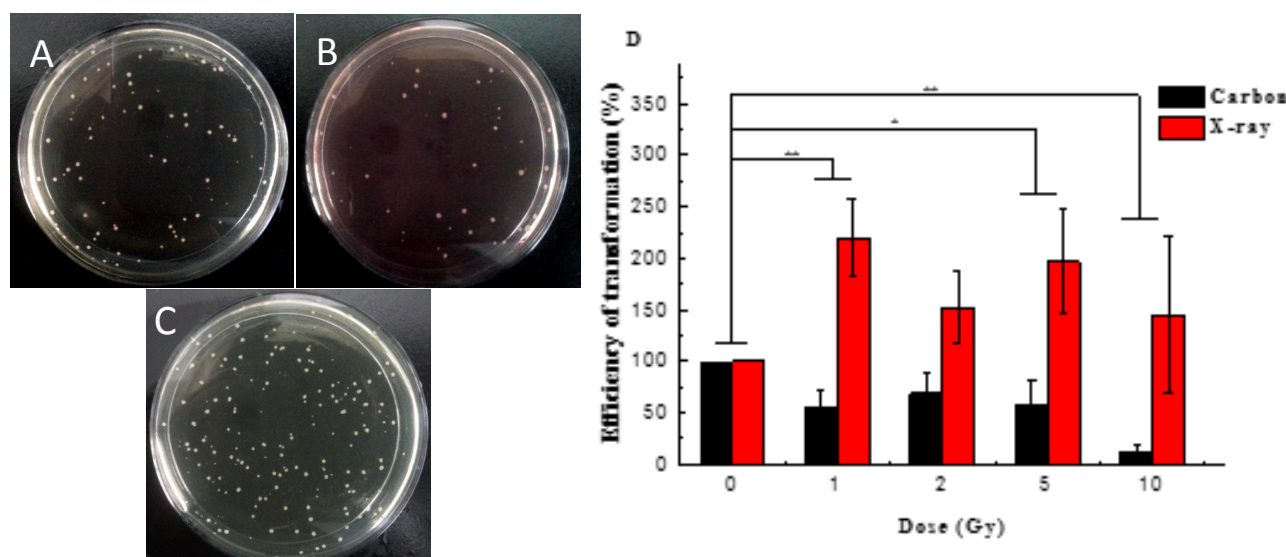


Figure 6. The efficiency of transformation of cells exposed to carbon ion or X-ray irradiation by means of soft agar colony formation assay. Colonies formed in soft agar derived from unirradiated control cell group (A), carbon ion irradiated cell group (B) and X-ray irradiated cell group (C). (D): The histogram of the efficiency of transformation of cells 30 d after exposure to carbon ions or X-rays. Each experiment was conducted at least three times (* $P < 0.05$, ** $P < 0.01$).

Carbon ions evokes more stable, more stem-like transformation colonies

The A549 cells with lower expression of CD133 were irradiated by carbon ions or X-rays at 2 Gy, and then plated cells, of an appropriate number, were cultured in a 100-mm-diameter dish at 37 °C, under an atmosphere containing 5% CO₂ in an incubator for two weeks. A total of six colonies of each irradiation group were collected, and subsequently cultured and harvested at days 30 and 50 for measurement of the CD133 expression level by flow cytometry. Interestingly, it was found that the amount of colonies with higher fractions of CD133⁺ expression cell subpopulation from carbon ion irradiation group was greater than that from the X-ray irradiation treatment group (figure 7A). Transformation of invasion capability of also

reflects the stem-like nature of the cells. Furthermore, we performed transformation colony and cell invasion experiments for the colonies picked at days 30 and 50. Figures 7B and 7E show that the transformation efficiencies and invasion abilities of colonies from the carbon ion irradiation group were also higher than those of colonies from the X-ray irradiation treatment group. In the carbon ion irradiation group, the fraction of CD133⁺ expression cell in colonies 1, 4, and 5 were higher and remained relatively stable (the fraction of CD133⁺ expression cells at day 50 was similar to that at day 30). This may suggest that, although the high-LET radiation killed more cells, including cancer stem-like A549 cells, it may have induced more stable, more stem-like colonies than low-LET radiation.

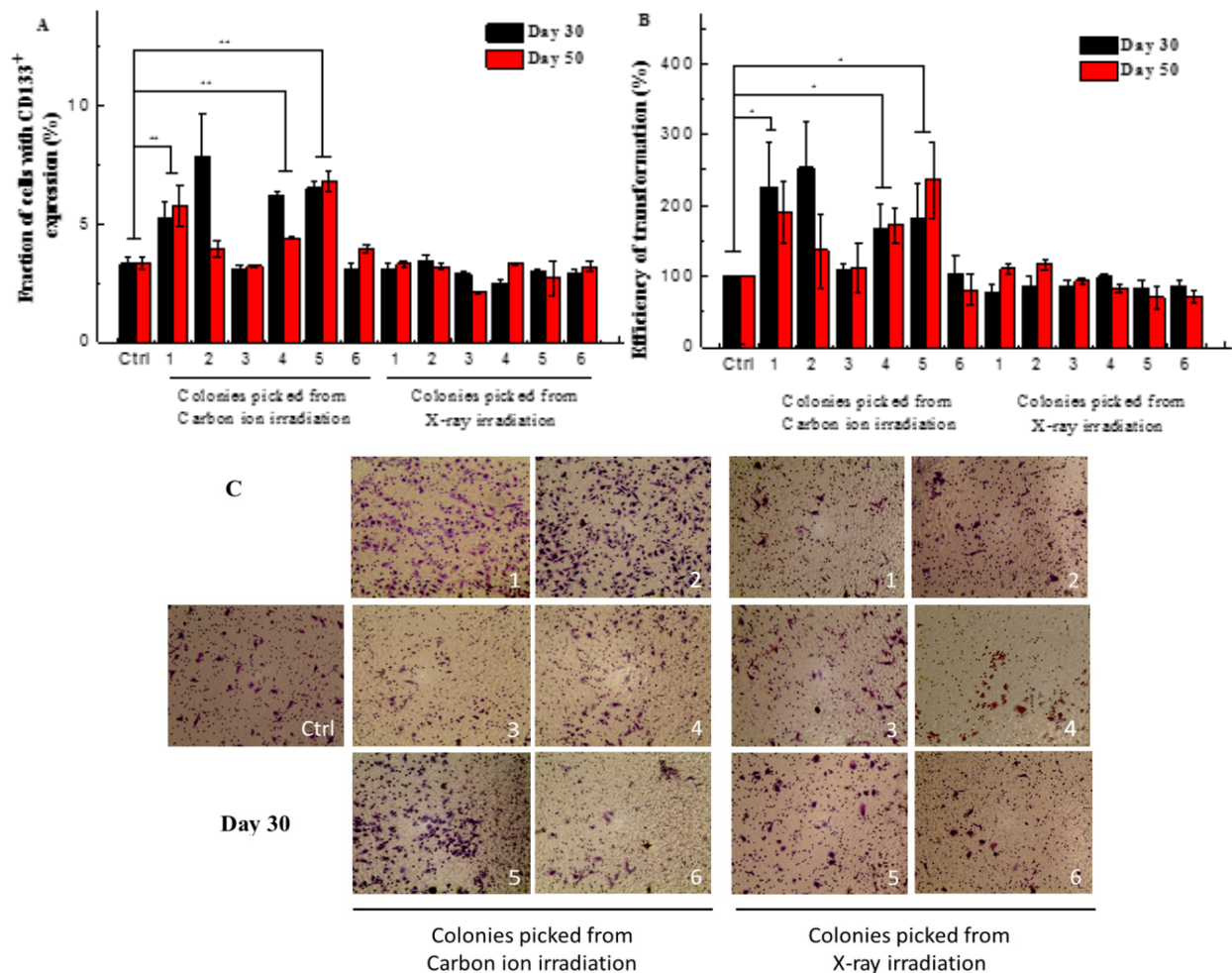


Figure 7. figure legend is described in next page.

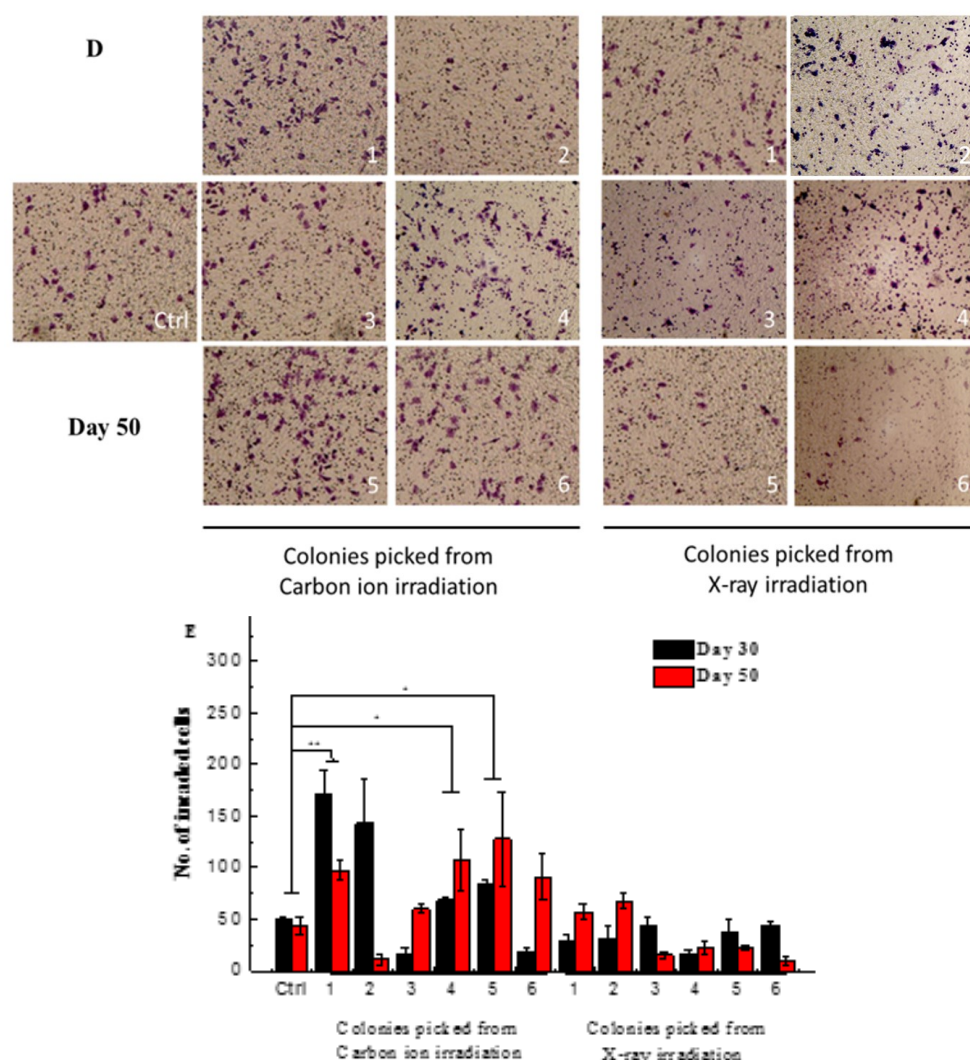


Figure 7. The analysis of stem-like feature for colonies picked from 2 Gy carbon ion or X-ray irradiation groups. The colonies were picked up 2 weeks after irradiation and then subsequently cultured and harvested at days 30 and 50 for further testing. The CD133⁺ expression levels in the long-term cultured cellular colonies were detected by flow cytometry (A). (B): The histogram of the efficiency of transformation of colonies. Representative images of cell invasion assay are shown (C and D). (E): The histogram of the invasion capacity of colonies picked from the carbon ion or X-ray irradiation group. Each experiment was conducted at least three times (Fisher-test, * P < 0.05, ** P < 0.01).

DISCUSSION

Radiotherapy using charged and/or high-LET particles has a long history, and was performed with protons for nearly 50 years and heavy ions for nearly 30 years^(40,41). In general, it is believed that the cellular damage induced by high-LET heavy ions radiation is more complex than that induced by X-rays, leading to more severe biological consequences including cell death, mutation, and transformation^(42,43). In the current study, the results also indicated that the

high-LET carbon ions exhibited a greater number of cells killed than in low-LET X-ray treatment.

It was reported that the expression of CD133 and Oct4 are higher in CSCs cells of human lung tumour^(34,36,44), therefore, the biomarkers of CD133 and Oct4 were mainly used to identify the induction of CSCs in A549 cells after exposure to IR. Our results showed that the fraction of CD133⁺ expression cells in the A549 cell population exposed to X-rays was a slightly higher than that in the carbon ion irradiation

group, and the same tendency was observed for Oct4⁺ expression. It suggests that heavy ions can kill more CSCs, however, the fractions of CD133⁺ expression cells increased slightly between 0.5 to 72 h after irradiation, while gradually returning to normal level over longer times, suggesting that it may be due to enrichment of tumour cells bearing CSC markers by irradiation in the short-term, and the number of CSCs/NSCCs phenotypic states returning towards equilibrium proportions in the longer-term. Recent studies have also proposed the concept of cancer stem cell plasticity in which these two states may not be definitive, but instead have a transitional capability of shifting from a non-CSC state to a CSC state, and *vice versa* ⁽⁴⁵⁾.

Colony formation assay in soft agar (transformation colony assay) and invasion assay are important end-points for determining oncogenic transformation and correlated with *in vivo* tumour growth of xenografts in mice ⁽⁴⁶⁾. They are also the good biological end-points at which to characterise the properties of CSCs. Our results demonstrated that the transformation efficiencies in high-LET carbon ion irradiation groups were lower than those in low-LET X-ray irradiation groups; however, the features of CSCs, and their stability in the transformed subpopulation from the high-LET carbon ion irradiation group, were higher than those from the low-LET X-ray irradiation group. These results suggest that high-LET irradiation has the potential to induce more stable cancer stem-like cells. Our on-going experiments showed that one cell colony, picked from the carbon ion irradiation group and with a higher expression of CD133, exhibited higher tumourgenesis in nude mice (data not shown). In addition, we also irradiated the normal cells (MRC5 and ES cells) to compare the transformation efficiency with cancer cells after IR, however, no transformed colony was observed in the normal cells after exposure to either low-LET X-rays or high-LET carbon ions (data not shown). It was argued by Dugan *et al.* that, starting with cells that are tumour-derived, immortalised, or in some way abnormal in the processing of radiation damage may already provide the kind of precondition that may be necessary for radiation-induced

instability ^(47,48) so that they have higher tumourgenesis. CSCs possess the capabilities of self-renewal and differentiation. They play vital roles in tumorigenesis, chemotherapy and radiotherapy resistance, and cancer metastasis. Generation of new CSCs in cancer cell population after irradiation may induce tumor recurrence and radioresistance.

In summary, heavy ions are more effective than X-rays in inducing cell damage. The intrinsic inter-conversion and dynamic equilibrium between CSCs and NSCCs exists in the A549 cell population after exposure to either high-LET heavy ions or low-LET X-rays. Irradiation can effectively kill cancer stem-like cells immediately, but in the long-term, the conversion of the dynamic equilibrium between CSCs and NSCCs entails a risk of tumour progression. The total oncogenic transformation efficiency induced by high-LET irradiation is lower, but some transformed colonies induced by heavy ions have higher cancer stem-like feature and are stable. Therefore, we need, in future research, to investigate secondary carcinogenesis when we use heavy ions as a stronger radiotherapy tool.

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Conflicts of interest: Declared none.

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