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This paper is dedicated to the memory of Professor Bronisław Filipowicz
Review

Carcinogen:DNA adducts in tobacco smoke-associated cancer of the upper respiratory tract*

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Mortality connected with tobacco smoke-associated laryngeal cancer in Poland markedly exceeds the relevant epidemiological data from other European countries. The main groups of genotoxic agents considered as potential carcinogens present in tobacco smoke are polycyclic aromatic hydrocarbons, aromatic amines, *N*-nitrosoamines and reactive oxygen species. Aromatic DNA adducts, N7-alkylated guanosines and oxidative DNA damage derived from tobacco smoke exposure were detected in laryngeal and oral (tumour and non-tumour) biopsies, and white blood cells of cancer subjects. Further, DNA lesions were analysed to estimate the significance of such confounders as intensity of smoking, subject's sex, age, topography of larynx, cancer staging and genetic factor. The number of cigarettes smoked per day was found to be the main determinant of an individual's DNA adduct level. The occurrence of DNA lesions was established as a reliable marker of former exposure to tobacco smoke genotoxicants. On the other hand, a comparison of DNA lesion levels in various regions of larynx indicates limited usefulness of DNA adduct analysis as an estimate of cancer risk. For a better risk estimation one has to take into account

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Abbreviations: PAH, polycyclic aromatic hydrocarbons; AA, aromatic amines; ROS, reactive oxygen species; TLC, thin-layer chromatography; GC/MS, gas chromatography/mass spectroscopy; SHBG, sex hormones binding globulin; GST, glutathion *S*-transferase; NAT, *N*-acetyltransferase; EH, epoxide hydroxylase.

DNA lesions in proto-oncogenes and tumour suppressor genes and the efficacy of DNA repair. Altogether, DNA adducts formation and removal has to be considered as a single stage in the multistep carcinogenesis.

Tobacco smoking is one of the main causative factors of cancer in human population [1]. It is estimated that at least one third of new cancers is connected with active or passive tobacco smoking. Respiratory tract is recognised as the primary target for carcinogenic constituents of tobacco smoke. The organ most frequently affected by tobacco smoking is lung. Cancers of the upper respiratory tract are not so common but their association with tobacco smoking is stronger. However, in the Polish male population laryngeal cancer is the third most frequent and its morbidity tends to increase since the early sixties reaching in the middle nineties the level of 9 deaths per 100000 men annually [2, 3]. Mortality connected with laryngeal cancer in Poland considerably exceeds the relevant epidemiological data from other European countries [4]. This corresponds well to the number of cigarettes sold in Poland that is at the top of the world selling list. Hence, tobacco smoke-related laryngeal cancer is recognised in Poland as both a medical and social problem. It is worth mentioning that morbidity and mortality in the female population all around the world is 5–20 times lower than in males.

Laryngeal cancer originates almost exclusively from exogenous exposure dominated by tobacco smoke [1, 5]. At this point it is necessary to note, that smoking is often accompanied by drinking and strong alcoholic drinks act synergistically, increasing the risk of laryngeal cancer [6]. Occupational exposures (asphalt laying, varnishing etc.) contribute also to the increased risk of laryngeal cancer but their significance is much smaller [5]. An alternative risk factor of cancer of the upper respiratory tract is infection with papilloma virus [7].

TOBACCO SMOKE-INDUCED DNA DAMAGE

Tobacco smoke contains about 4000 chemical substances including a number of carcinogens capable of interacting with DNA and other biomolecules. The compounds inducing DNA lesions are known as genotoxicants. The main groups of genotoxicants present in tobacco smoke are: polycyclic aromatic hydrocarbons (PAH), aromatic amines (AA), *N*-nitrosoamines and reactive oxygen species (ROS). A vast majority of genotoxicants require metabolic activation leading to reactive intermediates also known as ultimate carcinogens [8]. Metabolic activation is a multistep enzymatic process that alters both physical and chemical properties of the initial compound [9]. The enzymes responsible for metabolic activation belong to the cytochrome P450 family. The ultimate carcinogen is a water-soluble electrophilic reagent. At this point activated carcinogens become good substrates for detoxifying enzymes. The reactive electrophilic groups of the genotoxicants remaining in the cell can form covalent bonds with DNA bases. The relatively stable structures formed are known as carcinogen:DNA adducts. The preferred target for the interaction of DNA with the above mentioned tobacco smoke genotoxicants is guanine residue. The resulting DNA adduct structure is dependent on the electrophilic potential of a given genotoxicant. The main positions of interaction of guanine with metabolites of PAH, aromatic amines and *N*-nitrosoamines are shown on Fig. 1.

Some compounds present in tobacco smoke are highly oxidative and together with endogenous oxidation processes they are a source of reactive oxygen species [10]. The

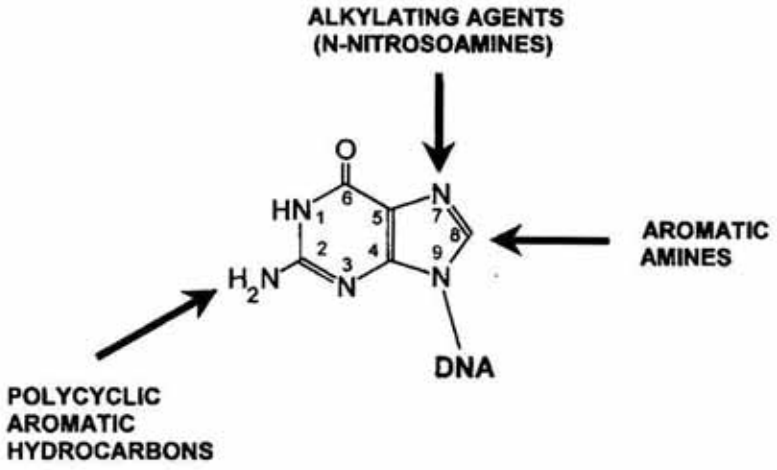

Figure 1. The preferable positions of interaction of guanine residue with metabolically activated polycyclic aromatic hydrocarbons, *N*-nitrosoamines and aromatic amines.

latter by a free radicals reaction can induce single and double DNA breaks as well as lesions of DNA purine and pyrimidine bases. Some of the base modifications are potentially mutagenic.

DNA lesions are removable by DNA repair discussed recently by Rzeszowska-Wolny and Widłak [11]. Only DNA lesions not removed fast enough can be turned into mutations and start neoplastic transformation of a cell.

Several techniques to analyse DNA adducts have been established, which explore specific properties of DNA adducts [12]. In contrast to the very weak immunogenic character of DNA, some DNA adducts are relatively potent antigens. Therefore, several polyclonal and monoclonal antibodies have been raised against several DNA adducts, which can be applied in classical radioimmunoassay or more sophisticated ELISA or USERIA techniques. Next, some DNA adducts are fluorescent, which permits the application of fluorescence spectrometry techniques including synchronous fluorescence spectrofluorimetry (SFS) which appeares to be highly successful in the analysis of PAH:DNA adducts. However, the technique most commonly used in the analysis of diverse types of DNA adducts, is ^{32}P-postlabelling. The technique was initially designed by Randerath *et al.* [13] for the analysis of "bulky" aromatic DNA adducts. In principle, it includes enzymatic DNA digestion to 3'-nucleotides, phosphorylation with [γ-^{32}P]ATP by T4 polynucleotide kinase, separation of the resulting mixture by chromatography and estimation of DNA adducts by radioactivity quantitation. Since then a number of modifications has been proposed to adjust the technique to particular types of DNA adducts and to increase sensitivity. Modifications include extra steps to enrich the DNA digestion mixture in the adducted nucleotides. Thin-layer chromatography (TLC) proposed originally for the separation of radiolabelled DNA adducts is often replaced by high pressure liquid chromatography (HPLC) or gas chromatography coupled with mass spectrometry (GC/MS). Recently, the detection limit has been improved to 1 DNA adduct per 10^8–10^9 normal nucleotides [14].

DNA adducts as markers of exogenous exposure

An aim of molecular epidemiology is to find a relationship between an exposure to a hazardous agent and the risk of developing disease (Fig. 2.). In contrast to the classical epidemiology the measurable items are biological markers of exposure at the molecular and subcellular (cytogenetic) level. DNA adducts can serve as molecular markers of exposure and they are recognised as a measure of carcinogen effectively interacting with the genetic material. In other words, the level of DNA adducts is recognised as an indicator of

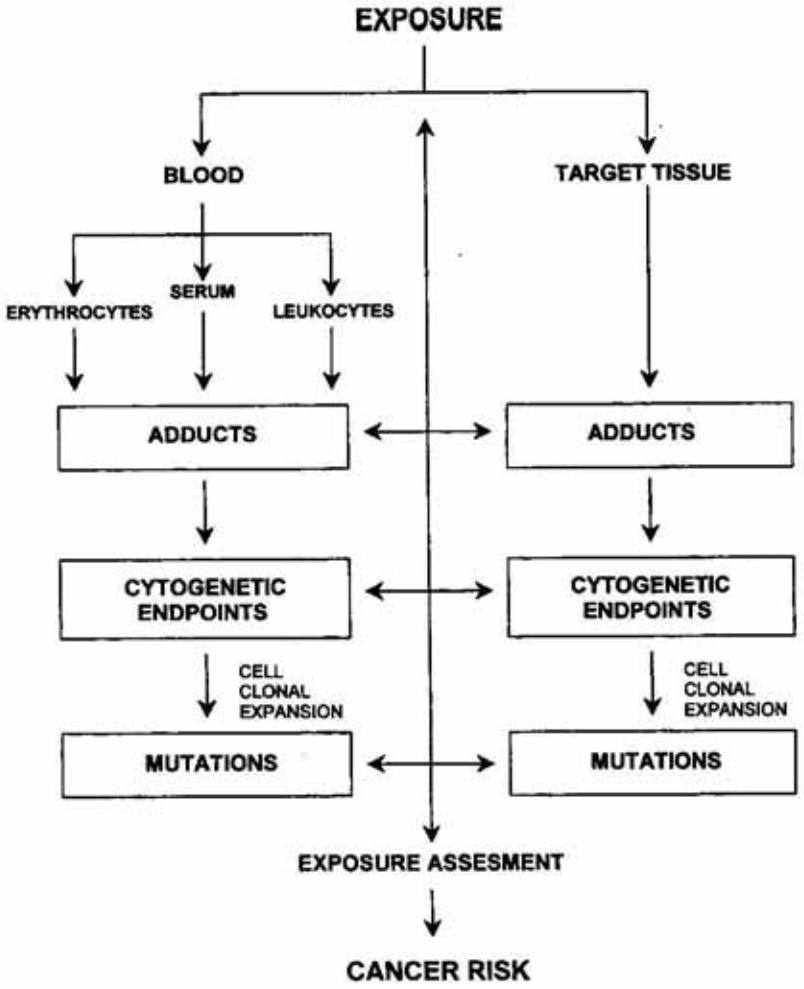

Figure 2. The concept of molecular epidemiology.

biologically effective dose of a carcinogen [8, 12, 15].

For a better estimation of risk of neoplastic transformation of a given tissue it is recommended to measure the biological marker in the cells derived from the carcinogen target tissue. The point is, however, that human target tissue usually is not available for an investigator. In practice, the only way to get access to the target tissue is to work with a post-surgery material. Because of such limitation, molecular epidemiologists have to work with alternative material (known as "surrogate tissue") attainable in a non-invasive way. Peripheral blood lymphocytes are in common use as the surrogate tissue. Whenever possible, a relationship between the occurrence of biological marker in the target and surrogate tissue is to be determined first.

Identification of DNA adducts in biopsies of the upper respiratory tract

Our research interest focused on so-called "bulky" DNA adducts, alkylated and oxidative modifications of DNA bases. Before reviewing the results, it is necessary to mention that detection of particular types of DNA adducts is strictly associated with the method applied to the analysis. It means that each type of DNA modification must be analysed separately.

Having access to the biological material removed in the course of surgical treatment of cancer, three types of cells were analysed in respect to DNA adducts detection and quantitation. The first were tumours developed on the mucosa of larynx or mouth cavity (tongue, tonsils, gum, bottom and upper floor of

mouth). As the surgery protocol always provides tumour removal together with the surrounding margin, the second studied tissue was an unchanged mucosa. The latter material was analysed by a histopathologist to confirm the absence of neoplastic cells. Finally, the way to compare DNA adducts profile in cancer and non-cancer subjects is the analysis of white blood cells. DNA from the listed tissues was analysed by a variant of ^{32}P-postlabelling assay relevant to the kind of DNA modification.

The classical ^{32}P-postlabelling assay improved in the course of our studies [16] for a better separation of "bulky" aromatic DNA adducts revealed on thin-layer chromatograms several radioactive spots believed to represent "bulky" aromatic DNA adducts [17, 18]. The main spot co-migrated with the benzo[*a*]-N^2-dGMP standard. According to the early interpretation of Randerath & Randerath [19] the spots located on the diagonal radioactive zone termed I-compounds represent PAH:DNA adducts. Alternative enrichment of the DNA adduct pool by P1 nuclease digestion or n-butanol extraction allows the differentiation between DNA adducts originated from an exposure to PAH or aromatic amines. The direct proof of DNA adducts originating from tobacco smoke-associated aromatic amines was presented by Flamini *et al.* [20] who detected 4-aminobiphenyl-DNA adducts in laryngeal biopsies by immunohistochemistry. The authors claimed that the level of 4-aminobiphenyl-DNA adducts can serve as a reliable marker of exposure to cigarette smoke.

It was established [21] that in the course of metabolic activation of PAH there is a considerable production of reactive oxygen species (ROS). The latter compounds were postulated to be responsible for oxidative DNA damage [10, 21]. Exploring this assumption, in a preliminary study we have found several DNA modifications, which can be attributed to ROS. In laryngeal DNA samples derived from cancer subjects (divided into tumour and non-tumour material) studied by the GC/MS technique, the following DNA modifications were identified: 8-oxoguanosine (8-OH-Gua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-oxoadenine (8-OH-Ade), 4,6-diamino-5-formamidopyrimidine (FapyAde), 5,6-dihydroxyuracil (5,6-diOH-Ura) and 5-hydroxycytosine (5-OH-Cyt). In tumour biopsies the mean levels of the studied oxidised bases were higher than in non-tumour mucosa. The differences were most pronounced for 8-OH-Gua (Jałoszyński *et al.*, manuscript in preparation).

N-nitrosoamines present in tobacco smoke are also capable of forming DNA adducts. After metabolic activation *N*-nitrosoamines become alkylating agents reacting with purines and pyrimidines. Using a variant of ^{32}P-postlabelling assay we detected N7-alkyl-dGMP in biopsies of laryngeal cancer subjects. The main radioactive spot contained N7-methyl-dGMP, but a contribution of N7-hydroxyethyl-dGMP cannot be excluded [22].

Recently, 1,N^2-propanodeoxyguanosine adducts detected in oral biopsies by the ^{32}P-postlabelling/HPLC assay have been claimed to be a potential new biomarker of tobacco smoke exposure. Their presence has been attributed to genotoxic activity of unsaturated aldehydes present in tobacco smoke [23].

DNA adduct level and its inter-individual variability

The levels of tobacco smoke associated DNA adducts were determined in some tissues of experimental animals and in several human tissues including peripheral blood (leukocytes, lymphocytes, granulocytes), respiratory tract (lung, bronchus, larynx, oral cavity) and organs distal in relation to tobacco smoke exposure (breast, cervix, kidney, liver, placenta etc.). Measurable DNA adducts levels were found in the majority of the studied material [14, 24]. In principle, DNA adducts level was higher in target tissue than in peripheral blood cells. To give an example concerning

Table 1. Average DNA adduct levels in tissues of larynx cancer subjects shown as the number of adducted nucleotides per 10^8 normal nucleotides.

The number of subjects analysed is given in parenthesis.

Group/Tissue	Aromatic DNA adducts × ± S.D.	N7-alkylguanines × ± S.D.
Larynx cancer subjects		
Larynx tumour – all	5.7 ± 5.1 (41)	26.2 ± 38.0 (44)
– smokers	6.5 ± 5.5 (30)	28.2 ± 36.3 (39)
– non-smokers	3.7 ± 0.7 (4)	11.3 ± 5.9 (5)
Larynx non-tumour – all	4.7 ± 4.9 (36)	22.7 ± 19.9 (33)
– smokers	4.8 ± 5.1 (30)	24.1 ± 18.9 (31)
– non-smokers	5.8 ± 3.0 (4)	15.3 ± 2.0 (3)
Leukocytes – all	2.1 ± 1.8 (12)	13.1 ± 5.6 (9)
– smokers	2.3 ± 2.1 (11)	13.6 ± 4.8 (8)
– non-smokers	nd	nd
Non-cancer controls		
Leukocytes – all	1.3 ± 2.1 (12)	9.2 ± 5.9 (15)
– smokers	nd	9.7 ± 5.9 (10)
– non-smokers	nd	5.1 ± 2.8 (5)

nd, not determined

the upper respiratory tracts we refer to our studies on laryngeal cancer subjects (Table 1). It is clearly seen that the level of N7-alkylguanosine exceeds aromatic DNA adducts roughly by an order of magnitude. DNA adduct levels in oral cavity are comparable with those found in laryngeal biopsies. Quantitation of oxidative DNA adducts is currently underway.

Aromatic DNA adducts and N7-alkylguanosines were analysed in the same material, which encouraged us to correlate the levels of two different DNA adducts formed under the same exposure. It was established that the formation of aromatic DNA adducts and N7-alkylguanosines proceeded on fairly independent ways [22]. For the explanation of this finding one has to remember that metabolic activation of PAH and *N*-nitrosoamines is catalysed by entirely different Cyt P450 dependent enzymes; also the removal of "bulky" aromatic and "small" alkylated DNA adducts occurrs by independent DNA repair mechanisms.

Besides average DNA adduct levels, large inter-individual differences were established for groups of subjects exposed to any particular carcinogen.

In our studies concerning the material derived from larynx cancer subjects, the analysis of DNA adduct levels demonstrated a variation of individual results ranging from 12-fold to 238-fold for aromatic DNA adducts [18] and from 18-fold to 748-fold for N7-alkylguanosine [22] in the studied tissues. The highest variation was observed in tumour cells and the lowest in blood leukocytes. The calculations concerned all the subjects independently of smoking status. Restricting the group to moderate smokers did not lead to a drastic narrowing of the range of inter-individual results. The results concerning levels of aromatic DNA adducts in biopsies from subjects with cancers of oral cavity were

much more coherent – the highest result exceeded the lowest one 135-fold.

The differences in DNA adduct levels were critically discussed by Hemminki [25] along the former assumption of Harris [8], who explained the actual level of DNA adducts as a result of counteracting processes of metabolic activation, detoxification and DNA repair. The capacity of each step is determined by the activity of specific enzymes involved in the metabolic process. Hence, it has been decided to study confounders modulating individual DNA adducts level.

Influence of tobacco smoking

As already mentioned in introduction, tobacco smoking is recognised as the main causative factor in larynx cancer aetiology. Hence, our attention was focused on the relationship between the intensity of tobacco smoking and the DNA adducts level in laryngeal cancer subjects. Concerning aromatic DNA adducts, there were higher levels found in biopsies of smokers than in non-smokers [17, 18]. Analysing N7-alkylguanosine content there was more data on smoking habits of the subjects [22]. Hence, subjects were divided (25) into non-smokers (including ex-smokers who quit smoking at least 5 years before surgery), moderate smokers (about 20 cigarettes per day) and heavy smokers (about 40 cigarettes per day); the average level of N7-alkylguanosine increased as shown in Fig. 3.

Biopsies from subjects with oral cavity cancer were also analysed in respect to the effect of tobacco smoking on aromatic DNA adducts formation. Generally, we observed the same regularity – more cigarettes smoked per day, more DNA adducts. However, in the group of heavy smokers we observed an unexpected decrease of DNA adducts level. To explain it we can only speculate about a cytotoxic effect caused by high exposure to tobacco smoke that could eliminate oral cavity cells containing highly adducted DNA. The average DNA adducts level was slightly lower in smokers of filter cigarettes than in smokers of cigarettes without filter. The genotoxic effect of tobacco smoke was better expressed in the mouth part than in laryngeal part of oral cavity (Pabiszczak, Ph.D. Thesis, 1999).

The analysis of tobacco smoke genotoxicity was further extended towards the claimed synergistic action of strong alcoholic beverages. DNA adducts level remained almost stable in blood leukocytes whereas in tumour and non tumour oral biopsies there was a re-

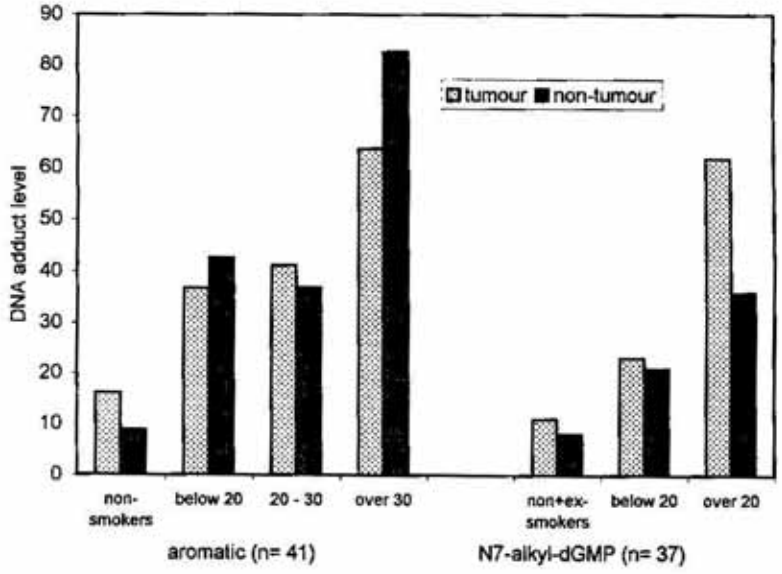

Figure 3. The effect of cigarette smoking on aromatic DNA adducts and N7-alkylguanosine levels (number per 10^8 normal nucleotides) in laryngeal tissues estimated by the ^{32}P-post-labelling technique.

The number of cigarettes smoked per day is shown below bars on the x axis.

markable increase of DNA adducts level, successively, from non-smokers/non-drinkers, smokers/non-drinkers to smokers/drinkers (Pabiszczak, Ph.D. Thesis, 1999).

Our findings are in agreement with the data of other authors, who have found a positive correlation between tobacco smoking and DNA adduct levels in white blood cells [26], bronchial tissue [26], oral mucosa [27] and lung [28]. However, it has to be acknowledged that there was also published a well-documented papers of van Schooten *et al.* [29] claiming a lack of influence of tobacco smoking on DNA adducts level.

Other confounders of DNA adduct level

Some other potential confounders modulating the level of DNA adducts in human laryngeal cells were studied. In contrast to tobacco smoking that can be regarded as an exogenous confounder, this part was designed to study the significance of individual endogenous confounders.

A weak positive correlation was established between a subjects' age (ranging from 38 to 78 years) and the level of N7-alkylguanosine [22]. In another study we found that in males an increase of the N7-alkylguanosine level was attributed mostly to a group of subjects over 70 years of age (unpublished). Lower efficiency of N7-alkylguanosine removal from senescent tissue compared with a young one was observed also by Gaubatz & Tan [30] in mouse kidney cells. Altogether, it fits the general phenomenon of reduced capacity of aged organisms to remove DNA lesions [31].

The question concerning the origin of the substantial difference in larynx cancer morbidity and mortality in males and females remains open. Several hypotheses were raised and experimentally tested. The first one explores the regulation of larynx function by hormones, but no sufficient experimental data have been provided yet. An alternative explanation of sex differences is related to exogenous exposure, and thus it refers to more frequent tobacco smoking and alcohol abuse in the male than in the female population. However, in leading industrialised countries it has been observed that adopting similar life style habits by men and women is not followed by drastic changes in larynx cancer epidemiology.

We have compared the levels of aromatic DNA adducts and N7-alkylguanosine measured in the groups of male and female moderate tobacco smokers [32]. Both in cancerous and non-cancerous larynx tissue the levels of DNA adducts were higher in males. The differences were even more pronounced in the case of N7-alkylguanosine, the proportion M/F reaching 3.74 in larynx tumour tissue. Our results are opposite to those of Ryberg *et al.* [33], who have found higher levels of aromatic DNA adduct in females' than in males' lung biopsies from lung cancer patients. When the data were adjusted to tobacco smoke exposure, the high level of DNA adducts was still more pronounced in females than in males. On the other hand, Phillips *et al.* [26] studying bronchial biopsies from patients undergoing pulmonary surgery have established that normal bronchial epithelium

Table 2. Average levels (number ± S.D. per 10^8 normal nucleotides) of aromatic DNA adduct estimated by ^{32}P-postlabelling/HPLC technique in the interaryteonid area (non-tumour) compared with other tumour and non-tumour larynx areas

Group	Number of subjects	Tumour	Non-tumour	Interarytenoid area
Males	28	46.4 ± 47.8	48.9 ± 47.2	55.8 ± 98.2
Females	5	27.5 ± 31.9	35.9 ± 44.7	30.2 ± 37.3
All	33	43.5 ± 45.9	46.9 ± 46.4	52.0 ± 91.7

cells from male-smokers contained 1.9-fold more aromatic DNA adducts than the female-smokers cells.

We think that the variable M/F proportions of the DNA adduct levels in lung, bronchus, nasal cavity, and larynx reflect a difference between lower and upper respiratory tract. Tobacco smoke exposure in larynx is direct and almost topical as opposed to non-direct one in the lower respiratory tract. Furthermore, the composition of tobacco smoke penetrating respiratory tract is subjected to changes. The organ specific activity of carcinogen-metabolising enzymes as well as the efficacy of DNA repair should also be taken into account. Nevertheless, the morbidity differences found at the epidemiological level seem to be recognisable also by methods used in molecular epidemiology.

Further, we have attempted to find a link between larynx cancer morbidity and the concentration of sex hormones in blood serum [32]. The serum concentrations of testosterone and sex hormones binding globulin (SHBG) were confronted with the average levels of aromatic DNA adducts in patient groups divided according to age. The only significant result was the association of a decreased level of SHBG with increased morbidity in males over 50 years old. Altogether, we failed to find any significant correlation between the analysed parameters. Hence, it was concluded that testosterone and SHBG concentration in serum are not proper markers of male predisposition to laryngeal cancer.

The relationship between the DNA adducts level and cancer progression was also studied having in mind its prognostic value [34]. The analysis was restricted only to N7-alkylguanosine. DNA adduct levels were calculated separately for the groups of the studied larynx cancer subjects categorised according to the disease progression estimated by the TNM staging system taking into account tumour growth (T), metastasis to adjacent lymph nodes (N) and long distance metastasis (M). The results seem to indicate a substantial role of DNA adducts in the early stage of carcinogenesis. Nonetheless, their significance in late stages of cancer cannot be excluded. The latter statement was deduced from the comparison of DNA adducts level between metastasis and non-metastasis samples. Metastasis to the adjacent lymph nodes is usually followed by remarkable worsening of general health of patients, which was not reflected by changes in the N7-alkylguanosine levels.

We have also compared DNA adducts in larynx tissues in primary and recurrent laryn-

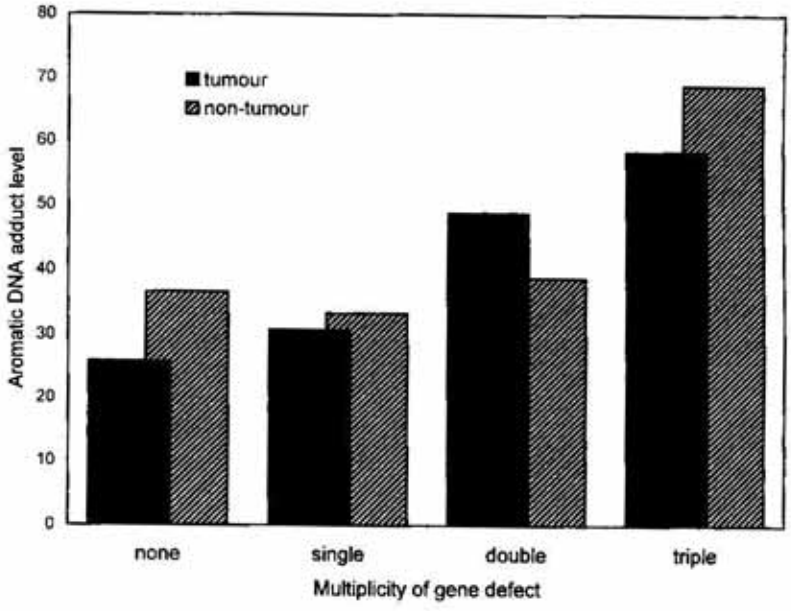

Figure 4. Aromatic DNA adduct level (number per 10^8 normal nucleotides) determined by the HPLC/^{32}P-postlabelling assay in laryngeal biopsies as a function of the number of defects of genes coding for detoxifying enzymes.

geal tumours. The average levels of aromatic DNA adducts and N7-alkylguanosine both in tumour and in non-tumour larynx tissue were higher in recurrent biopsies (unpublished). One can thus speculate on the progressive impairment of DNA repair in the course of tumourigenesis.

Finally, the impact of genetic status on DNA adducts level was taken into account. In yet another study we described the occurrence of gene defects of enzymes responsible for detoxification of carcinogens. The enzymes studied were: glutathione *S*-transferase (isoenzymes: GST M1, GST M3, GST P1 and GST T1), *N*-acetyltransferase (NAT2) and epoxide hydroxylase (EH) [35]. Detoxifying enzymes are not very selective and the diminished activity of a single enzyme can be compensated for by another one. However, a tendency to coincidence of gene defects was established in the group of over 200 larynx cancer subjects. We observed [36] that the level of aromatic DNA adducts in laryngeal tumour and non-tumour biopsies was increasing with the number of gene defects (Fig. 4). Thus, the significance of the genetic factor predisposing to an increased sensitivity to carcinogens was demonstrated.

DNA ADDUCTS AS A MEASURE OF CANCER RISK

Some carcinogens (e.g. PAH) are ubiquitous in the environment and humans are persistently exposed to their action. It explains a number of papers concerning the detection of carcinogen:DNA adducts without previous recorded exposure. Next, penetration of the human body by a carcinogen and its further processing, including transportation, leads to the occurrence of DNA adducts in many tissues, even distal to the exposure site [12, 14, 24].

In larynx cancer patients, DNA adducts have been detected in larynx tumour cells, in sections of larynx histopathologically recognised as normal and in peripheral blood lymphocytes. As expected, the level of DNA adducts in blood leukocytes was lower than in larynx tissue directly exposed to tobacco smoke. The proportion of DNA adducts level in tumour *versus* non-tumour cells of the same organ is an interesting matter. Fast proliferation of tumour cells but facilitates DNA repair according to the mechanism of transcription coupled DNA repair [37] but, on the one hand, there are numerous reports on DNA repair deficit in tumour tissue [31]. Comparing tumour biopsies and non-tumour larynx cells, we have found a significantly higher level of aromatic DNA adducts [18] and an insignificantly higher level of N7-alkylguanosine [22] in the former. When studying breast biopsies, Li *et al.* [38] found a fully comparable pattern of aromatic DNA adducts in tumour cells and in adjacent normal tissue. Other reports described relatively lower levels of DNA adduct in tumours than in normal adjacent tissues [39, 40].

To explore it further, we have compared levels of aromatic DNA adducts in a few sections of larynx (Table 2). HPLC/^{32}P-postlabelling method producing higher results than those obtained by TLC/^{32}P-postlabelling was applied. The attention was focused on interarytenoid section. The Clinic of Otolaryngology, Poznań Medical University, having performed surgery on about 3800 laryngeal cancers throughout four decades have found only one case of primary tumour location there. It was assumed that the extent of DNA modification at this location should be low. Contrary to expectation, DNA adducts level in the interarytenoid region remained high (J. Banaszewski, submitted for publication). The only interpretation possible is that the whole organ is subjected to DNA adducts formation and the presence of DNA adducts is not the only reason for entering multistage carcinogenesis. Another conclusion is that DNA adducts formation is a necessary step in carcinogenesis but not sufficient to turn it into tumourigenesis.

CONCLUDING REMARKS

The DNA adducts analysis described above concerns the whole genome. It is well known that many lesions along the genome remain ineffective, whereas some are still crucial for carcinogenesis. The latter include lesions in proto-oncogenes and tumour suppressor genes [8]. Mutations in the *p53* tumour suppressor gene are frequent in head and neck cancer [41]. Detection of mutations in the *p53* gene [42], chromosome rearrangements in the region of the *p53* and *p16* tumour suppressor genes [43], expression of the p53 protein and other biomarkers of cell proliferation [44] are other targets in the studies on the biology of laryngeal tumours but they remain outside the frames of DNA modifications and this review.

Altogether, DNA adduct studies in relation to carcinogenesis have to be taken as a single stage in the multistep process. To find a relationship between carcinogen exposure and cancer risk an investigator has to deal also with biomarkers relevant to other steps of carcinogenesis.

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