Genome Damage in Oropharyngeal Cancer Patients Treated by Radiotherapy

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Aim To estimate genome damage in oropharyngeal cancer patients before, during, and after radiotherapy and to measure the persistence of caused genome damage relevant in the evaluation of secondary cancer risk.

Methods DNA damage was evaluated in peripheral blood lymphocytes of 10 oropharyngeal cancer patients using alkaline comet assay, analysis of structural chromosome aberrations, and micronucleus assay. Blood samples were taken 2 hours before irradiation on day 1 of the first radiotherapy cycle, 2 hours after the application of the first dose, in the middle of the radiotherapy cycle, within 2 hours after the last received radiotherapy dose, and after 6 and 12 months after radiotherapy.

Results In most participants, the highest level of primary DNA damage was recorded in blood samples collected after the administration of first radiation dose (mean tail length 25.04 ± 6.23 μm). Most patients also had increased frequency of comets with long tail-nucleus (LTN comets) after the administration of the first radiation dose (mean, 10.50 ± 7.71 per 100 comets), which remained increased in the middle of radiotherapy (mean, 18.30 ± 27.62 per 100 comets). Later on, the levels of primary DNA damage as recorded by the comet assay, slightly diminished. The frequency of structural chromosome aberrations in lymphocytes gradually increased during the radiation cycle (26.50 ± 27.72 per 100 metaphases at the end of the therapy), as well as the frequency of micronuclei (mean total number of micronuclei 167.20 ± 35.69 per 1000 binuclear cells).

Conclusion Oropharyngeal cancer patients had relatively high levels of primary DNA damage in their peripheral blood lymphocytes even before therapy. The frequency of complex structural chromosome aberrations and the frequency of micronuclei increased with the progression of the radiation cycle and the doses delivered. As the frequency of chromosomal aberrations a year after radiotherapy mostly did not return to pre-therapy values, it represents an important risk factor related to the onset of second cancer.

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Squamous cell carcinoma of the oropharynx is an uncommon disease, the prevalence of which is about 5-10% of all newly diagnosed cancer cases in Europe and the United States (1). Pharyngeal carcinomas, like other tumors of the head and neck, are sentinel diseases of exposure to environmental factors. The development of oropharyngeal cancer is strongly associated with tobacco use and alcohol consumption, as well as with the exposure to several occupational carcinogens, vitamin deficiencies, and poor oral hygiene (2). Only a fraction of exposed individuals develop cancer in the head and neck region, which suggests that individual sensitivity to mutagens is an important endogenous risk factor that significantly contributes to the development of the disease (3-5). Although radiation is the mainstay of current therapy for oral cancer, the variability in intrinsic radiosensitivity significantly contributes to the outcome of the disease control. In general, >15% of nasopharyngeal cancer patients develop acute or late symptoms of enhanced radiosensitivity (6). It has been well documented that patients with head and neck malignancies are at considerable risk of developing a second primary tumor either within or outside the head and neck area (7).

Apart from the beneficial effect of radiotherapy, adverse consequences on normal tissue are almost always present. Following γ-irradiation, different types of lesions can be detected in the nuclear DNA. DNA single- and double-strand breaks and a plethora of modified nucleotides are induced by direct ionization of DNA and by free radical-mediated reactive oxidative species developed by the radiolysis of water (8). Although unrepaired DNA damage is useful in killing cancerous cells, it can be detrimental to normal cells, leading to the onset of secondary cancer (9). Growing evidence suggests that delayed radiation-induced damage, ie, induced genomic instability may also significantly contribute to the onset of secondary neoplasms (10).

The most extensively used biomarkers for the assessment of genotoxic and carcinogenic risks in humans involve cytogenetic endpoints such as chromosomal aberrations, sister chromatid exchanges, and micronuclei in mitogen-stimulated peripheral blood lymphocytes. In the last decade, the alkaline comet assay, as a relatively new biomarker, has also gained an increased application in clinical medicine (11,12). The majority of reports were concerned with either biomonitoring of increased levels of basal DNA damage in cancer patients or excess DNA damage caused by treatment with radiation or antineoplastic drugs (13-15).

Background genome damage in oropharyngeal cancer before radiotherapy has never been correlated with the rate of caused genome damage during and elimination of genome damage after radiotherapy. As in most cases, heavy drinkers with oropharyngeal cancer represent a unique population with genome burden related to alcohol consumption and possible genome instability related to cancer. Due to significant improvement of therapy efficiency in this type of cancer, the secondary cancer risk follow-up should be included in medical surveillance of these patients.

In this study, the levels of primary and residual DNA/chromosome damage in patients with oropharyngeal cancer were studied using the alkaline comet assay, analysis of structural chromosome aberrations, and cytokinesis-block micronucleus (CBMN) assay in peripheral blood lymphocytes. This study also investigated the susceptibility of cancer patients to radiation doses received in the course of radiotherapy, as well as possible inter-individual differences in the persistence of lymphocyte genome damage one year after irradiation.

Participants and methods

Participants

This study was performed in accordance with high standards of ethics and was approved by the Ethical Committee of the Zagreb University Hospital for Tumors, Zagreb, Croatia where the study participants were recruited. They were cancer patients who had not previously been treated with cytotoxic drugs or radiotherapy. Before entering the study, all participants were informed about the aim and the experimental details and gave their informed consent for voluntarily participation. Altogether 10 men (age range, 48-62 years) who had undergone excision of the primary oropharyngeal tumor and the unilateral neck dissection were selected. All of them were subjected to similar diagnostic procedures before operation (including chest x-ray) and to similar post-surgical treatments. All patients were regular and heavy smokers (they had smoked more than 20 cigarettes a day for over 10 years) and reported long-term alcohol consumption (average daily intake of 2-3 l of wine and other alcoholic drinks). They were free from other pathology and were not on medication that is known to cause DNA damage. None of them reported cancer cases in their family history. Histopathologically, all patients suffered from squamous cell carcinoma of the oropharynx, with positive nodes in the neck and negative borders. Patients were also classified according to the current Tumor Nodes Metastasis classification (16).

Adjuvant radiotherapy was scheduled to start within 4 to 6 weeks following surgery, when patients were in good clinical condition. Postoperative irradiation is recommended based on the tumor stage, tumor histology, and surgical findings after tumor resection. All patients were irradiated with photon beams from a ^{60}Co source in the area of the primary tumor and the neck. Total tumor dose applied after surgery was 62 Gy in 31 daily fractions. Two opposite lateral fields and one direct field (50Gy/25 fractions) to the neck were applied. Higher doses of radiation are required for microscopic disease to decrease the chance of locoregional failure because of interruption of the normal vasculature, scarring, and relative hypoxia in the tumor bed. Radiation doses received in the course of therapy were as follows: 2 Gy (second sampling, after the application of the first dose), 30 Gy (third sampling, in the middle of the radiotherapy cycle), and 62 Gy (fourth sampling, at the end of the radiotherapy cycle).

Methods

Blood sampling. Blood was taken by venipuncture. Further laboratory manipulations with blood samples and all investigations have been carried out in accordance with high standards of ethics.

Samples of venous blood (5 mL) were collected in heparinized vacutainer tubes (Becton Dickinson, New Jersey, NJ, USA) under sterile conditions. Altogether 6 blood samples were collected from each donor. Pretreatment blood sample (sample 1) was collected on day 1 of the first radiotherapy cycle, two hours before irradiation. Response of peripheral blood leukocytes to radiotherapy was evaluated on the blood sample taken within two hours after the application of the first dose (sample 2), in the middle of the radiotherapy cycle (sample 3), and within 2 hours after the last received radiotherapy dose (sample 4). One blood sample was taken 6 months (sample 5) and 12 months (sample 6) after radiotherapy.

All blood samples were handled in the same manner. After venipuncture they were coded, cooled to +4°C in the dark, and transferred to our laboratory. They were analyzed immediately after the arrival (one hour after collection at the latest) using the alkaline com-

et assay, the analysis of structural chromosome aberrations, and the CBMN assay.

Alkaline comet assay. All chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA). The comet assay was carried out under alkaline conditions, as described by Singh et al (17). Fully frosted slides were covered with 1% normal melting point agarose. After solidification, the gel was scraped off from the slide. The slides were then coated with 0.6% normal melting point agarose. When this layer had solidified, a second layer, containing the whole blood sample (4 μL) mixed with 0.5% low melting point agarose, was placed on the slides. After 10 minutes of solidification on ice, the slides were covered with 0.5% low melting point agarose. Afterwards, the slides were immersed for 1 hour 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 to 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 minutes to allow DNA to unwind and express alkali-labile sites. Denaturation and electrophoresis were performed at 4°C under dim light. Electrophoresis was carried out for the next 20 minutes at 25 V (300 mA). After electrophoresis, the slides were washed gently three times at 5-minute intervals with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 μg/mL) and covered with a coverslip. Slides were stored at 4°C in humidified sealed containers until analysis.

To prevent additional DNA damage, handling blood samples and all the steps included in the preparation of the slides for the comet assay 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.

The slides were examined at 250 × magnification using a fluorescence microscope (Zeiss, Gottingen, Germany), equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. The microscope was connected through a black and white camera to a computer-based image analysis system (Comet Assay II, Perceptive Instruments Ltd, Suffolk, UK). A total of 100 comets per patient 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. To avoid potential variability, one well-trained scorer performed all scorings of comets. Tail length (calculated from the midpoint of the head and presented in micrometers) and tail moment (calculated by the computer program) were used in this study as measures of DNA damage.

Analysis of structural chromosome aberrations. The analysis of structural chromosome aberrations was carried out using the standard procedure proposed by current International Programme on Chemical Safety guidelines for the monitoring of genotoxic effects of carcinogens in humans (18). Lymphocyte cultures were incubated in vitro in F-10 medium for 48 hours. To arrest dividing lymphocytes in metaphase, colchicine (0.004%) was added 3 hours prior to the harvest. Cultures were centrifuged at 1000 rpm for 10 minutes, the supernatant was carefully removed, and the cells were resuspended in a hypotonic solution (0.075 M KCl) at 37°C. After centrifugation, the cells were fixed with a freshly prepared fixative of ice-cold methanol/glacial acetic acid (3:1, v/

v). Fixation and centrifugation were repeated several times until the supernatants were clear. Cells were pelleted and resuspended in a minimal amount of fresh fixative to obtain a homogeneous suspension. The cell suspension was dropped onto microscope slides and left to air-dry. Preparations were made according to the standard procedure. Slides were stained with 5% Giemsa solution (Sigma). All slides were coded and scored blindly at 1000 × magnification under oil immersion. Structural chromosome aberrations were classified based on the number of sister chromatids and breakage events involved. Only metaphases containing 45-47 centromeres were analyzed. Microscope slides were coded and scored blindly. One hundred metaphases per sample (50 from each of two replicate cultures) were analyzed for total numbers and types of aberrations, as well as the percentage of aberrant cells.

CBMN assay. Lymphocyte cultures were incubated in F-10 medium for 72 hours, according to standard protocol for CBMN assay (19) with slight modifications. Cytochalasin B in final concentration 6 μg/mL was added into the culture after 44 hours. Following incubation, cultures were centrifuged at 600 rpm for 10 minutes, the supernatant was carefully removed, and the cells were gently resuspended in physiological saline at room temperature. After centrifugation, the cells were fixed with a freshly prepared fixative of ice-cold methanol/glacial acetic acid (3:1, v/v). Fixation and centrifugation were repeated several times until the supernatants were clear. Cells were pelleted and resuspended in a minimal amount of fresh fixative to obtain a homogeneous suspension. The cell suspension was dropped onto microscope slides and left to air-dry. Preparations were made according to standard procedure. Slides were stained with 5% Giemsa solution (Sigma). For micronuclei identification, the criteria of Fenech et al (20) were used. Scoring of micronuclei in 1000 binucleated cells was performed on coded slides at 1000 × magnification under oil immersion. Total number of micronuclei and their distribution were determined, along with the number of micronucleated cells.

Statistical analysis

Statistical analyses were carried out using Statistica 7.0 software (StatSoft, Tulsa, OK, USA). In alkaline comet assay, the extent of DNA damage was measured by the median (range) comet tail lengths and moments. Moreover, cells were classified as either "undamaged" or "damaged" by considering threshold levels indicating the LTN comets, ie, comets with the length over the 95th percentile of the distribution of the tail lengths among controls (21).

Since the distribution of data was not normal, statistical analysis was made using nonparametric methods.

Data on the comet assay, structural chromosome aberrations, and the CBMN assay gathered in different sampling times were evaluated by Friedman ANOVA test and Wilcoxon matched pairs test with downward adjustment of the α-level for multiple comparisons between pairs of measurements points. Differences in the prevalence of individual conditions were measured using χ^2 test and Cochran Q test. The level of statistical significance was set at $P<0.05$.

Results

Baseline DNA damage in peripheral blood leukocytes

Our results showed inter-individual differences in pre-therapy DNA damage in peripheral blood leukocytes of cancer patients (sample 1). Mean individual DNA migration ± standard deviation, ranged from 14.93 ± 2.57 μm to 32.02 ± 21.61 μm. For the whole studied group, mean tail length of 22.54 ± 4.58 μm

was recorded. Mean frequency of LTN comets among cancer patients was 4.80 ± 0.79, whereas their individual values varied between 3 and 6 per 100 comets measured in total. Mean individual tail moment values ranged from 2.99 ± 0.75 to 5.98 ± 4.39. Mean tail moment recorded for the whole group was 4.38 ± 0.99.

Post-irradiation DNA damage in peripheral blood leukocytes

The assessment of comet parameters in blood samples collected after administration of the first fraction of radiotherapy (sample 2) confirmed a positive response to the therapy in almost all patients. Among the patients inter-individual differences were observed. Mean individual DNA migration ranged from 17.94 ± 0.34 μm to 41.27 ± 2.40 μm. For the whole group, the mean tail length of 25.04 ± 6.23 μm was recorded. Mean frequency of LTN comets among cancer patients was 10.50 ± 7.71, while their individual values varied between 2 and 24 per 100 comets measured in total. Mean individual tail moment values were in range from 3.77 ± 0.10 to 7.39 ± 0.54. Mean tail moment recorded for the whole group was 4.80 ± 1.16.

The third blood sampling, which was performed in the middle of radiotherapy, also showed increased level of primary DNA damage in almost all patients. Mean individual DNA migration ranged from 19.13 ± 0.39 μm to 39.83 ± 2.32 μm. For the whole group, mean tail length of 26.81 ± 8.74 μm was recorded. Mean frequency of LTN comets among cancer patients was 18.30 ± 27.62, while their individual values varied between 0 and 90 per 100 comets measured in total. Mean individual tail moment values were in range from 3.87 ± 0.08 to 7.10 ± 0.52. Mean tail moment recorded for the whole group studied was 4.94 ± 0.96.

The values of comet parameters recorded in most of the blood samples collected after administration of the last fraction of radiotherapy (sample 4) did not substantially differ or were even lower than pre-therapy values. Mean individual DNA migration was in range from 15.85 ± 0.36 μm to 34.41 ± 0.92 μm. For the whole group, mean tail length of 22.37 ± 5.21 μm was recorded. Mean frequency of LTN comets among cancer patients was 13.90 ± 21.70, while their individual values varied between 0 and 66 per 100 comets measured in total. Individual tail moment values were in range from 3.55 ± 0.10 to 5.40 ± 0.22. Mean tail moment recorded for the whole group studied was 4.51 ± 0.63.

Six months following radiotherapy (sample 5), relatively high levels of primary DNA damage in peripheral blood leukocytes of cancer patients were detected. Mean individual DNA migration was in range from 20.13 ± 0.27 μm to 29.64 ± 1.68 μm. For the whole group, mean tail length of 24.86 ± 3.56 μm was recorded. Mean frequency of LTN comets among cancer patients was 11.00 ± 14.97, while their individual values varied between 0 and 35 per 100 comets measured in total. Mean individual tail moment values were in range from 3.86 ± 0.09 to 6.66 ± 0.41. Mean tail moment recorded for the whole group studied was 4.87 ± 1.22.

Decline of primary DNA damage in peripheral blood leukocytes of cancer patients was evident in blood samples taken one year following radiotherapy, when the values of all comet parameters returned to the baseline level. Individual DNA migration was in range from 14.67 ± 0.18 μm to 25.28 ± 1.25 μm. For the whole group studied, mean tail length of 17.11 ± 4.04 μm was recorded. Mean frequency of LTN comets among cancer patients was 1.33 ± 3.27, while their individual values varied between 0 and 8 per 100 comets measured in total. Mean individual tail moment values were in range from 2.96 ± 0.04 to 4.85 ± 0.23. Mean tail moment recorded for the whole group studied was 3.46 ± 0.70.

Taken together, the results obtained by the alkaline comet assay indicated that administration of adjuvant radiotherapy in patients with carcinoma of oropharynx caused an increase in DNA damage in their peripheral blood leukocytes up to the middle of the radiation cycle. Later on, the levels of DNA damage gradually decreased and a year after the end of radiotherapy they returned to lower values.

However, Friedman ANOVA showed no significant differences between six blood samplings (for comet tail lengths – $P = 0.063$, coefficient of concordance = 0.349, R = 0.219; for the frequency of LTN comets – $P = 0.345$, coefficient of concordance = 0.187, R = 0.025; and for the tail moments $P = 0.224$, coefficient of concordance = 0.232, R = 0.078).

The distribution of data regarding total number of long tailed nuclei recorded in different sampling times in peripheral blood lymphocytes of patients with carcinoma of oropharynx is shown in Figure 1.

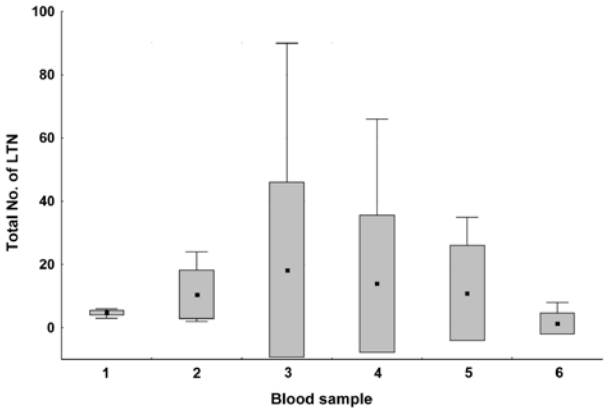

Figure 1. Distribution of the total number of long tailed nuclei (LTN) recorded in peripheral blood lymphocytes of patients with oropharyngeal carcinoma before (sample 1), during (samples 2-4), and six (sample 5) and 12 months (sample 6) after adjuvant radiotherapy. Dot represents mean, box represents mean ± standard deviation, and whiskers represent the range of the individual values measured (minimum-maximum).

Analysis of structural chromosome aberrations

Pre-therapy chromosomal damage in peripheral blood lymphocytes. There were inter-individual differences in pre-therapy chromosomal damage in peripheral blood lymphocytes of cancer patients (sample 1). Individual values for the total number of structural chromosome aberrations were in range from 1 to 5 aberrations per 100 metaphases analyzed, with the mean total number of structural chromosome aberrations 3.40 ± 1.43. Most of chromosomal aberrations recorded before radiotherapy were chromatid breaks, chromosome breaks, and acentric fragments. A presence of cells with only one type of structural chromosome aberrations was also observed.

Post-irradiation chromosomal damage in peripheral blood lymphocytes. After administration of the first fraction of radiotherapy (sample 2), an increase of chromosomal damage was observed in all patients. However, inter-individual differences among them were also noticed. Individual values for the total number of structural chromosome aberrations were in range from 3 to 9 aberrations per 100 metaphases analyzed, with the mean total number of structural chromosome aberrations 7.10 ± 2.08. The frequency of cells with more than one type of structural chromosome aberrations was also increased.

In the middle of radiotherapy (sample 3), an increase of chromosomal damage as compared with the samples was observed in the majority of the patients. Frequencies of acentric fragments and dicentric chromosomes increased. Considerable inter-individual differences were noticed, accompanied with an increased frequency of cells bearing more than one structural chromosome aberration. Individual values for the total number of structural chromosome aberrations were in range from 4 to 34 aberrations per 100 metaphases analyzed with mean total number of structural chromosome aberrations 17.50 ± 10.50 (median: 15.50).

The highest frequency of structural chromosome aberrations, as well as the frequency of aberrant cells was observed in blood samples collected after administration of the last frac-

tion of radiotherapy (sample 4). Individual values for the total number of structural chromosome aberrations were in range from 6 to 98 aberrations per 100 metaphases analyzed, with the mean total number of structural chromosome aberrations of 26.50 ± 27.72. Frequency of cells bearing more than one structural chromosome aberration was increased and these cells predominated over cells bearing only one structural chromosome aberration.

Six months following radiotherapy (sample 5), in the majority of patients, a decrease of chromosomal damage was detected. Individual values for the total number of structural chromosome aberrations were in range from 6 to 32 aberrations per 100 metaphases analyzed, with the mean total number of structural chromosome aberrations of 15.86 ± 9.34. Although their frequency decreased as compared with previous blood sampling, cells bearing more than one structural chromosome aberration predominated over cells bearing only one structural chromosome aberration.

In blood samples taken one year following radiotherapy (sample 6) the levels of chromosomal damage in peripheral blood lymphocytes were relatively high. Individual values for the total number of structural chromosome aberrations were in range from 5 to 49 aberrations per 100 metaphases analyzed, with the mean total number of structural chromosome aberrations of 19.83 ± 15.70. Chromosome damage in blood cells of some cancer patients did not return to baseline levels, both in terms of total number of structural chromosome aberrations and total number of aberrant cells.

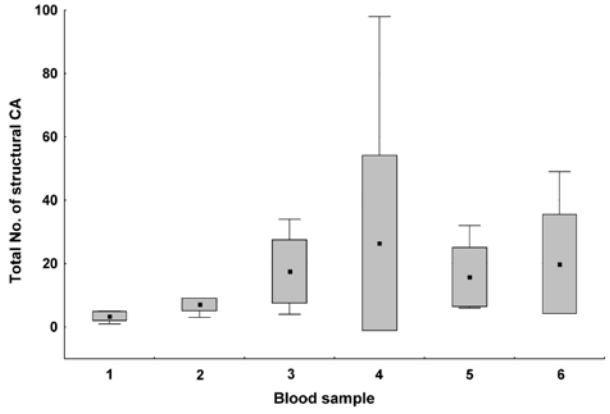

Figure 2. Distribution of the total number of structural chromosome aberrations (CA) recorded in peripheral blood lymphocytes of patients with oropharyngeal carcinoma before (sample 1), during (samples 2-4), and six (sample 5) and 12 months (sample 6) after adjuvant radiotherapy. Dot represents mean, box represents mean ± standard deviation, and whiskers represent the range of the individual values measured (minimum-maximum).

Friedman ANOVA revealed significant differences between six blood samplings in the total number of chromosome aberrations ($P = 0.006$, coefficient of concordance = 0.540, R = 0.448), frequency of acentric fragments ($P = 0.003$, coefficient of concordance = 0.610, R = 0.532), frequency of dicentric chromosomes ($P = 0.002$, coefficient of concordance = 0.650, R = 0.579), and number of cells with chromosome aberrations ($P = 0.033$, coefficient of concordance = 0.404, R = 0.285). Although the frequency of cells with 1 chromosome aberration did not significantly differ when six samplings were compared, significant difference was observed in the frequency of cells with more than one chromosome aberration ($P = 0.007$, coefficient of concordance = 0.531, R = 0.438).

Total number of structural chromosome aberrations recorded in different samplings in peripheral blood lymphocytes of patients with carcinoma of oropharynx is shown in Figure 2.

Cytokinesis-block micronucleus assay

Pre-therapy frequency of micronuclei in peripheral blood lymphocytes. There were inter-individual differences in pre-therapy frequency of micronuclei in peripheral blood lymphocytes of cancer patients (sample 1). Individual values for the total number of micronuclei were in range from 18 to 30 micronuclei per 1000 binuclear cells, with the mean total number of micronuclei of 23.60 ± 3.63. The frequency of micronucleated cells ranged between 18 and 28 per 1000 binuclear cells, with the mean total number of 23.00 ± 3.43 micronucleated

cells per 1000 binuclear cells. Most of binuclear cells contained a single micronucleus.

Post-irradiation frequency of micronuclei in peripheral blood lymphocytes . After administration of the first fraction of radiotherapy (sample 2) increased prevalence of micronuclei was observed in all patients. However, inter-individual differences among them were also noticed. Individual values for the total number of micronuclei were in range from 24 to 36 micronuclei per 1000 binuclear cells, with the mean total number of 29.00 ± 3.43 micronuclei. The frequency of micronucleated cells ranged between 24 and 32 per 1000 binuclear cells, with the mean of 28.60 ± 2.32 micronucleated cells per 1000 binuclear cells. Most of binuclear cells contained a single micronucleus.

In blood samples analyzed in the middle of radiotherapy cycle (sample 3) an increase in the total number of micronuclei was recorded as compared with the sample 2. There were also inter-individual differences noticed, accompanied with a higher rate of binuclear cells with more than 1 micronucleus. Individual values for the total number of micronuclei were in range from 68 to 164 micronuclei per 1000 binuclear cells, with the mean total number of micronuclei of 114.20 ± 26.64. The frequency of micronucleated cells ranged between 64 and 144 per 1000 binuclear cells, with the mean of 94.40 ± 22.17 (median: 90) micronucleated cells per 1000 binuclear cells. Most of binuclear cells contained only a single micronucleus.

In all patients, the prevalence of micronuclei, as well as the frequency of micronucleated cells was the highest in blood samples collected after administration of the last fraction of radiotherapy (sample 4). Individual values for the total number of micronuclei were in range from 134 to 250 micronuclei per 1000 binuclear cells, with the mean total number of micronuclei of 167.20 ± 35.69. The frequency of micronucleated cells ranged between 114 and 176 per 1000 binuclear cells, with the mean of 142.60 ± 17.99 micronucleated cells per 1000 binuclear cells. Most of binuclear cells contained a single micronucleus, but some also contained 2-4 micronuclei.

Six months following radiotherapy (sample 5), a decrease of micronuclei frequency was detected. Individual values for the total number of micronuclei were in range from 36 to 78 micronuclei per 1000 binuclear cells, with the mean total number of micronuclei of 59.71 ± 14.02. The frequency of micronucleated cells ranged between 34 and 76 per 1000 binuclear cells, with the mean of 55.71 ± 15.12 micronucleated cells per 1000 binuclear cells. Most of binuclear cells contained a single micronucleus, but some also contained 2-4 micronuclei.

In blood samples taken one year following radiotherapy, the micronuclei frequency further decreased, reaching values comparable to their pre-therapy values. Individual values for the total number of micronuclei were in range from 22 to 36 micronuclei per 1000 binuclear cells, with the mean total number of micronuclei of 29.00 ± 4.69. The frequency of micronucleated cells ranged between 22 and 36 per 1000 binuclear cells, with the mean of 28.00 ± 4.73 micronucleated cells per 1000 binuclear cells. Most of binuclear cells contained a single micronucleus, and some contained 2 micronuclei.

Friedman ANOVA revealed significant differences between six blood samplings for the total number of micronuclei ($P<0.001$, coefficient of concordance = 0.935, R = 0.922), total number of cells with micronuclei ($P<0.001$, coefficient of concordance = 0.929, R = 0.915), total number of cells with 1 micronucleus ($P<0.001$, coefficient of concordance = 0.928, R = 0.914), and number of cells with 2 micronuclei ($P<0.001$, coefficient of concordance = 0.835, R = 0.802).

Total number of micronuclei recorded in different sampling times in binuclear lymphocytes of patients with carcinoma of oropharynx is shown in Figure 3.

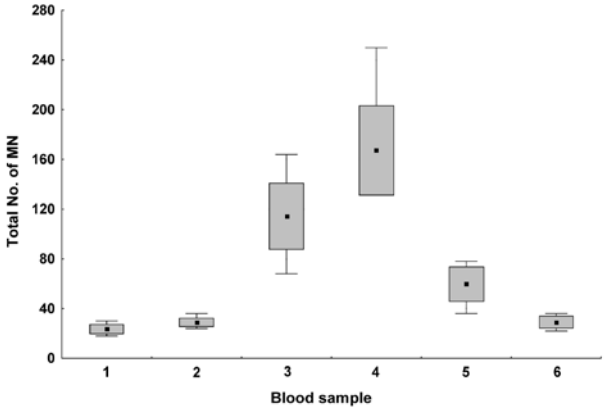

Figure 3. Distribution of the total number of micronuclei (MN) recorded in binuclear lymphocytes of patients with carcinoma of oropharynx before (sample 1), during (samples 2-4), and six (sample 5) and 12 months (sample 6) after adjuvant radiotherapy. Dot represents mean, box represents mean ± standard deviation, and whiskers represent the range of the individual values measured (minimum-maximum).

Discussion

The results obtained in this study showed higher levels of baseline genome damage in leukocytes of patients with carcinoma of the oropharynx than in Croatian general population (22). In some, the DNA damage measured by the alkaline comet assay was comparable to background values of the healthy population, but in others it was notably higher, even 2-fold higher than the normal values (23). Increased endogenous DNA/chromosome damage could be to some extent related to diagnostic exposures. However, considering that blood sampling was done approximately one month after radiological examination and the fact that cellular repair mechanisms efficiently remove most of the primary DNA damage induced by ionizing radiation within a few hours, it is doubtful whether the elevated levels of primary DNA damage, as recorded in some cancer patients before radiotherapy, were caused by diagnostic examination alone. Since the DNA damage detected by the alkaline comet assay represents a steady state between the 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. Therefore, it is likely that cancer patients have impaired DNA repair mechanisms, particularly those specialized for the removal of oxidative damage. For this reason, their baseline DNA damage was higher than in healthy participants. Other investigators also reported the probability that the neoplastic disease itself is associated with increased DNA damage, as well as that cancer patients have a more fragile DNA than healthy individuals (24,25). The background levels of DNA damage in leukocytes are mostly influenced by reactive oxygen species that are continuously generated under physiological conditions as an outcome of cellular metabolism, personal life-style, age, and daily environmental exposure to different mutagens (26). Potential influence of occupational exposure in our study is excluded, as the patients reported no exposure to known occupational mutagens. The contribution of smoking and alcohol consumption to the background DNA/chromosome damage is reported in oropharyngeal carcinoma as a sentinel disease of exposure to different external factors (27). All patients involved in our study were regular heavy smokers and reported long-term alcohol consumption. Alcohol consumption increases the level of DNA and chromosomal damage, mostly due to the metabolic conversion of ethanol to highly reactive acetaldehyde, an established genotoxic agent by most of the short-term assays (28,29).

Chromosome aberration assay and micronucleus assay showed a time-dependent occurrence and elimination of DNA and chromosomal damage before, during the course, and one year after radiotherapy. As comet assay did not show significant difference in mea-

sured parameters during the sampling period, it could be concluded that the applied version of the comet assay is not sufficiently sensitive for the purpose of such biomonitoring.

Inter-individual differences in response to radiotherapy were obviously influenced by different mutagen sensitivity and DNA repair capacity in the participants. Similar was also reported in previous investigations on cancer patients (5,25,30).

This study showed that prolonged exposure to therapy by ionizing radiation leads to some kind of adaptive response in peripheral blood leukocytes of the most of the treated patients. Small acute single doses of ionizing radiation produce damage in a very short time. Many of these are double-strand breaks of the DNA. The DNA double-strand breaks induced by acute, low radiation dose may be sufficient to activate induced resistance, which may develop in protective mechanism, ie, adaptive response. 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. Previous investigations also indicated that the human population exhibited heterogeneity in the adaptive response to ionizing radiations that might be, at least in part, genetically determined (31,32). The results of our study are also in agreement with these observations.

In patients with oropharyngeal carcinoma, adjuvant radiotherapy caused a steady increase in chromosomal damage throughout the radiation cycle. Despite significant elimination rate, a year after radiotherapy the levels of chromosomal damage mostly did not return to pretherapy values. It is also important to stress that the frequency of complex structural chromosome aberrations, such as dicentric, tricentric, quadricentric, and ring chromosomes, in this study was strongly time-dependent and correlated well with radiation doses.

The results obtained by the micronucleus assay correlated well with the levels of chromosomal damage. Micronuclei originate from chromosome fragments or whole chromosomes that fail to engage with the mitotic spindle and, therefore, lag behind when the cell divides (20). Results of earlier micronucleus studies in untreated and therapeutically exposed cancer patients showed this method as useful and important biomarker of genomic instability and a good cancer risk predictor (33).

In this study, peripheral blood lymphocytes were chosen as a model system as they were previously established as suitable biodosimeters that integrate the effects of exposure to exogenous and endogenous genotoxins, due both to the amount of agent metabolic capacity and the DNA repair capacity of the individual (34). Lymphocytes are also favored because of their easy availability, synchronous population, low frequency of spontaneous chromosomal aberrations, convenient culture methods, and ease of sample collection. The latter 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 stress.

It is known that lymphocytes consist of subpopulations with different life spans. About 90% are long-lived, with a half-life of about 3 years (some even have a lifespan of several decades), while the remaining 10% have a half-life of 1-10 days (35).

The levels of DNA/chromosome damage, as recorded in such surrogate cells, could indicate the following: 1) comparable levels of undesirable DNA/chromosome damage in other non-target cells, most likely correlated with secondary cancer risk and 2) possible levels of desirable DNA/chromosome damage in target tumor cells disseminated as micrometastases.

Our results showed that local fractionated radiotherapy delivered to cancer patients crit-

ically influenced the levels of primary DNA damage, induced chromosomal aberrations, and micronuclei in their peripheral blood lymphocytes. The large variation in DNA/chromosome damage observed in the course of study may result from selective elimination or no proliferation of cells with multiple chromosome aberrations and a subsequent replenishment with newly formed cells without aberrations. Frequencies of persistent DNA/chromosome damage will depend on the balance between the cell killing and related induction of proliferative responses in normal cell precursors (35). The decrease in the frequency of genetic damage, as detected during the follow-up period in this study, could probably be due to the elimination of damaged cells, dilution into the blood stream, or the instability of these cells to complete mitotic division; although radiation doses, sampling time, cell kinetics, and the individual sensitivity are factors that should be kept in mind when explaining this decline.

In conclusion, introduction of genotoxicological methods in medical surveillance of cancer patients before and after radiotherapy could be important in evaluating secondary cancer risk and, in case of cancers such as oropharyngeal carcinoma which may have initial genome burden, could be an important factor for individual therapy adjustment. Chromosome aberration assay and micronucleus assay are not expensive and time consuming methods, which could be run in any laboratory of clinical cytogenetics. In the future, application of fluorescent in situ hybridization will give focused insight in permanent genome damage of cancer patients after radio or chemotherapy yielding toward individual monitoring related with specific genomic rearrangements.

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