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Chromosomal Alterations and Mutagen Sensitivity in Human Mucosal Cells of the Oropharynx and Lymphocytes Caused by BPDE

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Abstract. *Background: 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 xenobiotics is not uniformly distributed over the DNA, as certain chromosomes and genes are more likely to be damaged than others. The DNA damaging effect of xenobiotics and the specific sites of chromosomal changes require further investigation. Materials and Methods: In order to evaluate mutagen sensitivity in macroscopically healthy mucosal tissue of 30 patients with (15) and without cancer (15) of the oropharynx, three different chromosomes (chromosomes 3, 5 and 8) involved in carcinogenesis of the oropharynx and one control chromosome (chromosome 1) were examined. After incubation with benz[a]pyren-7,8-diol-9,10-epoxide (BPDE), a tobacco-associated carcinogen, comet fluorescence in situ hybridization (FISH) was applied to assess DNA damage of these chromosomes. Furthermore, lymphocytes and macroscopically healthy mucosal cells of the oropharynx were assessed using FISH after their incubation with BPDE in order to evaluate loss and gain of DNA in these chromosomes. Results: BPDE caused significant DNA damage compared to the negative control in oropharyngeal mucosa cells of patients with and without carcinoma. No difference was observed between mutagen sensitivity of patients suffering from cancer of the oropharynx and patients without malignancy. In cells from patients suffering from squamous cell carcinoma, significantly higher DNA damage was found in chromosome 5 and 8 after incubation with BPDE and application of comet FISH. No difference was found in patients without cancer of the head and neck. After application of FISH, no difference in the amount of DNA was found in chromosomes 1, 3, 5 and 8, neither in lymphocytes nor in mucosal cells from both groups. No DNA gain or loss was detected. Conclusion: Our results confirm the higher sensitivity of chromosomes 5 and 8 of normal epithelial cells of oropharyngeal cancer patients to BPDE. These effects were shown in macroscopically healthy tissue of such patients for the first time. Therefore, we suggest that these are early onset effects in carcinogenesis of the head and neck. No such effect was shown for chromosome 3 and control chromosome 1.*

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Squamous cell carcinoma (SCC) is the most common epithelial malignancy of the head and neck, encompassing the oral cavity, oropharynx, hypopharynx, pharynx and larynx (HNSCC) (1). This region is one of the most important locations predisposed for tobacco-associated cancer. Tobacco-specific polycyclic aromatic hydrocarbons (PAHs) are known carcinogens causing DNA damage in human mucosal cells. A major representative of these compounds is benzo[a]pyrene (BaP). It is transformed by cytochrome-P 450 2E1 to benz[a]pyren-7,8-diol-9,10-epoxide (BPDE) in epithelial cells of the upper aerodigestive tract, where it mainly causes DNA adducts and single-strand breaks (2).

DNA damage caused by xenobiotics such as BPDE is not uniformly distributed over the DNA. As a result, certain chromosomes and genes are more likely to be damaged than others (3). Various changes in chromosomes, alleles, tumor-supressor and proto-oncogenes are described for carcinogenesis in the upper aerodigestive tract (4-8). 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 (9). 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 (10). Inactivation of tumor suppressor genes is in the contrary caused by DNA reduction, *e.g.* the loss of an allele.

DNA loss and gain is described for various chromosomes in cancer cells of the oropharynx. In our study, we examined chromosomes 3, 5 and 8, using chromosome 1 as a reference. Out of these, chromosomes 5 and 8 showed significant DNA loss and gain in cancer cells of the upper aerodigestive tract (11). We examined macroscopically healthy mucosa 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 BPDE. Furthermore, we used FISH to assess structural DNA loss and gain in mucosa metaphase chromosomes. Donor lymphocytes were used as control cells.

Materials and Methods

Comet and comet FISH in oropharyngeal mucosa cells

Biopsies. Tissue samples of macroscopically healthy oropharyngeal mucosa were harvested during surgery of oropharyngeal carcinoma (n=15, all male, average age 61 years), or tonsillectomy (n=15, all male, average age 51 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.

Cell separation and incubation. 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. Aliquots of 1×10^5 cells were incubated with BPDE (9 μM) (CAS-No. 58917-67-2; Midwest Research, Kansas, USA) for 60 min at 37˚C, the negative control was incubated with the solvent dimethylsulfoxide (DMSO, 166 mM, Merck, Darmstadt, Germany) followed by viability testing.

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 for the agarose layers. The slides were placed into a solution with 10% DMSO, 1% Triton-X®, 2.5 M NaCl, 10 mM Trizma-Base, 100 mM Na_2EDTA, 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.* (12) 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. The hybridization mixture added contained (per slide) hybridization buffer (formamide with dextran sulfate, 14 μl), DNA probes [2 μl, whole chromosome paint for Chr 1 (WCP 1 SpectrumOrange), Chr 3 (WCP 3 SpectrumGreen), Chr 5 (WCP 5 SpectrumOrange) and Chr 8 (WCP 8 SpectrumGreen), all Abbott, IL, USA)] and aqua bidest (4 μl). All probes hybridized to the centromere and 1p/1q, 3p/3q, 5p/5q and 8p/8q 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 2 x SSC and incubated for 10 min in 2 x SSC and 0.1 % detergent tergitol NP-40 in 2 x SSC for 5 min.

Staining and analysis. A volume of 10 μl DAPI (42 ng/ml; Abbott) with Antifade (Abbott) was applied after air-drying of the slides followed by storage of the slides 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 two slides per patient were analyzed.

FISH in lymphocytes

Preparation. Lymphocytes derived from heparinized blood samples of the donors were separated in equal amounts of Lymphoprep (Nycomed, Oslo, Norway) using density gradient centrifugation (20 min, 2000 U/min, 20˚C). Viability and cell count were investigated using trypan blue staining. Cell aliquots of 5×10^6/ml were stimulated for 72 hours with phythaemagglutinine (PHA; Biochrom, Heidelberg, Germany). BPDE (9 μmol/l / 4.5 μmol/l / 2.25 μmol/l) was added within the last hour of stimulation. DMSO served as negative control. After BPDE was washed from the cells and stimulation was stopped, colcemide (10 μg/ml, Sigma) was added in order to arrest mitosis in the metaphase and aliquots were resuspended in Joklik medium.

Fixation. The cells were incubated for 45 min in 8 ml KCl (37˚C, 0.075 mmol/l), following addition of ice-cold fixative (methanol and acetate 3:1). Fixation was repeated three times, until the cells were dropped onto prepared slides.

FISH. FISH was applied according to the protocol of Vysis/Abbott for co-denaturation of DNA (Abbott).

Staining. A volume of 10 μl DAPI (42 ng/ml) with Antifade was applied after air drying of the slides followed by storage of the slides at –20˚C protected from light.

Analysis. The extent of DNA loss or gain was measured by quantifying the relative amount of chromosomal DNA in relation to the entire DNA. Twenty cells per slide and two slides per patient were analyzed.

FISH in oropharyngeal mucosa cells

Preparation. Metaphase chromosomes were prepared in mucosa of the upper aerodigestive tract for the first time for quantification of DNA loss and gain. Mucosa cubes of 1 mm^3 were washed and

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

No.	Age (years)	Alcohol	Smoking	Diagnosis
1	30	0	27 py	OSAS
2	30	25 g/d	20 py	Chronic tonsillitis
3	34	100 g/d	30 py	Chronic tonsillitis
4	34	0 g/d	20 py	Chronic tonsillitis
5	42	75 g/d	20 py	Chronic tonsillitis
6	44	75 g/d	Not stated	OSAS
7	62	100 g/d	20 py	Peritonsillar abscess
8	62	25 g/d	120 py	OSAS
9	71	100 g/d	50 py	Chronic tonsillitis
10	73	75 g/d	50 py	Chronic tonsillitis
11	50	0 g/d	10 py	OSAS
12	50	25 g/d	0 py	OSAS
13	46	50 g/d	0 py	OSAS
14	71	300 g/d	30 py	Chronic tonsillitis
15	58	125 g/d	80 py	Chronic tonsillitis

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

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

No.	Age (years)	Alcohol	Smoking	Tumor localisation	Tumor classification/ grading*
1	43	400 g/d	27 py	Tonsil	pT2 pN0 cM0 G3
2	46	50 g/d	0 py	Vallecula	pT3 pN2c cM0 G2
3	50	25 g/d	20 py	Base of tongue	pT1 pN2a cM0 G2
4	50	0 g/d	10 py	Base of tongue	pT3 pN3 cM0 G2
5	50	25 g/d	0 py	Vallecula	pT3 pN1cM0 G1
6	51	100 g/d	30 py	Oropharynx	pT4 pN1cM0 G3
7	58	0 g/d	20 py	Base of tongue	pT1pNO cMO G1
8	58	125 g/d	80 py	Oropharynx	pT2 pN3 cM0 G3
9	60	75 g/d	20 py	Base of tongue	pT1 pN0 cM0 G1
10	62	75 g/d	not stated	Base of tongue	pT3 pN3 cM0 G2
11	62	100 g/d	20 py	Tonsil	pT3 pN2b cM0 G2
12	62	25 g/d	120 py	Tonsil	pT2 pN0 cM0 G2
13	71	100 g/d	50 py	Tonsil	pT1 pN1 cM0 G1
14	71	300 g/d	30 py	Base of tongue	pT2 pN3 M1 G2
15	73	75 g/d	50 py	Oropharynx	pT3 pN0 cM0 G2

py, Pack years; *according to UICC TNM classification.

incubated in penicillin/streptomycin (250 μg/ml) and amphotericin B (250 μg/ml) before transferring them into culture vessels (Sarstaedt, Nuembrecht, Germany). Cells were incubated in BEGM for 5-6 weeks until a dense layer of cells grew adherent to the container. Media were changed every 3 days. Cells were dissolved with trypsin/EDTA (10 μmol) and applied to prepared slides in a quadriperm vessel (Sarstaedt). After 10 days, the cells were adherent to the slides. Twenty-five μl of colcemide (10 μg/ml) were added to arrest mitosis in the metaphase. Slides were then processed as lymphocytes above.

Statistical analysis. Statistical analysis was performed using SPSS 16.0™ (Chicago, IL, USA). OTM values of cells from all patients of the non-tumor and tumor groups were compared (Mann-Whitney U test) as well as chromosomal tail moments of chromosome 1 *vs*. 3, 5 and 8 within each group (Wilcoxon test). No multivariant analysis was applied, as chromosome 1 served as reference. 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 in figures mark mild statistical outliers (between 1.5 and 3 times interquartile range (IQR)), while 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 the Olive tail moment (OTM: median DNA migration distance x relative amount of DNA in the tail of the comet). Although other investigators have different views, it is still considered the most informative measure in the comet assay (13). To describe the degree of chromosomal damage in the cell, the Munich chromosomal tail moment (MCTM) was implemented. The MCTM is the 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.

In the analysis of all 30 patients, as expected, BPDE caused significant ($p \leq 0.001$) DNA damage compared to the control incubated with DMSO. BPDE gave a median OTM of 18.88 (non-tumor) and 18.55 (tumor) compared to 1.81 (non-tumor) and 1.65 (tumor) with DMSO (Figure 1; Table III). No significant differences between the patient groups could be found comparing OTM values after treatment with DMSO ($p=0.345$) and BPDE ($p=0.970$), respectively.

The MCTMs for Comet FISH for chromosomes 1, 3, 5 and 8 in patients without carcinoma (n=15) are listed in Figure 2 with a median MCTM of 16.31 (Chr 1), 14.65 (Chr 3), 15.12 (Chr 5) and 14.99 (Chr 8). Damage of chromosome 1 and chromosomes 3, 5 and 8 caused by BPDE was compared using the Wilcoxon test. Differences in MCTM values were not statistically significant (Table IV).

However, cells from patients with carcinoma of the oropharynx (n=15) had median MCTM values of 18.53 (Chr 1), 23.15 (Chr 3), 22.19 (Chr 5) and 25.19 (Chr 8) (Figure 3). Statistical analysis showed significantly higher MCTM values for chromosome 5 and 8 compared to chromosome 1 (Table III). No significance was found for chromosome 3.

In the comparison of the FISH results in lymphocytes and mucosa cells, no DNA loss or gain was found at the BPDE concentrations used. This applies for both the investigated groups. Increasing concentrations of BPDE did not show any significant effect on DNA loss or gain (Figures 4-7).

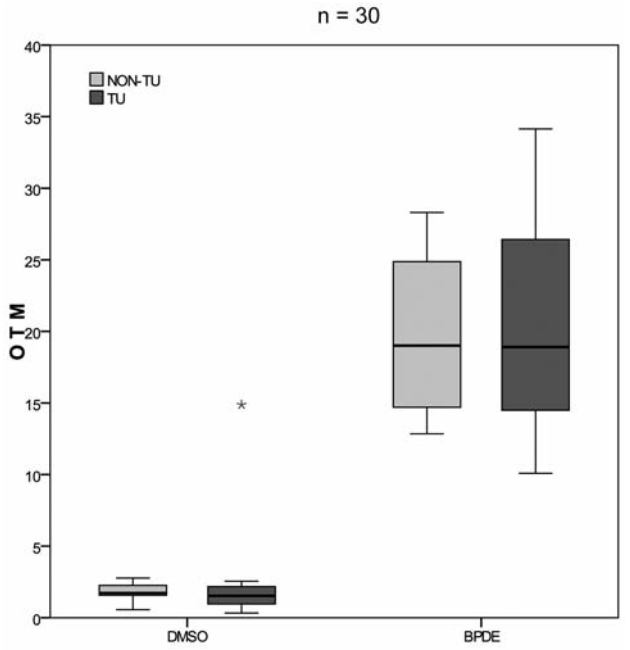

Figure 1. *Results of genotoxicity tests in human oropharyngeal mucosa cells after incubation with dimethyl sulfoxide (DMSO) as negative control and benzo[a]pyren-diolepoxide (BPDE). Box-plots show the lowest and highest values of OTM as well as the 1st and 3rd quartile and the median. * Mark statistical outliers. Values are given for the control group (NON-TU) and the group of patients with oropharyngeal carcinomas (TU).*

Table III. *Olive tail moment (OTM) values for the patients without (NON-TU) and with carcinoma of the oropharynx (TU) after incubation with DMSO as negative control and BPDE. p-Values using the Mann-Whitney test are listed comparing effects of DMSO and BPDE. OTMs of NON-TU and TU are also compared after incubation of cells with DMSO and BPDE respectively.*

	DMSO	BPDE	*p*-Value (DMSO *vs*. BPDE)
OTM (NON-TU)	1.81	18.88	≤0.001
OTM (TU)	1.65	18.55	≤0.001
p-Value (NON-TU *vs*. TU)	0.345	0.870	

Table IV. *MCTM values of chromosomes 1, 3, 5 and 8 for cells from patients without (NON-TU) and those with carcinoma of the oropharynx (TU) after incubation with BPDE. p-Values using the Wilcoxon test are listed comparing effects of BPDE on chromosome 1 vs. chromosome 3, 5 and 8 respectively.*

	Chr 1	Chr 3	Chr 5	Chr 8
MCTM (NON-TU)	16.31	14.65	15.12	14.99
p-Value *vs*. Chr 1	-	0.609	0.865	0.173
MCTM (TU)	18.53	23.15	22.19	25.19
p-Value *vs*. Chr 1	-	0.880	**0.020**	**0.002**

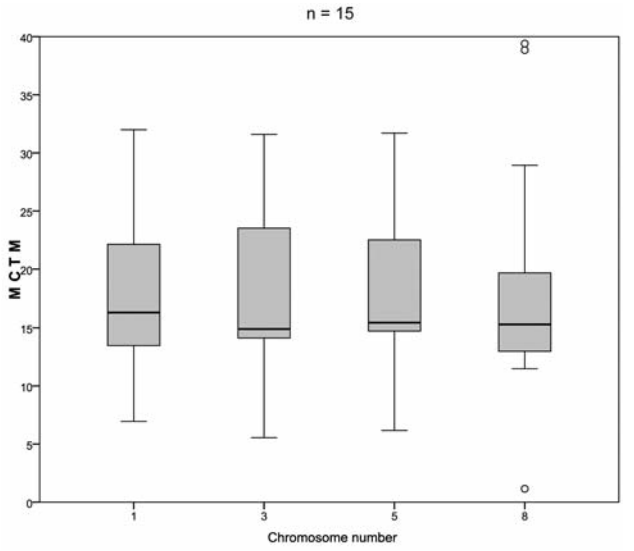

Figure 2. *Extent of DNA migration of chromosomes 1, 3, 5 and 8 induced in human oropharyngeal mucosa cells by benzo[a]pyren-diolepoxide (BPDE). MCTM describes the damage induced in each chromosome. Box-plots show the lowest and highest values of MCTM as well as the 1st and 3rd quartile and the median. ° Mark statistical outliers. Values are given for the control group of patients without oropharyngeal carcinoma (NON-TU).*

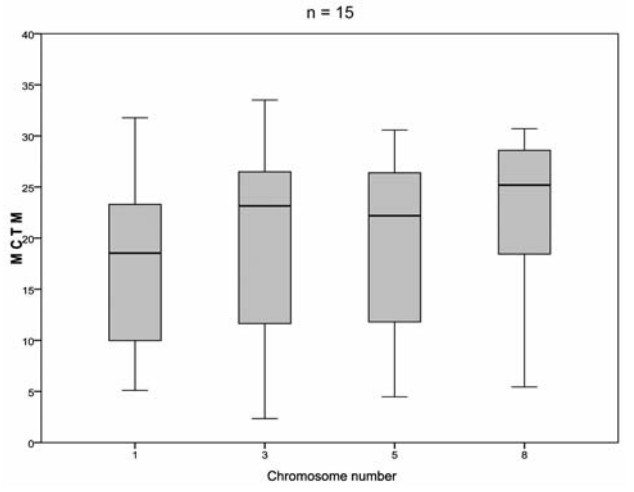

Figure 3. *Extent of DNA migration of chromosomes 1, 3, 5 and 8 induced in human oropharyngeal mucosa cells by BPDE. For the definition of the box-plots, see Figure 1. MCTM describes the damage induced in each chromosome. Values are given for the control group of patients with oropharyngeal carcinoma (TU).*

Discussion

HNSCC is the sixth most common cancer in the world and accounts for 90% of malignant neoplasias of the upper respiratory and aerodigestive system (14). Despite recent

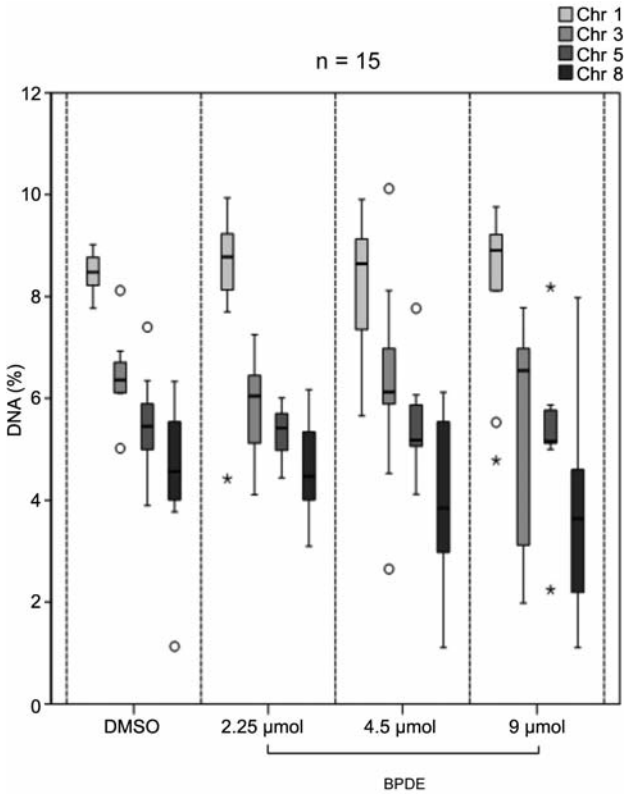

Figure 4. *DNA percentage of chromosomes 1, 3, 5 and 8 in lympocytes from patients without tumor of the oropharynx in relation to entire DNA. Values are given for control (DMSO), as well as all BPDE concentrations used. * And ˚ mark statistical outliers.*

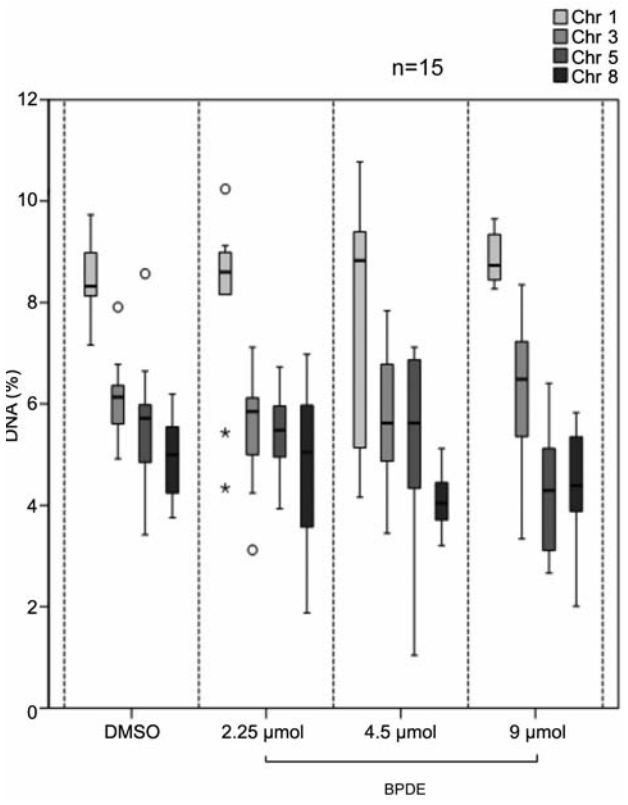

Figure 5. *DNA percentage of chromosomes 1, 3, 5 and 8 in lymphocytes of patients with tumor of the oropharynx in comparison to entire DNA. Values are given for control (DMSO), as well as all BPDE concentrations used. * And ˚ mark statistical outliers.*

advances in the management of locally advanced HNSCC, the overall survival of patients has improved only marginally over the past three decades (1). Many exogenous risk factors have been identified in the environment and working places (15, 16). Besides these factors, including tobacco smoke and alcohol consumption, the importance of endogenous risks is of growing interest in the multifactorial genesis of upper aerodigestive tract carcinoma. Mutagen sensitivity, as one of those risk factors, describes the sensitivity of organisms to environmental influences causing DNA damage (17). Unrepaired damage in epithelial cells may result in DNA transformation and mutation with subsequent tumor growth (18). To further analyze the carcinogenesis of HNSCC, detection of specific endangered genetic regions of interest in epithelial target cells is required.

The comet assay detects DNA damage such as strand breaks, alkali-labile sites and incomplete excision repair. Single epithelial cells of the upper aerodigestive tract have been used to investigate the genotoxicity of multiple environmental agents (15, 19) as well as DNA repair in epithelial cells and lymphocytes (20, 21). FISH was introduced in the 1980s. FISH-based methods have enhanced cytogenetic analysis of tumors and have continuously gained ground in clinical cytogenetic diagnostics (22).

However, most studies have focused examining the DNA as a whole and did not consider occurrence of DNA adducts and repair in different specific genomic regions. The combination of both methods enables region-specific studies on DNA damage and repair (12, 23, 24). In the present study, no differences in mutagen sensitivity after treatment with BPDE in oropharyngeal mucosa cells of patients with and without carcinoma of the oropharynx was observed. Furthermore, detection of chromosomal alteration revealed enlarged MCTMs and therefore DNA damage in chromosomes 5 and 8 compared to chromosome 1 in tumor patients, which indicates a higher sensitivity of these chromosomes to the tobacco carcinogen BPDE in this group. These findings correlate with previous studies, demonstrating higher strand break levels of chromosomes 5 and 8 in upper aerodigestive tract SCC cells (4). On the other hand, no increased DNA damage was found in chromosome 3. This fact did not correlate with previous

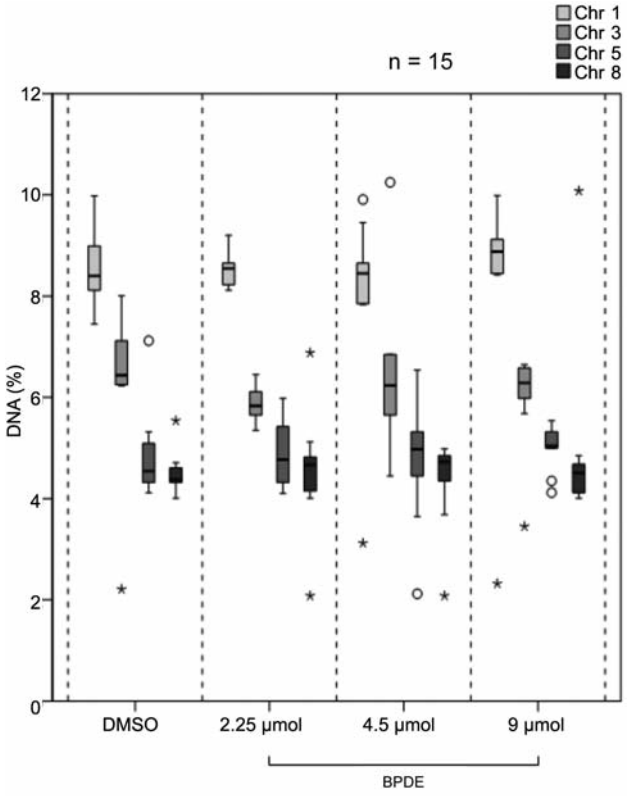

Figure 6. *DNA percentage of chromosomes 1, 3, 5 and 8 in mucosal cells of patients without tumor of the oropharynx in relation to entire DNA. Values are given for control (DMSO), as well as all BPDE concentrations used. * And ˚ mark statistical outliers.*

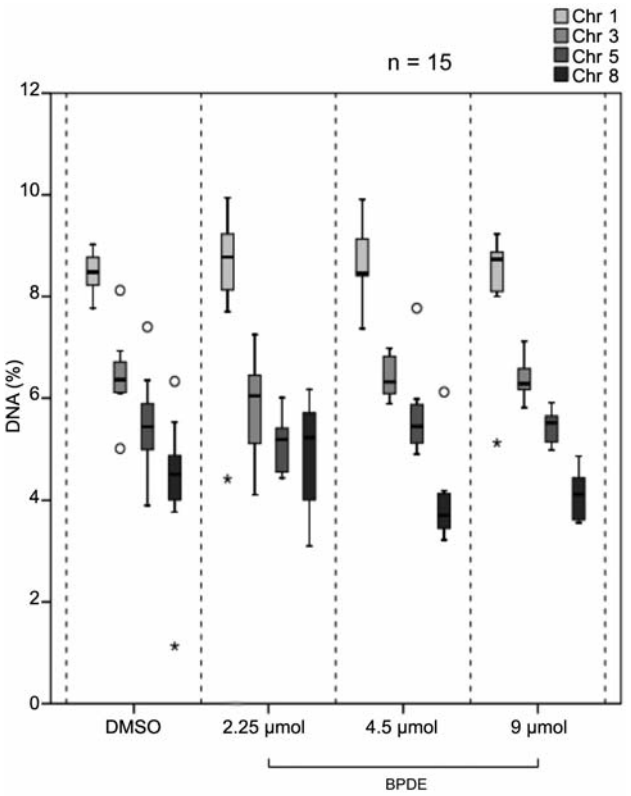

Figure 7. *DNA percentage of chromosomes 1, 3, 5 and 8 in mucosal cells from patients with tumor of the oropharynx in relation to entire DNA. Values are given for control (DMSO), as well as all BPDE concentrations used. * And ˚ mark statistical outliers.*

findings (4, 7, 25), indicating no enhanced mutagen sensitivity in this chromosome towards BPDE. 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. Furthermore, alterations in chromosomes 5 and 8 are involved during metastasis formation in tumor cells of patients with SCC of the upper aerodigestive tract (11).

In contrast to comet FISH, FISH enables to investigate DNA alteration in the metaphase of the cell. Inversions, deletions, transformations, DNA loss and gain can be detected by FISH. Oncological applications are various, as almost every genetic locus down to minimal size of 1 kb can be stained (26). In our study, we did not find any morphological alterations within the examined chromosomes. DNA loss or gain was not detected either in lymphocytes or in mucosa. Lymphocytes were used as control cells. Most studies using human cells are performed on cell cultures or peripheral blood lymphocytes. In a previous study using the comet assay, human peripheral blood lymphocytes and mucosal cells of the upper aerodigestive tract were found not to be equally sensitive to genotoxic agents and, at the level of the individual, the extent of DNA damage in lymphocytes was not comparable with the extent of DNA damage in mucosal cells of the upper aerodigestive tract (19). Thus, the *in vitro* sensitivity of lymphocytes to genotoxic agents is an unreliable predictor of DNA damage in target cells of inhalative or ingestive xenobiotics and for analysis of chromosomal alteration in upper aerodigestive tract epithelia. Nevertheless, we used lymphocytes as a control for morphological changes in the investigated chromosomes, as previous studies reported sensitivity of lymphocytes towards BPDE-induced DNA damage. For example, 3p21.3 was found to be a molecular target for BPDE damage in lymphocytes of patients with premalignant oral lesions (27).

We found higher sensitivity towards BPDE of chromosomes 5 and 8 in mucosal cells without morphological correlation in metaphase chromosomes. In previous studies, DNA loss and gain was described for specific regions on these chromosomes in cancer cells of the head and neck. DNA

amplification is described in the short arm of chromosome 5 (28), in particular in locus 5p13.33 (29). This allele encodes for *hTERT* (human telomerase reverse transcriptase) which is related to tumor induction in the head and neck (30). In various cell lines, overexpression of hTERT, depending on DNA alteration of 5p13.33, has been found (31).

For carcinogenesis in the upper aerodigestive tract, the short arm of chromosome 8 plays a significant role as well (32). Regions of interest are 8p21, 8p22 and 8p23.3 (33, 34). Studies report diverse tumor suppressor genes, some of which are not yet well defined (35, 36). A member of the tumor necrosis receptor family, KILLER/DR 5, is ascribed a role in carcinogenesis of the head and neck. Reduced expression is associated with a reduced apoptosis rate and increasing cell proliferation (37). DNA alteration in this region results in a worse prognosis of the disease (6).

Epithelial cells of normal oropharyngeal mucosa in patients with HNSCC demonstrated increased sensitivity to the effects of the mutagen BPDE of chromosomes 5 and 8. These would appear to be early onset effects in the carcinogenesis of HNSCC.

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