

Novel strategy for the development of radioresistance breast cancer cell line

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Abstract

Purpose: Fractionated radiation dose therapy is a routine procedure for the breast cancer treatment. However, despite of continuous improvement, tumor recurrence occurs in high proportion of the patients. There is a need of suitable radioresistance cell line system to study the properties of tumor recurrence. But, developing a radioresistant cancer cell line is a time requiring process. It requires a multiple radiation dosages for a period of three to four months. The present study aims for the new strategy to develop a radioresistant breast cancer MCF-7 cell line.

Methods: The cells were exposed to 4Gy dosage of γ rays prior to 10Gy dose and subjected for clonogenic assay. The cells survived in the clonogenic assay were pooled and named as RR-MCF-7. These cells were subjected to Bcl-2 analysis, apoptotic assay and LDH assay. **Results:** Developed cell line, RR-MCF-7 showed enhanced expression of Bcl-2 protein, which resulted in the declined apoptotic cell percentage. Though there was destabilization in the membrane of the irradiated cells, there was no significant difference observed in LDH level. Cloning efficiency of the 10 Gy exposed cells was found to be more when it was given a 4 Gy of prior dosage. The cells survived with this dosage showed the resistant character.

Conclusion: The approach followed in the current study for developing radioresistant cell line has reduced the time and dose requirement, also succeeded in obtaining resistant characters in it.

Keywords: Radioresistance, γ -radiation, Bcl-2, Breast cancer

1. Introduction

Fractionated radiation is used frequently in radiation therapy treatment to aid the recovery of normal cells or tissues, while the repair of tumor cells is generally less competent between fractions. However, the acquired radioresistance of cancer cells is thought to occur during the re-growth of the tumor during the long-term fractionated radiation.¹ Breast cancer derived tumor initiating cells (TICs) are relatively resistant to ionizing radiation and chemotherapy. This could be a major determinant of tumor recurrence following treatment.² TICs are known for decreased amount of reactive oxygen species (ROS) production in monolayer culture compare to non TICs.³ A radio-resistant cell bypasses the radiation induced DNA double strand breaks (DSBs) by ATM/DNA-PKcs dependent phosphorylation of histone

H2AX.⁴ Hyperphosphorylation of p53, RB and CHK2 are also responsible for the resistance property in cancer cells.⁵

Generation of radioresistant cells for the studies is a laborious process. It requires almost five months to develop radioresistant property in breast cancer cell MDA-MB 231.4 Gy Cobalt-60 dosage was used up to 13 times to achieve total dosage of approximately 50 Gy. These cells showed the over expressions of anti-apoptotic proteins such as Bcl-2, Bcl-XL, were considered as the sign of radioresistance.⁶ The study by Li *et al* has used total of 80 Gy X-ray radiation with 4 week interval after 10 Gy dosage on EC109 esophageal cancer cells. The study showed, there was lesser amount

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of apoptotic cells in radioresistant cells than control group when it was exposed to 12 Gy X-ray radiation.⁷

The present study focused on the new approach for the development of radio-resistant character in breast cancer cell line MCF-7.

2. Methods and Materials

2.1. Cell culture and maintenance

The MCF-7 cancer cell line were purchased from NCCS Pune, the cells were grown in MEM medium (Himedia) supplemented with 10% fetal bovine serum (FBS-Himedia) and maintained in a humidified 5% CO₂ atmosphere at 37°C.

2.2. Generation of radio-resistant character

MCF-7 cells (1×10^5) were plated in 25 cm³ culture flask and at 60% confluence it was irradiated with 4 Gy of Y-rays using blood irradiated at, CAART center, Mangalore University. Immediately following irradiation, the culture flask was renewed and cells were returned to the incubator. When the MCF-7 cells reached approximately 90% confluence, they were trypsinized, counted and passaged into new flasks. Now the cells were irradiated to 10 Gy and then it was subjected to clonogenic assay. The cells were maintained in triplicate, two for the colony counting and one for the cell recovery. After two weeks the cells were pooled from the colony and observed for radioresistant characters and it was named as radioresistant MCF-7 (RR-MCF-7).

2.3. Clonogenic assay

Post 10 Gy exposure the cells were seeded on to 25 cm³ flask for clonogenic assay. The flasks were incubated for 2 weeks at 37°C in CO₂ incubator. The colonies were fixed with pure ethanol and stained with 1% crystal violet, washed and air-dried. Colonies consisting of 35 or more cells were counted as clonogenic survivors. The surviving fraction (SF) was calculated by dividing the number of colonies by the number of cells seeded and then multiplying by the plating efficiency.

2.4. Apoptotic assay

MCF-7 and RR-MCF-7 cells were exposed to 4 Gy of radiation and 24 hours after incubation cells were trypsinized, pelleted by centrifuged at 1000 rpm for 5 min and washed with 1 ml of PBS. The cell pellets were then re-suspended in 25 µl of PBS and 2 µl of EB/AO dye mix. Stained cell suspension (10 µl) were placed on a clean glass slide and covered with a coverslip. Cells were viewed under 40x of fluorescent microscope (Olympus). Minimum 200 cells were counted and percentages of apoptotic cells were calculated.⁸

2.5. Membrane integrity

LDH is a soluble cytosolic enzyme present in most eukaryotic cells, released into culture medium upon cell death due to damage of plasma membrane. The increase of the LDH activity in culture supernatant is proportional to the number of lysed cells. LDH test was used to assess the cell membrane integrity.⁹ Hundred microlitres of supernatant was taken out from each flask for LDH assay following the instruction of the kit (Agappe). The absorbance at 340 nm was recorded on a Microplate Reader (Thermo scientific). The LDH leakage is expressed as U/L. The LDH level in the RR-MCF-7 group was compared with other experimental groups such as control, 4 Gy and 10 Gy exposed MCF-7 cells.

2.6. Total Bcl-2 Sandwich ELISA

Total Bcl-2 concentration in lysate of MCF-7 control cell and RR-MCF-7 cells were measured using PathScan® Total Bcl-2 Sandwich ELISA Kit (#12030 Cell signalling). Standard protocols were followed as given in the kit manual. The samples were read using microplate reader (Thermo scientific).

2.7. Western blotting

Western blottings using rabbit anti-human Bcl-2 antibody (#138800 Molecular Probes) was performed according to standard protocols. Total proteins were separated using SDS-PAGE (Life Technology) and it was transferred on to nitrocellulose membrane using Western blot instrument (iBolt-Life technology). HRP conjugated chromogenic detection was performed and images were captured. Beta actin (AM4302-Invitrogen) was used as a marker protein for analysis. The band intensity was measured using ImageJ software.

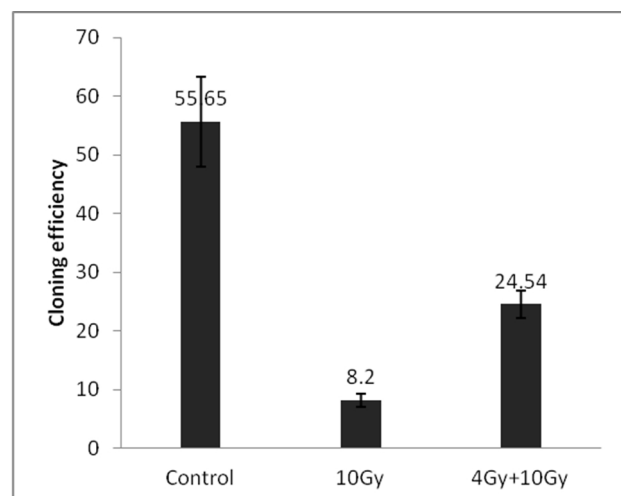


Figure 1: Comparison of clonogenic efficiency of 10 Gy and 4 Gy + 10 Gy with control.

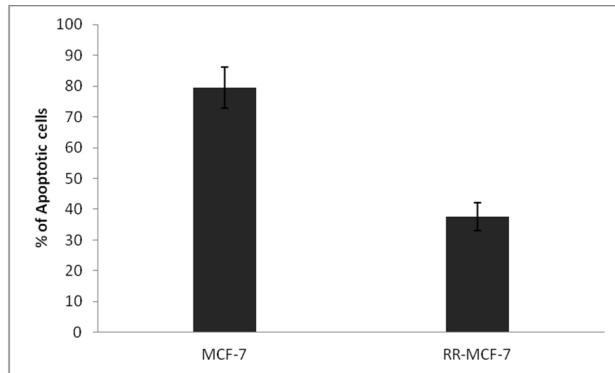


Figure 2: Percentage of apoptosis in Radioresistance cell line (RR-MCF-7).

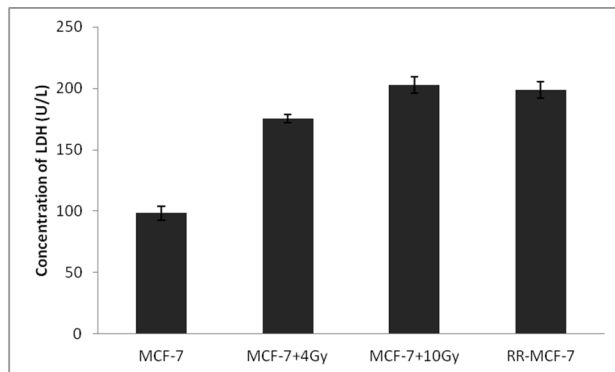


Figure 3: Membrane integrity: Concentration of leaked LDH in different irradiated samples

3. Results

Dosage of 4 Gy γ -radiation before 10 Gy exposure helped to enhance the cloning efficiency of the cells. Cloning efficiency was decreased up to 8.2 percent in 10 Gy exposed cell when compared to control (55.65%). It was comparatively increased up to 24.54% in 4 Gy+10 Gy group (Figure 1). The cells named RR-MCF-7 showed significantly ($P < 0.01$) lower apoptotic cell percentage than control group at 24 hr after 4 Gy exposure (Figure 2). The irradiated cells have lost the membrane integrity, which was measured by leaked LDH level in the cultured media. There was no significant ($P > 0.05$) in LDH level among irradiated groups but it was comparatively higher than control (98.35 ± 5.56) (Figure 3).

The reports of ELISA showed there was 2.43 folds higher Bcl-2 protein in the RR-MCF-7 group than control. The survived colonies in the clonogenic assay showed upto 4.79 ± 0.56 ng/ml of Bcl-2 protein. The cells exposed to 4 Gy and 10 Gy showed 2.99 ± 0.5 , 2.45 ± 0.9 ng/ml of Bcl-2 protein (Figure 4). It was significantly ($P < 0.05$) less when compared to the RR-MCF-7 group. Similar results observed in western blotting analysis. The obtained band intensity was significantly ($P < 0.05$) higher in RR-MCF-7 than control group (Figure 5).

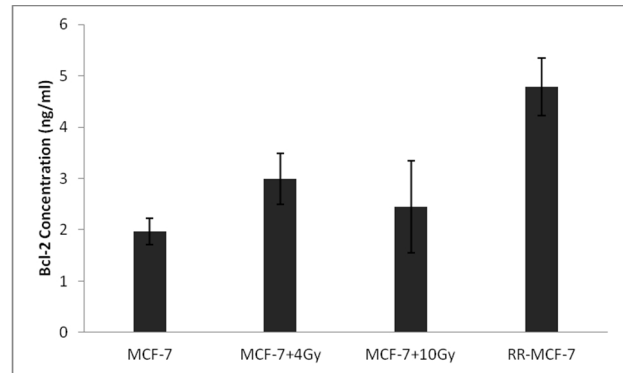


Figure 4: Comparison of Bcl-2 level in irradiated samples with RR-MCF-7.

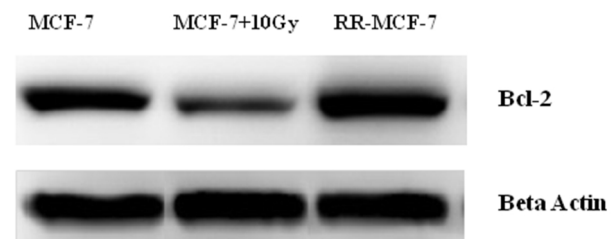


Figure 5: Comparison of Western blot band intensity.

4. Discussion

Radioresistance is a barrier in cancer treatment and affects the recovery process of patients. A long term exposure of cells to ionizing radiation induces an adaptive response that results in enhanced tolerance to the subsequent cytotoxicity of ionizing radiation.¹⁰ Cancer initiating cells and cancer stem cells are the major cause for the development of radioresistant character.¹¹ Fractionated radiation treatment is one of the well know method to develop radioresistance in cancer cell line. There were many other strategies were applied to develop radioresistant cancer cell lines^{12, 6, 7}. Fractionated radiation treatment has also reported to cause drug resistance in ovarian¹³ and ascites¹⁴ tumor cells. It can induce the expression of multi drug resistant gene and its protein.¹⁵ The present study focused on new method to develop radioresistant character in breast cancer cell line. The cells survived after the high dosage exposure may develop the characters of radioresistance and collection of clones of such cells from the clonogenic assay may give the population of uniform cells with these characters. Radioresistant cells are known for over expressing antiapoptotic protein such as Bcl-2.¹⁶

5. Conclusion

The present study showed the overexpression of Bcl-2 in RR-MCF-7 cells, which shows a sign of resistance character. However the radioresistant cells showed elevated LDH level when compared with non-irradiated

group. Further studies may be needed to check the membrane integrity in irradiated cells. Similar findings were highlighting in apoptosis process; percentages of apoptotic cells in 4 Gy exposed RR-MCF-7 cells were comparatively lesser than MCF-7 control group. Over expression of the Bcl-2 is the major reason for the inhibition of apoptosis process.

Conflict of interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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