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Enhancement of radiosensitivity of MCF-7 breast cancer cells subjected to X-ray or carbon-ion irradiations

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Abstract The sensitivity of cancer cells to radiation therapy varies based on cell cycle phase. Here we evaluated the differences between X-ray and carbon-ion irradiation with respect to cellular radiosensitivity and cancer cycle arrest in the breast cancer cell line, MCF-7. The cell survival rate, cell cycle distribution and the presence of apoptosis were measured by clonogenic assay and flow cytometry. BRCA1 and p21 protein levels were analyzed by Western blot, and the levels of *human telomerase reverse transcriptase* (*hTERT*) mRNA expression and telomere length were detected with real-time polymerase chain reaction. The results show a significant dose-dependent effects on survival rate, apoptosis and protein levels in the carbon-ion group of MCF-7 cells. Decreased proliferation was not observed at 2 Gy X-ray irradiation. There were significant differences in cellular cycle arrest, apoptosis percentages and BRCA1 and p21 protein expression between X-ray and heavy-ion groups. The results indicated that increasing in BRCA1 and p21 expression, and attenuation of *hTERT* gene expression induced by heavy-ion irradiation in MCF-7 cells might relate to mechanism of cellular radiosensitivity in G_2/M arrested phase.

Keywords Radiosensitivity · Cellular cycle arrest · BRCA1 · P21 · *hTERT* · Telomere length

1 Introduction

For many tumor entities, radiotherapy is a potent tool to achieve local control of tumor growth and severity. However, for patients with identical diagnoses and treatment paradigms, response to radiation is often heterogeneous [1]. Therefore, intrinsic radiosensitivity of tumor cells has been considered an important factor in determining how well a tumor responds to radiotherapy [2, 3].

Breast cancer is the leading type of cancer in women, accounting for 25 % of all cases. In 2012, it resulted in 1.68 million cases and 522,000 deaths [4]. Breast cancer is usually treated with surgery, often followed by chemotherapy or radiation therapy, or both. Radiotherapy is given after surgery to the region of the tumor bed and regional lymph nodes, to destroy microscopic tumor cells that may have escaped surgery. Radiation can reduce the risk of recurrence by 50 %–66 % when delivered in the correct dose and is considered essential when breast cancer is treated by removing only the lump (lumpectomy or wide local excision).

Ionizing radiation (IR), a potent inducer of DNA lesions, induces cell growth arrest [5]. Unrepaired or improperly repaired DNA lesions can lead to chromosomal truncations

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and translocations, ultimately leading to cell death. Cellular radiosensitivity is principally determined by three processes: (1) mitosis-linked cell death resulting from non- or mis-repaired DNA lesions, (2) radiation-induced differentiation resulting in a permanent cell cycle arrest, and (3) apoptosis [6].

Telomeres are specialized DNA–protein structures located at the ends of linear chromosomes. A critically short telomere can induce cellular senescence which plays a role in tumor suppression [7]. Telomere length stabilization is required for cellular immortalization, which is achieved in most human cancer cells through expression of human telomerase reverse transcriptase (*hTERT*), the catalytic subunit of telomerase [8, 9]. Telomerase is the key enzyme for stabilization and elongation of telomeres, a feat accomplished by adding TTAGGG repeats at the chromosome ends. Its activity is not detectable in most human somatic cells but is found in germ, immortalized and tumor cells [10].

MCF-7 breast cancer cells are hemizygous for *breast cancer 1 early onset* (*BRCA1*) mutation and p53 null. It was reported that MCF-7 cells are sensitive to low-dose irradiation [11], but are of a resistant phenotype for high-dose exposure. BRCA1 is part of a large complex of proteins involved in DNA damage recognition, repair and the maintenance of genomic stability. Lack of the BRCA1 protein in mice causes them to die early in embryogenesis as a result of a high level of genomic instability and cell proliferation arrest resulting from activation of the p53/p21 pathway [12]. p21 protein plays multiple roles not only as a cell cycle regulator in response to DNA damage, but also as a regulator of transcription, senescence, apoptosis and DNA damage repair [13].

X-rays are typical radiation for cancer treatment. Over 50 % of cancer patients benefit from a superior clinical outcome with at least one session of X-ray therapy during their treatments [14]. Heavy-ion beams are suitable for deeply seated cancer treatment, because of not only their high-dose localization around the Bragg peak, but also the high biological effect. The advantage of carbon-ion beams over X-rays is their higher linear energy transfer (LET). Compared to sparsely ionizing radiation like X-rays, high-LET particles have a higher relative biological effectiveness (RBE) because they produce an intense ionization along their track and cause severe damages to DNA which are more difficult to repair [15]. Over 20,000 cancer patients accepted heavy-ion therapy until 2014.

In this work, carbon-ion beams and X-rays were used to investigate the relationship between cycle phase and radiosensitivity in MCF-7 breast cancer cells, and the possible underlying mechanisms involved.

2 Experimental

2.1 Cell culture

The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and was maintained at 37 °C in a humidified atmosphere with 5 % CO_2 in Dulbecco's modified Eagle's medium with Ham's Nutrient F-12 mixture (DMEM-F12; Sigma-Aldrich, St. Louis, MO, USA) containing 2 mM L-glutamine, 5 % fetal calf serum (Hyclone; Logan, UT, USA), 100 U/mL streptomycin (BioReagent) and 100 μg/mL penicillin (BioReagent, Beijing, China). The experiment design is shown in Fig. 1.

2.2 Irradiation

MCF-7 cells were irradiated with 290 MeV/nucleon carbon ions at room temperature on the Heavy-Ion Medical Accelerator at the National Institute of Radiological Sciences (NIRS) in Chiba, Japan. The beam energy corresponds to an average LET of 13 keV/μm. The dose rates for delivery of 1.0 and 4.0 Gy were at 0.4 and 1.5 Gy/min, respectively.

The cells were also irradiated by X-rays from a linac (Pantak-320 S, Shimadzu, Japan) operated at 200 kVp and a dose rate of 1.8 Gy/min, using a filter of 0.5 mm aluminum + 0.5 mm copper. The dose rates were measured using an exposure rate meter (AE-1321 M, Applied Engineering Inc., Tokyo, Japan). This experiment was repeated three times.

2.3 Statistical analysis

The data were expressed in the mean ± standard deviation (*SD*). Analysis of variance (ANOVA) was used to determine statistical significance between irradiated and control groups, with $p \leq 0.05$ being selected as statistically significant.

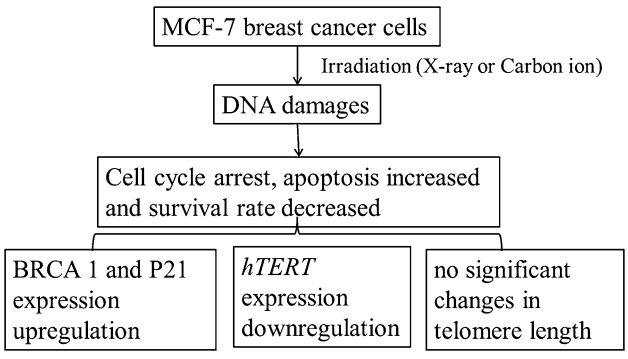

Fig. 1 Design of experiment

2.4 Clonogenic survival assay

Briefly, MCF-7 cells were trypsinized and seeded in 60-mm Petri dishes (300 cells per dish) for colony growth. After 14 days, the cells were fixed with cold methanol and stained with 1 % methylene blue (Sigma-Aldrich, St. Louis, MO, USA). Colonies with greater than 50 cells were counted as survivors [16].

2.5 Cell cycle analysis

The cells were trypsinized and washed with PBS and fixed with 75 % ethanol overnight. Then, they were treated with 20 μg/mL ribonuclease A (Sigma-Aldrich, St. Louis, MO, USA) and 50 μg/mL propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 min. DNA content was analyzed by flow cytometry with a Coulter Epics XL (Beckman Coulter, Inc., Fullerton, CA, USA) instrument using the System II program. Cell cycle distribution and apoptosis were analyzed by ModFit LT 3.0 software (Verity Software House Inc., Topsham, ME) [17].

2.6 Western blot analysis to detect BRCA1/p21 expression

After irradiation, MCF-7 cells were counted and then washed twice with ice-cold PBS before adding RIPA lysis buffer with phenylmethanesulfonyl fluoride, aprotinin and leupeptin (Sigma-Aldrich, St. Louis, MO, USA). Protein content was quantified by the bicinchoninic acid assay (BCA) method. Immunoblots were developed using the enhanced chemiluminescence detection system according to the manufacture's protocol. Integrated density value was measured using Alpha View (Nature Gene Corp. Medford, NJ, USA) software.

2.7 Monochrome multiplex quantitative PCR (MMQPCR) to measure telomere length

2.7.1 DNA, RNA and cDNA isolation and synthesis

Total RNA was extracted with the Trizol reagent (Life Technologies; Carlsbad, CA, USA) and purified with Qiagen RNeasy Mini kit (Qiagen, Düsseldorf, Deutschland). Genomic DNA isolation was performed using the total genome DNA isolation kit (Qiagen, Düsseldorf, Deutschland) according to the manufacturer's instructions. cDNA was synthesized with Qiagen cDNA synthesis kit (Qiagen, Düsseldorf, Deutschland) according to the manufacturer's instructions.

2.7.2 Monochrome multiplex quantitative PCR (MMQPCR)

Telomere length was measured by assaying for relative levels of *hTERT* using MMQPCR as described previously [18, 19]. The sequence of the four primers used is detailed in Table 1. SYBR Premix Ex Taq reagents (TaKaRa, Ishiyama, Japan) were used for RT-PCR.

3 Results and discussion

We examined the effect of radiation dose on MCF-7 cell proliferation using a clonogenic survival assay. The colony-forming units of MCF-7 cells 2 weeks after irradiation are shown in Fig. 2. The cell clonogenic survival was at 2 Gy of the X-ray, and ^{12}C groups are about the same. However, survival rate of the heavy-ion irradiation group decreased with increasing doses. The values in clonogenic survival assay of homogeneity of variances test were greater than 0.05, and two-way ANOVA test showed that they did not have interaction each other in clonogenic survival assay activity analysis ($F = 34.61$, $p < 0.001$).

Table 1 Primers used for PCR

Primer name	Primer sequence
hTERT	
S	CGGAAGAGTGTCTGGAGCAA
AS	GGATGAAGCGGAGTCTGGA
actin-S	GGGAATTCAAAACTGGAACGGTGAAGG
actin-AS	GGAAGCTTATCAAAGTCCTCGGCCACA
Telomere length	
Tel-S	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT
Tel-AS	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Albumin-Albu	CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG
Albumin-Albd	GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT

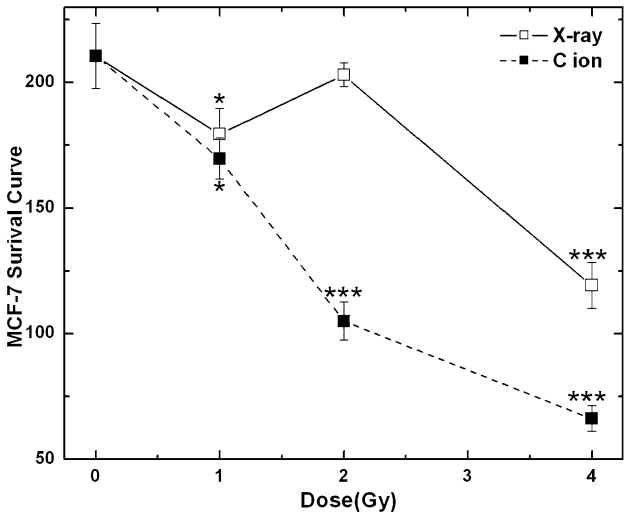

Fig. 2 MCF-7 cells survival rate by X-ray and carbon-ion irradiation (*$p < 0.001$; ***$p < 0.001$ vs. control group)

To determine whether or not the diminished clonogenic survival was due to cell cycle arrest after irradiation, we analyzed the mitotic phase profile of treated cells. The results show that the cell number in the G_0/G_1 phase increased significantly ($p < 0.05$ vs. control) with the dose in the X-ray irradiation group. The percentage was 53.1 % at 12 h after 4 Gy X-ray irradiation, whereas 61.5 % of cells arrested at G_2/M at 72 h after 4 Gy ^{12}C ion irradiation as shown in Fig. 3d–j. The results indicate that cellular cycle was arrested at G_2/M phase by ^{12}C ion irradiation ($F = 97.73$, $p < 0.001$ in G_0/G_1 phase; $F = 1.554$, $p = 0.094$ in S phase and $F = 6.31$, $p < 0.001$ in G_2/M phase).

The percentage of cells undergoing apoptosis increased with dose in the X-ray group except for 2 Gy ($p < 0.05$ vs. control). However, the apoptosis percentage increases with doses in the ^{12}C ion group (Fig. 3d–j). The homogeneity of variances test of apoptosis was greater than 0.05, and two-way ANOVA test showed that they did not have interaction each other ($F = 8.98$, $p < 0.001$).

Ionizing irradiations induce DNA lesions. Carbon-ion beams, of higher LET and RBE than low LET X-rays [20], can cause more serious DNA damage. Our results indicate that DNA lesions induced by X-ray [21] caused cell cycle arrest at the G_0/G_1 phase ($p < 0.05$ vs. control). Their repair may help to protect breast cancer cells from apoptosis, hence the radioresistance at 2 Gy. At 72 h after 2 Gy

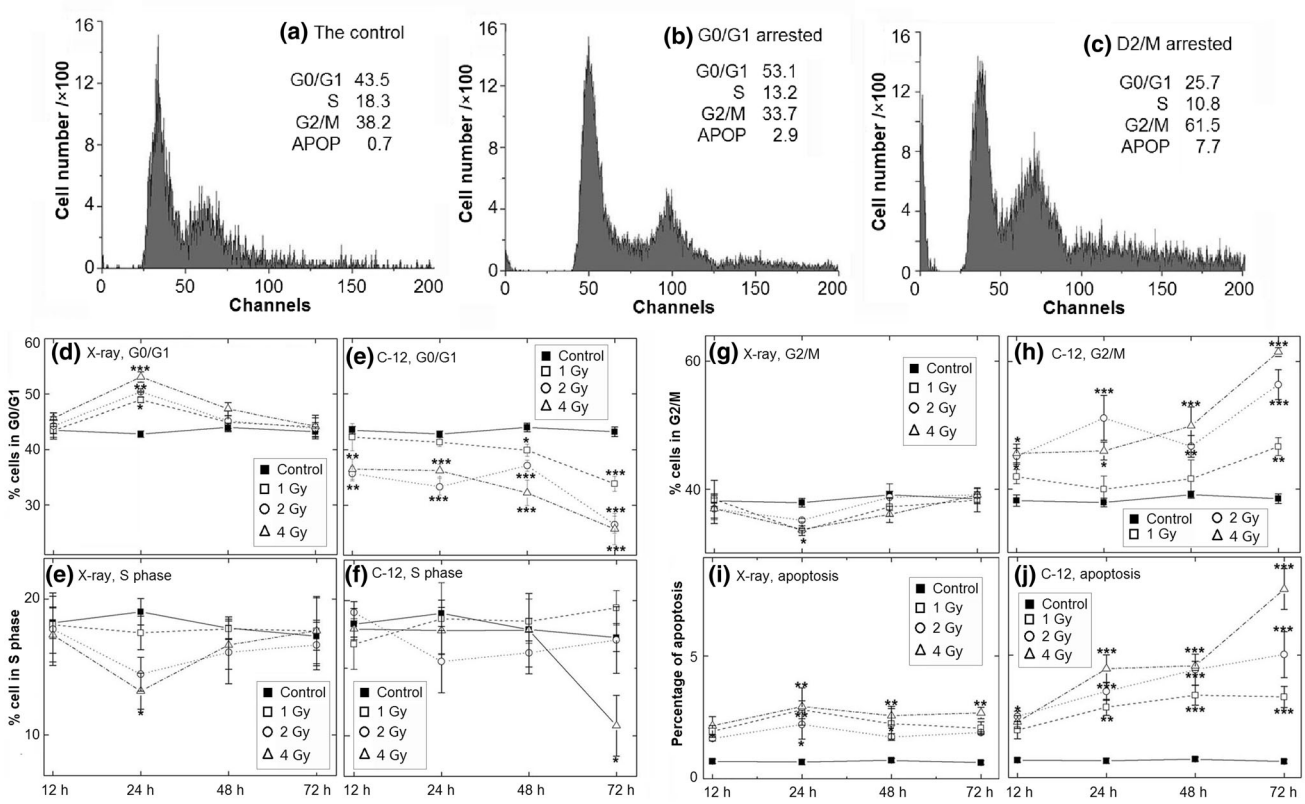

Fig. 3 Effect of ionizing irradiation on percentage of cells distributed in each phase of the cell cycle. **a–c** Representative FACS image of radiated and control MCF-7 cancer cells from three independent samples, which showed the X-rays G_0/G_1 and carbon-ion G_2/M phase cell cycle arrest. **d–j** Changes in the MCF-7 cellular cycle distribution and apoptosis induced by X-ray or carbon ions (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control group)

X-ray irradiation, cell cycle phase distribution of MCF-7 was similar to that of the control state, indicating the accomplishment of DNA damage repair-induced protection from apoptosis. On the contrary, it is hard to repair DNA damage induced by ^{12}C ion beams, hence the dose-dependent increase in apoptosis and cell cycle arrest at G_2/M phase. The results show that breast cancer cells are more susceptible to carbon-ion irradiation than X-ray irradiation and confirm that the cell cycle phase is paramount in determining radiosensitivity.

The expressions of BRCA1 and p21 proteins were similar (Fig. 4). The levels of both proteins expression increase with the ^{12}C ion irradiation dose, while they nearly reverted to those of the control at 72 h after X-ray irradiation (Fig. 4a). The values in BRCA1 and p21 protein expression of homogeneity of variances test were greater than 0.05, and two-way ANOVA test showed that they did not have interaction each other ($F = 35.18$, $p < 0.001$ of BRCA1 and $F = 18.14$, $p < 0.001$ of p21 protein).

The level of BRCA-1 and P21 was upregulated in cellular cycles G_2/M arrested phase. It is possible that they participated in cell cycle checkpoint activation in response to DNA lesions and assisted in coordinating the apoptosis and death of those treated cells. In our study, G_2/M cell

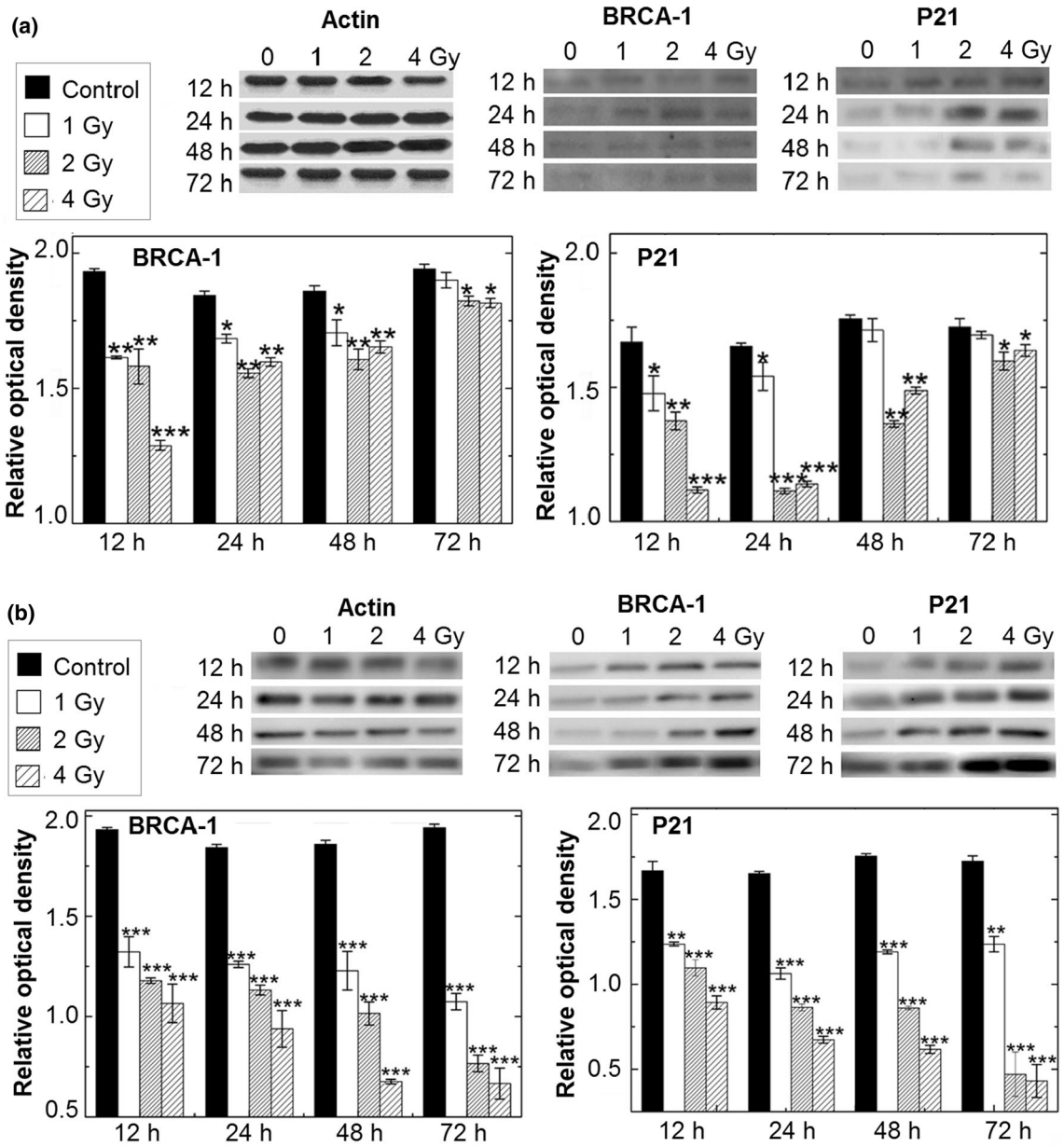

Fig. 4 BRCA1 and P21 protein expression in MCF-7 cells irradiated by (**a**) X-rays and (**b**) carbon-ion beam (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control group)

cycle arrest [22] caused by heavy-ion irradiation with the purpose of cell apoptosis and death occurred [23]. Overexpression of BRCA1 protein in G_2/M arrest phase has been shown to inhibit telomerase reverse transcriptase (*hTERT*) expression (Fig. 5) [24], which may lead to the upregulation of p21 [25], and all of them may be involved in the regulation of telomere length. Decreased telomere length could lead to cellular apoptosis and death.

The expression of *hTERT* mRNA was downregulated in the heavy-ion group (Fig. 5b), which became about 37.8 % lower than that of the X-ray-treated group at 72 h after 4 Gy irradiation, whereas no significant difference was found in *hTERT* expression levels in X-ray-treated group (Fig. 5a). The values in *hTERT* mRNA expression of homogeneity of variances test were greater than 0.05, and two-way ANOVA test showed that they did not have interaction each other ($F = 15.57$, $p < 0.001$).

In Fig. 6, the length of telomere did not change significantly in both radiation groups ($F = 0.585$, $p = 0.905$). In other words, neither type of irradiation treatment affects the telomere length.

Activated telomerase promotes tumor growth by modulating the expression of growth controlling genes and enhancing cell proliferation [26]. On the other hand, inhibition of telomerase activity in tumor cells induces telomere shortening and apoptosis. Thus, it is likely that the inhibition of proliferation and increase in apoptosis percentage inducing by irradiation may be mediated via the inhibition of telomerase. The attenuation of *hTERT* gene expression by irradiation may be part of the mechanism by which DNA lesions inhibit proliferation and induce apoptosis in cancer cells.

Surprisingly, the overall telomere length did not decrease after irradiation. This might, however, be explained by the appearance of fusion breakage fusion cycles that would lead to rearrangements, but not necessarily result in telomeric loss. It is possible that irradiation causes destabilization of the telomeric loop and uncapping of telomeres. This then leads to fusion breakage fusion cycles without net loss of telomere sequences. On the other hand, as mitotic cells divide, telomeres get shorter. After many rounds of cell division, telomeres become critically

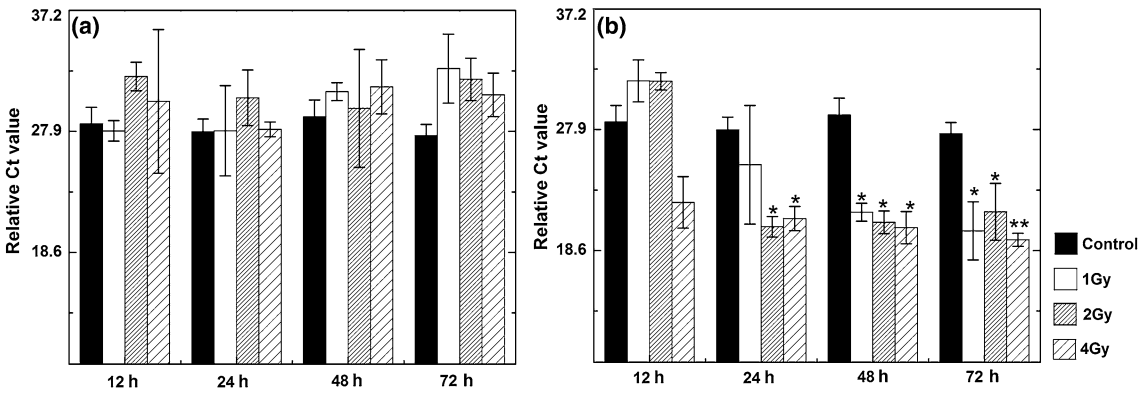

Fig. 5 Changes in expression of *hTERT* mRNA induced by (**a**) X-rays and (**b**) ^{12}C ions (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control group)

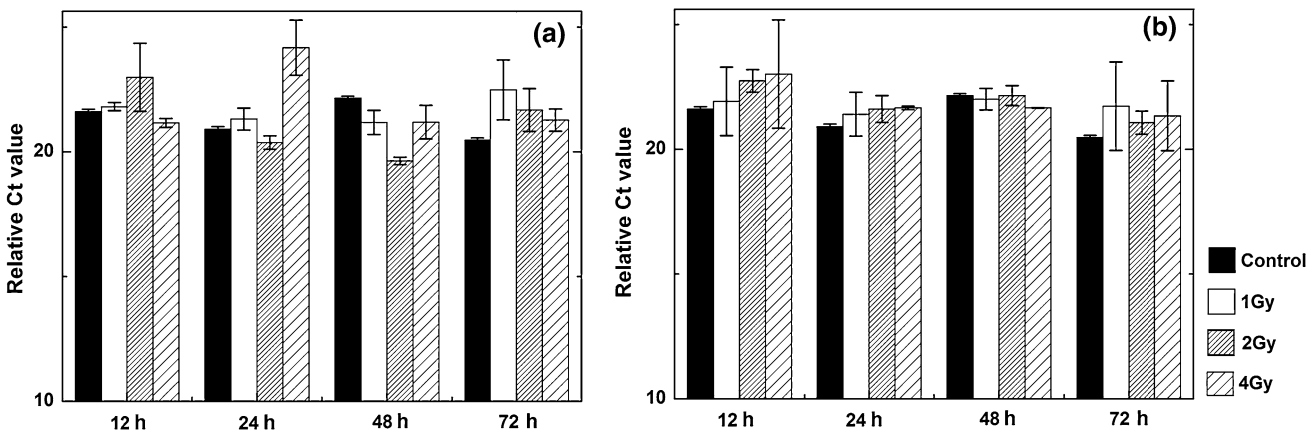

Fig. 6 Changes of levels of telomere length induced by (**a**) X-rays and (**b**) ^{12}C ions

short, which stops the cell from further division and it becomes senescent. In this case, cell cycle arrest caused by radiation, resulted in slowed cell division. Therefore, we did not detect changes in telomere length. However, more data at higher dose exposures and more time after radiation are needed to confirm or deny this model.

4 Conclusions

In general, increasing in BRCA1 and p21 expression, and the attenuation of *hTERT* gene expression by heavy-ion irradiation in MCF-7 cells might relate to cellular radiosensitivity in G_2/M arrested phase, and the changes of *hTERT* expression and telomere length are not directly related.

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