Free Radical Research, 2003 Vol. 37 (3), pp. 231–240

Oxidative DNA Base Modifications and Polycyclic Aromatic Hydrocarbon DNA Adducts in Squamous Cell Carcinoma of Larynx

PAWEŁ JAŁOSZYŃSKI[a,*], PAWEŁ JARUGA[b], RYSZARD OLIŃSKI[b], WIESŁAWA BICZYSKO[c], WITOLD SZYFTER[d], ESZTER NAGY[e], LENNART MÖLLER[e] and KRZYSZTOF SZYFTER[a,d]

[a]*Polish Academy of Sciences, Institute of Human Genetics, Strzeszyńska 32, 60-479 Poznań, Poland;* [b]*Department of Clinical Biochemistry, L. Rydygier Medical University, Bydgoszcz, Poland;* [c]*Department of Clinical Pathology, K. Marcinkowski University of Medical Sciences, Poznań, Poland;* [d]*Department of Otolaryngology, K. Marcinkowski University of Medical Sciences, Poznań, Poland;* [e]*Department of Biosciences, Karolinska Institute, Center for Nutrition and Toxicology, NOVUM Research Park, Stockholm, Sweden*

Accepted by Professor M. Dizdaroglu

(Received 30 October 2001; In revised form 28 August 2002)

Tobacco smoke, recognized as a major etiological factor for cancers of the upper aerodigestive tract, represents an abundant source of reactive oxygen species (ROS), which are believed to play a significant role in mutagenesis and carcinogenesis. An additional source of ROS in tissues exposed to tobacco smoke may be metabolic oxidation of polycyclic aromatic hydrocarbons (PAH). To investigate the relationships between oxidative DNA lesions and aromatic DNA adducts, six modified DNA bases 5-hydroxyuracil, 5-hydroxycytosine, 7,8-dihydro-8-oxoguanine, 7,8-dihydro-8-oxoadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine and the total level of PAH-related DNA adducts were measured in cancerous and the surrounding normal larynx tissues (68 subjects), using gas chromatography/isotope-dilution mass spectroscopy with selected ion monitoring and the ^{32}P-postlabeling-HPLC assay, respectively. The levels of oxidative DNA lesions in cancerous and adjacent tissue were comparable; the differences between the two types of tissue were significant only for 5-hydroxypyrimidines (slightly higher levels were observed in the adjacent tissue). Comparable levels of DNA lesions in cancerous and the surrounding normal tissues observed in the larynx tumors support a field cancerization theory. The surrounding tissues may still be recognized as normal by histological criteria. However, molecular alterations resulting from the chronic tobacco smoke exposure, which equally affects larynx epithelia, may lead to multiple premalignant lesions. Thus, a demonstration of similar levels of DNA damage in cancerous and the adjacent tissue could explain a frequent formation of secondary tumors in the larynx and the frequent recurrence in this type of cancer. A weak, but distinct effect of tumor grading and metastatic status was observed in both kinds of tissue in the case of 5-hydroxyuracil, 5-hydroxycytosine, 7,8-dihydro-8-oxoguanine, 7,8-dihydro-8-oxoadenine. This effect was displayed as a gradual shift in the data distribution toward high values from G1 through G2–G3 and from non-metastatic to metastatic tumors. Since the levels of oxidative DNA base modifications tended to increase with the tumor aggressiveness, we postulate that the oxidative DNA lesions increase genetic instability and thus contribute to tumor progression in laryngeal cancer. No associations between aromatic adduct levels and oxidative DNA lesions were present, suggesting that the metabolism of PAH does not contribute significantly to the oxidative stress in larynx tissues, remaining the tobacco smoke ROS as a major source of oxidative DNA damage in the exposed tissue.

Keywords: Larynx cancer; Tobacco smoke; Oxidative DNA damage; Polycyclic aromatic hydrocarbons

Keywords: B(a)P, benzo(a)pyrene; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; FapyAde, 4,6-diamino-5-formamidopyrimidine; GC/MS, gas chromatography/mass spectrometry; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-Ura, 5-hydroxyuracil; 8-oxoAde, 7,8-dihydro-8-oxoadenine; 8-oxodGuo, 7,8-dihydro-8-oxo-2′-deoxyguanosine; 8-oxoGua, 7,8-dihydro-8-oxoguanine; PAH, polycyclic aromatic hydrocarbons; ^{32}P-HPLC, ^{32}P-postlabeling assay with high-performance liquid chromatography; ROS, reactive oxygen species; TNM classification, T—the extent of the primary tumor, N—condition of the regional lymph nodes, M—the absence or presence of distant metastases

*Corresponding author. Tel.: +4861233011. Fax: +4861233235. E-mail: japawel@man.poznan.pl

ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576021000041014

INTRODUCTION

There is a considerable body of evidence demonstrating the role of reactive oxygen species (ROS) in the etiology of a number of human diseases including cancer (reviewed by Floyd[1]). An increase in the amount of the oxidative DNA lesions has been demonstrated in DNA exposed to ROS-generating systems *in vitro*.[2,3] Oxidatively modified DNA bases have also been found in the DNA of animal and human tissues under condition usually referred to as oxidative stress.[4–8] 7,8-Dihydro-8-oxoguanine (8-oxoGua), 2-hydroxyadenine, 7,8-dihydro-8-oxoadenine (8-oxoAde) and 5-hydroxycytosine (5-OH-Cyt) possess mispairing properties and were recognized as potent mutagens (reviewed by Oliński *et al*.[9]). In recent years a thorough qualitative and quantitative characterization of the oxidative DNA base modifications in cancerous tissues attracted particular interest since some of them may play a significant role in various stages of carcinogenesis. It has been shown that systems generating ROS can induce malignant transformation in a way comparable to potent tumor promoters.[10] Additional evidence for the role of ROS in cancer promotion and progression is a correlation between chronic inflammation, a condition associated with a local production of ROS, and increased risk of gastric, colonic, pancreatic and hepatic cancer (reviewed by Jackson and Loeb[11]). Also progression of breast cancer to the metastatic state seems to be linked to oxidative DNA damage.[12] In the case of tobacco smoke-related larynx cancer, generally the entire organ is exposed to genotoxic agents both before and during tumor development up to the late stages of cancer. Besides the early effects of ROS in tumor initiation and promotion, the genotoxic properties of tobacco smoke might contribute to the observed genomic instability of larynx cancer,[13] and thus may play a role in tumor progression and metastasis formation.

Tobacco smoke constitutes a complex mixture of chemicals including a number of genotoxic agents. Polycyclic aromatic hydrocarbons (PAH) of tobacco smoke have been studied in detail in terms of *in vitro* and *in vivo* metabolism, stereochemistry of the most reactive metabolites, prominent and minor products of reaction with DNA components, as well as chromatin region and DNA sequence preferences for DNA adduct formation[14–17] and mutagenic activities.[18,19] The presence of PAH-related DNA adducts in the tissues of tobacco smoke-associated tumors has been reported; the existing evidence indicates a significant role of this type of DNA lesions in tobacco smoke-induced mutagenesis and carcinogenesis.[20] B(a)B-DNA adducts have also been shown to be located at the mutagenic hotspots along the p53 gene, thus providing another explanation for cancerization by cigarette smoke.[21]

The oxidative properties of tobacco smoke have also been reported and thoroughly studied.[22] An elevated level of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodGuo) has been found in animal and human tissues, including the lung[4] and peripheral leukocytes[23,24] of smokers. Interestingly, the mutation spectra of PAH-related DNA adducts and some oxidative lesions are similar. Particularly, G → T transversion seems to be common for the two groups of mutagens.[18,19,25,26] Another argument for a need of investigations of mutual effects of PAH and ROS emerged from a recognition of the pathways involved in PAH metabolism. A model compound of this group, B(a)P, is oxidized in cytochrome P450-dependent reactions to form highly reactive diol-epoxide derivatives, which readily react with DNA bases.[27] It was demonstrated that significant amounts of reactive oxygen species are produced during this process,[28] which was also observed in exposed tissues. B(a)P-derived DNA lesions included strand breaks[29,30] and modified nucleosides: thymidine glycol[28,31] and 8-oxodGuo.[32,33] Appreciable activities of PAH-metabolizing enzymes (cytochromes CYP1A1 and CYP2C) were found in laryngeal mucosa microsomes,[34,35] in the tissue, which is topically exposed to tobacco smoke-derived PAH. Altogether, the above data strongly suggest a possible mutual effect of PAH-related DNA adducts and ROS-induced oxidative DNA base modifications in the development (initiation, promotion and progression) of the tobacco smoke-derived cancer.

Tobacco smoke has been proved to play a significant role in the etiology of the cancers of head and neck, with a particular impact on lung and larynx carcinogenesis.[20,36–38] The investigations on genetic and environmental factors that might be involved in larynx cancer biology contributed to our knowledge on several xenobiotic-metabolizing enzyme genotypes, loss of heterozygosity in particular gene *loci*, mutations in tumor suppressor genes, cytogenetic changes and chromosomal instability, and also provided well documented data on aromatic and alkylated DNA adducts possibly involved in the process of laryngeal carcinogenesis.[39] The oxidatively induced DNA base modifications in larynx tumors have not been investigated so far. The present study was undertaken to provide an insight into the ROS-related DNA lesion patterns and their levels in cancerous and the surrounding normal larynx tissues. Gas chromatography/mass spectrometry (GC/MS) was employed to measure the levels of 5-hydroxyuracil (5-OH-Ura), 5-OH-Cyt, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-oxoGua and 8-oxoAde in tumor and parallel adjacent tissue samples. PAH-related DNA adducts were quantified by means of ^{32}P-HPLC technique in the same tissue

samples to address the question whether the metabolism of PAH may be a significant source of ROS in larynx tissues. Larynx tumors of different grades of aggressiveness (including metastatic status) were analyzed to test a hypothesis assuming the accumulation of oxidative DNA lesions during the tumor development.

MATERIALS AND METHODS

Tissue samples were obtained at the Department of Otolaryngology at K. Marcinkowski University of Medical Sciences in Poznań, Poland, from 68 patients undergoing total or partial laryngectomy without being previously treated with chemo- or radiotherapy. Parallel tumor and normal adjacent tissue samples were collected from 44 patients, while from the remaining 24 only the adjacent tissues were available. The individual clinical data concerning patients' age (mean 60.3 ± 9.9, range of 36–85 years), gender, smoking habits and information on any other occupational or environmental exposure (when accessible) were supplemented with tumor-associated parameters: tumor stages (according to TNM classification, TNM System: T—the extent of the primary tumor; N—condition of the regional lymph nodes; M—the absence or presence of distant metastases) and tumor grading (according to G1–3 histopathologic characteristics). Individual smoking habits were described according to the following arbitrary classification: low <20, moderate 20–25 and heavy >25 cigarettes/day, ex-smokers, with cessation at least 1 year before surgery. The basic study group characteristics are compiled in Table I. A representative part of each tissue sample was subjected to a histopathological examination so as to exclude unsuitable specimens. The criteria for negative selection were: the presence of a recognizable inflammation process, the presence of large necrotic areas, the presence of tumor cells in adjacent tissue and less then 50% of cancer cells in the tumor sample. Before any further processing, the samples were stored at −80°C.

TABLE I The study group characteristics

Total number	68
Males	64
Females	4
Smoking status	
Non-smokers	0
Light	5
Moderate	31
Heavy	14
Ex-smokers	7
ND*	11
Tumor stages	
T1	1
T2	8
T3	41
T4	12
ND	6
Tumor grading	
G1	15
G2	32
G3	11
ND	10
Adjacent lymph node metastases	
N − †	40
N + ‡	22
ND	6

* Not determined. † Tumor stages $T_xN_0M_x$. ‡ Tumor stages $T_xN_{1-2}M_x$.

DNA Extraction

Tissue samples were homogenized on ice in digestion buffer containing 100 mM NaCl (POCh, Poland), 10 mM Tris–HCl (Sigma–Aldrich, Germany), 25 mM Na_2EDTA (POCh, Poland) and 0.5% sodium dodecyl sulfate (Sigma–Aldrich, Germany), pH 8 (ca. 1 ml/100 mg of tissue). The homogenates were then incubated overnight with 100 μg/ml of proteinase K (Sigma–Aldrich, Germany) at 37°C, under argon. Proteins were removed with chloroform/isoamyl alcohol (24:1, Merck, Germany and POCh, Poland, respectively); the extraction was repeated five times. DNA was precipitated with absolute ethanol (Romil, UK), washed with 70% ethanol and lyophilized. For quantity and purity estimation DNA was dissolved in distilled water and after spectrophotometric measurements DNA aliquots were lyophilized and stored at −20°C.

Analysis of Modified DNA Base by Gas Chromatography/Mass Spectrometry

From each DNA sample two aliquots of 50 μg were analyzed independently. The analysis by GC/MS was performed according to the previously described method;[40] the most important modification concerned trimethylsilylation conditions, as described below. Briefly, DNA was hydrolyzed under nitrogen with 60% formic acid (Sigma–Aldrich, Germany) at 140°C for 30 min. The hydrolyzed samples were lyophilized and trimethylsilylated with *N,O*-bis-(trimethylsilyl)trifluoroacetamide (Pierce, USA) for 180 min at room temperature, under nitrogen. As internal standards, ^{15}N- and ^{13}C-labeled analogues of the analyzed DNA bases were used. The volatile derivatives were analyzed with GC/MS using the selected-ion monitoring mode (gas chromatograph HP5870, mass spectrometer HP5972, Hewlett-Packard, USA).

P-HPLC Analysis of PAH-related DNA Adducts

The ^{32}P-postlabeling assay was performed as described previously.[41,42] Briefly, 10-μg aliquots of DNA were hydrolyzed with 800 mU of micrococcal

nuclease (Sigma, USA) and 16 mU of calf spleen phosphodiesterase II (Boehringer Mannheim, Germany). DNA adduct enrichment was achieved by the butanol extraction method. The vacuum-dried butanol extracts were labeled with 17.5 μCi [γ^{32}P] ATP (Amersham, USA) using 5 units of T4 polynucleotide kinase (USB, USA). The ^{32}P-HPLC analysis was performed by injecting the labeled mixture into the HPLC system, eluting with 0.5 ml/min of 2 M ammonium formate (Merck, Germany), 0.4 M formic acid (Merck, Germany) (pH 4.5) and a linear gradient of 0–35% acetonitrile (Merck, Germany) (0–70 min). The equipment used was Waters 600E System Controller, (Waters, UK) with a NewGuard RP18 precolumn and DELTA PAK $150 \times 3.9\,mm^2$ ID, C18, 5 μ, (25 + 28) columns (both from Waters, UK). The radioactive label was measured with an on-line flow scintillation counting method (FLO-ONE Beta A-200 Radioactive Flow Detector with a FLO-ONE\DATA II software, Radiomatic Instruments and Chemical Co. Inc., A Canberra Company, USA).

Data Analysis

Because of the nonnormality of most data distributions, median values and quartiles are presented rather than means and standard deviations. In order to achieve a better insight into an individual data distribution shape, additionally 10th and 90th percentile as well as outlier values were plotted in presented graphs. For the same reason a nonparametric statistical analysis was applied. The Wilcoxon test was used to calculate the statistical significance of differences in total level of DNA modifications between paired tumor and adjacent tissue data and the Mann–Whitney test was applied additionally to compare total tumor vs. total adjacent tissue data. The Mann–Whitney test was used to analyze the differences between metastatic (N+) and nonmetastatic (N−) groups and between two arbitrary chosen age groups: <60 years old and $=60$ years. The Kruskall–Wallis test was applied to evaluate the effect of tumor grading (groups G1, G2, G3). The associations between the variables under study were assessed using the Spearman correlation coefficient.

RESULTS

Oxidative DNA Base Modifications

Six DNA base modifications were chosen as markers of DNA oxidation: two pyrimidine derivatives (5-OH-Cyt, 5-OH-Ura) and four purine derivatives (8-oxoGua, 8-oxoAde, FapyGua and FapyAde). The results show high inter-individual differences in both tumor and adjacent tissue; moreover, the differences between the tissues seem to be shaped individually. The statistical analysis revealed no significant differences between tumor and adjacent tissue for 8-oxopurines and fapypyrimidines (Fig. 1), with median values for tumor and adjacent tissue, respectively: 8-oxoAde 14.8 vs. 14.2; 8-oxoGua 19.5 vs. 20.5; FapyAde 36.8 vs. 36.1 and FapyGua 54.3 vs. 51.2. Significant, but not striking differences were detected for 5-hydroxypyrimidines. Adjacent tissue displayed higher median values of modified bases and a remarkable shift of the data distribution toward high values (5-OH-Ura 2.0 vs. 2.3; 5-OH-Cyt 15.1 vs. 18.8 for tumor and adjacent tissue, respectively; both differences were significant at $p \leq 0.01$).

The relationship between the tumor grading and the levels of the DNA lesions is shown in Fig. 2. Gradual shifts of the data distribution toward high values from G1 through G2–G3 were observed for 5-OH-Ura, 5-OH-Cyt, 8-oxoAde and 8-oxoGua, both in the tumor and adjacent tissue. This tendency was confirmed by statistical analysis only in two cases representing adjacent tissue (5-OH-Ura, statistically significant increase at $p \leq 0.04$ and 8-oxoAde at $p \leq 0.04$). In the case of FapyAde and FapyGua neither significant differences between median values nor any trend were observed (data not shown).

Data divided according to the presence (N−) or absence (N+) of metastases in adjacent lymph nodes are plotted in Fig. 3. The subgroup N− represents clinical cases $T_nN_0M_n$, the subgroup N+ consists of combined cases $T_nN_1M_n$ and $T_nN_2M_n$. Shifts in data distribution towards high values were observed in a manner similar to the tendency found in tumor grading. This effect, although weak, was distinctly expressed for 5-OH-Ura, 5-OH-Cyt, 8-oxoAde and 8-oxoGua in the tumor tissue and only for 8-oxoAde and 8-oxoGua in the adjacent tissue. The shift was caused mainly by "tailing" of the data population beyond 75th percentile toward extreme values; the differences between median values were not significant. No differences between N− and N+ tumors were found in the case of FapyAde and FapyGua.

Several statistically significant correlations were found between the levels of oxidative DNA base modifications, both within one type of tissue, and between tumor and adjacent tissue as well. The correlating variables and calculated correlation coefficients are compiled in Table II. All correlations but one were positive; the one exception represented an association between 8-oxoGua in tumor and FapyGua in adjacent tissue ($R = -0.40$ at $p \leq 0.007$). When the presence of the remaining 15 positive correlations is concerned, this single negative value may be regarded as an artifact. Strong correlations were found between 5-OH-Cyt and 8-oxoGua in the tumor and between 5-OH-Cyt and 8-oxoGua in

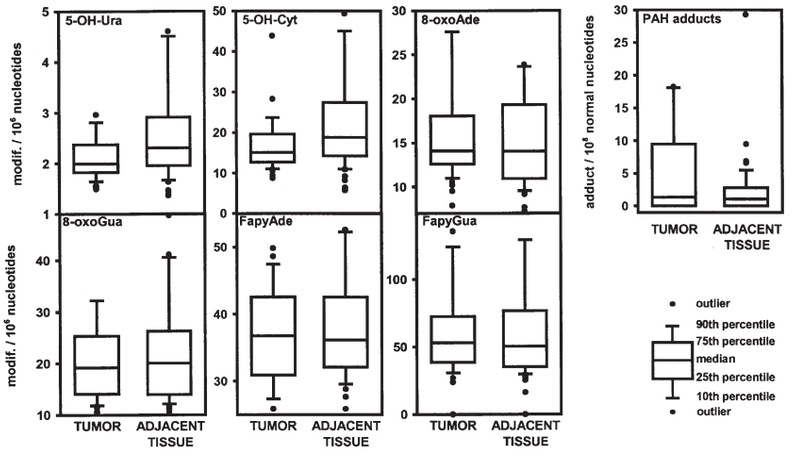

FIGURE 1 Oxidative DNA base modifications and PAH-related DNA adducts in tumor and adjacent tissues of larynx.

the adjacent tissue ($R = 0.73$ and 0.68 at $p \leq 1.1\text{E} - 8$ and 6.8E-10, respectively). Despite such a close relationship between 5-OH-Cyt and 8-oxoGua separately in the tumor and adjacent tissue, no correlation between the two different tissues was found. An association of levels of oxidatively induced DNA modifications between tumor and adjacent tissue was found only for 5-OH-Ura, 8-oxoAde and 8-oxoGua. Formamidopyrimidines participated in three relations only; the aforementioned negative correlation must be excluded, however.

Since larynx cancer is confined mostly to elderly individuals and the subjects were matched to constitute a rather consistent group so as to avoid multiplication of confounders, the assessment of age effects was undertaken only as a complementary analysis. Neither Spearman correlation coefficients analysis nor comparison between arbitrary selected groups (<60 years old) showed any significant associations between age and the level of modified DNA bases.

A similar situation was true for smoking habits—as shown in Table I; a considerable majority of the study group constituted smokers who declared to smoke 20 cigarettes per day or more. The arbitrary chosen categories were not sharply bordered with the exception of a very small group of ex-smokers. Moreover, the lack of any non-smokers in the study group made it impossible to divide the subjects into reasonable subgroups, and thus a thorough assessment of the effect of the smoking habit was not

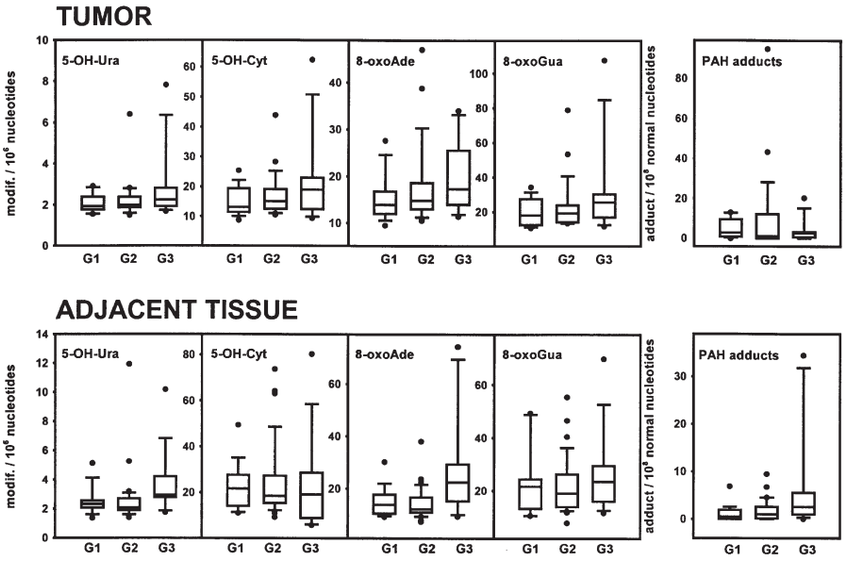

FIGURE 2 Tumor grading (G1–G3) and DNA lesions in tumor and adjacent tissues of larynx. Median values, 10th, 25th, 75th, 90th percentiles and outliers are shown (legend as in Fig. 1).

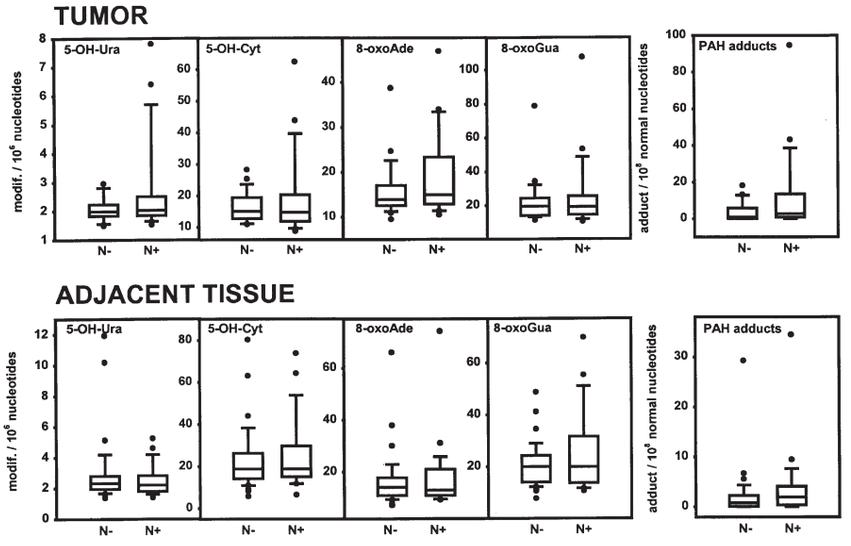

FIGURE 3 Tumor metastatic status and DNA lesions in tumor and adjacent tissues of larynx. Median values, 10th, 25th, 75th, 90th percentiles and outliers are shown (legend as in Fig. 1); "N − ", non-metastatic larynx tumors; "N + ", metastatic larynx tumors.

possible. Nonetheless, this confounder was taken into account when extremely high or outlier values were examined, however, no particular tendencies were found (data not shown). The same general remarks refer also to the PAH-related DNA adducts analysis presented below.

PAH-related DNA Adducts

The total number of aromatic DNA adducts was measured. Similar DNA adduct patterns were obtained for all subjects, with remarkable two peaks representing main guanine and adenine adducts (data not shown). The method allowed to detect a single adduct per 10^8 normal nucleotides, however, in the cases where clearly distinguishable peaks at proper retention times were visible, lower values were accepted.

Statistical analysis showed no significant difference in total DNA adduct levels between tumor and adjacent tissue (Fig. 1). As shown in Fig. 2, an increase in the DNA lesion levels from G1 to G3 in the adjacent tissue was found; the tendency was close to significant ($p \leq 0.07$). The effect of metastasis status was also confined to a non-significant shift in the data distribution; a weak increase from N− to N+ was observed for both kinds of tissues (Fig. 3).

The PAH-related DNA adduct levels were not significantly correlated with age, however, for the group of elder subjects (= 60 year old) a distinct "tailing" of data distribution was observed, with maximal value 34.5, whereas in <60 group the maximal value was only 6.9 DNA adducts/10^8 normal nucleotides.

The aromatic DNA adduct levels in tumor and adjacent tissue correlated at $R = 0.40$ and $p \leq 0.007$.

TABLE II Correlations between analyzed variables (only statistically significant Spearman correlation coefficients *R* are shown)

Variables			N	R	*p* level
5-OH-Ura, tumor	vs.	8-oxoAde, tumor	45	0.43	0.003
5-OH-Ura, tumor	vs.	5-OH-Ura, adj. tissue	43	0.48	0.001
5-OH-Ura, tumor	vs.	8-oxoAde, adj. tissue	43	0.31	0.038
5-OH-Cyt, tumor	vs.	FapyAde, tumor	45	0.41	0.005
5-OH-Cyt, tumor	vs.	8-oxoGua, tumor	45	0.73	<0.001
8-oxoAde, tumor	vs.	8-oxoGua, tumor	45	0.47	0.001
8-oxoAde, tumor	vs.	5-OH-Ura, adj. tissue	43	0.44	0.003
8-oxoAde, tumor	vs.	8-oxoAde, adj. tissue	43	0.51	<0.001
8-oxoGua, tumor	vs.	FapyGua, adj. tissue	43	-0.40	0.007
8-oxoGua, tumor	vs.	8-oxoGua, adj. tissue	43	0.49	<0.001
5-OH-Ura, adj. tissue	vs.	5-OH-Cyt, adj. tissue	63	0.45	<0.001
5-OH-Ura, adj. tissue	vs.	8-oxoAde, adj. tissue	63	0.53	<0.001
5-OH-Cyt, adj. tissue	vs.	8-oxoGua, adj. tissue	63	0.68	<0.001
FapyAde, adj. tissue	vs.	8-oxoGua, adj. tissue	63	0.40	0.005
8-oxoAde, adj. tissue	vs.	8-oxoGua, adj. tissue	63	0.58	<0.001
PAH adducts, tumor	vs.	PAH adducts, adj. tissue	44	0.40	0.007

No correlation was detected between the PAH-related adducts and any of the oxidative DNA base modifications.

DISCUSSION

Polycyclic aromatic hydrocarbons possess well recognized mutagenic and procarcinogenic properties. The model compound of this group, benzo(a)-pyrene, is known to form highly reactive epoxy-diol metabolites which bind to DNA bases. The predominant mutation, which is believed to be associated with the exposure to PAH, is a G → T transversion.[18,19] Interestingly, the same G → T transversion was demonstrated as a mutation typical for 8-oxoGua.[25,26] Mutation spectra found by several authors in tobacco smoke-associated cancers of lung and larynx include this particular alteration. Field *et al.*[43] reviewed p53 mutations in head and neck cancers and concluded that G → T transversions and G → A transitions constituted the most common alterations located at p53 hotspot region covering codons 238–248. The study of genetic alterations in this particular p53 region in larynx cancer revealed that the above-mentioned smoking-related mutations accounted for 46% of all changes.[44] The latter results were interpreted mostly in terms of the role of PAH in the formation of the observed mutations. However, the significant role of oxidatively induced DNA base modifications should also be accounted for.

The results of the analysis of PAH-related DNA adducts obtained in the present study were in agreement with former findings published elsewhere,[45,46] however, some already known associations were found to be weaker than literature-based expectations. In our opinion, the small number of subject employed in the study may be responsible for such an effect. Accordingly, our analyses and interpretations were focused mostly on the new data concerning the oxidative DNA base modifications in larynx tissues, and the PAH-related DNA adduct analysis was accomplished as a link between the well established and newly obtained knowledge.

Having analyzed the total of 68 larynx cancer patients (44 pairs: tumor-adjacent tissue, and additional 24 samples of adjacent tissue), we found no significant differences between cancerous and normal adjacent tissue in the case of 8-oxoGua, 8-oxoAde, FapyGua and FapyAde. The levels of 5-OH-Ura and 5-OH-Cyt were significantly higher in the adjacent tissue. The literature concerning similar analyses is rather scarce; the existing data concern tumors of breast, colon, stomach, ovary, brain, kidney, lung and liver, and in many cases such studies were limited to few subjects. Distinctly higher levels of the modified DNA bases were found in the tumor tissues in the case of cancers of colon, ovary, brain and kidney.[40,47,48] The data concerning lung, breast and stomach cancers are ambiguous; some authors found higher levels of the oxidative DNA base modifications (mainly 8-oxoGua) in tumor tissue;[40,49] recent data indicate the lack of differences between tumor and adjacent tissue.[4,50,51] The data concerning hepatocellular carcinoma show no difference between normal liver tissue and tumors.[52] The apparent discrepancies in the available tumor vs. adjacent tissue comparative data concerning the levels of modified DNA bases, cannot be easily explained in terms of general conclusions reducing this phenomenon to a single, basic mechanism. The results obtained in the present study comprise a relatively large study group (as compared to the existing literature data), and seem to support the opinion that differences between normal and cancerous tissues are shaped in an individual manner, and depend strongly on complex interactions between numerous factors taking their effect on observed levels of DNA modifications in each individual case. On the other hand, the comparable levels of DNA lesions in cancerous and adjacent tissue in some types of tumors strongly support a "field cancerization" theory (its application to the head and neck tumorigenesis has been thoroughly reviewed by Papadimitrakopoulou *et al.*[53]). In the case of larynx cancer, the surrounding tissue may still be recognized as a normal one by histological criteria, but molecular alterations resulted from the tobacco smoke exposure may lead to multiple premalignant lesions. Thus, a demonstration of extensive DNA damage in the adjacent tissue could explain a frequent recurrence in head and neck cancer that is the main reason of a low efficiency of a medical treatment. A practical income of such finding could meet surgeons' claims of an extension of operation area.

The presumed significant sources of ROS in the case of larynx cancer may be connected with: (i) tobacco smoke-associated oxidants, (ii) PAH metabolism-related generation of ROS, (iii) endogenous sources (by-products of an extensive aerobic metabolism of growing tumor). Tobacco smoke contains some 10^{17} oxidant molecules per single puff.[54] The larynx epithelium of smokers is exposed directly to both tobacco smoke ROS and PAH, and thus the formation of oxidative DNA lesions in larynx tissues may be significant. It happens unless the low molecular weight antioxidants, antioxidant enzymes and DNA repair systems are capable of protecting cells against detrimental effects of such an exposure. Larynx tumors belong to late-diagnosed cancers and are strongly associated with heavy tobacco smoking, which means that in the prevailing majority of cases both normal epithelium and tumor cells are

continuously exposed to genotoxic agents for a relatively long time. Besides of early effects of ROS in tumor initiation and promotion, the continuous and equal exposure of the whole larynx might contribute to the instability observed in cancer genome, and thus play a certain role in the tumor progression and metastasis formation. The same process might be responsible for the lack of differences in the levels of modified DNA bases between normal and cancerous tissues.

Another possible source of ROS in larynx tissues exposed to tobacco smoke may be a metabolic pathway responsible for PAH activation. The pathway consists of several oxidation steps and is carried out by CYP450-dependent enzymes. Several authors reported the formation of oxidative-type DNA lesions in cells and tissues exposed to B(a)P.[28,30,32] B(a)P-induced oxidative DNA damage was proposed as a possible mechanism for tumor promotion by this complete carcinogen.[31] There is also evidence showing a significant effect of tobacco smoke on an increase of 8-oxodGuo in DNA of cells exposed to tobacco smoke PAH *in vitro* or in the tissues of smokers.[23,24,55,56] Altogether, the literature data strongly suggest that PAH metabolism-related oxygen species might contribute to an overall oxidative stress in target tissues. Larynx tissues are known to possess potent capabilities of metabolizing PAH, expressed as a considerable activity of CYP1A1 and CYP2C.[34,35] In our studies, the lack of any correlation between oxidative DNA lesions and the level of PAH-related DNA adducts suggests that PAH metabolism-associated ROS do not contribute significantly to an overall level of oxidative DNA lesions neither in tumor, nor in the surrounding normal larynx tissue. Taking into account that endogenous ROS generated during an extensive aerobic metabolism should contribute to oxidative lesion levels in proliferating tumor cells rather than in normal, adjacent tissue, we suggest that tobacco smoke-related ROS play the most important role in the formation of oxidative base modifications in larynx tissue, both normal and cancerous. Thus, endogenous effects, if present, are probably masked by a massive external exposure. However, little is known about the differences between the two types of tissue in terms of an efficiency of antioxidant protection systems or oxidative DNA damage repair. Hence, further extensive studies are required to establish interactions between all factors that modulate the levels of the modified DNA bases in larynx.

Analyzing the associations between the studied parameters, we found no effect of age on the oxidative DNA lesions. However, larynx cancer is confined mostly to elder individuals, and the study group consisted of subjects between 36 and 85 years of age (average at about 60). The lack of significant age-related effects was also found for aromatic DNA adducts, even though an extremely high maximal value was found in the group of elder subjects (= 60 year old). We conclude that the number of subjects employed in the present study might have been still too scarce contributing this way to such a feeble effect. Accordingly, a larger group is required to examine any age-related effects on DNA base modifications.

Little is known about the effects of tumor staging or grading on the levels of oxidative DNA lesions. Malins *et al.*[12] analyzed FapyAde/8-oxoAde ratios in human breast cancer and found a near 2-fold increase in the oxidative damage in metastatic tumor DNA as compared to nonmetastatic tumors. The authors concluded that the progression of breast cancer to the metastatic state was significantly linked to the oxidative DNA damage. In our study, we found weak relationships between the level of modified pyrimidines and 8-oxopurines and tumor grading as well as the metastatic status. All the tendencies taken separately might represent random effects, but similar regularities in the data distribution patterns for 5-OH-Ura, 5-OH-Cyt, 8-oxoAde and 8-oxoGua suggest a true tendency. However, all gradual data distribution shifts toward high values from G1 through G2–G3 and from nonmetastatic to metastatic tumors were rather feebly expressed and statistically significant only for 5-OH-Ura and 8-oxoAde in the adjacent tissue. We postulate that a larger study group must be analyzed to detect significant relationships, and the tendencies found in our results should be confirmed in the case of other types of cancer. A discrepancy between the results obtained for the breast and larynx cancer may also reflect the tissue specificity. Recently, clear evidence has been provided, demonstrating a significant accumulation of chromosomal changes in metastatic tumors as compared to nonmetastatic tumors in larynx cancer subjects.[13] Therefore, oxidative DNA base modifications accumulated in advanced tumors may contribute to tumor progression and formation of metastases by increasing the genetic instability of cancerous cells. The detection of the massive oxidative DNA damage in tumor tissue may indicate increased cancer aggressiveness, and thus it may have a significant value for the therapeutic strategy.

Acknowledgements

The senior author is grateful to Magnus Zeisig and Lotta Jonsson for their invaluable help in solving methodological problems. This work was supported by Grant No. 4 P05A 058 16 from the Polish State Committee of Scientific Research and by Grant No. 131/1999 from the UICC International Cancer Technology Transfer Fellowship (ICRETT).

References

[1] Floyd, R.A. (1990) "Role of oxygen free radicals in carcinogenesis and brain ischemia", *FASEB J.* **4**, 2587–2597.

[2] Kennedy, L.J., Moore, K., Caulfield, J.L., Tannenbaum, S.R. and Dedon, P.C. (1997) "Quantitation of 8-oxoguanine and strand breaks produced by four oxidizing agents", *Chem. Res. Toxicol.* **10**, 386–392.

[3] Douki, T., Martini, R., Ravanat, J.-L., Turesky, R.J. and Cadet, J. (1997) "Measurement of 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-oxo-7,8-dihydroguanine in isolated DNA exposed to gamma radiation in aqueous solution", *Carcinogenesis* **18**, 2385–2391.

[4] Asami, S., Manabe, H., Miyake, J., Tsurudome, Y., Hirano, T., Yamaguchi, R., Itoh, H. and Kasai, H. (1997) "Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung", *Carcinogenesis* **18**, 1763–1766.

[5] Fiala, E.S., Conaway, C.C. and Mathis, J.E. (1989) "Oxidative DNA and RNA damage in the livers of Sprague–Dawley rats treated with the hepatocarcinogen 2-nitropropane", *Cancer Res.* **49**, 5518–5522.

[6] Abalea, V., Cillard, J., Dubos, M.P., Anger, J.P., Cillard, P. and Morel, I. (1998) "Iron-induced oxidative DNA damage and its repair in primary rat hepatocyte culture", *Carcinogenesis* **19**, 1053–1059.

[7] Bessho, T., Tano, K., Nishimura, S. and Kasai, H. (1993) "Induction of mutations in mouse FM3A cells by treatment with riboflavin plus visible light and its possible relation with formation of 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in DNA", *Carcinogenesis* **14**, 1069–1071.

[8] Kasprzak, K.S., Jaruga, P., Zastawny, T.H., North, S.L., Riggs, C.W., Olinski, R. and Dizdaroglu, M. (1997) "Oxidative DNA base damage and its repair in kidneys and livers of nickel(II)-treated male F344 rats", *Carcinogenesis* **18**, 271–277.

[9] Oliński, R., Jaruga, P. and Zastawny, T.H. (1998) "Oxidative DNA base modifications as factors in carcinogenesis", *Acta Biochim. Pol.* **45**, 561–572.

[10] Weitzman, S.A., Weitberg, A.B., Clark, E.P. and Stossel, T.P. (1985) "Phagocytes as carcinogens: malignant transformation produced by human neutrophils", *Science* **227**, 1231–1233.

[11] Jackson, A.L. and Loeb, L.A. (2001) "The contribution of endogenous sources of DNA damage to the multiple mutations in cancer", *Mutat. Res.* **477**, 7–21.

[12] Malins, D.C., Polissar, N.L. and Gunselman, S.J. (1996) "Progression of human breast cancers to the metastatic state is linked to hydroxyl radical-induced DNA damage", *Proc. Natl Acad. Sci. USA* **93**, 2557–2563.

[13] Kujawski, M., Sarlomo-Rikala, M., Gabriel, A., Szyfter, K. and Knuutila, S. (1999) "Recurrent DNA copy number losses associated with metastasis of larynx carcinoma", *Genes Chromosomes Cancer* **26**, 253–257.

[14] Rill, R.L. and Marsch, G.A. (1990) "Sequence preferences of covalent DNA binding by *anti*-(+)- and *anti*-(−)-benzo[a]-pyrene diol epoxides", *Biochemistry* **29**, 6050–6058.

[15] Obi, F.O., Ryan, A.J. and Billett, M.A. (1986) "Preferential binding of the carcinogen benzo[a]pyrene to DNA in active chromatine and the nuclear matrix", *Carcinogenesis* **7**, 907–913.

[16] Dittrich, K.A. and Krugh, T.R. (1991) "Mapping of (±)-*anti*-benzo[a]pyrene diol epoxide adducts to human c-Ha-ras1 protooncogene", *Chem. Res. Toxicol.* **4**, 277–281.

[17] Dittrich, K.A. and Krugh, T.R. (1991) "Analysis of site-specific binding of (±)-*anti*-benzo[a]pyrene diol epoxide to restriction fragments of pBR322 DNA via photochemical mapping", *Chem. Res. Toxicol.* **4**, 270–276.

[18] Yang, J.-L., Chen, R.-H., Maher, V.M. and McCormick, J.J. (1991) "Kinds and location of mutations induced by (±)-7b,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo(a)-pyrene in the coding region of the hypoxanthine (guanine) phosphoribosyltransferase gene in diploid human fibroblasts", *Carcinogenesis* **12**, 71–75.

[19] Rodriguez, H. and Loechler, E.L. (1993) "Mutational specificity of the (+)-anti-diol epoxide of benzo(a)pyrene in supF gene of an *Escherichia coli* plasmid: DNA sequence context influences hotspots, mutagenic specificity and the extent of SOS enhancement of mutagenesis", *Carcinogenesis* **14**, 373–383.

[20] Schantz, S.P. and Lee, N.K. (1996) "Tobacco carcinogenesis and head and neck cancer", In: Werner, J.A., Lippert, B.M. and Ruppert, H.H., eds, Head and Neck Cancer—Advances in Basic Research (Elsevier, Amsterdam), pp 9–20.

[21] Denissenko, M.F., Pao, A., Tang, M. and Pfeifer, G.P. (1996) "Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53", *Science* **274**(5286), 430–432.

[22] Pryor, W.A. (1997) "Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity", *Environ. Health Perspect.* **105**, 875–882.

[23] Kiyosawa, H., Suko, M., Okudaira, H., Murata, K., Miyamoto, T., Chung, M.-H., Kasai, H. and Nishimura, S. (1990) "Cigarette smoking induces formation of 8-hydroxydeoxyguanosine, one of the oxidative DNA damages in human peripheral leukocytes", *Free Radic. Res. Commun.* **11**, 23–27.

[24] Asami, S., Hirano, T., Yamaguchi, R., Tomioka, Y., Itoh, H. and Kasai, H. (1996) "Increase of a type of oxidative DNA damage, 8-hydroxyguanine, and its repair activity in human leukocytes by cigarette smoking", *Cancer Res.* **56**, 2546–2549.

[25] Grollman, A. (1992) "Structural basis for the mutagenic specificity of 8-oxoguanine-DNA", In: Sarma, R.H. and Sarma, M.H., eds, Structure and Function. Volume 1: Nucleic Acids (Adenine Press, New York), pp 165–170.

[26] Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S. and Loeb, L.A. (1992) "8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions", *J. Biol. Chem.* **267**, 166–172.

[27] Nebert, D.W. and Gonzales, F.J. (1987) "P-450 genes: structure, evolution and regulation", *Annu. Rev. Biochem.* **56**, 945–993.

[28] Leadon, S.A., Stampfer, M.R. and Bartley, J. (1988) "Production of oxidative DNA damage during the metabolic activation of benzo[a]pyrene in human mammary epithelial cells correlates with cell killing", *Proc. Natl Acad. Sci. USA* **85**, 4365–4368.

[29] Hanelt, S., Helbig, R., Hartmann, A., Lang, M., Seidel, A. and Speit, G. (1997) "A comparative investigation of DNA adducts, DNA strand breaks and gene mutations induced by benzo[a]pyrene and (±)-*anti*-benzo[a]pyrene-7,8-diol 9,10-oxide in cultured human cells", *Mutat. Res.* **390**, 179–188.

[30] Vaghef, H., Wisen, A.-C. and Hellman, B. (1996) "Demonstration of benzo(a)pyrene-induced DNA damage in mice by alkaline single cell gel electrophoresis: evidence for strand breaks in liver but not in lymphocytes and bone marrow", *Pharmacol. Toxicol.* **78**, 37–43.

[31] Frenkel, K., Donahue, J.M. and Banerjee, S. (1988) "Benzo(a)-pyrene-induced oxidative DNA damage: a possible mechanism for promotion by complete carcinogens", *Oxy-Radic. Mol. Biol. Pathol.*, 509–524.

[32] Kim, K.B. and Lee, B.M. (1997) "Oxidative stress to DNA, protein and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo(a)pyrene", *Cancer Lett.* **113**, 205–212.

[33] Canova, S., Degan, P., Peters, L.D., Livingstone, D.R., Voltan, R. and Venier, P. (1998) "Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive proteins in mussels exposed to waterborne benzo[a]pyrene", *Mutat. Res.* **399**, 17–30.

[34] Stern, S.J., Degawa, M., Martin, M.V., Guengerich, F.P., Kaderlik, R.K., Breau, R., McGhee, M., Montague, D., Lyn-Cook, B. and Kadlubar, F.F. (1993) "Metabolic activation, DNA adducts, and H-ras mutations in human neoplastic and non-neoplastic laryngeal tissue", *J. Cell. Biochem.*(Suppl. 17F), 129–137.

[35] Degawa, M., Stern, S.J., Martin, M.V., Guengerich, F.P., Fu, P.P., Ilett, K.F., Kaderlik, R.K. and Kadlubar, F.F. (1994) "Metabolic activation and carcinogen-DNA adduct detection in human larynx", *Cancer Res.* **54**, 4915–4919.

[36] Choi, S.Y. and Kahyo, H. (1991) "Effect of cigarette smoking and alcohol consumption in the aetiology of cancer of the oral cavity, pharynx and larynx", *Int. J. Epidemiol.* **20**, 878–885.

[37] Freudenheim, J.L., Graham, S., Byer, T.E., Marshall, J.R., Haughey, B.G., Swanson, M.K. and Wilkinson, G. (1992)

"Diet, smoking and alcohol in cancer of the larynx: a case-control study", *Nutr. Cancer* **17**, 33–45.
[38] Zatoński, W., Becher, H., Lissowska, J. and Wahrendorf, J. (1991) "Tobacco, alcohol, and diet in the etiology of laryngeal cancer: a population-based case–control study", *Cancer Causes Control* **2**, 3–10.
[39] Szyfter, K., Szmeja, Z., Szyfter, W., Hemminki, K., Banaszewski, J., Jaskuła-Sztul, R. and Louhelainen, J. (1999) "Molecular and cellular alterations in tobacco smoke-associated larynx cancer", *Mutat. Res.* **445**, 259–274.
[40] Oliński, R., Zastawny, T., Budzbon, J., Skokowski, J., Zegarski, W. and Dizdaroglu, M. (1992) "DNA base modifications in chromatin of human cancerous tissues", *FEBS Lett.* **309**, 193–198.
[41] Zeisig, M. and Möller, L. (1995) "32P-HPLC suitable for characterization of DNA adducts formed *in vitro* by polycyclic aromatic hydrocarbons and derivatives", *Carcinogenesis* **16**, 1–9.
[42] Möller, L., Zeisig, M. and Vodicka, P. (1993) "Optimization of an HPLC method for analyses of 32-P-postlabeled DNA adducts", *Carcinogenesis* **14**, 1343–1348.
[43] Field, J.K., Pavelic, Z.P., Spandidos, D.A., Stambrook, P.J., Jones, A.S. and Gluckman, J.L. (1993) "The role of the p53 tumor suppressor gene in squamous cell carcinoma of the head and neck", *Arch. Otolaryngol. Head Neck Surg.* **119**, 1118–1122.
[44] Zhang, L.-F., Hemminki, K., Szyfter, K., Szyfter, W. and Söderkvist, P. (1994) "p53 Mutations in larynx cancer", *Carcinogenesis* **15**, 2949–2951.
[45] Szyfter, K., Hemminki, K., Szyfter, W., Szmeja, Z., Banaszewski, J. and Yang, K. (1994) "Aromatic DNA adducts in larynx biopsies and leukocytes", *Carcinogenesis* **15**, 2195–2199.
[46] Szyfter, K., Banaszewski, J., Jaloszynski, P., Pabiszczak, M., Szyfter, W. and Szmeja, Z. (1999) "Carcinogen: DNA adducts in tobacco smoke-associated cancer of the upper respiratory tract", *Acta Biochim. Pol.* **46**, 275–287.
[47] Kondo, S., Toyokuni, S., Iwasa, Y., Tanaka, T., Onodera, H., Hiai, H. and Imamura, M. (1999) "Persistent oxidative stress in human colorectal carcinoma, but not in adenoma", *Free Radic. Biol. Med.* **27**, 401–410.
[48] Okamoto, K., Toyokuni, S., Uchida, K., Ogawa, O., Takenawa, J., Kakehi, Y., Kinoshita, H., Hattori-Nakakuki, Y., Hiai, H. and Yoshida, O. (1994) "Formation of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in human renal cell carcinoma", *Int. J. Cancer* **58**, 825–829.
[49] Malins, D. and Haimanot, R. (1991) "Major alterations in the nucleotide structure of DNA in cancer of the female breast", *Cancer Res.* **51**, 5430–5432.
[50] Nagashima, M., Tsuda, H., Takenoshita, S., Nagamachi, Y., Hirohashi, Y., Hirohashi, S., Yokota, J. and Kasai, H. (1995) "8-Hydroxydeoxyguanosine levels in DNA of human breast cancers are not significantly different from those of non-cancerous breast tissues by the HPLC-ECD method", *Cancer Lett.* **90**, 157–162.
[51] Farinati, F., Cardin, R., Degan, P., Rugge, M., Mario, F.D., Bonvicini, P. and Naccarato, R. (1998) "Oxidative DNA damage accumulation in gastric carcinogenesis", *Gut* **42**, 351–356.
[52] Shimoda, R., Nagashima, M., Sakamoto, M., Yamaguchi, N., Hirohashi, S., Yokota, J. and Kasai, H. (1994) "Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis", *Cancer Res.* **54**, 3171–3172.
[53] Papadimitrakopoulou, V.A., Shin, D.M. and Hong, W.K. (1996) "Molecular and cellular biomarkers for field cancerization and multistep process in head and neck tumourigenesis", *Cancer Metastasis Rev.* **15**, 53–76.
[54] Pryor, W.A., Hales, B.J., Bremovic, P.I. and Church, D.F. (1983) "The radicals in cigarette tar: their nature and suggested physiological implications", *Science* **220**, 425–427.
[55] Howard, D.J., Briggs, L.A. and Pritsos, C.A. (1998) "Oxidative DNA damage in mouse heart, liver, and lung tissue due to acute side-stream tobacco smoke exposure", *Arch. Biochem. Biophys.* **352**, 293–297.
[56] Howard, D.J., Ota, R.B., Briggs, L.A., Hampton, M. and Pritsos, C.A. (1998) "Environmental tobacco smoke in the workplace induces oxidative stress in employees, including increased production of 8-hydroxy-2′-deoxyguanosine", *Cancer Epidemiol. Biomark. Prev.* **7**, 141–146.