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ORIGINAL ARTICLE

Baseline and therapy-induced chromosome damages in peripheral blood lymphocytes of breast cancer patients assessed by the micronucleus assay

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Summary

Purpose: *Radiotherapy (RT) alone or in combination with chemotherapy (CT) leads nearly always to increase of DNA damage in cancer patients. The purpose of this study was to determine the variability rate and individual sensitivity of breast cancer (BC) patients to the applied RT and RT in combination with CT.*

Methods: *The analysed sample included 30 women with histologically confirmed BC. The frequency of micronuclei (MN) was estimated in peripheral blood lymphocytes (PBL) by using the cytokinesis-block micronucleus (CBMN) assay before the administered therapy and one month later.*

Results: *The mean therapy-induced MN value was significantly higher ($p < 0.001$) compared with mean baseline MN. Both therapies (RT and combined RT+CT) significantly increased the MN frequency in patients' lymphocytes ($p<0.001$), but without significant differences in the therapy-induced MN frequency between these two groups ($p > 0.05$). The administered therapy induced significant difference in cell kinetics ($p < 0.05$). The results showed a wide range of inter-individual variability in both baseline and the therapy-induced MN frequency.*

Conclusion: *The applied therapies increased the MN frequency in PBL in BC patients, and the presented data indicate absence of synergistic effect of these two therapies. None of the variation factors (age, smoking and therapy type) had influence on the noticed variability.*

Key words: breast cancer, chemotherapy, micronuclei, peripheral blood lymphocytes, radiotherapy

Introduction

BC remains the most common malignancy in females worldwide and, with over 1500000 new cases and over 400000 deaths annually, it represents an important global health problem [1]. In Serbia, BC ranks first in cancer incidence in women, with almost 4000 new cases and 1500 deaths per year [2]. In contrast with Western Europe, the mortality of BC patients in Serbia has increased in the last 30 years.

Molecular profiling has shown that BC is not a simple disease with a single tumorigenic pathway, but a rather heterogeneous one [3]. Out of all BC patients, 5-10% show inherited gene mutations [4] and 2% have a strong predisposition caused by the highly penetrable BRCA1 and BRCA2 genes [5].

In addition to endogenous factors in the etiology of BC, exogenous factors such as drinking habits [6], reproductive factors [7], age [8] etc play a significant role.

Tumor size, lymph node status, endocrine receptor status and human epidermal growth receptor 2 status (HER2) are standard parameters for therapeutic recommendations and outcome predictors for BC patients [9]. Surgery as a treatment of early-stage BC is usually followed by adjuvant RT and adjuvant CT. The aim of these two therapies is to prevent locoregional and/or distant disease relapse after surgery. Omission of RT is related to increased ipsilateral BC recurrence and with a small increase in mortality [10]. RT can also provide palliation in some metastatic localisations [11]. BC patients also receive adjuvant systemic therapies to achieve early eradication of possible micrometastases. Significant improvement in both disease-free survival (DFS) and overall survival (OS) has been confirmed in

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women who receive adjuvant chemotherapy [12-14].

Numerous population studies described genomic instability in various kinds of cancer including BC, before and after therapy, using a variety of assays (chromosome aberrations, MN, sister chromatid exchanges and Comet assay) [15-19].

MN in PBL are the cytogenetic biomarker which indirectly reflects chromosome damages. These are small nuclei originating from chromosome fragments or whole chromosomes, which may be induced by exposure to clastogenic and aneugenic agents, oxidative stress and genetic defects in the cell cycle [20]. Increased MN frequency has been observed in patients with different tumors [20-24] and in BC patients [19,25,26].

According to literature data that the MN assay could be used to assess the chromosome damage, the objective of the present study was to determine the variability rate and the individual sensitivity of BC patients to the administered RT and combined RT and CT, establishing their cytogenetic damages in PBL by using the CBMN test.

Methods

Patients

The analysed sample included 30 women with histologically confirmed BC, treated and followed at the Clinical Center in Kragujevac, Serbia, Department of Radiotherapy, during 2007-2008. The patient age ranged from 41 to 75 years (mean 55.10 ± 9.07) and they were divided in 2 age groups: 41-55 and 56-76 years. The study was approved by the Ethics Committee of Clinic of Kragujevac.

All patients received RT (daily dose 2 Gy, total dose 50 Gy over 5 weeks). Among the analysed patients, 17 (56.7%) received both RT and CT (cyclophosphamide 600 mg/m^2 methotrexate 40 mg/m^2, and 5-fluorouracil (600 mg/m^2 every 28 days).

Out of 30 analysed patients only 9 were smokers.

Blood samples were taken to determine MN frequencies before the beginning of RT and one month after the end of RT. In women who received CT, MN frequencies were determined before the beginning of RT.

Questionnaire

Before a blood sample was taken, each patient completed a detailed questionnaire containing diagnosis, age, education, employment, environmental exposures, diet, lifestyle characteristics, medical history and applied therapy.

Cytokinesis - block micronucleus assay (CBMN)

Micronuclei were prepared using the Fenech and Morley method [27]. Whole heparinised blood (0.5 ml) was added into 5 ml of PBMax Karyotyping (Invitrogen, California, USA), the complete medium for lymphocyte culture. All cultures were carried out in duplicate and incubated at 37° C for 72 h.

Forty-four h after PHA stimulation, cytochalasin B (Sigma, St. Louis, MO, USA) was added at a final concentration of 4 μg/ml. Cultures were harvested 28 h later. The cells were collected by centrifugation and resuspended in a cold (4° C) hypotonic solution (0.56% KCl). Then, the cells were fixed in freshly prepared acetic acid-methanol (3:1 vol/vol). The cell suspensions were dropped onto clean slides, air-dried and stained with 2% Giemsa for 12 min (Alfapanon, Novi Sad, Serbia). The scoring was performed using a light microscope (Nikon E50i) at ×400 magnification and following the criteria for MN scoring only in binucleated (BN) cells, as described by Fenech [28].

The MN frequencies were scored in 1000 BN lymphocytes of each patient. Five hundred viable cells from each patient were scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and to calculate the cytokinesis block proliferation index (CBPI) using the formula $CBPI = (M1 + [2 \times M2] + 3 \times [M3 + M4])/N$, where M1-M4 represent the number of cells with 1-4 nuclei respectively, and N is the total number of viable cells scored [29].

Statistical analysis

The results are shown as mean ± standard deviation (SD). Statistically significant difference between mean baseline and induced MN frequencies was determined using the Mann-Whitney U test. Levels of significance were $p < 0.05$ and $p < 0.001$. The relation between age, smoking habits, therapy treatment and MN and CBPI was determined by the Spearman's correlation coefficients.

Results

The results of MN analyses in PBL of patients before and one month after the end of the applied RT alone or RT combined with CT are shown in Tables 1-4.

Table 1 shows the individual baseline and therapy-induced MN frequency, as well as the CBPI of patients aged 41-75 years. The lowest assessed baseline MN frequency was 8/1000 BN cells, while the highest was 27/1000 BN. One month after the end of the applied

Table 1. Baseline and therapy-induced micronuclei frequency and cytokinesis block proliferation index in peripheral blood lymphocytes in patients with breast cancer

					Before therapy				After therapy			
Patient no.	*Age (years)*	*Smoking (+/-)*	*Therapy (RT/RT+CT)*	*Total number of CT cycles*	*No. of analysed cells*	*MN frequency/ analysed cells*	*MN/1000 BN cells*	*CBPI*	*No. of analysed cells*	*MN frequency/ analysed*	*MN/1000 BN cells cells*	*CBPI*
1	62	–	RT	–	1000	9	9	1.78	1000	50	50	1.91
2	52	–	RT	–	1000	12	12	1.4	1000	35	35	1.49
3	43	+	RT	–	1000	18	18	1.77	1000	38	38	1.68
4	61	–	RT	–	1000	12	12	1.53	1000	42	42	1.46
5	44	–	RT	–	1000	19	19	1.55	1000	42	42	1.42
6	69	–	RT	–	1000	20	20	1.62	1000	43	43	1.42
7	54	+	RT	–	1000	14	14	1.51	898	36	40.09	1.44
8	60	–	RT	–	1000	12	12	1.53	1000	37	37	1.46
9	51	–	RT	–	1000	19	19	1.62	1000	36	36	1.14
10	58	–	RT	–	1000	11	11	1.84	870	36	41.38	1.65
11	75	–	RT	–	1000	23	23	1.48	1000	75	75	1.39
12	55	+	RT	–	1000	27	27	1.55	1000	48	48	1.5
13	71	–	RT	–	1000	16	16	1.6	273	13	47.62	1.34
14	53	–	RT+CT	3	1000	15	15	1.46	1000	47	47	1.44
15	48	+	RT+CT	2	1000	16	16	1.31	1000	47	47	1.5
16	41	–	RT+CT	2	1000	8	8	1.48	1000	30	30	1.61
17	52	–	RT+CT	6	1000	10	10	1.75	1000	25	25	1.69
18	58	+	RT+CT	2	1000	13	13	1.66	1000	33	33	1.55
19	48	+	RT+CT	2	1000	8	8	1.7	1000	28	28	1.63
20	43	–	RT+CT	4	1000	11	11	1.84	1000	33	33	1.64
21	55	+	RT+CT	2	1000	23	23	1.64	1000	46	46	1.56
22	59	–	RT+CT	2	1000	19	19	1.59	1000	41	41	1.56
23	71	–	RT+CT	6	1000	22	22	1.64	1000	48	48	1.39
24	50	–	RT+CT	4	1000	16	16	1.56	1000	38	38	1.51
25	51	–	RT+CT	4	1000	12	12	1.6	904	53	58.63	1.45
26	60	–	RT+CT	4	853	16	17.76	1.85	1000	39	39	1.73
27	66	–	RT+CT	5	1000	18	18	1.7	620	29	46.77	1.59
28	45	+	RT+CT	2	1000	24	24	1.66	1000	67	67	1.66
29	53	–	RT+CT	6	1000	15	15	1.57	1000	38	38	1.48
30	45	+	RT+CT	6	1000	15	15	1.46	1000	41	41	1.33

RT: radiotherapy, CT: chemotherapy, MN: micronuclei, BN: binucleated, CBPI: cytokinesis-block proliferation index

therapy, the frequency of MN varied from 25 to 75/1000 BN cells. The CBPI values ranged from 1.31 to 1.85 before therapy, and from 1.14 to 1.91 after therapy.

The summarized results of MN frequency and CBPI are shown in Table 2. The MN frequency one month after the applied therapy was significantly higher compared with the baseline MN frequency ($p<0.001$). The applied therapy had a significant influence ($p<0.05$) on the cells kinetics, and the CBPI values dropped from 1.61 ± 0.13 to 1.52 ± 0.15.

Smokers had higher mean value of MN compared with nonsmokers, but without statistical significance ($p>0.05$). The same analysis a month after the end of therapy showed a statistically significant increase in both smokers and nonsmokers in relation to their baseline MN frequencies ($p<0.001$), but without statistical significance in their therapy-induced values ($p>0.05$). The CBPI values were lower in both smokers and nonsmokers after the end of therapy compared with the CBPI values before therapy. However, the reduction of CBPI values was statistically significant only in nonsmokers ($p<0.05$).

No significant difference of baseline MN frequency in relation to patient age was observed ($p>0.05$), but there was statistically significant difference ($p<0.001$) between the baseline and the therapy-induced MN frequency in both age groups (41-55 and 56-75 years). CBPI values dropped after the end of therapy, but they were statistically significant only in the older age group ($p<0.05$).

RT and combined RT+CT increased significantly the MN frequency ($p<0.001$). CBPI decreased significantly after the end of RT and combined RT + CT, but only after RT the decrease became statistically significant ($p<0.05$).

Table 3 shows the distributions of MN before and after treatment. Of the total analysed BN cells after therapy, 3.73% contained MN, a figure approximately 2.4 fold higher in comparison to the number of BN cells with MN recorded before therapy (1.46%). Generally, most of the cells with 1 MN were present both before

Table 2. Summarised results of micronuclei frequency and cytokinesis block proliferation index in the analysed patients (n=30)

Patients	*Patients, n*	*Before therapy (mean±SD)*	*After therapy (mean±SD)*
MN frequency (total)		15.86 ± 4.98	42.55 ± 10.47**
Smokers, n			
Yes	9	17.56 ± 6.06	43.12 ± 11.11**
No	21	15.13 ± 4.41	42.30 ± 10.45**
Mean age (years) 55.10 ± 9.07			
Age range (years)			
41-55	18	15.67 ± 5.32	40.98 ± 10.38**
56-75	12	16.15 ± 4.64	45.31 ± 10.57**
Therapy			
RT	13	16.31 ± 5.27	42.85 ± 11.78**
RT+CT	17	15.35 ± 4.82	41.26 ± 10.68**
CBPI (total)		1.61 ± 0.13	1.52 ± 0.15*
Smokers			
Yes	9	1.58 ± 0.14	1.54 ± 0.11
No	21	1.62 ± 0.13	1.51 ± 0.62*
Age range (years)			
41-55	18	1.58 ± 0.14	1.51 ± 0.14
56-77	12	1.65 ± 0.12	1.54 ± 0.17*
Therapy			
RT	13	1.60 ± 0.13	1.48 ± 0.18*
RT+CT	17	1.62 ± 0.14	1.55 ± 0.11

MN: micronuclei, CBPI: cytokinesis-block proliferation index, RT: radiotherapy, CT: chemotherapy
Mann-Whitney U test: statistical significance in relation to baseline frequency *$p < 0.05$, **$p < 0.001$

and after therapy, followed by the cells with 2 and 3 MN, while the BN cells that contained more than 3 MN were found only after the applied therapy. The percent of BN cells that contained 3 MN was slightly higher after the combined therapy in comparison to the percent after RT only (RT+CT 0.08% vs. RT 0.05%). Furthermore, BN cells with 4 MN and 5 MN were found in BN cells only after administering RT + CT.

The correlation between age, smoking habits and baseline MN were negative (r = –0.095, p = 0.635; r = –0.185, p=0.327). No significant correlation between age, smoking habits and baseline CBPI was found (r=0.098, p=0.625; r=0.046, p=0.808). The results of correlation analysis between age, smoking habits, type of therapy and therapy-induced MN were positive, yet without statistical significance (r=0.039, p=0.849; r=0.013, p=0.947; r=0.082, p=0.668). The correlation between age, smoking habits, therapy type and CBPI after the end of therapy was negative (r = –0.061, p = 0.764; r = –0.130, p = 0.492; r = –0.288, p = 0.123; Table 4).

Table 4. Results of Spearman's correlation analysis

	Before therapy		*After therapy*		*Therapy treatment (RT/RT+CT)*
	Age	*Smoking habits*	*Age*	*Smoking habits*	
MN r	–0.095	–0.185	0.039	0.013	0.082
p	0.635	0.327	0.849	0.947	0.668
CBPI r	0.098	0.046	0.013	–0.130	–0.288
p	0.625	0.808	0.947	0.492	0.123

RT: radiotherapy, CT: chemotherapy, MN: micronuclei, CBPI: cytokinesis-block proliferation index

Discussion

Human cancers are a spectrum of diseases in which genomic instability is the primary event leading to neoplastic transformation of cells [30]. Numerous studies confirmed increased levels of cytogenetic biomarkers in patients with different types of cancers, such as cervical [31], breast [32], lung cancer [33] etc. Among markers in PBL that can reflect chromosomal damages and cancer cells, MN are frequently used for assessing spontaneous chromosomal damage in cancer patients [24]. Moreover, during circulation, PBL pass through the radiation field, so MN in PBL can be a suitable biomarker for measuring the RT-induced level of chromosomal damage.

In the present study, the level of individual baseline as well as therapy-induced chromosomal damage in PBL was established in BC patients by applying the CBMN test. The results obtained before therapy revealed wide individual variations (8-27 MN per 1000

Table 3. Distribution of micronuclei before and after therapy

Treatment	*Total of analysed BN cells*	*BN cells with MN (%)*	*Distribution of MN (%)*				
			1 MN	*2 MN*	*3 MN*	*4 MN*	*5 MN*
Before therapy	29853	437 (1.46)	405 (1.36)	28 (0.09)	4 (0.01)		
After therapy	28565	1065 (3.73)	942 (3.30)	102 (0.36)	19 (0.07)	1 (0.004)	1 (0.004)
RT	12041	475 (3.94)	425 (3.53)	44 (0.37)	6 (0.05)		
RT+CT	16524	590 (3.57)	517 (3.13)	58 (0.35)	13 (0.08)	1 (0.01)	1 (0.01)

BN: binucleated, MN: micronuclei, RT: radiotherapy, CT: chemotherapy

BN cells) in MN frequency in PBL. The observed variability is in agreement with the report presented by Rajeswari et al. [34], where patients showed wide inter-individual variability in baseline chromosomal damage assessed in their leukocytes. As already known, the inter-individual variability in baseline chromosomal damage is a common feature in both healthy subjects and cancer patients [35] and in BC patients it might be at least caused by some of variation factors such as lifestyle, smoking habits, age, exposure to different mutagens, cancer stage etc. The study of Leal-Garza et al. [22] showed increased MN frequency with progressive stage of cervical cancer. Similar results were noted by Joseph et al. [36] in their study with thyroid cancer patients. To our knowledge, previous reports showed that age was one of the factors that influenced the MN frequency [37,38]. However, our results did not show any influence of age on the baseline MN frequency among patients ($p > 0.05$). Also, Jagetia et al. [39] and Santos et al. [40] in their studies did not observe such correlation.

Apart from age, the relevant literature contains controversial data regarding the effect of smoking on the baseline level of chromosomal damage. The results obtained in our study showed that smoking had no effect on the baseline micronuclei frequencies ($p > 0.05$), which is in accordance with the results of Santos et al. [40].

Similar to baseline MN frequency, a wide variation of MN frequencies (from 25 to 75) was observed in the analysed patients one month after the end of the applied therapy. This result suggests different sensitivity to RT in both healthy people [41] and cancer patients, which might be explained by differences in DNA repair capacity or polymorphisms in DNA repair genes [42], which may be important in the repair of DNA after ionizing radiation exposure.

Apart from the possibility to assess chromosomal damage, it is also possible to study cell kinetics (CBPI) using the CBMN test. Our study showed great individual variation of CBPI values before (1.31-1.85) and after the applied treatment (1.14- 1.91). Differences in CBPI values between appropriate groups (smokers vs. nonsmokers, age groups, RT vs. RT+CT) were not statistically significant either before or after the end of therapy ($p > 0.05$).

Generally, the data presented herein show that the applied therapy induced considerable chromosomal damage indicated by the elevated level of mean MN frequency in PBL. Application of therapy produced approximately 2.7-fold increase of the mean MN frequency in comparison to the mean value before therapy ($p<0.001$). Moreover, the applied therapy influenced the lymphocyte kinetics, resulting in a significant decrease of CBPI values ($p<0.05$). The results of our study are in concordance with the results obtained by Aristei et al. [19] in which RT induced an increase of MN frequency and CT increased sister chromatid exchange (SCE) in PBL of BC patients. In a recent study, Banerjee et al. [43] reported a similar observation about the effect of RT on the MN frequency in lymphocytes of BC patients. The same effect of RT on the MN frequency in cancer patients was reported by Lee et al. [44]. Apart from RT, anticancer drugs can evoke chromosomal damage and some of these chromosomal aberrations can be detected even 11 years after cessation of cytotoxic therapy [45].

Our results showed that in both age groups of patients (41-55 and 56-75 years), the applied therapy significantly increased the MN frequency ($p<0.001$). Concerning the MN frequency, we found no differences in the induced MN frequency between the 2 age groups. As no differences between age groups were obtained, even post-therapy, we concluded that there was no age sensitivity related to therapy. Our results are in disagreement with the results taken by Lisowka et al. [46] where a significant impact of age on the RT-induced aberrations in larynx cancer patients was found. In our study the applied therapy lowered the CBPI values in both age groups, but the decrease was statistically significant only in the older patient group ($p<0.05$).

The data presented herein revealed the same effect of therapy on the MN frequency was observed both in smoker and nonsmoker groups of patients ($p<0.001$). Our results show that smoking did not affect the MN frequency ($p>0.05$). These results were in agreement with earlier studies concerning the effect of smoking on the level of chromosomal damage in occupationally exposed people, such as radiology workers exposed to low level of ionizing radiation [47]. In a previous study, smokers in the group professionally exposed to radiation had lower MN frequency in contrast to nonsmokers [48]. Also, no effect of smoking was seen in radiation-induced aberrations in the study by Lisowska et al. [46]. Although the therapy lowered the CBPI values both in smokers and nonsmokers, a significant difference was noticed only in the group of nonsmokers ($p<0.05$).

Considering any possible synergistic effect of RT plus CT on the level of chromosomal damage we found that the combination of these two therapies induced chromosomal damage in PBL of patients, which was detected by increased MN frequency in relation to the baseline MN frequency ($p<0.001$). However, there were no statistically significant differences in the therapy-induced MN frequency between RT vs. RT+CT groups ($p >0.05$). These results might indicate absence of synergistic genotoxic effect of these two therapies. Legal et al. [49] demonstrated that CT, administered before RT, did not modify the yield of stable chromosomal aberra-

tions (SCAs) due to irradiation in comparison with the yield of SCAs after RT alone. Although we did not notice differences in MN frequencies induced by these two therapies, the PBL of patients that had undergone RT in combination with CT revealed a slightly higher number of BN cells with 3 MN (0.05% in the RT group vs. 0.08% in the RT+CT group). Furthermore, only in lymphocytes of 2 patients who received RT+CT, BN cells with 4 and 5 MN were found. It is known that the level of MN in cells is related with the level of chromosomal damage, i.e. BN cells with greater number of MN suffered from greater genome damage [50]. Therefore, the higher number of MN per BN cells, as well as the higher number of BN cells with 3 MN is the result of accumulation of DNA damage as consequence of the applied RT + CT. In addition to the increase of the MN frequencies, both therapies (RT and RT+CT) induced differences in cell kinetics by lowering the CBPI values, but with statistical significance ($p<0.05$) only in the RT group.

Spearman's correlation analyses showed no significant correlation between the analysed factors (age, smoking habits and type of therapy) and either baseline or induced MN frequency. Furthermore, Spearman's analyses showed that none of the previously mentioned factors affected significantly the CBPI values before or after the therapy.

In general, the results of MN distribution analyses showed that the applied therapy increased the number of BN cells with MN and the number of MN per BN cells. Cells with more than 3 MN were found only in the analyzed lymphocytes after the applied therapy.

In conclusion, the results obtained by using the CBMN assay indicated that RT as well as RT+CT significantly increased the MN frequency in the PBL of BC patients and had significant influence on the cell kinetics, indicating genotoxic and cytotoxic effect of these therapies. Although there were no differences between MN frequencies due to RT and RT + CT, BN cells with more than 3 MN as well as a slightly higher percentage of BN cells with 3 MN were noticed in patients who received the combined therapy. Moreover, the present study revealed wide individual variation in therapy-induced MN frequency among patients, which could indicate variable sensitivity to RT and CT. The data presented herein suggest that none of the variation factors (age, smoking habits and type of therapy) had any influence on the noticed variability.

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