

Radiosensitivity and Repair Kinetics of Gamma-Irradiated Leukocytes from Sporadic Prostate Cancer Patients and Healthy Individuals Assessed by Alkaline Comet Assay

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ABSTRACT

Background: Impaired DNA repair mechanism is one of the main causes of tumor genesis. Study of intrinsic radiosensitivity of cancer patients in a non-target tissue (e.g. peripheral blood) might show the extent of DNA repair deficiency of cells in affected individuals and might be used a predictor of cancer predisposition.

Methods: Initial radiation-induced DNA damage (ratio of Tail DNA/Head DNA), dose-response curves and kinetics of DNA repair in leukocytes from healthy volunteers and prostate cancer patients were assessed using alkaline comet assay after exposure to ⁶⁰Co gamma rays. **Results:** Results showed that higher levels of baseline and gamma rays induced DNA damage in leukocytes of prostate cancer cases than in controls. A similar dose response was obtained for both groups. After a repair time of 24 h following *in vitro* irradiation, samples from the healthy individuals showed no residual DNA damage in their leukocytes, whereas prostate cancer patients revealed more than 20%. Although similar initial radiosensitivity was observed for both groups, the repair kinetics of radiation induced DNA damage of leukocytes from prostate cancer cases and healthy subjects were statistically different. **Conclusion:** These results support the hypothesis that men affected by prostate cancer may have a constitutional genomic instability. *Iran. Biomed. J. 14 (3): 67-75, 2010*

Keywords: Leukocytes, DNA damage, Radiosensitivity, Prostate cancer, Comet assay

INTRODUCTION

Prostate cancer is the second most common cause of cancer death in men in most developed countries, and the incidence has increased significantly over recent years. Although its etiology is not fully understood, ethnicity/race and family history are associated with it, and incidence increases with age [1, 2].

Oxidative stress and accumulated genomic damage may contribute to prostate carcinogenesis [3, 4]. Genetic predisposition accounts for $\geq 10\%$ of all prostate cancers and is therefore considered a major risk factor, together with age and ethnic origin. Several epidemiological studies have suggested that familial clustering of prostate cancer may be associated with an increased frequency of breast and other cancers among relatives [5, 6].

Several DNA damage processing and repair pathways constitute a guard system that protects cells against genetic instability and tumorigenesis [7]. Both genetic instability and impaired DNA restitution have been pointed out as factors underlying increased susceptibility to malignancy [8, 9]. The biological importance of genetic instability and DNA repair mechanisms in cancer development are particularly well illustrated by the autosomal recessive disorders *Ataxia telangiectasia*, *Fanconi anaemia* and Nijmegen breakage syndrome. These chromosome breakage syndromes are characterized by various defects in DNA repair, predisposition to different forms of malignancy and increased radiosensitivity [10-13]. Apart from these rare syndromes, the deficient DNA repair capacity has been proposed to be a predisposing factor in familial breast cancer and in some sporadic breast cancer

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cases [14]. Genomic instability has also been described for various hereditary cancers including hereditary breast cancer [15, 16].

Several lines of evidence have proposed that prostate cancer may be another tumor related to deficient DNA single and double-strand break repair and multiple DNA repair pathways play important roles in prostate carcinogenesis [2, 17].

Since prostate cancer has also been associated with some of the genes which confer increased radiosensitivity, e.g. BRCA2, ATM and NBS1 [17], we investigated if prostate cancer patients exhibit a similar cellular phenotype as breast cancer patients.

There are few studies concerning the sensitivity of prostate cancer patients to the DNA-damaging agents [17, 18]. The micronucleus (MN) test (MNT) has not shown increased MN frequencies in sporadic prostate cancer patients after irradiation with 2 Gy gamma rays [17]. Recently, in a study which the alkaline comet assay was used to evaluate whether basal or H₂O₂-induced DNA damage is associated with prostate cancer risk, it has shown that the H₂O₂-induced DNA damage level was significantly higher in incident cases than controls [18].

The single-cell microgel electrophoresis assay (or comet assay) has been shown to be useful for the assessment of DNA damage and repair within epidemiologic studies, because it is a fast and reliable assay that needs only a small number of cells [19-22]. It has been shown that this method allows the discrimination of carriers of chromosome instability syndromes including *Ataxia telangiectasia* and *Fanconi anemia* [13, 23]. In addition, comet assay has been used to study the background and induced DNA damage in the peripheral blood lymphocytes from breast cancer patients [7, 15, 22, 24-28].

In the present study, we compared induction of DNA damage in peripheral blood leukocytes of prostate cancer patients and healthy volunteers after *in vitro* exposure with 1 Gy gamma rays using alkaline comet assay as well as the dose-response curves and DNA repair of their leukocytes.

MATERIALS AND METHODS

Study subjects, blood cells and irradiation. Thirty prostate cancer patients aged between 45 to 78 years (mean age = 65.23 ± 9.2) were recruited at 3 hospitals in Tehran (Iran) before radical prostatectomy. None of them had been treated with chemotherapy or radiotherapy. Thirty healthy

volunteers aged between 41 to 94 years (mean age = 61.53 ± 14) were selected as a matching group. The study was approved by the Ethical Committee of the School of the Medical Sciences of Tarbiat Modares University, Tehran. Patients gave their informed written consent and all donors completed a written questionnaire to obtain information related to their life style, such as smoking, dietary habits, medical history and exposure to chemical and physical agents.

Mononuclear cells were separated from heparinized blood samples by Ficoll (supplied by Blood Transfusion Organization of Iran, Tehran) centrifugation (2000 revolutions per minute (rpm), 20 min, 20°C), washed in phosphate buffered saline and resuspended in RPMI-1640 medium (Gibco, BRL, Long Island, NY, USA) containing 5% fetal calf serum (Gibco, BRL) for 1 day. Such a strategy is advisable because the isolation stress itself is sometimes sufficient to induce DNA damage that can be detected in the comet assay. Lymphocytes were suspended at 3×10^5 cells/ml and cultured in a 5% CO₂ incubator at 37°C.

Cells were irradiated on ice with various doses of gamma rays (at source to sample distance = 80 cm, room temperature $23 \pm 2^\circ\text{C}$) with a dose rate of 2.77 ± 0.11 Gy/min, generated from a ⁶⁰Co source (Theratron II 780C, Canada, AECL, Kanata, Ontario, Canada). To compare radiation-induced DNA damage between prostate cancer patients and controls, cells were irradiated with 1 Gy at similar irradiation condition described above. This radiation dose was selected using the dose-response curve constructed for a healthy individual showing intermediate levels of damage in cells. For dose-response curve construction, lymphocytes obtained from healthy and cancer individuals were irradiated with the dose ranging from 0.5 to 16 Gy. After exposure on ice, cells were immediately subjected to the comet assay. Those cells whose repair capacity was monitored were allowed to repair DNA damage.

DNA repair was assessed at 0, 15, 30, 60, 120, 180 minutes and 24 hours after 8 Gy irradiation by keeping cells in RPMI-1640 medium supplemented with fetal calf serum and antibiotics (penicillin/streptomycin) and at 37°C in an atmosphere containing 5% CO₂ before beginning the experiments. The rationale for using this dose of radiation was to induce enough DNA double-strand breaks and left unrepaired after 24 hours [22]. Viability of cells was determined by using trypan blue staining before and after treatment. Samples of cells were obtained, mixed with an equal volume of

0.4% trypan blue, and then counted on a hemocytometer slide (improved Neubauer) under a light microscope (Zeiss, Germany) with $\times 10$ objective lens to determine the number of viable cells. Only samples with greater than 95% viability were considered for the comet assay analysis. Cryopreserved lymphocytes of a healthy individual were used as an internal standard and assayed at several experimental dates.

Slide preparation. After irradiation, the samples were centrifuged at 0°C . The supernatant was poured off and the pelleted cells mixed with 100 μl of 37°C LMP agarose (Fermentas LQ, 0.75% $1 \times$ PBS, Ca^{2+} , Mg^{2+} free). The cell mixture was added to the double-windowed frosted slides (Sotooneh Co, Sari, Iran), precoated with 1% normal agarose in distilled water (Merck, Darmstadt, Germany) and immediately covered with a coverslip. The slides were placed on a tray and kept for 10 min on a cooling plate to solidify. After solidification, the coverslip was removed.

Alkaline comet assay. Slides were submersed in an alkaline lysis solution (2.5 M sodium chloride, 100 mM EDTA, 10 mM Tris base, 10% dimethylsulfoxide, 1% sodium Lauroyl Sarcosinate, and 1% Triton X-100, Merck, Germany, pH 10) at 4°C for 1 hour. Lysis was followed by unwinding step by immersing the slides in a freshly prepared alkaline solution, (0.3 M NaOH and 1mM EDTA, pH>13, Merck, Germany) in a horizontal gel electrophoresis tank (SEU-7305, Paya Pajouhesh, Iran) for 40 minutes at 4°C . Electrophoresis was done at 0.75 volt/cm at 4°C for 30 minutes. The slides were washed 3 times in neutralization buffer (400 mM Tris buffer, pH 7.5) and rinsed in absolute ethanol for 5 minutes and air dried.

Staining, microscopic analysis and experimental parameters. Slides were stained with 20 μl ethidium bromide (Merck, $2\mu\text{g}/\text{mL}$). Observations were made at magnification of $200\times$ using a Nikon E800 epifluorescence microscope (Japan) equipped with 546-516 wavelength band and a 590 nm barrier filter attached to a charge-coupled device camera. Images of more than 100 randomly selected cells were analyzed from two-coded slides each with two windows. Measurements were made by image analysis using CASP software (version 1.2.2). CASP is a tool to image analysis in comet assay and has been developed to work with either color, or gray-scale images of fluorescence-stained comets. The

ratio of the DNA intensity in the tail to that in the head region of the comet was used as a measure of DNA damage. Four experimental parameters were evaluated to characterize cellular radiation effects including: (1) Baseline DNA damage detectable in cells that had not been irradiated (DD_0), (2) Induced DNA damage (DD) measured directly after irradiation, (3) Net DNA damage which is calculated by subtracting the baseline DNA damage from DNA damage measured directly after irradiation ($\text{DD}-\text{DD}_0$), (4) Repair capacity was also estimated quantitatively at 15 min, 30 min, 1 h, 2 h, 3 h and 24 h after 8 Gy irradiation after standardization using the following equation adopted from Bergqvist *et al.* [29]:

$$\% \text{Standardized Residual DNA Damage (au)} = [(\text{DD at 15, 30, 60, 120, 180 min or 24 h after exposure} - \text{DD}_0 \text{ at 15, 30, 60, 120, 180 min or 24 h}) / (\text{DD at 0 min after exposure} / \text{DD}_0 \text{ at 0 min})] / 100$$

Standardizations were made by dividing the actual values obtained in the irradiated samples by the values obtained in the non-irradiated control cells included in the same electrophoresis run.

Statistical analysis. All statistical analyses were carried out using Graph Pad Prism software version 4. Differences between means of initial radio-induced DNA damage between groups were tested for significance with the two-sided, unpaired Student's *t*-test. To analyze the results of residual DNA damage between groups the non-parametric Mann-Whitney *U*-test was also used. Figures were drawn using Sigma Plot 2004 for Windows, version 10.0.

RESULTS

Initial radiosensitivity of prostate cancer patients and healthy controls. The ratio of the DNA intensity in the tail to that in the head region of the comet (Tail DNA/Head DNA) was used as a measure of DNA damage. In order to directly compare the effects of gamma irradiation in patients and controls, the baseline DNA damage value induced in gamma-irradiated samples was subtracted from the value of DNA damage in untreated control samples to determine the net induced DNA damage by radiation. Mean values of the ratio of Tail DNA/Head DNA obtained from normal healthy controls and prostate cancer patients are summarized and compared using student's *t*-test in Table 1.

Table 1. Mean values of baseline (DD_0), induced (DD) and net induced ($DD-DD_0$) DNA damage for controls and prostate cancer patients detected by alkaline comet assay. The ratio of the DNA intensity in the tail to that in the head region of the comet was used as a measure of DNA damage. Differences between the mean values were evaluated using student's *t*-test and the resulting two-sided *P* values are presented.

Measured Parameter	Tail DNA/Head DNA (mean \pm SD)		
	Healthy (n = 30)	Prostate cancer (n = 30)	<i>P</i> value (<i>t</i> -test)
Baseline (DD_0)	0.0785 \pm 0.0664	0.1246 \pm 0.1045	0.046
Induced (DD)	0.2121 \pm 0.1699	0.3184 \pm 0.2026	0.038
Net induced ($DD-DD_0$)	0.1383 \pm 0.1210	0.1902 \pm 0.1443	0.106

Induction of DNA damage was higher in prostate cancer patients than healthy controls ($P = 0.038$). Prostate cancer patients showed a higher net induced DNA damage but this failed to reach statistical significance ($P = 0.106$, Fig. 1). The Figure presents the range of assay parameters observed in the study subjects, as well as the medians and the 10th, 25th, 75th, and 90th percentiles. Non-irradiated cells of cancer patients exhibited noticeably higher baseline amounts of DNA fragmentation and the mean values of background DNA damage were statistically different in the prostate cancer patients and healthy individuals ($P = 0.046$). Induced DNA damage was higher in prostate cancer patients than healthy controls, although not statistically significant.

Net induced DNA damage showed nearly identical median (solid lines) and mean values (dotted lines) for the controls and the patients. In addition, a very similar range of distribution was found for the results of the controls and of the patients and is marked by the boundaries of the boxes that represent the 25th and 75th percentiles. Patients with results lying within the 25-75% range of the healthy controls were considered to show a "normal" cellular reaction to gamma irradiation. In addition, the patients exhibiting less damage in non-irradiated or irradiated cells than marked by the 25-75% range were also classified as normal. However, the data of some of the patients differed considerably from this normal range. All patients who exhibited results lying outside the 90th percentile of the healthy individuals were considered to have abnormal experimental markers or to show a "highly sensitive" cellular reaction to gamma rays. Regarding the background damage in non-irradiated cells, about 2 (6.7%) of the 30 controls and 5 (16.7%) of 30 prostate cancer patients have values higher than the basal cut point. Induced damage (DD) in irradiated cells yield about 3 (10%) of the 30 controls and 5 (16.7%) of 30 prostate cancer patients have values higher than the induced cut point. Regarding the net induced ($DD-DD_0$) damage, 3 (10%) of the 30 controls and 4 (13.3%) of 30 prostate cancer patients have values higher than the net induced cut point. Statistical analysis showed that there was no relationship between age and DNA damage of prostate cancer patients and healthy controls regarding baseline induced and net induced DNA damage.

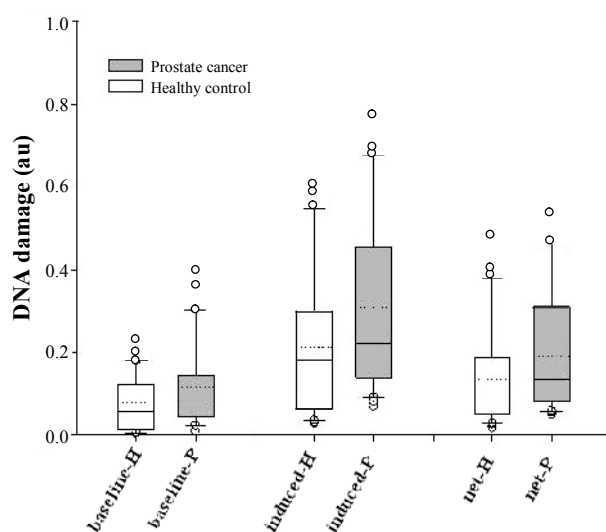


Fig. 1. Box plots showing baseline (DD_0), induced DNA damage (DD) and net induced DNA damage ($DD-DD_0$) for controls (H) and prostate cancer patients (P) measured by alkaline comet assay after exposure of 1 Gy gamma rays. The boxes extend from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th) and a dotted line at the mean value. The whiskers present the 10th and the 90th of the data. In addition, all values that were located outside the borderlines marked by the whiskers are presented as points.

Comparison of dose-response relationships in prostate cancer patients and healthy controls.

Figure 2 shows the dose-response curves of the 5 prostate cancer patients (mean age: 70 years, range 62 to 78) and 5 healthy controls (mean age: 65.7 years, range 56 to 71) under the alkaline comet assay

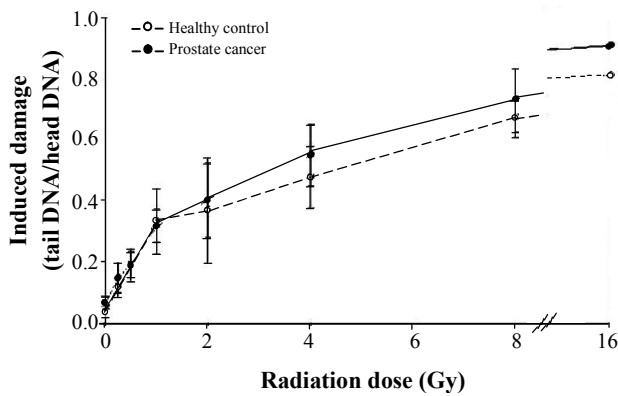


Fig. 2. Dose-response curves of DNA migration obtained for blood mononuclear cells from 5 prostate cancer patients (full lines) and 5 healthy donors (broken lines), processed immediately after *in vitro* exposure to various doses of gamma rays using alkaline comet assays. Each data point represents the mean \pm SD of induced DNA damage.

immediately after irradiation. The initial yield of DNA damage of both cancer patients and controls increased with radiation dose. Figure 2 demonstrates that marked differences can be observed when the initial radiation-induced DNA damage is analyzed in lymphocytes of different healthy people or cancer patients and this is particularly true for doses up to 2 Gy. The reduction in variability observed after 4 Gy is primarily due to the fact that at doses exceeding 4 Gy, only a marginal additional increase in the amount of DNA in the tail of the comet is obtained under our experimental conditions. Statistical analysis showed that there is no statistical difference between the dose-response curves of prostate cancer patients and healthy controls.

Study of repair kinetics. In order to investigate the rejoining of DNA breaks, the changes in the residual

DNA damage in 5 prostate cancer patients and 5 healthy volunteers at 0, 15, 30, 60, 120, 180 min, and 24 h after exposure to 8 Gy under the alkaline comet assay are shown in Figure 3. There were inter-individual differences in repair capacity of blood lymphocytes in both groups, but this variability was more marked among patients. Figure 3 and Table 2 shows, the radio-induced damage was less efficiently repaired among patients than among controls at 3 h and 24 h of analysis. Both statistical Student's *t*-test and Mann-Whitney analyses showed significant difference for 24 h ($P < 0.027$ and $P < 0.015$, respectively).

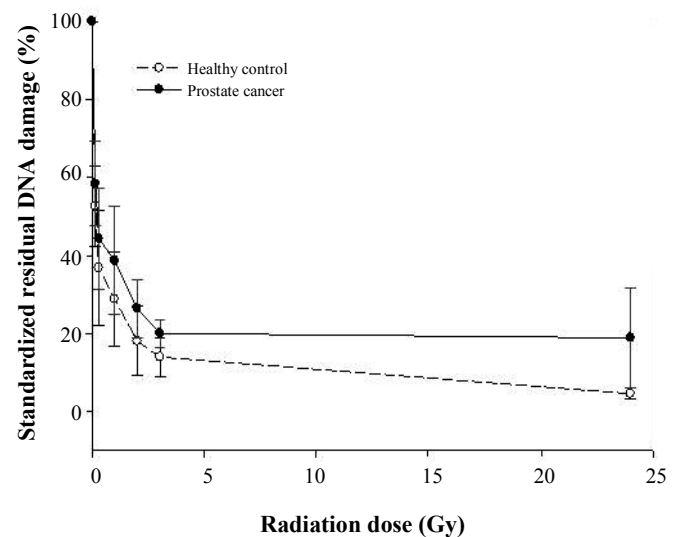


Fig. 3. Repair kinetics in blood mononuclear cells of 5 healthy donors and 5 prostate cancer patients after *in vitro* exposure to 8 Gy gamma rays (standardizations were made by dividing the actual values obtained in the irradiated samples by the values obtained in the non-irradiated control cells included in the same electrophoresis run). Repair assessed 15, 30, 60, 120, 180 min and 24 h of incubation time. Each data point represents the mean \pm SD of standardized residual DNA damage.

Table 2. Mean values of residual DNA damage obtained for blood samples from 5 healthy donors and 5 prostate cancer patients, processed immediately 15 min, 30 min, 1 h, 2 h, 3 h and 24 h after *in vitro* exposure to 8 Gy gamma rays using alkaline comet assay.

Time after irradiation	%Residual DNA damage (mean \pm SD)		Difference of residual DNA damage from controls (%)	P value	
	Controls (n = 5)	Prostate cancer cases (n = 5)		t-test	Mann-Whitney U-test
0 min	100	100	0		
15 min	52.66 \pm 10.44	58.36 \pm 10.86	10.82	0.4221	0.3095
30 min	36.80 \pm 14.58	44.29 \pm 13.00	20.35	0.4159	0.5476
60 min	28.79 \pm 11.95	38.68 \pm 13.78	34.35	0.2602	0.2222
120 min	18.09 \pm 8.87	26.36 \pm 7.56	45.71	0.1518	0.1508
180 min	13.95 \pm 4.85	20.07 \pm 3.56	60.00	0.0524	0.0952
24 h	4.46 \pm 1.48	18.88 \pm 11.80	303.00	0.0369*	0.0159*

*statistically significant

DISCUSSION

Under untreated conditions (without *in vitro* irradiation), leukocytes from prostate cancer patients have shown more DNA damage compared with the controls so that patients exhibited more than 1.5 times as DNA damage as that of the controls ($P = 0.046$) (Table 1 and Fig.1). Our results are in line with the results of other investigators who observed higher baseline values in breast and other cancer patients under the alkaline condition of comet assay, MNT and chromosomal aberrations as the study end point [16, 22, 26, 28, 30-34]. In contrast to our findings, other investigators using the alkaline comet assay [18] and MNT [17] have shown a similar background DNA damage in the peripheral blood lymphocytes from prostate cancer patients and control individuals. The reasons for the discrepancy between the findings of these studies and ours are not understood but might reside in the patients' and controls' cohorts, cancer stage, treatment prior to blood sampling, arbitrary determined cut-off values, study end points, experimental protocols as well as in inter-laboratory variability [7, 22].

The 90% range of values obtained for healthy controls was used to set the cut points to determine the range of normal radiation reaction. Using these cut points, the cases with marked DNA damage could be identified clearly. As shown in Table 1 and Figure 1, an increase in the induced DNA breaks after 1 Gy *in vitro* irradiation of both in untreated patients as well as in the controls, the lymphocytes of these patients display more radio-induced damage than the controls ($P < 0.038$). Regarding net induced DNA damage, results showed that the number of prostate cancer patients who exhibited results lying outside the 90th percentile of the controls was not more than healthy individuals (4/30 for prostate cancer patients and 3/30 for controls).

Lockett *et al.* [18] using alkaline comet assay showed that the H₂O₂-induced DNA damage level was significantly higher in prostate cancer cases than controls and prevalent cases. In contrast to our observation, Varga *et al.* [17] using MNT, demonstrated that with an automated MN scoring methodology, there was no significant difference in MN frequency between sporadic prostate cancer patients and healthy men overall. These results have been observed with and without irradiation of the cultures (baseline and induced MN frequencies) and also in the cells containing multiple MN. However, these two cytogenetic endpoints i.e. MN and comet assay, are not completely comparable because comet

assay shows the initial DNA damage whereas MN is the result of DNA damage after being subjected to repair processes and cell cycle checkpoints. The reasons for this discrepancy might also reside in the patients' and controls' cohorts, cancer stage, treatment prior to blood sampling, arbitrary determined cut off values, experimental protocols as well as in inter-laboratory variability.

The dose-response curves for DNA migration obtained just after irradiation showed an increase in DNA damage as a function of radiation dose (Fig. 2). Statistical analysis showed no difference between studied groups. In this context, we conclude that both groups had an analogous response when analyzed immediately after exposure. Other researchers reported the dose response of cells from unselected breast cancer patients was similar to that of control group using MNT and comet assay [7, 32].

The quantitative estimation of repair capacity in blood lymphocytes showed that most of the radiation induced damage in the healthy group was repaired within 3 h whereas prostate cancer patients revealed about 20% residual DNA damage in their leukocytes after a repair time of 24 h (Table 2 and Figure 3). These data indicate that the prostate cancer donors analyzed in this study may be partially deficient in repair of radio-induced DNA damage.

Increasing evidence suggests the roles of DNA damage/repair in human prostate cancer risk [2, 18, 35-39]. Previous studies showed that most of the DNA adducts generated by some prostate cancer related carcinogens, including polycyclic aromatic hydrocarbons, heterocyclic amines, and pesticides, are removed by the nucleotide excision repair pathway and there is a significant association between lower nucleotide excision repair capacity and prostate cancer risk [2]. It has shown that deficient nucleotide excision repair capacity enhances human prostate cancer risk [38]. The risk of prostate cancer is known to be elevated in carriers of germ line mutations in BRCA2, and possibly also in carriers of BRCA1 and CHEK2 mutations. These genes are components of the ATM-dependent DNA damage signaling pathways [40]. ELAC2, the first gene identified in hereditary prostate cancer [41] shows homology to PSO2 (DCLRE1A), a gene involved in the repair of DNA interstrand crosslinks [42]. Recently, several reports showed an association between prostate cancer risk and genetic variants of genes involved in DNA damage response, such as NBS1 [43], ATM (40) and BRCA2 [44]. In a recent report, Zhang *et al.* [45]

have shown that inhibition of p21-activated kinase 6 increases radiosensitivity of prostate cancer cells.

As far as we know there are no published results in the literature concerning the repair kinetics of radio-induced DNA damage in prostate cancer patients. Our results are in line with our recent findings [22] and of other researchers who described the difference between residual DNA damage at different intervals post irradiation among breast cancer patients and healthy controls using alkaline comet assay [32, 33]. In theory, differential 'adaptive responses' or 'damage-induced responses' to chronic exposure may result from genetic variations in drug metabolism and/or DNA repair. The evolving hypothesis is that subjects with lower cancer risk may have up-regulated detoxification and DNA repair enzymes in response to chronic exposures, and subjects with higher cancer risk lack this defense mechanism [18].

In summary, the present results suggest that elevated DNA damage may be associated with human cancer risk. Larger case-control and follow-up studies are warranted to further test the potential application of the alkaline comet assay in cancer risk assessment and prevention.

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