Radiosensitivity of peripheral blood lymphocytes from healthy donors and cervical cancer patients; the correspondence of in vitro data with the clinical outcome

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The reliability of a particular *in vitro* parameter as potential prognostic biomarker of individual radiosensitivity is still discussed. Therefore, several *in vitro* radiation-induced cellular endpoints including initial, oxidative and residual DNA damage and the rate of DNA repair were assessed in peripheral blood lymphocytes (PBL) from healthy donors and patients with carcinoma of the cervix using the alkaline single cell gel electrophoresis (the comet assay). PBL from cancer patients were analyzed three times during the course of therapy, prior, in the middle (25-27 Gy) and after the radiotherapy. Inter-individual differences in radiation-induced DNA damage and in the kinetics of strand break rejoining were determined within both groups. Significantly higher level of mean background and oxidative DNA damage was estimated in the cancer patient cohort than in the healthy subject group; however similar mean values of the initial DNA damage and the rate of DNA repair kinetics were found in both groups. No adaptive response was determined in PBL from cancer patients due to radiotherapy.

The acute radiation toxicity and the clinical outcome were scored according to the criteria as proposed by the National Cancer Institute. A substantial delay in DNA strand break rejoining was determined in cancer patients suffering from adverse side effects (G2+) in comparison to persons with no or very mild radiation toxicity (G0-G1) ($p<0.05$). The recurrence of disease has been associated with a lower initial DNA damage and slope value of dose-response effect, and increased rate of DNA repair. Results from this pilot study suggest that the residual DNA damage level might be a promising prognostic biomarker of acute radiation morbidity; however, further study is necessary to validate this finding.

Key words: Cervical cancer, Comet assay, Normal tissue damage, Tumor radioresponse, Clinical radiosensitivity,

Cancer of the cervix uteri is the second most prevalent cancer in women worldwide [1] and the fourth most common neoplasm in women in Europe [2]. The magnitude of this disease is probably much greatly important because the cervical cancer affects rather younger women than other malignancies; nearly ten percent of all cases are women younger than 35 years [3]. Although the cervical cancer incidence and mortality have been declining in many populations in the last few decades, paradoxically, upward trends have been reported in younger women in several countries including Slovakia [4].

Surgery, radiotherapy (RT) and cisplatin-based concurrent chemotherapy are the main treatment procedure employed in an effort to improve survival rate of the cervical cancer patients [5]. Despite the latest advances in RT treatment modalities such as optimization of treatment delivery schedules and technologic improvements in the physical targeting of ionizing radiation (IR), a number of patients relapse within the radiation field. A five-year overall and disease-free survival rates of patients with the carcinoma of the cervix is favourable at 60%, however, for patients with a regional or distant dissemination cancer is less encouraging [6]. Normal tissue adverse reactions to RT are the dose-limiting factors in delivery of radiation to cancer patient. Moreover, heterogeneity in patient response to RT, overreactions, and unresponsiveness to therapy, are the main obstacles to improve the patient outcome. The management of cervical cancer hence remains a critical medical need.

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An intense research effort has been made to characterize factors underlying the intrinsic cellular sensitivity to IR and develop assays to predict the clinical radiosensitivity of patients undergoing RT. Despite a good correlation between some endpoints (e.g. SF2, cytological approaches) and the clinical observations [7, 8], such measurements are not routinely used in a clinical practice because they are laborious and time consuming. A number of experiments with stable cell lines and patient cells with different sensitivity to IR have consistently demonstrated that the single cell gel electrophoresis (the comet assay) is able to detect heterogeneity in cellular response to radiation [9–13]. Since the reliability of particular *in vitro* parameter as potential prognostic biomarker of individual radiosensitivity is still discussed, this pilot study was established using a randomly selected group of 18 patients with squamous cell carcinoma of the cervix uteri who were treated with a uniform radiation regimen. Twenty-four age-matched healthy women were used as a reference (control group). Using the alkaline comet assay, several parameters were analyzed in non-irradiated and irradiated peripheral blood lymphocytes (PBL) from cervical cancer patients and healthy women. In addition to the primary objective of this prospective study to assess differences between healthy donors and cancer patients as a secondary goal the correspondence of various *in vitro* radiation-induced cellular endpoints with normal tissue and tumour radio-response to therapy was evaluated.

Materials and methods

Patients and therapy regimen. Women with histologically proven squamous cell carcinoma of the uterine cervix, grading 1-3, FIGO stage IB2-IVA, were involved in this prospective study (Table 1). Participants of the study were recruited at the Radiotherapy Department of the National Cancer Institute, Bratislava from June 2004 to November 2005. Approval by the local ethics committee was provided before recruitment of the patients and only persons providing written informed consent were included in this study. Each patient was asked to complete a questionnaire on her medical history and lifestyle, including medication, alcohol consumption, smoking habits etc. Radiotherapy consisted of external beam irradiation (EBRT) and intracavitary brachytherapy (BT) treatment. EBRT was performed with a 18-MV linear accelerator CLINAC 2100/2300C (Varian) in 1.8 or 2 Gy per fraction, five times a week. Clinical target volume was the tumor and lymphatics in pelvis plus para-aortic nodes, if involved. A conformal 4-field box technique was used for all patients. Total dose to the pelvis ranged from 40 – 60 Gy including boost doses to parametrial involvement, and total dose to the para-aortic nodes was 39.6 and 46 Gy, respectively. Intracavitary BT was performed using an Ir-192 high-dose-rate remote after-loading system. The prescribed dosage was 6 Gy per insertion to point A twice a week to a total dose of 12 – 24 Gy. All cervical cancer patients were treated by concomitant chemotherapy with cisplatin (n=17), one woman was medicated with 5-fluorouracil. Cisplatin was given by single weekly *i.v.* administration at 40 mg/m^2 for 3-8 weeks, and 5-FU at a dose of 500 mg weekly for 6 weeks. The mean patient age at the time of diagnosis was 51.8 years (range, 31 – 68 years). Eight of the patients were non-smokers, 7 were smokers and 3 ex-smokers.

Table 1. Tumor characteristics and therapy regimen of cervical cancer patients

Parameter	Number. of patients
FIGO* stage	
IB2	1
IIA	3
IIB	10
IIIB	3
IVA	1
Nodal status	
N0	3
N1	7
Nx	8
Metastatic status	
M0	18
Tumor radiation dose:	
40 – 46 Gy	11
47 – 50.4 Gy	7
Brachytherapy (^{192}Ir)	
no	1
yes	17
Parametrial boost	
no	12
yes	6
Para-aortal field boost	
no	16
yes	2
Chemotherapy	
cisplatin	17
5-fluorouracil	1

* FIGO, the International Federation of Gynecology and Obstretics classification

Assessment of normal tissue reactions to radiation. The acute radiation side effects were assessed during the therapy. The acute radiation morbidity was scored according to the Common Toxicity Criteria, version 3.0, of the National Cancer Institute [14]. Ten patients had no or very light adverse reactions to RT and were graded as 0-1 (G0–G1), 6 patients experienced side effects classified as grade 2 (G2: erythema, moderate edema, nausea/vomiting, diarrhea requiring drugs, abdominal pain, dysuria), and the adverse normal tissue reactions to RT of two patients were graded as 3 (G3: pitting edema, nausea/vomiting requiring parenteral support, abdominal pain, diarrhea requiring parenteral support, severe mucous or blood discharge, abdominal distention, dysuria, pelvis pain, hematuria). Development of at least one grade 2 reaction was considered as enhanced radiation toxicity and was taken as an indicator for acute clinical radiosensitivity.

Assessment of clinical outcome. Patients were regularly reviewed in 6-month intervals. Besides pelvic examination

and transvaginal ultrasonography performed during each follow-up visit, tumor markers (SCCA, CEA) were checked every 6 months and radiographic examinations (chest X-ray, abdominopelvic computed tomography or magnetic resonance imaging) was conducted yearly. Tumor response to RT was categorized by the degree of shrinkage at the end of RT. Complete response was characterized by shrinkage to <1% of the prior RT volume, partial response by ≥35% and stable disease by <35%. Local recurrence was confirmed if the disease was detected in the irradiated field (pelvis) and distant metastases if tumors occurred in the para-aortic lymph nodes or elsewhere outside the pelvis.

Control group. A group of 24 age-matched healthy women served as a control cohort. The healthy female donors had never been occupationally exposed to radiation and did not undergo any gynecological treatment. Only those women who provided written informed consent were included in the study. Each donor was asked to complete a questionnaire on her medical histories and lifestyles, including medication, alcohol consumption, smoking habits etc. The female donors were recruited at the National Blood Transfusion Station, Ružinov Hospital, Bratislava at the same period as the cancer patients. The mean age of the control cohort was 49.1 years with a range of 35 to 59 years. Four women were smokers, 6 ex-smokers and 15 female donors were non-smokers.

Blood collection and isolation of lymphocytes. Blood was taken from female donors and cervical cancer patients by venous puncture. From cancer patients, blood samples were taken three times during the course of therapy – prior to conventional radiotherapy, during dose regimen (after cumulative dose of 23-25 Gy) and 1 month after final treatment. Peripheral blood lymphocytes (PBLs) were separated from the heparinised blood by density-gradient centrifugation using the lymphocyte separation medium (PAA Laboratories GmbH) according to the manufacturer´s instructions. PBLs were washed twice with phosphate buffered saline (PBS, Ca^{2+}- and Mg^{2+}- free) and finally suspended in the freezing medium (90% FCS and 10% dimethyl sulphoxide) and stored frozen at -80°C until analysis.

Cell irradiation in vitro. Shortly prior to irradiation, PBLs were thawed, suspended in 0.75% LMP agarose to a final concentration $3x10^5$/ml and spread on a base NMP layer. Cells embedded in agarose were irradiated on slides using a 6 MV linear accelerator CLINAC 2100/2300C (Varian). The slides were kept in ice-cold Hank's solution during irradiation, then the slides were placed in ice-cold lysis solution (initial DNA damage) or in RPMI 1640 medium (kinetics of DNA repair, residual DNA damage), supplemented with 10% foetal calf serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively), at 37°C for specified times prior to lysis. PBLs from healthy donors were processed by the same mode.

Single cell gel electrophoresis. The procedure of Singh et al.[15], modified by Gábelová et al. [16] was used. Cells suspended in 0.75% low melting point (LMP) agarose were spread on base layer of 1.0 % normal melting point (NMP) agarose in phosphate buffer (PBS, Ca2+ and Mg^{2+} free). The slides were then placed in a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, pH 10 and 1% Triton X-100) at 4°C for 1 h to remove cellular proteins. After 40 min unwinding time in an electrophoretic box in alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH>13) at 4°C, a current of 25 V (300 mA) was applied for 30 min. The slides were removed, neutralised with 3x5 min washes with Tris-HCl (0.4 M, pH 7.5), and stained with 20 ml of ethidium bromide (EtBr, 10 mg/ml). The samples were examined with the Olympus BX51 fluorescence microscope by image analysis using the software Komet 5.5 (Kinetic Imaging Ltd.). The percentage of DNA in the tail (% tail DNA) was used to assess the extent of DNA damage. A total of 100 nucleoids (triplicate of slides) were scored per each sample in one electrophoretic run. PBLs of one healthy female were used as a reference sample; lymphocytes were irradiated and treated in parallel with the patient and healthy cohort samples in each experiment. Data of the reference sample were used to calculate experimental variability and the cut-point for exclusion of the experimental data from the statistical analysis [17].

The oxidative DNA damage was detected using repair specific DNA endonucleases, formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (endo III) according to the procedure of Collins et al. [18]. After lysis, the slides were washed 3 times for 5 min in endonuclease buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, pH 8.0) and then incubated with endo III (45 min.) or Fpg (30 min) diluted in endonuclease buffer to working concentrations. The slides were then transferred to an electrophoretic box and processed as described above. The relative level of oxidative damage was expressed as net endo III- and Fpg-sensitive sites by subtracting the value of DNA damage determined in nucleoids incubated with endonuclease buffer alone.

The level of residual DNA damage was calculated according to Aka et al. [19]. The amount of DNA damage remaining at a given repair time (tx) was defined as follows:

$$RD = \frac{TD_{(tx)} - TD_{(pre)}}{TD_{(0)} - TD_{(pre)}};$$

where: RD is residual DNA damage, $TD_{(0)}$ is the % tail DNA immediately after irradiation, $TD_{(pre)}$ is the % tail DNA prior to irradiation, $TD_{(tx)}$ is the % tail DNA at a given time x (15, 30, 60, 90, and 120 min) after irradiation.

Statistical analysis. Data are given as mean values with ±SD. The inter-patient variation and differences between healthy donors and cancer patients were evaluated by the Student´s *t*-test, the Wilcoxon rank sum test and one-way analysis of variance (*ANOVA*). The threshold of statistical significance was set at $p<0.05$. Dose-response data were fitted by a linear regression and the slopes of the dose-response curves were determined. A nonlinear (exponential) regression model was proposed and

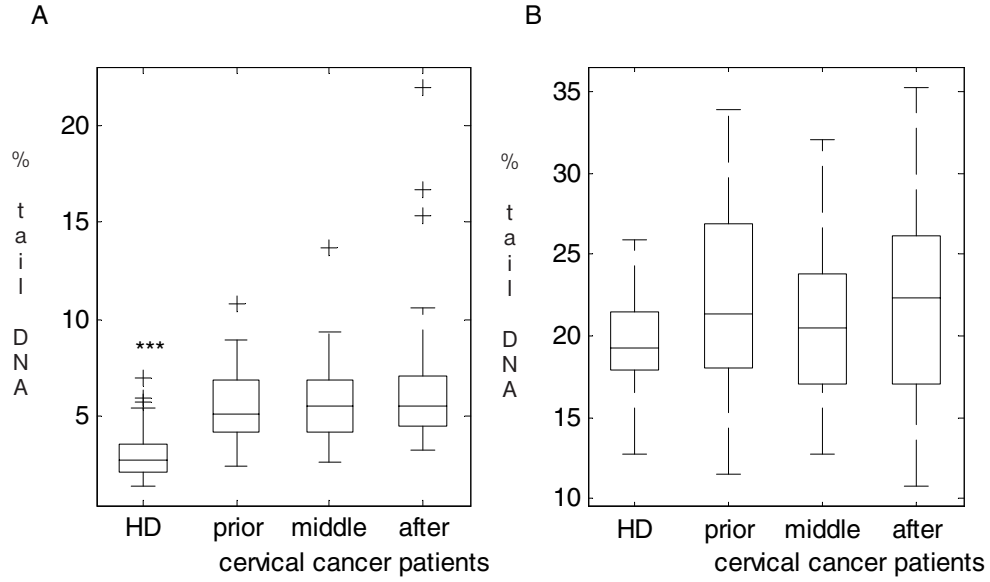

Figure 1. Boxplots of background (A) and initial DNA damage after a dose of 2Gy (B) determined in PBLs from healthy donors (HD) and cervical cancer patients during the course of therapy. The boxes have lines at the lower quartile, median, and upper quartile values. The whiskers show the extent of the rest of the data. Whiskers extend from the box out to the most extreme data value within 1.5 * IQR (the inter quartile range). Symbol '+' denotes the data with values beyond the ends of the whiskers (the outliers). The background DNA damage of healthy donors is significantly different from that of the cancer patients prior, in the middle and after the therapy, Student t-test as well as the Wilcoxon rank sum test for difference in means (medians) of healthy donors and the cancer patients resulted in small p-values, p < 0.001 (***) in all cases. The initial DNA damage (after a dose of 2Gy) of healthy donors was not proven to be significantly different from that of the cancer patients.

applied to evaluate the rate of DNA repair kinetics. Coefficient C, the main indicator of DNA repair kinetics, was estimated by nonlinear least squares method by using the model:

$y_{it} = A_i + B_i \exp(-C_i t) + e_{it}$;

where: y_{it} is DNA damage of individual patient with index i; and *t* is a specified time after irradiation (0 – 120 min).

Results

Healthy donors and cancer patients. An inter-individual variation in the basal and initial DNA damage was found within both the healthy cohort and patient group (Fig. 1). The background DNA damage detected in PBLs from cancer patients was significantly higher in comparison to healthy women ($p<0.001$); however, it did not change substantially during the course of therapy (Fig. 1A). Cell-irradiation with a dose of 2 Gy resulted in a variable but statistically significant increase in DNA strand breakage in both groups (Fig. 1B); however in contrast to basal DNA damage, no significant differences in the level of DNA fragmentation were found between control cohort and cancer patient group (mean values: 19.3±2.9 *vs*. 22.2±5.5, respectively). Moreover, approximately equal mean values of initial DNA damage were detected during and after final treatment (20.85±4.76 vs. 21.83±5.98, respectively).

A substantially larger inter-individual variation and significantly higher levels of oxidative DNA damage were ascertained in cancer patient group compared to healthy donor cohort (Table 2). The RT had only negligible effect on the oxidative DNA damage lesions; insignificant differences

Table 2 The mean oxidative DNA damage levels in healthy donors and cervical cancer patients during the course of radiotherapy

	healthy donors				caner patients			
	background		30 min after RT		background		30 min after RT	
	endo III	Fpg	endo III	Fpg	endo III	Fpg	endo III	Fpg
prior to RT	1.278±0.026	1.292±1.569	1.731±2.301	2.724±2.997	3.891±4.300[a]	6.324±7.143[a]	7.454±6.691[b]	10.177±8.362[b]
during RT	-	-	-	-	2.106±2.812	3.534±4.514	6.176±5.245	7.378±7.081
post RT	-	-	-	-	2.532±1.251	4.352±3.634	5.299±3.542	6.141±5.609

Significantly different from healthy donors, Student t-test, [a]$p<0.05$, [b]$p<0.01$

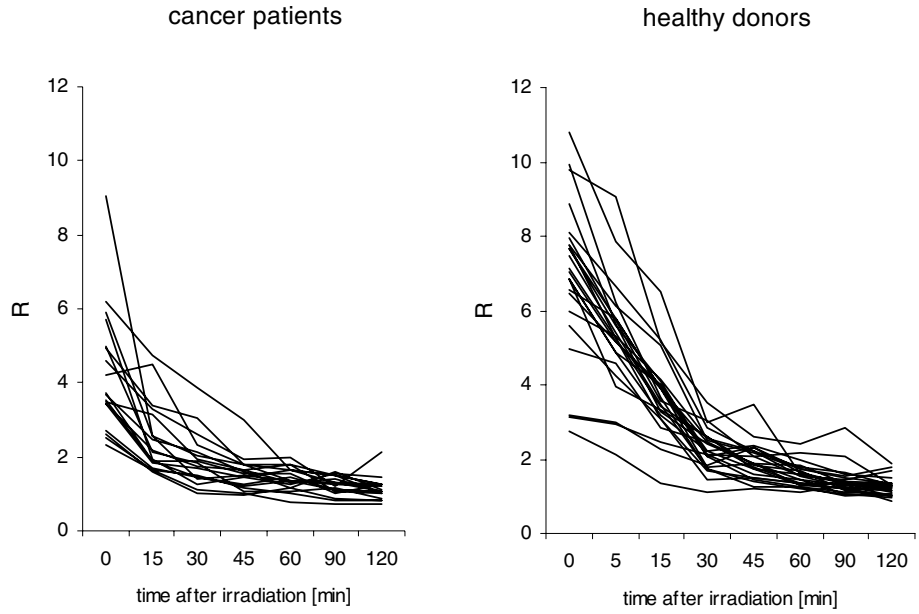

Figure 2. Kinetics of DNA repair in PBLs from cancer patients and healthy donors irradiated with a dose of 2 Gy. R – the ratio of % tail DNA in irradiated to nonirradiated cells at specified times after irradiation.

in the mean values of endo III- and Fpg-sensitive sites were detected during and after final treatment. Nearly two-fold higher mean levels of endo III- and Fpg-sensitive sites were detected 30 min after irradiation in both groups.

Although a wide heterogeneity was found in the rate of DNA damage repair within the group of healthy women and cancer patients, the strand break levels in irradiated lymphocytes had returned to near the basal values within 120 min after irradiation in both groups (Fig. 2). Values are presented in relative units as ratio of % tail DNA in irradiated to nonirradiated cells to give the yield of induced DNA damage. The curves of DNA repair kinetics in PBLs from cancer patients are slighter than in healthy donors due to higher level of basal DNA damage. A mild but negligible intra-individual variation in the rate of DNA repair kinetics was determined in PBLs from cancer patients during and after therapy (data not shown). In order to compare the DNA repair efficiency between patients and donors, individual data on % tail DNA damage were fitted by a nonlinear (exponential) regression and the rate of repair kinetics was expressed as coefficient C. Figure 3 shows the probability density of the coefficient C distribution for healthy and cancer patient subjects. A clear shift in the coefficient C values was estimated for controls and patients; the mean coefficient C value for healthy women (C = 0.0563, 95% CI 0.0450 – 0.0676) was higher than for cancer patients (C = 0.0385, 95% CI 0.0321 – 0.0449). Moreover, a bimodal shape of the probability density determined in both groups suggests that as the healthy cohort so the patient group is a non-homogeneous population consisting from two distinct subpopulations. The distribution of the coefficient C of both cohorts are best separated at the threshold value $C_T = 0.04092$.

The quantity of residual DNA damage (RD) in PBLs from both donors and patients were calculated at several time intervals after irradiation. Although a trend towards higher level of mean RD was found in patient group prior to therapy compared to control cohort up to 60 min after irradiation, it did not reach a statistical significance (data not shown). Likewise to DNA repair kinetics, only insignificant intra-individual variability in RD values was found in PBLs from patients under therapy (data not shown).

Normal tissue radiation toxicity and the comet assay results. Based on the clinical acute toxicity criteria to radiotherapy (see Material and methods), the patient cohort was divided in 2 subgroups, patients with no or very mild side effects (G0-G1), and radiosensitive patients (G2+) with radiation morbidity classified as grade 2 and more. No significant differences in the basal and initial DNA damage, and the rate of DNA repair kinetics (coefficient C) were determined between patients with acute radiation toxicity and patients without any adverse reaction to radiotherapy prior, during and after therapy (data not shown). More than twofold higher level of oxidative DNA lesions (both endo III- and Fpg-sensitive sites) was determined in non-irradiated PBLs from radiosensitive patients (G2+) compared to patients with no or very mild radiation toxicity (G0-G1), this difference was, however, insignificant due to extensive inter-individual variation within both groups (data not shown). This phenomenon was less evident in irradiated PBLs 30 min after exposure. A good correlation was found between the acute radiation

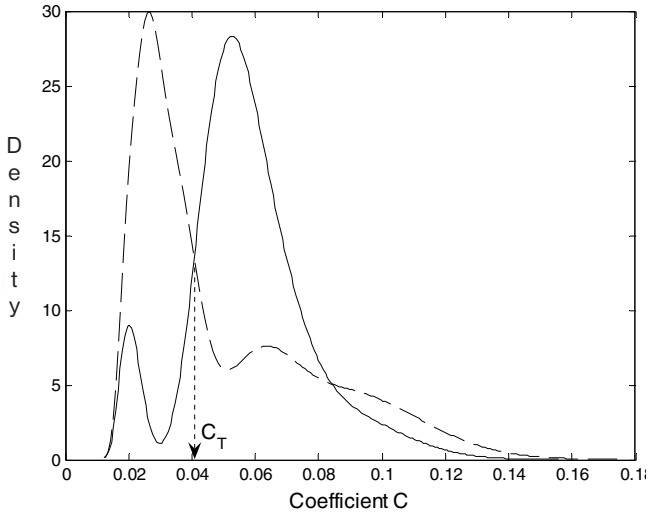

Figure 3. Distribution of the coefficient C for individual healthy donor (solid line) and cancer patient prior to radiation (dashed line), the threshold value C_T = 0.04092.

toxicity and residual DNA damage in irradiated PBLs from cancer patients prior to treatment (Fig. 4). A substantial delay in DNA strand break rejoining was determined in cancer patients suffering from adverse side effects (G2+) in comparison to persons with no or very mild radiation toxicity (G0-G1) 15 min and 30 min after irradiation ($p<0.05$ and $p<0.01$, respectively). This phenomenon was not detected in irradiated PBLs from cancer patients during and after therapy (data not shown). The late radiation toxicity was not evaluated in this study.

Correlation between the clinical response to radiotherapy and the comet assay data. The clinical response of cancer patients to radiotherapy was analyzed in 6-month intervals after the final treatment. From 18 patients involved in this pilot study only one woman did not response to radiotherapy at all and died before 12 months follow-up while a remission was certified in the rest of patient cohort at the end of treatment (Table 3). Six months after therapy, 2 patients, based on the histological examination were in complete remission, while progression of disease was determined in further 3 women from whose two died before 1-year follow-up due to locoregional failure. Eighteen months after therapy, a regional recurrence of the disease was estimated in another patient. Based on the clinical outcome (see Material and methods) the cancer patient cohort was stratified in 3 subgroups; patients with complete remission (CR), patients in partial remission (R), and patients with progression of the disease (P). The data from the comet assay experiments were correlated only with clearly definite clinical response to therapy, i.e. patients in CR and P, since the clinical outcome of patients in remission can change progressively within 5 year follow-up. From the clinical standpoint, both relapse of the disease as well as complete remission can be expected within this group.

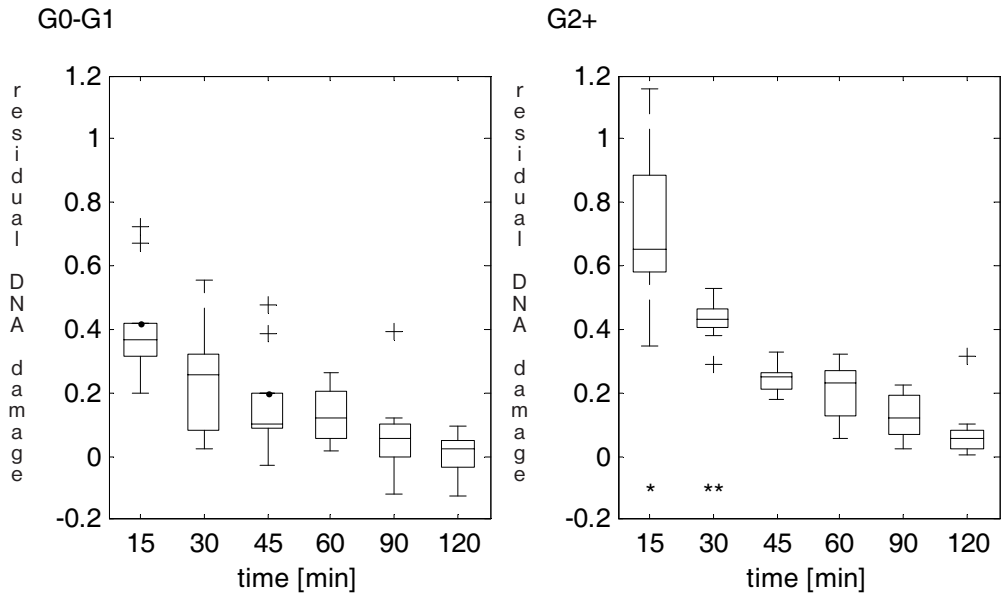

Figure 4. Boxplots of the residual DNA damage in PBLs from cancer patients without adverse reactions to radiation (G0-G1) and patients suffering from acute radiation toxicity (G2+). PBLs from cancer patients were irradiated *in vitro* with a dose of 2 Gy and the residual DNA damage (RD) was determined at a given time interval after irradiation. The boxes have lines at the lower quartile, median, and upper quartile values. The RD of G0-G1 cancer patients 15 and 30 min after irradiation is significantly lower than that of G2+ cancer patients, Student t-test as well as the Wilcoxon rank sum test for difference in means (medians), $p < 0.05$ (*), and $p < 0.01$ (**). The RD of G0-G1 cancer patients 45 – 120 min after irradiation was not proven to be significantly different from that of G2+ cancer patients.

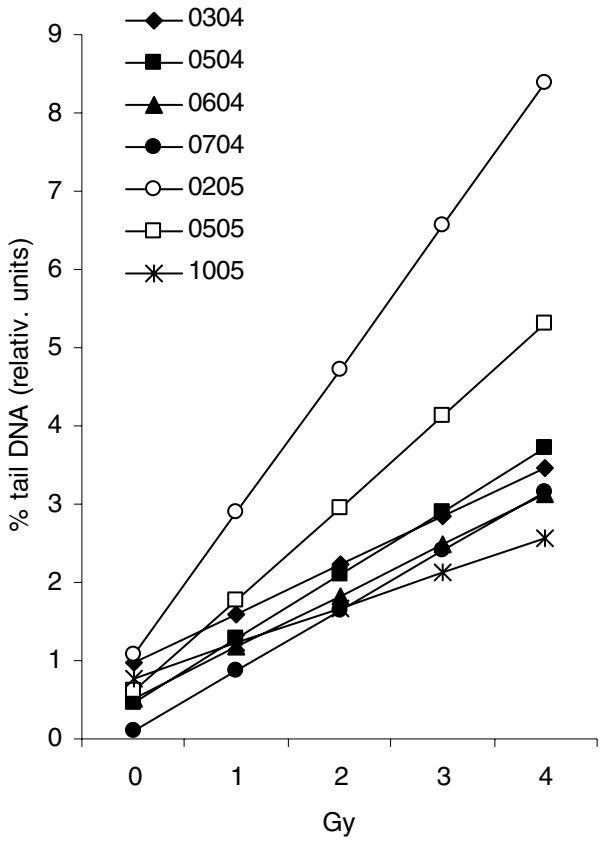

Figure 5. Dose-response curves for initial DNA damage in PBLs from cancer patients irradiated with 0 – 4 Gy. Dose-response data were fitted by the linear regression. Dose-response curves of 0205 and 0505 patients significantly different from other, *ANOVA*, p=0.05.

In general, no relationship was determined between the level of basal, oxidative and residual DNA damage, and the patient clinical outcome (data not shown). However, a good correlation was found between the initial DNA damage after a dose of 2 Gy and the clinical response to therapy (Table 3). After in vitro irradiation with 2 Gy, a significantly higher level of DNA fragmentation was detected in PBLs from cancer patient in CR compared to patients in progression ($p<0.01$). In addition, an association was found between the rate of DNA repair kinetics (coefficient C) and the clinical outcome. Lower C values were detected for patients in CR compared with patients in progression (Table 3).

In order to analyse the individual *in vitro* susceptibility to radiation in more detail, the level of initial DNA damage upon irradiation with 0 – 4 Gy was evaluated in PBLs from cancer patients prior to therapy. A considerable variation in dose-response curves for DNA breakage was detected within the patient cohort. Very steep dose-response curve was found in irradiated PBLs from two cancer patients (ID 0205 and 0505) in comparison to five others (ID 0304, 0504, 0604, 0704 and 1005), whose PBLs were substantially more resistant to ionising irradiation (Fig. 5). Responsiveness of the rest of patient cohort to radiation displayed intermediate sensitivity in comparison to above mentioned cancer patients (data not shown). The slopes of dose-response curves of individual cancer patients were used to characterize the personal *in vitro* radiosensitivity (Table 3). An association was found between the slope value and patient clinical outcome ($p=0.05$). The slope values were higher in patients with complete remission than in persons with progression of the disease.

Discussion

Although numerous studies have shown the critical role of DNA repair in intrinsic radiosensitivity and cancer susceptibility [20–24], methods for direct measuring of DNA damage and repair have not been routinely applied clinically to predict radiation susceptibility of cancer patients undergoing radiotherapy. In close cooperation with the hospital, the National Cancer Institute, a pilot study was initiated with emphasis on searching out potential cell-based prognostic biomarkers which after incorporation into radiation treatment planning could aid to improve outcomes.

Comparison between healthy women and cancer patients have revealed significantly higher level of mean basal DNA damage ($p<0.001$) in cervical cancer patient cohort in comparison to healthy control group (Fig. 1A). These data are in line with the finding of Ban et al. [25] who have analyzed the background level of DNA damage in cells from cervical cancer patients and healthy donors. Consistent with our results, significantly higher steady-state DNA damage were determined in non-irradiated PBL from patients with bladder cancer [26], haematological malignancies [27], oral squamous cell carcinoma [28], breast cancer [29–31], head and neck cancer [32] and patients with various forms of cancer undergoing chemotherapy [33] compared with healthy controls. The increased DNA damage level in untreated cells from cancer patients might suggest a spontaneous genetic instability which is a hallmark of several cancer-prone clinical syndromes (e.g. Nijmegen breakage syndrome, NBS; ataxia telangiectasia, AT; Fanconi anemia, FA) and hereditary forms of cancer (familiar adenomatous polyposis, FAP; hereditary nonpolyposis colorectal cancer, HNPCC; hereditary breast cancer, BC). Irradiation of PBL with a therapeutic dose of 2 Gy resulted in variable but statistically significant increase in DNA strand breakage in both groups (Fig. 1B), however, insignificant variation in the level of DNA fragmentation was found between control cohort and cancer patient group. These data are in line with Djuzenova et al. [34] and Iwakawa et al. [35]. A trend toward increased basal oxidative DNA damage was found in PBL from cancer patient group compared to healthy donor cohort (Table 2). On an individual basis, a few patients were detected with relatively high level of oxidative DNA damage while others had comparable oxidative DNA damage level with healthy women. An elevated oxidative DNA

Table 3 Correlation between the clinical outcome and the comet assay data

Patient ID	clinical outcome[a] 6 m	12 m	18 m	initial DNA damage	coeficient C (95% CI)[c]	slope of dose-response curve
0205	CR	CR	CR	29.48±2.35**	0.0212 (0.0072 – 0.0352	1.828
0505	CR	CR	CR	30.49±0.93**	0.0256 (0.0099 – 0.0413)	1.174
0304	P	P	P	20.00±1.22	0.0362 (-0.0033 – 0.0758)	0.621
0504	P	exitus	-	20.96±0.84	0.0751 (0.0485 – 0.1016)	0.816
0604	P	exitus	-	15.98±2.31	0.0282 (0.0010 – 0.0555)	0.655
0704	R	exitus	-	14.91±0.53	0.0351 (0.0164 – 0.0538)	0.764
0804	R	R	P	17.77±2.02	0.0275 (0.0034 – 0.0515)	1.055
0104	R	R	R	22.90±2.16	0.0657 (0.0176 – 0.1138)	0.870
0204	R	R	R	26.36±2.16	0.0259 (-0.0116 – 0.0633)	n.d.[d]
0404	R	R	R	23.07±1.58	0.0198 (-0.0116 – 0.0511)	0.597
0105	R	R	R	15.07±3.70	0.0901 (0.0206 – 0.1597)	0.596
0305	R	R	R	23.56±1.80	0.1131 (0.0589 – 0.1673)	0.740
0405	R	R	R	20.40±2.52	0.0555 (0.0315 – 0.0795)	0.966
0605	R	R	R	30.25±0.95	0.0405 (0.0315 – 0.0795)	1.210
0705	R	R	?[b]	30.85±2.72	0.0300 (0.0131 – 0.0470)	0.876
0805	R	R	?	14.89±0.63	0.0393 (0.0064 – 0.0723)	1.093
0905	R	R	?	18.77±0.60	0.0981 (0.0352 – 0.1610)	0.588
1005	R	R	?	23.63±2.69	0.0646 (0.0155 – 0.1137)	0.447

[a]CR – complete remission, P – progression of the disease, R – remission

[b] – early to analyse

[c]CI – 95% confidence interval

[d]n.d. – not determined, sample unavailable

**significantly different from patients in progression, Student *t*-test, $p<0.01$

damage level in cells from cancer patients compared to healthy controls were detected by Blasiak et al [29]. Increased level of oxidative DNA damage (8-oxodG) have been demonstrated in human cancer tissues in comparison with the cancer-free tissue [36]. Substantially higher oxidative DNA damage values found in PBL from cancer patients 30 min after irradiation compared to healthy donors might suggest an impairment of oxidative damage removal in cancer patients. Individual variations in the kinetics of DNA strand break repair were observed to be striking for both healthy volunteers and cancer patients (Fig. 2). Although the mean rate of DNA strand break rejoining (coefficient C) estimated for healthy women was higher than for cancer patients (Fig. 3), nearly all strand breaks were repaired within 120 min after irradiation in both groups. The calculation of coefficient C takes into account both the residual DNA damage level at specified time intervals after irradiation as well as the basal DNA damage level therefore such approach could more reliably characterize individual radiosensitivity. Fractionated radiotherapy had only negligible effect on the basal, initial, and oxidative DNA damage level and DNA repair capacity of PBL (data not shown). Consistent with these data, Muller et al., [37] found negligible effect of radiotherapy on the repair capacity of PBL from cancer patients.

Normal tissue adverse radiation toxicity is the rate-limiting factor in the delivery of ionizing radiation to cancer patients. The incidence of early morbidity of carcinoma of the cervix is most frequently seen in the rectum and urinary bladder [38]. Based on our preliminary results, a substantial delay in DNA strand break rejoining was determined in radiosensitive cancer patients (G2+) in comparison to patients with no or very mild side effects (G0-G1) 15 min and 30 min after irradiation (Fig. 4). These data suggest that impairment of DNA repair pathway could be a potential risk factor for radiation morbidity. Consistent with our data, Muller et al. [39] observed high amounts of residual damage for many but not all tumor patients who had experienced severe side-effects in their normal tissue during or after radiotherapy and Widel et al. [40] have detected significantly higher level of micronuclei in samples from cervical cancer patients who suffered from acute and/or late normal tissue reactions. On the other hand, West et al. [7] pointed out that SF2 parameter is a highly significant prognostic factor for the probability of developing any radiation morbidity following radiation therapy in carcinoma of the cervix.

PBL are frequently used as an easy-to-obtain surrogate tissue because of the simplicity of collection in a standardized, patient-convenient manner and with the rationale that genetic factors affecting radiosensitivity in a particular organ should be reflected globally in all cell types. Experiments on PBL from patients with rare genetic syndromes characterized by an enhanced cellular and clinical radiosensitivity, such as NBS [24], AT [41, 9] and FA [41], support this assumption and reveal that lymphocyte radiosensitivity might be a good prog-

nostic biomarker. Based on this hypothesis, the correspondence of the clinical outcome and in vitro comet assay data were analysed. Data from this pilot study have suggested a correlation between the initial DNA damage and the clinical response to therapy (Table 3). Significantly higher level of DNA fragmentation was detected in PBLs from cancer patient (prior to treatment) in complete remission compared to patients in progression ($p<0.01$). In addition, an association was found between the rate of DNA repair kinetics (coefficient C), the slope value and patient clinical outcome. Despite of the fact that only a small group of patients was analyzed these data positively suggest a substantial impact of DNA repair on radiation susceptibility of patients with carcinoma of the cervix.

Our data revealed that the comet assay data obtained from in vitro measurements could have a prognostic value and could aid after incorporation into radiation treatment planning to improve the patient outcome. Recently, Buffa et al. [42] have shown that incorporation of biological in vitro measurements (SF2 and colony-forming efficiency) into a tumor control probability model increased the local control and survival of patients with carcinoma of the cervix. Further study involving larger number of cancer patients undergoing radiotherapy is necessary to perform in order to verify the reliability of potential biomarkers determined in this pilot study. In addition, the association between polymorphisms in genes involved in radiation-induced DNA damage repair (e.g. XRCC1, XRCC3, XRCC4, hOGG1 etc.) and radiation susceptibility should be analyzed.

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