

Original Article

Combination of carbon-ion beam and dual tyrosine kinase inhibitor, lapatinib, effectively destroys HER2 positive breast cancer stem-like cells

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Abstract: To investigate whether carbon-ion beam alone, or in combination with lapatinib, has a beneficial effect in targeting HER2-positive breast cancer stem-like cells (CSCs) compared to that of X-rays, human breast CSCs derived from BT474 and SKBR3 cell lines were treated with a carbon-ion beam or X-rays irradiation alone or in combination with lapatinib, and then cell viability, spheroid formation assays, apoptotic analyses, gene expression analysis of related genes, and immunofluorescent γ -H2AX foci assays were performed. Spheroid formation assays confirmed that ESA+/CD24- cells have CSC properties compared to ESA-/CD24+ cells. CSCs were more highly enriched after X-ray irradiation combined with lapatinib, whereas carbon-ion beam combined with lapatinib significantly decreased the proportion of CSCs. Carbon-ion beam combined with lapatinib significantly suppressed spheroid formation compared to X-rays combined with lapatinib or carbon ion beam alone. Cell cycle analysis showed that carbon ion beam combined with lapatinib predominantly enhanced sub-G1 and G2/M arrested population compared to that of carbon-ion beam, X-ray treatments alone. Carbon-ion beam combined with lapatinib significantly enhanced apoptosis and carbon-ion beam alone dose-dependently increased autophagy-related expression of Beclin1 and in combination with lapatinib greatly enhanced ATG7 expression at protein levels. In addition, a large-sized γ H2AX foci in CSCs were induced by carbon ion beam combined with lapatinib treatment in CSCs compared to cells receiving X-rays or carbon-ion beam alone. Altogether, combination of carbon-ion beam irradiation and lapatinib has a high potential to kill HER2-positive breast CSCs, causing severe irreparable DNA damage, enhanced autophagy, and apoptosis.

Keywords: Heavy-ion radiation, breast cancer stem cell, lapatinib

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and is the leading cause of cancer-related deaths in worldwide [1]. BC is rapidly increasing in many Asian countries and has become the fifth-leading cause of cancer deaths in Japan [2-4]. BCs represent a group of highly heterogeneous lesions consisting of morphologically distinct subtypes with different molecular and biochemical signatures [5-7].

Tumors that overexpress the receptor tyrosine kinase HER2 (HER2-positive tumors) tend to

be higher grade tumors that are more likely to spread and thus are more aggressive than other types of BCs [8]. Approximately 10~15% of women diagnosed with BC will have a HER2-positive disease. HER2-positive type BCs can be treated with anti-HER2 drugs such as trastuzumab, and new studies shows that trastuzumab improves the long-term survival of patients with HER2-positive BCs [9, 10]. Although the clinical outcomes of patients with HER2-positive BC are much improved by trastuzumab treatment, these patients are more than twice as likely to develop a recurring cancer [11-13].

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Because BCs are highly heterogeneous both in their pathology and in their molecular profiles, the treatment of BCs should be designed according to their different subtypes in order to achieve desirable outcome [14-16]. Breast cancer stem-like cell (CSC) populations have recently been identified based on the cell membrane markers CD44+/CD24- or ESA+/CD24-cells [17-19]. It has been demonstrated that CSCs are shown to be radioresistant owing to their high DNA repair capacity and upregulated survival pathways, which protect them from various cellular stresses, including radiation. It is therefore very important to develop novel therapeutic strategies that target CSCs in order to improve patient's survival [20-22].

Carbon-ion beams have a well-localized energy deposition, releasing enormous amount of energy in a well-defined range with insignificant scatter in surrounding tissues. As such, carbon-ion beams can induce more complex DNA damage and have been shown to be more effectively in killing radioresistant cancer cells, with less cell-cycle and oxygen dependencies, compared to conventional radiation [23-25]. Recently, a phase I clinical trial for early stage BC treatment using carbon-ion beam radiotherapy was started. However, one of critical problems was the elevated doses of radiation, especially with aggressive subtypes like HER2-positive tumors, used in carbon-ion radiotherapy, because of its side effects on skin, ribs, and lungs. Lapatinib is a very low molecular weight, dual inhibitor of the intracellular tyrosine kinase domain of HER1 (or EGFR) and HER2, and therefore has potential activity against brain metastases originating from HER2-positive BCs [26-29]. In this study, we reasoned that carbon-ion beam combined with molecular targeting drugs may reduce the doses of irradiation needed to effectively destroy BC cells. The combination of lapatinib and heavy ion radiotherapy may open new perspectives in the fight against this challenging BC subgroup which suffers from limited therapy options and poor prognosis.

Recently, we have reported that carbon-ion beam irradiation has a marked effect on colon and pancreatic CSCs [30, 31] and also shown that carbon ion beam combined with DNA damaging drugs has increased efficacy in killing radioresistant CSCs [32-34]. Considering the fact that lapatinib has been reported to be

effective in treating HER2-positive metastases [26-29], the present study examined the effects of carbon-ion beam irradiation alone or in combination with lapatinib on putative HER2-positive breast CSCs survival, DNA repair, and expression changes of various cell death-related genes compared to that of X-ray irradiation. To the best of our knowledge, this is the first study to show that heavy-ion radiation combined with lapatinib has an advantage in targeting HER2-positive breast CSCs when compared to carbon-ion beam alone or conventional X-rays.

Materials and methods

Cell lines and reagents

Human HER2-positive breast cancer cell lines, BT474 and SKBR3 were purchased from American Type Culture Collection (Manassas, VA). Unsorted cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Beit-Heamek, Israel), 100 unit/mL penicillin and 100 µg/mL streptomycin (Invitrogen) at 37 °C with 5% CO₂-in-air. The medium was changed every other day. CSCs and non-CSCs isolated from BT474 and SKBR3 cells were cultured with Cancer Stem Cell *medium* (Heidelberg, Germany). Lapatinib was purchased from Sigma Japan. The lapatinib solutions were diluted in DMSO immediately before use. Antibodies used in the present study were as follows: mouse anti-human CD24-FITC (BD Pharmingen™ Cat No. 555427), mouse anti-human CD326 (Miltenyi Biotec, Cat No. 130-091-253), monoclonal anti-phospho-histone H2AX (Ser139) (γH2AX, abcam, ab26350), LC3 (CST #4108), Beclin1 (CST #3738) and ATG7 (CST #2631).

Spheroid formation assays

Spheroid formation ability assay for ESA+/CD24- and ESA-/CD24+ cells sorted from BT474 and SKBR3 cells were performed as described previously [20]. In brief, 3000 cells per well were plated in a Low Cell Adhesion 96-well plate (SUMILON, Sumitomo Bakelite, Tokyo, Japan) for 1-week and then the sphere area dimension was estimated. The data is presented as the average size using WinRoof 5.6 software (Mitani Corporation, Tokyo, Japan) after 1-week incubation.

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Irradiation

Cells were irradiated with carbon-ion beams (accelerated by the HIMAC). Briefly, the initial energy of the carbon-ion beams was 290 MeV/n, 50 KeV/ μ m, center of 6 cm Spread-Out Bragg Peak (SOBP). As a reference, cells were also irradiated with conventional 200 kVp X-ray (TITAN-320, GE Co., USA).

Cell viability assay

For the analysis of cell viability, a CellTiter-Glo luminescent cell viability and trypan blue staining assays were used. The CellTiter-Glo[®] Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. In brief, a single reagent (CellTiter-Glo[®] Reagent) directly added to cells which cultured in multiwell plate with serum-supplemented medium and estimated by GloMax[®] Discover System (Promega, Wisconsin, USA). Cell viability was also tested by trypan blue exclusion test, which based on the principle that live cells exclude trypan blue dye and do not stain, whereas dead or dying cells will be stained. In brief, dilute the cells by preparing a 1:1 dilution of the cell suspension using 0.4% Trypan Blue solution and added to the Counting Slide Chamber and then estimated by using an Olympus Automated Cell Counter model R1 (Olympus, Tokyo, Japan).

Fluorescence-activated cell sorting (FACS) analysis

FACS analysis for the cells irradiated with X-rays or carbon ion beams was performed with *BD FACS Aria* (Becton Dickinson, San Jose, CA, USA) as described previously [23, 27]. In brief, the cells were prepared and labeled with conjugated anti-human ESA-PE and CD24-FITC. Isotype matched immunoglobulin served as control. Cells were incubated for 20 min at each step and were washed with 2% FBS/PBS between steps. The percentage of ESA+, and CD24+ present was assessed after correction for the percentage of cells reactive with an isotype control.

Apoptotic analysis

The apoptosis was analyzed using Annexin-V/PI doubling staining flow cytometry assay with

Annexin V-FITC Apoptosis Detection Kits, according to the commercial procedure available (R&D Systems, Minneapolis, MN USA). Briefly, after 24 h of irradiation cells were harvested by trypsinization, washed in PBS and labeled fluorescently for detection of apoptotic and necrotic cells by adding 100 μ L of binding buffer and 1 μ L of Annexin V-FITC to each sample. Samples were mixed gently and incubated at room temperature in the dark for 15 min. Immediately before analysis by flow cytometry (BD FACSCalibur Flow Cytometry System), 1 μ L of propidium iodide (PI, 1 mg/mL; Cedarlane Laboratories, Hornby, Ontario, Canada) were added to each sample. A minimum of 10,000 cells within the gated region was analyzed.

Cell cycle analysis

After harvesting and washing cells with phosphate-buffered saline (PBS), fix in ice-cold 70% ethanol (ethanol in distilled water) while vortexing, then stained with propidium iodide (1 μ g/mL, Sigma) in the presence of RNase A according to the manufacturer's protocol, and then analyzed using a BD FACS Calibur flow cytometer (BD Biosciences). A minimum of 10,000 cells was counted for each sample, and data analysis was performed with CellQuest software.

Real time reverse transcription polymerase chain reaction (RT-PCR) analysis of various gene expression related to apoptosis and autophagy

RNA was purified using the Qiagen RNeasy kit, including on-column DNase treatment to remove genomic DNA. cDNA was prepared with the RT² First Strand Kit (SABiosciences, Frederick, Maryland, USA). A PCR specific for apoptosis, autophagy related genes was performed (RT² SYBR Green/ROX qPCR Master Mix; SABiosciences) in 96-well microtiter plates on a LightCycler[®] 96 system (Roche, Basel, Switzerland). For data analysis, the $\Delta\Delta$ Ct method was applied using the RT PCR software package and statistical analyses performed ($n = 3$). This package uses $\Delta\Delta C_T$ -based fold change calculations and the Student's *t*-test to calculate two-tail, equal variance *p*-values. The fold changes were calculated using the equation $2^{-\Delta\Delta C_T}$. If fold change was greater than 1, the result was considered as fold-upregulation. If fold change was less than 1, the negative inverse of the result was considered as fold-

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Table 1. The primer sequences for real-time PCR

Gene	Primer sequence
GAPDH	
Forward	5'-TGAACGGGAAGCTCACTGG-3'
Reverse	5'-TCCACCACCCTGTTGCTGTA-3'
Bax	
Forward	5'-CAAACGGGTGCTCAAGGCC-3'
Reverse	5'-GCACTCCCGCCACAAGAT-3'
Bcl-2	
Forward	5'-ATGTGTGTGGAGAGCGTCAACC-3'
Reverse	5'-TGAGCAGAGTCTTCAGAGACAGCC-3'
Caspase-3	
Forward	5'-TCATTATTCAGGCCTGCCGTGTA-3'
Reverse	5'-TGGATGAACCAGGAGCCATCCTTT-3'
LC3-II	
Forward	5'-GATGTCCGACTTATTCGAGAGC-3'
Reverse	5'-TTGAGCTGTAAGCGCCTTCTA-3'
Beclin-1	
Forward	5'-AGCTGCCGTTATACTGTTCTG-3'
Reverse	5'-ACTGCCTCTGTGTCTTCAATCTT-3'
ATG-7	
Forward	5'-GCTG CTAATTC TGCAATGATGT-3'
Reverse	5'-GCAAGCTCACTAGG CTGCAGAACC-3'

downregulation [26, 27]. The primer sequences used in this study was shown as **Table 1**. GAPDH used as a housekeeping gene in this study.

Western blotting analysis

Cells were irradiated with carbon-ion beam alone or in combination with lapatinib and incubated for 96 h. The cells were harvested and lysed with Denaturing lysis buffer (Minute Total Protein Extraction Kit, Invent Biotechnologies). The extracted proteins were separated by sodium-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 1% (v/v) non-fat dried milk in Tris-buffered saline with 0.05% Tween 20 and with the appropriate antibodies. Primary antibodies were used at a 1:1000 dilution, and secondary antibodies were used at a 1:5000 dilution. Immunoreactive protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences) and scanned.

Visualizing autophagy by fluorescence microscopy

The cells which cultured in chamber slide were stained with Cyto-ID according to the manufac-

turer's protocol. In brief, cells were washed with PBS (supplemented with 5% FBS) and then mixed with Cyto-ID Dual Detection Reagent solution (Cyto-ID Green Detection Reagent and Hoechst 33342). The cells were then incubated at 37°C for 30 min (Cyto-ID) in the dark, then washed twice with PBS to remove the free dyes. The autophagic cells stained by Cyto-ID green fluorescence were observed using fluorescence microscope.

γ H2AX immunofluorescence assay

γ H2AX Immunofluorescence assay was performed as described previously [26, 27]. In brief, cultured cells grown on plastic chamber slides (Lab-Tek. Nunc, USA) were fixed in 4% formaldehyde for 15 min at room temperature. Then the cells were permeabilized in 0.2% Triton X-100 and blocked with 10% goat serum, then incubated with mouse monoclonal anti-phospho-histone H2AX (Ser139) (γ H2AX) at 37°C in PBS with 10% goat serum and washed with PBS. The cells were incubated with the Alexa 488 anti-rabbit secondary antibody at 37°C in PBS with 10% goat serum and washed in PBS. Cover glasses were mounted in ProLong® Gold antifade reagent with DAPI (Invitrogen). Fluorescence images were captured using an Olympus DP70 fluorescence microscope for analysis. All treatment groups were then assessed for γ H2AX foci via sequential imaging through each nucleus. A minimum of 100 cells in each treatment group were counted. Nuclear γ H2AX foci size was estimated by ImageJ 1.45 software (NIH).

Statistical analysis

One-way analysis of variance (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.

Results

Cell viability after carbon ion beam or X-ray irradiation alone or in combination with lapatinib

Morphological changes of BT474 cells as shown in **Figure 1A**, 24 h after carbon-ion beam combined with lapatinib greatly destroyed the cells

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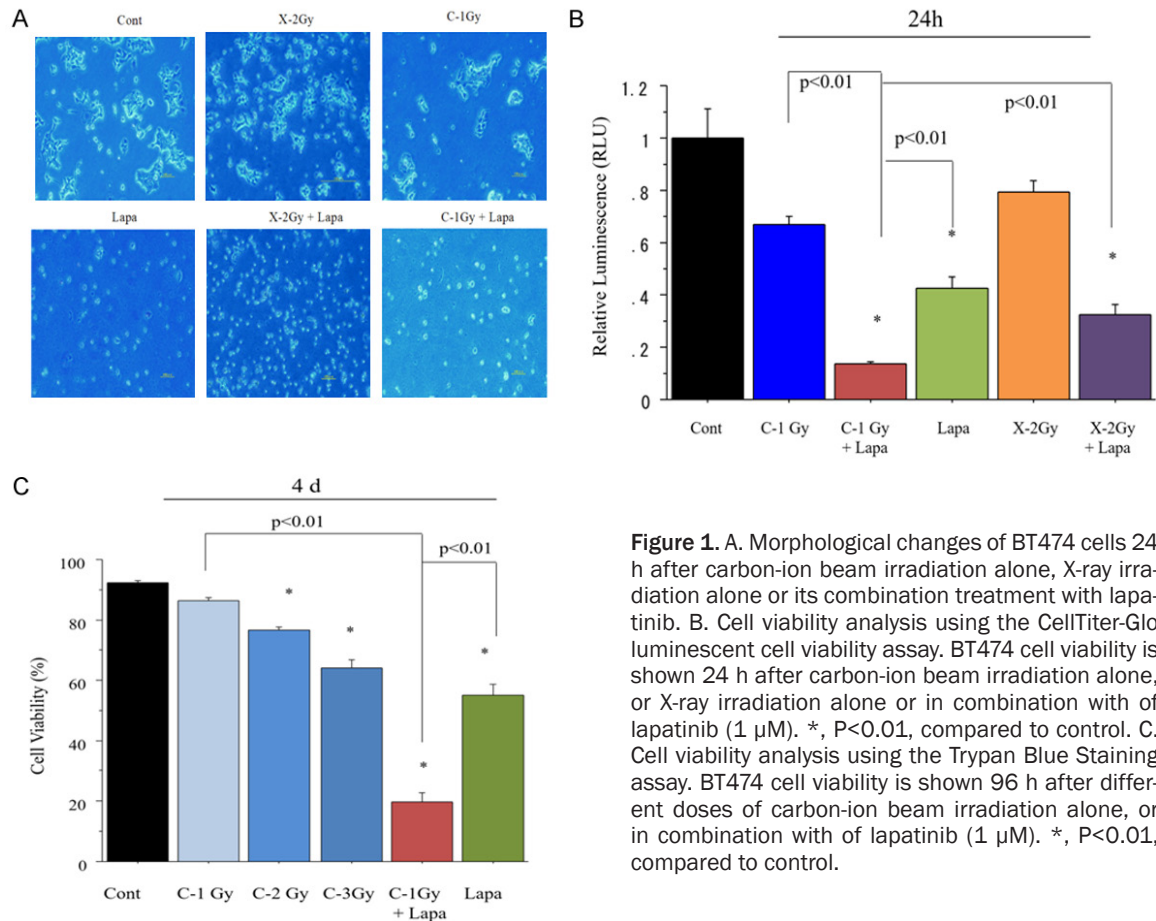


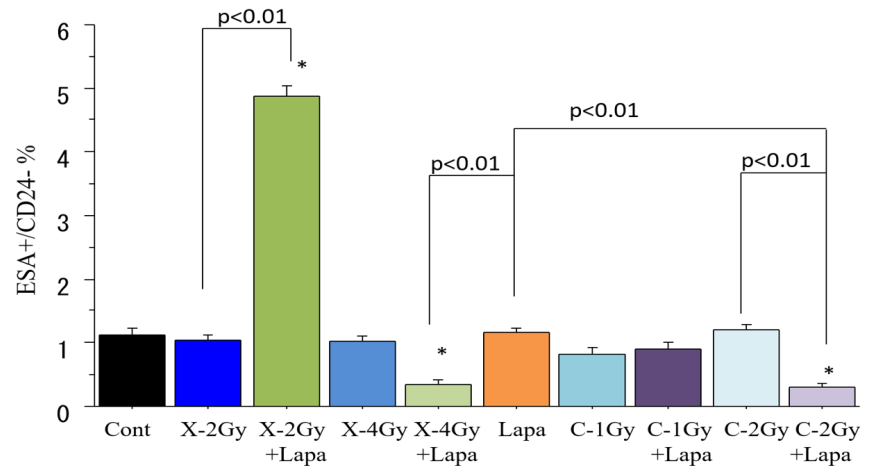
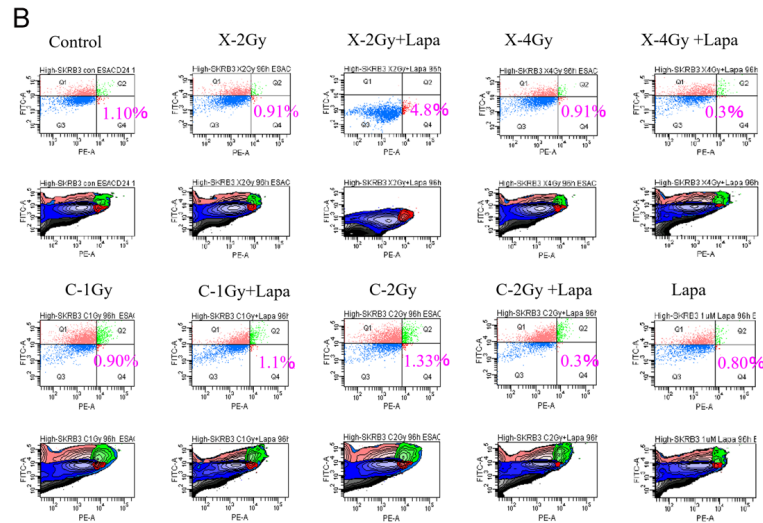
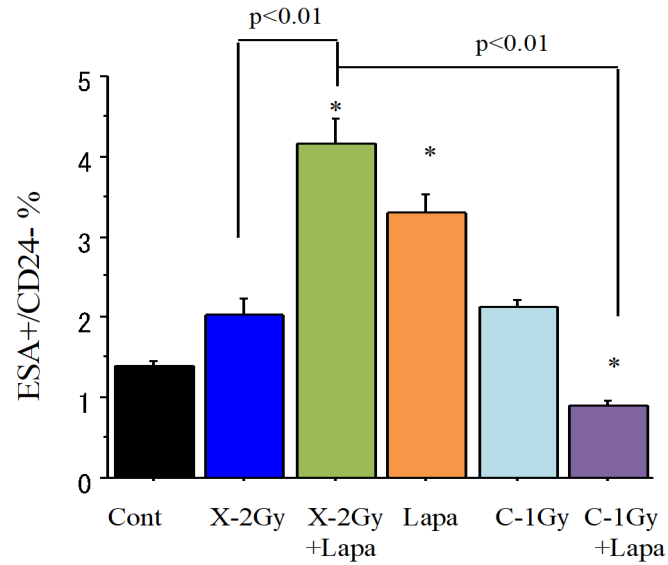
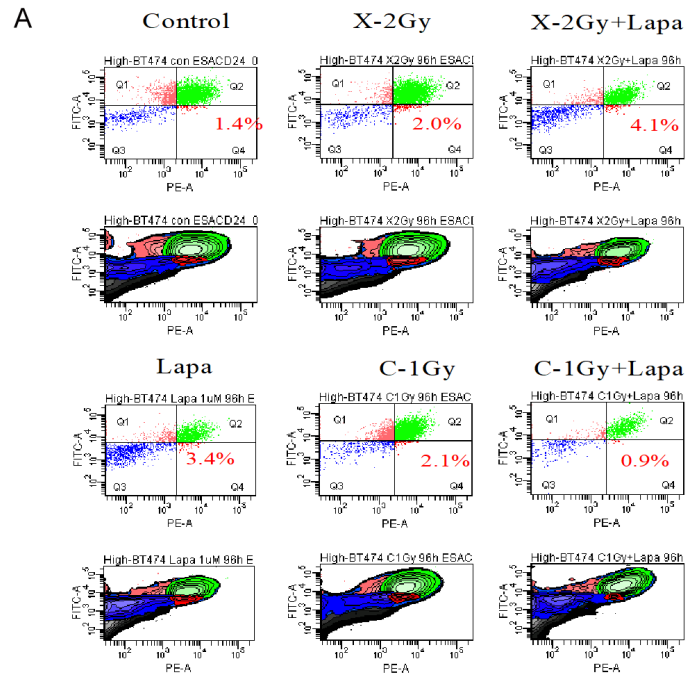
Figure 1. A. Morphological changes of BT474 cells 24 h after carbon-ion beam irradiation alone, X-ray irradiation alone or its combination treatment with lapatinib. B. Cell viability analysis using the CellTiter-Glo luminescent cell viability assay. BT474 cell viability is shown 24 h after carbon-ion beam irradiation alone, or X-ray irradiation alone or in combination with lapatinib (1 μ M). *, P<0.01, compared to control. C. Cell viability analysis using the Trypan Blue Staining assay. BT474 cell viability is shown 96 h after different doses of carbon-ion beam irradiation alone, or in combination with lapatinib (1 μ M). *, P<0.01, compared to control.

compared to that of carbon-ion beam alone, X-ray irradiation alone or its combination treatment with lapatinib. According to our and other previous reports, the relative biological effectiveness (RBE) values are around 2.0, and in this study, we compared a 1 Gy dose of carbon-ions with a 2 Gy of X-rays to evaluate their biological effects. At first, BT474 cell viability was performed using the CellTiter-Glo luminescent cell viability assay. As shown in **Figure 1B**, cell viability was significantly decreased after 1 Gy of carbon-ion beam, or X-rays alone, and it was predominantly decreased after 1 Gy of carbon-ion beam in combined with 1 μ M lapatinib compared to 2 Gy of X-rays combined with lapatinib or with lapatinib alone. To further confirm the cell killing effects of carbon-ion beam alone or its combination with lapatinib, we also performed Trypan Blue Staining assay. As shown in **Figure 1C**, cell viability was suppressed by carbon-ion beam irradiation alone dose-dependently and it was significantly further decreased by carbon-ion beam irradiation combined with lapatinib.

Changes in the proportion of ESA+/CD24- cells after carbon-ion beam or X-ray irradiation alone or in combination with lapatinib

Percentage changes of cancer stem like ESA+/CD24- CSCs 96 h after carbon-ion beam, or X-ray irradiation alone or in combination with 1 μ M of lapatinib were investigated by FACS analysis. As shown in **Figure 2A**, the proportion of ESA+/CD24- cells in BT474 cells was significantly increased after 2 Gy of X-rays combined with lapatinib or with lapatinib alone, whereas 1 Gy of carbon-ion beam combined with lapatinib significantly decreased the proportion of ESA+/CD24- cells. Interestingly, the percentage of ESA+/CD24- cells in SKBR3 cells was significantly increased when 2 Gy of X-rays combined with lapatinib was used, but no changes were observed with 2 Gy of X-rays alone or with 1 Gy of carbon-ion beam combined with lapatinib. However, the proportion of ESA+/CD24- cells was predominantly decreased with either 4 Gy of X-rays or 2 Gy of carbon-ion beam combined with lapatinib (**Figure 2B**).

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Figure 2. A. Percentage changes of ESA+/CD24- cells by FACS analysis 96 h after carbon-ion beam or X-ray irradiation alone or in combination with 1 μ M of lapatinib in BT474 cells. Lapatinib was added prior to irradiation and treated for 96 h. B. Percentage changes of ESA+/CD24- cells by FACS analysis 96 h after carbon-ion beam or X-ray irradiation alone or in combination with 1 μ M of lapatinib in SKBR3 cells. Lapatinib was added prior to irradiation and treated for 96 h. *, P<0.01 compared to non-CSCs. All experiments were performed in triplicate (n = 3).

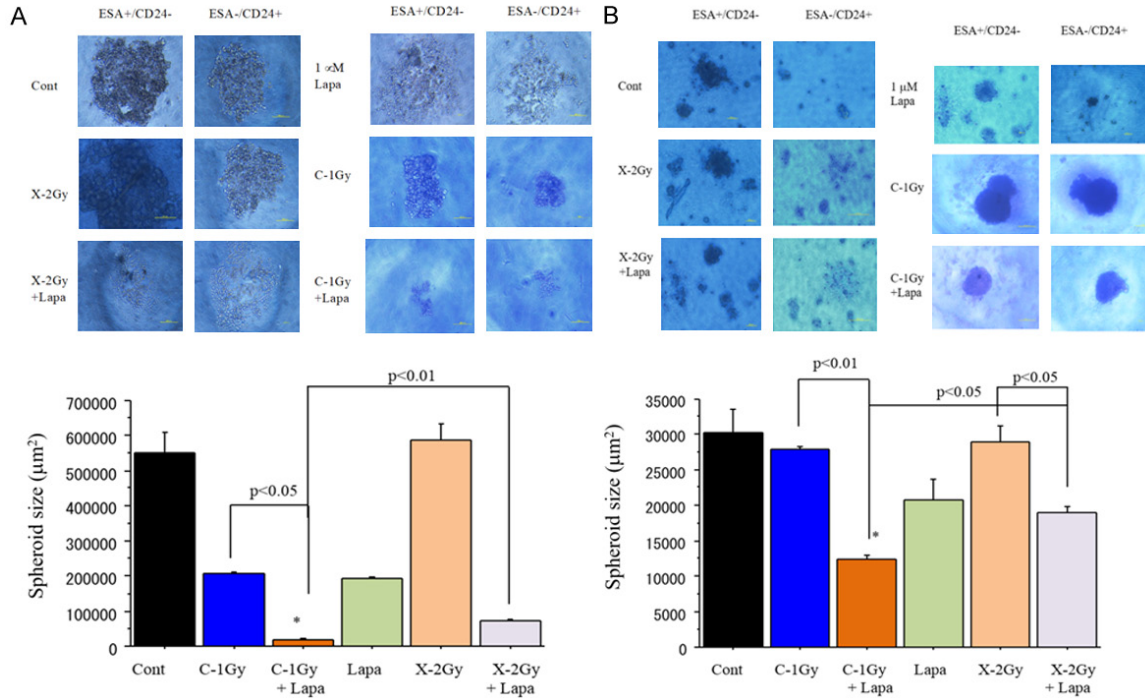


Figure 3. Spheroid formation of cancer stem-like cells (CSCs) (ESA+/CD24-) and non-CSCs (ESA-/CD24+) isolated from SKBR3 (A) and BT474 (B) cells. Cells were cultured for one week for spheroid formation ability analyses. *, P<0.01, #, P<0.05 compared to spheres formed from the Control. All experiments were performed in triplicate (n = 3).

Spheroid formation ability of ESA+/CD24- and ESA-/CD24+ cells after carbon-ion beam or X-ray irradiation alone or in combination with lapatinib

Under experimental condition with a stem cell medium, only cancer cells with self-renewal ability are expected to grow and maintain their spheroid morphology. Thus, spheroid formation assays have been widely used to evaluate cancer stem cell properties. To investigate the ability to form spheroid bodies, isolated ESA+/CD24-CSCs and non-CSC ESA-/CD24+ cells were cultured in 96-well round-bottomed Sumilon cell tight spheroid plates. After being in culture for 1 week, the ability of ESA+/CD24-cells to form spheroid bodies was significantly higher than that of ESA-/CD24+ cells (Figure 3). To examine the effects of lapatinib on radiosensitization to X-rays and carbon ion beams, spheroid formation ability assays of cancer

stem-like ESA+/CD24- cells and non-cancer stem-like ESA-/CD24+ cells after irradiation with an X-rays or carbon-ion beam alone or in combination with lapatinib were performed. As shown in Figure 3A, we found that the tumor spheroid formations of stem-like ESA+/CD24-cells isolated from SKBR3 cell line was significantly reduced by carbon-ion beam irradiation, and with lapatinib alone, but not by X-ray irradiation, and it was extremely decreased by carbon-ion beam combined with lapatinib. In contrast, no spheres were formed in non-stem-like ESA-/CD24+ cells after X-rays or carbon-ion beam, either alone or in combination with lapatinib. We have also examined the spheroid formation ability of ESA+/CD24- cells isolated from the BT474 cell line and found that carbon-ion beam alone did not reduced spheroid size but carbon-ion beam combination with lapatinib significantly reduced not only spheroid size and but also the numbers. In contrast,

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X-ray irradiation alone, lapatinib alone did not reduce the spheroid size and the numbers, even in combination with lapatinib (**Figure 3B**).

Apoptosis analyses after carbon-ion beam or X-ray irradiation alone or in combination with lapatinib

Induction of apoptosis in BT474 cells was analyzed using the Annexin V-FITC Apoptosis Detection kits 10 days after carbon-ion beam or X-ray irradiation alone or in combination with lapatinib. The data showed that there is no clear dose-dependent response with carbon-ion beam irradiation, however, only 1 Gy was sufficient to significantly increase apoptosis (**Figure 4A**). In comparison, there is a clear dose-dependent response with X-ray irradiation, but more than 4 Gy is needed to significantly increase apoptosis (**Figure 4B**). Interestingly, carbon-ion beam in combination with lapatinib synergistically enhanced apoptosis, whereas no such action was observed using X-rays combined with lapatinib.

Cell cycle analyses after carbon-ion beam or X-ray irradiation alone or in combination with lapatinib

Cell cycle analyses of BT474 cells 4 days after a carbon-ion beam, or X-rays alone or in combination with 1 μ M of lapatinib were performed. Lapatinib was added prior to irradiation and constantly applied for 4 days, and then the cell cycle distribution (sub G1, G1, S and G2/M phase) was measured using an FACS Calibur. As shown in **Figure 5**, carbon-ion beam irradiation combined with lapatinib was more effective in inhibiting cell cycle progression (sub-G1 arrest) and inducing cell death (apoptosis/necrosis) than X-rays alone or X-rays combined with lapatinib. We have also performed cell cycle analyses in SKBR3 cells and obtained the same results (data not shown).

γ H2AX foci formation in ESA+/CD24- and ESA-/CD24+ cells after carbon-ion beam or X-rays alone or in combination with lapatinib

To examine the role of lapatinib radiosensitization on DNA damage and repair in stem-like ESA+/CD24- cells, the γ -H2AX foci formation analysis was performed, which marks DNA double-strand breaks (DSB). As shown in **Figure 6A**, carbon-ion beam and X-rays significantly increased the number of γ H2AX foci, with no

observable differences between them, and this number was significantly increased further using carbon ion beam combined with lapatinib. However, the size of foci (clustered DSB) was frequently found in cells treated by carbon-ion beam combined with lapatinib compared to carbon-ion beam alone, X-rays alone, lapatinib alone, or X-rays combined with lapatinib (**Figure 6A**). We also examined the nuclear γ H2AX foci formed in non-stem like ESA-/CD24+ cells 24 h after a carbon-ion beam or X-ray irradiation alone or in combination with 1 μ M of lapatinib. We found that many more numbers of γ H2AX foci were present after carbon-ion beam treatment alone compared to X-ray alone, and either carbon ion beam or X-ray irradiation combined with lapatinib enhanced γ H2AX foci formation (**Figure 6B**).

Expression changes of various genes in CSCs after carbon-ion beam alone or X-rays alone or in combination with lapatinib measured by quantitative real-time RT-PCR analysis

To quantitatively examine multiple gene expression changes in CSCs (ESA+/CD24-) and non-CSCs (ESA-/CD24+) isolated from BT474 cells, quantitative real-time RT-PCR analysis was performed according to the manufacturer's protocol. The data shows that irradiation with a carbon-ion beam combined with a constant treatment of 1 μ M of lapatinib for 72 h significantly increased the expressions of apoptosis-related caspase 3 but not Bax, and increased Bcl2 expression in both non-CSC and CSCs. Interestingly, expression of autophagy-related genes varied, LC3 was significantly increased only in non-CSCs, but Beclin1 expression was predominantly enhanced in both CSCs and non-CSCs after carbon-ion beam irradiation alone or in combination with lapatinib. In addition, no changes in ATG7 expression were found after treatment with carbon-ion beam alone, or X-rays alone or combined with lapatinib when compared to carbon-ion beam alone, or lapatinib alone in both non-CSC and CSCs (**Figure 7A, 7B**). We have examined above autophagy-related genes at protein levels and found that expressions of ATG7 and Beclin1 and were increased in a dose-dependent manner by carbon-ion beam alone and only ATG7 expression was further enhanced by combination treatment of carbon-ion beam and lapatinib, whereas expression of Beclin1 was undetectable (**Figure 7C**). Furthermore, we investigated

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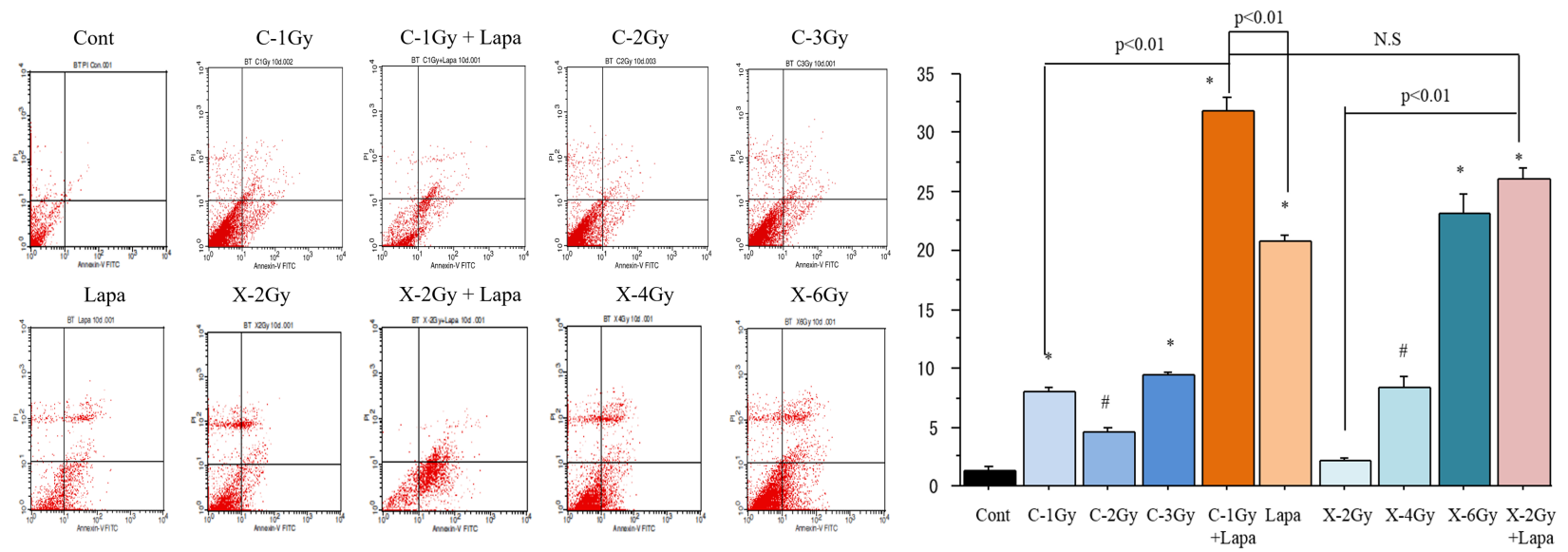


Figure 4. Apoptosis analysis of BT474 cells 10 days after a carbon-ion beam irradiation alone or in combination with 1 μ M of lapatinib using the FITC Annexin-V-PI detection kit. *, $P < 0.01$, #, $P < 0.05$ compared to control. All experiments were performed in triplicate ($n = 3$).

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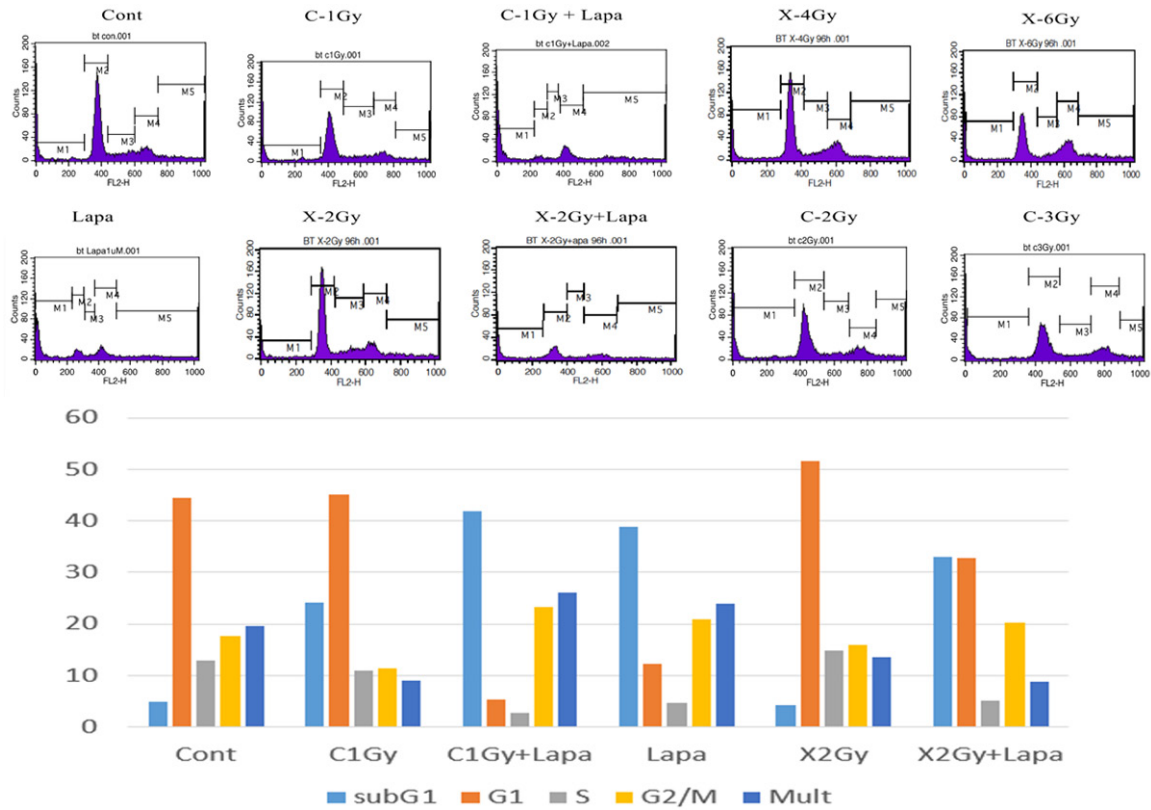


Figure 5. Cell cycle analyses of BT474 cells 4 days after carbon-ion beam, or X-rays irradiation alone or in combination with 1 μ M of lapatinib. Lapatinib was added 2 h prior to irradiation and continued for 4 days, and the cell-cycle distribution (subG1, G1, S and G2/M phases) was measured by flow cytometry. Carbon-ion beam combined with lapatinib significantly inhibited cell-cycle progression (sub-G1 arrest) and induced cell death (apoptosis/necrosis). All experiments were performed in triplicate ($n = 3$), and the averages of the three separate experiments are shown.

autophagosomes using Cyto-ID Autophagy Detection Kit. The cells treated with autophagy inducer rapamycin which used as a positive control. We found that 24 h after carbon-ion beam irradiation alone effectively induced autophagy and in combination with lapatinib seems to induce more strongly (Figure 7D).

Discussion

Although the anti-proliferative and pro-apoptotic effects of lapatinib have been reported [35, 36], the molecular mechanism of radiosensitization and modes of cell death by lapatinib combined with high linear energy transfer (LET) carbon-ion beam on HER2-overexpressing breast cancer cells have not been elucidated. In this study, we found that the HER2 positive breast cancer cell viability was significantly decreased by using carbon-ion beam, or by lapatinib alone, and it was further decreased by using carbon-ion beam in combination with

lapatinib when compared to X-ray irradiation alone, X-rays combined with lapatinib also reduced cell viability, but was less effective than using carbon-ion beam together with lapatinib. This is partially consistent with previous reports showing that lapatinib inhibits cell growth, enhances antibody-dependent cellular cytotoxicity and potentiates radiation-induced cell death of HER2-overexpressing cancer cells [37-39].

In the present study, the percentages of cancer stem-like ESA+/CD24- cells (CSCs) in BT474 cell line showed a tendency to increase after either 2 Gy of X-rays or 1 Gy of carbon-ion beam, and it was significantly increased after 2 Gy of X-rays in combination with 1 μ M of lapatinib. In contrast, 1 Gy of carbon ion beam combined with lapatinib significantly decreased the proportion of CSCs in BT474 cells. The proportional changes of CSCs in SKBR3 cells were not affected by 2 Gy of X-rays or 1 Gy of carbon-ion

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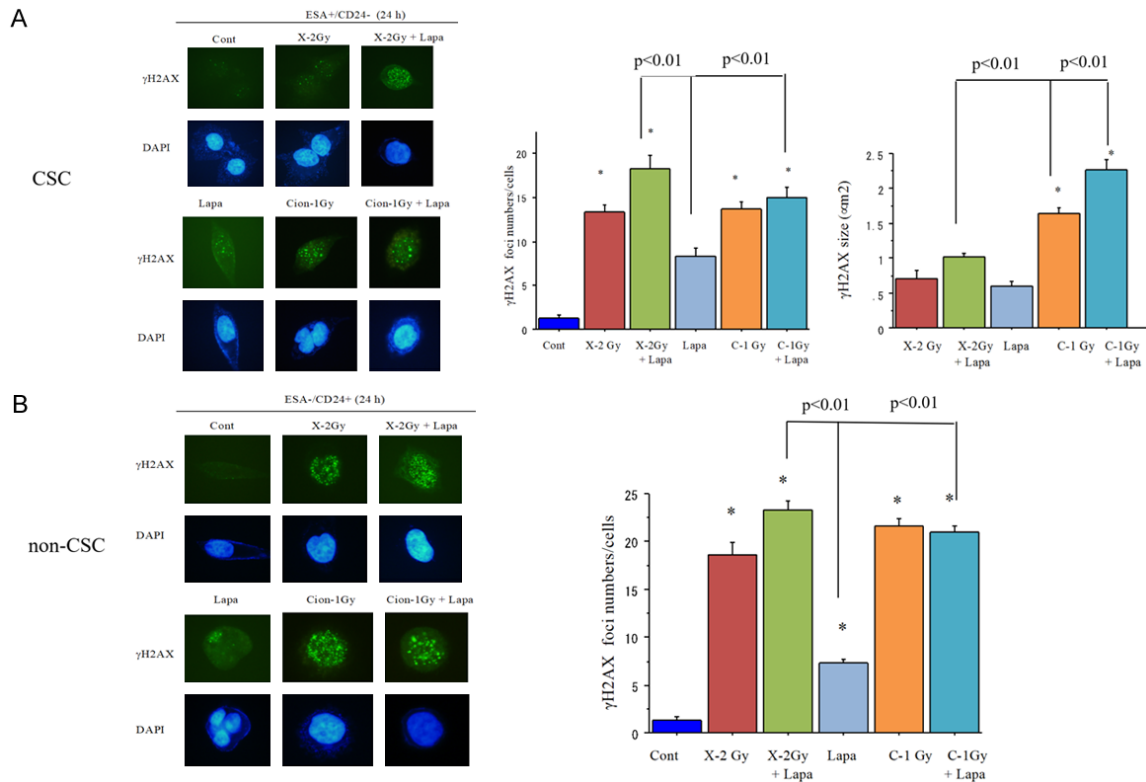


Figure 6. A. Quantification and representative photos of nuclear γ H2AX foci formation in CSCs (ESA+/CD24-) isolated from BT474 cells 24 h after a carbon-ion beam, or X-ray irradiation alone or in combination with 1 μ M of lapatinib. Quantification of nuclear γ -H2AX foci larger than 1.5 μ m² is also displayed. Lapatinib was added prior to irradiation and continued for 2 h. Data represents mean \pm SD. * $P < 0.05$ compared to control. B. Quantification and representative photos of nuclear γ H2AX foci formation in non-CSCs (ESA-/CD24+) isolated from BT474 cells 24 h after carbon-ion beam, X-ray irradiation alone or in combination with 1 μ M of lapatinib. Lapatinib was added 2 h prior to irradiation and continued for 24 h. Data represent mean \pm SD. * $P < 0.05$ compared to control. All experiments were performed in triplicate (n = 3).

beam, or with 1 μ M of lapatinib alone. However, it was significantly increased by 2 Gy of X-ray irradiation in combination with lapatinib but not 1 Gy of carbon-ion beam in combination with lapatinib, whereas it was significantly decreased by 4 Gy of X-rays or 2 Gy of carbon-ion beam combined with lapatinib. This finding suggests that CSC proportions can be reduced when lapatinib is combined with slightly increasing doses of radiation. This is partially consistent with ours and other previous reports [30-34, 38-40]. At first, to confirm the CSC properties of ESA+/CD24- cells, we sorted this population from SKBR3 cells and examined its spheroid formation capability compared to sorted non-CSC ESA+/CD24- cells. The data showed that ESA+/CD24- cells have a significantly higher spheroid formation capability than ESA-/CD24+ cells, indicating that ESA+/CD24- cells have CSC properties. This is in line with previously reports that ESA+/CD24- is a marker for breast

CSC [18, 33]. We also examined and confirmed that demonstrate that ESA+/CD24- cells have CSC properties compared to ESA-/CD24+ cells sorted from the BT474 cell line based on its high spheroid formation ability (data not shown). Furthermore, the data shows that spheroid formation of CSCs was significantly inhibited by carbon-ion beam, or by lapatinib alone, and it was extremely suppressed by carbon-ion beam combined with lapatinib when compared to X-rays alone or X-rays combined with lapatinib. This suggests that breast CSCs are significantly radiosensitized when carbon-ion beam is combined with lapatinib.

To determine apoptosis induction after long-term treatments of carbon-ion beam, or X-ray irradiation alone or in combination with lapatinib, we analyzed the apoptosis using the Annexin V-FITC Apoptosis Detection Kits 10 days later. The data showed that carbon-ion

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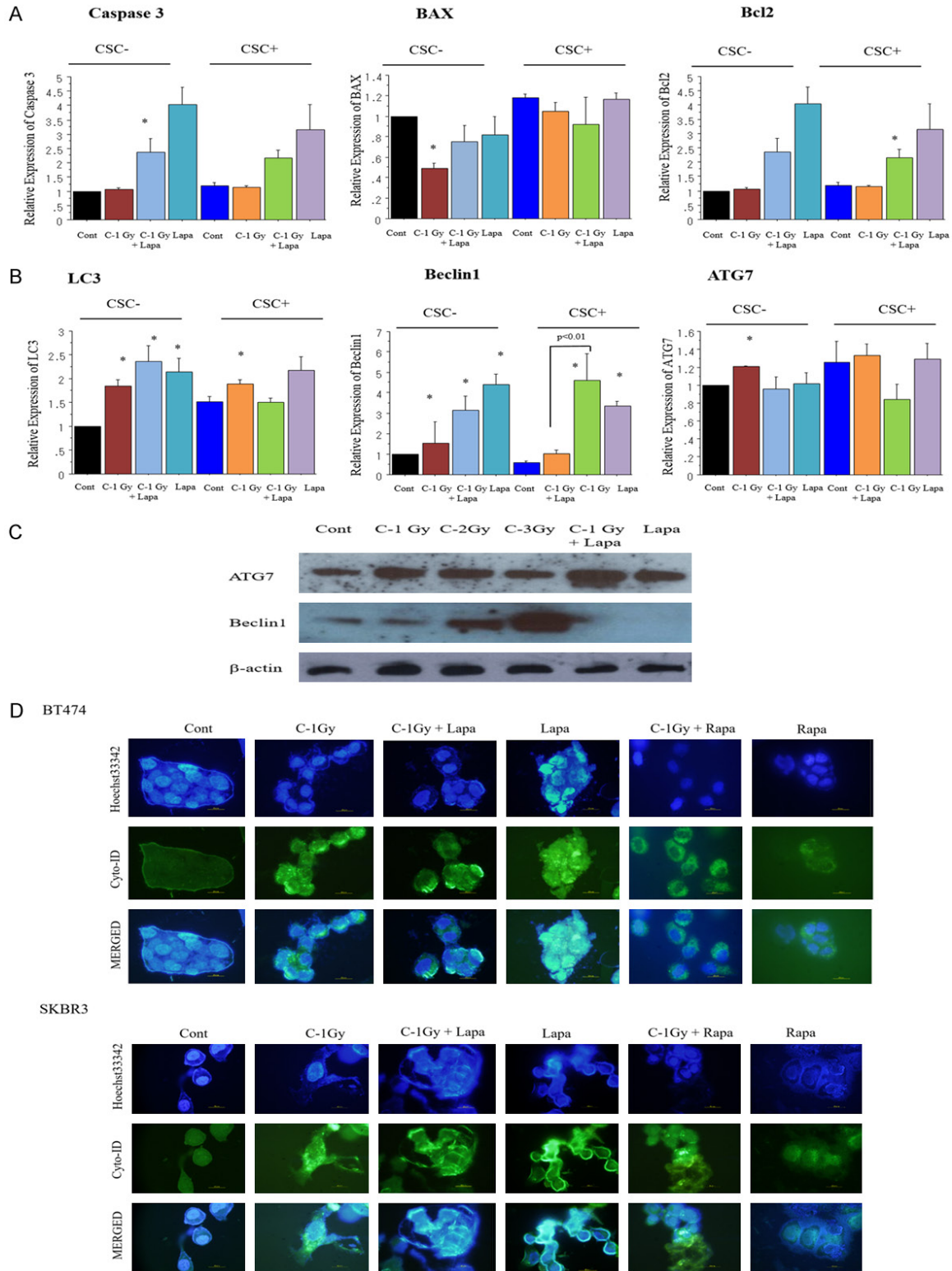


Figure 7. Quantitative real time RT-PCR analysis of apoptosis (A) and autophagy-related (B) genes in CSCs and non-CSCs isolated from BT474 cells 96 h after a carbon-ion beam, or X-ray irradiation alone or in combination with 1 μ M of lapatinib. Lapatinib was added 2 h prior to irradiation and continued for 96 h. *, $P < 0.01$, compared to control. All experiments were performed in triplicate ($n = 3$). (C) Western blotting analysis of ATG7 and Beclin1 in BT474 cells 96 h after a carbon-ion beam alone or lapatinib alone, or in combination with lapatinib. (D) Visualizing autophagy by fluorescence microscopy. The autophagic cells stained by Cyto-ID dual detection reagent solution (Cyto-ID Green Detection Reagent and Hoechst 33342) and were observed using fluorescence microscope.

beam irradiation alone can increase apoptosis, although there is no dose-related response, whereas X-ray irradiation alone increased apoptosis in a dose-dependent manner. Interestingly, carbon ion beam in combination with lapatinib dramatically enhanced apoptosis compared to carbon-ion beam alone, lapatinib alone, X-rays alone or in combination with lapatinib. The cell cycle distribution of BT474 and SKBR3 cells 4 days after a carbon-ion beam, or X-ray irradiation alone or in combination with 1 μ M of lapatinib was analyzed by flow cytometry. Carbon-ion beam combined with lapatinib more significantly inhibited cell cycle progression (sub-G1 arrest) and induced cell death (apoptosis/necrosis) compared to carbon-ion beam alone, X-rays alone or X-rays combined with lapatinib in both BT474 and SKBR3 (data not shown) cells. This is in line with previous reports showing that lapatinib induces apoptosis in various cancer cells [35, 36].

There is increasing evidence indicating that high LET particle irradiation can induce complex DNA damage such as clustered DNA lesions [41-44]. In this study, the number of double strand breaks (DSBs) visualized by γ H2AX foci formation in non-CSCs and CSCs was significantly increased by carbon-ion beam, X-ray irradiation alone, or in combination with lapatinib with no significant difference among the treatments. However, significantly larger-sized γ H2AX foci were only induced by carbon-ion beam irradiation, and carbon-ion beam combined with lapatinib induced more large-sized γ H2AX foci, suggesting that a higher complexity of clustered DSB was induced by carbon ion beam in combination with lapatinib. These results reveal that the greater complexity of DSBs induced by carbon-ion beam combined with a molecular target drug which potentially leads to increased mutagenicity and decreased reparability of the damaged site [43, 44]. Taken together, our results are the first to show that carbon-ion beam in combination with lapatinib synergistically enhanced cell killing of HER2-positive CSCs. This is partially consistent with previous reports that HER2 is preferentially expressed in the CSC population, and HER2 targeting drug trastuzumab found to effectively reduced tumor sphere formation and CSC markers [10, 40].

A number of studies showed that CSC subpopulations are shown to be radioresistant com-

pared to non-CSC subpopulations and cross-talk between autophagy signaling and apoptosis signaling was considered as one of this resistant mechanisms [41]. In the present study, we found that after treatment with carbon-ion beam combined with lapatinib for radioresistant CSCs, apoptosis-related gene expression of caspase 3 but not Bax, and autophagy-related genes like LC3 and Beclin1 showed significant enhancements compared to that of carbon ion beam, X-ray alone or X-ray combined with lapatinib, suggesting that carbon-ion beam combined with lapatinib may have more power to induce multiple cell death. Altogether, carbon-ion beam combined with lapatinib appear to show enhanced effects in inducing apoptosis and autophagy related gene expression at the mRNA levels in vitro in the disruption of HER2-positive breast CSCs. Cyto-ID autophagy flux immunofluorescence analysis indicated that 24 h after carbon-ion beam irradiation alone effectively induced autophagy and combination treatment with lapatinib seems to be induced autophagy more strongly. Furthermore, western blot data showed that carbon-ion beam dose-dependently increased expression of ATG7 and Beclin1, however carbon-ion beam in combination with lapatinib only enhanced ATG7 expression, whereas expression of Beclin1 was lost. We speculated that relatively long-term treatment (96 h) with lapatinib and/or in addition with carbon-ion beam may strongly killed most of tumor cells and degraded some proteins. It has been reported that lapatinib radiosensitized BCs are accompanied with apoptosis and autophagy [38-40], and lapatinib induces autophagic cell death and combined treatment with rapamycin, a autophagy activator, or with radiation further increased autophagy in HER2 positive breast cancer cells [45-52]. It has also demonstrated that Tat-Beclin1 peptide inhibits HER2 positive human breast cancer xenograft with robust autophagy induction [53]. Based on above literature and our present findings, it is implying that carbon ion beam in combination with lapatinib may enhance cell death via autophagy induction [54]. However, the deep molecular mechanisms of carbon ion beam irradiation alone or lapatinib combination treatment-induced apoptosis, autophagy and their interaction need to be further studied. In addition, it has been demonstrated that overexpression of the HER2 gene results in the formation of a

ligand-independent HER2 homodimer and initiate downstream signaling cascades, such as the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, which regulate cell proliferation and survival [55]. We plan to further investigate the effects of carbon-ion beam irradiation in combination with lapatinib on these of signaling pathways in the near future.

In summary, relatively low doses of carbon-ion beam combined with lapatinib have promising advantages in targeting putative HER2-positive breast CSCs by inducing complex DNA damage, increased apoptosis, autophagy, and subsequent cell death then by using carbon-ion beam alone.

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Disclosure of conflict of interest

None.

Abbreviations

BC, breast cancer; HER2, human epidermal growth factor receptor-2; ER, estrogen receptor; PR, progesterone receptor; CSC, cancer stem-like cell; HIMAC, heavy ion medical accelerator in Chiba; DMEM, dulbecco's modified eagle's medium; ANOVA, analysis of variance; SOBP, spread-out bragg peak; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; LET, linear transfer energy; RBE, relative biological effectiveness; DSB, double strand breaks.

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