

Evaluation of the types and frequency of unstable chromosomal aberrations induced in lymphocytes of breast cancer patients before and after radiotherapy

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ABSTRACT

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Background: There is not yet an appropriate biomarker to predict or follow radiosensitivity of Breast cancer (BC) patients during or after radiotherapy. The aim of this study was to monitor chromosomal aberrations (CA) induced before and during radiotherapy in peripheral blood lymphocytes of BC patients. **Materials and Methods:** Age-matched twenty normal healthy individuals and 20 invasive ductal BC patients were enrolled in this study. A blood sample was obtained from normal healthy women and BC patients before and after the first, two and four weeks after radiotherapy. Lymphocyte microculture was initiated in 4.5ml complete RPMI-1640 medium. Cells were harvested 50 hours after culture initiation. Cells were harvested based on standard protocols. Hundreds of well-spread mitoses were scored under a light microscope with a magnification of x1000 for various types of CA. Data were statistically analyzed and $p<0.05$ was considered a significant difference. **Results:** Results indicated a higher frequency of CA in lymphocytes of un-irradiated BC patients compared to healthy normal individuals, although not statistically significant ($p>0.05$). High frequencies of CA were observed in lymphocytes of BC patients after radiotherapy, significantly different from the un-irradiated group ($p<0.01$). The increase in the frequency of CA was increased with increasing radiation dose. **Conclusion:** Genome instability may contribute to high background and radiation-induced CA in lymphocytes of BC patients. However, there is also the possibility of a radio-adaptation of cells during the course of radiotherapy. Results imply that dicentric chromosomes might be valuable cytogenetic bioindicators to monitor the response of BC patients to radiotherapy.

INTRODUCTION

One of the most serious life-threatening events after radiation therapy is developing a new second cancer or subsequent malignant neoplasms (SMNs), causing premature death after radiotherapy⁽¹⁾. Radiation therapy (RT) is a common and effective way of treatment for several types of malignant tumors. About 70% of patients suffering from cancer are treated with radiation therapy⁽²⁾. Breast cancer (BC) is the most common and second leading cause of death among women worldwide⁽³⁾. About 15% of breast cancer is familial and the rest (85%) is sporadic, expressed as different subtypes. Current approaches fail to provide a single molecular marker for breast cancer detection, treatment response, and prognosis prediction. RT is an effective tool in the management of BC and has been used as a routine protocol after breast-conserving surgery (BCS) for controlling local tumors and decreasing the risk of

locoregional recurrence⁽⁴⁾. About 50% of patients with malignant breast tumors receive RT and most patients seem to tolerate it, but some suffer severe adverse effects induced by the therapy. Unfortunately, early During or shortly after therapy, treatment of side effects such as mild erythema, ulceration, etc. occur in a different part of the skin, which are reversible⁽⁵⁾. Late adverse outcome happens six months to several years after treatment, including subcutaneous fibrosis, atrophy, and vascular damage that could be permanent⁽⁶⁾. RT response is not the same among different patients. A variety of factors are substantial in this phenomenon, including inflammatory interactions, oxidative stress, genetic background, variants in genes involved in response to radiation-induced DNA damage, age and environmental conditions⁽⁷⁾, or late adverse side effects of this therapy in normal tissues are undeniable⁽⁸⁾. Induction of double-strand breakage (DSB) in the genome is one of the most deleterious

effects of IR, which if not repaired accurately, leads to genomic instability, chromosome aberrations and eventually may lead to mutagenesis and carcinogenesis (9). In clinical radiotherapy, RT responses in patients may be in a broad range from latent to severe and sometimes lethal; thus, it is important to develop powerful diagnostic techniques to predict patients' responses to tumor therapy and also patients prone to radiation-related toxicity before RT (10).

This response variability may be caused by various genes involved in response to radiation-induced DNA damage (11). Therefore, it is important to develop and implement new diagnostic methods for predicting cancer treatment responses and identifying patients susceptible to radiation-related toxicity. The toxicity reactions of normal tissues to ionizing radiation brings limit the efficiency of RT. Unfortunately, an appropriate protocol to prevent or treat these side effects has not yet been developed. Therefore, the inherent radiosensitivity of normal cells is supposed to be a serious problem in the management of many cancers, including breast cancer RT (12). Currently, the terms radiation sensitivity and susceptibility are being debated (13, 14). Some authors believe that radiosensitivity should be related to tissue reactions following cell death, while radiation susceptibility is the proneness to develop radiation-induced cancer (13). According to the estimation of the International Commission on Radiological Protection, between 5% and 15% of the population may be carriers of genetic mutations conferring those more radiosensitive (15). Radiosensitivity is caused by extrinsic (radiation dose) and intrinsic factors (genetic factors) which the second account for almost 80% of normal tissue responses. At present, our knowledge of molecular pathways involved in adverse responses to cancer treatment agents is fairly poor.

Biomarkers are such potent tools but their capability for recurrences prediction after

RT for BC is limited (16). Moreover, the identification of (predictive) biomarkers of radiation sensitivity could also be relevant for cancer patients treated with radiotherapy. With the growing interest in personalized medicine, treatment plans could be better tailored to individual patients based on their personal radiation sensitivity. To date, chromosomal aberrations have been widely accepted as biomarkers of exposure to ionizing radiation. Moreover, it has previously been shown that the frequency of radiation-induced chromosomal aberrations is associated with overall cancer risk (17, 18), suggesting their potential use as indicators for individual radiosensitivity.

Moreover, there is not yet a cellular or molecular predictor of radiation toxicity during and after radiotherapy. This study aims to evaluate the radiation response of peripheral blood lymphocytes

during and after radiotherapy, where the patient receives various doses of radiation in terms of chromosomal aberration induction.

Currently, standard post-BCS fractionation is performed 5–6 weeks of daily treatments of 1.8–2 Gy/d (19). Ionizing radiation used in RT is a known carcinogen and can generate different DNA lesions such as DSBs in tumor cells and normal adjacent tissues. Breast cancer radiosensitivity refers to the inherent sensitivity of cells or tissues to IR, which is a multifactorial feature related to several factors; among them, genetic factors have a dramatic role. Studies have revealed genomic instability in hereditary BC and other hereditary cancers occur. Data suggest that some BC patients have a significantly increased chromosomal radiosensitivity (CRS) (20, 21, 22). CRS in the lymphocytes of patients could be a potential marker for low penetrance genes related to breast cancer development. It is estimated that almost 10% of normal individuals and 40% of unselected BC patients have increased radiosensitivity (22). Several parameters impact tumor response to IR, including total dose, fractionation, tumor potential doubling time, hypoxia and innate radiosensitivity.

It was clarified before that alternation in DNA repair capacity and genome instability can increase susceptibility to cancer development and enhance radiosensitivity, which means the reaction of normal tissues to IR and tumor cells. According to this information, it can be concluded which biomarkers that predict radiosensitivity, in addition to the identification of hypersensitive patients to IR before administration of RT, could be possibly used for early detection of breast cancer in the population at risk as well. Elevated inherent radiosensitivity is a major cause of adverse side effects of radiotherapy and chemotherapy for cancer patients. Although the underlying nature of radiosensitivity is not clearly known yet; insufficiency and impaired repair mechanisms of DNA damage may be the prime cause (23). The biological importance of genomic instability and DNA repair mechanisms in cancer development are well illustrated by several heritable genetic disorders known as chromosomal instability syndromes. These syndromes are characterized by various defects in DNA repair, predisposition to various forms of malignancies and increased radiosensitivity. It has been suggested that individuals who are genetically susceptible to cancer, manifest impaired DNA damage repair by exhibiting increased DNA radiosensitivity. Although possible associations between genetic markers and radiosensitivity have been found, the strong association between a specific marker and even markers has not yet been established, probably due to inadequate knowledge of the molecular pathology of adverse reactions induced by radiotherapy. In terms of carcinogenesis, radiosensitivity might

potentiate the effects of ionizing radiation and increase the frequency of radiation-induced cancer.

There are methods allowing radiosensitivity assessment of cancer patients and susceptible individuals. Cytogenetic methods have been shown to be the appropriate and suitable method for radiation-induced late toxicity assessment in cancer patients. A molecular method such as assessment of genetic or epigenetic modification via candidate's gene approaches or whole-genome methods has also been shown to be powerful approaches for radiosensitivity detection. However, these methods are too expensive for routine procedures and unaffordable for the majority of patients. Our knowledge of mechanisms leading to higher radiosensitivity of normal tissues has been relatively poor until now.

However, it has been estimated that 70% of this feature results from genome instability and defective repair of radiation-induced DSB⁽²⁴⁾. Ionizing radiation-induced foci (IRIF) are usually produced after IR at the site of produced DSBs. γ -H2AX is an important part of IRIF formation, which act as a chromatin platform generated on a 2-Mb size chromatin domain involving DSBs and gather related factors to DNA damage repair mechanisms. Recent studies revealed that some γ -H2AX foci remain at the site of DSBs even after their repair has been completed⁽²⁵⁾. The exact role of remaining IRIF even after completion of repair is currently unknown. However, it's been suggested that they could possibly have a role in remaining chromatin alternations, late repair and misrejoining of DSB, apoptosis, the activity of several kinases and phosphatases, and checkpoint signalling⁽²⁵⁾.

It is shown a significantly elevated chromosomal radiosensitivity (CRS) in some BC patients⁽²⁶⁾. CRS of lymphocytes of these patients could be a potential marker for low penetrance genes related to breast cancer development. It is estimated that almost 10% of normal individuals and over 40% of unselected BC patients' exhibit increased inherent radiosensitivity⁽²²⁾. A subgroup of these populations is AT heterozygotes which can make a correlation between high radiosensitivity and predisposition to cancer⁽²⁷⁾ and BC patients with a known mutation in BRCA1 or BRCA2 high penetrance genes or those with a positive family history have an increased CRS than a healthy population⁽²⁸⁾.

Cytogenetic assays are among the most common approaches used in radiation exposure of cells including G₂ chromosomal radiosensitivity^(20, 22, 29) and the G₀ micronucleus induction assay^(20, 21, 29). In G₂-assay, the number of chromatid aberrations is measured within peripheral blood lymphocytes or other types of cells in the G₂ phase of the cell cycle exposed to IR. It might also reveal the correlation between radiosensitivity and genetic susceptibility to cancer as this condition usually leads to a higher chromosomal aberration and a hypersensitivity to IR

as well. G₀ micronucleus assay measures small extracellular bodies called MNs which have been formed of chromosomes lagging during anaphase or partial breaks in chromosomes and the first interphase after cell division, and these structures can be identified and scored⁽³⁰⁾. However, none of those mentioned above methods are persistent and specific to radiation. For example, any chemicals or ROS-inducing agents are able to induce DSB and micronuclei. Some types of chromosomal aberrations are specific to radiation response, such as dicentric chromosomes expressed following exposure to ionizing radiation in G₀ or G₁ phases of the cell cycle. In this study, lymphocytes exposed to radiation in G₀ phase of the cell cycle were evaluated for the presence of chromosomal aberrations at different time intervals before and during radiotherapy when the patients were exposed to various doses of radiation.

MATERIALS AND METHODS

The present study was based on the analysis of chromosome aberrations observed in peripheral blood lymphocytes obtained from untreated and radiotherapy-treated breast cancer patients and healthy donors. Peripheral venous blood were obtained from 20 breast cancer affected women the aged between 28-67 years (mean age \pm SD; 43.7 \pm 9.04) before starting radiotherapy and after 1, 2 and 4 weeks of completion of radiotherapy, i.e., after receiving a radiation dose of 10, 20 and 40 Gy. All the patients were irradiated by a 6-MV photon beam from a medical linear accelerator (Elekta Synergy-Platform; Stockholm, Sweden). The prescribed radiation doses for patients with breast cancers were 5000 cGy at 200 cGy per fraction five days a week. Demographic information of patients was obtained and shown in table 1.

All patients were non-smokers and had no previous history of irradiation exposure to ionizing radiation. A similar number of healthy controls were included in the study to compare the background frequency of chromosomal aberrations in breast cancer patients and normal healthy controls. The age of the control group was between 29-65 (mean age \pm SD; 41.9 \pm 10.1). All healthy donors were non-smokers, with no previous history of radiotherapy, chemotherapy, antibiotic use, or infectious disease. The Ethics Committee of the NIMAD approved the study with registration number IR.NIMAD.REC.1398.165. All patients and healthy donors provided their informed consent before participating in the study.

Lymphocyte cultures

Peripheral blood samples were obtained by venipuncture from healthy donors and from

untreated patients before radiotherapy and from all patients during radiotherapy (after 1st week, 2nd week and 4th week; i.e., after receiving radiation doses of 10 Gy, 20 and 40 Gy). Obtained whole blood were transferred into sterile tubes containing heparin as anticoagulant, and then used for lymphocytes culture. A microculture for lymphocytes with 0.4 ml whole blood was initiated in 4.5 ml culture media containing RPMI-1640 (Gibco, BRL) supplemented with 15% foetal calf serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) (Gibco). 0.1 ml phytohaemagglutinin was added to the culture to stimulate G₀ lymphocytes for division. Culture vessels were left in a 37 °C incubator.

To take individual variability and radiation-induced mitotic delay, all cultures were harvested at 50 hours post culture initiation. Two hours before harvesting cells were treated with colcemid (20 µl/ml). Metaphase cells were prepared according to the standard method (hypotonic 0.075 M/l KCl treatment followed by fixation in methanol plus glacial acetic acid, 3:1) and stored at 4 °C. Cell suspensions were dropped onto pre-cleaned slides and air-dried. Slides were stained in 5% Giemsa for 10 minutes. The frequency of chromosomal breaks and exchanges was evaluated in 100 well spread metaphases of unirradiated or irradiated cells under a light microscope (Ziess, Germany) with a magnification of x1000. Prototype photomicrographs showing normal metaphase and metaphases with different types of chromosomal aberrations are shown in figure 1.



Figure 1. Giemsa stained metaphase with and without chromosomal abnormalities. (A), normal metaphase; (B), metaphase showing chromatid type gap and breaks; (C), metaphase showing chromosome type breaks and exchanges (dicentric and ring chromosomes). Magnification, x1000.

Statistical analysis

All data were analysed and depicted using Graphpad Prism software (version 4). The overall aberration yields scored in lymphocytes from patients and healthy donors followed Poisson distribution. Therefore, the overall aberration yields in the patient and healthy donor groups were compared as two means of Poisson distributions using the Student's t-test for infinite degrees of freedom. The groups comparing individual values were also tested by one-way non-parametric analysis of variance (ANOVA). P values of <0.05 were considered to be significant.

RESULTS

Demographic information of patients enrolled in the study is presented in table 1. As seen, the mean age of control and breast cancer patients is nearly similar with no statistically significant difference. Other molecular pathology data indicate variations in patients that were studied in this investigation except that all were common in invasive ductal carcinoma. Therefore, obtained data were analyzed for all patients irrespective of their pathological differences.

Table 1. Demographic information of normal healthy subjects and breast cancer patients.

Characteristics	Numbers	Age (mean ± SD)
Control	20	41.9 ± 10.1
Patients	20	43.7 ± 9.04
Pathological information		
<i>Tumour type</i>	Invasive ductal carcinoma	
<i>Tumour size</i>	<4 cm ≥4 cm	12 8
<i>Tumour grades</i>		
I-II	7	
III-IV	13	
<i>ER status</i>	Negative Positive	8 12
<i>PR status</i>		
Negative	9	
Positive	11	
<i>Her2 status</i>	Negative Positive	5 15
<i>Staging(clinical)</i>		
I-II	9	
III	11	
<i>Mean level of ki-67</i>	%≥ (14) <14	12 8

Cytogenetic findings

Detailed results of the study of 20 BC patients before and after radiotherapy, as well as 20 normal individuals, are shown in table 2 and depicted in figures 2 - 4. As seen in table 2 and figure 2, the

frequency of background gap was about twice more in lymphocytes of breast cancer patients compared to control. However, this increased background frequency of gaps was not statistically significant ($p>0.05$). Moreover, the frequency of induced gaps during the course of radiotherapy was not so pronounced after receiving high doses of radiation, although the frequency was significantly different from the background frequency in patients ($p<0.01$). Gaps were excluded from the total number of aberrations because some researchers consider gaps as a technical artifact⁽³¹⁾.

Table 2. Detailed data were obtained from the study. Gaps were excluded from the total number of aberrations. Values indicate mean \pm SD.

Subjects	No. of samples	Mean age \pm SD	Total no. of cells scored	gaps	Breaks	Dicentric and Ring	Total no. of aberrations
Normal	20	41.9 \pm 10.1	2000	0.4 \pm 0.49	0.4 \pm 0.58	0.05 \pm 0.21	9
BC patients	20	43.7 \pm 9.04	2000				
Before RT	20		2000	0.95 \pm 0.92	1.4 \pm 1.16	0.2 \pm 0.4	32
RT 10 Gy	20		2000	1.2 \pm 0.75	7.05 \pm 1.63	4.05 \pm 0.97	222
RT 20 Gy	20		2000	1.85 \pm 0.96	11.85 \pm 3.2	5.95 \pm 0.92	356
RT 40 Gy	20		2000	2.35 \pm 1.39	17.15 \pm 3.18	8.3 \pm 1.14	509

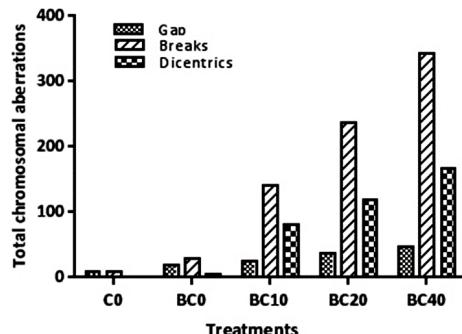


Figure 2. Frequency of total chromosomal aberrations observed for lymphocytes of healthy individuals, non-irradiated breast cancer patients and lymphocytes of breast cancer patients after receiving various doses of radiation during the course of radiotherapy. C0=Control; BC0=non-irradiated BC patients; BC10, BC20, BC40= BC patients receiving doses of radiation from 10-40 Gy.

The frequency of background simple chromosomal breaks was also higher in BC patients compared with normal controls but not statistically significant ($p>0.05$). The frequency of chromosome breaks increased with increasing radiation dose after radiotherapy, statistically significant with non-irradiated BC lymphocytes ($p<0.01$) and statistically significant between treatment groups ($p<0.05$) (figures 3 and 4). However, the increase in the frequency of breaks was not dose-dependent. The mean number of breaks after 10 Gy was 7 breaks /100 cells, whereas, after 40 Gy, the frequency of breaks was 17. In a dose-dependent manner, it was

expected to induce about 28 breaks/100 cells (figure 4).

A similar observation was made for the frequency of dicentric chromosomes before and after radiotherapy. NO statistical significance was observed for background frequency of dicentric in normal individuals and un-irradiated BC lymphocytes, although the frequency was higher in lymphocytes of BC patients. The frequency of dicentrics increased with increasing radiation dose but was not dose-dependent. The frequency of dicentric was statistically significant for each treatment time compared to non-irradiated BC patients ($p<0.01$) (figure 4).

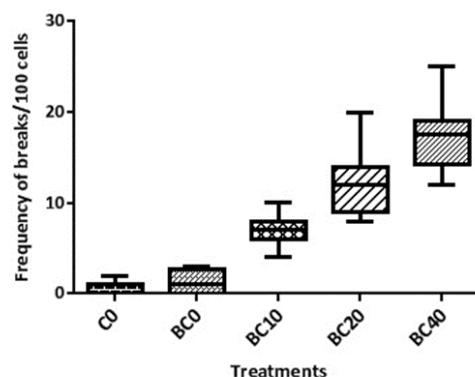


Figure 3. Frequency of chromosome breaks in lymphocytes of normal healthy individuals and breast cancer patients before and after radiotherapy. Box plots show a median number of breaks in the box as a horizontal line, 75 percentile as a bar above the box and 25 percentile below the box. C0=Control; BC0=non-irradiated BC patients; BC10, BC20, BC40= BC patients receiving doses of radiation from 10-40 Gy.

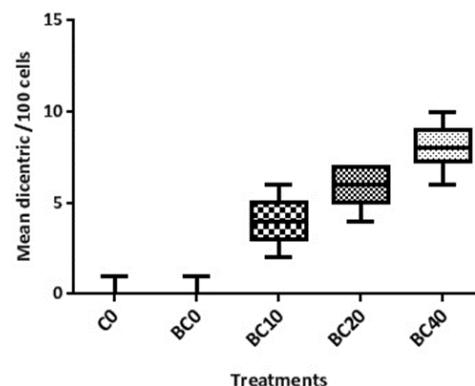


Figure 4. Frequency of dicentrics in lymphocytes of normal healthy individuals and breast cancer patients before and after radiotherapy. Box plots show a median number of dicentrics in the box as a horizontal line, 75 percentile as a bar above the box and 25 percentile below the box. C0=Control; BC0=non-irradiated BC patients; BC10, BC20, BC40= BC patients receiving doses of radiation from 10-40 Gy.

To establish a relationship between the frequency of chromosomal aberrations (either breaks or dicentric chromosomes) with molecular pathological markers such as PR, ER and Her2, each marker was statistically analyzed. There was no statistically significant ($P>0.05$ for all groups) observation for the

frequency of chromosomal aberrations with the pathological markers.

DISCUSSION

The use of cytogenetic tests to monitor the frequency of radiation-induced chromosomal aberrations dates back to the 1960s when Tough *et al.* described chromosomal aberrations in the blood of patients who had undergone radiotherapy to treat ankylosing spondylitis⁽³²⁾. To date, chromosomal aberrations not only have been widely accepted as biomarkers of exposure to ionizing radiation but is considered to be associated with overall cancer risk^(17, 18) suggesting that they may also be used as indicators for individual radiosensitivity. Mechanistic evidence supporting the role of chromosomal alterations in the development of cancer has been available for a long time, and epidemiological data showed that various markers of DNA repair⁽³³⁾ or especially the frequency of chromosomal aberrations in peripheral lymphocytes, might be an independent marker of cancer susceptibility⁽³⁴⁾.

In the present study, we analysed spontaneous and radiation-induced chromosomal aberrations in 20 patients with breast cancer during the course of radiotherapy by Giemsa-staining. In Giemsa-stained metaphases, genomic yields of dicentric chromosomes and excess acentric fragments were evaluated separately (table 1, figure 1). While the spontaneous rate of chromosome breaks and dicentric chromosomes did not vary between the studied breast cancer patients and healthy subjects, the rate of spontaneous excess acentric fragments was significantly increased in the patient group. This finding is similar to the results reported for breast, prostate, testicular and lung cancers (e.g.³⁵⁻³⁹). Overall, our healthy control group was well age-matched and, therefore, our data indicate that a subgroup of breast cancer patients with significantly increased chromosomal instability might exist.

Several studies have been performed on inducing CAs in human lymphocytes by radiation. Legal *et al.* (2002)⁽⁴⁰⁾ reported increased CAs frequency after RT and chemotherapy in lymphocytes of patients with breast carcinoma. Our results are in accordance almost with the previous studies available so far and mentioned above. In fact, in most studies radiation has been shown similar effects. However, most of the studies after radiotherapy focused on residual chromosomal aberrations after radiotherapy. Our study is more similar to the study performed by Cavusoglu *et al.* (2009)⁽³⁸⁾ with lung cancer patients. The frequencies of observed chromosomal aberration were higher in this study compared to the present report. The reason might be the larger radiation exposure field during lung radiotherapy

compared to breast cancer, especially when only one breast is under treatment. However, the frequencies of aberrations were close to the observation of Cavusoglu *et al.* it is expected that a higher frequency of chromosomal aberrations to be seen in the lymphocytes of these patients. Consequently, exposure to gamma-radiation during RT increases the frequency of CAs, and this condition is a significant risk for health. These damages may be developed secondary diseases such as leukaemia and anaemia^(41, 42). Several studies have been performed on inducing CAs in human lymphocytes by radiation. Legal *et al.* (2002)⁽⁴⁰⁾ reported increased CAs frequency after RT and chemotherapy in lymphocytes of patients with breast carcinoma. A similar study compared CAs in human sperm and lymphocytes before and after in vivo radiation treatment of 13 cancer patients. As a result, it was demonstrated that there were no abnormalities in sperm or lymphocytes before RT. However, following RT there was an increase in the frequency of numerical and structural chromosomal abnormalities in both lymphocytes and sperm⁽⁴³⁾.

The association between increased rates of radiation-induced chromosome aberrations in peripheral lymphocytes and a predisposition to cancer might be based on deficiencies in the DNA repair system maintaining the integrity of the genome. Depending on the type of the induced lesion, different repair mechanisms will be activated. DNA double-strand breaks are a hallmark of ionizing radiation effects, which will activate specific repair pathways, mainly homologous recombination and non-homologous end-joining. Misrepair of DNA DSB manifests as chromosomal aberrations (table 1, figure 2-4) or micronuclei. Increased spontaneous frequency of micronuclei in lymphocytes of untreated cancer patients has already been reported^(30, 44).

However, our main findings that is associated with impaired DNA repair is not the only reason for chromosomal instability before radiotherapy and that instability persists after radiotherapy are strongly supported by various publications^(45, 46), which provide evidence for the existence of imbalance in the oxidative stress/antioxidant status in breast cancer. At present, our knowledge of molecular pathways involved in relation to adverse responses to cancer treatment agents is fairly poor. Hence, by identification of these molecular mechanisms, it'll be possible to enhance the output of treatment technologies and then increase the survival of cancer patients.

CONCLUSION

From the results obtained, it appears that breast cancer patients show a trend to be more sensitive to radiation than the other cancer groups. Their normal

tissue hypersensitivity sensitivity might be associated with genome instability and DNA repair defects in these patients. Moreover, the trend of chromosomal aberrations was not dose-dependent as expected probably due to the radioresistance cells experience during the course of radiotherapy. These results need to be confirmed in a larger cohort of patients.

Limitation of the study

Although, the aim of this study was to assess radiation-induced chromosomal instability in the course of radiotherapy of breast cancer patients, however, if the number of studied individuals could be higher, it would have been possible to correlate the frequency of chromosomal aberrations with molecular pathology markers such as ER, PR and Her2.

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Conflicts of interest: None to declare.

Ethical considerations: The Ethics Committee of the NIMAD approved the study with registration number IR.NIMAD.REC.1398.165. All patients and healthy donors provided their informed consent before participating in the study.

Authors' contribution: HM; conceived the idea, analysed and interpreted data, drafted and finalised the manuscript. RRP and SM were involved in experimentation and data collection. ML; was involved in sampling and patient management.

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