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Comparative study of chemo-sensitivity expressed as micronuclei in lymphocytes of breast cancer patients, their unaffected first degree relatives and normal controls as a possible prognostic marker

M. Salimi*

Department of Medical Genetics, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

► ***Original article***

Corresponding authors:
Dr. Mahdieh Salimi,
Fax: *+ 98 21 44787395*
E-mail: *salimi@nigeb.ac.ir*

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ABSTRACT

Background: Genomic instability is one of primary causes for malignant cell transformation. In this study induced genomic instability expressed as micronuclei in breast cancer (BC) patients with different stages of the disease compared with their unaffected first degree relatives (FDR) and normal unrelated controls was investigated. ***Materials and Methods***: The background and net micronucleus frequency as well as other cellular damages induced after in vitro treatment with 25 µg/ml of bleomycin were evaluated using cytokinesis block micronucleus-cytome assay in peripheral blood lymphocytes of 120 Iranian individuals comprised of 40 BC patients, 40 FDRs and 40 normal control groups. Considering the protocol for each person total of 1,000 binucleated cells with well-preserved cytoplasm were blind scored on coded slides. ***Results:*** The net frequency of micronuclei was dramatically higher in breast cancer patients compared with controls. Also the net micronucleus (MN) frequency was significantly higher in FDRs compared with normal unrelated control. Considering cancer stages and clinical parameters, our results showed that the higher net frequency of MN was observed in higher stages and distant metastasis. ***Conclusion:*** This higher MN frequency both background and bleomycin induced in FDR compared with control group, clearly demonstrates that MN frequencies are determined by genetic factors to a major part and MN frequencies represent an intermediate phenotype between molecular DNA repair mechanisms and the cancer phenotype and affirms the approaches that are made to utilize them as predictors' cancer risk . Also the association between MN frequency and metastasis proposes it as a possible prognostic marker.

Keywords: *Breast cancer, DNA damage, CBMN-Cytome assay, radiomimetic agent, prognostic biomarker.*

INTRODUCTION

Breast cancer is the most common type of female's malignancies in women worldwide [1]. It was suggested mutation in genes involved in the processing of DNA damage and repair known as low penetrance genes may be involved in predisposition to breast cancer [1, 3].

DNA damage repair pathways are important in genome maintenance. Genomic instability expressed as aneuploidy and chromosomal rearrangements could be closely related to tumor development and tumor progression [4,5]. It was reported that highly aneuploid breast tumors generally progressed faster, on the average four times, and were clinically more aggressive than their counterparts without aneuploidy [5]. It was reported that chromosomal rearrangements such as deletions, duplications and amplifications were found to be much more frequent in the highly aneuploid breast tumors than in the diploid ones [6]. High-resolution

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microarray-based CGH data have verified and extended these findings by identifying chromosomal regions, novel specific patterns and degree of rearrangements related to aggressive tumor behaviors [7]. These data clearly indicate that genomic instability may considered as an important factor for tumor development and progression.

The cytokinesis-block micronucleus cytome (CBMN cyt) assay is a technique frequently used for measuring DNA damage, spindle defects, cytostasis, and cytotoxicity in variety tissue types, including lymphocytes. DNA damages expressed as micronuclei are scored specifically in once-divided binucleated cells arrested at cytokinesis in this technique. The micronucleus (MN) is an established biomarker for genomic instability indicating chromosome breakage and/or whole chromosome loss. The nuclear bud (NBUD) is considered as a biomarker of elimination of amplified DNA and/ or DNA repair complexes and the nucleoplasmic bridge (NPB) is a biomarker of DNA misrepair and/or telomere end-fusions. These three biomarkers, i.e., MN, NPB and NBUD, are the end points evaluated with this technique. Cytostatic effect is measured via nuclear division index (NDI) considering the proportion of mono-, bi-, and multinucleated cells. The cytotoxicity effect which is detectable via this technique determines apoptotic and/or necrotic cell ratios [8, 9].

It has been proposed that bleomycin sensitivity can be used as a biomarker for assessing risk of mutagen sensitivity. In exposure effect studies, lymphocytes are excellent available biological systems with a relatively long life span that accumulate DNA damages [9, 10].

Genomic instability may determine by genetic factors to a major part. In the present study the back ground and bleomycin induced MN frequency was assessed as a representor of genomic instability in breast cancer patients comprised of different stages of the disease compared with unaffected first degree relatives and unrelated normal control in order to find out if MN frequency could be a representor of an intermediate phenotype between molecular DNA repair mechanisms and the cancer phenotype.

MATERIALS AND METHODS

Study population

The study was carried out as a case control study in a group of 120 Iranian females (40 ductal carcinoma breast cancer patients, 40 unaffected first degree relatives of the studied patients and 40 unaffected matched controls).

The protocol was approved by the Ethical Committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran. Patients and controls signed a written informed consent letter before enrolment. Table 1 shows clinical and analytical data for test and control groups.

About 5 ml of blood was collected from each donor (breast cancer, unaffected first-degree relatives of patients and normal control, all were female) by venipuncture into heparinized tubes. Breast cancer patients were collected from patients referred to ‘‘Imam Khomeini hospital, Tehran, Iran’’. All donors completed a written questionnaire to obtain information related to their life style, such as dietary habits, medical history and exposure to chemical and physical agents. The inclusion criteria for our patient samples were histopathological diagnosis of ductal carcinoma and availability of immunohistochemistry (IHC) results for HER-2, ER and PR status and other pathologic diagnostic information. Receiving chemotherapy or radiotherapy a month before recruitment and any history of familial breast disease or malignancy considered as an exclusion criteria in our study. The control group individuals was matched based on sex, age, dietary, life style and smoking habit with test group and none of them has received chemo or radio agent under any circumstances.

The patients were distributed into three groups according to tumor stage (stage II to IV), which was determined by a pathologist in compliance with common standards. Details of the patient clinicopathological parameters are presented in table 1.

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Table 1. Characteristics of breast cancer patients and controls.

	Patient N (%)	Control N (%)	First degree relatives
number	40	40	40
Age (years)			
Mean	47.2±12.6	48.5±16.4	44.6±10.8
Range	27-84	25-80	20-75
Stage at diagnosis			
Stage II	21 (52.5%)		
Stage III	13(32.5%)		
Stage IV	6(15%)		
Lymph node status			
N0	17(42.5%)		
N+	23(57.5%)		
Distance metastasis			
yes	6[1bone, 5 lung] (15%)		
No	34(85%)		
Hormone receptor status (IHC)			
ER and/or PR positive	32(80%)		
ER and PR negative	8(20%)		
HER-2 status (IHC)			
+ + +	11(27.5%)		
Negative	25(62.5%)		
Triple-negative breast cancer	4(10%)		
Menopause status			
Yes	21(52.5%)	19(47.5%)	11(27.5%)
No	19(47.5%)	21(52.5%)	29(72.5%)
Smoking			
Yes	10(25%)	13(32.5%)	9(22.5%)
No	30(75%)	27(67.5%)	31(77.5%)
Pregnancy at term			
Yes	33(82.5%)	32(80%)	25(62.5%)
No	7(17.5%)	8(20%)	15(37.5%)
HRT			
Yes	9(22.5%)	8(20%)	7(17.5%)
No	31(77.5%)	32(80%)	33(82.5%)

HRT hormone replacement therapy

Cell culturing, bleomycin treatment and CBMN cyt assay

Blood samples were drawn by venipuncture into sodium-heparin vacutainers and processed within 3 hr after retrieved at the hospital. For each individual, four lymphocyte cultures were set up by adding 0.5 mL of whole blood into 4.5 mL of RPMI 1640 medium supplemented with 15% Fetal Bovine Serum (FBS), 1% antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) and 0.15 mL phytohaemagglutinin were also added to the cultures (all provided by Gibco Life Technologies, Paisley, UK). Two cultures were treated with bleomycin at a dose of 25 µg/ml final concentration, 24 hours prior to harvesting of the cells. The other two were used to assess background levels of MN. The duration for bleomycin treatment and optimal dose was

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earlier established in a set of experiments on human lymphocytes.

Lymphocytes (bleomycin treated and untreated) were cultured at 37˚C for 72 hr. After 44 h, cytochalasin B [GIBCO, 6 µg/mL] was added to the culture to arrest cells at cytokinesis. At 72 hr of incubation, cultures were harvested by centrifugation at 120g for 8 min followed a brief hypotonic treatment (2–3 min in 0.075 M KCl at 37˚C). The cells were centrifuged, then fixed and washed in methanol/ acetic acid (3:1 v/v) solution three times. The resulting cells were resuspended and dropped onto clean slides. Slides were coded and stained with 10% of Giemsa (Merck, Darmstadt, Germany) in phosphate buffer (pH 6.8) for 5 min [8].

Scoring and data evaluation

The scoring criteria established by Fenech [8] were used for CBMN Cyt assay analysis. To determine the frequency of CBMN assay endpoints (micronuclei, nucleoplasmic Bridge and nuclear buds) as well as apoptosis and necrosis a total of 1,000 binucleated cells with well-preserved cytoplasm were blind scored on coded slides. In addition, a total of 500 lymphocytes were scored to determine the percentage of cells with one, two, or more nuclei in order to calculate the nuclear division index (NDI). To better visualize the response to bleomycin treatment, a net nuclear aberrations value (micronuclei, nucleoplasmic Bridge and nuclear buds), was estimated by calculating the difference between nuclear aberrations after 25 µg/ml of bleomycin treatment and the background nuclear aberrations values.

Statistical Analyses

Statistical computations were performed using the SPSS version 16.0 (SPSS, Chicago, IL). The comparison of the data between patient and control groups was carried out using an analysis of variance (ANOVA) test. A Student's *t-test* was performed for comparisons between two groups. For all analyses, differences were accepted as statistical significant at $p<0.05$. Numerical data are presented as mean ± standard deviation (SD).

RESULTS

Characteristics of the study populations

Table 1 summarizes the demographic and clinical data for the different groups of patients and control. The data are presented as mean ± standard deviation (SD). There were no significant differences in the distribution of body mass index, age of menarche, number of children (data not shown) and smoking habits and use of hormone replacement therapy. There was a positive correlation between age and MN frequency in all groups. The breast cancer patient group was divided in subgroups based on clinical stages, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status also lymph node involvement and metastasis situation were considered.

CBMN assay in the studied populations

The background and bleomycin-induced MN frequency as well as nuclear buds and nucleoplasmic bridges in binucleated peripheral blood lymphocyte and micro nucleated cell frequency in breast cancer, unaffected first degree relatives of breast cancer patients (FDR) and control groups are summarized in table 2.

Our data showed that the background frequency of micronuclei was significantly higher in breast cancer (BC) group compared with both unaffected FDR and control groups ($p\leq0.001$). Also the mean MN frequency was higher in unaffected FDR group compared with normal control ($p\leq0.01$). Somehow similar trend of MN formation was observed when the frequency of micronuclei was analyzed per 1000 binucleated cells following bleomycin treatment. After bleomycin treatment the mean frequency of micronuclei was increased 10.9 times than background in breast cancer group compared with FDR and normal control which was 6.01 and 5.48 times, respectively. The better visualization was done by calculating the net MN frequency, 248.65±79.45, 59.5±8.1, 38.2±8.97, for breast cancer patients, FDR and control groups respectively ($p<0.001$).

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Table 2. Background and bleomycin -induced cytogenetic (**A**), cytostatic (**B**) and cytotoxicity (**C**) parameters in the breast cancer, first degree relatives and control groups.

A.	Breast cance (mean±SD)	First degree relatives(mean±SD)	Control (mean±SD)
Cytogenetic			
Background MN	25.23±9.01 *a/*c	12.08±5.03 **a	8.5 ±2.17 *b
bleomycin induced MN	276.5±81.6 *a/*c	72.69±9.06 **a	46.58±12.36 *b
Net MN	248.65±79.45 *a/*c	59.5±7.89 **a	38.2±8.97 *b
Background Micronucleated cell	21.05±6.54 *a/*c	12.02±5.01 **a	8.12±2.02 *b
bleomycin induced Micronucleated cell	246.02±86.43 *a/*c	66.23±8.83 **a	40.08±11.76 *b
Net Micronucleated cell	239.02± 79.65 *a/*c	53.86±8.12 **a	32.06±11.21 *b
Background NBUD frequency	4.36±1.85 *a/*c	1.36±1.42	1± 0.82
bleomycin induced NBUD frequency	11.63±2.17 *a/*c	2.54±1.1	2.5±1.17
Net NBUD frequency	8.45±3.61 *a/*c	1.32±1.21	1.5±0.92
Background NPB frequency	5±2.12 *a/*c	2.56±1.67	1.84±1.04
bleomycin induced NPB frequency	16.02±3.11 *a/*c	5.12±2.45	4±1.63
Net NPB frequency	9.94±2.87 *a/*c	3.76±2.87	2.28±1.57
B.	**Breast cancer (mean±SD)**	**First degree relatives (mean±SD)**	**Control (mean±SD)**
Cytostatic			
Background NDI	1.9±0.31	2.1±0.3	1.9± 0.31
bleomycin induced NDI	1.97±0.16	1.98±0.17	1.96±0.17
C.	**Breast cancer (mean±SD)**	**First degree relatives (mean±SD)**	**Control (mean±SD)**
Cytotoxicity			
Background apoptosis	8.06±2.02 **a	7.78±1.76 **a	5±1.13
bleomycin induced apoptosis frequency	12.32±1.22 **a	12±2.03 **a	7.08±1.7
Net apoptosis frequency	4.42±2.13 **a	4.06±2.61 **a	2.04±1.83
Background necrosis frequency	4.34±1.35	4.06±1.34	3±1.48
bleomycin induced necrosis frequency	7.98±2.24	8±1.87	7.2±1.6
Net necrosis frequency	3.84 ±1.83	3.94±1.47	4±2.4

MN: Micronuclei, **PBL**: peripheral blood lymphocyte, **SD**: standard deviation, **NBUD**: nucleoplasmic bud, **NPD**: nucleoplasmic bridge, **NDI**: nuclear dividing index: (mononucleated cells + 2X binucleated cells + 3X trinucleated cells + 4 X tetranucleated cells)/total number of cells

[a] *t*-test with control group as reference

[b] ANOVA test : C versus first degree relatives and breast cancer groups.

[c] ANOVA test : BC versus first degree relatives and C groups.

*P≤ 0.001 ; **P≤0.01

As shown in figure 1(A), when the breast cancer patients stratified according to the metastasis situation (metastasis to lymph node (LN+/LN-) and distant metastasis) the frequency of micronuclei was significantly higher in breast cancer patients with distance metastasis (p≤0.001) whereas no significant differences were observed between LN+ and LN- (p>0.05). As shown in figure 1-B, when our data in breast cancer patients stratified based on TNM staging, the higher net MN frequency was observed in stage IV compared with other clinical stages (p≤0.01). There was no significant difference between other stages (stages II and III).

Mean of net micronucleus frequency in breast cancer patients categorized according to immuno histochemistry (IHC) results for hormone receptors which commonly used in clinical practice were shown in table 3. Our data showed that, the frequency of net micronuclei was not significantly different between ER +/ER - or PR+/PR- or HER2+/ HER2- breast cancer patients (p>0.05) whereas the mean of net MN

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frequency was significantly lower in triple negative (ER-, PR-, HER2-) breast cancer patients compared with non- triple negative ones (p≤0.001).

Our data showed that the background, bleomycin-induced and net frequency of both nucleuplasmic buds and nucleoplasmic bridges were significantly higher in breast cancer patients group compared with FDR and normal control groups (p<0.001) (table 2).

The rate of apoptosis in breast cancer group was significantly higher than other groups, when the FDR group was considered, our data showed that the frequency of apoptosis was higher in FDR group compared with normal control ones (p<0.001) whereas no statistically significant difference was shown between net necrosis rate among all three test and control groups (p>0.05) (table 2).

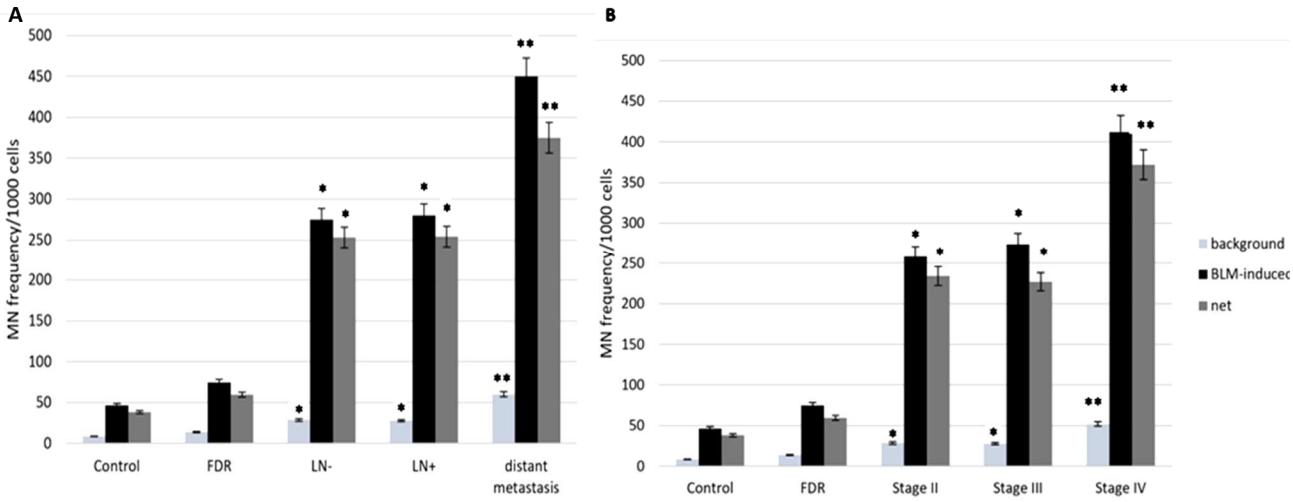

Figure 1. Micronucleus frequency in different breast cancer groups based on metastasis situation (**A**) and TNM staging (**B**) compared with unaffected first degree relatives and normal control groups. C: control, FDR: unaffected first degree relatives, LN -/+: lymph node negative/positive, MN: micronucleus, * Statically significant compared with C and FDR groups (P≤ 0.001) **Statically significant compared with C, FDR and other groups (P≤ 0.001).

Table 3. Mean of background, bleomycin - induced and net micronucleus frequency in breast cancer group compared with first degree relatives and control groups. Patients were grouped considering IHC studies commonly used in clinical practice.

	Background MN frequency (mean ± SD)	bleomycin-induced MN frequency (mean ± SD)	Net MN frequency (mean ± SD)	P value
Control	8.5 ±2.17	46.58±12.36	38.2	
FDR	12.08±5.03	72.69±9.06	59.5±7.89	
ER +	25.4 ± 9.8	259.6 ± 100.1	238.5 ± 98.6	*
ER -	26.8 ± 11.43	243.4 ± 98.73	224.8 ± 96.5	*
PR +	23.8 ± 9.21	285.3 ± 84.83	262.8 ± 104.5	*
PR -	27.4 ± 11.09	252.85 ± 101.65	238.42 ± 106.3	*
HER2 +	26.3 ± 7.89	272.4 ± 96.65	257.4 ± 60.27	*
HER2 -	25.7 ± 9.38	276.82 ± 78.53	252.3 ± 90.6	*
TN	25.8 ± 7.84	192.3 ± 41.23	142.6 ± 73.6	*/ **
NTN	27.9 ± 9.65	304 ± 68.96	272.64 ± 38.55	*/ **

IHC: immunohistochemistry, ER: estrogen receptors, PR: progesterone receptor, HER-2: human epidermal growth factor 2, TN: triple negative, NTN: none triple negative, FDR: First degree relatives

* Statically significant compared with C and FDR groups (P≤ 0.001).

**Statically significant compared with C, FDR and TN / NTN groups (P≤ 0.001).

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DISCUSSION

CBMN assay has been applied to examine the effect of variety of factors such as genetics, lifestyle, dietary and environmental on chromosomal stability and mitotic function [11-13]. The frequency of baseline or background micronuclei has been considered as a good indicator for genetic instability but an individual's instability is better determined when cells are challenged with the defined dose of a standard genotoxic agent, such as a radio mimetic agent like bleomycin. Chromosome sensitivity to bleomycin measured by the MN assay, may considered as an important biomarker of genomic instability and cancer predisposition [9, 11-15]. Each individual responses to external stimulation may be different via activating a series of biological responses therefore sensitivity to bleomycin can be considered as an individual susceptibility measurement to chemical and physical insults [9]. Our data demonstrated that the frequency of DNA damages expressed as nuclear aberrations was significantly higher in the breast cancer patients group compared to FDR and normal controls. We considered different CBMN assay endpoints in our study (table 2) and the frequency of micronuclei was chosen as a biomarker of effects. This biomarker, MN, may be considered as a well surrogate marker of cancer risk [16].

Our results showed that the frequency of both baseline and bleomycin -induced micronuclei was significantly higher in BC patients and FDR groups compared with normal control group (table 2). This result was somehow in line with other studies have reported the higher frequency of micronuclei in cancer patients compared with normal unaffected individuals [17-22]. The micronuclei scoring as a biomarker on fine needle aspiration cytology smears of breast carcinoma was done and confirmed the association of high MN frequency and breast cancer [2, 23]. Micronucleus assay in buccal smears of breast carcinoma patients showed that micro nucleated cells are significantly increased in buccal cells of the breast carcinoma cases [24, 25]. From our data and literature review, we may conclude that the increased number of MN in different sample types of BC raises the possibility that the genetic damage in breast cancer patients is generalized and predicted MN scoring could be used in biomonitoring of DNA damage and early detection of high risk cases of carcinoma of breast in future. In contrast Bolognesi *et al*. reported no significant role of micronucleus frequency as a biomarker of breast cancer risk/ susceptibility [26].

The higher net frequency of micronuclei in FDR group compared with control was somehow in line with a study reported that the FDRs of patients having head and neck cancer showed significantly higher chromosomal damage in terms of MN frequencies in lymphocytes when compared with those of controls. They concluded that this phenomena was reflecting an increased susceptibility to head and neck cancer in FDRs [27]. In another study higher frequency of sister chromatid exchange and micronuclei were reported in breast cancer patients compared with normal control group [28].

This higher MN frequency both background and bleomycin induced in FDR compared with normal control group, clearly demonstrates that MN frequencies are determined by genetic factors to a major part. The strong reflection of the genetic background supports the idea that MN frequencies represent an intermediate phenotype between molecular DNA repair mechanisms and the cancer phenotype and affirms the approaches that are made to utilize them as predictors' cancer risk [29]. Since Candidate genes for increased breast cancer risk are those involved in DNA damage repair pathways, and mutations in these genes are characterized by increased chromosomal radio sensitivity, bleomycin treatment as a radiomimetic agent was done in order to find the induced genomic instability in test and control groups. Bleomycin treatment to induce DNA damages has some advantages compared with other methods such as irradiation. The most important one is its systemic effect that affects all the cells equally. The other advantage is being more accessible in every laboratory

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than irradiation tools. Our results showed that the variability observed before the bleomycin treatment (background damage) was correlated with the level of damage observed after treatment in all the study groups. But the portion of MN frequency after bleomycin treatment in breast cancer showed higher increase compared with other studied groups. This phenomenon may be due to higher genomic instability and lower DNA damage repair capacity in breast cancer patients. When the breast cancer patients categorized according to the tumor hormone receptor situation the background, bleomycin -induced and net MN frequency was not significantly different in all the breast cancer groups with ER/PR/HER2 positive or negatives, except the triple negative (ER -, PR - and HER2-) ones which showed the lower frequency of net micronuclei compared with non-triple negative ones (table 2). The lower MN frequency after in vitro bleomycin treatment could be due to bleomycin resistance of triple negative breast cancer group. We may conclude that this bleomycin resistance may be due to capability of triple negative tumors to repair double strand breaks (DSBs) induced by radiomimetic agents such as bleomycin. This repair process may be due to function of the long noncoding RNA LINP1, that was recently reported to regulate repair of DNA double-strand breaks in triple-negative breast cancer and over expressed in triple negative breast cell line [30].

In conclusive, our results provided the evidences that high micronucleus frequency measured in lymphocytes challenged with *in vitro* bleomycin treatment could be considered as genomic instability indicator that may have variety of applications. For instance our study showed for the first time that metastatic breast cancers were introduced with higher bleomycin -induced MN frequency and triple negative ones with lower induced MN frequency. It may represent MN frequency as a cytogenetic prognosis biomarker detectable in blood. Also more genomic instability expressed as MN frequency in unaffected first degree relatives of breast cancer patients that indicate genetic factors importance, was reported for the first time. Implementation of micronucleus assay in blood as possible cytogenetic biomarker in clinical level may potentially enhance the quality of breast cancer management.

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