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Evaluation of the *in vivo* genotoxic effects of gamma radiation on the peripheral blood leukocytes of head and neck cancer patients undergoing radiotherapy

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ABSTRACT

The present study aimed to evaluate the genotoxic effects of ionizing radiation on non-target cells of Head and Neck Squamous Cell Carcinoma (HNSCC) patients exposed to various cumulative doses of gamma rays during radiotherapy. The ten patients (P1–P10) were treated with cobalt 60 gamma radiation (External Beam Radiotherapy) for a period of five to six weeks with a daily fraction of 2 Gy for 5 days each week. The genotoxic effects of radiation (single strand breaks – SSBs) in these patients were analyzed using the alkaline single cell gel electrophoresis (SCGE) technique, with the Olive Tail Moment (OTM) as the critical parameter. A sample of each patient's peripheral blood before starting with radiotherapy (pre-therapy) served as the control, and blood collected at weekly time intervals during the course of the radiotherapy served as treated (10, 20, 30, 40, 50 and 60 Gy) samples. *In vivo* radiosensitivity of these patients, as indicated by SSB's after the cumulative radiation doses at the various times, was assessed using Student's *t*-test. Significant DNA damage relative to the individual patient's pre-therapy baseline data was observed in all patients. Inter-individual variation of the genotoxic effects was analyzed using two-way ANOVA. The correlation between doses for the means of smoker and non-smoker patients was calculated using the Pearson test. The results of this study may indicate the need to reduce the daily radiotherapy dose further to prevent genotoxic effects on non-target cells, thus improving safety. Furthermore, these results may indicate that the estimation of DNA damage following exposure to a gamma radiation, as measured by the comet assay in whole blood leukocytes, can be used to screen human populations for radiation-induced genetic damage at the molecular level.

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1. Introduction

Radiotherapy is the most important non-surgical modality for the curative treatment of cancer. In 2004, in the United States alone, nearly 1 million of the 1.4 million people who developed cancer were treated with radiation. Of the 10.1 million people diagnosed with cancer worldwide each year [1], approximately 50% require radiotherapy, 60% of whom are treated with curative intent. In general, approximately 50% of cancer patients receive radiation therapy for their disease management [2]. Radiotherapy is also highly cost effective, accounting for only 5% of the total cost of cancer treatment [3]. Ionizing radiation is one form of radiotherapy treatment of cancer. The most frequent type of radiotherapy treatment for HNSCC patients is external beam radiation with gamma rays. This therapeutic intervention is considered as a double-edged sword, with both benefits and risks, because it has been classified

as a potent human carcinogen [4]. Radiation exposure causes DNA strand breakage, chromosomal aberrations, mutations and overall genetic instability [5]. Genetic integrity is maintained by an intricate network of DNA repair proteins [6]. Defects in this complex machinery are linked with familial predisposition to cancer and other diseases [7].

The aim of radiation therapy is to eliminate malignant cells while maintaining the integrity of the normal cells by employing an optimal dose of radiation. It is generally acknowledged that ionizing radiation kills mammalian cells by inducing damage to the nuclear DNA, although the ultimate cause of cell death in terms of DNA damage is controversial [8]. Several types of DNA damage and repair processes are induced by ionizing radiation. The sensitivity of both tumor cells and healthy tissues depends on the cell type and its proliferation and metabolic status [9,10]. The sensitivity also depends on intracellular scavenger concentrations and genetically determined factors [11–13].

Blood leukocytes are often employed to test genetically determined radiation sensitivity, mainly because they are readily available [14]. It is essential to study *in vivo* DNA damage in cancer patients who undergo radiotherapy to prevent or reduce the

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side effects of radiation exposure. Ionizing radiation is mutagenic and carcinogenic by virtue of its ability to damage DNA in cells, and thus, radiation therapy is also associated with an increased risk of incidence of secondary malignancies in cancer patients [15]. Hence, determination of radiation-induced DNA damage in humans has potential value for risk assessment. Monitoring of patients under radiotherapy for DNA damage could therefore contribute to the optimization of irradiation conditions and biological dosimetry. Peripheral white blood cells are often used as non-target cells for biological dosimetric studies. However, several studies have reported the use of whole blood rather than isolated lymphocytes for population studies [16]. Additional purification and culturing of the lymphocytes does not provide a definitive advantage because it is not clear at present whether any subtype would more closely approximate the effects on the target tissue [17]. We therefore evaluated whole blood in the present study.

The alkaline comet assay has become a popular technique for detecting a range of types of DNA damage during the last decade, and its usage in clinical practice has also increased rapidly [18,19]. Here, we compared the inter-individual differences in gamma radiation-induced damage of single strand breaks (SSBs) in peripheral blood leukocytes of HNSCC patients with different lifestyles. The DNA damage was determined by SCGE and subjected to statistical analysis.

2. Materials and methods

2.1. Subjects of study

Study participants with newly diagnosed HNSCC were recruited at Goa Medical College, Department of Radiation Oncology, Goa, India from a group of HNSCC patients who had not previously been treated with chemotherapy or radiotherapy. The population studied comprised 10 volunteer subjects (2 females and 8 males) diagnosed with SCC of the tongue, oropharynx, vocal cord and pyriform fossa. The patients gave their informed written consent and also provided information related to their lifestyles, such as their smoking status, medical history and exposure to chemical/physical agents, in responses on a specific questionnaire. Detailed patient data are provided in Table 1. Subsequent laboratory procedures involving the subjects and all investigations were carried out in accordance with a high standard of ethics under the guidelines of the Institutional Ethics Committee, Goa Medical College, Goa, India.

2.2. Radiotherapy

All patients underwent standardized external-beam partial-body irradiation with curative intent for localized tumors after a planned computed tomography scan and computer-generated distribution evaluation-assisted target localization and beam arrangement. An External Beam Radiotherapy Cobalt 60 source (Plate 1) was used for all patients, comprising lateral fields of the head and neck, with an average 1.25 MeV γ -ray beam of 2 Gy per day to the target area (Table 1). They received γ -radiation for five week(s) at a 10 Gy dose per week, up to a cumulative tumor dose of 54–66 Gy.

2.3. Sample collection and processing

Peripheral blood sampling was performed by venipuncture. Venous blood (5 ml) was collected in heparinized Vacutainer tubes (Becton Dickinson, NJ, USA) under sterile conditions. The peripheral blood of each patient collected prior to the initiation of radiotherapy was marked as his/her control sample. Seven samples were collected from each donor, one prior to the initiation of radiotherapy (control – 0 Gy) and the remaining six at weekly interval irradiations of 10 Gy for six weeks (treated). The pre-therapy blood sample (0 Gy) was collected on day 1 of the first radiotherapy cycle, 2 h prior to irradiation. The response of the peripheral blood leukocytes to the radiotherapy was evaluated in a blood sample collected within 1 h of the last dose of irradiation of the first week *i.e.* 10 Gy (after 5th day). Further blood samples were collected and handled in the same manner at various intervals of treatment *i.e.* after completion of the 20 Gy (2nd week), 30 Gy (3rd week), 40 Gy (4th week), 50 Gy (5th week) and 60 Gy (6th week) of irradiation. The blood samples, collected in vacutainer tubes, were coded and transported to the laboratory in an ice box. They were processed immediately (within a maximum of 1 h after collection) and subjected to further analysis by the alkaline single cell gel electrophoresis (SCGE) assay.

2.4. Single cell gel electrophoresis assay

The alkaline SCGE assay was performed according to the methods of Singh et al. [20] and Tice [21], with slight modifications. Fully frosted microscope slides (Fisher Scientific, cat no.: 12-544-5CY, USA) were coated with a thin layer of 1% normal melting-point agarose (LMA) and cooled to 4 °C. Subsequently, 20 μ l of whole blood was mixed with 100 μ l of 0.5% low melting agarose, and this suspension was pipetted onto the pre-coated slides and covered with a cover slip. The preparation was chilled for 5 min in the dark in a refrigerator at 4 °C, and after solidification of the suspension, the cover slip was removed.

2.4.1. Alkaline lysis

Slides with blood cells embedded in LMA were submersed in an alkaline cold (4 °C) lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris base pH 10, 1% Triton X-100, 10% DMSO) at pH 10 and maintained at 4 °C for 4 h. They were then placed in an alkaline electrophoresis buffer of pH 13 (1 mM Na₂EDTA/300 mM NaOH) for 25 min to induce unwinding of DNA strands. The slides were then transferred to an electrophoresis tank with fresh alkaline electrophoresis buffer, and electrophoresis was performed at a field strength of 1.33 V/cm for 25 min at 4 °C (20V/125 mA). Following electrophoresis, the samples were neutralized by incubation in 0.4 M Tris, pH 7.4, for 5 min.

2.4.2. Staining, microscopic analysis and experimental parameters

DNA was stained by placing 20 μ l/ml ethidium bromide on the agarose, which was then covered with a cover slip and incubated for 5 min in the dark. From the time of placing the suspended cells on the slides through the electrophoresis, the cells were protected from additional DNA damage resulting from direct exposure to visible light by performing all steps in the dark at 4 °C. The DNA damage was visualized by observing the cells under 20 \times objective magnification of an epifluorescent microscope (Olympus BX 53, Japan) equipped with an excitation filter of 510–560 nm and an emission filter of 590 nm. One hundred comet images were recorded for each sample (2 slides, 50 images from each). The cells were analyzed by the image analysis software CASP [22]. OTM, which is the product of the percent tail DNA and the distance between the center of gravity of the head and tail, was used as a measure of DNA damage [23]. The OTM for each image was used as the variable of interest.

2.5. Statistical analysis

Intra-individual variation between the control and treated samples was analyzed using Student's *t*-test, and inter-individual variation was analyzed by two-way analysis of variance (ANOVA). The correlation coefficient (r^2) between the smokers and non-smokers was calculated using the Pearson test. The results are presented as the mean \pm SD, and the values of $P < 0.001$, 0.01 and 0.05 were regarded as statistically significant.

3. Results

The DNA damage present in the peripheral leukocytes of the patients prior to radiotherapy and after various dose intervals of treatment (10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy and 60 Gy), measured as OTM values using the CASP software, is presented in Table 1. All the patients exhibited dose-dependent increases in DNA damage. The baseline (pre-therapy) DNA damage in peripheral leukocytes at 0 Gy (control) indicates inter-individual variation. The smoker (S) patients exhibited a high percentage of DNA damage (7 ± 2.2 – 16.4 ± 4.3), which was significantly higher than that of the non-smoker (NS) patients (0.1 ± 0.04 – 7.5 ± 2.5).

The OTM value at 10 Gy ranged from a minimum of 0.4 ± 0.2 (P2) to a maximum of 18.6 ± 8.2 (P7). The values for smokers after the 10 Gy dose ranged from 12.9 ± 4.9 to 18.6 ± 8.2 and were significantly greater than the values for non-smokers (0.4 ± 0.2 – 16 ± 2.5). The values of DNA damage at 20 Gy ranged from a minimum of 1.8 ± 0.7 (P1) to a maximum of 35.1 ± 16.1 (P6). All of the patients showed significant ($P < 0.001$) increases in DNA damage relative to their respective pre-therapy control values. The 20 Gy dose effects varied from 16.1 ± 2.5 (P5) to 35.1 ± 16.1 (P6) in smokers and from 1.8 ± 0.7 (P1) to 29.4 ± 17.9 (P10) in non-smokers.

In addition to the significant increase of DNA damage at 30 Gy in all patients relative to their respective pre-therapy values, elevated DNA damage was observed in smokers (24.8 ± 9.4 – 53.1 ± 15.4) compared with non-smokers (7.4 ± 2.6 – 52.3 ± 15.8). All patients showed a significant increase with respect to their individual control values at 40 Gy of irradiation. The OTM values for smokers

Table 1
Details and clinical data of HNSCC patients.

Patient no.	Sex/age (Y)	Habits smoker (S)/non-smoker (NS)	Control Prior to treatment 0 Gy	Treatment duration (dose in Gy)					
				I week 10 Gy	II week 20 Gy	III week 30 Gy	IV week 40 Gy	V week 50 Gy	VI week 60 Gy
P1	F/45	NS	0.2 ± 0.1	0.5 ± 0.3*	1.8 ± 0.7 [†]	7.4 ± 2.6*	16.1 ± 2.6*	30.9 ± 10.4**	48.4 ± 14.4***
P2	M/46	NS	0.1 ± 0.04	0.4 ± 0.2*	1.9 ± 0.9 [†]	8.1 ± 3.1*	16.1 ± 4.6*	39.6 ± 21.3**	60.2 ± 24.9***
P3	M/37	NS	7.5 ± 2.5	16 ± 2.5*	24.7 ± 10.1 [†]	52.3 ± 15.8**	58.6 ± 25.1**	63.8 ± 15.1***	64.9 ± 9.4***
P4	F/56	NS	2.0 ± 1.0	8.2 ± 3.2*	16.2 ± 4.8 [†]	36.8 ± 18.7 [†]	56.9 ± 21.2**	64.6 ± 15.8***	67.6 ± 14.3***
P5	M/47	S	7.6 ± 2.7	12.9 ± 4.9*	16.1 ± 2.5*	24.8 ± 9.4*	55.9 ± 22.1**	63.5 ± 46.6***	62.9 ± 14.5***
P6	M/62	S	8.1 ± 3.1	16.8 ± 4.4*	35.1 ± 16.1 [†]	33.6 ± 13.8*	58.4 ± 23.4**	61.5 ± 23.1***	64.4 ± 20.4***
P7	M/42	S	16.4 ± 4.3	18.6 ± 8.2*	27.9 ± 16.8*	46.2 ± 27.5**	62.7 ± 22.1**	69.3 ± 45.7***	71.9 ± 14.7***
P8	M/69	S	7.0 ± 2.2	15.9 ± 2.8*	27.9 ± 16.9*	53.1 ± 15.4**	67.3 ± 21.9**	72.88 ± 39.1***	82.5 ± 24.8***
P9	M/71	S	9.4 ± 4.6	14.8 ± 4.0*	27.9 ± 16.10*	52.8 ± 24.7**	53.5 ± 22.3**	66.9 ± 21.8***	75.5 ± 40.7***
P10	M/37	NS	6.8 ± 3.8	12.9 ± 6.7*	27.9 ± 16.11*	43.1 ± 22.3 [†]	55.2 ± 22.1**	63.9 ± 22.3***	68.3 ± 33.4***

Data for different doses (10, 20, 30, 40, 50 and 60 Gy) were compared with baseline data (0 Gy) patient wise by Student's *t*-test. Each reading indicates the mean ± SD value. Notes: (1) Student's *t*-test. (2) Two way ANOVA; inter-individual variation showed significant (rows: $P < 0.0001$; $F = 17.56$ and column: $P < 0.001$; $F = 119.83$) difference by two way ANOVA. Abbreviations: P, patient; S, smoker; NS, non-smoker; M, male; F, female; RT, radiotherapy; Y, year; Gy, gray.

* Statistically significant differences between dose and OTM at $P < 0.05$.

** Statistically significant differences between dose and OTM at $P < 0.01$.

*** Statistically significant differences between dose and OTM at $P < 0.001$.

ranged from 53.5 ± 22.3 to 67.3 ± 21.9 , and non-smokers showed values from 16.1 ± 2.6 to 58.6 ± 25 .

Prominent DNA damage was observed in all patients at 50 Gy too. The OTM values in smokers ranged from 61.5 ± 23.1 to 72.8 ± 39.1 , and in non-smokers, they ranged from 30.9 ± 10.4 to 64.6 ± 15.8 . The DNA damage in patients induced after 60 Gy of irradiation (sixth week of treatment) ranged from 62.9 ± 14.5 to 82.5 ± 24.8 in smokers and from 48.4 ± 14.4 to 68.3 ± 33.4 in non-smokers.

A comparison of the DNA damage in the patients exposed to various doses of gamma rays is shown in Fig. 1. These data indicate the inter-individual variation of radiation-induced genetic damage in HNSCC patients.

Fig. 2 shows the average value of the DNA damage in smoker patients and non-smoker patients. A statistically significant difference between smokers and non-smokers was found for the pre-therapy samples as well as for the 10 Gy and 60 Gy doses. However, there were no statistically significant differences between the patients of the two groups at irradiation doses between 20 and 50 Gy. Interestingly, a dramatic increase in DNA damage was noted in both the smoker and non-smoker patients in the doses from 20 Gy to 30 Gy.

4. Discussion

The dose-dependent increase in DNA damage observed in the present study in the entire group of gamma-irradiated HNSCC patients compared to their own pre-therapy baseline data indicates that gamma radiation caused considerable DNA damage in the form of SSBs in the peripheral leukocytes of these HNSCC patients at all the dose intervals of treatment. Further, the significantly greater DNA damage observed in the smoker patients compared to the non-smoker patients at the pre-therapy level and at 10 Gy and 60 Gy of irradiation may indicate a higher sensitivity of the smokers to gamma radiation at these doses. However, the failure of gamma irradiation, at doses of 20–50 Gy, to cause greater DNA damage in smokers compared to non-smokers may indicate the radio-protective effect of tobacco at irradiation doses of 20–50 Gy in smoker HNSCC patients.

The WHO [24] reported that the annual mortality associated with cigarette smoking was 3 million in 1990, was 4 million in 1998 and is expected to rise up to 8.4 million in 2020. Cigarette smoking is responsible for the vast majority of lung cancers and is associated

with cancers of the mouth, pharynx, larynx, esophagus, tongue, stomach, pancreas, kidney, colon and uterine cervix [25]. Cigarette smoke contains over 4000 chemical compounds, including over 50 known carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines, aromatic amines and trace metals [26]. Many of the chemicals found in cigarette smoke are genotoxic, and therefore, chromosome damage appears to be an excellent biomarker for determining the effect of exposure to smoking [27]. The genotoxic effects of tobacco smoke have recently been reviewed by De Marini [27], and smoking is a well-documented cause of cancer [1].

The comet assay is being used regularly to measure DNA damage associated with tobacco smoking. Hoffmann et al. [28], based on a meta-analysis of numerous related studies, provided evidence that smoking damages the DNA of peripheral blood cells, as measured by the SCGE. The significantly higher levels of DNA damage in the smoker patients compared to non-smoker patients at the pre-therapy level, as indicated by comet assay in the present study, may indicate that this assay is an ideal biomarker for studying the genotoxic effect of cigarette smoke.

The pre-therapy/baseline DNA damage in peripheral leukocytes of patients at 0 Gy of irradiation showed various levels of SSBs in different individuals, indicating inter-individual variations. These inter-individual variations may be mainly related to their lifestyle factors (including smoking habits) as well as various other inherited factors. Further, the large differences in the pre-treatment (0 Gy) levels of DNA damage between the smokers and non-smokers suggested that these variations may be due to the patient's lifestyle and/or genetic sensitivity. Genotoxicity in a cell is primarily dependent on the dose of the mutagen and/or its metabolites reaching the target area; however, the net damage is also dependent on the efficiency of DNA repair in the affected cells in the target area [29]. A thorough analysis of the baseline frequency of DNA damage is also important because this information has a direct bearing on the utility of these measurements for biological dosimetry, especially in cases where individuals are suspected to have been overexposed but no pre-exposed background DNA damage frequency is available.

The significant increase in DNA damage observed in all patients irradiated with 10 Gy indicated the mutagenic effect of gamma radiation at this dose. Compared to baseline damage, the damage caused by the 10 Gy dose was significantly higher in smokers (P5–P9) than in three of the non-smoker patients (P1, P2 and P4). However, higher DNA damage observed in the non-smokers P3

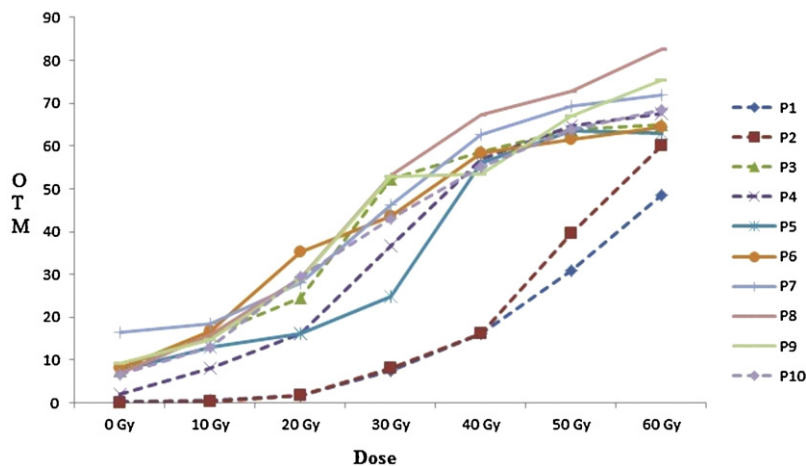


Fig. 1. Gamma radiation-induced DNA strand breaks in leukocytes measured as OTM values, shown in HNSCC patients in non-smokers (–) and smokers (–).

and P10 may have been due to their extreme sensitivity to gamma irradiation. Additional DNA damage observed at the 20 Gy dose suggested a positive response to the therapy. The further increases in DNA damage in patients irradiated up to 30 Gy indicated a dose-dependent increase in SSBs within the range of 0–30 Gy. The results obtained in the present study indicate that radiotherapy is accompanied by significantly increased levels of primary DNA damage in peripheral blood leukocytes. These findings are in agreement with reports of other authors who investigated the impacts of radio- and chemotherapy on non-target cells in cancer patients [30,31]. Earlier reports that radiotherapy patients show a wide variation of response in non-target cells is consistent with similar findings in the present study [32]. Although a significant proportion of this variation can be due to treatment-related factors such as dose inhomogeneity, there is increased evidence showing that the major factors determining these differences are related to intrinsic factors [33].

It is well known that, in addition to direct damage to DNA, ionizing radiation also causes indirect DNA-damaging effects due to the formation of free radicals as a result of the ionization of oxygen. In the most common form of radiation therapy, the most significant radiation effect occurs through free radicals. Our observations

that prolonged exposure to gamma radiation (40–60 Gy) during the course of radiotherapy leads to a gradual decline in the intensity of DNA damage may be due to saturation in DNA damage in peripheral blood leukocytes which can be measured through comet assay. Human population's exhibit heterogeneity in the adaptive response to ionizing radiation that might be genetically determined [34].

The decrease in DNA damage observed in the present study in the smoker HNSCC patients compared with the non-smoker HNSCC patients irradiated at 20–50 Gy may indicate some form of radio-protective or shielding effect of tobacco (nicotine) against radiation-induced DNA damage, possibly related to an anti-apoptotic property of nicotine on targeted/non-targeted cells. However, this may have important implications for radiotherapy because nicotine induces protection against apoptosis in targeted/non-targeted cells, which could indicate a reduced likelihood of effective treatment in smokers. The same anti-apoptotic response was reported by Wright et al. [35] in the context of chemotherapeutic drugs. Browman et al. [36] reported that patients with head and neck cancer who continue to smoke have lower rates of response and survival than patients who do not smoke. However, the significant damage observed at the end of the exposures (after 60 Gy) may indicate that there is less resistance in leucocytes of

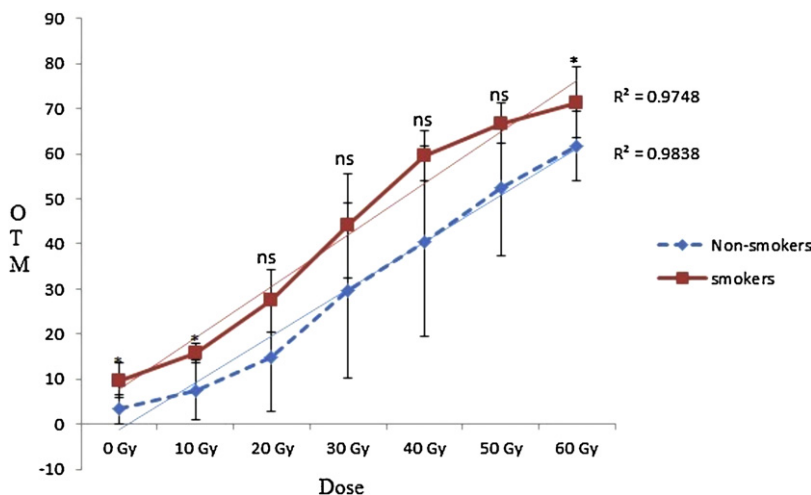


Fig. 2. The Pearson correlation test demonstrates a correlation between gamma radiation-induced DNA damage in leukocytes of HNSCC smoker and non-smoker patients. The different doses were compared with baseline data, and t-tests were used to assess differences between smokers and non-smokers before and after radiotherapy. Each point indicates the mean ± SD of smokers and non-smokers. Note: * represent statistically significant differences between smoker and non-smoker patients at $P < 0.05$; ns indicates a non-significant difference.

smoker patients, which may imply that any nicotine effect is suppressed and that the response is dominated by the effects of the radiation.

The presence of significantly increased levels of DNA damage caused by ionizing radiation is desirable in cancer cells but not in other non-target cells. Although the majority of the lesions induced by ionizing radiation are successfully repaired within a relatively short time after exposure [20,21], a part of the DNA damage may still remain unrepaired. Our data supplements the earlier reports of the genotoxic effect of radiation on the circulating leukocytes of HNSCC patients. The therapeutic exposure to ionizing radiation may therefore lead to the induction of secondary cancer in the exposed non-target cells or tissues. Sensitive techniques, such as the alkaline comet assay employed in the present study, may permit detection of genotoxic effects induced *in vivo* by radiotherapy and could also be used in pooled analyses to evaluate the side effects of radiotherapy. Nevertheless, taking into account the high variability found between and within individuals, further knowledge is needed regarding the fundamental aspects of the comet assay and the kinetics of formation and disappearance of the damage indicated by the comet assay after therapeutic radiation exposure to patients *in vivo*.

Conflict of interest

Authors do not have any conflicts of interest.

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