Coll. Antropol. **31** (2007) 3: 837–845
Original scientific paper

Evaluation of DNA Damage in Radiotherapy-Treated Cancer Patients Using the Alkaline Comet Assay

Marija Gamulin[1], Vera Garaj-Vrhovac[2] and Nevenka Kopjar[2]

[1] Department of Oncology, University Hospital, Zagreb, Croatia
[2] Mutagenesis Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia

A B S T R A C T

The aim of this study was to evaluate primary DNA damage and the dynamics of the repair of radiotherapy-induced DNA lesions in non-target cells of cancer patients. This study included patients diagnosed with different solid tumors who received radiotherapy. The levels of DNA damage were evaluated using the alkaline comet assay on peripheral blood leukocytes. Altogether four blood samples per patient were collected: before and after receiving the first dose of radiotherapy, in the middle of radiotherapy cycle, and after the last dose of radiotherapy. The results indicate that after the first radiation dose significantly increased levels of DNA damage were recorded in almost all cancer patients compared to their baseline values. Specific patterns of DNA damage were recorded in samples analyzed in the middle of radiotherapy and after receiving the last dose, indicating the possibility of adaptive response in some patients. Our results indicate that persistence of post-irradiation damage in peripheral blood leukocytes (and possibly in other non-target cells) of cancer patients that are strong determinants for the secondary cancer risk. Moreover, the alkaline comet assay was confirmed as a rapid and sensitive assay for the assessment of genome damage after in vivo irradiation.

Key words: *cancer, comet assay, leukocytes, DNA damage and repair*

Introduction

The recent literature from the field of clinical oncology and radiotherapy still does not explain sufficiently the question of the ratio of damages to non-tumor cells and tissues after radiation treatment. Ionizing radiation is a proven mutagen that, besides damaging tumor tissue, also caused genome damage in other cells. Genome damage caused by radiation or other mutagens is the most frequently monitored in peripheral blood lymphocytes[1]. In the lymphocytes of oncological patients treated with radiotherapy it is extremely important to study the processes of DNA repair as well as qualitative and quantitative changes in various cytogenetic biomarkers. All these factors may indicate the individual (over) sensitivity to radiotherapy and a potential risk for the appearance of secondary tumors. Research has undoubtedly shown that individual cytogenetic and molecular-biological tests are useful indicators or biomarkers, applicable for short-term[2–5] or long-term cytogenetic monitoring in oncological patients[6–8]. The results of previous cytogenetic studies indicate the increase of the frequency of unstable chromosome aberrations and the increase in the number of micronuclei in peripheral blood lymphocytes in oncological patients treated with radiation[9,10]. Furthermore, the results of sensitive molecular-biological tests, such as the comet assay, indicate the increase in the level of primary DNA damage in peripheral blood lymphocytes of the patients with malignant tumors in comparison with healthy population[3,11–13].

The alkaline comet assay has become popular way of detecting a variety of types of DNA damage during last decade and its usage in clinical practice also increases rapidly[14,15]. The basic principle of the comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. When viewed under microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of anode. Among them

Received for publication July 17, 2006

various versions of the assay, the alkaline (pH of the unwinding and electrophoresis buffer >13) method enables the detection of a broad spectrum of DNA damage. It can detect double- and single-strand breaks, alkali-labile sites and single-strand breaks associated with incomplete excision repair. Under certain conditions, the assay can also detect DNA-DNA and DNA-protein cross linking, as well as apoptotic and necrotic cells[16–18].

In the present study the alkaline comet assay was applied to survey the levels of primary DNA damage in peripheral blood leukocytes collected from 10 cancer patients who were post-surgically treated with primary or adjuvant radiotherapy. The objective of our investigation was also to study the dynamics of the repair of radiotherapy-induced DNA lesions in non-target cells and to estimate the potential value of the alkaline comet assay as a possible predictor of response to treatment.

Patients and Methods

Patient population

Study participants were recruited at The University Hospital for Tumors (Zagreb, Croatia), from a group of cancer patients who had not previously been treated with cytotoxic drugs or radiotherapy. The research included ten patients (four female and six male; aged 35–79 years) with solid tumors of the head and neck, prostate, uterus, lungs, breasts, brains and testes. Detailed patient data is quoted in Table 1.

In our study two patients were diagnosed with an invasive breast carcinoma G2, hormone receptor positive, HER2/neu negative, T2N1M0 (stage II). Conceptually, the adjuvant treatment of breast cancer (except lobular carcinoma [LCIS]) includes the treatment of local disease with surgery, radiation therapy (RT), or both, and the treatment of systemic disease with cytotoxic chemotherapy or hormonal therapy[19,20]. After radical operation – mastectomy and ipsilateral lymphnode dissection the patients wish was to be treated with radiotherapy and subsequent hormonotherapy with tamoxifen. Radiotherapy was done with direct field to the chest whole and supraclavicular region with the total tumor dose of 45 Gy in 18 daily fractions (Figure 1.).

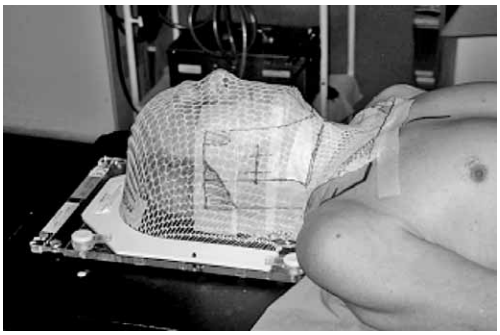

Fig. 2. Carcinoma of the oropharynx – lateral and anterior portals for treating patient with primary tumor and cervical lymph nodes.

Two patients had a squamous cell carcinoma T2 N1 M0 (Stage III) of the oropharynx, which is extremely rich in lymphatics. Postoperative irradiation is recommended based on the tumor stage, tumor histology, and surgical findings after tumor resection. One of them was treated with adjuvant radiotherapy with a total tumor dose of 66 Gy in 33 daily fractions after operation – excision of the primary tumor and the unilateral neck dissection (Figure 2.). Higher doses of radiation (>60 Gy) are required for microscopic disease to decrease the chances of locoregional failure because of interruption of the normal vasculature, scarring, and relative hypoxia in the tumor bed. The other patient was treated with primary radiotherapy (RT) after a tumor biopsy with a total tumor dose of 70 Gy in 35 daily fractions, with 2 opposite lateral field and one direct field to the neck, because he wanted to be treated with radiotherapy only[19,21].

Two patients had an adenocarcinoma of the prostate T2 N0 M0, Gleason score 6 and PSA 10 ng/mL (Stage II). The combination of the Gleason score, PSA level and stage can effectively stratify patients into categories associated with different probabilities of achieving a recovery. The primary treatment options for initial therapy for localized prostate cancer includes radical prostatectomy or radiotherapy[19,22]. After prostate biopsy our patients were treated with primary curative radiotherapy with a total tumor dose of 70 Gy in 35 daily fractions with 4 radiotherapy fields.

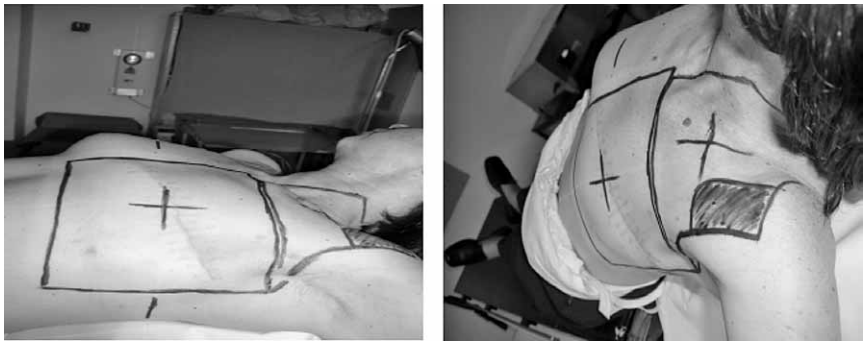

Fig. 1. Example for irradiation of the chest wall and regional lymphatic portal.

One patient was diagnosed with endometrial cancer G3 stage IIIA. This form of cancer is usualy treated with pelvic RT with or without vaginal brachytherapy, whole abdominopelvic radiotherapy[19,23]. Our patient was treated with pelvic RT 50 Gy in 25 daily fractions with 2 opposite fields and with vaginal brachytherapy.

One patient was diagnosed with partial operabile patohistology brain meningioma. Meningioma is a benign tumor, usually with a well-defined plane separating them from the surrounding brain parenchyma. In general, total extirpation is the therapy of choice. But subtotal resection or recccurent tumor needs to be treated with radiotherapy[19,24]. Our patient was treated with fractionated external beam radaition therapy – 2 opposite fields to include tumor volume and the free margins with total tumor dose 60 Gy in 30 daily fractions.

One patient was diagnosed with unresectabile non-small cell lung cancer T4N0M0 (stage IIIB). This form of cancer is usualy treated with radiotherapy + chemotherapy[19,25]. Our patient was treated with radiotherapy because chemotherapy is contraindicated (cardiovascular disease). Radiotherapy after tumor biopsy with total tumor dose of 66 Gy in 33 daily fractions was applied in the primary tumor and regional lymph nodes.

One patient was diagnosed with seminoma. Early stage seminoma is a radiosensitive tumor. Between 15% and 20% of seminoma patients stage I, relapse during surveillance if they do not receive adjuvant radiation therapy after orchiectomy. The median time to relapse is approximately 12 months, but relapses do occur more than 5 years following. Patients with disease in stages IA, IB, and IS are treated with radiation (25–30 Gy) to the infradiaphragmatic area including para- aortic lymph nodes[19,26]. Our patient was treated with 25Gy in 16 daily fractions (Figure 3).

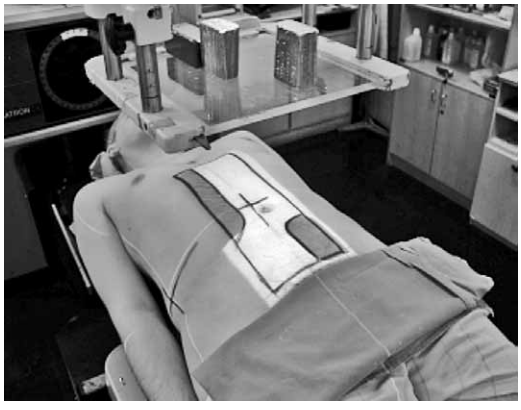

Fig. 3. Example of paraaortic and pelvic irradiation portals for treatment testicular seminoma.

Blood sampling

Blood sampling was made by venepuncture; a way of acquisition usually accepted as non-traumatic for patients. Further laboratory handlings with blood samples and all investigations have been carried out in accordance with a high standard of ethics.

Samples of venous blood (5 ml) were collected in heparinized vacutainer tubes (Becton Dickinson, N.J., USA) under sterile conditions. The current study used each cancer patient as his / her own control. Altogether four blood samples were collected from each donor. Pre-treatment blood sample (I) was collected on day 1 of the first radiotherapy cycle, two hours prior to the irradiation. Response of peripheral blood leukocytes to the radiotherapy was evaluated on blood sample taken within two hours after the application of the first dose (II), as well as in the middle of the radiotherapy cycle (III) and within 2 hours after the last received radiotherapy dose (IV).

All blood samples were handled in the same manner. After venepuncture, they were coded, cooled at +4°C in the dark and transferred in laboratory. They were processed immediately after transportation (within a maximum of one-hour period after collection) by means of the alkaline comet assay.

The alkaline comet assay

All chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA). The comet assay was carried out under alkaline conditions, basically as described by Singh et al.[27]. Fully frosted slides were covered with 1 % normal melting point (NMP) agarose. After solidification, the gel was scraped off from the slide. The slides were then coated with 0.6 % NMP agarose. When this layer had solidified a second layer containing the whole blood sample (4 μl) mixed with 0.5 % low melting point (LMP) agarose was placed on the slides. After 10 minutes of solidification on ice, slides were covered with 0.5 % LMP agarose. Afterwards the slides were immersed for 1 h in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1 % Na-sarcosinate, pH 10) with 1 % Triton X-100 and 10 % dimethyl sulfoxide added fresh to lyse cells and allow DNA unfolding. The slides were then randomly placed side by side in the horizontal gel-electrophoresis tank, facing the anode. The unit was filled with freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and the slides were set in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at 4°C under dim light. Electrophoresis was carried out for the next 20 min at 25 V (300 mA). After electrophoresis the slides were washed gently three times at 5-min intervals with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20μg/ml) and covered with a coverslip. Slides were stored at 4°C in humidified sealed containers until analysis.

To prevent additional DNA damage, handling with blood samples and all steps included in the preparation of slides for the comet analysis were conducted under yellow light or in the dark. Furthermore, to avoid possible position effects during electrophoresis two parallel replicate slides per sample were prepared. Each replicate was processed in a different electrophoretic run.

Slides were examined at 250x magnification using a fluorescence microscope (Zeiss, Germany), equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Microscope was connected through a black and white camera to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd., U.K.). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets. A total of 100 comets per subject were scored (50 from each of two replicate slides). As a measure of DNA damage in this paper tail length (calculated from the midpoint of the head and presented in micrometers) and tail moment were used.

Statistics

Statistical analyses were carried out using Statistica 5.5 software (StatSoft, Tulsa, USA). Each subject was characterized for the extent of DNA damage by considering the mean (± standard error of the mean), median and range for the comet tail lengths / moments measured. In order to normalize distribution and to equalize the variances, a logarithmic transformation of data was applied. Differences between comet parameters measured in individual blood samples were compared using a *t*-test for dependent samples. The level of statistical significance was set at $p<0.05$.

Results

Using a computer-based image analysis system, we measured DNA migration in peripheral blood leukocytes from cancer patients before and after radiotherapy. Individual data on DNA damage recorded in peripheral blood leukocytes are reported in Table 2. Box and whisker plots on Figure 4 show the distribution of the comet tail length values measured in individual patients.

The baseline DNA damage in peripheral blood leukocytes

The results indicate inter-individual differences in background, pre-therapy DNA damage in peripheral blood leukocytes of cancer patients (samples I), considering the two main parameters of the alkaline comet assay. As indicated in Table 2, DNA migration was in range from 15.73±0.19 μm (patient No. 2, with breast carcinoma) to 37.85±3.12 μm (patient no. 9, with lung cancer). Corresponding tail moment values were in range 13.11±0.19 to 34.11±2.97. Range (min.–max.) of the tail lengths measured among cancer patients was 10.90 to 117.31 μm, and range of the tail moments was 8.89 to 113.12 (Table 2). The majority of differences in pre-therapy DNA damage levels may be explained with different life-style factors as well as different sensitivity to diagnostic treatment prior to the operation and radiotherapy.

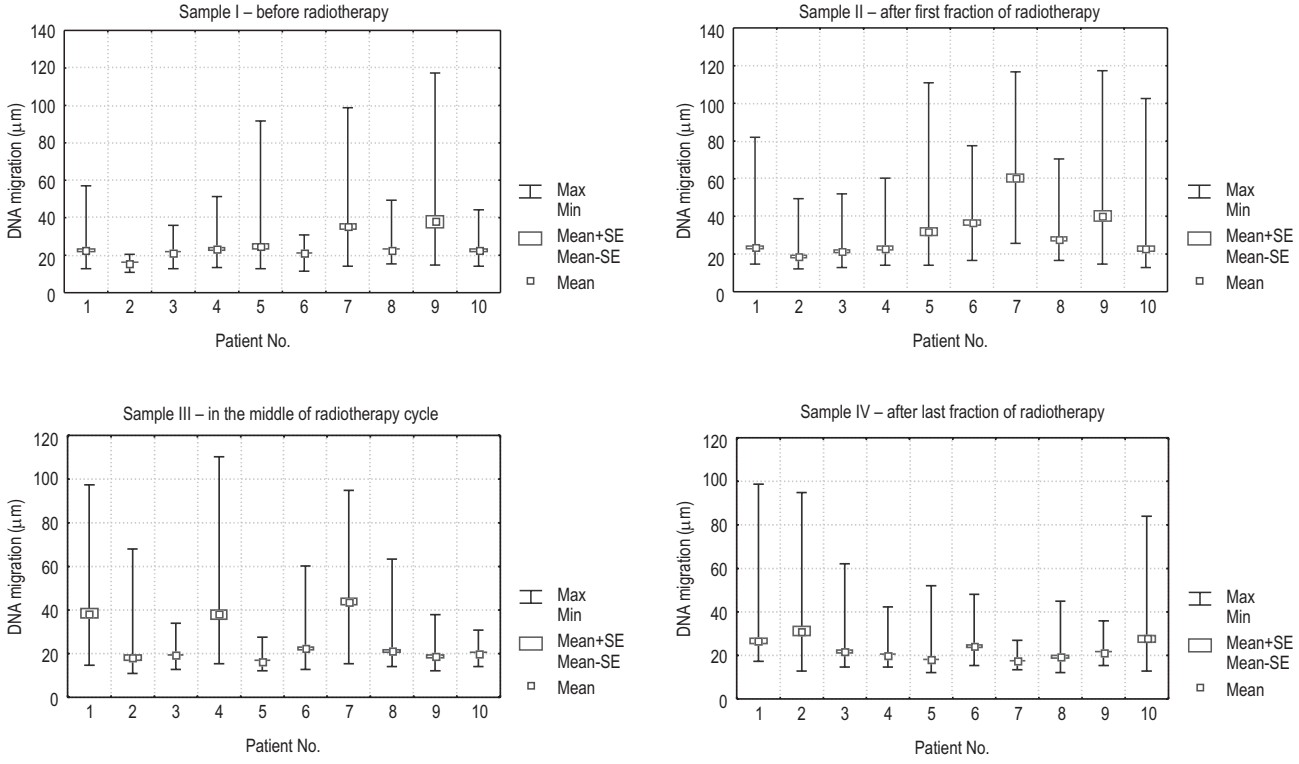

Fig. 4. Individual results of the alkaline comet assay on peripheral blood leukocytes of cancer patients (numbered as 1–10) before (sample I) and after administration of radiotherapy (samples II–IV). Box and whisker plots showing the distribution of the comet tail length values measured. DNA migration was evaluated on 100 comets per sample and expressed in micrometers.

TABLE 1
ANAMNESTIC AND CLINICAL DATA ON CANCER PATIENTS INVOLVED IN THE STUDY

Patient code	Gender Age	Smoking Alcohol	Medical exposures	Diagnosis	TNM or stage	Radiotherapy
1	F, 79	N =	mam; chest and bone X	breast carcinoma	T2N1M0	45 Gy / 18 fr.
2	F, 76	N –	mam; chest and bone X	breast carcinoma	T2N1M0	45 Gy / 18 fr.
3	M, 56	S +	chest X	carcinoma of the oropharynx	T2N1M0	70 Gy / 35 fr.
4	M, 54	S +	chest X	carcinoma of the oropharynx	T2N1M0	66 Gy / 33 fr.
5	M, 67	N –	bone scan,chest X and CT abd	adenocarcinoma of the prostate	T2N0M0	70 Gy / 35 fr.
6	M, 70	N –	bone scan,chest X and CT abd	adenocarcinoma of the prostate	T2N0M0	70 Gy / 35 fr.
7	F, 70	N –	Chest X	endometrial cancer	Stage IIIA	50 Gy / 25 fr.tt. 10 Gy / 1 fr.bt.
8	F, 69	N –	CT brain	brain meningeoma	benign tumor	60 Gy / 30 fr.
9	M, 66	S –	Chest X end CT	lung cancer	T4N0M0	66 Gy / 33 fr.
10	M, 35	S –	Chest X CT abd	seminoma	T1N0M0	25 Gy / 16 fr.

F – female; M – male; S – smoker; N – nonsmoker; TNM – Tumor Nodes Metastasis classification (NCCN 2005); T1 tumor size < 2 cm, T2 between 2 and 5 cm, T3 > 5 cm and T4 tumor of any size with direct extension to chest wall or skin; N – nodes metastasis; N0 absence, N1 presence of nodes metastasis; M – metastasis; M0 absence, M1 presence of metastasis; fr – fraction; tt – teleradiotherapy; bt – brachytherapy.

Post-irradiation DNA damage in peripheral blood leukocytes

The assessment of two comet parameters in blood samples collected after administration of the first fraction of radiotherapy (samples II) confirmed a strong positive response to the therapy ($p<0.05$, t-test for dependent samples) in almost all patients. Exceptions were patients No. 1, 3, 4 and 10 (Table 2). Among the patients studied considerable interindividual differences were observed. These differences reflect the impact of radiation doses applied, but also indicating different susceptibility and genome sensitivity. DNA migration was in range from 18.56±0.58 μm (patient No. 2, with breast carcinoma) to 60.30±2.17 μm (patient no. 7, with endometrial carcinoma). Corresponding tail moment values were in range 15.69±0.56 to 54.82±2.12. Range (min.–max.) of the tail lengths measured among cancer patients was 12.18 to 117.31 μm, and range of the tail moments was 9.65 to 108.46 (Table 2).

Specific patterns of DNA damage were recorded in blood samples analysed in the middle of radiotherapy cycle (samples III). In majority of the patients studied, a decrease of DNA damage as compared to samples II were observed. Exceptions were patients No. 1 and 3, in whose blood samples significant increase of DNA damage was observed (Table 2). Among the patients studied considerable interindividual differences were also observed. DNA migration was in range from 16.36±0.24 μm (patient No. 5, with adenocarcinoma of the prostate) to 43.950±1.67 μm (patient no. 7, with endometrial carcinoma). Corresponding tail moment values were in range 13.87±0.23 to 39.99 ± 1.64. Range (min.–max.) of the tail lengths measured among cancer patients was 10.99 to 110.25 μm, and range of the tail moments was 7.58 to 103.01 (Table 2).

The values of both comet parameters recorded in most of the blood samples collected after administration of the last fraction of radiotherapy (samples IV) indicate the possibility of the adaptive response. Namely, they did not substantially differ, or were even lower as compared to the pre-therapy values; exceptions were patients No. 1, 2, 6 and 10 (Table 2). DNA migration was in range from 17.48±0.25 μm (patient No. 7, with patient no. 7, with endometrial carcinoma) to 31.17±1.98 μm (patient no. 2, breast carcinoma). Corresponding tail moment values were in range 14.94±0.25 to 27.18±1.80. Range (min.–max.) of the tail lengths measured among cancer patients was 12.18 to 98.72 μm, and range of the tail moments was 9.87 to 92.92 (Table 2).

The results obtained indicate that radiotherapy is accompanied by significant DNA damage in peripheral blood leukocytes. Both parameters measured by the alkaline comet assay are sensitive indicators of the individual response to radiation treatment, and point to the individ-

TABLE 2
RESULTS OF THE ALKALINE COMET ASSAY ON PERIPHERAL BLOOD LEUKOCYTES OF THE CANCER PATIENTS TREATED WITH RADIOTHERAPY

Patient code	Sample No.	Radiation dose (Gy) received	DNA migration – tail length (μm)				Tail moment			
			Mean±SE	Min.	Max.	Med.	Mean±SE	Min.	Max.	Med.
1	I	0	22.89±0.72	12.82	57.05	21.15	19.53±0.65	10.33	50.71	18.15
	II	2.5	23.44±0.90	14.74	82.05	21.47	20.28±0.82	11.94	73.16	18.31
	III	22..5	38.41±2.08[b]	14.74	97.43	28.53	33.80±1.92[b]	12.23	92.14	24.74
	IV	45	26.64±1.05[c]	17.31	98.72	23.72	23.10±0.99[c]	14.16	92.92	20.47
2	I	0	15.73±0.19	10.90	20.51	15.38	13.11±0.19	8.89	17.82	13.05
	II	2.5	18.56±0.58[a]	12.18	49.36	17.31	15.69±0.56[a]	9.65	45.29	14.20
	III	22..5	18.15±1.06	10.90	67.95	14.74	15.14±1.00	7.58	63.08	11.95
	IV	45	31.17±1.98[c]	12.82	94.87	22.11	27.18±1.80[c]	10.40	81.27	19.14
3	I	0	21.40±0.50	12.82	35.90	19.87	18.02±0.45	9.85	31.05	16.70
	II	2	21.71±0.71	12.82	51.92	19.23	18.52±0.66	10.10	46.47	16.61
	III	34	19.24±0.42	12.82	33.97	18.59	16.49±0.39	10.41	26.85	15.66
	IV	70	21.83±0.80	14.74	62.18	19.23	18.89±0.73	11.89	54.76	16.72
4	I	0	23.08±0.74	13.46	51.28	21.15	19.99±0.70	10.92	46.03	18.32
	II	2	23.22±0.83	14.10	60.26	20.51	20.10±0.78	11.34	56.23	17.78
	III	34	37.89±2.01[b]	15.38	110.25	33.01	33.47±1.85[b]	12.48	103.01	28.75
	IV	70	20.27±0.47[c]	14.74	42.31	19.23	17.55±0.44[c]	11.99	38.81	16.36
5	I	0	24.25±1.39	12.82	91.66	19.87	21.08±1.25	9.81	83.82	17.26
	II	2	32.94±2.28[a]	14.10	110.90	22.11	28.21±2.17[a]	11.44	104.92	19.31
	III	34	16.36±0.24[b]	12.18	27.56	16.03	13.87±0.23[b]	8.03	24.06	13.70
	IV	70	17.94±0.51	12.18	51.92	16.67	15.32±0.48[c]	9.87	47.20	14.37
6	I	0	21.03±0.41	11.54	30.77	20.51	17.87±0.38	9.22	26.59	17.32
	II	2	36.90±1.27[a]	16.67	77.56	33.97	32.29±1.19	12.90	69.99	29.80
	III	34	22.46±0.78[b]	12.82	60.26	20.51	19.36±0.73	9.73	55.31	17.96
	IV	70	24.29±0.55[c]	15.38	48.08	23.72	21.19±0.52	12.67	43.50	20.47
7	I	0	35.36±1.46	14.10	98.72	32.37	30.52±1.38	11.93	88.75	28.17
	II	2	60.30±2.17[a]	25.64	116.66	60.58	54.82±2.12[a]	21.21	110.54	54.78
	III	26	43.95±1.67[b]	15.38	94.87	43.91	39.99±1.64[b]	12.56	90.19	39.59
	IV	60	17.48±0.25[c]	13.46	26.92	17.31	14.94±0.25[c]	10.55	23.30	14.72
8	I	0	22.90±0.57	15.38	49.36	21.79	19.69±0.52	12.85	44.23	18.59
	II	2	27.78±1.03[a]	16.67	70.51	24.36	24.33±0.97[a]	13.79	67.68	21.14
	III	30	21.10±0.65[b]	14.10	63.46	19.87	18.45±0.59[b]	11.81	56.32	17.03
	IV	60	19.50±0.63[c]	12.18	44.87	17.31	16.78±0.58[c]	9.67	39.45	14.88
9	I	0	37.85±3.12	14.74	117.31	22.44	34.11±2.97	12.57	113.12	19.41
	II	2	40.47±2.80[a]	14.74	117.31	28.53	35.49±2.56[a]	11.72	108.46	23.89
	III	32	18.72±0.48[b]	12.18	37.82	17.63	15.96±0.44[b]	10.05	33.43	15.02
	IV	66	21.29±0.36[c]	15.38	35.90	20.51	18.56±0.34[c]	12.79	32.17	18.02
10	I	0	22.91±0.77	14.10	44.23	19.87	19.57±0.70	11.12	38.53	17.11
	II	1.5	22.99±1.26	12.82	102.56	20.51	19.69±1.14	9.94	92.41	17.50
	III	12.48	20.25±0.35	14.10	30.77	19.87	17.76±0.35	11.06	28.60	17.13
	IV	26	27.69±1.50[c]	12.82	83.97	22.76	24.10±1.39[c]	10.49	46.81	19.66

100 comets per each sample were scored. F – female; M – male; SE – standard error of the mean; Med. – median; a – significantly different as compared to the sample I; b – significantly different as compared to the sample II; c – significantly different as compared to the sample III ($p<0.05$, t-test for dependent samples).

ual capacity for DNA repair of radiotherapy-induced damage in non-tumor cells. Specific patterns of DNA damage recorded in samples analyzed in the middle of radiotherapy and after receiving the last dose indicate the possibility of adaptive response in some patients. Furthermore, diverse DNA damage patterns observed at the end of the radiotherapy indicate that patients with different types of solid tumors considerably differ according to the genome sensitivity.

Discussion

Radiotherapy constitutes a major part of the treatment of cancer patients. It may be used as the primary therapy, but often is combined with surgery and chemotherapy or hormone therapy. Since radiotherapy works by damaging the DNA of cancer cells, therapeutic interventions lead to the unavoidably exposure of different non-target cells in treated patient. Because only a proportion of treated population will develop a secondary cancer in the future, the biomonitoring of patients after successful therapy becomes extremely important. The main goal is to discover sensitive subpopulations of patients, often with inherited genome instability, which is a prerequisite to the increased risk of secondary carcinoma.

The most extensively used biomarkers for the assessment of genotoxic and carcinogenic risks involve cytogenetic endpoints as chromosomal aberrations, sister chromatid exchanges, and micronuclei in mitogen-stimulated peripheral blood lymphocytes [28, 29]. Most of them, however, are limited with the need for the cell proliferation *in vitro,* as DNA damage must be processed into microscopically visible lesions [30]. Using novel and more sensitive techniques DNA damage could be estimated directly, in single cells and without previous cultivation. Such a powerful technique is the alkaline comet assay that becomes widely accepted as a new tool in the field of genetic toxicology and clinical medicine during the last decade [15].

In the present study, the alkaline comet assay was applied for the evaluation of background and radiation-induced DNA damage in cancer patients who were subjected to radiotherapy after surgically removed solid tumors. The results obtained indicate that radiotherapy is accompanied by significantly increased levels of primary DNA damage in peripheral blood leukocytes, and consequently in other non-target cells or tissues. These findings are in agreement with reports of other authors who investigated the impacts of radio- or chemotherapy on non-target cells in cancer patients[2,3,31–33]. Previous investigations have shown that following radiotherapy patients show a wide variation in response of both tumor and normal tissues[33]. Similarly was observed in our study. Although a significant proportion of this variation can be attributed to treatment-related factors, such as dose inhomogeneity, there is increased evidence showing that the major factors determining these differences are related to intrinsic biological factors[34]. The assessment of background DNA damage in patients involved in the present study also confirmed this assumption. We found out that pre-therapy levels of DNA damage in peripheral blood leukocytes of cancer patients were substantially different. In some patients DNA damage was comparable with 'normal' values recorded among healthy population, while in other it was notably increased, and even doubled compared to controls. Since the DNA damage detected by the alkaline comet assay represents a steady state between induction of lesions and their repair, 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 inter-individual variation is certainly related to age and some life-style factors (especially smoking habits and alcohol consumption), as well as their previous medical, i.e. diagnostic, exposures [5, 35-37], while the other part is related to inherited biological factors. The observed diversity in background DNA damage among cancer patients was difficult to explain, because they suffered from different solid tumors. However, many authors who applied the alkaline comet assay in biomonitoring 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 healthy population[11–13].

As observed in our study, radiotherapy critically influenced the levels of DNA damage in cancer patients, as detected by the alkaline comet assay. 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 that can be easily converted into strand breaks during alkaline denaturation and therefore sensitively detected[17,38,39]. It is well known that, besides direct ionization of DNA, ionizing radiation also causes an 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 through free radicals. Ionizing radiation deposits energy that injures or destroys cells in the area being treated (the »target tissue«) by damaging their genetic material, making it impossible for these cells to continue to grow. Although radiation damages both cancer cells and normal cells, the latter are able to repair them and function properly. Small frequent doses of radiation allow healthy cells time to grow back, repairing damage inflicted by the radiation. On the other hand, cancer cells generally are undifferentiated and often have a diminished ability to repair sub-lethal damage compared to most healthy differentiated cells. Consequently, cells with inherited or accumulated DNA damage will die or reproduce more slowly. For these reasons, radiation therapy is usually given daily. The dose delivered depends primarily on tumor type, but also on many other factors such as whether radiation is given alone or with chemotherapy, before or after surgery, the success of surgery and its findings and many other reasons. The typical dose for a solid epithelial tumor may range from 50 to 70 grays (Gy) or more, while lymphomas might receive doses closer to 20 to 40 Gy given in daily doses that in adults typically are 1.8 to 2 Gy per fraction[40].

We observed that prolonged exposure to ionizing radiation during the course of radiotherapy leads to the some kind of adaptive response in peripheral blood leukocytes in majority of treated patients. Other authors also reported the same phenomenon. Small acute single doses of ionizing radiation produce damages in a very short time. Many of these are double-strand breaks of the DNA. Under normal conditions cells have to cope with almost 10^6 damages due to reactive oxygen species produced by normal metabolic activity[41,42]. The DNA double strand breaks induced by the acute, low radiation dose may be sufficient to activate induced resistance, which may protect cells even against damage due to metabolism. The adaptation induced by low doses of radiation is attributed to the induction of a novel efficient chromosome break repair mechanism that if active at the time of challenge with high doses would lead to less residual damage[43]. 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[44]. The results of our study are also in agreement with these observations.

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[40].

The presence of significantly increased levels of DNA damage, as caused by ionizing radiation, is žde sirable' in cancer cells, but not in other non-target cells. Although the majority of lesions induced by ionizing radiation are successfully repaired in relatively short time after exposure[27,45], a part of DNA damage still remains unrepaired. 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[39,42]. If unrepaired DNA damage persisted in non-cancer cells over long periods of time, it presents an increased risk for development of secondary cancers.

Early detection of repair-deficient patients may provide arguments for stricter follow-up and prevention in the management of many human cancers. Sensitive techniques, as the alkaline comet assay employed in the present study, may help in detection of genotoxic effects induced *in vivo* by radiotherapy. Nevertheless, taking into account the high variability found between and within individuals, further knowledge of the fundamental aspects of the comet assay and on the kinetics of formation and disappearance of comets after patient radiation exposure *in vivo* are needed.

REFERENCES

1. CHENG L, WANG LE, SPITZ MR, WEI Q, Cancer Lett, 166 (2001) 155. — 2. VAGHEF H, NYGREN N, EDLING C, BERGH J, HELLMAN B, Mutat Res, 395 (1997) 127. — 3. BRAYBROOKE JP, HOULBROOK S, CRAWLEY JE, PROPPER DJ, O'BYRNE KJ, STRATFORD IJ, HARRIS AL, SHUKER DEG, TALBOT DC, Cancer Chemother Pharmacol, 45 (2000) 111. — 4. KOPJAR N, GARAJ-VRHOVAC V, MILAS I, Teratogen Carcinogen Mutagen, 22 (2002) 13. — 5. BLASIAK J, ARABSKI M, KRUPA R, WOZNIAK K, RYKALA J, KOLACINSKA A, MORAWIEC Z, DRZEWOSKI J, ZADROZNY M, Mutat Res, 554 (2004) 139. — 6. JAGETIA GC, JAYAKRISHAN A, FERNANDES D, Mutat Res, 491 (2001) 9. — 7. CARBONEL, E, DEMOPOULOS NA, STEFANOU G, PSARAKI K, PARRY EM, MARCOS R, Anti-Cancer Drug, 7 (1996) 514. — 8. DHILLON VS, DHILLON IK, Cancer Genet Cytogenet, 100 (1998) 143. — 9. NEUBAUER S, DUNST J, GEBHART E, Radiother Oncol, 43 (1997) 189. — 10. DUFFAUD F, ORSIERE T, DIGUE L, Mutat Res, 439 (1999) 259. — 11. LIU X, ZHAO J, ZHENG R, Mutat Res, 539 (2003)1.— 12. PALYVODA O, POLANSKA J, WYGODA A, RZEZOWSKA-WOLNY J, Acta Biochim Polo, 50(1) (2003) 181. — 13. SÁNCHEZ P, PEÑARROJA R, GALLEGOS F, BRAVO JL, ROJAS E, BENÍTEZ-BRIBIESCA L, Arch Med Res, 35 (2004) 480. — 14. OLIVE PL, DURAND RE, JACKSON SM, LE RICHE JC, LUO C, MA R, LAREN DB, AQUINO-PARSONS C, THOMSON TA, TROTTER T, Acta Oncol, 38(7) (1999) 839. — 15. FAUST F, KASSIE F, KNASMÜLLER S, BOEDECKER RH, MANN M, MERSCH-SUNDERMANN V, Mutat Res, 566 (2004) 209. — 16. ROJAS E, LOPEZ MC, VALVERDE M, J Chromatography, B 22 (1999) 225. — 17. SINGH NP, Mutat Res, 455 (2000) 111. — 18. HARTMANN A, AGURELL E, BEEVERS C, BRENDLER-SCHWAB S, BURLINSON B, CLAY P, COLLINS A, SMITH A, SPEIT G, THYBAUD V, TICE RR, Mutagenesis, 18 (2003) 45. — 19. CHA O, CLIFORD KS, PEREZ CA, BRADY LW, Radiation Oncology (.Lippincott-Raven Willkins, Philadelphia, 2002). — 20. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology.Breast Cancer-v.2. — 21. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology.Head and Neck Cancers-v.2. — 22. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology.Prostate Cancer-v.2. — 23. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology.-Uterine Cancers-v.2. — 24. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology.Central Nervous System Cancers-v.2. — 25. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology. Non-Small Cell Lung Cancer-v.2. — 26. NATIONAL COMPREHENSIVE CANCER NETWORK 2005 Clinical Practice Guidelines in Oncology. Testicular Cancer-v.1. — 27. SINGH NP, MCCOY MT, TICE RR, SCHNEIDER LL, Exp Cell Res, 175 (1988) 184. — 28. TUCKER JD, PRESTON RJ, Mutat Res, 365 (1996) 147. — 29. HAGMAR L, BONASSI S, STRÖMBERG U, MIKOCZY Z, LANDO C, HANSTEN IL, HUICI MONTAGUD A, KNUDSEN L, NORPPA H, REUTERWALL C, TINNERBERG H, BRØGGER A, FORNI A, HÖGSTEDT B, LAMBERT B, MITELMAN F, NORDENSON I, SALOMA S, KERFVING S, Mutat Res, 405 (1998) 171. — 30. KASSIE F, PARZEFALLW, KNASMÜLLER S, Mutat Res, 463 (2000) 13. — 31. ALAPETITE C, THIRION P, DE LA ROCHEFORDIERE A, COSSET MJ, MOUSTACHI E, Int J Cancer, 83 (1999) 83. — 32. LEPRAT F, ALAPETITE C, ROSELLI F, RIDET A, SCHLUMBERGER M, SARASIN A, SUAREZ H, MOUSTACHI E, Int J Radiat Oncol Biol Phys, 40(5) (1998) 1019. — 33. DE ALMODÓVAR JMR, GUIRADO D, NÚÑEZ MI, LÓPEZ E, GUERRERO R, VALENZUELA MT, VILLALOBOS M, DEL MORAL R, Radiother Oncol, 62 (2002) 327. — 34. ALSBEIH G, MALONE S, LOCHRIN C, GIRARD A, FERTIL B, RAAPHORST GP, Int J Radiation Oncology Biol Phys, 46(1) (2000) 134. — 35. BAJPAYEE M, DHAWAN A, PARMAR D, KUMAR PENDEY A, MATHUR N, SETH PK, Mutat Res, 520 (2002) 83. — 36. SPEIT G, WITTON-DAVIES T, HEEPCHANTREE W, TRENZ K, HOFFMAN H, Mutat Re, 542 (2003) 33. — 37. MENDOZA-NÚÑEZ VM, SÁNCHEZ-RODRÍGUEZ MA, RETANA-UGALDE R, VARGAS-GUADARRAMA LA, ALTAMIRANO-LOZANO MA, Mech Ageing Development, 122 (2001) 835. — 38.WARD JF, Proc Nucl Acid Res Mol Biol, 35 (1988) 95. — 39. LITTLE JB, Mutaganesis, 21(3) (2000) 397. — 40. AnonymOus, Radiotherapy,

Available from http://en.wikipedia.org/wiki/ Radiotherapy, accessed August 20, 2005. — 41. FEINENDEGEN LE, Human Exp Toxicol, 18 (1999) 426. — 42. PFEIFFER P, GOEDECKE W, OBE G, Mutagenesis, 15(4) (2000) 289. — 43. WOLF S, Environ Health Perspect, 106(1) (1998) 277. — 44. BOSSI A, OLIVIERI G, Mutat Res, 211 (1989) 13. — 45. TICE RR, The single cell gel / comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In:Phillips DH, Vennit S (Eds), Environmental Mutagenesis (Bioscientific, Oxford, 1995).

V. Garaj-Vrhovac

Institute for Medical Research and Occupational Health, Mutagenesis Unit, Ksaverska c. 2, HR-10000 Zagreb, Croatia
e-mail: vgaraj@imi.hr

PRIMJENA ALKALNOG KOMET-TESTA U PROCJENI OŠTEĆENJA DNA U BOLESNIKA LIJEČENIH RADIOTERAPIJOM

S A Ž E T A K

Istražene su razine primarnih oštećenja izazvane radioterapijskim liječenjem u DNA ne-tumorskih stanica bolesnika s novotvorinama te je procijenjena dinamika njihova popravka. Istraživanjem je obuhvaćena skupina bolesnika s različitim solidnim tumorima koji su liječeni radioterapijom. Razine primarnih oštećenja DNA u leukocitima periferne krvi procjenjivane su s pomoću alkalnog komet-testa. Analizirani su uzorci krvi izuzeti: prije i nakon primjene prve doze zračenja, u sredini ciklusa radioterapije te nakon primitka posljednje doze zračenja u ciklusu. Rezultati istraživanja upućuju na značajno povišene razine oštećenja DNA u usporedbi s njihovim osnovnim vrijednostima u gotovo svih bolesnika nakon primitka prve doze zračenja. U uzorcima analiziranim sredinom te po završetku ciklusa radioterapije uočeni su specifični obrasci oštećenja DNA koji ukazuju na mogući adaptivni odgovor u nekih bolesnika. Dobiveni rezultati upućuju na postojanost oštećenja u DNA leukocita periferne krvi bolesnika s novotvorinama (a moguće i u drugim ne-tumorskim stanicama) nakon završenog zračenja, koji su snažni predskazatelji rizika od pojave sekundarnih novotvorina. Nadalje, potvrđena je primjenjivost komet-testa kao osjetljivog i brzog testa za procjenu oštećenja genoma nakon ozračivanja u uvjetima *in vivo*.