Coll. Antropol. **34** (2010) 2: 455–465
Original scientific paper

Cytogenetic Follow-Up in Testicular Seminoma Patients Exposed to Adjuvant Radiotherapy

Marija Gamulin[1], Nevenka Kopjar[2], Mislav Grgić[1], Snježana Ramić[3], Tomislav Viculin[3], Marija Petković[4] and Verica Garaj-Vrhovac[2]

[1] Department of Oncology, Zagreb University Hospital Center, Zagreb, Croatia
[2] Mutagenesis Unit, Institute for Medical Research and Occupational Health, Zagreb, Croatia
[3] The University Hospital for Tumors, Zagreb, Croatia
[4] Department of Oncology and Radiotherapy, Rijeka University Hospital Center, Rijeka, Croatia

A B S T R A C T

Early stage testicular seminoma is a radiosensitive tumor. Its incidence has significantly increased during the last decade especially in the young population. Although the therapy for testicular seminoma gives very satisfying results, the evaluation of genome damage caused by the therapy is of a great importance in order to recognize possible related health risks. The present study was performed on ten patients diagnosed with seminoma stage I; pT1/2N0M0S0, treated with adjuvant radiotherapy (a radiation dose of 25 Gy divided in 16 fractions) after orchidectomy. To assess the possible existence of an increased baseline DNA/chromosome damage in patients we also selected the appropriate control group of ten healthy men. The levels of primary DNA/chromosome damage in peripheral blood lymphocytes, as well as the dynamics of their repair were studied using the alkaline comet assay, chromosome aberration and cytokinesis-block micronucleus assay. Altogether four blood samples per patient were collected in the course of the therapy: before and after receiving the first dose of radiotherapy, in the middle of the radiotherapy cycle, and after the last dose of radiotherapy. Other two follow-up blood samples were collected six and twelve months after the cessation of therapy. As observed, the administration of the first radiation dose significantly increased the levels of DNA damage in almost all 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 an adaptive response in some patients. The levels of chromosomal aberrations and the incidence of micronuclei also increased in the course of therapy but gradually declined during the follow-up period. Our results confirmed the existence of post-irradiation damage in peripheral blood lymphocytes (and possibly in other non-target cells) of cancer patients which may represent a risk for the secondary cancer development. Considering that the majority of patients with testicular cancer are of a younger age, they represent a population deserving special attention. As cytogenetic screening may detect high-risk individuals, it might be useful in regular medical monitoring of seminoma patients after the successful therapy.

Key words: *testicular seminoma, lymphocytes, DNA damage and repair, comet assay, chromosome aberrations, micronuclei, genetic instability*

Introduction

Testicular cancer is relatively rare and accounts for 1% of all male cancers. It is the most common malignancy in men aged between 15–34, and its incidence has more than doubled over the last 30–40 years[1,2]. About 40% of testicular cancer are pure seminoma. Testicular cancer incidence in Croatia is on the rise. Age-standardized incidence rates in Croatia have increased by 278% (from 3.39 *per* 100,000 men in 1994 to 12.83 *per* 100,000 men in 2005)[3].

Ionizing radiation is a proven mutagen[4]. It is important to study the processes of DNA repair as well as qualitative and quantitative changes in cytogenetic biomarkers in seminoma patients. Short-term and long-term

Received for publication April 29, 2009

monitoring of cytogenetic biomarkers of testicular cancer patients after being treated with adjuvant irradiation is important to recognize radiosensitive patients with a high risk for secondary tumor development[5,6]. The results of previous cytogenetic studyes indicate the increase of frequency of cytogenetic biomarkers, such as the number of micronuclei, unstable chromosome aberrations in cancer patients treated with radiation therapy in comparison with the healthy population[7–11].

In this study, we investigated the level of primary and residual chromosome damage in peripheral blood lymphocytes collected from the patients with testicular seminoma. As sensitive biomarkers the alkaline comet assay, analysis of structural chromosome aberrations and cytokinesis-block micronucleus assay were used. We also investigated inter-individual differences in the persistence of the genome damage 6 months and one year after the adjuvant radiotherapy. We also tried to evaluate whether the results obtained by the cytogenetic endpoints might be useful as prognostic factors in the monitoring of the testicular seminoma patients after radiotherapy.

Subjects and Methods

Subjects

Investigation was performed in accordance with the high standards of ethics. Before entering the study all subjects were informed about the aim and the experimental details and gave their signed consent for voluntarily participation. The work was approved by the national ethical committees on human experimentation and complies with the principles laid down in the Declaration of Helsinki.

Patients

The study population consisted of ten men diagnosed with seminoma of testes (stage I; pT1/2N0M0S0), subjected to surgical removal of tumors (orchidectomy) and treated with adjuvant radiotherapy. Their median age was 35 years (range: 23–49 years). Patients were interviewed using a standardized questionnaire that covered personal anamnesis data, along with occupational, medical, and other variables known to influence cytogenetic endpoints. Three patients were smokers. All patients were non-alcoholics, with no previous history of malignant diseases or therapy by antineoplastic drugs or radiation. Patients were not exposed to physical or chemical agents in their living or working environment which could influence the results of cytogenetic screening. Anamneses and clinical data collected from patient records are presented in Table 1.

The same diagnostic protocol was applied for all patients. Before the operation, they underwent a standard diagnostic procedure (physical examination, blood tests and tumor markers: AFP, bHCG, LDH, chest X-ray, CT of the abdomen and pelvis). Adjuvant radiotherapy to para-aortic and ipsilateral iliac nodes was scheduled to start within four weeks following the surgery, when patients were in good clinical condition.

Patients were treated with external beam radiotherapy (from the linear accelerator) using the radiation dose of 25 Gy divided in 16 daily fractions. Two opposite antero – posterior (AP-PA) fields of photons (energy: 15MV) were applied to para-aortal and ipsilateral iliacal lymph nodes.

Radiation doses during the treatment were 1.56 Gy / 1 fr. after the 1st radiation (second blood sampling); 12.5 Gy / 8 fr. in the middle of radiotherapy (third blood sampling); and 25 Gy / 16 fr. at the end of radiotherapy (fourth blood sampling).

When radiotherapy was completed, patients underwent standard diagnostic procedures and were regularly monitored during the following year using conventional clinical protocols.

Control group

Ten volunteer male blood donors of the similar age (range: 22 to 50 years), and life-styles were selected as the control group. All of them were healthy at the moment of blood sampling and interviews. They were not occupationally exposed to genotoxic agents. None of them reported alcohol consumption, medicine intake, the presence of known inherited genetic disorders, family history of testicular cancer or chronic diseases. Among them there were 7 non-smokers and 3 smokers. For the one-year period prior to the blood sampling, control subjects had not been subjected to ionizing or non-ionizing radiation for diagnostic or therapeutic purposes.

Methods

Blood sampling

Samples of venous blood (5 mL *per* each sampling time) were collected in heparinized vacutainer tubes (Becton Dickinson, N.J., USA). Patients were sampled six times throughout the study. Using blood samples collected before therapy, individual baseline values for each method were estimated.

The pre-treatment blood sample (I) was collected on day 1 of the first radiotherapy cycle, two hours prior to the radiation. The response of peripheral blood leukocytes to the radiotherapy was evaluated on blood samples 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). Two blood samples were taken 6 months (V), respectively 12 months (VI) after radiotherapy.

The blood samples from the age-matched healthy men were taken simultaneously during the study (a balanced collection design was used).

All blood samples were taken in the morning. 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 maxi-

mum of one-hour period after collection) by alkaline comet assay, and cell cultures were launched for the analysis of structural chromosome aberrations (CA) and the cytokinesis-block micronucleus assay (CBMN) following the recommendations by International Atomic Energy Agency (IAEA, 2001), ICPS guidelines (Albertini et al., 2000) and the HUMN project[12].

The comet assay was carried out under alkaline conditions, as described by Singh et al.[13]. All chemicals, if not specified, were purchased from Sigma Chemicals (St. Louis, MO, USA). Two replicate slides *per* sample were prepared. Agarose gels were prepared on fully frosted slides coated with 1% and 0.6% normal melting point (NMP) agarose. Blood samples (5 μL) were mixed with 0.5% low melting point (LMP) agarose, placed on the slides and covered with a layer of 0.5% LMP agarose. The slides were immersed for 1 h in freshly prepared ice-cold 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 (Kemika). Alkaline denaturation and electrophoresis were carried out at 4 °C under dim lights in freshly prepared electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0). After 20 min of denaturation, the slides were randomly placed side by side in the horizontal gel-electrophoresis tank, facing the anode. Electrophoresis at 25 V lasted another 20 min. After electrophoresis, the slides were gently washed with a neutralisation buffer (0.4 M Tris-HCl, pH 7.5) three times at five-minute intervals. Slides were stained with ethidium bromide (20 μg/mL) and stored at 4 °C in humidified sealed containers until analysis. Each slide was examined using a 250x magnification fluorescence microscope (Zeiss) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The total of 100 comets *per* sample were scored (50 from each of two replicate slides). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells and superimposed comets. Using a black and white camera, the microscope image was transferred to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd.). To avoid the variability, one well-trained scorer scored all comets. As a measure of DNA damage, tail length and tail moment were chosen. Moreover, cells were 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 control samples[14].

Chromosome aberration analysis was performed according to International Atomic Energy Agency guidelines (IAEA, 2001). In brief, cultures were incubated *in vitro* for 48 h in F-10 medium (Sigma) with 20% calf serum (Sigma) and stimulated by phytohaemagglutinin (PHA; Apogent). To arrest dividing lymphocytes in metaphase, colchicine (0.004%) was added 3 h prior to the harvest. Preparations were made according to the standard procedure. 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 analysed. One hundred metaphases *per* sample (50 from each of two replicates) were analysed for the total number and types of aberrations, as well as the percentage of aberrant cells.

Cytokinesis-block micronucleus assay (CBMN) was performed using lymphocyte cultures according to the standard protocol, with minor modifications (Fenech and Morley, 1985). Lymphocyte cultures were incubated in vitro in F-10 medium for 72 h. Cytochalasin B in final concentration 6 μg/mL was added to the culture at 44 h. Preparations were made according to the standard procedure. Slides were stained with 5% Giemsa solution (Sigma). For MN identification the criteria of Fenech et al.[12] were used. MN scoring was performed on coded slides at 1000x magnification under oil immersion. Altogether 1000 binuclear (BN) cells *per* each sample were scored. Total number of MN and their distribution were

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

Patient code	BW (kg) / BH (cm)	BMI	Age	Familial history of cancer	Smoking habit
T1	75/183	22.1	33	–	–
T2	80/175	25.0	41	–	–
T3	80/187	22.9	23	–	+
T4	89/184	24.4	49	+	–
T5	76/170	25.3	40	+	–
T6	103/185	30.1	26	–	–
T7	116/175	37.2	37	–	–
T8	90/180	27.1	37	–	+
T9	65/183	19.4	26	+	–
T10	70/177	22.2	31	+	+

BW – body weight; BH – body height; BMI – body mass index

determined, along with the number of micronucleated cells.

Statistical Analyses

Statistical analyses were carried out with the commercial programme Statistica 7.0 (StatSoft, Tulsa, USA). The extent of DNA damage, as recorded by the alkaline comet assay, was analysed considering the parameters of descriptive statistics: mean (±standard deviation), 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. Normality of distribution was tested by using the Shapiro-Wilks' W-test. Since the distribution of variables was not normal, nonparametric methods were used in further analyses. Differences between groups of independent variables were analyzed using the Mann-Whitney U-test and Kruskall-Wallis test. Friedman ANOVA test and Wilcoxon matched pairs test with the downward adjustment of the α-level for multiple comparisons between pairs were used to determine differences between the groups of dependent variables. The level of significance of correlation between the 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

Alkaline Comet Assay

Distribution of comet tail lengths measured in blood samples I–VI collected from seminoma patients throughout the study is shown in Figure 1.

Individual values of comet tail lengths measured in pre-treatment blood samples (I) ranged between 16.58±0.29 μm (T1) and 31.05±1.79 μm (T2). The group mean value of tail lengths was 22.56±1.52 μm and the median 21.14 μm. Tail moments measured in the same samples ranged between 3.40±0.08 (T4) and 5.61±0.13 (T8). The group mean value of tail moments was 4.44±0.26 and the median 4.36.

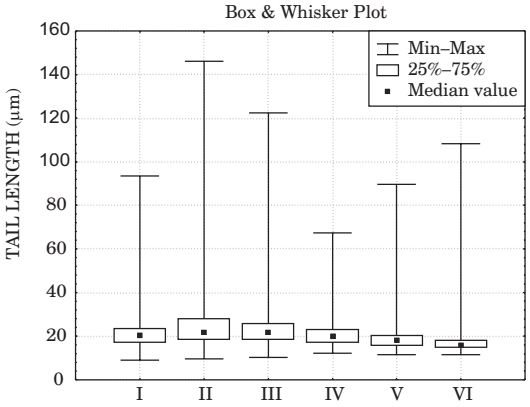

Fig. 1. The distribution of comet tail lengths in peripheral blood leukocytes of testicular seminoma patients (N=10). Pre-treatment blood sample (I) was collected on day 1 of the first radiotherapy cycle, two hours prior to the irradiation. The response of peripheral blood leukocytes to the radiotherapy was evaluated on blood samples collected 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). Two follow-up blood samples were taken 6 months (V), respectively 12 months (VI) after the cessation of radiotherapy.

The response of peripheral blood leukocytes to the radiotherapy was evaluated on blood samples taken within two hours after the application of the first dose (samples II). The values of comet tail lengths measured in these blood samples ranged between 14.92±0.40 μm (T1) and 62.07±2.97 μm (T5). The group mean value of tail lengths was 28.17±4.56 μm and the median 22.11 μm. Individual tail moments were 3.22±0.11 (T1) to 9.85±0.56 (T5). The group mean value of tail moments was 5.01±0.60 and the median 4.54.

In the middle of the radiotherapy cycle (sample III) individual values of comet tail length ranged between

TABLE 2
RESULTS OF THE COMET ASSAY AND CYTOGENETIC ENDPOINTS IN THE REFERENT POPULATION

Parameters	$\bar{X}$±SD	Range of individual values
Comet tail length (μm)	13.79±1.07	12.45±0.72 – 15.26±1.24
LTN *per* 100 comets	1.90±2.77	0 – 7
Structural CA *per* 100 metaphases	0.55±0.37	0 – 1
Chromatid breaks	0.35±0.33	0 – 1
Chromosome breaks	0.10±0.31	0 – 1
Acentric fragments	0.10±0.21	0 – 1
Cells with structural CA	0.55±0.37	0 – 1
MN *per* 1000 binuclear cells	3.89±2.47	1 – 9
Micronucleated cells	3.44±1.88	1 – 7
with 1 MN	3.00±1.41	0 – 5
with 2 MN	0.44±0.73	0 – 2

17.49±0.41 μm (T9) and 29.82±1.17 μm (T6). The group mean value of tail lengths was 24.17±1.13 μm and the median 24.64 μm. Corresponding tail moments were between 3.68±0.07 (T9) and 5.25±0.10 (T4), with the group mean value of tail moments 4.46±0.16 and the median 4.52.

After the last received radiotherapy dose (samples IV) the values of comet tail length ranged between 18.68±0.27 μm (T10) and 25.56±0.64 μm (T9), with the group mean value 21.10±0.60 and the median 20.69. Tail moments ranged between 3.75±0.07 (T3) and 6.14±0.11 (T6). The group mean value of tail moments was 4.86±0.29 and the median 4.39.

In blood samples taken 6 months (V) after radiotherapy the values of comet tail lengths ranged between 17.55±0.41 μm (T5) and 21.74±1.19 μm (T8), with the group mean value 19.51±0.51 and the median 19.59. Corresponding tail moments were 3.29±0.06 (T5) to 4.88±0.12 (T7). The group mean value of tail moments was 3.99±0.17 and the median 3.88.

The last blood sampling was done 12 months after radiotherapy (sample VI). In these blood samples tail lengths ranged between 15.19±0.22 μm (T9) and 24.18±1.66 μm (T10). The group mean value of tail lengths was 17.18±0.18 μm and the median 16.53 μm. Corresponding tail moments were between 3.31±0.06 (T4) and 4.36±0.22 (T10), with the group mean value 3.49±0.11 and the median 3.37.

The statistical evaluation of the data (Friedman ANOVA test) confirmed the differences observed among the patients and the sampling times as significant (tail length: $p<0.001$; coefficient of concordance=0.411; average r=0.346; tail moment: $p=0.008$; coefficient of concordance=0.311; average r=0.234).

The incidence of LTN comets was evaluated in parallel with tail lengths and tail moments. The distribution of LTN comets in six blood samples (I–VI) is shown in Figure 2. The baseline frequency of LTN was 3–5/100 comets, with the mean value 4.40±0.22 and the median 4.50. Our results indicate the differential response of patients to therapeutic irradiation. Some patients, for instance T4 and T5, showed a marked increase of LTN following the administration of a single fraction of radiation. Individual values recorded in blood samples II ranged between 0–48 LTN/100 comets, with the mean value 13.90±5.79 and the median 5. In the majority of patients the incidence of LTN increased in the middle or at the end of the radiotherapy cycle. In blood samples III individual values of LTN ranged between 0–46 LTN/100 comets, with the mean value 17.40±4.95 and the median 14.50. In blood samples collected at the end of the radiotherapy cycle there were 8.10±4.16 LTN/100 comets recorded (median: 2.00 range 0–37 LTN/100 comets). In blood samples collected six months following the cessation of the therapy a relatively high number of LTN was observed: 6.00±4.16 LTN/100 comets (median: 7.00, range 0–13 LTN/100 comets). We assume that the increase of LTN incidence in these blood samples was also related to the diagnostic exposure, since before fifth blood sampling the patients underwent diagnostic examinations. During the next six months the number of LTN gradually decreased and in most patients it returned to pre-therapy levels. The group mean value was 1.20±0.92 LTN/100 comets (median: 0, range 0–9 LTN/100 comets).

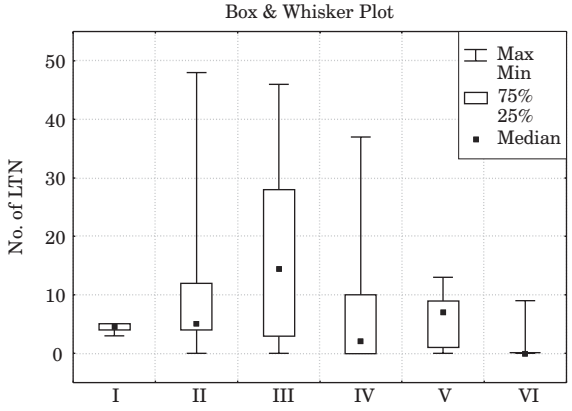

Fig. 2. The distribution of LTN comets (LTN-long tailed nuclei) in blood samples collected from testicular seminoma patients (N=10) before (I), in the course (II,III) and after radiotherapy (IV), as well as during the follow-up period (V, VI).

The statistical evaluation of the data (Friedman ANOVA test) confirmed the differences regarding LTN observed between the patients and the sampling times as significant ($p<0.002$; coefficient of concordance=0.368; average r=0.298).

The levels of primary DNA damage in healthy men were more uniform than in seminoma patients and therefore would not be reported individually. Detailed data obtained for the control group are shown in Table 2. The statistical evaluation by the Mann-Whitney test indicates that difference between the pre-therapy values of comet tail lengths in seminoma patients and the matched healthy controls was highly significant ($p=0.0002$). Similar results were obtained for the LTN comets ($p=0.0413$).

Analysis of Structural CA

The total number of structural chromosome aberrations recorded in pre-treatment blood samples (I) ranged between 1% (T1, T2, T3, T9) and 4% (T6). The total number of structural chromosome aberrations recorded in blood samples taken after the application of the first dose ranged between 3% (T9) and 12% (T8). In the middle of the radiotherapy cycle (sample III) the total number of structural chromosome aberrations ranged between 3% (T9) and 40% (T4). After the last received radiotherapy dose (samples IV) the total number of structural chromosome aberrations ranged between 11% (T8) and 86% (T4). In blood samples taken 6 months (V) after radiotherapy the total number of structural chromosome aberrations ranged between 8% (T8) and 48% (T2). In blood samples taken one year after cessation of radiotherapy (sample VI) the total number of structural chromosome aberrations ranged between 5% (T3, T8) and 40% (T10). Among the patients studied, marked inter-in-

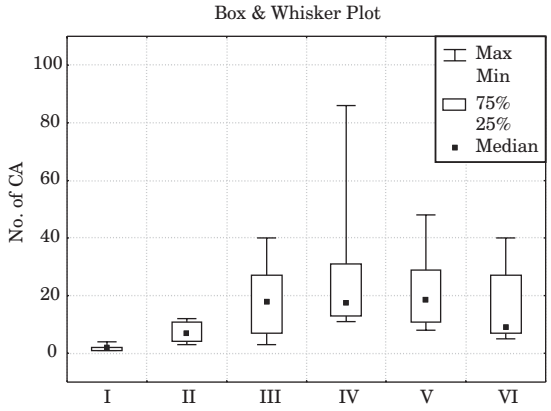

Fig. 3. The distribution of structural chromosome aberrations (CA) in blood samples collected from testicular seminoma patients (N=10) before (I), in the course (II,III) and after radiotherapy (IV), as well as during the follow-up period (V, VI).

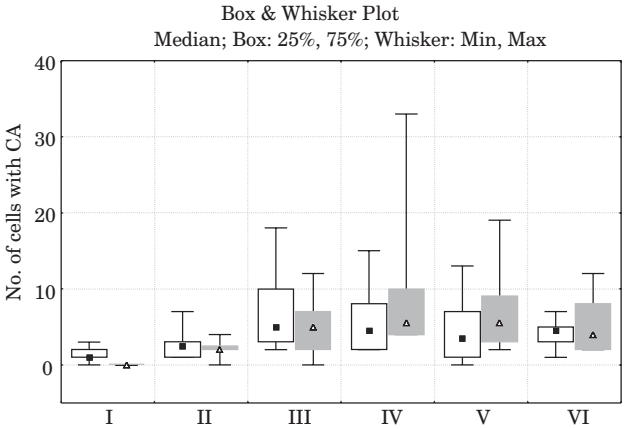

Fig. 4. The distribution of cells with one aberrations (white box) and more than one aberration (grey box) in blood samples collected from testicular seminoma patients (N=10) before (I), in the course (II,III) and after radiotherapy (IV), as well as during the follow-up period (V, VI).

dividual differences were observed. The incidence of chromosome aberrations (CA) in six blood samples (I–VI) is given in Figure 3.

The total number of aberrant cells recorded in pre-treatment blood samples (I) ranged between 1% (T1, T2, T3, T9) and 4% (T6). After the administration of the first radiation dose (samples II) there were between 2% (T9) and 9% aberrant cells recorded (T2). In the middle of the radiotherapy cycle (sample III) the total number of aberrant cells ranged between 3% (T9) and 25% (T4). After the last received radiotherapy dose (samples IV) the total number of aberrant cells ranged between 6% (T6) and 48% (T4). During the follow-up period the number of aberrant cells decreased. In blood samples taken 6 months (sample V) after radiotherapy the total number of aberrant cells ranged between 2% (T8) and 23% (T4). In blood samples taken one year after the cessation of radiotherapy the total number of aberrant cells ranged between 4% (T3, T8) and 17% (T10). Among the patients studied, marked inter-individual differences were observed.

In pre-treatment blood samples of almost all patients aberrant cells contained only one structural chromosome aberration. Cells with multiple aberrations were recorded in later blood samplings (especially in the middle of the radiotherapy cycle and after the last radiation dose administered), when their distribution among the patients was similar. Distribution of aberrant cells with one and those with more then one aberration in samples I–VI is displayed in Figure 4. Figure 5 shows the correlation between the cells with chromosome aberrations and micronucleated cells established in blood samples I–VI.

The statistical evaluation of the data (Friedman ANOVA test) confirmed the differences regarding the total number of structural chromosome aberrations observed between the patients and the sampling times as significant ($p<0.001$; coefficient of concordance=0.647; average r=0.608). The incidence of unstable aberrations was also significant: acentric fragments ($p<0.001$; coefficient of concordance=0.699; average r=0.666) and dicentric chromosomes ($p<0.001$; coefficient of concordance=0.543; average r=0.342). The similar was observed for the incidence of aberrant cells ($p<0.001$; coefficient of concordance=0.592; average r=0.547), the incidence of cells containing only one aberration ($p=0.005$; coefficient of concordance=0.336; average r=0.262) and the incidence of cells with multiple aberrations ($p<0.001$; coefficient of concordance=0.642; average r=0.602).

Detailed data obtained for the control group are reported in Table 2. The statistical evaluation by the Mann-Whitney test indicates that pre-therapy values of structural CA and aberrant cells in seminoma patients were significantly higher than in the matched healthy controls ($p=0.0009$). However, in both groups we recorded similar types of CA, mainly chromatid breaks. Chromosome break was recorded in only one control subject and acentric fragments in two of them.

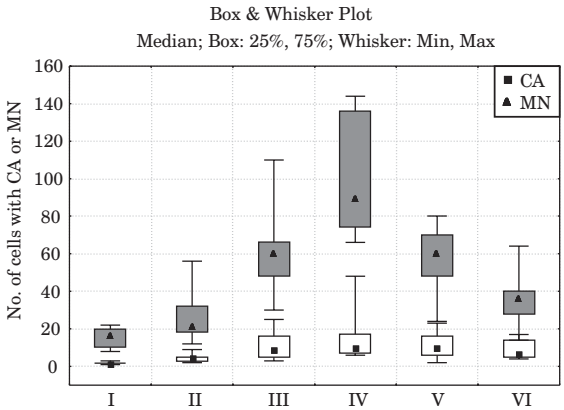

Fig. 5. The correlation between the total number of cells with CA and micronucleated cells in blood samples collected from testicular seminoma patients (N=10) before (I), in the course (II,III) and after radiotherapy (IV), as well as during the follow-up period (V, VI).

Micronucleus assay

The incidence of micronucleated cells in pre-treatment samples ranged between 8/1000 cells (T3) and 22/1000 cells (T2). After the administration of the first radiation dose (samples II) there were between 12 (T3) and 56 micronucleated cells /1000 cells recorded (T8). In the middle of the radiotherapy cycle (sample III) the total number of micronucleated cells ranged between 30/1000 cells (T10) and 110/1000 cells (T8). After the last received radiotherapy dose (samples IV) the total number of micronucleated cells ranged between 66/1000 cells (T5) and 144/1000 cells (T8). Six months after radiotherapy (samples V) the total number of micronucleated cells ranged between 24/1000 cells (T9) and 80/1000 cells (T2). One year after radiotherapy (sample VI) the total number of micronucleated cells ranged between 14/1000 cells (T9) and 64/1000 cells (T2). The distribution of micronucleated cells in blood samples I–VI is displayed in Figure 6.

Among the studied patients marked inter-individual differences were observed. Micronucleated cells in pre-treatment blood samples of many patients contained more than one MN. As observed, such cells appeared more frequently in later blood samplings (especially in the middle of the radiotherapy cycle and after last radiation dose administered). In all patients and sampling times micronucleated cells which contain one MN predominated over cells which contain more then one MN. In two patients (T4 and T6) micronucleated cells with 4 micronuclei were also recorded.

Distribution of MN in blood samples I–VI is shown in Figure 7. The total number of micronuclei in pre-treatment sample ranged between 8 MN/1000 cells (T3) and 22 MN/1000 cells (T2 T4, T9). After the administration of the first radiation dose (samples II) there were between 12 MN (T3) and 66 MN/1000 cells recorded (T8). In the middle of the radiotherapy cycle (sample III) the total number of micronuclei ranged between 34 MN/1000 cells (T10) and 128 MN/1000 cells (T8). After the last received radiotherapy dose (samples IV) the total number of micronuclei ranged between 86 MN/1000 cells (T7) and 200 MN/1000 cells (T8). Six months after radiotherapy (samples V) the total number of micronuclei ranged between 28 MN/1000 cells (T9) and 100 MN/1000 cells (T8). One year after radiotherapy (sample VI) the total number of micronucleated cells ranged between 14 MN/1000 cells (T9) and 70 MN/1000 cells (T4).

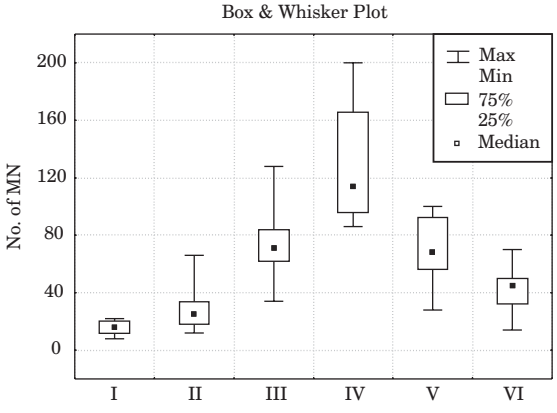

Fig. 6. The distribution of MN in blood samples collected from testicular seminoma patients (N=10) before (I), in the course (II,III) and after radiotherapy (IV), as well as during the follow-up period (V, VI).

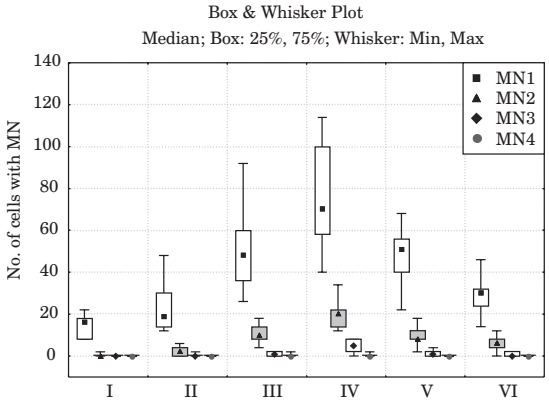

Fig. 7. The distribution of micronucleated cells containing 1–4 MN in blood samples collected from testicular seminoma patients (N=10) before (I), in the course (II,III) and after radiotherapy (IV), as well as during the follow-up period (V, VI).

The statistical evaluation of the data (Friedman ANOVA test) confirmed the differences regarding the total number of micronuclei observed between the patients and the sampling times as significant ($p<0.001$; coefficient of concordance=0.954; average r=0.949). Similar was observed for the incidence of micronucleated cells ($p<0.001$; coefficient of concordance=0.922; average r=0.913), micronucleated cells containing one MN ($p< 0.001$; coefficient of concordance=0.877; average r=0.863) and micronucleated cells containing two MN ($p<0.001$; coefficient of concordance=0.888; average r=0.876).

Detailed data obtained for the control group are reported in Table 2. The statistical evaluation by the Mann-Whitney test indicates that pre-therapy values of MN in seminoma patients were significantly higher than in the matched healthy controls ($p=0.0004$). The similar result was obtained for the micronucleated cells ($p=0.0002$). In both groups micronucleated cells with 1MN predominated over those with 2 MN.

Discussion

Radiotherapy is one of the most evident examples of intentionally induced DNA damage. Recent progress in this field has improved the prognosis of cancer patients, but in turn has brought about many complications. Attention is especially focused on the secondary cancer following radiotherapy, for which the risk is substantial[15]. Since only a portion of the treated population would develop a secondary cancer in the future, the biomonitoring of patients after the successful therapy becomes essen-

tial. The main idea is to detect sensitive subpopulations of patients, often with inherited genome instability, which is a precondition to the increased risk of secondary carcinoma.

In the present study, the alkaline comet assay, chromosome aberration and micronucleus assay were applied for the evaluation of background and radiation-induced DNA damage in patients diagnosed with seminoma who were subjected to adjuvant radiotherapy. As known, 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 orchidectomy. The median time to relapse is approximately 12 months, but relapses may occur more than 5 years following the therapy[2,4].

The role of biomarkers as prognostic indicators for genotoxic and carcinogenic risks is extensively investigated. Among them, cytogenetic endpoints as chromosomal aberrations, sister chromatid exchange frequency, and micronucleus frequency were mostly used[16,17]. During the last decade, the alkaline comet assay also gained importance in clinical medicine as a sensitive tool that enables the estimation of primary DNA damage directly and in single cells[18].

In this study the peripheral blood lymphocytes were chosen as a model system as they are the favored cells for most of the biomarker assays and established earlier as suitable biodosimeters that integrate the effects of exposure to exogenous and endogenous genotoxins[19]. Furthermore, lymphocytes seemed to be suitable because of their easy availability, synchronous population, low frequency of spontaneous chromosomal aberrations, convenient culture methods and simplicity of sample collection[20]. The last was particularly important in our study, as we were able to collect the blood samples from the patients using minimal invasive procedure without putting them in additional emotional or physical distress.

Our results showed that local fractionated radiotherapy delivered to seminoma patients critically influenced the levels of primary DNA damage, induced chromosomal aberrations and micronuclei in their peripheral blood lymphocytes. These findings are in agreement with the reports of other authors who investigated the impacts of radio- or chemotherapy on non-target cells in other cancer patients[21–25].

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 may be easily converted into strand breaks during alkaline denaturation and therefore sensitively detected by the alkaline comet assay[26–28]. In addition to the direct ionization of DNA it also causes an indirect ionization through reactive oxygen species. The largest part of the radiation effect in radiotherapy is mediated by free radicals. In such conditions clusters of lesions or multiply damaged sites are also formed and they significantly contributed to the lethality of ionizing radiation. It is believed that the more complex the lesion, the less likely repair or correct repair would occur[29].

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 themselves and function properly. Small frequent doses of radiation allow healthy cells time to repair damage inflicted by the radiation. However, cancer cells often are undifferentiated and have a lesser ability to repair sub-lethal damage compared to most healthy differentiated cells. As a result, cells with accumulated DNA damage would die or proliferate more slowly. For these reasons, radiation therapy is typically delivered daily. The dose administered depends mainly on the tumor type, but also on other factors: whether radiation is given alone or with chemotherapy, before or after surgery, the success of the surgery and its findings and many other reasons. The typical dose for patients with seminoma in stages IA, IB, and IS is 20–30 Gy, delivered to the infra diaphragmatic area including para-aortic and iliac lymph nodes[2]. The patients involved in this study were treated with 25 Gy in 16 daily fractions.

Previous investigations have shown that after the radiotherapy patients show a wide variation of responses of both tumor and normal tissues[25]. Our results also sustain these reports. Although a significant portion of inter-individual variations may be attributed to treatment-related factors, such as dose distribution, dose inhomogeneity and the patient size, an increased evidence show that the major factors determining these differences are related to intrinsic biological factors and genetic predisposition[30,31].

The assessment of background DNA damage in patients involved in the present study also confirmed this assumption. We found out that the pre-therapy levels of DNA damage in peripheral blood leukocytes of cancer patients were substantially different. In some patients DNA damage was comparable to background values recorded in the age-matched healthy men, both from the control group and from the healthy Croatian population studied earlier[32], while in others it was significantly increased. Since the DNA damage detected by the alkaline comet assay stands for a balance between the induction of lesions and their repair, a lower damage level in an individual may be the result of an actually lower number of lesions or of a high efficiency of repair[33]. One part of the inter-individual variation is certainly related to age and some life-style factors (especially smoking habits), as well as their previous medical, i.e. diagnostic, exposures[34–38], while the other part is related to inherited biological factors. Other authors who applied the alkaline comet assay in biomonitoring 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[39–41].

We observed that prolonged exposure to ionizing radiation during the radiotherapy cycle leads to possible

adaptive response in peripheral blood leukocytes in the 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[42,43]. 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 which, if active at the time of challenge with high doses, would lead to less residual damage[44]. 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]. The results of our study are also in agreement with these observations.

DNA double strand breaks induced by ionizing radiation, if non- or misrepaired, lead to chromosomal aberrations. From the results obtained here the existence of a wide variability before treatment in the baseline frequency of CA among cancer patients is evident. Our results provide evidence that local radiotherapy induced significant levels of chromosome aberrations such as dicentrics and acentric fragments in circulating blood lymphocytes. The analysis of the CA frequency in blood samples collected in the course of radiotherapy, and also in the follow-up, indicates a wide variability. This result is in agreement with previous reports[38,46]. These studies also indicated that the short time interval between deliveries of irradiation would result in complex time-dependent patterns of DNA damage. The similar was noted in our study as well. When individual values were compared with their own control, no specific pattern was observed. In some patients the rate of dicentrics declined with time elapsing after exposure, while in others it was not observed. Obviously, there are many factors which contributed to the non-uniform response to irradiation. Heavily damaged cells which are more prone to death, disappeared and are replaced with new cells. Consequently, the level of chromosome aberrations as recorded six months and one year after the last treatment showed a tendency to lower values. This decline in most subjects reached the mean value similar to that observed in the pre-treatment. The recovery of induced CA in lymphocytes of cancer patients after the cessation of radiotherapy has also been observed in other studies[38].

The results of the CBMN assay performed on the same blood samples correlated well with the analysis of structural chromosome aberrations. Radiation-induced increase of micronuclei frequency was observed in cultures of all the patients studied. The large variation in micronuclei frequencies observed in the course of the study may result from the selective elimination or mitotic arrest of cells with multiple chromosome aberrations and subsequent replenishment with newly formed cells without aberrations. In this study we observed a decline of MN frequency during the follow-up period. In some patients, however, MN levels were still slightly higher than those observed before treatment. Frequencies of persistent MN would depend on the balance between the amount of cytogenetic damage, cell killing and related induction of proliferative responses in normal cell precursors[47]. The decline to baseline may be the result of the repair of induced lesion, replacement of damaged lymphocytes from a pool of imbalanced precursors or a combination of these processes[38].

All together the results of the present study demonstrate how adjuvant therapeutic irradiation influenced the biomarker levels. Therefore, we assume that the data reported here might be potentially interesting to the medical researchers working in the area of radiotherapy. It is well-known that the results obtained in studies on small populations are often complex to interpret, mainly due to inter-individual variability. Although we also discovered a certain degree of inter-individual variability in the patient population, the results obtained for the referent group speak in favour of the fact that the biomarkers were properly selected and confirm that they did not vary with time.

As expected, the damage inflicted by radiotherapy in seminoma patients was a reversible effect and regardless of what assay is being used, it increased from baseline and returned to baseline (or near baseline) after adjuvant radiotherapy ended. Our results clearly show that all biomarkers employed were able to sensitively respond to different doses of radiation delivered throughout the radiotherapy. With respect to their own specificity, the comet assay and both cytogenetic assays confirmed sensitive during the post-radiation and the follow-up period too. We observed that even a long time after the therapeutic radiation ended all endpoints were able to detected subtle differences between DNA/chromosome damage in lymphocytes of the seminoma patients involved in the study. These findings speak in favour of their use for biomonitoring purposes in cancer patients after the successful therapy.

Another interesting observation of this study is that biomarkers in seminoma patients reach a peak at different times since the start of radiotherapy. As capacities for the repair of DNA lesions significantly differ among individuals, it is not surprising that cytogenetic biomarkers reach a peak later than the comet assay. Different responses to irradiation might also be related to an adaptive protection that develops as a physiological stress response relatively slowly, within a few hours after radiation and may last from several weeks to months[48]. It is also possible that seminoma patients who were exposed daily to low doses of radiation had different efficiencies of chromosome break repair mechanisms.

Our findings suggest that the responses to therapeutic irradiation should be studied on individual basis. As the outcome of the radiation exposure depend both on the physical dose and the individual radiosensitivity, the knowledge of individual radiation sensitivity is extre-

mely important for the individualization of radiotherapy. To be exact, it allows the therapist to apply either higher tumor doses (and improve the local tumor control without harming the normal tissue) or lower tumor doses (and avoid severe side effects in the normal tissue) than it is usually done[49]. In the present study we discovered that the biomarkers employed had complementary values in the assessment of radiotherapy-induced primary and residual DNA damage in patients with seminoma. Taken together, these endpoints might be successfully employed to a study of individual radiosensitivity.

Regardless of the benefits of radiotherapy, therapeutic exposure to ionizing radiation may also lead to the induction of secondary cancers in the treated area. The levels of DNA damage, as recorded in lymphocytes, might correlate with the levels of the damage produced by therapy in other non-target cells or tissues. Although the majority of lesions induced by ionizing radiation are successfully repaired in a relatively short time after exposure[13,50,51], a part of DNA damage still remains unrepaired. It is assumed that radiation-induced reciprocal translocations and dicentrics in lymphocytes are formed in equal proportion. Cells that carry unstable aberrations, such as dicentrics, disappear in the subsequent mitosis, and dicentrics yields decrease rapidly after irradiation. The lesions in DNA that are not repaired may result in DNA mutations, RNA mutations leading to mutated proteins and blocks to DNA replication, biological consequences which may result in the onset of cancer[29]. In this view, translocation frequency which is eliminated in the decades after exposure presents an increased risk for developing secondary cancers.

The results of the present study point to the increases in the frequency of genetic damage induced by radiotherapy in the patients with seminoma of testes, indicating an increased risk of secondary malignancies development. Literature brings evidence of the persistent genome damage in somatic cells of patients with a long survival after the successful chemo/radiotherapy. The presence of a higher number of structural chromosomal aberrations in some cancer patients as detected during the follow-up period points to chromosome instability. The patients with an unstable genome, as seems to be the case in the present study, are more susceptible to secondary cancers. 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[52].

Early detection of repair-deficient patients may provide arguments for a stricter follow-up and prevention in the management of many human cancers. As the majority of patients with testicular cancer are 20–30 years of age when the tumor is discovered, genetic changes induced by therapy in their genome may not only cause secondary cancers in cured patients, but perhaps also transgenerational genetic diseases. Sensitive techniques, as those employed in the present study, undoubtedly might help in detection of genotoxic effects induced *in vivo* by radiotherapy. As cytogenetic screening may detect high-risk individuals, it might be useful in regular medical monitoring of seminoma patients after the successful therapy.

Acknowledgements

This investigation was partially supported by the Croatian Ministry of Science, Education and Sports (Grant No. 0022020 followed by 0022-0222148-2125). The authors gratefully acknowledge the cooperation of all the volunteers who participated in this study.

REFERENCES

1. HUYGE E, PLANTE P, THONNEAU PF, Eur Urol, 51 (2007) 621. — 2. NATIONAL COMPREHENSIVE CANCER NETWORK NCCN. Clinical Practice Guidelines in Oncology. Testicular Cancer-v.1.2009.Accessed: August 2008. — 3. GAMULIN M, ZNAOR A, FUCIC A, J Clin Oncol, 26 (Suppl) (2008) 1518. — 4.CHA O, CLIFORD KS, PEREZ CA, BRADY LW, Radiation Oncology: Management Decisions. (Lippincott-Raven Wouldkins, Philadelphia, 2nd edition, 2002) — 5. KOPJAR N, GARAJ-VRHOVAC V, MILAS I, Teratogen Carcinogen Mutagen, 22 (2002) 13. — 6. 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. — 7. NEUBAUER S, DUNST J, GEBHART E, Radiother Oncol, 43 (1997) 189. — 8. DUFFAUD F, ORSIERE T, DIGUE L, Mutat Res, 439 (1999) 259. — 9. GAMULIN M, KOPJAR N, GRGIĆ M, RAMIĆ S, BIŠOF V, GARAJ-VRHOVAC V, Croat Med J, 49 (2008) 515. — 10. LIU X, ZHAO J, ZHENG R, Mutat Res, 539 (2003) 1. — 11. PALYVODA O, POLANSKA J, WYGODA A, RZEZOWSKA-WOLNY J, Acta Biochim Polon, 50 (1) (2003) 181. — 12. FENECH M, CHANG WP, KIRSCH-VOLDERS M, HOLLAND N, BONASSI S, ZEIGER E, Mutat Res, 534 (2003) 65. — 13. SINGH NP, MCCOY MT, TICE RR, SCHNEIDER EL, Exp Cell Res, 175 (1998) 184. — 14. BETTI C, DAVINI T, GIANNESSI L, LOPRIENO N, BARALE R, Mutat Res, 307 (1994) 323. — 15. BYRNE J, Teratology, 59 (1999) 210. — 16. TUCKER D, PRESTON RJ, Mutat Res, 365 (1996) 147. — 17. 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, SALOMAA S, KERFVING S, Mutat Res, 405 (1998) 171. — 18. KASSIE F, PARZEFALL W, KNASMÜLLER S, Mutat Res, 463 (2000) 13. — 19. MOHERNWEISER HW, JONES IM, Mutat Res, 400 (1998) 15. — 20. JAGETIA GC, JAYAKRISHAN A, FERNANDES D, Mutat Res, 491 (2001) 9. — 21. VAGHEF H, NYGREN N, EDLING C, BERGH J, HELLMAN B, Mutat Res, 395 (1997) 127. — 22. 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. — 23. ALAPETITE C, THIRION P, DE LA ROCHEFORDIERE A,COSSET MJ, MOUSTACHI E, Int J Cancer, 83 (1999) 83. — 24. LEPRAT F, ALAPETITE C, ROSELLI F, RIDET A, SCHLUMBERGER M, SARASIN A, SUAREZ H, MOUSTACHI E, Int J Radiat Oncol Biol Phys, 40 (1998) 1019. — 25. DE ALMODÓVAR JMR, GUIRADO D, NÚÑEZ MI, LÓPEZ E, GUERRERO R, VALENZUELA MT, VILLALOBOS M, DEL MORAL R, Radiother Oncol, 62 (2002) 327. — 26. SINGH NP, Mutat Res, 455 (2000) 111. — 27.WARD JF, Proc Nucl Acid Res Mol Biol, 35 (1988) 95. — 28. LITTLE JB, Mutagenesis, 21(3) (2000) 397. — 29. HARRISON L, MALYARCHUK S, Pathophysiol, 18 (2002) 49. — 30. ALSBEIH G, MALONE S, LOCHRIN C, GIRARD A, FERTIL B, RAAPHORST GP, Int J Radiat Oncol Biol Phys, 46

(1) (2000) 134. — 31. BARBER JBP, BURRIL W, SPREADBOROUGH AR, LEVINE E, WARREN C, KILTIE AE, ROBERTS SA, SCOTT D, Radiotherapy Oncology, 55 (2000) 179. — 32. KOPJAR N, ŽELJEŽIĆ D, GARAJ-VRHOVAC V, Acta Biochim Polon, 53 (2006) 321. — 33. WOJEWÓDZKA M, KRUSZEWSKI M, IWANENKO T, COLLINS AR, SZUMIEL I, Mutat Res, 440 (1999) 19. — 34. BLASIAK J, ARABSKI M, KRUPA R, WOZNIAK K, RYKALA J, KOLACINSKA A, MORAWIEC Z, DRZEWOSKI J, ZADROZNY M, Mutat Res, 554 (2004) 139. — 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 Res, 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. CARBONELL E, DEMOPOULOS NA, STEFANOU G, PSARAKI K, PARRY EM, MARCOS R, Anti-Cancer Drugs, 7 (1996) 514. — 39. LIU X, ZHAO J, ZHENG R, Mutat Res, 539 (2003) 1. — 40. PALYVODA O, POLAŃSKA J, WYGODA A, RZEZOWSKA-WOLNY J, Acta Biochim Polon, 50 (2003) 181. — 41. SÁNCHEZ P, PENARROJA R, GALLEGOS F, BRAVO JL, ROJAS E, BENÍTEZ-BRIBIESCA L, Arch Med Res, 35 (2004) 480. — 42. FEINENDEGEN LE, Hum Exp Toxicol, 18 (1999) 426. — 43. PFEIFFER P, GOEDECKE W, OBE G, Mutagenesis, 15 (2000) 289. — 44. WOLF S, Environ Health Perspect, 106 (Suppl 1) (1998) 277. — 45. BOSSI A, OLIVIERI G, Mutat Res, 211 (1989) 13. — 46. RIGAUD O, GUEDENEY G, DURANTON I, LEROY A, DOLOY MT, MAGDELENAT H, Mutat Res, 242 (1990) 17. — 47.TATES AD, VAN DAM FJ, NATARAJAN AT, ZWINDERMAN AH, OSANTO S, Mutat Res, 307 (1994) 293. — 48. FEINENDENGEN LE, Brit J Radiol, 78 (925) (2005) 3. — 49. MÜLLER W-U, BAUCH T, STÜBEN G, SACK H, STREFFER C, Radiat Environ Biophys, 40 (2001) 83. — 50. 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). — 51. GAMULIN M, GARAJ-VRHOVAC V, KOPJAR N, Coll Antropol, 31 (3) (2007) 837. — 52. BENDER MA, PRESTON J, LEONARD RC, PYATT BE, GOOCH PC, SHELBY MD, Mutat Res, 204 (3) (1988) 421.

M. Gamulin

Department of Oncology, Zagreb University Hospital Center, Kišpatićeva 12, 10 000 Zagreb, Croatia
e-mail: marija.gamulin@zg.t-com.hr

CITOGENETIČKI NADZOR BOLESNIKA SA SEMINOMOM TESTISA LIJEČENIH ADJUVANTNOM RADIOTERAPIJOM

S A Ž E T A K

Seminomi testisa su osjetljivi na terapiju zračenjem. Incidenca ovog karcinoma značajno se povećala tijekom posljednjeg desetljeća osobito u mlađoj populaciji. Iako je terapija seminoma testisa vrlo uspješna, od velike je važnosti izučiti oštećenje genoma nastalo tijekom tereapije i prepoznati moguće zdravstvene rizike. Istraživanje je provedeno na skupini od 10 bolesnika s dijagnosticiranim seminomom stadija I; pT1/2N0M0S0, koji su nakon operativnog zahvata orhidektomije liječeni adjuvantnom radioterapijom na područje paraaortalnih i istostranih ilijačnih limfnih čvorova. Bolesnici su primili ukupnu dozu zračenja od 25 Gy podijeljenu u 16 dnevnih frakcija. Kako bismo utvrdili postoji li u bolesnika povećana nestabilnost genoma prije radioterapije, usporedili smo ih s referentnom skupinom od 10 zdravih muškaraca odgovarajuće dobi. Primjenom komet-testa u alkalnim uvjetima, analize kromosomskih aberacija i mikronukleus testa u limfocitima periferne krvi istražene su razine primarnih oštećenja DNA te oštećenja kromosoma, kao i dinamika njihova popravka. Svakom ispitaniku uzeta su četiri uzorka krvi za vrijeme terapije: prije i nakon što su primili prvu frakciju zračenja, u sredini i nakon završenog ciklusa zračenja. Dva su uzorka krvi uzimana za vrijeme post-terapijskog praćenja bolesnika, šest mjeseci i godinu dana nakon završene radioterapije. Utvrđeno je da primjena već prve frakcije zračenja u gotovo svih bolesnika izaziva značajan porast razine oštećenja DNA u odnosu na pred-terapijske vrijednosti. U uzorcima krvi analiziranim u sredini ciklusa, te nakon primitka zadnje doze zračenja uočeni su specifični obrasci oštećenja koji ukazuju i na mogući adaptivni odgovor u nekih bolesnika. Za vrijeme radioterapije porasle su i razine strukturnih oštećenja kromosoma i učestalost mikronukleusa u limfocitima, a njihove se vrijednosti uglavnom snižavaju tijekom post-terapijskog razdoblja. Dobiveni rezultati potvrđuju postojanost post-radijacijskih oštećenja u limfocitima (a moguće i u drugim ne-ciljnim stanicama) koja predstavljaju potencijalni rizik za pojavu sekundarnih karcinoma. Kako je većina bolesnika u doba kad im se postavlja dijagnoza karcinoma testisa mlađe dobi, oni predstavljaju osjetljivu populaciju koja zahtijeva posebnu pozornost. Budući da se citogenetičkim metodama mogu otkriti pojedinci pod povišenim rizikom, ove metode mogu biti korisne u redovitom zdravstvenom nadzoru nad bolesnicima sa seminomom testisa nakon provedene adjuvantne radioterapije.