

## Biological Effectiveness of Carbon-Ion Radiation on Various Human Breast Cancer Cell Lines

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### Abstract

**Introduction:** Carbon-ion radiotherapy (C-ion RT) is known as a highly effective local treatment and its relative biological effectiveness (RBE) has been evaluated for various types of malignant tumors. There are only a few studies on C-ion radio sensitivity in breast cancer, and there has been no evaluation by subtypes. To estimate the impact of C-ion RT for breast cancer, RBE of C-ion beams of various types of human breast cancer cell lines was evaluated by comparison with X-rays.

**Methods:** Six human breast cancer cell lines with different subtypes, Luminal-human epidermal growth factor receptor 2 (HER2)-negative (MCF-7), Luminal-HER2-positive (BT-474), Her2-enriched (SK-BR-3), Basal-like (MDA-MB-468, HCC1937) and ductal carcinoma in situ (MCF10DCIS.com) were used. Radio sensitivities were assessed with survival curves created from colony-forming assay (CFA) and high-density surviving assay (HDS). An X-ray generator was used with 200 kV, 20 mA. The Heavy Ion Medical Accelerator in Chiba (HIMAC) was used for C-ion irradiation, with 290 MeV/u, mono-peak, linear energy transfer (LET) of 80 KeV/μm

**Results:** CFA was not suitable for BT474, SK-BR-3, MDA-MB-468, and HCC1937 because of their low plating efficiency. The differences between the D10 values on HDS were large with X-ray, and the survival curve shoulders for MCF7, MDA-MB-468, and MCF10DCIS.com were wide. On the other hand, the differences between the D10 values were small with C-ion beams, and the survival curves were linear without shoulders for all cell lines except a small shoulder with MCF10DCIS.com. The RBE value of C-ion beams was 2.3 to 3.6, median 2.9 in all cell lines by CFA and HDS.

**Conclusion:** RBE around 3 by C-ion beams was seen in many types of ductal cancer. The small survival curve shoulder on MCF10DCIS.com suggested that non invasive ductal carcinoma is relatively more resistant than invasive cancer.

**Keywords:** Breast cancer, Carbon-ion radiotherapy, Radio sensitivity, Relative biological effectiveness

### Abbreviations:

C-ion RT: Carbon-ion Radiotherapy; RBE: Relative Biological Effectiveness; HER2: Human Epidermal Growth Factor Receptor 2; CFA: Colony-Forming Assay; HDS: High Density Surviving Assay; HIMAC: The Heavy Ion Medical Accelerator in Chiba; LET: Linear Energy Transfer; NIRS: The National Institute of Radiological Sciences; APBI: Accelerated Partial Breast Irradiation; ASTRO: The American Society for Radiation Oncology; ER: Estrogen Receptor; PgR: Progesterone Receptor; DMEM: Dulbecco's Modified Eagle's Minimum Essential Medium; FBS: Fetal Bovine Serum; PS: Penicillin/Streptomycin; RPMI: Roswell Park Memorial Institute; PBS: Phosphate-Buffered Saline; PE: Plating Efficiency

### Introduction

Radiotherapy with X-rays plays an important role in the treatment of breast cancer for breast irradiation of breast-conserving therapy, post-mastectomy regional radiotherapy, and recurrent tumor radiotherapy. However, the treatment effects are limited, as the local control rate is attenuated by post-operative micro residual tumor cells. If more effective radiotherapy would be available, its role would rise in importance.

Carbon-ion radiotherapy (C-ion RT), one of the types of heavy particle radiotherapy, with high biological effect and good dose distribution, has been reported to be effective for locally advanced tumor and radio resistant tumor [1]. Good dose distribution has the potential to cure tumors without problematic adverse effects on surrounding normal tissue. Relative biological effectiveness (RBE), which is the effective ratio compared to photon, reportedly about 3, is also advantageous compared to proton with RBE of about 1.1 [2]. The National Institute of Radiological Sciences (NIRS) has been carrying

out C-ion RT using the Heavy Ion Medical Accelerator in Chiba (HIMAC) since 1994, treating more than 8000 various malignant tumors that had been considered difficult to treat by conventional therapy [3]. NIRS has reported good local control with C-ion RT in adenocarcinoma, melanoma and sarcoma, known radio resistant tumors. C-ion RT facilities in other countries have also reported good results [3]. The usefulness of C-ion RT has been recognized, and the number of C-ion RT facilities has been gradually increasing throughout the world. In most of those countries, breast cancer is the most common cancer among women.

Breast cancer had not been considered a good candidate for C-ion RT because of the increased skin dose in both whole breast and regional irradiation. Also, relatively better treatment results had already been obtained in early-stage tumor with breast-conserving therapy combined with systemic therapy [4]. However, in recent years, breast-conserving therapy for certain types of early breast cancer could be carried out without whole breast irradiation, and sufficient control can be obtained by partial breast irradiation to the tumor bed following lumpectomy. In the concept of partial breast irradiation, as the radiation field contains a relatively small amount of normal tissue, radiotherapy can be conducted within a few days without consideration of adverse effects on normal tissue. Therefore, accelerated partial breast irradiation (APBI) can usually be performed within 5 days. In European countries and the United States, breast-conserving surgery with APBI is a part of clinical practice for patients with low risk factors. According to the consensus guidelines of the American Society for Radiation Oncology (ASTRO), patients aged 60 years or older, less than 2-cm solitary primary tumor, no axillary lymph node metastasis, estrogen receptor (ER) positive, human epidermal growth factor receptor 2 (HER2) negative, invasive ductal carcinoma, or common type histology are good candidates for APBI [5]. The proportion of good candidates for APBI is about 15% of all breast cancer patients, some 9000 patients per year in Japan.

Particle radiotherapy application for breast cancer has been reported from only a few facilities, using post-operative proton radiotherapy to the tumor bed as APBI or whole breast irradiation [6-8]. Even though Japan has thirteen particle therapy facilities, no study of breast cancer has been reported except from our institute. In respect to the higher anti-tumor effect than photon on other malignant tumors including radio resistant tumor, as well as our experience with metastatic breast cancer, C-ion RT for primary breast tumor is considered to be satisfactory in comparison with X-ray irradiation. Therefore, our institute has conducted radical radiotherapy of APBI with C-ion RT without lumpectomy. Radical C-ion RT of breast tumor will be able to deliver a lower physical burden of treatment, higher quality of life, and a cosmetic outcome compared with standard breast-conserving therapy. The Research Center for Charged Particle Therapy of NIRS had been preparing the clinical application of C-ion RT for low-risk stage I breast cancer since 2011. The breast cancer clinical research committee designed the protocol for clinical trials of C-ion RT for low-risk stage I breast cancer, and started the protocol treatment from May 2013 [9].

There are only a few studies on the C-ion radiosensitivity of breast cancer [10], and these did not evaluate the radiosensitivity of each subtype of invasive cancer and non-invasive cancer to X-rays and C-ion beams. Therefore, in order to support the utility of breast cancer treatment with C-ion beams, this study was conducted to evaluate the radiosensitivity of each subtype of invasive and non-invasive human breast cancer cell lines to C-ion beams and X-rays.

## Methods

### Cells

Six human breast cancer cell lines, MCF-7, BT-474, SK-BR-3, MDA-MB-468, HCC1937, and MCF10DCIS, derived from different subtypes were purchased from ATCC (Manassas, VA, USA). The statuses of ER, progesterone receptor (PgR), and HER2 of individual cell lines are shown in Table 1. MCF-7 and MDA-MB-468 were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS) (HyClone, UT, USA), 1% penicillin/streptomycin (PS)

(Gibco, MD, USA) and 1% L-glutamin (Gibco). BT-474, SK-BR-3, and HCC1937 were cultured in Roswell Park Memorial Institute (RPMI) medium (Nissui) supplemented with 10% FBS, 1% PS and 1% L-glutamin. For MCF10DCIS, cells were cultured in DMEM Nutrient Mixture F-12 media (Gibco) supplemented with 5% FBS and 1% PS. Expression profiles of ER, PgR and HER2 in 6 breast cancer cell lines were confirmed by Western blot analysis (Figure 1). Primary antibodies (Abs) for human ER (sc-543, Santa Cruz, Dallas TX, USA), PgR (NCL-L-PGR-312, Novocastra, Newcastle upon Tyne, UK), HER2 (sc-284, Santa Cruz) and  $\alpha$ -tubulin (B-5-1-2, Sigma-Aldrich, St. Louis, MI, USA) with HRP-conjugated anti-mouse/rabbit IgG (Dako, Glostrup, Denmark) were used.

### Irradiation

Cells were irradiated with C-ion beams accelerated by HIMAC at NIRS. The initial energy of C-ion beams was 290 MeV/u, and the LET value was 80 keV/ $\mu$ m; a mono-energetic beam with a narrow Bragg Peak was applied at a depth of 10 cm, and cells were irradiated with 0, 0.5, 1, 1.5, 2, 2.5 or 3 Gy. For a comparison with the C-ion beams, 200-kV X-rays with 0, 1, 2, 3, 4 or 6 Gy were used. X-rays were produced by PANTAC HF320-S X-ray generator (Shimadzu, Kyoto, Japan) at 200 kV, and 20 mA, and filtered with 0.5 mm Al and 0.5 mm Cu. All irradiations were carried out at a dose rate of approximately 1 Gy/min at room temperature. Cells were cultured on Falcon T25 flask (BD Falcon, NJ, USA) for 2 to 3 days before irradiation and cell cultures at about 50 to 60% confluence were irradiated.

### Colony-forming assay (CFA)

After irradiation, cells were immediately rinsed with phosphate-buffered saline (PBS) (Nissui) and detached with 0.1% trypsin (Gibco). The cell densities were counted with a hemacytometer, and plated onto 150-mm, 100-mm or 60-mm diameter plastic dishes. Colonies were fixed and stained with 1% methylene blue in 30% methanol after a 7-14-day incubation period, the period fitting the respective cell lines for colony formation. Colonies consisting of more than 50 cells were scored as surviving colonies. The plating efficiencies for colony formation on a plastic dish ranged from 0% to 44% in each cell line.

### High-density survival assay (HDS)

The HDS assay was carried out as described by Kuwahara et al. with a modification [11,12]. Briefly, cells with about 50 to 60% confluence were irradiated, and the cells were kept in culture for another 3 days. Cells of each flask were then trypsinized and 1/8 of cells for MCF-7, BT-474, SK-BR-3, MDA-MB-468, HCC1937, or 1/24 of cells for MCF10DCIS were plated onto new T25 flasks and subcultured for a

further 5 days. The number of cells was counted with a hemacytometer at 8 days after exposure to radiation.

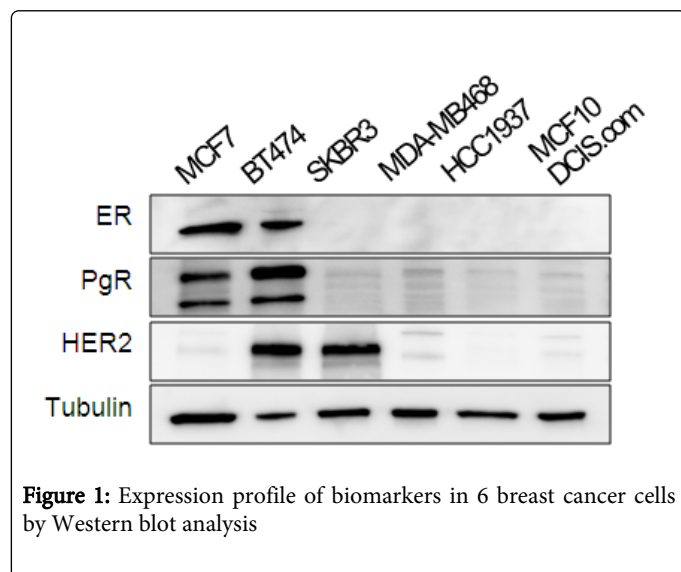
## Results

### Expression profiles of ER, PgR and HER2

Expressions of ER, PgR and HER2 in 6 breast cancer cell lines were confirmed by Western blotting (Figure 1), and the data are summarized in Table 1.

| Subtype                  | ER | PgR | HER2 | Cell identification number |
|--------------------------|----|-----|------|----------------------------|
| Luminal-HER2negative     | +  | +   | -    | MCF7                       |
| Luminal-HER2 positive    | +  | +   | +    | BT474                      |
| HER 2                    | -  | -   | +    | SK-BR-3                    |
| Basal-like               | -  | -   | -    | MDA-MB-468                 |
|                          |    |     |      | HCC1937                    |
| Ductal Carcinoma in situ | -  | -   | -    | MCF10DCIS.com              |

**Table 1:** Names, characteristics, cell identification numbers of human breast cancer cell lines examined



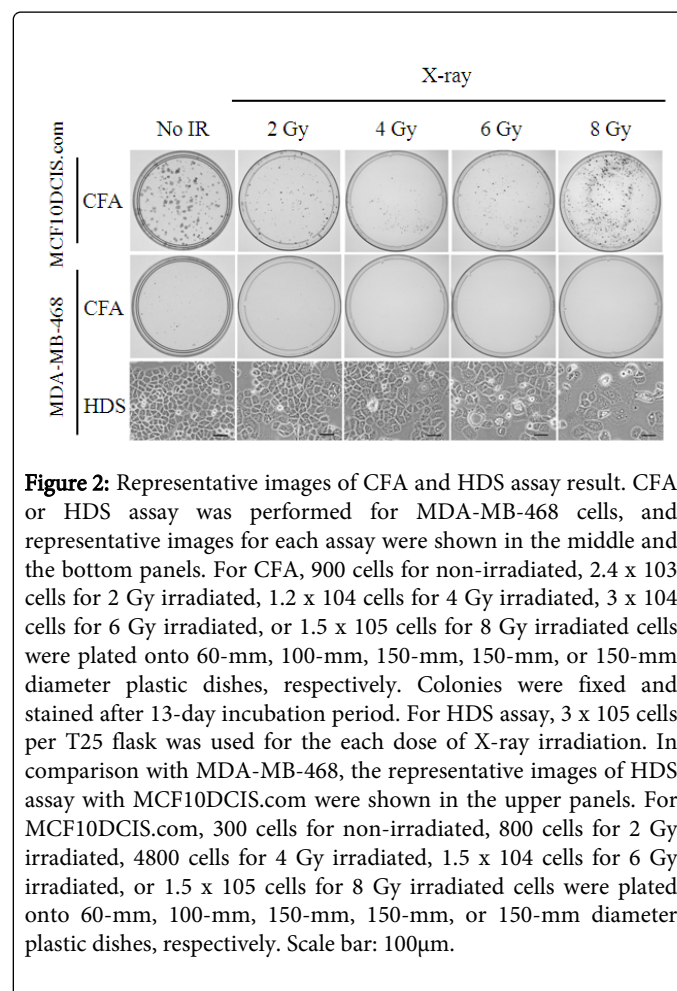
**Figure 1:** Expression profile of biomarkers in 6 breast cancer cells by Western blot analysis

### Plating efficiency of cells

Plating efficiency (PE) of MCF-7, SK-BR-3, MDA-MB-468 and MCF10DCIS.com was 29, 15, 5.8 and 48%, respectively (Table 2). Countable colonies were not formed in BT474 and HCC1939, thus cell-survival curves by CFA were unable to be determined for those cell lines. Colonies were formed for SK-BR-3 and MDA-MB-468, colonies were formed, but the data were not stable within repeated assessments, because of their low PE.

Representative images of CFA and HDS assay result were shown in Figure 2. Because the plating efficiency of MDA-MB-468 is much lower than the one of MCF10DCIS.com, three times more cells, 900 cells, were needed to form the countable colonies. In addition, the

countable colonies were unable to be formed in irradiated MDA-MB-468 although much higher number of cells were plated onto the dishes compared to those used for the MCF10DCIS.com, which were  $2.4 \times 10^3$  cells for 2 Gy irradiated,  $1.2 \times 10^4$  cells for 4 Gy irradiated,  $3 \times 10^4$  cells for 6 Gy irradiated, or  $1.5 \times 10^5$  cells for 8 Gy irradiated cells. On the other hand, much fewer cell densities were enough to form the colonies for the MCF10DCIS.com, which were 800 cells for 2 Gy irradiated, 4800 cells for 4 Gy irradiated,  $1.5 \times 10^4$  cells for 6 Gy irradiated, or  $1.5 \times 10^5$  cells for 8 Gy irradiated cells. Thus, in contrast to the MCF10DCIS.com, MDA-MB-468 is not suitable to use the CFA for the determination of surviving fraction.



**Figure 2:** Representative images of CFA and HDS assay result. CFA or HDS assay was performed for MDA-MB-468 cells, and representative images for each assay were shown in the middle and the bottom panels. For CFA, 900 cells for non-irradiated,  $2.4 \times 10^3$  cells for 2 Gy irradiated,  $1.2 \times 10^4$  cells for 4 Gy irradiated,  $3 \times 10^4$  cells for 6 Gy irradiated, or  $1.5 \times 10^5$  cells for 8 Gy irradiated cells were plated onto 60-mm, 100-mm, 150-mm, 150-mm, or 150-mm diameter plastic dishes, respectively. Colonies were fixed and stained after 13-day incubation period. For HDS assay,  $3 \times 10^5$  cells per T25 flask was used for the each dose of X-ray irradiation. In comparison with MDA-MB-468, the representative images of HDS assay with MCF10DCIS.com were shown in the upper panels. For MCF10DCIS.com, 300 cells for non-irradiated, 800 cells for 2 Gy irradiated, 4800 cells for 4 Gy irradiated,  $1.5 \times 10^4$  cells for 6 Gy irradiated, or  $1.5 \times 10^5$  cells for 8 Gy irradiated cells were plated onto 60-mm, 100-mm, 150-mm, 150-mm, or 150-mm diameter plastic dishes, respectively. Scale bar: 100µm.

### Dose-response and RBE values of cells

Dose-response curves of each cell survival following irradiation by 200-kV X-rays and C-ion beams by CFA and HDS are shown in Figure 3. The irradiation dose-response curves for cell survival by HDS were shifted to be more resistant than those observed by CFA in both MCF-7 and MCF10DCIS.com cell lines. Therefore, the parameters of the dose-response curves, such as the D10 values, could not be simply compared among the cell lines. However, the HDS data showed that the cells were more sensitive to C-ion beams compared to X-ray irradiation and similar RBE values calculated by D10 were obtained by the two assays (Table 2). The dose-response curve of X-ray irradiation obtained by HDS also indicated that the survival curve shoulders for MCF7, MDA-MB-468 and MCF10DCIS.com were broad. On the

other hand, the survival curves of C-ion beams were linear without significant shoulders for the analyzed cell lines except MCF10DCIS.com cells. The RBE values obtained by HDS ranged from 2.4 to 3.4 among the 6 breast cancer cell lines.

| Cell          | Plating Efficiency (%) | Doubling Time |       | D10 Value |           | RBE |
|---------------|------------------------|---------------|-------|-----------|-----------|-----|
|               |                        | (h)           | Assay | X-ray     | C-ion     |     |
| MCF-7         | 29.3 ± 7.9             | 24            | CFA   | 3.0 ± 0.4 | 1.0 ± 0.1 | 3   |
|               |                        |               | HDS   | 4.7 ± 0.5 | 1.5 ± 0.1 | 3.1 |
| BT474         | <1.0                   | 48            | CFA   | NC        | NC        | NC  |
|               |                        |               | HDS   | 7.6 ± 0.7 | 3.2 ± 0.3 | 2.4 |
| SK-BR-3       | 15.0 ± 6.6             | 30            | CFA   | NC        | NC        | NC  |
|               |                        |               | HDS   | 6.5 ± 1.0 | 1.9 ± 0.4 | 3.4 |
| MDA-MB-468    | 5.8 ± 4.4              | 24            | CFA   | NC        | NC        | NC  |
|               |                        |               | HDS   | 5.0 ± 0.2 | 1.8 ± 0.1 | 2.8 |
| HCC1937       | <1.0                   | 40            | CFA   | NC        | NC        | NC  |
|               |                        |               | HDS   | 3.0 ± 0.5 | 0.8 ± 0.1 | 3.3 |
| MCF10DCIS.com | 47.5 ± 8.9             | 12            | CFA   | 2.5 ± 0.1 | 1.1 ± 0.2 | 2.3 |
|               |                        |               | HDS   | 6.0 ± 0.1 | 2.3 ± 0.1 | 2.6 |

**Table 2:** Summary of D10 and RBE of each cell line

## Discussion

It is well known that CFA is a cell-survival assay that determines the ability of a single cell to grow into a colony [13]. However, the CFA method is not suitable for determining the surviving ability of cells, which require cell-cell interaction to survive. Prior to the experiment, low PE of breast cancer cells on CFA was not expected. The breast cancer cell lines used in our series had lower PE compared with other cancer cell lines. PE of about 90% was previously reported in colon cancer and pancreatic cancer cell lines [14,15], but in the six breast cancer cell lines, the highest PE was the 48% of MCF10DCIS.com, and BT474 and HCC1939 did not form countable colonies. The cell lines with higher PE, MCF10DCIS.com and MCF-7 (30% PE) showed good reproducible data. On the other hand, the 6% PE of MDA-MB-468 and 15% of SK-BR-3 indicated instability, and no dose-dependency could be confirmed, even with repeated examinations.

The MTT cell proliferation assay was then performed. This assay is a colorimetric method to measure the enzymatic activity that is reduced to formazan purple pigment *in vivo*. Cells are usually seeded quite thinly for a small volume in 96-well plates when using the MTT assay, but breast cancer cells could not grow under this condition. When we seeded cells with enough density to grow, the well became

full immediately and the growth curve reached a plateau, meaning that we could not obtain a growth curve with this assay. This behavior is not seen in the usual passage, meaning that some breast cancer cells need surrounding cells to survive. It was thought that the cause of low PE was low cell density.

We had searched for other assays that could evaluate radio sensitivity in a manner similar to the usual culture, and we found articles on HDS, reportedly effective for evaluating the radiosensitivity of cell lines with low PE [11,12].

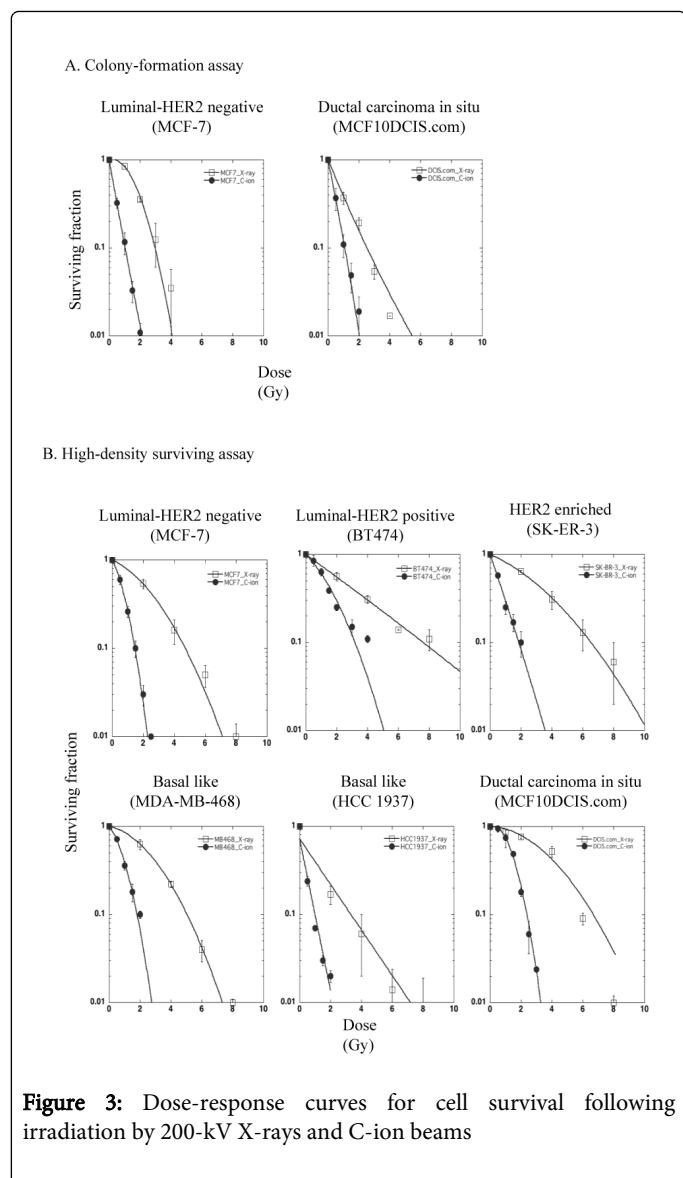
In the beginning, we tried this assay for MCF-7, for which we had already obtained proper data with CFA, and obtained data that correlated with CFA with good reproducibility. HDS uses a T25 flask and reseed in mid-flow, and this type of assay matches cells such as breast cancer, with cell density affecting cell growth. We were able to obtain stable data of radio sensitivity of BT474, SK-BR-3, HCC1937, and MDA-MB-486 by HDS, although they were not obtainable by CFA. However, the RBE values of 2.3 with MCF10DCIS.com (DCIS) by CFA and 2.6 by HDS may show some differences between CFA and HDS.

By comparing the RBE values of the respective cell lines by HDS assay, it is suggested that MCF10DCIS.com would be relatively resistant compared to the other cells. In addition, MCF10DCIS.com cells indicated a small survival curve shoulder by C-ion irradiation, suggesting the existence of some cell-specific properties affecting cell growth or death. The differences between CFA and HDS were considered to be caused by the communication of cells surrounding cancer cells, as the surrounding cells might possibly affect the survival of cells. We think that a cell-dense condition similar to real tumor by HDS was able to increase the potential for a high reliability of the results.

RBE values of 2.4 with BT474 (Luminal-HER2-positive) and 3.3 with HCC1937 (Basal-like) were only available by HDS. Although the RBE value of BT474 was smaller than that of HCC1937, however, we could not conclude that the radio sensitivity of Luminal-HER2 positive type was less than that of the other type.

The RBE values of C-ions have been extensively examined by various researchers and totally reviewed in the article by Ando et al. [16]. They reviewed 54 papers presenting 506 RBE values. They summarized that the LET of C-ions was distributed between 11 and 500 keV/μm, and RBE ranged from 0.2 to 9.6, with mean and standard deviation of 2.22 and 1.20, respectively. A peak of RBE values was found at around 80-200 keV/μm. Relatively high RBE values were reported in malignant melanoma, uterine cancer, brain tumor and fibroblast, while low values were reported in hepatoma and salivary gland tumor. Friedrich et al. systematically analyzed RBE and related quantities using a database of cell-survival experiments.

The review by Ando et al. [16] included 3 breast cancer cell lines examined by Matsuzaki et al. [10]. They irradiated YMB-1, OCUB-M and CRL-1500 at LET 80 keV/μm. YMB-1 is an ER- and PgR-positive cell line with RBE of 2.17 and D10 values of X-rays and C-ions of 2.91 and 1.34 Gy, respectively. OCUB-M is an ER- and PgR-negative cell line with RBE of 2.89 and D10 values of X-ray and C-ions of 4.74 and 1.64 Gy, respectively. CRL-1500 is an ER- and PgR-positive cell line with RBE of 2.73 and D10 values of X-ray and C-ion of 3.66 and 1.34 Gy, respectively.



**Figure 3:** Dose-response curves for cell survival following irradiation by 200-kV X-rays and C-ion beams

There was no significant difference between the RBE values of breast cancer cell types of Matsuzaki and our results. The data were not sufficient to draw a final conclusion, but it was presumed that C-ion was effective regardless of the type of breast cancer cells. Follow-up study by other researchers will be needed to confirm our results.

As the report of other adenocarcinoma cell lines, Suzuki et al. reported an RBE value of 2.37 of lung cancer cell (A-549) [15]. Other adenocarcinomas of prostate, rectum, lung, uterus and hepatocellular carcinoma were reported with similar RBE values and better clinical treatment results than with conventional photon irradiation at NIRS.

From the present study, we can at least conclude that the usefulness of C-ion RT for breast cancer was similar to that for other carcinomas that we have been treating since 1994. We believe that there was value in conducting the clinical trial of C-ion RT for localized breast cancer.

## Conclusion

Carbon-ion beams were effective against any type of breast cancer cell lines. An RBE value of around 3 with C-ion beams has been recognized in any type of breast cancer cells. The RBE values of BT474 (Luminal-HER2-positive), MDA-MB-468 (Basal like) and MCF10DCIS.com were slightly smaller than 3, and we think further study will untangle the difference of radiosensitivity in type of breast cancer.

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