Original Article

Evaluation of Relationship between Intrinsic Radiosensitivity (Survival Fraction at 2 Gy) and Gamma-H2AX Test and Apoptosis of Lymphocytes in Breast Cancer Patients

Abstract

Background: Radiotherapy is one of the routine treatment strategies for breast cancer (BC) patients. Different responses of the patient to radiation due to different intrinsic radiosensitivity (RS) were induced to the researcher try to introduce a standard assay for the prediction of RS. Clonogenic assay is recognized as a gold standard method in this subject but because of some of its disadvantages, it is needed for alternative assays. In this study, two assays were evaluated for this reason in ten BC patients with different RSs. **Methods:** The peripheral blood of 10 volunteers with BC was obtained, and the peripheral blood mononuclear cells were extracted. After exposed with 2 Gy, survival fraction at 2 Gy (SF2) was calculated by clonogenic assay. γ-H2AX assay was performed for all patients, and apoptosis assay was evaluated for three represented categorized patients. **Results:** RS of patients showed SF2 and categorized in three groups (high, medium, and low RS). Double-strand breaks (DSBs) were decreased in high radiosensitive patients, but the residual DSBs were clearly higher than other two groups. It is shown that the repair system in these patients is lower active than others. Apoptosis frequency in patient 4 is highly active which could induce the enhancement of her RS. **Conclusion:** γ-H2AX and apoptosis assays could predict the intrinsic RS, but evaluation of them separately is not sufficient for this aim. It is necessary to consider all the parameters together and consideration of the combination of assays could fit a better prediction of intrinsic RS.

Keywords: *Apoptosis, H2AX, radiosensitivity*

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Introduction

Important cancer in women worldwide is breast cancer (BC) that about 21% of all cancer cases.[1] Radiotherapy is one of the routine treatment strategies for these patients. Often even after similar treatment, different radiation complications are displayed in patients. It was suggested that variation in the intrinsic cellular radiosensitivity (RS) is responsible for this difference.[2-6]

It was recommended that differences in DNA damage and repair processing due to differences RS have proportion to substantial of BC patients.[5,7]

In Ataxia telangiectasia, Fanconi anemia, and Nijmegen breakage syndrome are the autosomal recessive disorders, that is, caused by genetic instability and DNA repair mechanisms in the development of tumor particularly. These syndromes are considered to increase RS tendency of different forms of malignancy by DNA repair mismatch.[5]

If the individual RS and subsequent risk of radiation side effects were known before radiotherapy, the potential injuries could be decreased by dose reduction in patients with high RS, on the other hand, the possibility of treatment could be increased in normal and resistant patients.[8,9] This objective is a controversial area in radiobiology that what assays could be adequate to determine differences in individual RS. However, no standard test for this aim has been yet introduced.[10-12]

The number of chromosomal aberrations, micronuclei, sister chromatid exchanges, and DNA fragmentation have the capacity to be analyzed in numerous population-based studies by some gene expression and comet assay, etc.[5]

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The peripheral blood mononuclear cells (PBMCs) because taken from patients easily with large numbers of cells can be used for measuring individual RS. Clonogenic assay was a gold standard test for examining cell survival. Evaluation of the ability to form colony in PBMCs could be a potential predictive assay before treatment planning.

While clonogenic assay has provided useful information, it has several important disadvantages, including requiring at least 4 weeks for colony formation, slowness, labor-intensive, and requirement of considerable technical expertise. Furthermore, the success percentage in measuring survival fraction at 2 Gy (SF2) is around 70% interpreting it as inappropriate for common clinical use. The limitations of this method caused to need for the advance of new rapid, predictive assays for radiation responses as the alternative testing of cellular endpoints.[10,13]

The double-strand break (DSB) is an apparent candidate marker that is related to clonogenic cell survival and has been correlated in some studies. The phosphorylation of histone H2AX at the sites of DSBs was an assay that showed a linear correlation with radiation. This assay could be a further beneficial marker of DNA repair and integrity.[14,15] As shown by,[16] fundamental expression of histone γ-H2AX may indicate an interruption of the genetic instability and DNA damage repair mechanism. Apoptotic cell frequency is other marker that could show RS in different patients.[13]

The relationship between DNA damage, apoptosis, and RS is still unclear. There have been no reports of an assay that can be predicted individual radiation response in BC patients routinely.[10,17]

This study recognized *in vitro* predictive assays for radiation-induced toxicity in 10 BC patients. Intrinsic RS (SF2) of peripheral blood lymphocytes was compared with the results of DNA damage and radiation-induced apoptosis *in vitro* assays in patients.

Materials and Methods

Subjects

PBMCs were obtained from ten BC patients. The study was approved by the Mashhad University of Medical Sciences Ethics Committee (IR.MUMS.MEDICAL.REC.1398.533), and the patients gave informed written consent. Any patients were not treated before sampling. The immunohistochemistry[18] of cancer cells was performed according to the American Society of Clinical Oncology/College of American Pathologists guidelines 2020.

Blood sampling and isolation of cells

PBMCs were separated from the heparinized blood samples by density-gradient centrifugation using Ficoll (inno-train, Germany) according to the manufacturer's instructions. Physiological phosphate-buffered saline (PBS, Sigma) was used for washing PBMCs twice and finally resuspended in the DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively). The samples were put in incubator at 37°C and humidified atmosphere enriched with 5% CO2 incubator.

In vitro X-ray irradiation

The isolated PBMCs with 1×10^6 cells/ml cell density were adjusted and were placed at 37°C in a 5% CO2 incubator. The X-ray irradiation (2 Gy) was performed at a dose rate of 2 Gy/min using a 6 MV Elekta Precise linear accelerator. Nonirradiated cells were preserved similarly but at a zero radiation dose.

Clonogenic assay with dilution assay

PBMCs were suspended at 10^5 cells/mL in DMEM, supplemented 10% FBS (Gibco), 1% heat-inactivated human serum (taken from a normal person), 1% phytohemagglutinin (Gibco), and 10 U/mL human recombinant interleukin-2 (Immune Teb Padideh, Iran). The lymphocytes were then plated, using 17 and 20 cell densities per dose 0 and 2 Gy into 96-well Terasaki plates (SPL). After irradiation, negative wells in each plate were scored by an inverted phase contrast microscope between days 10 and 14 of cell culture. Colony-forming efficiencies were then measured as described previously.[19] Briefly, this index was estimated as -ln(n/t)/c, n equal to the number of wells without colony, *t* is the total wells seeded, and c is the cell number of seeded in each well.

DNA double-strand breaks and repair kinetics analysis

DNA DSBs and repair kinetics analysis were measured by phosphorylation of histone H2AX (γH2AX) at three different times after irradiation of patient's sampling. H2AX phosphorylation detection kit (Millipore, USA) was performed for analysis of γH2AX in PBMCs. First, cells were washed with PBS twice and then fixed with 20 min incubation on ice in the presence of formaldehyde/methanol solution. The fixation buffer was removed with PBS washing. Then, 50 µl of the ×1 permeabilization solution and 3.5 µl of anti-phosphorylated histone H2AX (Ser139) FITC-conjugate were added to the cellular pellet. After 20 min incubation on ice, cells were suspended with 100 µl of PBS solution. Accordingly, fluorescence intensities were measured with a two-color FACSCalibur flow cytometer (Becton Dickinson, USA), and data were analyzed using FlowJO software (version V10, Flowjo, USA).

Apoptotic parameters with Annexin-v/PI assay

Apoptotic and necrotic cells were estimated by the Annexin V/PI staining kit (Mahboub Bioresearch Company, MBR, Tehran, Iran). 1×10^6 cells per well were seeded on a 6-well plate and exposed to 2 Gy irradiation. After 24 h, the cells were trypsinated and then washed with 200 µl of binding buffer and centrifuged at 1200 rpm for 5 min. Subsequently, 10 µl of Annexin V/FITC reagent was

added to the cells and incubated for 15 min in the dark at room temperature. After 5 min incubated with 3 µl PI reagent, free reagents were removed by PBS. Analysis of the flow cytometer proximately quantified the number of viable, early, late apoptotic, and necrotic cells (BD FACSCALIBUR™ Becton Dickinson, USA). FlowJo V10 software (Flowjo, USA) was used for data analyzing.

Statistics

All statistical analyses were performed using GraphPad Prism version 8 (San Diego, CA). The threshold of statistical significance was set at $P < 0.05$. The Pearson correlation coefficient was measured to evaluate grouped dependence. The graphs are rowed with Microsoft Excel (version 2019).

Results

The immunohistochemistry and pathological information of the patients

Immunohistochemistry staining was performed on frozen BC tissue removed during biopsy. HER2 receptors and hormone receptors (estrogen and progesterone receptors on cancer cells surface are shown in Table 1.

Sensitivity of patient's lymphocyte to X-rays determined by clonogenic assay

The SF2 value for each patient is given in Table 2. There was a wide variation in the obtained SF2 values with a mean of 51.09 ± 11.85. All patients were categorized into three groups, high, medium, and low RS. The mean ± standard deviation of each group is shown in Table 2.

DNA double-strand breaks and repair kinetics

The DNA damage and its repair kinetics were evaluated up to 24 h after exposure to 2 Gy of X-rays *in vitro*. The DNA damage was assessed by the geometric mean of γH2AX at 30 min after radiation. Over time to 3 and 24 h, the repair pathways were activated, and many DSBs were repaired. The geometric mean at 24 h displayed the residual DSBs and cell repair kinetics. The geometric mean of γH2AX at three times is shown in Figure 1 for three groups. The shift of the signal intensity from the change in the amount of DSBs for three represented patients is shown in Figure 2.

Two parameters of DSB and residual DSB were calculated for comparison with SF2. Table 2 shows the number of these parameters for patients.

DSBs induced by radiation were calculated using the formula:[19]

$$\%DSBs = [\text{Geometric mean of } \gamma\text{H2AX (30min } post - irradiation) - \text{Geometric mean of } \gamma\text{H2AX (control)}]*100$$

Residual DSBs is the amount of unrepaired damage at subsequent times of γH2AX assessment which is calculated as follows:

Table 1: Results of immunohistochemistry and pathological information of three breast cancer patients

	Age	Grade	ER	PR	HER2	Ki67 (%)
Patient 1	35	3	−	−	Negative	70
Patient 2	51	3	+	+	Negative	50
Patient 3	44	2	+	+	Positive	30
Patient 4	56	2	+	+	Borderline	10
Patient 5	48	2	+	−	Negative	15
Patient 6	66	2	+	+	Negative	10
Patient 7	54	2	+	−	Negative	20
Patient 8	52	3	−	−	Negative	70
Patient 9	48	2	+	+	Negative	20
Patient 10	58	2	−	−	Positive	10

ER – Estrogen receptor; PR – Progesterone receptor; HER2 – Human epidermal growth factor receptor-2

Table 2: The results of survival fraction at 2 Gy and measurement of gamma-H2AX assay parameters for all patients

	SF2%	Percentage DSBs	Percentage residual DSB
High radio sensitivity			
Patient 1	41.8	51.55249	28.10707
Patient 2	41.61	255.625	38.63081
Patient 3	38.81	142.0253	0
Patient 4	42.91	83.02304	69.59331
-	40.74±1.6	149.7342	22.2459
Medium radio sensitivity			
Patient 5	47.86	273.5051	46.18231
Patient 6	46.38	387.3418	14.37908
Patient 7	48.9	447.7528	20.95358
-	47.71±1.2	284.3141	11.7775
Low radio sensitivity			
Patient 8	74.29	237.9092	38.3538
Patient 9	64.52	69.21529	0
Patient 10	70.13	462.1622	0
-	69.646±4.9	256.4289	12.7846

DSBs – Double-strand breaks; SF2 – Survival fraction at 2 Gy

$$\%\text{Residual}\,DSBs = [\gamma H2AX\,24\,h) - (\gamma H2AX\ (\text{control})] / [\gamma H2AX\,30\ min) - (\gamma H2AX\ (control)]*100$$

Radiation-induced apoptosis

The Annexin V-FITC binding analysis and PI staining were performed for quantification of the cell death-necrosis and apoptosis-, and apoptosis parameters were estimated. As shown in Figure 3, the percentage of apoptotic cells was about 10.5%, 7.4%, and 6.4% without any radiation for patients 8, 4, and 5, respectively. After exposed 2 Gy, the number of apoptotic cells was increased to 12.6%, 11.1%, and 8.6%, respectively.

As regards, patient 4 has higher RS than patient 8 on the basis of their SF2, so enhancement of its apoptotic cells was to be expected.

The correlation of survival fraction at 2 Gy with gamma-H2AX parameters

The Pearson correlation coefficients were measured between SF2% and DSB and Residual DSB parameters. The mid-positive correlation was showed between SF2% and DSBs (r = 0.531) and high negative correlation with residual DSBs (r = −0.621).

The difference between apoptotic cells in the group with and without radiation was calculated and correlated with SF2%. The high negative correlation was shown between them (r = −0.6644).

Discussion

Despite the advances in RT effects on normal tissue, complications cannot be avoided. The occurrence of side effects during treatment is one of the problems in radiotherapy that could even lead to stopping the treatment. The difference in individual RS is the main cause of complications.[11,20] Different factors (e.g., DNA repair capacity, age, diet, and lifestyle) are reason for differences in individual RS. The toxicity risk and improvement of the treatment efficacy could be predicted with estimating the intrinsic component of RS before RT. Nowadays, clonogenic assay is the gold standard test to evaluate intrinsic RS, but this assay has different disadvantages that require alternative tests. Clonogenic assay for measuring SF2 takes about 20 days. It is very important to special treatment planning performed at low time, therefore, need to alternate and development of more fast assay of estimating cellular RS.[10,13]

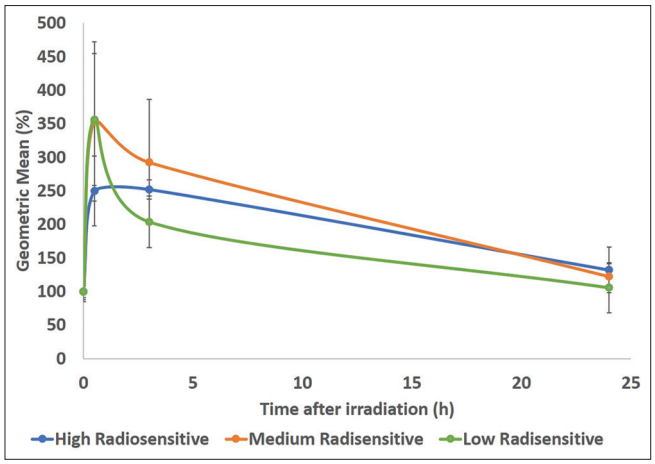

Figure 1: Geometric means of gamma-H2AX assay at three times for categorized breast cancer patients

For this purpose, ten patients with BC selected that have different RS. After the isolation of peripheral blood cells, DNA damage was measured using the histone γ-H2AX and radiation-induced apoptosis.

DNA damages in patients with low RS clearly were higher than the others, but residual DSBs were decreased after 24 h. The low radiation sensitivity of these patients could be attributed to the high ability of DNA damage repair system. Residual DSBs after 24 h were considerably decreased consequence of strong restoration. Previous studies were reported different results on this subject. Some studies were presented that γ-H2AX assay could show acceptable findings to use as a predictive biomarker before radiotherapy. Pouliliou and Koukourakis in conclusion of a strong review presented that γ-H2AX parameters correlated with early and late response of radiotherapy and these could be used as a predictor of radioresistance in radiation combinations with drugs, radiation fractionation, and biologically targeted agents.[21]

Bahreyni Toossi *et al*. in 2022 suggest that DNA DSBs and repair kinetics with γ-H2AX assay are predictive biomarkers to recognize BC patients with high RS clinically. In their study, DSBs and repair kinetics in BC patients were compared with acute normal tissue complications induced by radiotherapy. They emphasized that there is a relationship between these parameters.[19]

Djuzenova *et al*. presented that the γ-H2AX foci number was significantly increased in BC patients in comparison with healthy volunteers in both initial and residual DNA damage. They reported γ-H2AX assay could be used for screening the RS in BC patients.[22]

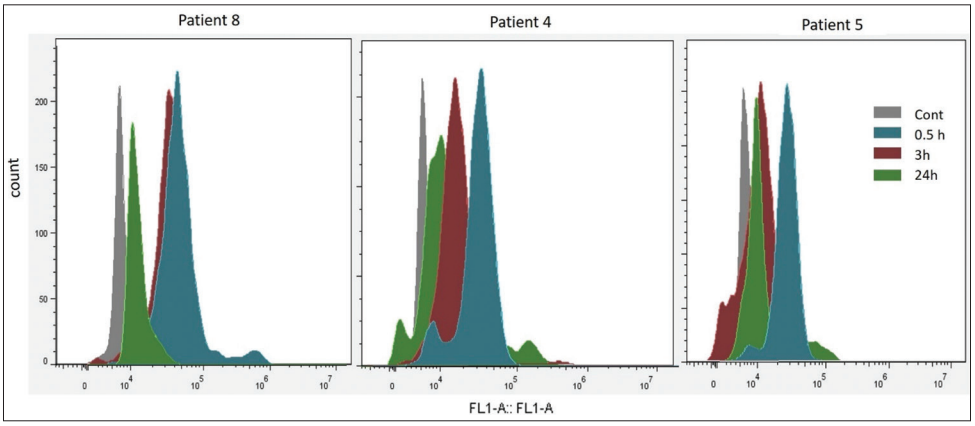

Figure 2: The shift of signal intensity of gamma-H2AX at three times after radiation for three represented breast cancer patients

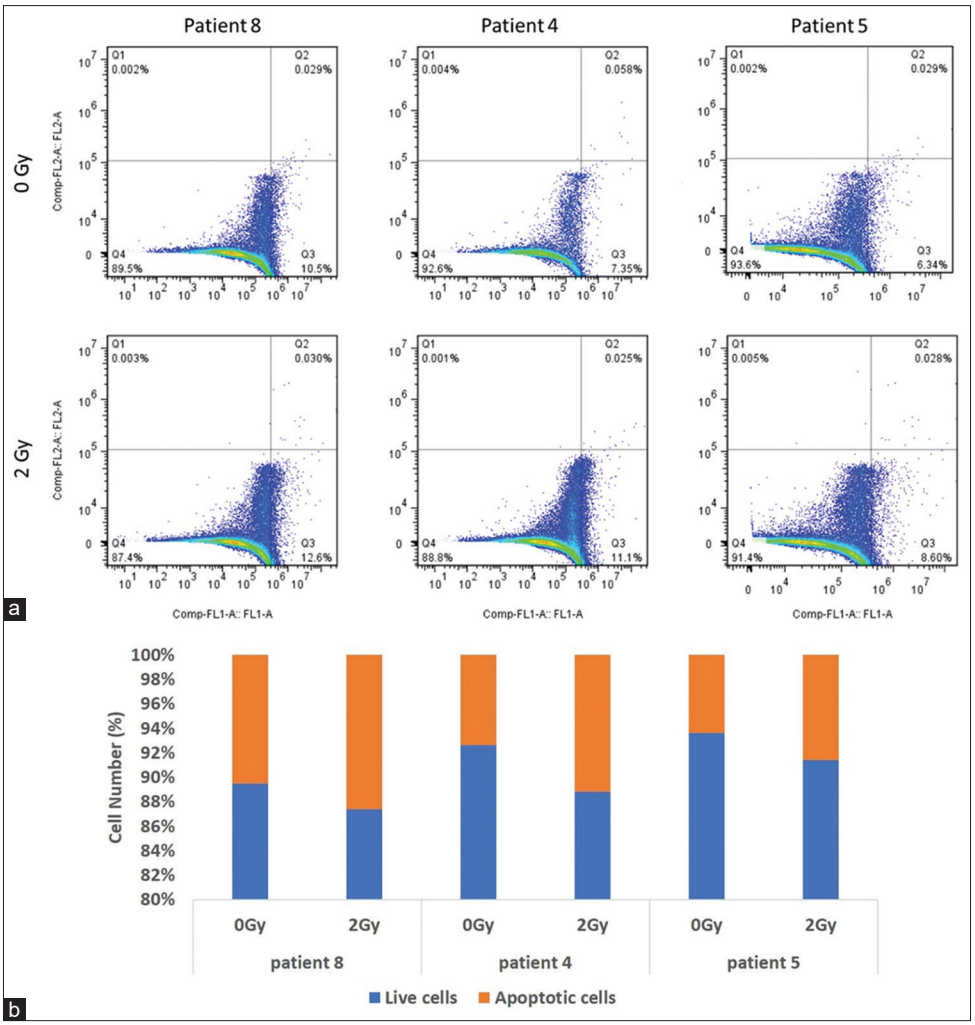

Figure 3: (a) Dot plot of Annexin V/PI assay for three breast cancer (BC) patients at 0 and 2Gy. (b) The percentage of the number of lived and apoptotic cells in BC patients at 2 radiation doses

Conversely, some researcher presented evidence for a lack of association between γ-H2AX and normal tissue RS. Finnon *et al*. in a study performed on 31 BC patients and 28 healthy volunteers represented that there is no deceptive correlation between the frequency of positive cells at 6 and 24 h in each sample. They suggested that the relationship between different indices of RS is not well known and required to legalize signatures and then move to future studies.[23]

Chua and Rothkamm presented in their review article that DNA damage-related markers such as γ-H2AX have some of the limitations and confounding effects that restricted their clinical use.[10]

This assay could predict RS and normal tissue complications successfully in other cancers, such as prostate cancer. Nuijens *et al*. in 2021 by the evaluation of 179 prostate cancer patients reported that DNA DSB repair quantified by γ-H2AX correlated with late radiation toxicity in prostate cancer patients.[24] However, in 2022, the same auteurs evaluated 198 prostate cancer and did not display a correlation between toxicity at long-term follow-up and γ-H2AX. They emphasized that it is better to γ-H2AX assay validated to use as a predictive marker within a population of prostate cancer patients.[25]

On the basis of our results, the number of DSBs alone is not enough to decide initial RS, but it is necessary to consider both parameters – DSBs and residual DSBs. In compromise of our findings, Lobachevsky *et al*. suggested an RS map algorithm that could combine the results of γ-H2AX assay and clinical RS.[26]

Other variations in DNA damage response processes may also contribute to DSB repair and prediction of individual normal tissue tolerance to RT. Dunne *et al*. presented that there was a complex relationship between the surviving fraction at 2 Gy (SF2) and the percentage of apoptotic cells, 24 h after the same dose. They demonstrated that the apoptotic frequency at this time is clearly increased with a reduction of SF2, but not fit with any simple mathematical relationship. They also revealed different background apoptosis frequency independent of cell type.[13]

In agreement, we found different background apoptosis frequencies in different BC patients. On the other hand, increasing of apoptotic cells was related to the amount of intrinsic RS (SF2).

However, in some studies, low radiation-induced apoptosis level was displayed in lymphocytes of patients with marked adverse RT effects. They attributed this result to impaired response to cellular damage.[10,27] Conversely, Finnon *et al*. investigated the apoptosis in 59 BC patients and showed no relationship between apoptosis and a number of other lymphocytes RS endpoints with the development of late effects.[23] Benlloch *et al*. in 2022 evaluated the SF2 and apoptosis in two cervical and pharyngeal cell lines. They reported that apoptotic cells even after 6 Gy radiation were not increased significantly.[28]

This study was performed at the time of endemic coronaviruses that already all health and medical services were concentrated on this virus treatment. It was difficult to find a volunteer's patient under these conditions. As sending of the test's kits were delivered late with high cost that limited this study.

We recommended evaluating these assays with a greater number of patients to use in clinical cases.

Conclusion

The γ-H2AX assay could predict the RS of BC patients while should be noted that evaluation of both primary and residual DSBs parameters is necessary for accurate assessment of individual RS. The apoptotic cells are correlated with RS but less than γ-H2AX assay parameters. Finally, although DSBs formation, repair and residual DSBs and apoptosis are important pathways after irradiation, their examination alone is not sufficient for RS prediction. It is necessary to consider a set of these parameters to best fit the results with intrinsic RS.

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Conflicts of interest

There are no conflicts of interest.

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