

# Chromosomal radiosensitivity in head and neck cancer patients: evidence for genetic predisposition?

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The association between chromosomal radiosensitivity and genetic predisposition to head and neck cancer was investigated in this study. In all, 101 head and neck cancer patients and 75 healthy control individuals were included in the study. The G<sub>2</sub> assay was used to measure chromosomal radiosensitivity. The results demonstrated that head and neck cancer patients had a statistically higher number of radiation-induced chromatid breaks than controls, with mean values of 1.23 and 1.10 breaks per cell, respectively ( $P < 0.001$ ). Using the 90th percentile of the G<sub>2</sub> scores of the healthy individuals as a cutoff value for chromosomal radiosensitivity, 26% of the cancer patients were radiosensitive compared with 9% of the healthy controls ( $P = 0.008$ ). The mean number of radiation-induced chromatid breaks and the proportion of radiosensitive individuals were highest for oral cavity cancer patients (1.26 breaks per cell, 38%) and pharynx cancer patients (1.27 breaks per cell, 35%). The difference between patients and controls was most pronounced in the lower age group ( $\leq 50$  years, 1.32 breaks per cell, 38%) and in the non- and light smoking patient group ( $\leq 10$  pack-years, 1.28 breaks per cell, 46%). In conclusion, enhanced chromosomal radiosensitivity is a marker of genetic predisposition to head and neck cancer, and the genetic contribution is highest for oral cavity and pharynx cancer patients and for early onset and non- and light smoking patients.

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Enhanced chromosomal radiosensitivity has been observed in a large number of patients with cancer-prone genetic diseases such as ataxia–telangiectasia and Nijmegen breakage syndrome (Gatti, 2001). Several studies have shown that enhanced chromosomal radiosensitivity is also present in a significant proportion of cancer patients (Scott *et al*, 1994, 1998, 1999; Parshad *et al*, 1996; Patel *et al*, 1997; Terzoudi *et al*, 2000; Baria *et al*, 2001, 2002; Buchholz and Wu, 2001; Papworth *et al*, 2001; Riches *et al*, 2001; Baeyens *et al*, 2002, 2005; Ban *et al*, 2004; Howe *et al*, 2005; Kolusayin Ozar and Orta, 2005; Mozdarani *et al*, 2005; Distel *et al*, 2006; Lisowska *et al*, 2006; Varga *et al*, 2006). The majority of data have been collected in studies considering breast cancer patients whose lymphocytes were irradiated in the G<sub>2</sub> phase of the cell cycle (Scott *et al*, 1994, 1999; Parshad *et al*, 1996; Patel *et al*, 1997; Terzoudi *et al*, 2000; Baria *et al*, 2001; Buchholz and Wu, 2001; Riches *et al*, 2001; Baeyens *et al*, 2002, 2005; Howe *et al*, 2005; Djuzenova *et al*, 2006; Docherty *et al*, 2007). In a high number of these studies, it was found that approximately 40% of breast cancer patients show an increased G<sub>2</sub> chromosomal radiosensitivity (Scott *et al*, 1994, 1999; Baria *et al*, 2001; Buchholz and Wu, 2001; Riches *et al*, 2001; Baeyens *et al*, 2002, 2005). As it was also shown that healthy first-degree relatives of breast cancer patients have an enhanced G<sub>2</sub> sensitivity (Parshad *et al*, 1996; Patel *et al*, 1997), it has been

suggested that this elevated *in vitro* radiosensitivity may be a marker of low-penetrant cancer-predisposing genes (Roberts *et al*, 1999).

There is also indirect evidence for the existence of low-penetrant predisposing genetic factors for head and neck cancers (Sturgis *et al*, 2004). Although tobacco and alcohol consumption play a major role in the aetiology of head and neck squamous cell carcinoma (HNSCC), several large family studies demonstrated a three- to eightfold increased risk of HNSCC in first-degree relatives of patients with HNSCC (Copper *et al*, 1995; Foulkes *et al*, 1995, 1996). Furthermore, only a fraction of frequently exposed individuals develop HNSCC and up to 20% of all HNSCC patients develop the disease without any exposure to the main risk factors (Sturgis *et al*, 2004). Finally, data emerging from case–control studies using several phenotypic and genotypic assays support the hypothesis of genetic susceptibility for HNSCC (Sturgis and Wei, 2002). Until recently, most of the studies evaluating phenotypic assays as possible marker for head and neck cancer susceptibility focused on the analysis of chromosomal damage in lymphocytes after mutagen exposure (Schantz and Hsu, 1989; Schantz *et al*, 1989; Bondy *et al*, 1993; Spitz *et al*, 1993; Cloos *et al*, 1996; Wang *et al*, 1998; Wu *et al*, 1998; Zhu *et al*, 2002; Szekely *et al*, 2005; Xiong *et al*, 2007). Studies analysing chromosomal damage after *in vitro* irradiation are, however, limited and often comprise a low number of patients (Terzoudi *et al*, 2000; Papworth *et al*, 2001; Ban *et al*, 2004; Distel *et al*, 2006; Lisowska *et al*, 2006). Nevertheless, both Terzoudi *et al* (2000) and Lisowska *et al* (2006) reported enhanced chromosomal radiosensitivity in larynx cancer patients,

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and Papworth *et al* (2001) observed increased chromosomal radiosensitivity in young head and neck cancer patients.

In the present study, we investigated the association between chromosomal radiosensitivity and genetic predisposition to head and neck cancer using the G<sub>2</sub> assay for peripheral blood lymphocytes. An analysis of the data according to tumour histology and tumour location was performed. In addition, the influence of age of onset of the disease and the effect of smoking habits on genetic predisposition was investigated.

## MATERIALS AND METHODS

### Population

The study population consisted of 101 head and neck cancer patients recruited from the Radiation Oncology Department of the Ghent University Hospital between January 2003 and October 2005. All patients were newly diagnosed, previously untreated and histologically confirmed. The cases included 83 squamous cell carcinomas (SCCs), 12 adenocarcinomas (ACs) and 6 other or unknown carcinomas. A total of 13 patients had cancer of the oral cavity, 37 patients suffered from pharyngeal cancer, 29 patients had laryngeal cancer and 22 patients were diagnosed with sinus

cancer or cancer of unknown primary. The population consisted of 84 men and 17 women, and the mean age of the patients at the time of treatment was 60 years (range: 33–91 years) (Table 1).

From each patient in the study, a heparinised blood sample was taken before therapy and the G<sub>2</sub> assay was performed on the sampling day. At the same time, parallel cultures from healthy individuals were started. The samples of the healthy control individuals were collected at the Occupational Medicine Department of the Ghent University Hospital. Details of the control population are described in Table 1. The study was approved by the Ethical Committee of the Ghent University Hospital. All study participants signed an informed consent form and completed a detailed questionnaire on smoking and drinking habits. Smoking was quantified by the degree of tobacco consumption categorised as pack-years (1 pack-year = 20 cigarettes per day for 1 year). Alcohol exposure was determined by the number of alcoholic drinks per week. For four patients and two control individuals, no smoking and drinking data were available.

### G<sub>2</sub> assay

The G<sub>2</sub> assay procedure of the Paterson Institute (Manchester) was applied with some minor changes. Briefly, heparinised blood was kept at room temperature before culturing within 4 h after preleaving. To a tissue culture flask (25 cm<sup>2</sup>), 0.5 ml blood was added to 4.5 ml complete RPMI-1640 culture medium supplemented with 10% fetal calf serum (Invitrogen, Merelbeke, Belgium), 1% L-glutamine, 50 U ml<sup>-1</sup> penicillin and 50 µg ml<sup>-1</sup> streptomycin. The lymphocytes were stimulated to divide with 1% phytohemagglutinin (Invitrogen). Per donor two cultures were started, one served as control, the other for *in vitro* irradiation. After 70 h incubation in a CO<sub>2</sub> incubator at 37°C, the cultures were irradiated with a dose of 0.4 Gy <sup>60</sup>Co rays at 37°C. At 30 min post irradiation, 75 µl colcemid (final concentration 0.15 µg ml<sup>-1</sup>; Sigma-Aldrich, Bornem, Belgium) was added to block the cells at metaphase, and at 90 min post irradiation the cultures were arrested by putting them on ice for 5 min. The cells were harvested by centrifugation of the samples, and the cell pellets were resuspended in 5 ml of 0.075 M KCl for 15 min on ice. After the hypotonic shock, the cells were fixed three times in 5 ml cold methanol/acetic acid (3:1). Finally, cells were dropped on clean slides and stained with 4% Azur B SCN solution (Polylab, Merksem, Belgium). Fifty well-spread metaphases were analysed by two independent scorers on coded slides for the appearance of chromatid breaks. All types of single chromatid breaks where a clear discontinuity was present were scored (Bryant *et al*, 2002).

**Table 1** Characteristics of the study population

Characteristic	Controls (%) (n = 75)	Patients (%) (n = 101)
Sex		
Male	41 (54.7)	84 (83.2)
Female	34 (45.3)	17 (16.8)
Mean age ± s.d.	45 ± 13.7	60 ± 10.0
Anatomic tumour location		
Oral cavity	—	13 (12.9)
Pharynx	—	37 (36.6)
Larynx	—	29 (28.7)
Other and unknown	—	22 (21.8)
Tumour histology		
Squamous cell carcinoma (SCC)	—	83 (82.2)
Adenocarcinoma	—	12 (11.9)
Other and unknown	—	6 (5.9)
Mean pack-years ± s.d.	7 ± 11.6	33 ± 21.5
Mean drinks per week ± s.d.	6 ± 5.4	28 ± 27.9

**Table 2** Overview of the G<sub>2</sub> data

	Head and neck cancer patients						
	Controls	All	Tumour histology		Tumour location		
			SCC	AC	Oral cavity	Pharynx	Larynx
Population size	75	101	83	12	13	37	29
Mean ± s.d. <sup>a</sup>	1.10 ± 0.18	1.23 ± 0.20	1.23 ± 0.20	1.21 ± 0.24	1.26 ± 0.23	1.27 ± 0.24	1.18 ± 0.14
Range <sup>a</sup>	0.64–1.54	0.75–1.81	0.75–1.81	0.90–1.76	0.94–1.76	0.75–1.81	0.96–1.43
P-value <sup>b</sup>		<0.001	<0.001	0.166	0.021	<0.001	0.024
Cut-off value <sup>c</sup>	1.33						
% > cut off value	9	26	28	25	38	35	17
P-value <sup>d</sup>		0.008	0.005	0.261	0.018	0.002	0.419
OR (95% CI)	Ref <sup>e</sup>	3.37 (1.37–8.26)	3.72 (1.49–9.29)	3.24 (0.71–14.82)	6.07 (1.56–23.69)	5.26 (1.88–14.74)	2.02 (0.59–6.98)
P-value <sup>f</sup>		0.008	0.005	0.130	0.009	0.002	0.265

AD, adenocarcinoma; CI, confidence interval; OR, odds ratio; SCC, squamous cell carcinoma. <sup>a</sup>Number of chromatid breaks per cell. <sup>b</sup>Mann–Whitney test. <sup>c</sup>90th percentile of the control population. <sup>d</sup>χ<sup>2</sup> test. <sup>e</sup>Ref, reference: control individuals with ≤ 1.33 breaks per cell were used as reference. <sup>f</sup>Logistic regression.

## Statistical analysis

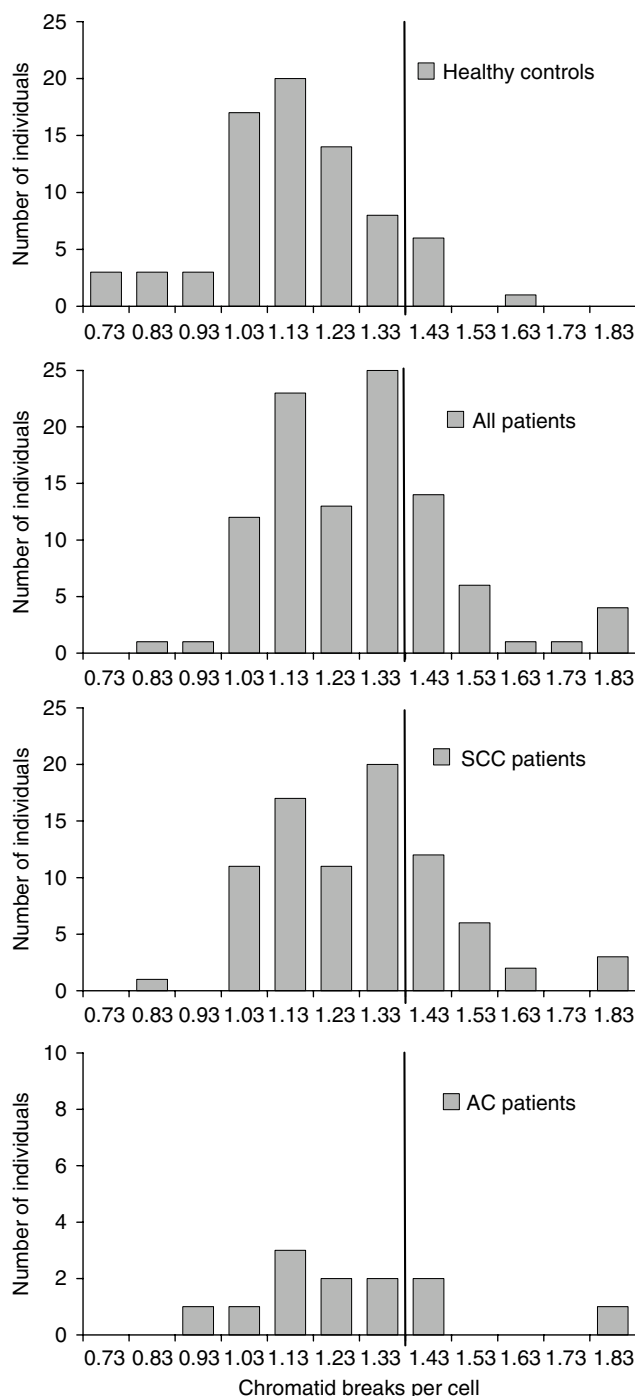
Statistical analysis was performed by SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). For the comparison of the mean  $G_2$  scores between different groups, the Mann–Whitney test was applied. Differences in the proportions of radiosensitive individuals in the different populations were compared using the  $\chi^2$  test. Unconditional logistic regression analyses were performed to calculate ORs (odds ratios) and 95% CIs (95% confidence intervals). Pearson correlation coefficients were used to study the correlation between aberration frequencies, age, smoking and drinking.

## RESULTS

Chromosomal radiosensitivity  $G_2$  data were collected for 75 healthy individuals and 101 head and neck cancer patients. For each sample, the spontaneous yield of chromatid breaks was subtracted from the yield in irradiated cells to obtain the radiation-induced aberration yield.

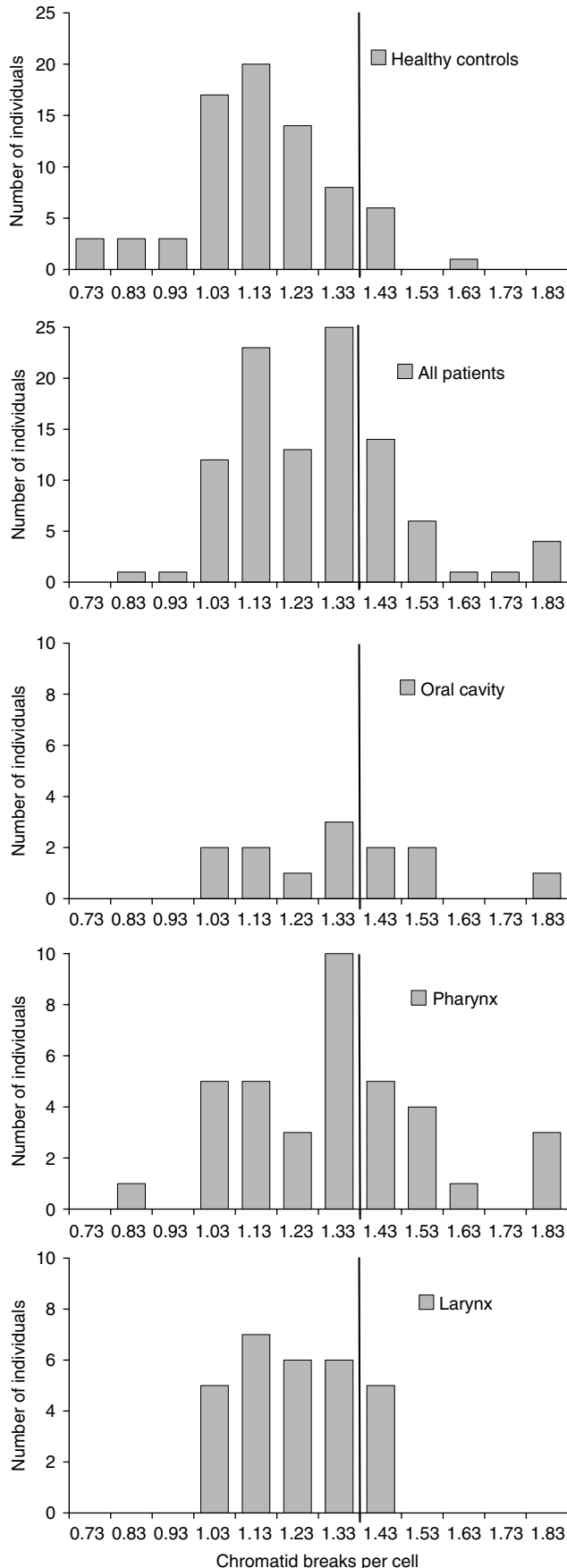
The results for the head and neck cancer patients and for the healthy individuals are summarised in Table 2 and graphically displayed in Figures 1 and 2. The average yield of radiation-induced chromatid breaks per cell was 1.10 for healthy individuals and 1.23 for all head and neck cancer patients ( $P < 0.001$ ). According to tumour histology, patients with SCC and AC were considered. Patients with SCC had 1.23 breaks per cell ( $P < 0.001$ ), whereas those with AC had 1.21 breaks per cell ( $P = 0.166$ ). Using the 90th percentile of the  $G_2$  scores of the healthy individuals as a cutoff value for chromosomal radiosensitivity (1.33 breaks per cell), 26% of all cancer patients, 28% of SCC patients and 25% of AC patients were radiosensitive compared with 9% of the healthy individuals ( $P = 0.008$ , 0.005 and 0.261, respectively). This corresponds, respectively, to ORs with respect to cancer incidence of 3.37 ( $P = 0.008$ ), 3.72 ( $P = 0.005$ ) and 3.24 ( $P = 0.130$ ) for individuals with  $G_2$  scores exceeding 1.33 breaks per cell (Table 2). According to anatomic tumour location, three patients subgroups were considered: (1) patients with oral cavity cancer, (2) patients with pharyngeal cancer and (3) patients with laryngeal cancer. Patients from each subgroup had significantly more chromatid breaks per cell compared with the individuals from the healthy control group ( $P = 0.021$ ,  $< 0.001$  and 0.024, respectively). On the basis of the 90th percentile cutoff value, 38% of the oral cavity cancer patients, 35% of the pharynx cancer patients and 17% of the larynx cancer patients were radiosensitive ( $P = 0.018$ , 0.002 and 0.419, respectively). Consequently, individuals with increased chromosomal radiosensitivity had a six-, five- or two-fold increased risk to develop oral cavity cancer, pharynx cancer or larynx cancer ( $P = 0.009$ , 0.002 or 0.265), respectively.

For further analyses, the patients were stratified according to age, smoking habits and alcohol intake, and chromosomal radiosensitivity was evaluated in the subsequent subgroups. The results of these analyses are presented in Table 3. No significant effect of age was seen on radiation-induced aberrations in individual patients and controls ( $P > 0.100$ ), but stratified analysis showed the highest mean  $G_2$  scores for the youngest patients. Patients with ages  $\leq 50$ , between  $> 50$  and  $\leq 70$ , and  $> 70$  years had 1.32, 1.22 and 1.18 breaks per cell, respectively. Except for the oldest patient group ( $P = 0.269$ ), these results were significantly different from the control population ( $P = 0.004$  and  $< 0.001$ , respectively). Using the cutoff value of the healthy control group, 38% of the patients with age  $\leq 50$  years ( $P = 0.009$ ) were radiosensitive. Consequently, radiosensitive individuals  $\leq 50$  years had a six-fold increased risk for developing head and neck cancer ( $P = 0.007$ ). There was also no correlation between smoking and radiation-induced aberrations in patients and controls ( $P > 0.130$ ). Nevertheless, the highest mean  $G_2$  scores were found for non- and light smoking patients. Patients with  $\leq 10$ , between  $> 10$  and  $\leq 25$ , and  $> 25$  pack-years had 1.28, 1.22 and 1.21 breaks per cell,



**Figure 1** Distribution of radiation-induced  $G_2$  chromatid breaks in healthy individuals ( $n = 75$ ), all head and neck cancer ( $n = 101$ ), SCC ( $n = 83$ ) and AC patients ( $n = 12$ ). The vertical line represents the 90th percentile cutoff point.

respectively. The results of all subgroups defined according to smoking habits were statistically significant compared with the control population ( $P = 0.006$ , 0.007 and 0.001, respectively). On the basis of the cutoff value of the healthy control group, 46% of the patients with  $\leq 10$  pack-years ( $P = 0.003$ ) were radiosensitive. Accordingly, an OR of 8.33 ( $P = 0.002$ ) was found for non- and light smoking individuals. A similar trend was not seen for alcohol intake in the patient population, nor for any of the three parameters in the control population.



## DISCUSSION

The association between  $G_2$  chromosomal radiosensitivity and genetic predisposition to head and neck cancer was investigated in the present study. Moreover, an analysis of the data according to tumour histology and tumour location was performed, and the influence of age of onset of the disease and the effect of smoking habits on genetic predisposition were investigated.

Analysis of the data shows that head and neck cancer patients have a significantly higher mean number of *in vitro* radiation-induced chromatid breaks per cell than healthy individuals (1.23 vs 1.10 breaks per cell,  $P < 0.001$ ). The observed elevated chromosomal radiosensitivity was independent of tumour histology and tumour location. Patients with SCC and AC, as well as patients with oral cavity, pharynx and larynx cancers, all showed an increased mean number of radiation-induced chromosomal aberrations compared with the control group. Except for the AC patients, all results were statistically significant. With respect to laryngeal cancer, these results are in agreement with the findings of Terzoudi *et al* (2000) and Lisowska *et al* (2006), who both reported enhanced chromosomal radiosensitivity in larynx cancer patients. This increase was statistically significant in the latter study, but in the study of Terzoudi *et al* (2000), the statistical significance was not mentioned. In the study of Lisowska *et al*, a very different experimental protocol was used compared with the one applied in the present study. Although five times higher irradiation doses were used (2 vs 0.4 Gy in the present study), five times lower mean  $G_2$  values were obtained for larynx cancer patients (0.22 vs 1.18 breaks per cells in the present study). This discrepancy is at least partly due to the fact that in the study of Lisowska *et al*, chromatid breaks were analysed in metaphase cells 4.5 h post irradiation, whereas in the present study chromatid breaks were analysed in metaphase cells 1.5 h post irradiation. An exponential decline of chromatid breaks with time after irradiation has been demonstrated in the past by our research group and supports the observed differences in absolute chromatid break numbers in both studies (Vral *et al*, 2002). Concerning various head and neck cancer patients, Papworth *et al* (2001) reported a nonsignificant increase in mean radiation-induced chromosomal aberrations in these patients. Next to the positive association found between high levels of radiation-induced chromatid breaks and increased risk for head and neck cancer, several studies also reported a positive association between high frequencies of chromatid breaks after bleomycin or benzo[*a*]pyrene diol epoxide (BPDE) exposure and elevated risk for head and neck cancer (Cloos *et al*, 1996; Wang *et al*, 1998; Wu *et al*, 1998; Zhu *et al*, 2002; Szekely *et al*, 2005; Xiong *et al*, 2007). As radiation as well as bleomycin and BPDE cause DNA damage, both assays are linked to DNA repair capacity. Consequently, the observed enhanced chromosomal radiosensitivity in cancer patients indicates that suboptimal repair of DNA damage contributes to the genetic susceptibility to head and neck cancer. Alternatively, the observed enhanced chromosomal radiosensitivity may also result from a less efficient  $G_2/M$  checkpoint, as it has been shown that  $G_2$  checkpoint abrogation comprises repair of chromosome damage and that defects in the  $G_2/M$  checkpoint are associated with increased cancer risk (Terzoudi *et al*, 2005; Wu *et al*, 2005; Zheng *et al*, 2005).

Although in this study the mean number of radiation-induced aberrations was significantly higher in the studied cancer population compared with the healthy control population, an overlap exists between both groups. Therefore, a cutoff value,

**Figure 2** Distribution of radiation-induced  $G_2$  chromatid breaks in healthy individuals ( $n = 75$ ), all head and neck cancer ( $n = 101$ ), oral cavity cancer ( $n = 13$ ), pharynx cancer ( $n = 37$ ) and larynx cancer patients ( $n = 29$ ). The vertical line represents the 90th percentile cutoff point.

**Table 3** Stratification analysis of G<sub>2</sub> scores in head and neck cancer patients according to age and smoking

Subgroup	Population size	Mean G <sub>2</sub> score <sup>a</sup>	P-value <sup>b</sup>	% > cutoff value <sup>c</sup>	P-value <sup>d</sup>	OR (95% CI)	P-value <sup>e</sup>
<b>Age (years)</b>							
≤50	16	1.32	0.004	38	0.009	5.83 (1.63–20.89)	0.007
>50 to ≤70	71	1.22	<0.001	27	0.009	3.55 (1.39–9.08)	0.008
>70	14	1.18	0.269	7	0.788	0.75 (0.09–6.60)	0.793
<b>Smoking (PY)</b>							
≤10	13	1.28	0.006	46	0.003	8.33 (2.18–31.78)	0.002
>10 to ≤25	25	1.22	0.007	24	0.113	3.07 (0.92–10.22)	0.068
>25	59	1.21	0.001	20	0.120	2.48 (0.91–6.77)	0.076

CI, confidence interval; OR, odds ratio; PY, pack-years. <sup>a</sup>Number of chromatid breaks per cell. <sup>b</sup>Mann–Whitney test, compared with the control population. <sup>c</sup>Cutoff value = 90th percentile of the control population = 1.33 breaks per cell. <sup>d</sup> $\chi^2$  test, compared with the proportion of control individuals with a G<sub>2</sub> score > cutoff value. <sup>e</sup>Logistic regression, control individuals with ≤1.33 breaks per cell were used as reference.

above which an individual can be considered as radiosensitive, was determined (Scott *et al*, 1999). With this value, the proportion of radiosensitive individuals can be compared between different populations. As in most studies, the 90th percentile of the normal population was considered as cutoff point. Using this threshold for chromosomal radiosensitivity, 26% of the head and neck cancer patients were radiosensitive compared with 9% of the healthy individuals ( $P=0.008$ ). A high proportion of radiosensitive cases (31%) among various head and neck cancer patients have also been reported by Papworth *et al* (2001). Subdividing our large patient group according to anatomic location of the tumour revealed that the high G<sub>2</sub> scores were mainly attributable to the oral cavity and pharyngeal cancer patients of which 38 and 35%, respectively, were radiosensitive ( $P=0.018$  and 0.002). Of the laryngeal cancer patients, in contrast, only 17% of the patients were radiosensitive ( $P=0.419$ ). In the study of Lisowska *et al* (2006), a higher and significant proportion of radiosensitive individuals (40%) among larynx cancer patients was reported. Because a very different experimental protocol is used by Lisowska *et al*, compared with the present study, this could explain the difference in the number of radiosensitive larynx cancer patients.

In this study, no correlation has been found between radiation-induced aberration frequency and age, pack-years of smoking and drinks per week in patients and controls. Concerning age, this has also been published in other studies (Scott *et al*, 1999; Riches *et al*, 2001; Vral *et al*, 2007). For smoking and drinking exposure, no data are available in the literature for their effect on the level of radiation-induced aberrations. It has, however, been shown that age, smoking and alcohol intake do not influence mutagen-induced chromosomal aberrations (Cloos *et al*, 1996). Despite the lack of correlation on individual level, grouped analysis showed that patients ≤50 years and with ≤10 pack-years had the highest mean number of chromatid breaks. On the basis of the 90th percentile cutoff value, 38% of the patients aged 50 years or less were radiosensitive ( $P=0.009$ ). Similar proportions of *in vitro* radiosensitive young head and neck cancer patients have also been

reported by Papworth *et al* (2001) (38%, ≤45 years). Our patient group included only five patients ≤45 years but 60% of them were *in vitro* radiosensitive ( $P=0.008$ ). These results indicate that the genetic contribution, as measured by *in vitro* radiosensitivity, to head and neck cancer risk is higher for early onset cases. This finding for head and neck cancer patients differs from that for breast cancer patients in that no age dependence for G<sub>2</sub> sensitivity could be found in the latter group (Baeyens *et al*, 2005). As cancer at young age is a hallmark of heredity, this indicates that individuals at risk for early onset head and neck cancer possess a higher number of low-penetrant predisposing genes or a number of highly penetrant predisposing genes involved in DNA repair pathways compared with late-onset head and neck cancer patients and early onset breast cancer patients. Concerning tobacco exposure, it was found that non- and light smoking patients had the highest mean number of radiation-induced chromatid breaks. This indicates the existence of a larger genetic predisposition in non- and moderately tobacco-exposed individuals and supports the possibility of important gene–environment interactions in the development of head and neck cancer (Xiong *et al*, 2007).

In conclusion, this study supports the fact that chromosomal radiosensitivity of lymphocytes is a marker of genetic predisposition to head and neck cancer. Analyses of different subgroups revealed that the genetic contribution is highest for oral cavity and pharynx cancer patients and is more pronounced for early onset and non- and light smoking patients.

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