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Chromosomal Alterations in Mini Organ Cultures of Human Oropharyngeal Mucosa Cells Caused by Hydrogen Peroxide

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Abstract. *Background: Oxidative stress is a known exogenous risk factor for carcinogenesis in the head and neck. In addition to exogenous risk factors, the development of head and neck cancer is based on genetic alterations and individual mutagen sensitivity. DNA damage caused by reactive oxygen species (ROS) is not uniformly distributed over the DNA, as certain chromosomes and genes are more likely to be damaged than others. The ability to repair damaged DNA sufficiently is a necessity in order to prevent carcinogenesis. The DNA-damaging effect of ROS, the specific sites of chromosomal changes and DNA repair in those regions require further investigation. Materials and Methods: In order to evaluate DNA damage in macroscopically healthy mucosal tissue of 37 patients with (15) and without cancer (22) of the oropharynx, four different chromosomes (chromosomes 3, 5, 8 and 11) involved in carcinogenesis of the oropharynx were examined. After incubation with H_2O_2, comet FISH was applied to assess DNA damage of these chromosomes. The extent of DNA repair was evaluated in the same samples after a 24-hour repair period. Results: H_2O_2 caused significant DNA damage in oropharyngeal mucosal cells of patients with and without carcinoma. DNA fragmentation of all chromosomes investigated in the two groups was comparable. No differences were observed between mutagen sensitivity of patients suffering from cancer of the oropharynx and those without malignancy for any of the observed chromosomes. On the other hand, chromosomal DNA damage of these specific chromosomes was significantly higher than the average DNA fragmentation of the entire DNA in both groups. DNA repair led to a significant decrease in DNA damage in all groups. Controls tend to have a better DNA repair in all of the analysed chromosomes. However, these differences were not significant. Conclusion: The increased chromosomal DNA fragmentation in comparison to that of the entire DNA indicates the contribution of the investigated chromosomes to carcinogenesis in the oropharynx. DNA repair in those chromosomes might play a role in carcinogenesis of the oropharynx, but further investigations are warranted.*

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Oxidative stress is defined as an imbalance between cellular antioxidative and pro-oxidative factors in favour of the latter. In the tumor microenvironment, reactive oxygen species (ROS) mainly arise from metabolic products of macrophages and endothelial cells (1). In the upper aerodigestive tract, ROS also derive from exogenous sources such as cigarette smoke and environmental pollutants (2). Oxidative stress caused by ROS can damage lipids, proteins, enzymes, carbohydrates in cells and tissues, resulting in diverse damage of human cells. Concerning DNA, oxidative stress may lead to base modifications, DNA-protein cross links and strand breaks (3). DNA fragmentation caused by ROS is not uniformly distributed over the DNA. As a result, certain chromosomes and genes are more likely to be damaged than others (4). Various changes in chromosomes, alleles, tumorsupressor and proto-oncogenes are described for carcinogenesis of the upper aerodigestive tract (5-9). The development of cancer in the oropharynx is based on the occurrence of multiple genetic alterations. Healthy cells profit from a balanced interplay of tumor suppressor genes and oncogenes. Malignant transformation may be caused by a defect of these genes (10). However, genetic alterations can also be followed by a limitation of tumor suppressor genes or activation of proto-oncogenes to oncogenes. These mechanisms lead to uninhibited cell growth and therefore to the development of tumors. Oncogene activation is often triggered by addition of DNA, *e.g.* by chromosomal translocation or inversion (11). Inactivation of tumor suppressor genes on the contrary is caused by DNA reduction, *e.g.* the loss of an allele (12).

Effective DNA repair is a crucial factor in ensuring a low steady-state level of oxidative DNA damage. The DNA repair machinery is essential in protecting the integrity of the genome (13). Damage caused by ROS is mainly repaired by a pathway known as base excision repair (BER) (14). It is commonly accepted that poor DNA repair is associated with an increased risk of developing oropharyngeal cancer (15, 16).

In our study, we examined chromosomes 3, 5, 8 and 11, all of which are involved in the carcinogenesis of head and neck cancer (8, 17). We examined macroscopically healthy mucosal cells of the oropharynx and applied comet fluorescence *in situ* hybridization (FISH) to the investigated chromosomes in order to evaluate DNA damage in these cells after incubation with H_2O_2. Tissue from patients without oropharyngeal carcinoma served as control in order to evaluate potential differences in susceptibility or DNA repair capacity in the tumor group.

Materials and Methods

Biopsies. Tissue samples of macroscopically healthy oropharyngeal mucosa were harvested during surgery of oropharyngeal carcinoma (n=15; 12 male, 3 female, average age 56.3 years), or tonsillectomy (n=22, all male, average age 45.2 years) respectively. Only mucosa that was resected for surgical reasons was used to avoid additional stress for the patients. All donors were informed about the experiments and had signed a written consent statement. In addition, a comprehensive questionnaire was obtained to evaluate any former risk factors such as tobacco and alcohol consumption.

Mini organ cultures (MOC). Specimens were dissected into cubes of 1 mm^3, excluding deeper layers, and washed three times in bronchial epithelial cell basal medium (BEGM; Promocell, Heidelberg, Germany). Cubes were placed in 24-well plates coated with 0.75% Agar Noble (Difco, Detroit, MI, USA) and dissolved in Dulbecco's modified Eagle's medium (Gibco, Eggenstein, Germany). 10% Fetal calf serum (Gibco), non-essential amino acids (Gibco) and amphotericin B (Gibco). BEGM (250 μl/well) was added and the plates were incubated at 37˚C, 5% CO_2 and 100% relative humidity. After 14-20 days, MOC were completely coated with epithelium. BEGM was replaced every second day during cultivation. Multiwell plates were changed every week.

Cell separation and incubation. MOC were incubated with H_2O_2 at a concentration of 200 μM for 30 minutes at 37˚C and washed twice. In order to evaluate DNA repair capacity, a subset of the samples then underwent cultivation for another 24 h before DNA damage assessment. Aqua bidest served as the negative control. After microscopic preparation, all biopsies underwent enzymatic digestion [10 mg hyaluronidase (Boehringer, Mannheim, Germany); 10 mg collagenase (Roche, Mannheim, Germany); 50 mg protease (Sigma, Steinheim, Germany)] for 45 min at 37˚C. To preserve the physiological character of the samples, no metabolic activation was used before the incubation period. Viability was tested with trypan blue staining.

Comet assay. The cells were resuspended in 0.7% low-melting agaroses (Biozym, Hameln, Germany) and applied onto slides (Langenbrinck, Emmendingen, Germany), frosted at the long edges and covered with 0.5% normal melting agaroses (Biozym), to provide stability of the agarose layers. The slides were placed into a solution with 10% dimethysulfoxide (DMSO), 1% Triton-X®, 2.5 M NaCl, 10 mM Trizma-Base, 100 mM Na2EDTA and 1% N-lauroylsarcosine sodium salt for 1 h. The slides were then placed into a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany), positioned close to the anode and covered with alkaline buffer solution containing 300 mM NaOH and 1 mM Na_2EDTA at pH 13.2. After a 20-min DNA unwinding period, electrophoresis was started at 0.8 V/cm and 300 mA for 20 min, followed by neutralization (400 mM Trizma base, pH 7.5; Merck, Darmstadt, Germany).

Comet-FISH. For hybridization, the protocol of McKelvey-Martin *et al*. (18) was used with only minor changes. After neutralization and treatment with SSC-buffer (0.3 M NaCl, 30 mM sodium citrate), the slides were dehydrated with alcohol (70, 85 and 100%) and dried at 37˚C. Hybridization mixture was added, containing hybridization-buffer (all quantities are listed per slide) (formamide with dextran sulfate, 14 μl), DNA probes [2 μl, Chr 3 (WCP 3 SpectrumGreen), Chr 5 (WCP 5 SpectrumOrange), Chr 8 (WCP 8 SpectrumGreen) and Chr 11 (WCP 11 SpectrumOrange, all Abbott, Il, USA)] and Aqua bidest (4 μl). All probes hybridized to the centromere and 3p/3q, 5p/5q, 8p/8q and 11p/11q arms, respectively. After coverage and sealing of the prepared slides and incubation at 74˚C for 5 min on a precision hot plate, the slides were placed into a wet chamber for 12-16 h at 37˚C. Before detection of probes, the slides were washed three times each in 50% formamide in 2xSSC and incubated for 10 min in 2xSSC and 0.1% detergent NP-40 tergitol in 2xSSC for 5 min.

Table I. *Characteristics of donors of mucosa without carcinoma*.

No.	Age (years)	Alcohol consumption (g/d)	Smoking (py)	Diagnosis
1	37	0	0	OSAS
2	28	25	0	Chronic tonsillitis
3	45	0	20	Chronic tonsillitis
4	33	0	0	Chronic tonsillitis
5	62	0	0	Chronic tonsillitis
6	34	75	20	OSAS
7	62	100	20	Peritonsillar abscess
8	65	0	0	OSAS
9	48	0	0	Chronic tonsillitis
10	31	75	50	Chronic tonsillitis
11	35	25	5	OSAS
12	48	10	0	OSAS
13	38	50	0	OSAS
14	36	300	30	Chronic tonsillitis
15	63	125	80	Chronic tonsillitis
16	37	0	0	Chronic tonsillitis
17	33	0	0	Chronic tonsillitis
18	40	25	0	Chronic tonsillitis
19	61	25	40	Chronic tonsillitis
20	52	45	20	Chronic tonsillitis
21	47	10	0	Chronic tonsillitis
22	59	25	0	Chronic tonsillitis

py, Pack years; OSAS, obstructive sleep apnea syndrome.

Table II. *Characteristics of donors of mucosa with carcinoma.*

No.	Age (years)	Gender	Alcohol consumption (g/d)	Smoking (py)	Localization	Classification/grading*
1	61	♂	400	27	Base of tongue	pT1 pN1 cM0 G3
2	57	♂	50	0	Base of tongue	pT4 pN2c cM0 G2
3	45	♀	25	20	Base of tongue	pT2 pN2a cM0 G2
4	52	♂	0	10	Base of tongue	pT1 pN3 cM0 G2
5	45	♂	25	0	Base of tongue	pT2 pN2 cM0 G1
6	58	♂	100	30	Oropharynx	pT4 pN2c cM0 G3
7	54	♂	0	20	Base of tongue	pT1pNO cM0 G1
8	51	♂	125	80	Oropharynx	pT2 pN3 cM0 G3
9	56	♂	75	20	Base of tongue	pT3 pN0 cM0 G1
10	68	♂	75	0	Base of tongue	pT3 pN3 cM0 G2
11	60	♀	100	20	Tonsil	pT3 pN2b cM0 G1
12	77	♀	25	120	Tonsil	pT3 pN0 cM0 G1
13	49	♂	100	50	Tonsil	pT4 pN1 cM0 G3
14	50	♂	300	30	Base of tongue	pT1 pN1 M1 G3
15	62	♂	75	50	Oropharynx	pT3 pN2a cM0 G3

♂, Male; ♀, female; py, pack years; * according to UICC classification.

Staining and analysis. 10 μl 4',6-Diamidino-2-phenylindole (DAPI) (42 ng/ml, Abbott, Ill, USA) with Antifade (Abbott) was applied after air-drying of the slides followed by storage at –20˚C protected from light. DNA fragmentation was visualized using a fluorescence microscope and digital analysis (Comet++, Kinetic Imaging™; Liverpool, UK). Twenty cells per slide and 2 slides per patient were analyzed. Olive tail moments (OTM: median DNA migration distance x relative amount of DNA in the tail of the comet) and Munich chromosomal tail moments [MCTM: product of the median chromosomal migration distance and the chromosomal fluorescence in the tail of the comet divided by the entire chromosomal fluorescence measured in a cell (20)] were calculated to quantify DNA damage. DNA repair capacity (DRC) was calculated as described elsewhere (19).

Statistical analysis. Statistical analysis was performed using SPSS 16.0™ (SPSS Inc., Chicago, IL, USA). OTM values of all patients of the control and tumorgroups were compared by Mann-Whitney *U*-test and MCTM values for chromosome 3, 5, 8 and 11 within each group by Wilcoxon test. No multivariant analysis was applied, as the OTM served as reference. No control chromosome was used as discussed in prior studies (20). The general level of acceptance of significance was $p \leq 0.05$. Bonferroni correction was used where necessary. Standard box-plots (lower quartile, median, upper quartile) were used to illustrate the results. Dots mark mild statistical outliers [between 1.5 and 3 times interquartile range (IQR)], asterisks mark extreme statistical outliers (more than 3 times IQR).

Results

Patient characteristics (age, gender, diagnosis, grading, smoking and alcohol habits) are presented in Tables I and II. Combining the comet assay and FISH, human upper aerodigestive tract cells were used to identify DNA damage and specific alterations by hybridizing genetic sites of interest. DNA damage was quantified using OTMs. Although controversially discussed, it is still considered the most informative measure in the comet assay (21). Cell viability verified by use of the trypan blue staining test was constantly >90%, thus excluding major cytotoxic effects.

In the analysis of all patients, as expected, H_2O_2 caused significant DNA damage compared to that in MOCs treated with Aqua bidest ($p \leq 0.001$). Median OTM values were 11.8 for the control group and 10.2 for patients with oropharyngeal carcinoma on treatment with H_2O_2, while they were 1.8 and 1.6 respectively on rteatment with Aqua bidest. Only OTMs >2 are considered to reflect relevant DNA damage (22). No difference between the groups could be observed (Figure 1).

The median MCTM values for patients without carcinoma were 18.3 (Chr 3), 17.6 (Chr 5), 17.1 (Chr 8) and 17.4 (Chr 11). The OTM for DNA fragmentation of the entire DNA was 11.8. All of the investigated chromosomes showed significantly higher fragmentation than that of the entire DNA ($p \leq 0.001$ for all chromosomes) (Figure 2). Similar results were found for the group with oropharyngeal carcinoma: the median MCTM for the chromosomes were 17.6 (Chr 3), 18.2 (Chr 5), 15.8 (Chr 8) and 18.8 (Chr 11), while the median OTM was 10.2. Again, all chromosomes showed significantly higher damage than that of the entire DNA ($p \leq 0.002$ for all chromosomes). Statistical analysis between the two groups showed no significant differences in chromosomal or DNA damage (Figure 2).

Twenty-four hours of DNA repair led to a significant reduction of DNA fragmentation in MOCs of the tumor and control group. OTM was reduced from 11.8 to 2.0 in tumor MOCs and 10.2 to 1.7 in controls MOCs, resulting in a DRC of 79.9% and 83.4% respectively ($p \leq 0.001$ for both groups) (Figure 1). All the investigated chromosomes showed significantly lower MCTMs in both groups (Figure 3)

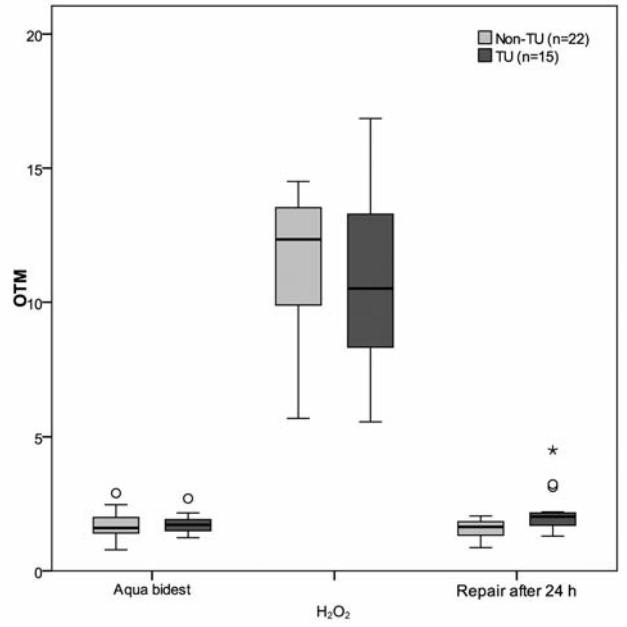

Figure 1. *Results of genotoxicity tests in human oropharyngeal mucosa cells after incubation with aqua bidest as negative control and hydrogen peroxide (H_2O_2) and DNA repair after 24 hours. Box-plots show the lowest and highest values of OTM as well as the 1st and 3rd quartile and the median; * and ° mark statistical outliers. Values are given for the control group (Non-TU) and the group of patients with oropharyngeal carcinoma (TU).*

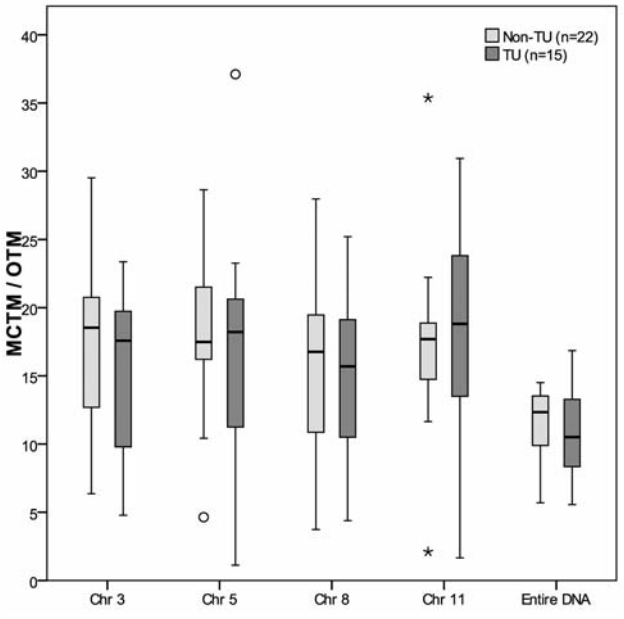

Figure 2. *Extent of DNA migration of chromosome 3, 5, 8 and 11 and entire DNA induced in human oropharyngeal mucosa cells by H_2O_2. For the definition of the box-plots, see Figure 1. MCTM describes the damage induced in each chromosome, OTM in entire DNA. Values are given for groups with (TU) and without (Non-TU) oropharyngeal carcinoma.*

($p \leq 0.001$ for all chromosomes in both groups). DNA repair was slightly more effective in the control group, but this difference was not significant for any of the investigated chromosomes.

Discussion

Head and neck squamous cancer (HNSCC) is the sixth most common cancer in the world and accounts for 90% of malignant neoplasias of the upper respiratory and aerodigestive system (23). Despite recent advances in the management of locally advanced HNSCC, the overall survival of patients has improved only marginally over the past three decades (24). Besides exogenous risk factors, such as smoking and alcohol consumption, the importance of endogenous risk factors is of growing interest in the multifactorial genesis of upper aerodigestive tract carcinoma (20). Mutagen sensitivity, as one of these contributors to risk, describes the sensitivity of organisms to environmental agents causing DNA damage (25). Oxidative stress, as a result of disturbances in the balance between ROS and the efficiency of antioxidant mechanisms, may cause DNA damage (26). Carcinoma cells are characterized by persistent oxidative stress and high levels of ROS. Human tumor cells *in vitro* have a

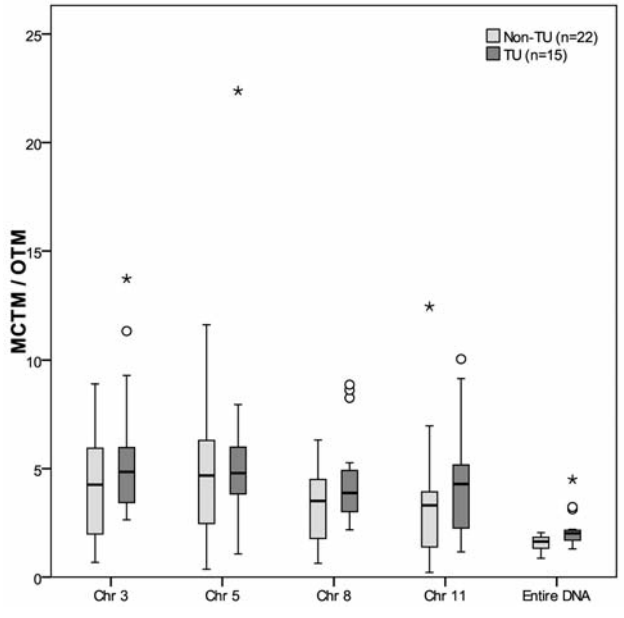

Figure 3. *Extent of DNA migration of chromosome 3, 5, 8 and 11 and entire DNA induced by H_2O_2 in human oropharyngeal mucosa cells after a 24-h repair period. For the definition of the boxplots, see Figure 1. MCTM describes the damage induced in each chromosome, OTM in entire DNA. Values are given for groups with (TU) and without (Non-TU) oropharyngeal carcinoma.*

much enhanced production of hydrogen peroxide relative to nontransformed cell lines (27). This leads to an enhanced induction of DNA damage. Unrepaired damage in epithelial cells may result in DNA transformation and mutation with subsequent tumor growth (28). To further analyze the carcinogenesis of HNSCC, detection of specific endangered genetic regions of interest in epithelial target cells is required. However, most studies in the past have focused on examining the DNA as a whole and did not consider occurrence of DNA adducts and repair in different specific genomic regions, even though DNA damage is not uniformly distributed over the DNA (4). The combination of the comet assay and FISH enables region-specific studies on DNA damage and repair (18, 20, 29). In the present study, no differences in mutagen sensitivity after treatment with H_2O_2 in oropharyngeal mucosal cells of patients with and without carcinoma of the oropharynx was observed. Furthermore, detection of chromosomal alterations revealed higher MCTMs and therefore DNA damage in all chromosomes compared to the values for the entire DNA, indicating a higher susceptibility of these specific chromosomes in both investigated groups. These findings correlate with previous studies demonstrating higher strand break levels of chromosome 3, 5, 8 and 11 in upper aerodigestive tract squamous cell carcinoma cells (30). As macroscopically healthy mucosa of the oropharynx was investigated in this study, our results argue for an early onset involvement of these chromosomal alterations in carcinogenesis of the head and neck.

On the other hand, efficient repair mechanisms comprise a critical component of the protection against DNA mutations (31). Most damage is removed by repair enzymes before it can interfere with the process of DNA replication and introduce mutation. Individual variation in the capacity for DNA repair is therefore likely to be an important factor in determining cancer risk. In general, oxidative stress creates 8-oxo-7,8-dihydroguanine (8-oxoGua) and single-strand breaks in the DNA (26). These defects are mainly repaired by BER. A lesion-specific glycosylase removes the base and the resulting apurinic/apyrimidinic site is converted to a break. Small gaps are filled in by DNA polymerase plus ligase. Deficiency in these mechanisms is associated with increasing risk of developing cancer of the head and neck (14).

In our study, the extent of DNA repair after 24 hours was comparable in both groups. No significant differences in the decrease of OTMs after the repair period were observed. Furthermore, levels of DNA repair were similar in all investigated chromosomes. Although DNA repair tended to be slightly more efficient in the control group compared to the tumor group, these differences were not significant. This may be due to the small patient numbers included in the study.

Conclusion

Mutagen sensitivity and DNA repair capacity play important roles in the endogenous risk concerning carcinogenesis. In our study, the susceptibility towards DNA damage of the chromosomes 3, 5, 8 and 11 was higher than that of DNA as a whole in both patients with and those without HNSCC. DNA repair was efficient in both groups. A slightly better chromosomal DRC in control patients was not significant, most likely due to the low patient numbers. DNA repair in these chromosomes might play a role in carcinogenesis of the oropharynx, but further investigations are warranted.

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