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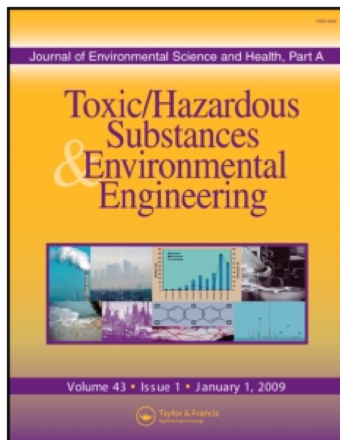

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DNA and cytogenetic damage in white blood cells of postmenopausal breast cancer patients treated with radiotherapy

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The primary and residual genome damage and its elimination rate were evaluated in peripheral blood lymphocytes of breast cancer patients treated with adjuvant radiotherapy after surgical removal of the tumor by mastectomy or quadrantectomy. The levels of DNA/chromosome damage were estimated before, throughout, as well as after six months, respectively one year after the radiotherapy, using the alkaline comet assay, the chromosome aberration analysis and the cytokinesis-block micronucleus assay. The marked individual differences in the baseline genome damage were observed in patients, which additionally increased until the end of the radiotherapy cycle. The levels of DNA/cytogenetic damage slowly declined during post-irradiation period; although in the majority of subjects they did not return to pre-therapy levels. In addition to the well-established comet parameters, the long-tailed nuclei were also proved as a useful indicator of individual DNA damage and response to radiation. One of the most important observation was that older breast cancer patients, irradiated after mastectomy, had higher values of almost all parameters evaluated. We found positive correlations between the comet assay parameters and the cytogenetic biomarkers that confirmed their complementary value in the assessment of the radiation sensitivity/susceptibility in elderly breast cancer patients. The specific patterns of DNA damage observed in the majority of subjects after a prolonged exposure to ionizing radiation indicate the possibility of adaptive response. Such results may also be linked to the hormesis theory and support previous observations, but the underlying mechanisms should be further investigated on a much larger population.

Keywords: White blood cells, DNA damage, comet assay, chromosome aberrations, micronuclei, breast cancer, radiotherapy.

Introduction

Breast cancer is principally a disease of older women and the risk increases with age. As the life expectancy has increased, this will become a frequent disease affecting the lives of older women, especially in more developed countries.[1] The success of cancer therapy today is no longer measured by the healing of the primary cancer alone, but also by the quality of life and health risks after radio- and chemotherapy. Thanks to new therapies, operated breast cancer shows a longer time before relapse, as well as a greater survival rate.[2] Since conventional anticancer therapies are mostly not cancer tissue-specific, the genome damage in non-target tissues represents an unavoidable side-effect whose level and rate of elimination are still unknown.

Cytogenetic methods with human peripheral lymphocytes have been extensively used in previous biomonitoring studies on breast cancer patients. Recent studies mostly focused on biomonitoring of the increased level of the basal DNA damage in breast cancer patients[3–6], and their female relatives[7] or excessive DNA damage caused by the treatment with antineoplastic drugs or radiation.[8–13]

The objective of this study was to evaluate and compare the levels of primary and residual DNA/cytogenetic damage inflicted by adjuvant radiotherapy in peripheral blood lymphocytes of post-menopausal women diagnosed with breast cancer. Patients were cytogenetically monitored over the one-year period after the successful surgery (by mastectomy and quadrantectomy) and subsequent radiotherapy. Genome damage was assessed using the alkaline comet assay, analysis of chromosome aberrations (CA), and

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cytokinesis-block micronucleus (CBMN) assay. Repeated analyses performed before, during, and after the therapy aimed to: (i) estimate the baseline level of the genome damage in elderly subjects diagnosed with breast cancer; (ii) investigate individual differences in sensitivity to therapeutic doses of ionizing radiation; (iii) estimate the persistence and elimination rate of genome damage; and (iv) evaluate the sensitivity and predictivity of genotoxicological methods and their potential use as additional tools in biomonitoring and the regular follow-up on cancer patients after therapy.

Patients and methods

Patients

Study population consisted of twenty post-menopausal women diagnosed with breast cancer, subjected to surgery and treated with adjuvant radiotherapy at the University Hospital for Tumors (Zagreb, Croatia). Before entering the study they were informed about the aim and the experimental details and they signed their consent for voluntarily participation. Their median age was 73 years (range 57–80 years). Patients were interviewed using a standardized questionnaire that covered personal anamnestic data, along with occupational, medical, and other variables known to influence cytogenetic endpoints. All patients were non-smokers and non-alcoholics, with no previous history of malignant diseases or therapy by antineoplastic drugs or radiation. They also were not exposed to physical or chemical agents in their living or working environment which mayinfluence the results of cytogenetic screening. Detailed anamnestic and clinical data collected from patient records are reported in Table 1.

The same diagnostic protocol was applied for all patients. Before the operation, they had undergone a standard diagnostic procedure (blood screening, CA 15–3, mammography, locoregional breast and abdomen ultrasound, gynaecological examination and a chest X-ray). Patients had undergone breast surgery either by a total mastectomy with axillary lymph node dissection or a partial breast resection, i.e., quaderantectomy. Histopathologically, they suffered from ductal breast cancer (*carcinoma ductale invasivum*). The distribution of patients according TNM (Tumor Nodes Metastasis) classification established by the National Comprehensive Cancer Network[14] is presented in Table 1. T is defined as 1–4 with respect to the tumor size (T1 tumor size < 2 cm, T2 between 2 and 5 cm, T3 > 5 cm and T4 tumor of any size with direct extension to the chest wall or skin), N for the absence (N0) or presence (N1) of nodes metastasss and M for the presence or absence of metastasss. Table 1 also reports data on estrogen and progesterone receptors, as well as HER2/neu status of tumor. Adjuvant radiotherapy was scheduled to start within 4 weeks following the surgery, when patients were in good clinical condition.

Patients subjected to mastectomy were treated with external beam radiotherapy (by a linear accelerator) using the radiation dose of 45 Gy divided in 18 fractions. Direct electron field of 9 MeV was also applied to the thoracal chest wall. The same dose was applied to ypsilateral axillo-supraclavicular region using direct combination of electrons (energy: 16MeV; 2/3 tumor dosage-TD) and photons (energy: 6MV; 1/3 TD). Radiation doses during treatment were 2.5 Gy/1.fr. after the 1st radiation (second blood sampling); 22.5 Gy/9 fr. in the middle of radiotherapy (third blood sampling); and 45 Gy/18 fr. at the end of radiotherapy (fourth blood sampling). After the breast-conserving surgery, i.e., quadrant resection, the patients were treated with radiation doses in ranges of 50 Gy–60 Gy by photons from the linear accelerator, with energy of 6 MV, and LINAC electrons 12 MeV. All patients received adjuvant treatment on the breast area where the tumor was, with tangential photon rays of 6 MV energy and the tumor dosage (TD) of 50 Gy/25 fractions (fr.) + a "boost" with TD 10Gy/5 fractions on the tumor bed with a direct electron field of 12 MeV energy. Radiation doses during the treatment were 2 Gy/1.fr. after the 1st radiation (second blood sampling); 30 Gy/15 fr. in the middle of radiotherapy (third blood sampling); and 60 Gy/30 fr. at the end of radiotherapy (fourth blood sampling).

For each patient, based on the data specified by the manufacturer of the radiation source and personal data, the percentages of irradiated body volume were calculated. When radiotherapy was completed, patients underwent standard diagnostic procedures and were regularly monitored during the following year using conventional clinical protocols.

All described procedures were in accordance with the high ethical standards. The work was approved by the relevant Research Ethics Committees on human experimentation (The University Hospital for Tumors and the Institute for Medical Research and Occupational Health; Zagreb, Croatia) and complies with the principles laid down in the Declaration of Helsinki.

Methods

Blood sampling: In order to determine the genome damage, venous blood was drawn six times throughout the study. Samples of the venous blood (5 ml per each sampling time) were collected in heparinized vacutainer tubes (Becton Dickinson, N.J., USA) under sterile conditions. The current study employed each breast cancer patient as her own control. Using blood samples collected before therapy, individual baseline values for each method were estimated.

Pre-treatment blood sample (I) was collected on day 1 of the first radiotherapy cycle, 2 hours prior to the irradiation. The response of the peripheral blood leukocytes to the radiotherapy was evaluated on the blood sample taken within two hours after the application of the first dose (II), as well

Table 1. Anamnestic and clinical data of breast cancer patients involved in the study.

Code	*Age*	*BW/kg BH/cm*	*BMI*	*TNM/stage*	E_r	P_r	*HER2*	BV_{irr}(%)
m_1	79	59/150	21.7	T4N1M0/IIIB	+	+	−	4.2
m_2	79	69/161	22.1	T1N1M0/IIA	−	+	−	4.1
m_3	76	84/156	30.0	T1N1M0/IIA	+	+	−	3.9
m_4	77	85/163	27.5	T2N1M0/IIB	−	+	−	3.7
m_5	75	60/163	18.1	T2N1M0/IIB	+	+	−	3.2
m_6	75	85/163	27.5	T2N0M0/IIA	+	+	−	3.2
m_7	78	62/158	20.3	T4N0M0/IIIB	−	+	−	3.0
m_8	80	68/154	24.2	T1N1M0/IIA	+	+	−	3.0
m_9	76	85/163	27.5	T2N1M0/IIB	+	+	−	2.6
m_{10}	76	70/168	20.3	T2N1M0/IIB	−	+	+	2.1
q_1	58	93/167	30.5	T1N0M0/I	+	+	−	4.3
q_2	59	69/170	22.0	T1N0M0/I	+	+	−	3.9
q_3	57	68/168	22.4	T1N0M0/I	−	+	−	3.6
q_4	58	58/150	23.0	T1N0M0/I	−	+	−	3.1
q_5	66	69/160	23.4	T1N0M0/I	−	+	−	3.1
q_6	68	57/164	17.4	T1N0M0/I	+	+	−	2.6
q_7	68	93/167	29.5	T1N0M0/I	+	+	−	2.2
q_8	69	59/165	17.8	T1N0M0/I	−	+	−	1.9
q_9	70	76/150	29.8	T1N0M0/I	+	+	−	1.8
q_{10}	62	63/166	19.7	T1N0M0/I	+	−	−	1.7

Abbreviations: m–mastectomy; q–quadrantectomy; BW–body weight; BH–body height; TNM – classification with respect to the tumor size (T), the presence or absence of nodes metastases (N) and metastases (M); E_r–estrogen receptor; P_r–progesteron receptor; HER2–HER2/neu (Human Epidermal Growth Factor Receptor 2); BV_{irr}–irradiated body volume.

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as in the middle of the radiotherapy cycle (III) and within 2 hours after the last received radiotherapy dose (IV). Two blood samples were taken 6 months (V), respectively 12 months (VI) after radiotherapy.

All blood samples were handled in the same manner. After venepuncture, they were coded, cooled at +4°C in the dark and transferred to our laboratory. They were processed immediately after transportation (within a maximum of 1-hour period after the collection) by means of the alkaline comet assay, the analysis of structural chromosome aberrations (CA) and the cytokinesis-block micronucleus (CBMN) assay following the recommendations by International Atomic Energy Agency[15] and ICPS guidelines[16] as well as the HUMN project.[17]

The comet assay was carried out under alkaline conditions.[18] Preparation of agarose microgels was done as previously described.[12] Two replicate slides per sample were prepared. Each slide was examined using a 250 x magnification fluorescence microscope (Zeiss) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A total of 100 comets per sample (50 per slide) were scored using a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd. As the measure of DNA damage, tail length and tail moment were chosen. Cells were also classified as either "undamaged" or "damaged" by considering threshold levels indicating the comets with a long-tailed nucleus (LTN), i.e., the length over the 95th percentile of the distribution of the tail lengths among the control samples.[19]

Chromosome aberration analysis was performed according to standard procedure described in IAEA guidelines.[16] Slides were stained with 5% Giemsa solution (Sigma). All slides were coded and scored blindly at 1000x magnification under oil immersion. Structural chromosome aberrations were classified based on the number of sister chromatids and breakage events involved. Only metaphases containing 45–47 centromeres were analyzed. One hundred metaphases per sample (50 from each of two replicates) were analyzed for the total number and types of aberrations, as well as the percentage of aberrant cells.

Cytokinesis-block micronucleus (CBMN) assay was performed according to the standard protocol[20]. Cytochalasin B in the final concentration 6 μg/ml was added to cultures at 44 h. Slides were stained with 5% Giemsa solution (Sigma). MN scoring was performed on coded slides at 1000x magnification under oil immersion. Altogether 500 binuclear (BN) cells per each sample were scored. For MN identification the criteria of[17] were used. The total number of MN and their distribution were determined, along with the number of micronucleated cells.

Statistical analysis

Statistical analyses were carried out with a commercial programme Statistica 7.0 (StatSoft, Tulsa, USA). The extent of DNA damage, as recorded by the alkaline comet assay, was analyzed considering the parameters of descriptive

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statistics: mean (± standard error of the mean), median and range of the comet parameters. Variance homogeneity was tested by the Lindman's test prior to the analysis of correlation and between-group differences. The normality of distribution was tested by using the Shapiro-Wilks' W test. Since distribution of variables was not normal, in further analyses nonparametric methods were used. Differences between groups of independent variables were analyzed using the Mann–Whitney U-test and Kruskal–Wallis test. Friedman ANOVA test and Wilcoxon matched pairs test with downward adjustment of the α-level for multiple comparisons between the pairs were used to determine differences between groups of dependent variables. The level of significance of correlation between variables and the correlation trend were analyzed using the Spearman Rank Order Correlation Test. Statistical significance was defined as p<0.05 in all analyses.

Results

Detailed anamnestic and clinical data on patients are reported in Table 1. Individual results are listed in Table 2 (mastectomy) and Table 3 (quadrantectomy). Table 4 reports mean group values and the results of comparisons made between two studied cohorts.

Alkaline comet assay

Patients irradiated after mastectomy. Individual results of the alkaline comet assay on peripheral blood leukocytes are shown in Table 2. We observed differences in pre-therapy levels of DNA damage and detected a significant negative correlation ($P < 0.01$) between the comet parameters and the body mass index (which informs on the patient's physical condition). The administration of the first fraction of irradiation caused a noticeable increase of DNA damage. In the middle of the radiotherapy cycle majority of the patients had increased DNA damage as compared to the previous sampling. A significant positive correlation ($P < 0.05$) between the age and the comet parameters indicates a greater DNA instability in older patients. The levels of DNA damage recorded in most patients after the administration of the last fraction of irradiation pointed to the adaptive response. Six months after radiotherapy, DNA migration notably decreased. Significant reduction in the frequency of LTN indicates the efficacy of DNA repair processes (Fig. 1a). A year after the radiotherapy we detected low levels of primary DNA damage in most patients.

Statistical significance of the results was confirmed by Friedman ANOVA (tail length $P < 0.001$; LTN $P = 0.003$; tail moment $P = 0.004$).

Patients irradiated after quadrantectomy. Individual results of the of the alkaline comet assay in peripheral blood leukocytes are shown in Table 3. Pre-therapy levels of DNA damage among the patients varied and were significantly higher in those with positive estrogen receptors ($P < 0.05$). Irradiation caused an increase of DNA damage in almost all patients. In the middle of the radiotherapy cycle 50% of patients (mostly older ones) had higher levels of

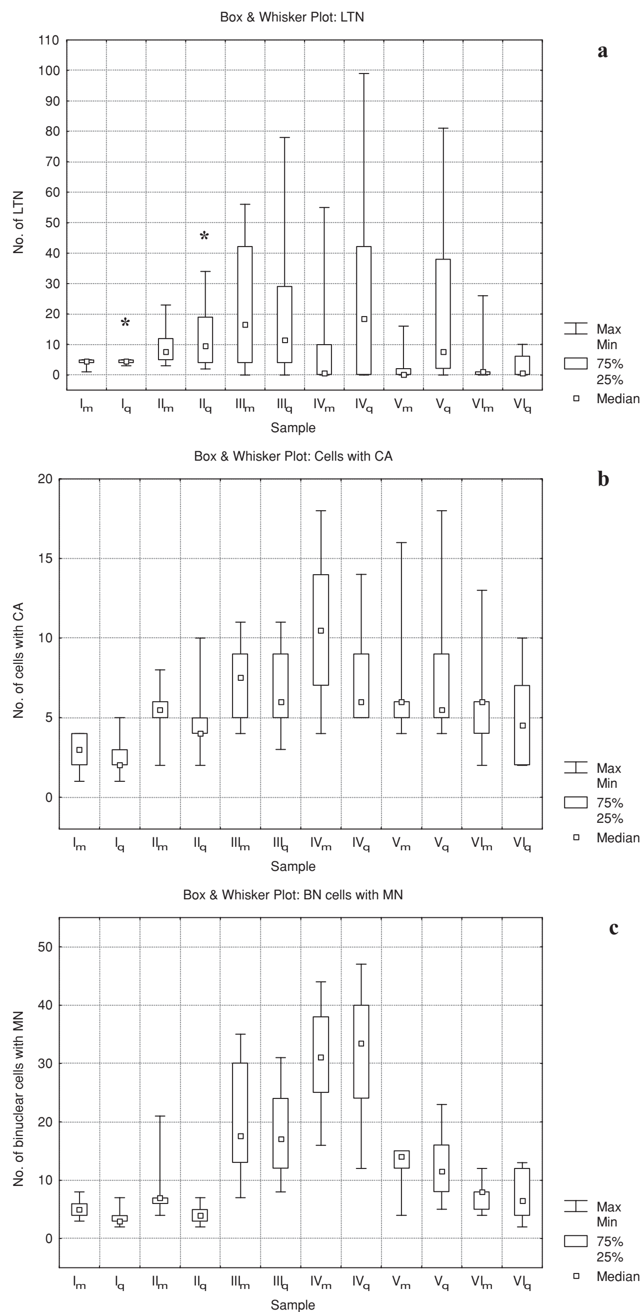

Fig. 1. The distribution of: (a) long-tailed nuclei (LTN), (b) lymphocytes with chromosome aberrations (CA) and (c) binuclear lymphocytes with micronuclei (MN) in peripheral blood of patients irradiated after mastectomy (m) or quadrantectomy (q). I-VI represent blood samplings: before radiotherapy (I), in the course of radiotherapy (II-IV), 6 months (V), and one year after radiotherapy (VI). *Statistically significant differences (Mann-Whitney U test, $P < 0.05$).

Table 2. Individual results of the alkaline comet assay and cytogenetic endpoints in peripheral blood lymphocytes of breast cancer patients treated with adjuvant radiotherapy after mastectomy (m).

		Alkaline comet assay														
		DNA migration – tail length (μm)				*Tail moment*			*Analysis of structural CA*						*CBMN assay*	
Code		*Mean ± S.E.*	*Med.*	*Range*	*LTN*	*Mean ± S.E.*	*Med.*	*Range*	*ΣaC/CA*	b_1	b_2	*af*	*dc*	*other*	*ΣMN/BN*	*Distribution 1–2–3 MN*
m_1	I	20.11 ± 0.70	17.95	12.18-52.56	5	3.64 ± 0.07	3.25	2.51–5.74	3/3	1	1	1	—	—	4/4	4–0–0
	II	23.65 ± 0.71	22.44	15.38–64.74	7	4.88 ± 0.11	4.75	3.07–8.16	6/6	—	1	5	—	—	21/21	21–0–0
	III	45.31 ± 1.95	41.35	17.31–102.56	56	6.84 ± 0.20	6.31	3.80–15.04	7/11	1	1	4	5	—	36/34	32–2–0
	IV	22.95 ± 1.23	19.87	14.74–73.08	10	4.63 ± 0.27	3.87	2.51–17.48	4/5	1	—	2	2	—	18/16	14–2–0
	V	30.72 ± 1.70	25.00	16.67–92.95	16	5.90 ± 0.28	5.10	2.58–19.51	4/10	—	—	5	3	2r	4/4	4–0–0
	VI	14.86 ± 0.48	13.46	10.26–38.46	1	2.95 ± 0.08	2.61	1.32–6.29	6/9	—	—	5	2	2r	8/8	8–0–0
m_2	I	22.66 ± 0.78	21.15	12.82–59.61	5	3.92 ± 0.11	3.82	1.89–9.09	3/4	1	—	2	1	—	5/5	5–0–0
	II	22.92 ± 0.85	21.15	13.46–82.05	5	4.31 ± 0.12	3.85	2.52–10.86	8/12	4	2	3	3	—	7/7	7–0–0
	III	39.31 ± 2.20	28.53	14.74–112.82	42	5.99 ± 0.40	4.48	3.14–27.91	9/11	2	1	4	4	—	17/15	13–1–0
	IV	27.20 ± 1.27	23.72	17.31–99.36	14	4.84 ± 0.24	4.40	3.10–20.77	16/28	—	—	19	8	1tc	40/38	36–2–0
	V	16.47 ± 0.23	16.03	11.54–21.79	—	4.64 ± 0.08	4.43	3.05–8.36	16/28	—	—	27	10	1tc	17/15	13–2–0
	VI	19.23 ± 0.36	18.59	12.82–30.77	—	4.03 ± 0.08	3.83	2.28–6.42	13/28	—	—	21	7	—	11/10	9–1–0
m_3†	I	15.77 ± 0.19	15.70	10.90–20.51	1	3.39 ± 0.06	3.20	1.64–15.16	2/2	—	—	2	—	—	8/8	8–0–0
	II	18.21 ± 0.49	16.99	12.82–40.38	23	3.67 ± 0.11	3.24	2.53–8.17	5/5	3	1	1	—	—	9/9	9–0–0
	III	17.51 ± 0.93	14.74	10.90–67.31	17	3.48 ± 0.17	3.12	1.78–13.56	4/9	—	—	6	3	—	34/30	27–2–1
	IV	30.98 ± 2.04	21.47	13.46–103.84	55	4.61 ± 0.20	3.88	2.51–16.03	8/10	1	—	7	1	1r	35/44	43–1–0
m_4	I	20.97 ± 0.42	19.87	15.38–39.74	4	5.00 ± 0.09	5.02	3.67–7.64	3/3	2	—	1	—	—	4/4	4–0–0
	II	23.35 ± 0.98	20.51	15.38–80.13	12	4.95 ± 0.09	5.03	3.18–7.57	5/6	—	—	4	2	—	6/6	6–0–0
	III	19.63 ± 0.26	19.55	14.74–25.64	—	3.89 ± 0.06	3.77	3.04–5.55	5/9	—	—	7	1	1tc	24/22	20–2–0
	IV	17.10 ± 0.22	16.67	13.46–25.00	—	3.69 ± 0.06	3.79	2.52–5.75	11/12	1	—	9	1	1r	34/34	34–0–0
	V	15.23 ± 0.18	15.06	11.54–19.87	—	3.20 ± 0.06	3.19	1.98–4.53	4/4	2	—	1	1	—	14/14	14–0–0
	VI	17.25 ± 0.26	17.31	11.54–23.72	—	3.35 ± 0.05	3.18	1.87–4.49	2/2	—	—	1	—	1r	12/12	12–0–0
m_5	I	49.87 ± 2.57	42.31	17.95–120.51	5	7.03 ± 0.29	5.90	3.74–17.37	4/4	2	—	1	1	—	5/5	5–0–0
	II	52.67 ± 2.37	48.72	17.31–116.66	3	8.38 ± 0.31	7.58	3.17–18.76	6 /7	—	—	6	1	—	6/6	6–0–0
	III	19.75 ± 0.23	19.23	14.10–26.92	—	4.14 ± 0.07	4.33	2.55–7.05	7/12	2	1	9	—	—	22/20	18–2–0
	IV	19.40 ± 0.83	17.95	13.46–80.77	—	4.11 ± 0.11	3.84	2.50–8.37	13 /15	—	—	11	4	—	33/31	29–2–0
	V	16.72 ± 0.29	16.67	12.18–28.20	2	3.67 ± 0.09	3.78	1.95–5.75	6/9	1	—	7	1	—	13/13	13–0–0
	VI	23.56 ± 0.74	22.44	12.82–55.13	—	4.37 ± 0.10	4.39	2.50–8.74	6/15	2	—	9	3	1tc	5/5	5–0–0
m_6	I	18.03 ± 0.76	16.03	10.90–62.82	5	4.27 ± 0.22	3.79	2.54–21.32	1/1	—	—	1	—	—	5/5	5–0–0
	II	21.50 ± 0.41	21.15	12.82–38.46	3	5.47 ± 0.15	5.09	2.54–13.60	2/3	—	—	2	1	—	7/7	7–0–0
	III	21.97 ± 0.52	20.83	13.46–49.36	4	5.07 ± 0.12	5.03	2.60–9.22	8/10	—	—	7	3	—	14/13	12–1–0
	IV	15.94 ± 0.25	16.03	11.54–22.44	—	4.79 ± 0.08	4.97	3.12–6.36	10/14	1	—	10	3	—	33/31	29–2–0
	V	18.23 ± 0.21	17.95	14.74–23.72	—	3.98 ± 0.07	3.85	3.09–5.78	5/8	1	—	4	3	—	15/15	15–0–0
	VI	15.46 ± 0.24	15.06	11.54–25.64	—	3.29 ± 0.07	3.19	2.43–5.84	2/4	—	—	2	2	—	8/8	8–0–0
m_7	I	21.50 ± 1.02	19.55	14.10–78.20	4	4.90 ± 0.19	4.48	3.13–20.51	1/1	—	—	1	—	—	7/7	7–0–0
	II	23.61 ± 0.94	21.15	15.38–62.18	9	4.40 ± 0.09	4.41	2.58–8.20	6/6	3	—	3	—	—	7/7	7–0–0
	III	24.52 ± 0.59	23.08	16.03–41.02	16	4.88 ± 0.11	4.51	2.59–7.69	9/15	2	—	8	5	—	37/35	33–2–0
	IV	18.06 ± 0.33	17.31	12.18–30.13	—	4.51 ± 0.08	4.42	3.09–6.95	6/8	1	—	6	1	—	45/41	37–4–0
	V	19.35 ± 0.43	18.59	12.18–41.67	2	4.19 ± 0.10	3.86	1.95–7.71	5/10	—	—	6	4	—	15/15	15–0–0
	VI	24.60 ± 0.57	23.40	17.31–48.72	11	4.29 ± 0.08	4.38	3.11–7.73	7/8	1	—	5	2	—	8/7	6–1–0
m_8	I	18.53 ± 0.59	17.31	12.18–57.05	4	3.59 ± 0.08	3.24	1.98–7.38	2/2	2	—	—	—	—	3/3	3–0–0
	II	19.17 ± 0.43	17.95	13.46–41.02	6	3.95 ± 0.09	3.82	2.54–8.44	3/5	2	1	2	—	—	5/4	3–1–0
	III	27.42 ± 1.11	24.36	13.46–75.84	45	4.98 ± 0.18	4.47	1.91–10.66	5/9	—	—	5	3	1r	8/7	6–1–0
	IV	17.93 ± 0.41	17.31	12.18–48.72	1	3.48 ± 0.06	3.22	2.51–5.77	14/34	1	—	22	11	—	23/21	19–2–0
	V	17.69 ± 0.20	17.31	14.10–23.06	—	4.33 ± 0.06	4.41	3.06–6.37	6/6	2	—	3	1	—	9/8	7–1–0
	VI	18.21 ± 0.32	17.96	12.18–27.66	1	4.01 ± 0.07	3.85	2.57–5.62	4/7	1	—	3	3	—	4/4	4–0–0
m_9	I	18.92 ± 0.31	18.59	12.18–32.69	4	4.06 ± 0.09	3.86	2.46–7.14	4/4	2	1	1	—	—	6/6	6–0–0
	II	19.03 ± 0.42	17.95	11.54–41.02	8	4.08 ± 0.11	3.84	1.96–9.48	8/8	2	5	1	—	—	7/7	7–0–0
	III	22.92 ± 0.66	22.44	12.18–69.87	33	6.23 ± 0.14	6.21	3.74–15.77	8/10	1	—	7	2	—	14/14	14–0–0
	IV	16.19 ± 0.27	16.67	10.90–22.44	—	4.62 ± 0.08	4.44	2.49–6.40	7/11	2	—	5	3	1r	26/25	24–1–0
	V	17.15 ± 0.26	17.31	9.62–32.05	1	3.62 ± 0.06	3.79	1.89–5.12	9/17	—	—	13	3	1r	15/15	15–0–0
	VI	16.24 ± 0.34	16.03	10.26–39.10	1	3.42 ± 0.07	3.22	1.92–5.81	6/11	—	—	1–	1	—	8/8	8–0–0
m_{10}	I	21.53 ± 0.32	21.15	14.74–32.69	5	4.37 ± 0.07	4.42	3.06–7.11	4/4	4	—	—	—	—	5/5	5–0–0
	II	23.92 ± 0.41	23.08	17.95–41.02	17	4.97 ± 0.08	4.99	3.68–7.03	5/7	5	1	1	—	—	6/6	6–0–0
	III	22.73 ± 0.75	21.79	14.10–83.33	12	5.49 ± 0.13	5.65	0.09–10.92	11/35	11	7	11	4	2r	13/13	13–0–0
	IV	19.85 ± 0.34	19.23	15.38–30.77	4	4.38 ± 0.09	4.42	3.06–7.01	18/48	6	8	18	13	1tc,2r	32/31	30–1–0
	V	15.12 ± 0.21	14.74	10.26–21.79	—	3.05 ± 0.05	3.15	1.88–4.53	6/15	5	2	6	2	—	12/12	12–0–0
	VI	24.61 ± 0.92	21.79	14.10–64.10	26	4.22 ± 0.10	3.87	2.57–7.01	5/12	4	—	6	2	—	4/4	4–0–0

Note. Blood samples were collected before radiotherapy (I), in the course of radiotherapy (II–IV), as well as 6 months (V), and 12 months (VI) after radiotherapy. In the alkaline comet assay 100 comets per each sample were measured; the analysis of structural CA was performed on 100 first *in vitro* metaphases per sample; incidence of micronuclei (MN) was evaluated on 500 binuclear cells per sample. Abbreviations: TNM – classification with respect to tumor (T) size, the presence or absence of nodes metastases (N) and metastases (M); Irr% - percentage of the irradiated body volume; S.E. – standard error; Med. - median; LTN – long tailed nuclei; ΣaC– total number of cells with CA; ΣCA- the total number of chromosome aberrations per 100 metaphases; b_1 – chromatid break; b_2 – chromosome break; af – acentric fragment; dc – dicentric chromosome; tc– tricentric chromosome; r – ring chromosome; ΣBN – the total number of binuclear cells with micronuclei; ΣMN - the total number of micronuclei per 500 binuclear cells. †- the patient died soon after the termination of radiotherapy.

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Table 3. Individual results of the alkaline comet assay and cytogenetic endpoints in peripheral blood lymphocytes of breast cancer patients treated with adjuvant radiotherapy after quadrantectomy (q).

	Alkaline comet assay														
	DNA migration – tail length (μm)				*Tail moment*			*Analysis of structural CA*						*CBMN assay*	
Code	*Mean ± S.E.*	*Med.*	*Range*	*LTN*	*Mean ± S.E.*	*Med.*	*Range*	*ΣaC/CA*	b_1	b_2	*af*	*dc*	*other*	*ΣMN/BN*	*Distribution 1–2–3 MN*
q_1 I	25.11 ± 0.52	24.36	15.38–39.10	4	4.93 ± 0.13	4.51	3.02–8.42	4/2	2	—	—	—	—	3/3	3–0–0
II	25.95 ± 0.83	24.04	15.38–73.08	9	4.74 ± 0.13	4.46	2.47–11.97	5/6	—	1	2	3	—	6/5	4–1–0
III	17.55 ± 0.30	16.99	12.18–28.85	—	4.45 ± 0.07	4.44	3.06–6.81	11/6	3	—	1	2	—	13/11	9–2–0
IV	17.67 ± 0.36	16.67	12.82–33.33	—	3.85 ± 0.08	3.82	1.94–6.32	5/7	2	—	4	1	—	26/20	17–3–0
V	25.22 ± 0.60	24.36	12.82–50.00	4	5.14 ± 0.13	5.00	2.43–9.77	9/7	1	—	5	1	—	8/7	6–1–0
VI	14.67 ± 0.23	14.74	10.26–23.08	—	2.90 ± 0.06	2.59	1.90–4.48	2/9	3	—	5	1	—	5/5	5–0–0
q_2 I	19.06 ± 0.28	19.23	12.82–25.64	3	4.10 ± 0.07	3.77	3.04–6.34	2/3	3	—	—	—	—	2/2	2–0–0
II	22.77 ± 0.35	22.44	16.67–32.69	34	5.00 ± 0.11	5.06	3.07–7.73	4/5	—	—	3	2	—	2/2	2–0–0
III	21.86 ± 0.84	19.23	13.46–72.43	16	4.40 ± 0.15	3.89	1.95–12.79	9/14	2	1	10	1	—	13/12	11–1–0
IV	29.97 ± 1.82	23.72	15.38–107.69	42	5.56 ± 0.31	4.50	2.62–20.04	6/9	2	—	6	1	—	28/26	24–2–0
V	19.59 ± 0.72	17.31	13.46–58.97	11	3.70 ± 0.09	3.76	2.48–7.41	5/5	—	—	2	1	2r	8/8	8–0–0
VI	15.90 ± 0.27	16.03	11.54–27.56	1	3.24 ± 0.06	3.17	1.94–5.10	4/5	—	—	4	—	1tc	4/4	4–0–0
q_3 I	21.68 ± 0.53	20.51	15.38–50.00	5	4.15 ± 0.07	3.87	3.04–5.76	5/5	3	2	—	—	—	3/3	3–0–0
II	23.49 ± 0.60	21.79	17.31–51.92	4	4.67 ± 0.07	4.47	3.11–6.46	4/5	—	—	2	3	—	3/3	3–0–0
III	20.94 ± 0.88	18.59	12.82–72.43	5	4.37 ± 0.29	3.85	1.38–26.76	7/12	—	—	6	5	1r	20/20	20–0–0
IV	19.76 ± 0.36	19.87	13.46–33.33	1	4.22 ± 0.09	3.90	1.97–7.05	5/10	—	1	4	5	—	39/37	35–2–0
V	17.72 ± 0.25	17.31	13.46–27.56	—	3.90 ± 0.08	3.82	2.54–5.78	6/11	1	—	6	3	1r	10/9	8–1–0
VI	15.42 ± 0.20	15.38	10.90–21.79	—	3.18 ± 0.07	3.16	1.90–5.14	5/5	—	—	3	2	—	4/4	4–0–0
q_4 I	17.36 ± 0.33	16.67	12.82–35.90	4	3.37 ± 0.07	3.21	1.95–5.60	1/1	—	—	—	1	—	3/3	3–0–0
II	21.40 ± 0.68	19.23	14.74–48.72	23	3.82 ± 0.07	3.81	2.49–5.82	2/2	—	—	1	1	—	4/4	4–0–0
III	18.27 ± 0.34	17.63	12.18–33.33	4	3.74 ± 0.07	3.82	2.54–5.81	8/10	2	1	4	3	—	8/8	8–0–0
IV	29.98 ± 0.81	28.85	17.31–59.61	78	6.04 ± 0.16	5.74	3.15–13.38	9/12	1	1	6	4	—	13/12	11–1–0
V	23.58 ± 0.57	22.44	14.10–48.72	38	4.95 ± 0.13	4.72	2.51–10.43	5/6	3	—	3	—	—	6/5	4–1–0
VI	16.28 ± 0.26	16.03	10.90–25.00	1	3.31 ± 0.06	3.17	2.36–4.50	3/5	1	—	2	2	—	2/2	2–0–0
q_5 I	18.85 ± 0.19	18.59	14.47–23.08	4	4.00 ± 0.05	3.79	3.06–5.14	1/1	—	—	1	—	—	3/3	4–0–0
II	18.48 ± 0.36	17.95	14.10–34.61	7	3.80 ± 0.07	3.76	2.50–5.80	3/5	—	—	4	1	—	4/4	4–0–0
III	26.70 ± 1.50	23.08	15.38–105.77	68	5.30 ± 0.16	5.05	3.21–14.36	3/7	—	—	5	2	—	16/15	14–1–0
IV	22.73 ± 0.61	20.51	15.38–51.28	40	4.93 ± 0.10	4.50	3.05–8.34	7/12	1	—	9	1	1r	25/24	23–1–0
V	20.70 ± 0.73	17.95	12.18–43.59	30	3.93 ± 0.12	3.80	1.90–8.58	4/6	2	—	3	1	—	17/15	13–2–0
VI	18.25 ± 0.33	17.31	11.54–30.13	10	3.82 ± 0.08	3.79	1.93–6.46	2/4	—	—	2	2	—	12/12	12–0–0
q_6 I	22.73 ± 0.53	22.11	12.18–37.82	5	4.88 ± 0.12	4.95	1.96–8.71	2/2	2	—	—	—	—	2/2	2–0–0
II	24.33 ± 1.03	20.19	11.54–56.41	19	4.90 ± 0.22	3.83	2.42–11.34	4/4	2	—	—	2	—	3/3	3–0–0
III	29.08 ± 1.95	19.23	12.82–101.92	29	4.68 ± 0.25	3.87	2.56–20.68	5/8	4	—	1	3	—	30/30	30–0–0
IV	19.29 ± 0.18	19.23	14.74–23.08	—	3.55 ± 0.05	3.71	2.50–5.04	6/8	3	—	4	1	—	39/35	33–3–0
V	20.35 ± 0.50	19.23	12.82–46.15	2	4.41 ± 0.13	3.86	2.56–12.31	7/7	1	—	5	1	—	8/8	8–0–0
VI	15.63 ± 0.20	15.38	12.18–23.72	—	3.18 ± 0.06	3.17	1.97–6.44	2/3	—	—	2	1	—	5/5	5–0–0
q_7 I	22.05 ± 1.22	17.95	14.74–80.13	5	4.20 ± 0.13	3.84	2.55–8.54	3/3	2	1	—	—	—	5/5	5–0–0
II	28.47 ± 1.63	23.08	16.03–91.66	10	4.98 ± 0.12	4.49	3.08–9.29	10/12	6	—	4	2	—	5/6	4–1–0
III	32.57 ± 1.16	28.85	15.38–62.82	7	5.41 ± 0.14	5.13	3.07–10.10	3/4	—	—	1	3	—	26/24	22–2–0
IV	17.44 ± 0.48	16.67	12.18–46.15	—	3.75 ± 0.08	3.80	2.53–5.78	14/20	—	—	11	9	—	43/41	39–2–0
V	20.67 ± 1.07	18.59	11.54–73.72	3	4.26 ± 0.20	3.80	1.92–18.04	9/13	4	—	6	3	—	17/16	15–1–0
VI	16.01 ± 0.27	16.03	11.54–27.56	—	3.25 ± 0.06	3.18	1.94–5.10	7/11	4	—	2	4	1r	12/12	12–0–0
q_8 I	15.26 ± 0.23	15.38	10.26–21.15	5	3.12 ± 0.07	3.18	1.83–5.12	2/3	—	—	2	1	—	7/7	7–0–0
II	15.70 ± 0.35	14.74	10.90–28.85	13	3.23 ± 0.11	3.13	1.90–9.13	4/7	2	—	4	1	—	7/7	7–0–0
III	21.72 ± 0.41	20.51	14.74–39.10	78	4.68 ± 0.10	4.46	2.52–7.74	5/10	—	—	7	3	—	31/35	28–0–0
IV	83.70 ± 3.13	89.10	19.59–137.18	99	10.96 ± 0.46	9.47	4.37–22.71	6/9	2	—	4	3	—	47/53	43–2–2
V	25.71 ± 0.89	22.44	14.10–66.67	81	4.49 ± 0.10	4.46	2.54–7.57	4/6	—	—	5	1	—	16/16	16–0–0
VI	15.13 ± 0.24	14.74	10.90–23.72	6	3.15 ± 0.05	3.14	2.44–5.68	15/15	7	—	4	4	—	13/13	13–0–0
q_9 I	19.58 ± 0.22	19.87	12.18–23.08	5	4.29 ± 0.06	16.55	10.23–20.07	3/3	2	—	1	—	—	3/3	3–0–0
II	18.42 ± 0.28	17.95	14.74–35.26	4	3.80 ± 0.08	3.73	2.42–7.30	7/11	10	—	1	—	—	4/4	4–0–0
III	20.68 ± 0.84	17.95	11.54–59.61	20	5.34 ± 0.15	5.05	3.16–11.73	9/12	3	1	5	3	—	20/15	11–3–1
IV	22.65 ± 0.45	21.79	14.74–35.26	36	4.54 ± 0.11	4.43	2.47–7.04	10/19	—	2	13	3	1r	42/40	38–2–0
V	30.16 ± 1.91	25.00	12.82–135.25	63	5.04 ± 0.23	4.50	2.45–18.17	18/23	2	—	17	4	—	23/23	23–0–0
VI	17.59 ± 0.38	17.31	12.18–41.02	6	3.96 ± 0.11	3.84	2.52–8.92	7/2	—	—	14	6	—	13/13	13–0–0
q_{10} I	27.12 ± 0.59	26.28	16.03–39.10	3	4.65 ± 0.10	4.46	2.50–7.03	2/2	2	—	—	—	—	4/4	4–0–0
II	24.13 ± 0.50	23.08	17.95–55.77	2	4.43 ± 0.10	4.44	2.56–8.83	4/6	—	1	2	3	—	6/5	4–1–0
III	15.19 ± 0.15	14.74	12.82–21.15	—	3.14 ± 0.05	3.19	1.89–4.96	5/6	3	—	1	2	—	19/19	19–0–0
IV	16.38 ± 0.23	16.03	12.82–26.28	—	3.60 ± 0.07	3.80	2.51–5.73	5/7	2	—	4	1	—	34/32	30–2–0
V	21.54 ± 0.53	20.83	13.46–41.67	1	4.18 ± 0.09	3.87	2.49–6.47	5/7	1	—	5	1	—	14/15	13–1–0
VI	15.79 ± 0.21	15.38	11.54–23.72	—	3.08 ± 0.05	3.15	1.89–4.43	7/9	3	—	5	1	—	8/8	8–0–0

Note. Blood samples were collected before radiotherapy (I), in the course of radiotherapy (II–IV), as well as 6 months (V), and 12 months (VI) after radiotherapy. In the alkaline comet assay 100 comets per each sample were measured; analysis of structural CA was performed on 100 first *in vitro* metaphases per sample; incidence of micronuclei (MN) was evaluated on 500 binuclear cells per sample. Abbreviations: TNM – classification with respect to tumor (T) size, the presence or absence of nodes metastasis (N) and metastasis (M); Irr% – the percentage of irradiated body volume; S.E. – standard error; Med. – median; LTN – long tailed nuclei; ΣaC – the total number of cells with CA; ΣCA – the total number of chromosome aberrations per 100 metaphases; b_1 – chromatid break; b_2 – chromosome break; af – acentric fragment; dc – dicentric chromosome; tc– tricentric chromosome; r – ring chromosome; ΣBN – the total number of binuclear cells with micronuclei; ΣMN – the total number of micronuclei per 500 binuclear cells.

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Table 4. The comparison between the two cohorts of breast cancer patients (mastectomy vs. quadrantectomy).

		Mastectomy				*Quadrantectomy*			
Parameter	*Sample*	*Mean*	*S.E.*	*Med.*	*Range*	*Mean*	*S.E.*	*Med.*	*Range*
Tail length (μm)	I	22.79	3.08	20.54	15.77–49.87	20.88	1.13	20.63	15.26–27.12
	II	24.80	3.17	23.13	18.21–52.67	22.31	1.22	23.13	15.70–28.47
	III	26.11	2.87	22.83	17.51–45.31	22.46	1.72	21.33	15.19–32.57
	IV	20.56	1.58	18.73	15.94–30.98	27.96	6.38	21.21	16.38–83.70
	V	18.52	1.59	17.15	15.12–30.72	22.52*	1.16	21.12	17.72–30.16
	VI	19.34	1.31	18.21	14.86–24.61	16.07	0.34	15.85	14.67–18.25
Tail moment	I	4.42	0.34	4.17	3.39–7.03	4.17	0.19	4.18	3.12–4.93
	II	4.91	0.42	4.64	3.67–8.38	4.34	0.20	4.55	3.23–5.00
	III	5.10	0.34	5.03	3.48–6.84	4.55	0.23	4.56	3.14–5.41
	IV	4.37	0.15	4.56	3.48–4.84	5.10	0.70	4.38	3.55–10.96
	V	4.06	0.29	3.98	3.05–5.90	4.40	0.16	4.33	3.70–5.14
	VI	3.77*	0.17	4.01	2.95–4.37	3.31	0.10	3.21	2.90–3.96
No. of CA	I	2.80	0.39	3.00	1.00–4.00	2.50	0.37	2.50	1.00–5.00
	II	6.50	0.75	6.00	3.00–12.00	6.10	0.99	5.00	2.00–12.00
	III	13.10	2.50	10.50	9.00–35.00	9.40	0.98	10.00	4.00–14.00
	IV	18.50	4.34	13.00	5.00–48.00	11.10	1.55	9.50	5.00–20.00
	V	11.89	2.42	10.00	4.00–28.00	9.20	1.72	7.00	5.00–23.00
	VI	10.67	2.54	9.00	2.00–28.00	8.00	1.81	5.00	3.00–20.00
No. of af	I	1.00*	0.21	1.00	0–2.00	0.60	0.27	0.00	0–2.00
	II	2.80	0.55	2.50	1.00–6.00	2.20	0.47	2.00	0–4.00
	III	6.80*	0.70	7.00	4.00–11.00	4.30	0.93	4.50	1.00–10.00
	IV	10.90	2.10	9.50	2.00–22.00	6.40	1.09	5.00	3.00–13.00
	V	8.00	2.62	6.00	1.00–27.00	5.40	1.38	5.00	2.00–17.00
	VI	6.89	2.02	5.00	1.00–21.00	3.90	1.19	2.50	1.00–14.00
No. of dc	I	0.20	0.13	0.00	0–1.00	0.20	0.13	0.00	0–1.00
	II	0.70	0.33	0.00	0–3.00	1.50*	0.34	1.50	0–3.00
	III	3.00	0.52	3.00	0–5.00	2.50	0.43	3.00	0–5.00
	IV	4.70	1.39	3.00	1.00–13.00	2.80	0.85	2.00	0–9.00
	V	3.11	0.93	3.00	1.00–10.00	1.70	0.40	1.00	0–4.00
	VI	2.44	0.65	2.00	0–7.00	2.30	0.58	2.00	0–6.00
No. of MN	I	5.20*	0.47	5.00	3.00–8.00	3.50	0.48	3.00	2.00–7.00
	II	8.10*	1.47	7.00	5.00–21.00	4.50	0.52	4.00	2.00–7.00
	III	21.90	3.33	19.50	8.00–37.00	20.00	2.62	19.50	8.00–35.00
	IV	31.90	2.50	33.00	18.00–45.00	34.20	3.62	36.50	13.00–53.00
	V	12.67	1.32	14.00	4.00–17.00	13.80	1.74	15.50	6.00–23.00
	VI	7.56	0.94	8.00	4.00–12.00	7.80	1.36	6.50	2.00–13.00

Legend: S.E. – standard error; Med. – median; af – acentric fragments; dc – dicentric chromosome. Blood samples were collected before radiotherapy (I), in the course of radiotherapy (II–IV), as well as 6 months (V), respectively 12 months (VI) after radiotherapy. *– statistically significant differences (Mann–Whitney U-test, $P < 0.05$).

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DNA damage than in the previous sampling. The values of the comet parameters recorded after the administration of the last fraction of radiotherapy indicate a possibility of the adaptive response. Six months after the radiotherapy the levels of DNA damage were reduced, but mostly did not reach the pre-therapy value. We also noticed a significant positive correlation between the age and tail moment ($P < 0.05$).

The frequency of LTN gradually decreased after the radiotherapy (Fig. 1a). In majority of subjects a decline of primary DNA damage was evident in blood samples taken one year following the radiotherapy. High levels of DNA damage recorded in some subjects were possibly affected by radiological diagnostic procedures performed before the last blood sampling. A significant positive correlation ($P < 0.05$) between the body mass index and the comet parameters indicated that better physical conditions in older women during the post-radiation period significantly contributed to the lower levels of DNA damage in their white blood cells.

The comparison of the results for different sampling times by Friedman ANOVA revealed statistically significant differences: tail length $P < 0.001$; LTN $P = 0.048$; tail moment $P = 0.003$.

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Analysis of structural CA

Patients irradiated after mastectomy

Individual results of the analysis of structural chromosome aberrations (CA) in peripheral blood lymphocytes are listed in Table 2. Before radiotherapy patients had 1 to 4 structural CA (mostly chromatid breaks and acentric fragments) per 100 metaphases. The administration of radiotherapy caused an increase of chromosomal damage (especially acentric fragments and dicentric chromosomes) in all patients. In the middle of the radiotherapy cycle an additional increase of chromosomal damage was observed in the majority of patients. In two of them we detected ring chromosomes. We observed considerable individual differences in chromosome damage among the patients. Incidence of structural CA and the frequency of aberrant cells were highest after the administration of the last fraction of irradiation. In next six months chromosomal damage was reduced in majority of patients and continued to drop (Table 2; Fig. 1b).

Friedman ANOVA confirmed highly significant ($P < 0.001$) individual differences between the time-defined measuring points for the total number of CA, the frequency of acentric fragments, the frequency of dicentric chromosomes, the total number of cells with CA and the frequency of cells with > 1 CA. Furthermore, for the frequency of cells with 1 CA $P = 0.049$. The differences between the pairs of measurements were not significant after adjusting the α-level (Wilcoxon matched pairs test).

Patients irradiated after quadrantectomy

As reported in Table 3, in this group of patients we obtained comparable results. In pre-therapy samples a significant positive correlation ($P < 0.05$) between the age and the incidence of acentric fragments was noticed. The administration of the first fraction of irradiation caused chromosomal damage in all patients, which continued to increase in the middle of the radiotherapy cycle. At this time we also found an increased frequency of cells which contained more then one structural CA. The number of structural CA and the frequency of aberrant cells were the highest after the administration of the last fraction of irradiation. Six months after the radiotherapy, the levels of chromosomal damage in the majority of patients was reduced and continued to drop in the next period.

Friedman ANOVA confirmed highly significant ($P < 0.001$) differences between the time-defined measuring points for the total number of aberrations, the total number of acentric fragments, the total number of cells with CA and the frequency of cells with >1 CA. For dicentric chromosomes $P = 0.002$, while for the frequency of cells with 1 CA $P = 0.084$. The differences between the pairs of measurements were not significant after adjusting the α-level (Wilcoxon matched pairs test). Figure 1b displays the incidence of cells with CA in blood samples analyzed during the one-year period.

Cytokinesis-block micronucleus assay

Patients irradiated after mastectomy

Individual results of the CBMN assay on peripheral blood lymphocytes are listed in Table 2. Pre-therapy values ranged between 3–8 micronuclei per 500 binuclear cells. Most of the binuclear cells contained only one MN. The frequency of MN increased in almost all patients after the administration of the first fraction of irradiation. In the middle of the radiotherapy cycle we observed a significant increase in the total number of MN compared to the previous sampling and a higher rate of binuclear cells with more than 1 MN. The incidence of MN and the frequency of micronucleated cells were the highest in blood samples collected after the administration of the last fraction of irradiation. Six months after the radiotherapy, the number of MN decreased in the majority of patients. At this time we also recorded a significant positive correlation ($P < 0.05$) between the frequency of tricentric chromosomes and the cells that contained two MN. Although the incidence of MN was reduced in next months, one year after the radiotherapy it did not return to pre-therapy level in the majority of patients.

Friedman ANOVA test showed a statistically significant difference ($P < 0.001$) between the time-defined measuring points for the total number of binuclear cells with MN, the total number of MN, the total number of cells with 1 MN and the total number of cells with 2 MN. The differences between the pairs of measurements were not significant after adjusting the α-level (Wilcoxon matched pairs test). Figure 1c displays the incidence of binuclear cells with MN in blood samples analyzed during the one-year period.

Patients irradiated after quadrantectomy

Individual results of the CBMN assay on peripheral blood lymphocytes are listed in Table 3. Before radiotherapy, binuclear cells in all subjects contained only one MN, while total number of MN ranged between 2–7 MN/500 cells. Application of the first fraction of irradiation caused an increase of MN frequency. We found a significant positive correlation ($P < 0.05$) between frequency of cells with 2 MN and comet parameters, as well as between the number of cells that contained 1 MN and tricentric and ring chromosomes. Total number of micronucleated cells was also in significant positive correlation with total number of CA, indicating that an excess of CA resulted in MN. The same trend was observed up to the end of radiotherapy cycle. In all samples analyzed in the course of radiotherapy (except after the administration of first fraction) we found a significant positive correlation ($P < 0.05$) between age and the

incidence of MN or cells that contained MN. In samples analyzed six months and one year after radiotherapy we observed a gradual decrease of MN frequency, accompanied by a lower incidence of cells that contained more than 1 MN.

Friedman ANOVA confirmed a statistically significant difference ($P < 0.001$) between the time-defined measuring points for the total number of binuclear cells with MN, total number of MN, total number of cells with 1 MN and total number of cells with 2 MN. The differences between the pairs of measurements were not significant after adjustment of the α-level (Wilcoxon matched pairs test). The incidence of binuclear cells with MN in blood samples analyzed during the one-year period is shown on Figure 1c.

Comparison between two cohorts (mastectomy vs. quadrantectomy)

An overall comparison between two cohorts also revealed interesting results (Table 4). Patients irradiated after mastectomy were older (median age: 77 years), compared with patients irradiated after quadrantectomy (median age: 64 years) and soon after the end of radiotherapy one of them died. Older patients mostly had higher values of the comet parameters. However, 6 months after the termination of therapy, quadrantectomised patients had significantly increased comet tail length ($P = 0.007$) and LTN ($P = 0.013$) (Table 4). As displayed on Figure 1a, this cohort of patients also had a much broader range of highly damaged cells (LTN) during the study, which was not surprising, since they received more fractions and a higher dose of irradiation. In contrast, 1 year after the radiotherapy patients irradiated after mastectomy had a significantly higher value of tail moment ($P = 0.018$) (Table 4), which was possibly influenced by radiological diagnostic procedures performed before the last blood sampling.

Older patients irradiated after mastectomy had significantly higher frequency of acentric fragments prior to radiotherapy ($P = 0.048$) and in the middle of the radiotherapy cycle ($P = 0.047$) (Table 4). On the contrary, patients irradiated after quadrantectomy had significantly higher frequency of dicentric chromosomes in samples analyzed after the administration of the first fraction of irradiation ($P = 0.028$).

Baseline frequency of MN and binuclear cells that contained MN were significantly higher in thr cohort of older patients irradiated after mastectomy ($P = 0.013$). After the administration of the first fraction of irradiation both values remained significantly higher as compared to the other cohort studied ($P = 0.024$ frequency of MN; $P = 0.048$ frequency of BN cells with MN). It was also observed that their frequency of BN cells with 1 MN also significantly increased ($P = 0.038$).

Discussion

Various risk and prognostic factors related with the onset of breast cancer were described. Nevertheless, the inclusion of any additional biomarker, able to indicate an increased individual risk, maybe potentially helpful in medical monitoring of cancer patients, following the surgery and/or radiotherapy.

The present study was aimed to evaluate the usefulness of the results obtained by the alkaline comet assay and cytogenetic biomarkers in the follow-up of post-menopausal breast cancer patients before, during and after the radiotherapy. The strengths of this study were that it was population based and prospective, with the same subjects being followed continuously over the one-year period. In addition, selection of appropriate biomarkers enabled a simultaneous assessment of the actual levels of DNA damage present in white blood cells at the moment of blood sampling (detectable by the comet assay) together with the fraction of DNA damage that resulted in fixed mutations, which persist at least one mitotic cycle (detectable by the analysis of structural chromosome aberrations and micronuclei).

We observed that pre-therapy levels of DNA damage in peripheral blood leukocytes of cancer patients were substantially different. In some patients DNA damage was comparable to 'normal' values recorded in the healthy population[21], while in others it was notably increased, and even doubled, compared to healthy control subjects. Since DNA damage detected by the alkaline comet assay represents a steady state between the induction of lesions and their repair, the lower damage level in an individual may be the result of an actually lower number of lesions or of a high efficiency of repair. One part of the individual variation is certainly related to age. Previous biomonitoring studies with the same endpoints also reported age-related increase of DNA/cytogenetic damage in white blood cells.[22–25] Background DNA damage in cancer patients was also influenced by inherited biological factors, as well as recent and previous medical exposures. Moreover, older subjects typically have more disease states, take more medication, experience more drug interactions, have more variability in the nutritional status and in the underlying chronic health status. Other authors who applied the same biomarkers in monitoring of cancer patients, reported similar results and found that the presence of malignant tumors itself caused significantly increased levels of DNA damage as compared to the healthy population.[3,5,8,26]

Adjuvant radiotherapy is an important treatment modality after surgery for breast cancer. As observed in the present study, the administration of radiotherapy critically influenced the levels of DNA damage in white blood cells of breast cancer patients, as detected by all endpoints used. It was not surprising, because ionizing radiation causes a wide variety of DNA damage, ranging from single- and double-strand breaks in DNA, as well as DNA base modifications, oxidative damage and alkali-labile lesions. Besides

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direct ionization of DNA, ionizing radiation also causes indirect ionization when free radicals, formed as a result of the ionization of oxygen, damage the DNA. In the most common forms of radiation therapy, the largest part of the radiation effect is by free radicals. All the above mentioned lesions may be sensitively detected by the alkaline comet assay and cytogenetic techniques as used here.

In the course of the study we separately monitored patients irradiated after mastectomy and those irradiated after quadrantectomy. Generally speaking, in both studied cohorts the levels of the genome damage gradually increased until the end of the radiotherapy cycle. Later on, the levels of DNA/cytogenetic damage slowly declined during one year post-irradiation period; although in the majority of subjects they did not return to pre-therapy levels. However, one of the most important observation of our study was that older breast cancer patients irradiated after mastectomy had higher values of almost all parameters evaluated (although not all differences were considered statistically significant). This observation of the more susceptible cohort of patients showed an increased risk that has to be carefully monitored during the post-irradiation period. Our findings are also in agreement with the reports of other authors who investigated the impacts of radio- or chemotherapy on non-target cells in breast cancer patients.[8,9,27] Previous investigations have shown that following radiotherapy patients show a wide variation in response of both tumor and normal tissues.[27,28] The similar was observed in this study. The observed differences in response to radiation certainly depended on different doses, types and duration of the radiation exposure, intrinsic biological factors[29], the rate of appearance of new lymphocytes in peripheral blood[30], but also on the sensitivity of the biomarker employed.

The present study confirmed the usefulness of the comet assay in the follow-up of irradiated patients. Using that biomarker, we were able to show differences in the individual sensitivity to therapeutic irradiation. In addition to two well-established comet parameters (tail length and tail moment), the long-tailed nuclei (LTN) were also proved a useful indicator of individual DNA damage and/or response to radiation. Our study showed a significant contribution of LTN or highly damaged comets with exceedingly long tails in interpreting the comet test results. The values of this parameter were in significant correlation with the sampling times and received radiation doses, and notably decreased a year after the treatment. The importance of this biomarker was also recognized in other studies.[10,19,21]

We also found positive correlations between the increased levels of primary DNA damage, as detected by comet assay and chromosome aberrations and micronuclei, originated from unrepaired DNA lesions. According to literature, the response to radiotherapy is generally characterized by the increase in CA and MN frequency in lymphocytes, which, after reaching their peak, gradually decrease over the next few weeks, most probably with the clearance of severely damaged cells or because these cells may not complete mitosis.[31] Literature brings evidence of the stability of genome damage in somatic cells of patients who had a long survival after the successful chemo/radiotherapy. Aberrations detected in these patients are mostly balanced rearrangements, probably without greater functional importance. However, chromosome breaks may occur at the loci of important genes which then remain modified in the target tissue. These sites are important as potential sources of new neoplastic transformations.[32]

In our study a significant increase in the frequency of dicentric chromosomes and acentric fragments was detected shortly (within two hours) after the administration of the first fraction of irradiation. Dicentric chromosomes, as typical radiation exposure biomarkers, are in correlation with the appearance of translocations, stable chromosome aberrations, which are the basis for secondary neoplasms. The elimination of dicentric chromosomes observed in this study is in accordance with the results described elsewhere. Although the studies differ somewhat in sampling times after radiation, the increase in dicentrics is similar, just as the results after one year, with evident individual differences in eliminating dicentrics in both studies. Huber et al.[33]also investigated translocation frequency and observed significant individual differences in the dynamics of their appearance and disappearance. Obviously, there is a need for further research of the causes which lead to this variability.

The results obtained by CBMN assay in this study also correlated well with the levels of the recorded primary and chromosomal damage. The cytokinesis-block micronucleus assay in human lymphocytes is one of the most commonly used methods for measuring DNA damage because it is relatively easier to score micronuclei than chromosome aberrations. Micronuclei originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and therefore lag behind when the cell divides.[17] Therefore, the MN assay may be used to show both clastogenic and aneugenic effects. The distinction between these phenomena is important, since the exposure studied often induces only one type of MN. The proportion of centromere-positive MN in human lymphocytes increases with age, which primarily reflects an age-dependent micronucleation, especially of female X chromosome.[34]

An increased micronucleus frequency in older women reported also other authors.[35] Nevertheless, among cancer patients an elevated frequency of both acentromeric and centromeric micronuclei was frequently reported.[3,28] The data gathered in the present CBMN study indicate that the administration of adjuvant radiotherapy in patients with breast carcinoma caused a significant increase of MN frequency in their peripheral blood lymphocytes until the end of the radiation cycle. Later on the rate of MN gradually decreased and a year after the end of the radiotherapy in the majority of patients it returned to the similar, or slightly higher level as their pre-therapy values. These results were in accordance with earlier CBMN studies on untreated and

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therapeutically exposed cancer patients, and confirmed the usefulness of the method as an important biomarker for genomic instability and cancer risk prediction.

Regardless of the benefits of radiotherapy, therapeutic exposure to ionizing radiation may also lead to the induction of secondary cancers in the treated area. In order to spare interstitial tissue (such as skin or organs which radiation must pass through in order to treat the tumor) several angles of exposure are utilized such that the radiation beams overlap on top of each other at the tumor, providing a much larger absorbed dose there than in the surrounding, healthy tissue.[12]

The presence of significantly increased levels of DNA damage, as caused by ionizing radiation, is 'desirable' in cancer cells, but not in other non-target cells. Most genome damage caused by ionizing radiation is basically repairable and the majority of lesions are successfully repaired in a relatively short time after the exposure.[18] The most cytotoxic lesions are double-strand breaks (DSBs). It is now accepted that misrepaired DSBs are the principle lesions of importance in the induction of both chromosomal abnormalities and gene mutations.[36] Some cells die due to the high level of damage while unstable and stable genome damage remains longer in some cells. Cells with stable genome damage may be detected decades after radiotherapy and are a potential source for the development of secondary tumors.[37]

The results of our study show the importance of applying biomarkers to obtain data on the actual risk for individuals exposed to radiation, especially in relation to the allocation and volume of radiation exposure. An important advantage of cytogenetic biomarkers is that they measure individual DNA damage summarizing total biological effects, including all external and intrinsic factors of each person. By applying genotoxicological biomarkers we may also predict qualitative changes on the genome level as a consequence of radiation. The observed individual differences may be detected in persons with inherited genome instability. Early detection of repair-deficient patients may provide arguments for a stricter follow-up and prevention in the management of many human cancers.

Thus, in order to reduce the potential risk of developing secondary neoplasms after radiation therapy, some kind of continuous biomonitoring of successfully treated patients would be required, especially by introducing sensitive techniques, such as the alkaline comet assay and the cytogenetic methods employed in the present study, but also fluorescent *in situ* hybridization, which maydetermine the frequency of stable genome damage induced *in vivo* by radiotherapy. Generally speaking, radiation induced DNA damage and genomic instability increase with absorbed dose. However, at low doses the reduction of damage from endogenous sources by adaptive protection may be equal to or outweigh radiogenic damage induction[38]. We observed that extended exposure to small doses of ionizing radiation during the course of radiotherapy caused some kind of adaptive response in leukocytes in the majority of treated patients. For that reason, the findings of the present study may be also associated with the "hormesis theory." This refers to the effects of low level ionizing radiation (generally in the range of 1–50 cGy) which stimulate metabolic detoxification and repair networks, resulting in beneficial effects on the cell, organism or species.[39] This phenomenon was well-described by other radiobiologists.[38,40–42] Small acute single doses of ionizing radiation produce DNA damage (among them also harmful double-strand breaks) in a very short time. Under normal conditions cells have to cope with almost 10^6 damages due to reactive oxygen species produced by normal metabolic activity.[36] The DNA double-strand breaks induced by an acute, low radiation dose may be sufficient to activate the induced resistance, which may protect cells even against the damage due to metabolism.

Contrary to the immediate initiation of repair after the DNA damage has occurred, the adaptive protection develops as a physiological stress response relatively slowly within a few hours and may last from several weeks to months.[38] Our results, especially those obtained by the alkaline comet assay, also sustain those reports.

The adaptation induced by low doses of radiation is attributed to the induction of efficient chromosome break repair mechanisms,[38,43] but also to a temporary inhibition in the DNA synthesis which provides a longer time for irradiated cells to recover.[44]

It also may stimulate different free radical scavengers, glutathione and superoxide dismutase, so irradiated cells would be more resistant to any further exposures. As known, stimulation of detoxification of ROS appears to reach the maximum at about 4 h after irradiation and lasts for several hours or even weeks, depending on tissue and cell types.[38]

Previous investigations also indicate that the human population exhibits heterogeneity in the adaptive response to ionizing radiations that might be, at least in part, genetically determined.[45] Protection against high-dose induced chromosomal aberrations and/or micronuclei formation in human lymphocytes varied between individuals and cell types; it was absent in some individuals and is probably genetically determined. This adaptive response probably involves a several-fold enhancement of the DNA repair rate.[38]

In our study we did not measure the DNA repair processes directly, but the outcomes of all endpoints used were in accordance with previous surveys.

Conclusions

The present study reveals information that might be potentially interesting to the medical researchers working in the area of radiotherapy. First, we found positive correlations between the comet assay parameters and cytogenetic biomarkers that confirmed their complementary

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value in the assessment of radiation sensitivity/ susceptibility among elderly breast cancer patients. Such results show that the responses to irradiation should be studied on individual basis. It was also established that the studied biomarkers might reveal additional data potentially useful in regular medical monitoring of cancer patients after radiotherapy. Finally, we observed that the prolonged exposure to ionizing radiation during the course of radiotherapy caused some kind of adaptive response in peripheral blood leukocytes in the majority of treated patients. This findings support previous observations, but also calls for additional investigation to further elucidate the underlying mechanisms.

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