

DNA DOUBLE STRAND BREAKS REPAIR AND APOPTOSIS INDUCTION IN PERIPHERAL BLOOD LYMPHOCYTES OF HEAD AND NECK CANCER PATIENTS

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Aim: To evaluate the generation and repair of DNA double strand breaks (DSBs) as a critical factors that define the efficiency of radiation therapy of cancer patients. **Methods:** Peripheral blood lymphocytes obtained from 18 patients with head and neck squamous cell carcinoma (HNSCC) and 18 healthy donors were studied. The efficiency of DSBs repair after genotoxic treatment with hydrogen peroxide and γ -radiation were examined by neutral comet assay. MTT assay was used for cell viability analysis and Annexin V-FITC kit specific for kinase-3 was employed to determine apoptosis. **Results:** Lymphocytes from HNSCC patients were sensitive to genotoxic treatment and displayed impaired DSBs repair. Finally, as a consequence of this finding we have evidenced higher rate of apoptosis induction after γ -radiation treatment of lymphocytes from HNSCC patients than those from healthy controls. **Conclusions:** DSBs repair and increased apoptosis in cells of patients with head and neck cancer is relevant for efficient therapy of HNSCC.

Key Words: DSBs, DNA repair, apoptosis, hydrogen peroxide, γ -radiation, head and neck cancer.

The tumor response to genotoxic treatment may depend on differences in DNA repair capacity of cancer cells [1]. Double-strand breaks (DSBs) are the most deleterious DNA lesions and the cells capacity to restore DSBs is known to affect cancer sensitivity to therapeutic treatment [2, 3]. The clinical response to DSBs varies widely and likely depends on different cancer cell populations [4]. Since DSBs are major lethal lesion caused by anti-cancer drugs and radiation, their processing in association with head and neck cancer treatment become still not fully elucidated.

Head and neck squamous cell carcinomas (HNSCC) comprise about 6% of all malignant neoplasms. The major risk factors are smoking and alcohol consumption. Overall the survival rate has improved, but is still low, especially in developing countries [5]. The choice of healing method depends on localization and clinical stage of neoplasm. Radiotherapy as the single treatment is usually chosen in very early stages of HNSCC [6]. Routine treatments are chemotherapy and radiotherapy alone or in combination. In early-stage disease (T1–2, N0–1) the use of both these methods leads to health restoration in 60–90% of cases. However, in advanced stages the results are worse. Percentages of local adversity exceed 60% and rise in conjunction with stage increase and the risk of distant metastasis [7]. Standard practice is to offer postoperative adjuvant radiation therapy to patients at high risk for recurrence within HNSCC, the hope being that overall survival will

increase [8]. In this work, using peripheral blood lymphocytes we investigated the DSBs repair ability which could account for clinical sensitivity to anti-cancer treatment of head and neck cancer patients. Neutral comet assay was used to examine repair of DSBs in examined cells. The cell viability and DSBs capability to apoptosis induction was also estimated after hydrogen peroxide and γ -radiation treatment.

MATERIALS AND METHODS

Patients. Eighteen patients with HNSCC were enrolled in the study (10 men and 8 women; mean age 61 ± 9). The lymphocytes were collected from peripheral blood of patients before they had received any chemotherapy or radiation therapy for their primary disease. The diagnosis was made after histopathological examination of patient biopsies. 18 healthy subjects without cancer (14 men and 4 women; mean age 58 ± 12) were also used as a blood donors. Moreover, controls were selected based on family history, in order to exclude familiar predisposition to cancer development. Prior to examination, the patients and control subjects did not receive other medications such as antibiotics or steroids. Despite of 3 years younger controls, there were no statistical differences in age of analyzed patient subjects and control group ($P = 0.402$). Patient and control subjects enrolled in the examination were non-smokers. Other exclusion criteria for the patient and control subjects enrolled in the examination was alcohol consumption. All patient and control subjects were recruited from two medical units of the Head and Neck Neoplasm Surgery Department, the Department of Otolaryngology and Oncology, both of the Medical University of Lodz, Lodz, Poland. All subjects included in the study were unrelated Caucasians and lived in the Lodz district, Poland. The study

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Abbreviations used: DSB – DNA double strand breaks; HNSCC – Head and Neck Squamous Cell Carcinoma; MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole.

was approved by the Local Ethic Committee and written consent was obtained from each patient or healthy blood donor before enrolling into the study.

Lymphocytes isolation and DNA damage induction. Peripheral blood lymphocytes from blood of healthy donors and HNSCC patients were isolated by centrifugation (15 min, $280 \times g$) in a density gradient of histopaque-1077 (Sigma, Poland). Lymphocytes accounted for about 92% of leukocytes in the obtained cell suspensions as judged by the characteristic shape of their nucleus. The final concentration of lymphocytes was adjusted to $1\text{--}3 \times 10^5$ cells/ml by adding EMEM medium (ATCC, USA) to the single cell suspension.

After isolation lymphocytes were incubated for 10 min on ice with different concentrations of hydrogen peroxide (from 2.5 to 50 μM). After incubation cells were washed in EMEM medium by centrifugation for 15 min at $280 \times g$. Alternatively, the lymphocytes were irradiated using ^{60}Co source at the dose of 5 to 50 Gy at 37 °C in growth medium. Then, to examine DNA repair, cells were harvested immediately (time 0) and 30, 60, 120 and 240 min thereafter, and placed on ice to stop the repair reaction.

MTT assay for cell survival. MTT test was used to quantitatively determine the survival of lymphocytes that had been treated with genotoxic agent. Cell suspensions (1.5×10^6 /ml of growth medium) were incubated with hydrogen peroxide or irradiated, and 4 days later cell viability was evaluated by the MTT assay. Briefly, cells were plated onto 96-well plates in 200 μl growth medium and 20 μl of 10 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide) reagent was added to each well. After incubation at 37 °C for 4 h, the supernatant was removed and an aliquot of 200 μl solution containing 10% SDS and 0.04 N HCl was added to dissolve the water-insoluble formazan salt. One hour later, the difference OD_{650 nm}–OD_{570 nm} was measured with an ELISA reader (Bio-Rad, Hercules, CA, USA). Based on OD value, cell resistance to drugs was presented as a percentage of the survival of untreated cells estimated as 100% of controls.

Double strand breaks repair assay. DNA damage and repair were measured by the single cell gel electrophoresis method (comet assay). The comet assay was performed under neutral conditions according to the procedure of Wojewodzka *et al.* [9] with some modifications. Cell suspension in 0.75% LMP agarose dissolved in PBS was spread onto microscope slides (Superior, Germany) pre-coated with 0.5% normal-melting agarose. The cells were then lysed for 1 h at 4 °C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After lysis, the slides were placed in a buffer consisting of 100 mM Tris, 300 mM sodium acetate, pH = 9 for 20 min. After that, the slides were placed in an electrophoresis unit in the electrophoretic solution consisting of 100 mM Tris, 300 mM sodium acetate pH = 9. Electrophoresis was conducted at 4 °C (the temperature of the running buffer

did not exceed 12 °C) for 60 min at the electric field strength of 0.4 V/cm (50 mA). To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark.

Comet assay analysis. The objects were observed at 200 \times magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to COHU 4910 video camera (Cohu, San Diego, CA, USA) equipped with UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, Czech Republic). Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells, and the mean percentage of DNA in comet tail (Tail DNA %) was calculated. This quantity is positively correlated with the level of DNA breakage in a cell and was taken as an index of DNA damage in each sample.

Detection of apoptosis. Annexin V-FITC (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen, San Jose, CA, USA) assay specific for phosphatidylserine was used to quantitatively determine the percentage of cells within a population that are undergoing apoptosis. After cells genotoxic treatment with hydrogen peroxide or γ -radiation, apoptosis rate was evaluated 4 h later in growth medium at 37 °C. For staining procedure cells were washed twice with cold PBS then resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml. 100 μl of solution (1×10^6 cells/ml) were transferred to a 5 ml culture tube. 5 μl of annexin V-FITC and 5 μl of propidium iodide was added. The cells were gently vortexed and incubated for 15 min at 25 °C in the dark. After incubation 400 μl of 1X binding buffer was added to each tube. Cells were analyzed with a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA) using the CellQuest program, then percentage of necrotic cells, apoptotic cells, non-apoptotic cells and cells at early stage of apoptosis were measured.

Statistical analysis. The data of MTT test were expressed as mean \pm SD and the values from comet assay in this study were expressed as mean \pm SEM of three separate experiments from each analyzed patient and control. If no significant difference between variations was found, as assessed by Snedecor — Fisher test, the difference between means was evaluated by applying Student's *t*-test. Otherwise, the Cochran-Cox test was used. The data was analyzed using STATISTICA (StatSoft, Tulsa, OK) statistical package.

RESULTS

Cell survival under genotoxic treatment. Fig. 1 shows mean percentage of survival of lymphocytes from HNSCC patients and healthy donors used as a control measured by MTT test 4 days after incubation with increasing concentrations of hydrogen peroxide (from 2.5 μM to 50 μM) or irradiated with increasing doses of γ -radiation (from 5 Gy to 50 Gy).

Lymphocytes from HNSCC patients were sensitive to genotoxic treatment, and it was found that hydrogen peroxide treatment induced statistically

significant differences at all concentrations from 2.5 μM to 25 μM ($P < 0.001$) (see Fig. 1; upper panel). Cells from cancer patients demonstrated higher sensitivity to hydrogen peroxide compared with those from healthy controls. Survival of control cells decreased to 83% for 10 μM concentration while survival of lymphocytes from HNSCC patients decreased to 29%. At the highest dose used in further experiments (50 μM) the average survival of the control cells was 22% and for lymphocytes from HNSCC patients it was 6%.

Lymphocytes from HNSCC patients were also sensitive to γ -radiation, and it was found that increasing doses induced statistically significant differences between patients and control lymphocytes ($P < 0.001$) (see Fig. 1; lower panel). There was a dose-dependent (from 25 Gy to 50 Gy) decrease in cell viability of both patients and control lymphocytes. The average viability of the control cells for 25 Gy was 90% and for 50 Gy 78% while for lymphocytes from HNSCC patients average viability was 83% and 54% respectively.

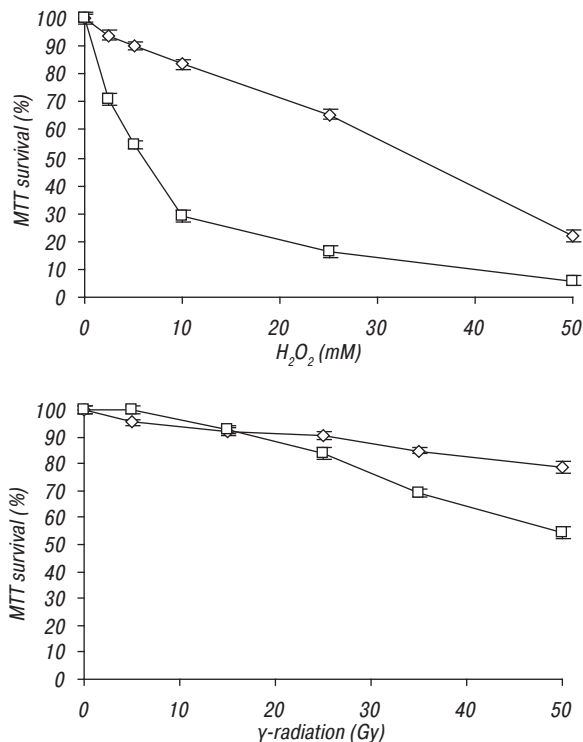


Fig. 1. Survival rate of peripheral blood lymphocytes obtained from HNSCC patients (\square) and control subjects (\diamond) measured by MTT assay after treatment with 2.5, 5, 10, 15, 25, 50 μM of H_2O_2 (upper panel) or 5, 15, 25, 35, 50 Gy of γ -radiation (lower panel). Error bars denote SD

The kinetic of DNA double strand breaks repair.

Fig. 2 shows DNA damage presented as percentage of DNA in comet tail (Tail DNA %) lymphocytes from HNSCC patients and healthy donors after genotoxic treatment with hydrogen peroxide at the concentrations of 5 μM and 10 μM or irradiated with dose of 5 Gy and 25 Gy immediately after exposure as well as 30, 60, 120 and 240 min thereafter. In all cases, DNA damage in the control cells was constantly low, indicating that preparation and subsequent processing of the cells did not introduce significant damage to their DNA.

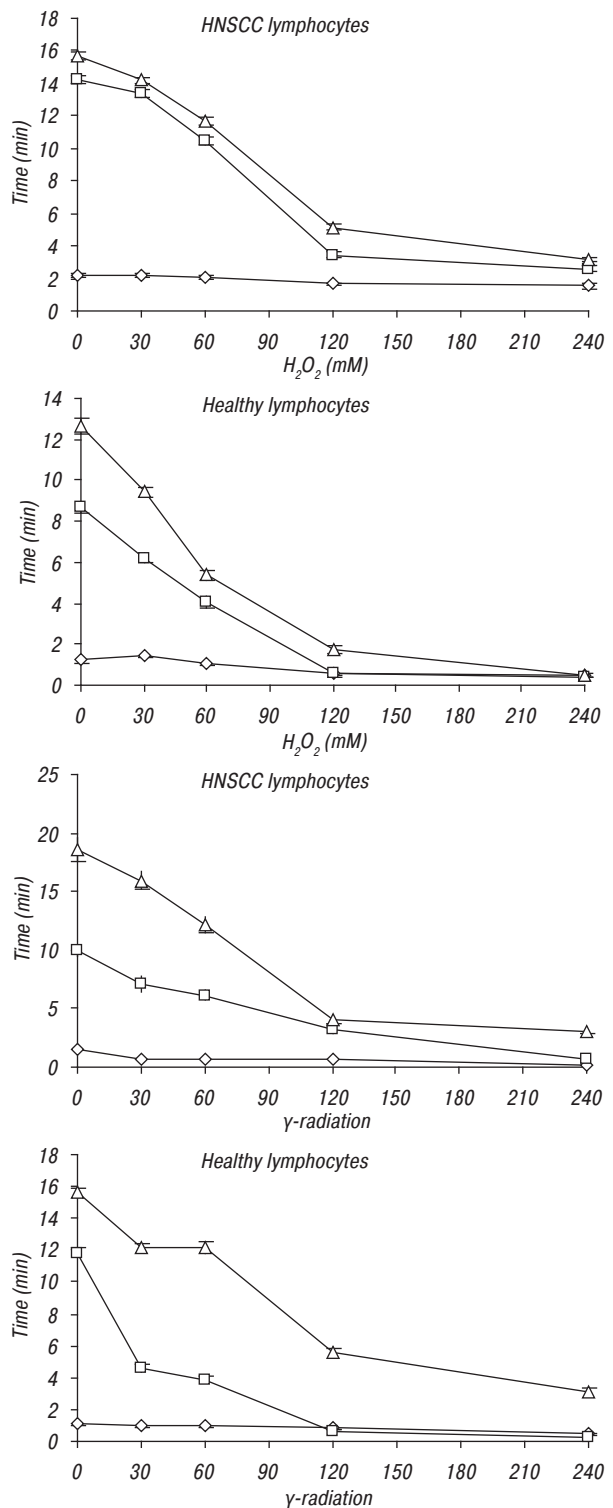


Fig. 2. Time course of the repair of DNA damage in peripheral blood lymphocytes obtained from HNSCC patients (left rows) and control subjects (right rows) measured by neutral comet assay. The graph displayed comet DNA tails percentages of cells after treatment with 5 μM (\square) and 10 μM (Δ) of H_2O_2 , (upper panel) or 5 Gy (\square) and 25 Gy (Δ) of γ -radiation (lower panel) according to not treated control cells (\diamond). In each sample % of tail DNA was measured in 100 cells. Error bars denote SEM

Lymphocytes from healthy donors treated with 5 μM of hydrogen peroxide were able to completely repair DNA damage ($P > 0.05$) during 120 min post-treatment incubation repair, while cells treated with 10 μM hydrogen peroxide recovered after 240 min ($P > 0.05$) (see Fig. 2; upper panel). Lymphocytes from

HNSCC patients treated with 5 or 10 μM of hydrogen peroxide were not able to completely repair DSBs even after 240 min post-treatment incubation ($P < 0.001$).

Healthy lymphocytes exposed to 5 Gy of ionizing radiation were able to completely repair DNA damage ($P > 0.05$) during 120 min of post-treatment incubation repair (see Fig. 2; lower panel). Exposition to 25 Gy caused damage that has not been completely repaired within 240 min ($P < 0.001$). Efficiency of DNA repair in lymphocytes from HNSCC patients exposed to 5 Gy after 120 min post-treatment incubation was only 67%, and cells completely recovered after 240 min ($P = 0.01$). The dose of 25 Gy caused damage that HNSCC cells could not completely repair even after 240 min ($P < 0.001$).

Apoptosis induction. The data of apoptosis analysis is shown in Fig. 3. In each experiment percentage of living cells not treated with any genotoxic agent was no less than 72% and below 2% of necrotic cells. Cells at early apoptosis (membrane integrity is present) were also measured apart from apoptotic cells (end-stage apoptosis and death). It was found that cells at early stage of apoptosis after genotoxic treatment represented no more than 33% of analyzed populations but after γ -radiation treatment percentage of end-stage apoptosis raised in a dose-dependent manner up to 26%. It was observed that hydrogen peroxide at both concentrations (5 μM and 10 μM) induced stronger activation of apoptosis in lymphocytes from HNSCC patients than from healthy controls ($P < 0.001$) (see Fig. 3; upper panel). The data analysis of cells within a population that were actively undergoing apoptosis after treatment with 5 μM hydrogen peroxide showed 1.3% of control lymphocytes and 21.5% of HNSCC lymphocytes, undergoing early apoptosis at 5.2% and 33.4% respectively. Analysis of cells within a population that were actively undergoing apoptosis after treatment with 10 μM of hydrogen peroxide showed 10.8% of control lymphocytes and 19.5% of HNSCC lymphocytes, undergoing early apoptosis at 8.8% and 33.2% respectively. It was also shown in the lower panel of Fig. 3 that γ -radiation induced stronger apoptosis in HNSCC lymphocytes than in control lymphocytes at the dose of 15 Gy and 25 Gy ($P < 0.001$). Percentage of apoptotic cells after irradiation with 15 Gy for control lymphocytes was 8.4% while for HNSCC lymphocytes was 19% and for dose of 25 Gy was 12.8% and 26.2% respectively. Percentage of cells at early apoptosis after irradiation with 15 Gy for control lymphocytes was 12.5% while for HNSCC lymphocytes was 30.9% and for the dose of 25 Gy was 13% and 30.3% respectively.

DISCUSSION

DNA double-strand breaks are the major intracellular lethal lesion. In response to DSBs, cells must trigger cascade of biological pathways to promote repair of the DNA damage in order to survive and restore genome integrity. To elucidate the molecular mechanisms involved in cellular responses to clinical radiation, it is informative to clarify the roles of DSBs [10].

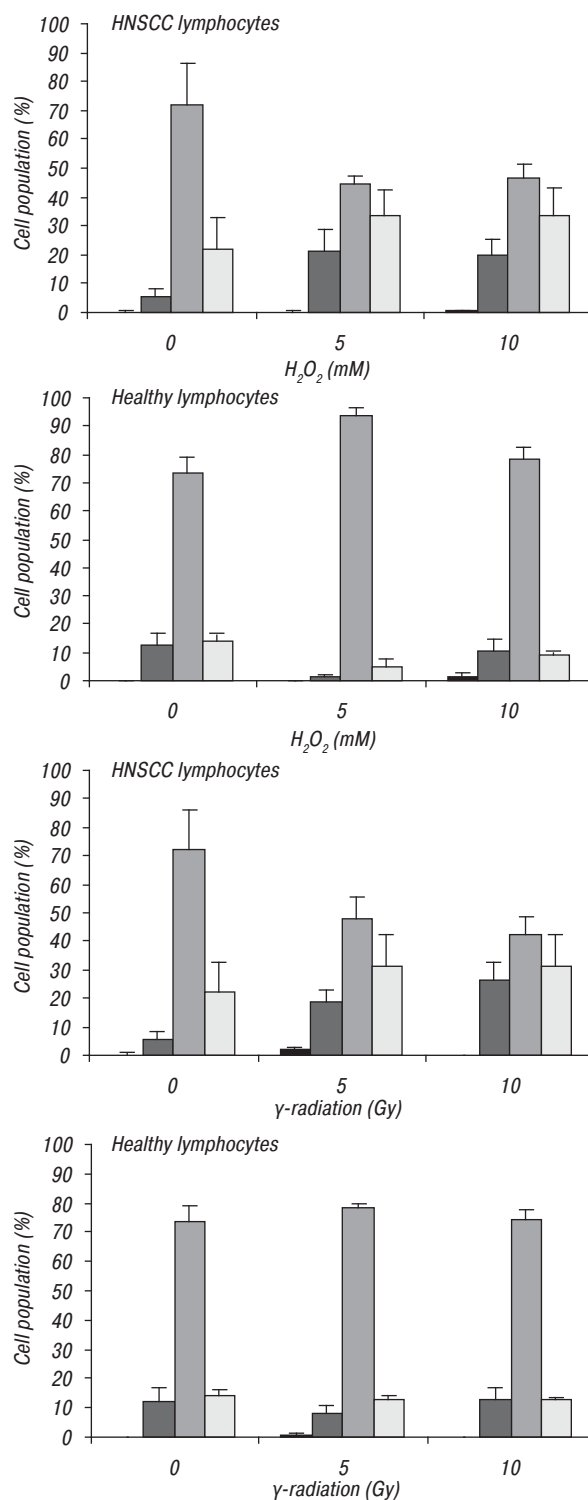


Fig. 3. Apoptosis induction in peripheral blood lymphocytes obtained from HNSCC patients (left row) and control subjects (right row) treated with 5 μM and 10 μM of H_2O_2 (upper panel) or 15 Gy and 25 Gy of γ -radiation (lower panel). The graph displayed percentage of necrotic cells (■), apoptotic cells (■), non-apoptotic cells (□) and cell in early stage of apoptosis (□). Error bars denote SD

In eukaryotes, there are at least two major DSBs repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR) [11]. In the treatment of HNSCC radiation is chosen alone or is offered combined with chemotherapy to patients at high risk metastasis or the development of a new primary head

and neck cancer. However, in advanced stages of this disease the outcomes worsen contemporaneously with the progression of disease and metastasis development. The molecular basis for successful therapy in HNSCC patients are still debatable [7].

Previously, alkaline comet assay has been used to examine the association of DNA repair capacity in peripheral white blood cells with risk of various cancers [12–14] including HNSCC [15–17]. Palyvoda *et al.* [15] measured DNA damage at six time points between 0 and 180 min after γ -radiation exposure in HNSCC patients and healthy controls. The study showed increased baseline damage and decreased repair in HNSCC patients. Iwakawa *et al.* [16] showed that residual DNA damage after 15 min of repair was significantly higher in HNSCC patients compared to healthy controls. Saha *et al.* [17] examined peripheral white blood cells and showed that during first 15 min the repair was also slower in HNSCC patients than in healthy controls. Recently, we had examined the DNA damage level in peripheral blood lymphocytes from HNSCC patients and healthy controls by alkaline comet assay [18]. We analyzed time-course of DNA repair within 240 min of incubation after cells treatment with γ -radiation. Eventually, we have evidenced a higher level of DNA damage and decreased efficiency of its repair in HNSCC cancer cells. Therefore, our future goal is an investigation of DSBs processing in association with apoptosis in head and neck cancer patients. Higher sensitivity to DSBs is likely to be caused by apoptosis induction what in turn may be connected to effective cancer therapy.

It has been shown a wide range of DSBs sensitivity between different cell populations including cancer and normal cells [4]. There is a growing number of evidences that the susceptibility to radiation is caused by the genetic predisposition including mutations and/or polymorphisms in genes involved in cellular responses to genotoxic treatment [19]. Recently Rube *et al.* [20] has been reported increased radiosensitivity associated with genetically determined DSBs repair. In this study the highly sensitive gammaH2AX-foci approach was used to test DNA double-strand break repair of blood lymphocytes analysed in a preclinical mouse model. In our study using human peripheral blood lymphocytes examined by neutral comet assay, we have reported that the molecular basis for successful therapy of head and neck cancer patients might be their sensitivity to DSBs. Using MTT assay, it was found that HNSCC lymphocytes were killed by lower concentration of hydrogen peroxide than control cells. γ -radiation caused the larger HNSCC cells death in comparison with lymphocytes from healthy controls. Moreover, it was found that γ -radiation caused higher apoptosis induction in lymphocytes from HNSCC patients than that of healthy donors. In connection to higher genotoxic susceptibility, impaired repair of DSBs caused by γ -radiation was evidenced in lymphocytes from HNSCC patients. We conclude that in comparison to healthy donors,

HNSCC lymphocytes are highly sensitive to genotoxic treatment and the molecular basis for this finding may be impaired DSBs repair. Furthermore, we have found an increased induction of apoptosis in the peripheral blood lymphocytes of HNSCC patients following the genotoxic exposures. That might confirm that the deficiency of DSBs repair and increased apoptosis in cells of patients with head and neck cancer is relevant for efficient therapy of HNSCC.

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