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Altered genotoxicity in mucosal cells of head and neck cancer patients due to environmental pollutants

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Abstract The complexity of carcinogenesis in squamous cell cancer (SCC) of the upper aerodigestive tract requires examining environmental risk factors, including mutagen sensitivities to xenobiotics. Three environmental, occupational, and habitual pollutants – dibutylphthalate (DBP), diisobutylphthalate (DiBP), and *N*′nitrosodiethylamine (NDELA) – were submitted to genotoxicity testing on mucosal biopsy specimens of tumor and nontumor patients in vitro. The single-cell microgel electrophoresis (Comet) assay was applied to detect DNA strand breaks in human epithelial cells of the pharynx and larynx from nontumor patients, patients with SCC of the oropharynx and patients with SCC of the larynx. Genotoxicity was found for DBP, DiBP, and NDELA in cells derived from nontumor and tumor patients. With respect to phthalates, Olive tail moment (OTM) levels were higher in patients with SCC of the oropharynx and SCC of the larynx ($P < 0.01$), the latter showing even more pronounced genotoxicity for DiBP. Testing epithelial cells of the patients with either oropharyngeal or laryngeal SCC for NDELA demonstrated results similar to the nontumor patients. Present findings indicate heterogeneous mutagen sensitivities to some but not all xenobiotics.

Keywords Genotoxicity · Carcinogenesis · Aerodigestive squamous cell carcinoma · Phthalates · Nitrosamines

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Introduction

The complexity of carcinogenesis in squamous cell cancer (SCC) of the upper aerodigestive tract suggests a role of such risk factors as environmental chemicals and specific susceptibilities. Dibutylphthalate (DBP) and diisobutylphthalate (DiBP) are used as plasticizers in a wide range of synthetic materials, including polyvinyl chloride, and to improve the chemical properties of paints and lubricants. They can be found in toys, teething rings, medical products, and food wrappings [1, 2]. Because phthalates can emerge from these products, they have become nearly ubiquitous environmental pollutants. In a study measuring plasma phthalate levels after extracorporal membrane oxygenation in infants, levels were clearly elevated after treatment [13], although the significance of this is unclear. Despite reports about possible promoting influences in a human breast cancer cell line [3] and hepatocarcinogenesis in an animal model [11], there is no information concerning the genotoxic impact on human epithelial cells in tumor patients. Minor but regularly occurring breaks in DNA strands due to phthalates have been observed in human mucosal cells taken from the upper aerodigestive tract in otherwise healthy individuals [16].

Nitrosamines, such as *N*′nitrosodiethylamine (NDELA), are present in tobacco smoke and foods. They are used in the preservation of meat, cheese, beer, and many other products. Although a carcinogenic risk has been associated with nitrosamines [20], data on the genotoxic effects on the human mucosa of the pharynx and larynx as a possible compounding factor in carcinogenesis are rare.

In the present study we compared susceptibilities to DBP, DiBP, and NDELA in nontumor patients to those in patients with SCC of the oropharynx or larynx.

Materials and methods

Macroscopically healthy mucosal biopsy specimens were taken from the oropharynx of 18 patients with SCC of the oropharynx

Table 1 Characteristics of nontumor patients (controls, NT) and patients with SCC of the oropharynx (TO) or larynx (TL)

Patient no.	Age (years)	Sex	Tumor site	Tumor stage	Tobacco (pack-years)[a]	Alcohol (g/d)
NT						
1	68	M	25	150		
2	34	M	15	50		
3	49	M	0	25		
4	36	F	0	15		
5	33	M	15	25		
6	78	M	50	25		
7	53	M	12	25		
8	47	F	–	–		
9	28	F	0	10		
10	38	M	–	10		
11	62	F	35	25		
12	56	M	35	10		
13	32	M	10	25		
14	33	M	0	0		
15	31	F	0	25		
16	32	F	10	0		
17	25	F	0	0		
18	24	M	0	25		
19	68	F	30	15		
20	30	F	20	25		
21	42	F	0	0		
22	27	F	–	–		
23	33	F	0	10		
24	31	F	0	10		
25	14	F	0	0		
26	18	F	3	10		
27	17	F	3	0		
28	19	M	1	0		
29	33	M	15	25		
30	19	F	0	0		
TO						
31	55	M	Tonsil	G3	40	100
32	46	M	Tonsil	G2	–	–
33	39	M	Tonsil	G2	0	10
34	56	M	Lateral oropharynx	G3	70	50
35	48	M	Tonsil	G3	45	10
36	52	M	Lateral oropharynx, tongue	G3	–	–
37	56	M	Lateral oropharynx	G3	70	50
38	45	M	Tonsil	G2	–	–
39	63	M	Soft palate	G3	30	200
40	46	M	Lateral oropharynx	G3	25	75
41	58	M	Soft palate	–	–	–
42	48	M	Tonsil	G3	60	125
43	74	M	Lateral oropharynx	G3	0[b]	50
44	39	M	Glosso-tonsillar recess	G2	20	0[b]
45	60	M	Tonsil	G2	90[b]	50
46	74	M	Glosso-tonsillar recess	G1	100	150
47	55	M	Glosso-tonsillar recess	G2	40	0[b]
48	78	M	Lateral oropharynx, tongue	G3	35	50
TL						
49	64	M	Supraglottic	G3	160	100
50	52	M	Supraglottic	G3	–	–
51	56	M	Supraglottic	G3	50	75
52	55	M	Supraglottic	G3	–	–
53	62	M	Supraglottic	G3	–	–
54	61	M	Supraglottic	G3	0[b]	50
55	46	M	Supraglottic	G3	25	75
56	51	M	Glottic	G3	–	–
57	58	M	Supraglottic	G3	40	75
58	59	M	Supraglottic	G3	40	200
59	65	M	Supraglottic	G3	0	500
60	60	M	Glottic	G2	80	25

[a] 1 pack-year = 20 cigarettes/day × 1 year
[b] History of prior abuse 7–20 years ago, without current intake

(group TO), 12 patients with SCC of the larynx (group TL) and the oropharynx in 30 nontumor subjects (group NT). Characteristics of the patients are depicted in Table 1. Oropharyngeal mucosa was obtained during tonsillectomy and represented parts of the glossopharyngeal arch. Laryngeal epithelia were taken from the supraglottis during laryngectomy. No other mucosa was removed, so that no additional risk was posed to the patient. Patients were otherwise healthy and had signed an informed consent statement approved by the Ethics Committee of the Medical College, Ludwig Maximilians University of Munich.

Cell cultures

Tissue biopsy specimens were transferred to the laboratory in Joklik-Modified Eagle Medium (without L-glutamine and $NaHCO_3$; Linaris, Bettingen, Germany). To quantify genotoxic impact, the alkaline Comet assay was applied to demonstrate DNA damage [8, 16, 17, 19].

All samples underwent enzymatic digestion with collagenase P, hyaluronidase from bovine testes (Boehringer-Mannheim, Germany) and pronase E type XIV from *Streptomyces griseus* (Sigma, Deisenhofen, Germany) for 45 min in a 37 °C shaking water bath. Using trypan blue staining, viability and cell count were performed and the composition of the mucosal cell population was defined to ensure predominance of epithelial cells [9].

Cell aliquots of 5×10^4 were incubated for 60 min with 354 µmol/ml DBP (Sigma), 354 µmol/ml DiBP (Fluka, Buchs, Switzerland), and 50 µmol/ml NDELA (Sigma). The directly alkylating agent *N'*methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, 0.07 µmol/ml; Fluka, Buchs, Switzerland) was used as the positive control, whereas 166 µmol/ml dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany), the solvent for all substances tested in this study, served as the negative control. The concentrations of all substances used were based on dose-response curves that were determined using either lung epithelia of rodents (NDELA, MNNG: Kuchenmeister et al., German Cancer Research Center, personal communication) or human oropharyngeal mucosa cells (DBP, DiBP).

Comet assay

Special slides were designed for the Comet assay with a frosting of 5 mm along the long edges (76 × 26 mm; Langenbrinck, Emmendingen, Germany), prepared with 85 µl 0.5% normal melting agarosis (Biozym, Hameln, Germany). After the incubation period, the viability of the cells was reexamined using trypan blue staining. After obtaining viabilities of 80%–100%, the remaining aliquots were resuspended with 75 µl 0.7% low melting agarosis (Biozym) and applied to the prepared slides. Alkaline lysis with 10 ml DMSO, 1 ml Triton-X, and 89 ml alkaline lysis buffer followed for 1 h. The slides were then dried and placed into a horizontal gel electrophoresis chamber (Renner, Dannstadt, Germany), positioned close to the anode and covered with alkaline buffer solution containing 10 mM NaOH and 200 mM Na_2EDTA at pH 13.2. After a 20-min DNA "unwinding" period, electrophoresis was started with 25 V and 300 mA for 20 min. Following neutralization (Trizma base, pH 7.5; Merck), cells were stained with ethidium bromide (Sigma). All slides were examined under a DMLB microscope (Leica, Heerbrugg, Switzerland) with an adapted charge-coupled device camera (Cohu, San Diego, Calif., USA).

Depending on the degree of strand-break induction, the DNA fragments demonstrated different types of migration within the electric field, creating so-called comets. The head of the comet contained the remaining unbroken DNA, whereas its tail represented the fragmented DNA after induced strand breaks. The comets were measured using an image analysis system (Komet 3.1; Kinetic Imaging, Liverpool, UK). To quantify the DNA damage induced, we used Olive tail moment (OTM), which is the percentage of DNA in the tail of the comet multiplied by the migration distance [19]. As described previously, undamaged cells were defined as having average tail moments less than 2.0 [8].

Fully frosted slides were used previously to stabilize the agarosis layers embedding the cells. For the present study, slides were designed as being only partially frosted along the edges in order to hold the agarosis layers in solid contact throughout the procedure and improve digital analysis in terms of speed and sensitivity by eliminating background noise to allow for a higher specificity.

Statistical analysis

For statistical analysis, the SPSS 8.0 package was used. Because of very skewed distributions, genotoxic impacts were compared between the NT groups vs. groups TO and NT vs. TL by using the Mann-Whitney *U* test. To compare impacts of a substance (DBP, DiBP, or NDELA) with the DMSO control the Wilcoxon test was used. To control for possible confounding factors such as age (age < 50 years vs. > 50 years), alcohol (daily consumption < 25 g vs. 25–75 g vs. > 75 g), and tobacco consumption (0–5 pack-years vs. 5–50 pack-years vs. > 50 pack-years), the GLIMStat 3.2.3 program was applied in several multiple linear regression analyses comparing nontumor patients with tumor patients (oropharynx and larynx). To achieve a complete case situation for all 60 cases, we substituted missing values by means for the OTMs and by the mean category for alcohol and tobacco. Skewed measures on OTMs were adjusted for with logs of the dependent variables. To compare the best and worst case data situation, analyses were performed with all 60 cases as well as with a smaller data set of 49 cases, in which all cases with any missing values were omitted. The two approaches were compared with respect to possibly confounding covariates.

Results

Comparing the genotoxic impacts of DBP, DiBP, NDELA and MNNG with DMSO controls within groups using the Wilcoxon test showed that all results achieved *P* values of less than 0.001, indicating that DBP, DiBP and NDELA produced significant DNA damage on mucosal cells in all groups of patients (Fig. 1). Rank statistics regarding OTM indicated that only DBP and DiBP produced significant differences between groups TO, TL, TO plus TL, and the NT donors (Table 2). These multiple tests were still significant ($P < 0.003$) after correction with the Bonferroni-Holm method. Corrected results for DMSO, NDELA and MNNG were not significantly different between the groups.

In order to check for possible covariates in all 60 patients (age, tobacco and alcohol consumption), a multiple linear regression analysis was performed. None of these factors proved to have a significant influence on the OTM values measured. As a result the *U* test suffices for testing between the groups.

Although mean age in the NT group was 35 years and that in the TO group 53.5 years and the TL group 56.8 years, age was not a significant factor on OTMs in a multiple linear regression analysis. Gender was not equally distributed in the NT, TO and TL groups, but analysis showed no significant differences ($P_{DMSO} = 0.42$, $P_{DBP} = 0.29$, $P_{DiBP} = 0.35$, $P_{NDELA} = 0.39$, $P_{MNNG} = 0.06$). Furthermore, differences of OTM values in the NT group of smokers vs. nonsmokers were not significant ($P_{DMSO} = 0.77$, $P_{DBP} = 0.29$, $P_{DiBP} = 0.84$, $P_{NDELA} = 0.90$, $P_{MNNG} = 0.87$). Testing the alcohol drinkers (> 2 5 g/d) vs. the nondrinkers (< 25 g/d) in the NT group also failed to show any signif-

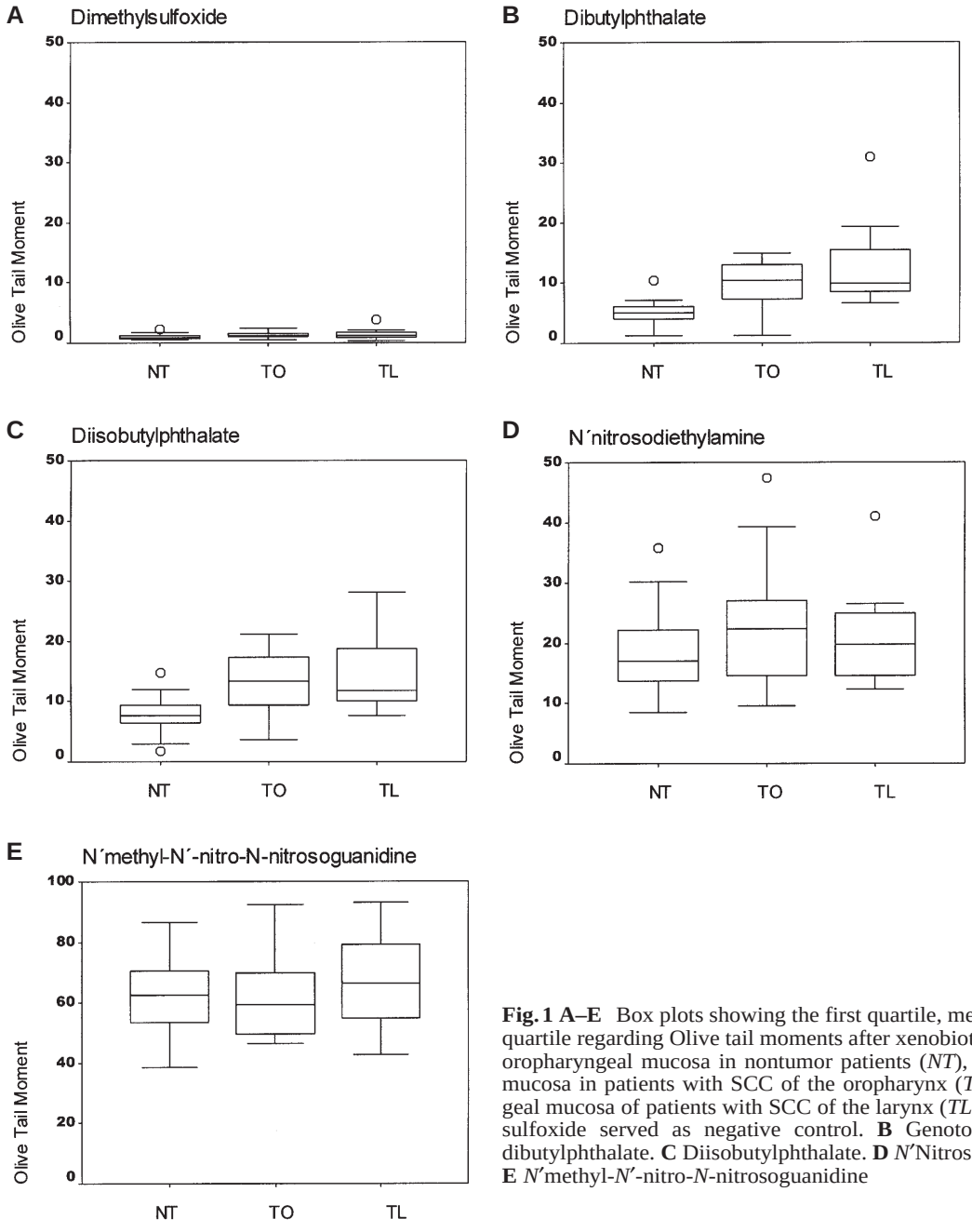

Fig. 1 A–E Box plots showing the first quartile, median, and third quartile regarding Olive tail moments after xenobiotic exposure on oropharyngeal mucosa in nontumor patients (*NT*), oropharyngeal mucosa in patients with SCC of the oropharynx (*TO*), and laryngeal mucosa of patients with SCC of the larynx (*TL*). **A** Dimethylsulfoxide served as negative control. **B** Genotoxic impact of dibutylphthalate. **C** Diisobutylphthalate. **D** *N*′Nitrosodiethylamine. **E** *N*′methyl-*N*′-nitro-*N*-nitrosoguanidine

icant differences except for DiBP (P_{DMSO} = 0.91, P_{DBP} = 0.51, P_{DiBP} = 0.02, P_{NDELA} = 0.80, P_{MNNG} = 0.56).

Discussion

Although mutagen sensitivity is an individual constitutional factor [6], there are differences as to the specific mutagens tested. Whereas some mutagens show their mutagenic capacity in patients with SCC only, as has been shown for benzo(a)pyrene [9], DBP, DiBP and NDELA had a mutagenic impact on tumor and nontumor patients. Nevertheless, differences were found among our groups studied. An increased sensitivity was demonstrated for DBP and DiBP in the tumor groups, and this was even more pronounced for DiBP in patients with laryngeal cancer. This supports findings reported by Cloos et al. [5] describing varying mutagen sensitivities to bleomycin tested on lymphocytes from patients with SCC of the oral cavity or larynx.

Slight differences between the DMSO control groups may have been caused by unspecified technical reasons. Results appeared to be higher in the TO tumor group than in the nontumor control patients, although this difference was not significant. Data of patients with high DNA damage levels (OTM > 2.5) after DMSO treatment (OTM_{DMSO} patient no. 31, 3.6; no. 55, 4.8; no. 57, 3.9), were reevaluated. However, the values for DBP, DiBP, NDELA and

Table 2 Statistical results comparing OTM levels of nontumor patients (*NT*) and patients with SCC of the oropharynx (*TO*) or larynx (*TL*) after xenobiotic exposure using the Comet assay, *Q1* first quartile of OTM levels, *Q3* third quartile of OTM levels

Agent	*n*	Q1	OTM		*P* values of rank tests	
			Median	Q3	Between groups[a]	Corrected[b]
DMSO						0.11
NT	30	0.7	0.9	1.3	–	
TO	18	1.0	1.3	1.6	0.034	
TL	12	0.9	1.2	1.8	0.229	
TO + TL	30	1.0	1.2	1.6	0.035	
DBP						0.003
NT	29	4.0	5.1	6.1	–	
TO	18	7.3	10.4	13.1	0.001	
TL	12	8.6	9.9	15.5	0.001	
TO + TL	30	8.4	10.1	13.7	0.001	
DiBP						0.003
NT	30	6.5	7.6	9.3	–	
TO	18	9.3	13.4	17.3	0.001	
TL	12	10.0	11.8	18.8	0.001	
TO + TL	30	10.0	12.5	17.5	0.001	
NDELA						0.35
NT	28	13.7	17.1	22.2	–	
TO	18	14.6	22.4	27.1	0.118	
TL	10	14.5	19.8	25.0	0.482	
TO + TL	28	14.6	20.1	26.7	0.136	
MNNG						0.99
NT	30	53.4	62.7	70.6	–	
TO	18	49.5	58.5	69.7	0.701	
TL	11	54.3	66.3	80.6	0.375	
TO + TL	29	49.6	62.3	73.8	0.850	

[a] DMSO (control) vs. DBP, DiBP, NDELA and MNNG
[b] The Bonferroni-Holm correction was used since these three tests (per substance) were dependent

MNNG were not higher than in the other patients. Thus, it was not possible to elucidate "hypersensitive patients." An OTM level of 2.5 reflected the experience of several laboratories [8, 18, 19] and our own as a limit for undamaged DNA.

All five multiple linear regression analyses showed that possibly confounding covariates, such as age, alcohol and tobacco had no significant influence in our study groups. Differences in genotoxic impacts in tumor vs. nontumor patients were independent of these factors.

Most environmental toxic chemicals and carcinogens need to be metabolically activated to reveal their toxic or carcinogenic effects. Cytochrome-P450 enzymes (CYP) are hemoproteins used for this purpose to catalyze the biotransformation of various xenobiotics. Individual CYPs have substrate preferences, although their spectra overlap. The metabolic activation or detoxification of all the substances tested in our study depends on the CYP system. CYPs demonstrate genotypic and phenotypic polymorphisms.

Because the individual capability to metabolize toxicants can be altered by the presence of variant alleles or phenotypes, genetic polymorphism and variant phenotypes of CYPs have been proposed as biomarkers describing susceptibility to environmental carcinogens [10]. This may help to explain the varying mutagen sensitivities in the different groups of patients in this study. Other predisposing genetic factors for oropharyngeal and laryngeal cancer may be, for example, a lack or polymorphism of the glutathione S-transferase μ-enzyme or a polymorphism in the alcohol dehydrogenase-3 enzyme, as reported by Coutelle et al. [7] and Kihara's group [14].

Up to now, there has been little information concerning a possible genotoxicity of phthalates tested on human mucosa samples [16]. Nevertheless, our results suggest a possible mechanism of tumor initiation. Furthermore, phthalates are classified as peroxisome proliferators, since they are capable of mediating changes in gene expression and metabolism of xenobiotics. This capability may result in another carcinogenic pathway, but this has been demonstrated only in nonhuman liver cells [21]. Depending on the test systems and concentrations used, phthalates seem to either promote or inhibit carcinogenesis in the rat liver [11, 23]. Blom et al. [3] showed such phthalates as DBP, contributing to tumor proliferation in a human breast cancer cell line, due in part to the phthalates' xenoestrogenic impact [3]. Considering the widespread environmental presence of phthalates, a possible compounding influence on the initiation of head and neck tumors in patients may occur.

NDELA is presently classified as a potential carcinogen by the International Agency for Research on Cancer [12]. Nevertheless, a tumor-initiating factor has been shown for

nitrosamines in animal tests [15]. Little information has been available concerning their genotoxic impact on humans to date. Thus, it is important for us to find DNA damage in oropharyngeal and laryngeal mucosa, being the first organ of contact for nitrosamines in tobacco smoke and foods. Our data failed to show significant differences between tumor and nontumor patients, but this might be due to the higher values of OTMs in all patients compared to the phthalates, which could blur differences caused by genetic determination. However, the results suggest equal genotoxic sensitivities in all groups to this nitrosamine.

MNNG was used as a positive control and induced complete DNA damage on all mucosal cells at a concentration as low as 0.07 μmol/ml. By directly alkylating cellular DNA, this was the substance with the largest genotoxic potential in our test setting. NDELA and MNNG showed only a slight or no difference in mutagen sensitivity between tumor and nontumor patients. Considering the high genotoxic potential of *N*′nitroso-compounds demonstrated in some previous studies (e.g., [20]), detection of endogenic-based variations might be more difficult.

Individual exposure to genotoxic substances tested in our study, as well as intrinsic susceptibilities can act in concert to modulate cancer risk. The intrinsic susceptibilities may also be influenced by varying degrees of DNA maintenance capabilities among our patient groups [6]. The interindividual differences within the groups may not only be based on previous environmental effects on the patients, but could also be due to molecular changes in seemingly macroscopically healthy mucosa in the tumor patients. These findings would be compatible with the concept of "field cancerization," which describes carcinogen-induced changes throughout the upper aerodigestive tract mucosa of head and neck cancer patients [22]. For this reason the effects of early molecular stages of tumor progression on the extent of DNA damage must be considered. Such transforming factors are, e.g., inactivation of the p53 gene or specific alterations of proto-oncogenes and tumor suppressor genes [4].

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