



DOI: 10.19187/abc.20174110-15

Inherent Radiation Sensitivity of Lymphocytes of Triple Negative (TN) and Luminal A: A Comparison Between Patients with Breast Cancer and Normal Individuals as Assayed by the Micronucleus Test

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ARTICLE INFO

Received:
23 November 2016
Revised:
02 February 2017
Accepted:
03 February 2017

Keywords:
TNBC,
Luminal A,
lymphocytes,
micronucleus,
breast cancer,
inherent Radio-sensitivity

ABSTRACT

Background: About 83% of patients with breast cancer (BC) undergo radiation therapy. These patients show various degrees of mild to acute reactions during and after the completion of treatment. The aim of this study was to compare inherent radiosensitivity of gamma-irradiated G0-lymphocytes between BC patients and normal individuals using cytokinesis blocked micronucleous assay.

Methods: Three to 4 mL blood was drawn in heparinized syringes from patients and normal individuals. A portion of the sample was irradiated with gamma rays at a dose of 300 cGy. Irradiated and non-irradiated samples were cultured in complete RPMI-1640 culture medium. A standard cytokinesis-blocked micronucleus assay protocol was followed for the preparation of binucleate lymphocytes. Slides were prepared and stained in Giemsa. Thousand binucleate cells were scored for the presence of micronucleus (MN). Data were statistically analyzed using SPSS software.

Results: The results showed that the background frequency of micronuclei in both groups of control and Luminal A (LA) patients was nearly similar and relatively low but was significantly higher in triple negative BC (TNBC) patients. Significantly different ($P < 0.01$). The irradiation of lymphocytes led to a high frequency of MN in control and LA patients, relatively higher in LA patients ($P < 0.001$); but the frequency of MN was considerably lower in TNBC patients after irradiation.

Conclusions: The results indicated radio-sensitivity of LA patients but radio-resistance in TNBC patients. This different reaction of lymphocytes of patients with BC might be due to different status of genome instability in these patients.

Introduction

Excluding skin cancers, breast cancer (BC) is the most common malignancy in women (29%). Likewise, this malignancy claims the death of 14% of all cancer-based cases. BC ranks second as a cause of cancer death in women following lung cancer.¹

Approximately, 10% to 15% of breast cancers are triple negative, in which case cancerous cells lack estrogen (ER), progesterone (PR), and

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HER-2 receptors. Generally, preoccupation with this type of cancer is weaker than the other types, also, they are resistant to treatment.² More confusingly, there is a high probability that the cancer returns and causes metastasis. The majority of patients are categorized as Luminal. In such patients, cancerous breast cells resemble Luminal cells in mammary glands. Luminal A has the best prognosis and the lowest probability of recurrence. These patients are ER+ and can be treated by hormone therapy.^{3,4}

The main treatments in breast cancer include surgery, radiotherapy, chemotherapy and hormone therapy. The radiotherapy usage rate for breast cancer is about 83%.⁵ In radiotherapy using X or gamma rays, the effort is made to damage cancer cells while sparing the normal cells. Normally, the prescribed dose for each radiotherapy session depends on the radiobiological reaction of both tumor and normal cells to radiation.⁶ It is generally believed that individual differences in the radio-sensitivity of normal tissue among patients with breast cancer stem from individual genetic differences and innate cellular-sensitivity depending on the function of various genes responsible for regulating cell cycle and DNA repair.⁷

Patients with BC show different biological reactions from mild to acute for ionizing radiation during radiotherapy.⁸ Also, upon the completion of radiotherapy, there may be side effects, such as fibrosis or secondary cancers in the radiotherapy area.⁹ In fact, the final result of radiotherapy depends on the tolerance dose of normal tissues around the tumor. The highest level of normal cells' tolerance has a one-to-one relation to the maximum dosage of tumor radiation.¹⁰ Studies have shown that 10% of the general public are sensitive to radiation and that 15% of the patients treated by radiotherapy are also sensitive to ionizing radiation.^{6,11} It was shown that approximately 40% of patients with BC are sensitive to radiation.¹¹⁻¹³

The aim of this study was to examine inherent radio-sensitivity of triple negative patients with breast cancer compared with that in Luminal A, patients with BC, and control health individuals. To this end, cytochalasin-blocked micronucleus (MN) assay was done on gamma irradiated G0-lymphocytes of patients with BC. MN, observed as discrete element with similar feature to nucleus in cytoplasm of binucleate lymphocytes, representing an acentric chromosomal fragment or lagging chromosome in anaphase, is a well approved method for the study of radio-sensitivity. Although there are other well established cytogenetic methods, such as G2 chromosomal assay for radio-sensitivity assessment, MN assay is a faster and easier method compared to other cytogenetic techniques.¹⁴⁻¹⁶

Methods

Study subjects

Fifteen triple negative BC (TNC) patients with the mean age of 53.3 ± 10.39 (age range 37-78), 15 patients with Luminal A with the mean age of 45.8 ± 10.9 (age range 32-62), and 30 normal individuals including 20 females with mean age of 39.6 ± 11.6 (aged 26-63) and 10 males with mean age of 43.8 ± 10.02 (age range 35-55) were enrolled in the present study. This study was approved by the Ethical Committee of Cancer Institute of Tehran University of Medical Sciences.

All donors completed a written questionnaire to obtain information related to their life style including their dietary habits, medical history and exposure to chemical and physical agents. Therefore, all samples were screened to exclude radiation exposure, smoker, antibiotic therapy and virus infection at least one month prior to sampling.

Patients with breast cancer were not under chemo- or radiotherapy at the time of sampling. Regarding blood sampling, written informed consent was obtained from the patients and normal individuals. Three to 5 mL blood was drawn in heparinized syringes via venipuncture from both normal and the patient individuals. Blood sample collected from each volunteer was aseptically transferred to 2 tubes; hence, one of them was used as control and the other irradiated with gamma rays.

Irradiation

Irradiation of samples was done with gamma rays generated from a cobalt-60 machine (Theratron 780C, Canada). Blood samples were irradiated with a dose of 300 cGy at ambient room temperature in an irradiation field of 10×10 cm and at a source to sample distance of 80 cm.

MN assay

Full details of this assay have been outlined elsewhere with minor modification.^{17,18} Briefly, whole-blood culture was initiated for each blood sample. To each culture vessel 0.5 ml of the blood was added to 4.5 ml complete RPMI-1640 culture medium supplemented with 15% fetal calf serum, 1% L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin (all materials from Gibco BRL). The lymphocytes were stimulated to proliferate with 1% phytohemagglutinin (PHA, Life Technologies GmbH, Frankfurter, Germany, final concentration 1 μ g/mL). Vessels were incubated at 37°C. Forty-four hours later, cytochalasin B (Sigma) was added at a final concentration of 6 μ g/mL. After further incubation, cells were harvested at 72 h post-stimulation. After centrifugation and removal of culture medium cell suspension was exposed to hypotonic shock with 0.075 M KCl, followed by fixation, three times, in methanol:acetic acid (3:1, v/v) solution. For preparation of slides, cells were



dropped on clean glass slides and stained with 5% Giemsa (in phosphate buffer) for 5 minutes. The slides were then coded and randomized for analysis. Overall 1000 binucleated cells (BNCs) per slide were scored for the presence of micronucleus (MN) based on the standard criteria described previously.¹⁶

Figure 1 shows example of binucleate cells with or without micronuclei observed in this study.

Statistical analysis

The data were analyzed using the SASS 16.0 statistical program for Windows (SASS Inc., Chicago, IL, USA). The results were statistically analyzed using Kolmogorov-Smirnov test for normal distribution, then analyzed using Student's t-test and analysis of variance (ANOVA). $P < 0.05$ was considered as statistically significant.

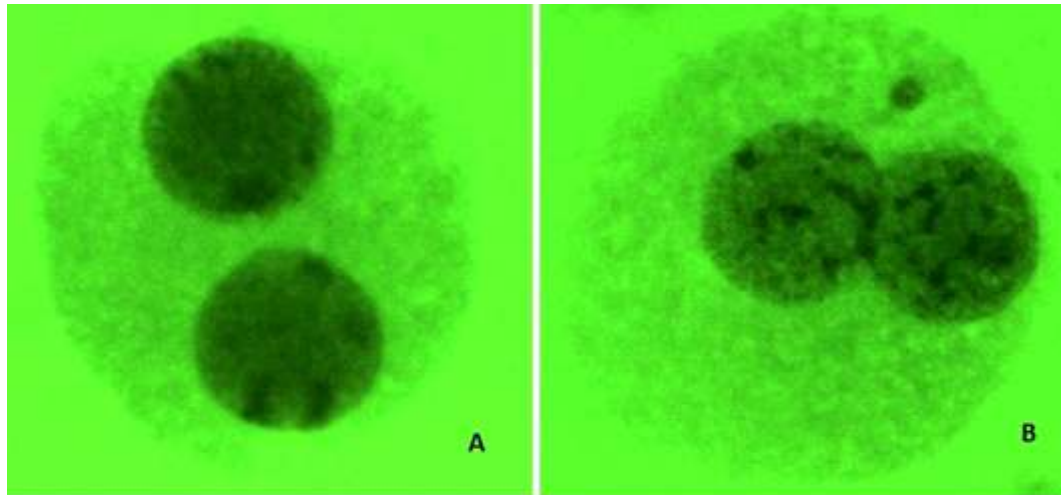


Figure 1. Photomicrograph showing a cytokinesis blocked binucleate lymphocyte without MN (A) and with one micronucleus (B) (Magnification x1000)

Results

Results of the study is summarized in Table 1 and shown in Figure 2. The total background frequency micronuclei in the control group was 19.9 per 1000 binucleate lymphocytes. The frequency of background micronuclei in lymphocytes of LA breast cancer patients was significantly higher than in control group ($P < 0.01$). However a significantly higher background level of MN was seen in lymphocytes of TNBC patients compared to both control and LA groups ($P < 0.001$) (Table 1, Figure 2-A).

Irradiation of lymphocytes with 300 cGy gamma rays led to a considerable increase in the frequency of MN in lymphocytes of control and LA groups. The mean frequency of radiation induced MN for control

group was 145.7 micronuclei per 1000 binuclei cells (87 to 280 for females and 112 to 260 micronuclei cells for males). There was no statistical difference between the frequency of MN induced in lymphocytes of females and males ($P > 0.1$). The Frequency of radiation induced MN in lymphocytes of Luminal A patients was higher than in control group which was statistically significant ($P < 0.01$).

However, in our surprise, the frequency of radiation induced MN in lymphocytes of TNBC patients was considerably lower than both control and Luminal A groups (Mean 100 MN/1000 binuclei) (Figure 2-B). Net induced MN was calculated by subtracting background frequency from radiation induced MN, as shown in Figure 2-C.

Table 1. The average frequency of MN per binucleate lymphocyte observed in 3 groups before and after irradiation with 3 Gy of gamma rays

Gender	Number of Samples	Age	Frequency of Background MN /cell	Frequency of IR induced MN/cell	P-value	Frequency of Net induced MN/cell	P-value
Control Group					>0.1		>0.1
Female	20	39.6 ± 10.8	0.02 ± 0.01	0.15 ± 0.06		0.13 ± 0.06	
Male	10	43.8 ± 7.5	0.02 ± 0.01	0.14 ± 0.04		0.13 ± 0.05	
Total	30	41.0 ± 9.77	0.02 ± 0.01	0.15 ± 0.05		0.15 ± 0.06	
TNBC					<0.001		<0.001
Female	15	53.5 ± 10.3	0.07 ± 0.014	0.10 ± 0.03		0.04 ± 0.03	
Luminal A					<0.001		<0.001
Female	15	45.8 ± 10.9	0.04 ± 0.02	0.19 ± 0.07		0.15 ± 0.05	

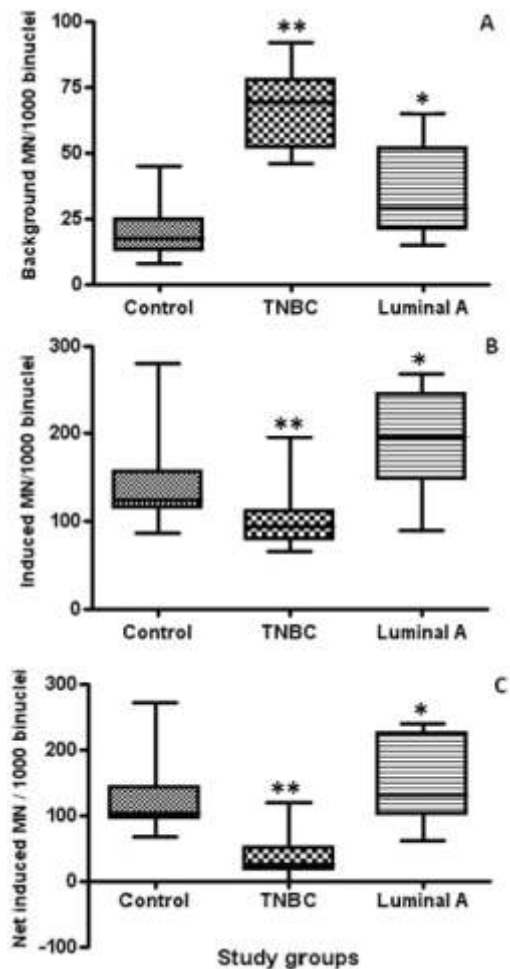


Figure 2. Box plots showing baseline micronucleus frequency (panel A), radiation induced MN (panel B) and Net induced MN frequency (panel C) for controls, TNBC, and Luminal A BC patients. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th). The whiskers present the 10th and the 90th of the data. Moreover, “*” denote $P < 0.001$ compared to control group; “**” denote $P < 0.001$ compared to control and LA group.

Discussion

The present study describes different status of inherent radio-sensitivity of 2 types of BC lymphocytes; ie. TNBC and LA compared to healthy individuals. MN is evaluated in cytochalasin-blocked binucleate lymphocytes as an indicator of cytogenetic damage induced by gamma-radiation. Radio-sensitivity is a feature of some genetic conditions like ataxia telangiectasia expressed as elevated levels of background and radiation induced chromosomal abnormalities.

Previous studies have shown that lymphocytes of about 45% of breast cancer patients show hypersensitivity to ionizing radiation when irradiated at G2 phase of the cell cycle.¹⁹⁻²¹ However, this value drops to about 25% when lymphocytes of breast cancer patients is irradiated at G0 phase of the cell cycle.^{13,19,20} As shown in Figure 2, we found that the frequency of radiation induced MN in lymphocytes of Luminal A BC patients was much

higher than normal individuals indicating the hypersensitivity of these patients to ionizing radiation.

The reason behind hypersensitivity of lymphocytes of patients with BC might be due to inefficient repair capacity of damaged DNA. Patel *et al.* have shown inefficient DNA repair capacity in patients with BC, using G2 assay and counting the number of chromatid aberrations in several time intervals.²² Similarly, other investigators have shown defective DNA repair capacity in cancer prone individuals.^{23,24} Further research also indicated that genome of individuals with cancer susceptibility and patients with BC generate more DNA damage and increase radio-sensitivity because of defective DNA repair mechanisms compared to normal individuals.^{11,25,26} The elevated radio-sensitivity may lead to complications during or after radiotherapy of these patients as well as recurrence of secondary cancers due to its damaging effect on normal surrounding tissues.

However, as shown in Figure 1-A, the background frequency of MN in TNBC patients was much higher than in other two studied groups ($P < 0.001$). Surprisingly, after irradiating the lymphocytes of TNBC patients, a dramatic decrease in the frequency of MN was observed. This observation might be indicative of resistance of lymphocytes in these patients to ionizing radiation. This result might be a plausible reason for the resistance of these patients to conventional chemo- and radiotherapy. The mechanism by which this resistance is happening is not clearly understood, but it is much similar to the observation of adaptive response induced by low doses of ionizing radiation in radiation workers.¹⁷

It was previously shown that high background frequency of MN is observed in lymphocytes of radiation workers exposed to very low doses of ionizing radiation below dose limit. Exposure of these lymphocytes to higher doses of ionizing radiation led to a considerable reduction in frequency of MN compared to non-exposed lymphocytes.¹⁷ This observation might indicate that the existence of high background chromosomal abnormalities expressed as MN in lymphocytes as well as other tissues including tumor in patients with BC might keep cellular repair machinery activated to respond further insult to DNA.

In conclusion, unlike other patients with BC, TNBC patients do not show inherent radio-sensitivity and rather they somehow show radio-resistance.

Acknowledgment

The authors express their special thanks to all patients and normal individuals for their voluntary contribution in this project.



Conflicts of Interest

The authors declare no potential conflicts of interests.

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