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4-Aminobiphenyl–DNA adducts in laryngeal tissue and smoking habits: an immunohistochemical study

Giovanna Flamini[1,5], Gianpiero Romano[1], Giuseppe Curigliano[1], Alessandro Chiominto[2], Giovanni Capelli[3] , Alma Boninsegna[1], Carlo Signorelli[1], Luca Ventura[2], Regina M.Santella[4], Alessandro Sgambato[1] and Achille Cittadini[1]

[1]Institute of General Pathology-Centro di Ricerche Oncologiche 'Giovanni XXIII', Catholic University School of Medicine, Largo F. Vito, 1, 00168 Rome, [2]Laboratory of Pathology, S. Salvatore Hospital, 67100 L'Aquila, [3]Institute of Hygiene, Catholic University School of Medicine, Largo F. Vito, 1, 00168 Rome, Italy and [4]Division of Environmental Health Sciences, Columbia School of Public Health, 701 West 168th Street, New York 10032, USA

[5]To whom correspondence should be addressed
Email: ibipg@rm.unicatt.it

4-Aminobiphenyl (4-ABP)–DNA adducts and *p53* overexpression were evaluated in laryngeal biopsies from 38 patients by immunohistochemical methods. Samples were categorized as tumors (n = 9), polyps (n = 28) or normal tissue (n = 1). 4-ABP–DNA adducts were evaluated with a quantitative immunoperoxidase method using monoclonal antibody 3C8 in both the lesion and adjacent tissue. Relative staining intensity data showed a log-normal distribution and values found in adjacent tissue from smokers were significantly higher (median: 173.5, geometric mean: 159.9) than those measured in adjacent tissue from non-smokers (median: 75.5, geometric mean: 7.40). Statistical significance was assessed both by non-parametric testing on raw data (P = 0.0007 on rank sum test) and by parametric testing on log-transformed data (P = 0.0002 on an unpaired t-test). Furthermore, relative staining intensity in the lesional tissue showed the same significant difference between smokers and non-smokers in patients affected by polyps, whereas no significant difference was detected in patients with laryngeal tumors. Overexpression of *p53*, also measured with an immunoperoxidase method, was observed in 44% of the malignant tumors and in 3.5% of the polyps. This work demonstrates that 4-ABP–DNA adducts can be evaluated in laryngeal tissue and are related to smoking exposure.

Introduction

Larynx cancer represents 4.5% of all human tumors and 30% of respiratory tract neoplasias (1). There is no strict geographic localization, but incidence in males is usually higher than in females (2). This sex difference is probably related to exposure to exogenous factors, since epidemiological studies suggest 50–70% of deaths from larynx cancer are attributable to tobacco smoking (3–5). In these cases, cancer risk correlates with factors such as number and type of cigarettes smoked, and the risk from cigarette smoking is amplified in alcohol drinkers (6,7).

Carcinogens in cigarette smoke include polycyclic aromatic hydrocarbons (PAH*), nitrosamines, benzene and aromatic amines (8). Most studies on tobacco smoking genotoxicity in the respiratory tract have focused on PAH and *N*-nitrosamines (9–11), with less importance attributed to aromatic amines such as 4-aminobiphenyl (4-ABP). 4-ABP is metabolically activated to several electrophilic intermediates, all capable of interacting with DNA to form adducts (12). The predominant one is *N*-(2′-deoxyguanosin-8-yl)-4-ABP accounting for ~70% of the total adducts formed (13). As covalent modification of DNA appears to be a critical event in chemical carcinogenesis, DNA adduct quantification is considered a useful means of assessing exposure to chemical carcinogens.

Exposure to 4-ABP and metabolic activation to a reactive intermediate was initially detected by evaluating hemoglobin adducts and higher levels were observed in cigarette smokers versus non-smokers (14,15). In recent years, methods have also been developed for quantitation of 4-ABP–DNA damage and include immunoassays, ^{32}P-postlabeling-HPLC and gas chromatography-mass spectroscopy (GC-MS) (16). These methods require the isolation of bulk DNA from tissues and thus do not allow a direct evaluation of adducts at the morphological level. In addition, relatively large amounts of material are required, which is limiting for routine applications.

A monoclonal antibody, 3C8, highly specific to 4-ABP–DNA adducts, which does not cross-react with the DNA adducts of several other aromatic amines, including 1-aminopyrene, 8-nitro-1-aminopyrene and 6-nitro-1-aminopyrene, was recently obtained (17). With this antibody a quantitative immunofluorescence method for detecting adducts was developed and validated in 4-ABP-treated mice by analysis of DNA from the same tissues by GC/MS (17). Data were also obtained by our group on 4-ABP–DNA adducts in a series of patients with T1 bladder cancer using an immunoperoxidase method with the same antibody (18).

In recent years, larynx cancers have been characterized by *p53* tumor suppressor gene product alterations, which confer a selective proliferative advantage (19,20). The product of the *p53* gene is a nuclear phosphoprotein fundamentally involved in cell-cycle regulation, which accumulates to a detectable level due to a lowered turnover and prolonged half-life. This is most frequently the result of gene abnormalities (e.g. mutations, deletions). *p53* overexpression is now considered a tumor progression marker in which inactivating mutations bring about irreversible functional damage to the protein product (21–23). Moreover, it has been observed that *p53* mutation patterns may be generally associated with carcinogen exposure and recent exciting data were obtained on the relationship with cigarette smoking (24).

The objective of our work was to evaluate, by an immunohistochemical method, 4-ABP–DNA adducts and *p53* overexpression in lesional and adjacent normal larynx tissues from smokers and non-smokers.

***Abbreviations:** 4-ABP, 4-aminobiphenyl; PAH, polycyclic aromatic hydrocarbons; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography/mass spectroscopy; PBS, phosphate buffered saline.

Materials and methods

Larynx tissues

Larynx tissues (both from biopsies and/or surgery) were obtained from 38 patients, diagnosed between January and December 1995 in the Department of Pathology at the University of L'Aquila. The group consisted of 23 men aged 27–83 years (mean ± SD 55 ± 14 years) and 15 women aged 25–68 years (45 ± 14 years), all without reported occupational exposure to 4-ABP. Eighteen patients were aged below 50 and 20 were over 50 years of age. Twenty patients were current alcohol consumers and 18 were not. One case (male, 40 years) was diagnosed as normal, 28 samples contained benign lesions (polyps) and nine were squamous cells carcinomas. Excluding the normal case, polyps and tumors were evaluated for 4-ABP adducts in both the lesion and surrounding tissue.

Information on ever/never smoking and alcohol consumption was obtained from the medical charts for all 38 patients. Patients were classified as smokers, if they were currently smoking at the time of diagnosis ($n = 24$). Patients smoking <200 cigarettes during their lifetime or who never used any other tobacco-related product were classified as non-smokers ($n = 14$).

Immunoperoxidase detection of 4-ABP–DNA adducts

Sections (5 μm) of archival formalin-fixed, paraffin-embedded larynx tissues were placed on slides coated with poly-L-lysine (Sigma Chemical Co., St Louis, MO). 4-ABP–DNA adducts were analyzed as previously described (18,25). After deparaffinization, slides were washed with phosphate buffered saline (PBS), treated with RNase (100 μg/ml Sigma) at 37°C for 1 h and washed again. They were subsequently treated with proteinase K (10 μg/ml, Sigma) at room temperature for 10 min to remove histone and non-histone proteins from DNA and to increase antibody accessibility. In order to denature DNA, the samples, after washing in PBS, were incubated with 4 N HCl for 10 min and with 50 mM TRIS base for 5 min at room temperature. This method does not alter adduct evaluation, as proved in previous studies (17). After washing with PBS, they were incubated with 0.3% H_2O_2 in methyl alcohol at room temperature for 30 min to quench endogenous peroxidase activity. Non-specific binding was blocked with 1.5% normal horse serum, and an incubation with the monoclonal anti-4-ABP–DNA antibody 3C8 diluted 1:5 in PBS with 1.5% horse serum was performed overnight at 4°C. The sections were then incubated with a biotinylated horse anti-mouse secondary antiserum (Vector Laboratories, Burlingame, CA), and the reaction was visualized with the ABC complex followed by diaminobenzidine (Vector). The samples were dehydrated in serial ethyl alcohol solutions and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Liver tissue from a mouse treated with 80 mg/kg 4-ABP and killed 24 h later was used as a positive control. Normal larynx tissue, adjacent to a polyp, from a non-smoker patient of the series under study, was used as a negative control.

In order to demonstrate staining specificity, a subset of slides from smokers and non-smokers were pretreated with DNase (Sigma) (100 μg/ml for 1 h at 37°C) before staining or stained with a non-specific antibody (8G1; 1:10 dilution of hybridoma supernatant) that recognized DNA damage produced by the photo-activated drug 8-methoxypsoralen (26) or with the specific 3C8 monoclonal antibody pre-absorbed with 4-ABP–DNA (1 μg/ml for 20 min at room temperature). Because of the limited number of slides from each patient, these controls could not be run for all samples. Depending on the width of the section, the tissue area was explored in terms of 5–10 fields, within each of which 100 randomly selected cells were analyzed, and this was repeated for each tissue compartment (adjacent, polyp and tumor tissue). An image analyzer (Vidas Plus, Kontron Elektronik, Software Ibas 2.0) was used to measure the relative intensity of nuclear staining. The image was obtained in black and white and the optical density was measured as absorbance indicator. As all the measured sections were of the same thickness (5 μ) this parameter was not taken into account. The image analyzer expressed the results for each sample as the average ± SD optical density (IOD) multiplied by 1000.

Immunoperoxidase detection of p53 overexpression

Immunohistochemical staining for *p53* was performed essentially as for 4-ABP except that pre-treatment with RNase, proteinase K and HCl was omitted. A microwave antigen retrieval pre-treatment was performed utilizing 0.01 M sodium citrate, pH 6.0, and a domestic microwave for 10 min (27). The anti-*p53* CM 1 polyclonal antiserum was utilized at a 1:100 dilution (28). The sections were weakly counterstained with Harris hematoxylin. A colon carcinoma with documented *p53* nuclear immunostaining was used as a positive control. Normal larynx epithelium was used as a negative control.

Statistical analysis

Single observations of the adduct level are presented as the average ± SD values of optical density that were obtained as described above. The statistical analysis was performed on the average measures of optical density. Normality of raw and log-transformed data were assessed by the Wilk–Shapiro *W*-test.

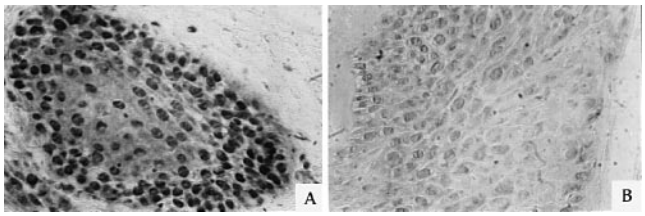

Fig. 1. Immunoperoxidase staining for 4-ABP–DNA adducts of larynx tissue from a smoker (**A**) and a non-smoker (**B**) (mean relative staining intensity: 430 ± 47 and 52 ± 25 respectively) (×200).

Since data were compatible with a lognormal distribution, on the raw data a non-parametric test was applied (the rank sum test) and on the log-transformed data, a *t*-test (unpaired) assuming equal variances, was performed.

Results

Immunoperoxidase detection of 4-ABP–DNA adducts

Representative staining patterns for 4-ABP–DNA adducts in polyp surrounding tissues from a smoker and a non-smoker are illustrated in Figure 1A and B respectively. Specific nuclear staining is observed in the smoker, whereas a much weaker staining can be detected in the non-smoker sample.

The staining controls were run on tissues from different subjects, because of the limited number of slides available for each individual. It has to be underlined that all the controls have been performed in polyp surrounding tissues. Treatment with a non-specific antibody able to recognize DNA damage produced by 8-methoxypsoralen decreased staining from 430 ± 47 to 45 ± 13 in a smoker and from 91 ± 23 to 38 ± 11 in a non-smoker. Pre-absorption of the primary antibody with 4-ABP–DNA before use decreased staining from 200 ± 38 to 30 ± 8 in a smoker and from 106 ± 24 to 35 ± 10 in a non-smoker. Pre-treatment of slides with DNase also decreased mean relative staining from 206 ± 34 to 36 ± 9 in a smoker and from 149 ± 28 to 34 ± 9 in a non-smoker, as did the omission of the primary antibody performed in a smoker (from 190 ± 15 to 48 ± 14).

The Shapiro–Wilk *W*-test that was applied to staining average values in lesional and adjacent tissue showed that the raw data were not normally distributed, whereas the log-transformed values were compatible with a normal distribution. Thus, it can be stated that average optical density values follow a lognormal distribution. When comparing the smoker and non-smoker groups, a higher staining level for 4-ABP–DNA adducts was observed in the larynx tissue adjacent to the lesion of smokers (median: 173.5; 1st quartile: 99.5; 3rd quartile: 209.0; geometric mean: 159.9; range: 63.0–430.0) compared with non-smokers (median: 75.5; 1st quartile: 44.0; 3rd quartile: 129.0; geometric mean: 74.0; range: 34.0–165.0). The difference in staining level between smokers and non-smokers was statistically significant both in the rank-sum test on the raw data ($P = 0.0007$) and in the unpaired *t*-test on log-transformed data ($P = 0.0002$).

The same results were obtained for adduct staining levels in the polyp lesional tissue: polyps of smokers showed higher values than non-smokers that were statistically significant both on the rank-sum test for raw data ($P = 0.0043$) and on the *t*-test for log-transformed data ($P = 0.0019$). The difference in the adduct staining levels between smokers and non-smokers in tumor tissue was not evaluated due to the small number of samples (only two tumor cases in the non-smoker group).

Distributions of adduct staining levels in lesional and adja-

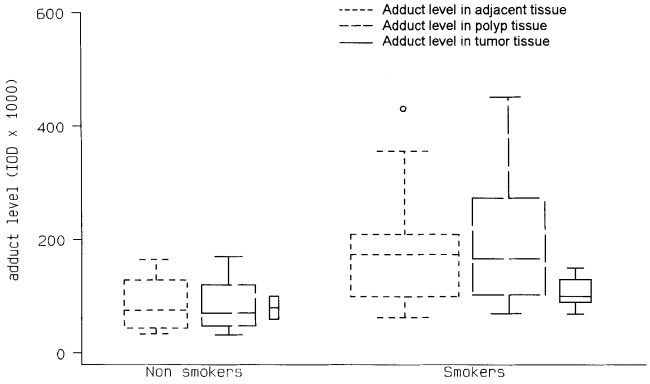

Fig. 2. The relative intensity distribution of 4-ABP–DNA adducts in adjacent, polyp and tumor tissue of smokers and non-smokers. The lower and upper edges of the box are the 25 and 75 percentiles. Median values are shown by the line within the box. The width of each box is proportional to sample number.

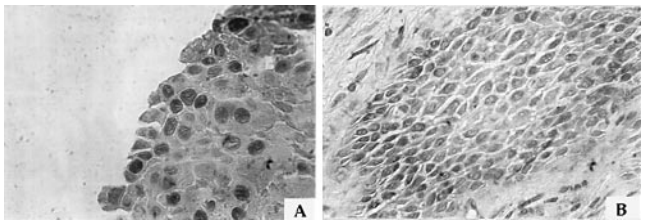

Fig. 3. Immunohistochemical nuclear overexpression of p53 protein from a tumor (**A**) and a polyp (**B)** sample (×200).

Table I. Immunohistochemical staining for 4-ABP–DNA adducts in larynx tissue by smoking habit

	Surrounding tissue	Polyp	Tumor
Non-smokers	86 ± 47 (14)	87 ± 50 (12)	80 ± 28 (2)
Smokers	*182 ± 96 (24)	*193 ± 112 (16)	**154 ± 50 (7)

*$P < 0.05$, **$P = 0.1$ (Kolmogorov–Smirnov test versus non-smokers).

cent tissues are reported in Figure 2 in relation to smoking habit. When adducts in lesion and adjacent tissue in each single patient were evaluated, no difference was found in the polyp group. Some difference was detected in the cancer group, where the lesion stained invariably fainter than the adjacent tissue, and also showed staining intensity distribution variability. No significant correlations were found between adduct levels and patients' sex, age and alcohol consumption habit.

Immunoperoxidase detection of p53 overexpression

Nuclear overexpression of *p53* was observed in four (44%) of the nine malignant tumors and in one (3.5%) of the 28 polyps. Representative staining of a tumor and a polyp are shown in Figure 3A and B respectively. Weak cytoplasmic staining was detected in a few samples. The highest 4-ABP–DNA adduct level observed within the overall series of patients (430 ± 47) was shown to correspond to the only *p53* positive case in the benign lesion group. No relationship was found between 4-ABP relative staining intensity and *p53* overexpression.

Discussion

4-ABP is a carcinogenic and mutagenic aromatic amine found in air pollution, azo dyes and cigarette smoke, for which it is considered an exposure marker (29).

We previously studied 4-ABP–DNA adducts in bladder tumors and demonstrated a significantly higher mean adduct level in smokers than non-smokers using the same immunoperoxidase method with the monoclonal antibody, 3C8, as used in the present study (18). Here, 4-ABP–DNA adducts were evaluated in larynx tissues with proliferative abnormalities, including polyps and malignant tumors, from smokers and non-smokers. Our aim was to determine whether in larynx tissue 4-ABP–DNA adducts, detected by an immunohistochemical method, are a useful marker for discriminating smokers from non-smokers.

Our results in lesion adjacent tissues clearly demonstrate that in larynx tissue 4-ABP–DNA adducts are formed and that a statistically significant difference is detectable when comparing smokers with non-smokers. The data also show that the 3C8 antibody is sufficiently sensitive for evaluating 4-ABP–DNA adducts. An ~6- and 5-fold range in DNA adducts was detected in the smoker and non-smoker groups respectively, most likely due to inter-individual genetic variation in carcinogen activation and detoxification and/or DNA damage repair (30–32). The low staining observed in the non-smoker group was also observed in our studies of T1 bladder cancer patients (18) and may be the result of exposure to nitropolycyclic aromatic hydrocarbons.

A recent report (33) suggested that lung tissue of non-smokers contains adducts resulting from exposure to 4-nitrobiphenyl, which can be metabolically reduced to *N*-hydroxy–ABP, an intermediate in the oxidative metabolism of 4-ABP.

When the polyp group was analyzed by smoking habit, a significant difference was observed, suggesting a substantial similarity in metabolism and/or repair of adducts between normal and benign proliferative tissues in the larynx. In contrast, in tumors, adduct levels were not related to smoking status, perhaps as a result of a lower mean adduct level in tumor tissue because of rapid cell turnover and accelerated DNA metabolism (34) and/or reduced carcinogen metabolism (35). Others have also shown lower adduct levels in larynx tumors compared with normal adjacent tissue (36).

At the individual level, no apparent difference between the two compartments was detected in polyps, whereas in tumors the mean lower intensity was the result of a variable staining, probably due to tumor heterogeneity.

A significant correlation between smoking status and *N*7-alkylguanine adducts in larynx tissue by ^{32}P-postlabeling was reported by Szyfter *et al.* (10). In contrast with our observation, these authors also detected a non-significantly higher mean adduct level in tumors compared with adjacent tissue. Such a discrepancy may be due to the relatively small number of patients in both studies and to tumor heterogeneity. In another study by Degawa *et al.* (11) on larynx tumors, a significant correlation was also found between adduct levels and smoking status by using ^{32}P-postlabeling with both the butanol extraction and the nuclease P1 enhancement methods. Moreover, the authors observed a positive correlation between adducts and P450 levels but not *N*-acetyltransferase activity. On the basis of these data, they suggested that aromatic amine adducts were not present. However, in a recent comparison of 4-ABP–DNA adduct detection by postlabeling, an immunoassay by an

alternate method to that used here, and GC/MS, a good correlation was observed between all three methods but the postlabeling method was found to underestimate adducts by 20- to 60-fold (33). The authors suggested that the underestimation by postlabeling may be due to the resistance of lung DNA to digestion. The immunohistochemical method used in the present study was validated by analysis of liver tissues from 4-ABP-treated mice by GC/MS (17). The limit of sensitivity is estimated to be about one adduct/10^7 nucleotides. Thus, adduct levels in the larynx tissue may be in the range of $1/10^{6-7}$. However, this value is an estimate. While antibody 3C8 did not cross-react with the other aromatic amine C-8 guanine adducts tested (17), it is possible that, given the complex exposure of humans, other adducts may be present and recognized. While this possibility makes absolute quantitation difficult, the data strongly indicate that 4-ABP–DNA adducts are present in larynx tissue. All the samples were analyzed for *p53* in order to evaluate its overexpression in these proliferative lesions. In agreement with other studies, *p53* overexpression was observed in 44% of the cancer cases (19,20). In the benign lesion group of 28 patients, only one had nuclear *p53* localization (3.5%), this sample also presented the highest 4-ABP–DNA adduct level of all the tested cases. A direct link between a defined chemical carcinogen (benzo[*a*]pyrene) and *p53* mutational spectrum has recently been demonstrated in lung cancer (24). In our previous study on bladder cancer, where a significant correlation between adduct levels and smoking status was found, a significant correlation was also demonstrated with respect to *p53* overexpression. About this point, it has to be noted that in bladder tumors, *p53* alterations are not a specifically late event, as they may also be detected in the early stages of the disease (37). On the contrary, in the present study on larynx, no significant relationship between adduct levels and mutated *p53* has been observed. The only case in our series of a benign lesion presenting a very high adduct level, associated with *p53* positivity in a smoker, might indicate a possible connection between adduct formation rate in a proliferating tissue and *p53* alteration probability. In any case, at least from our data, polyps in larynx tissue do not seem very good candidates for undergoing *p53* modifications. Probably, a larger series of patients is needed to obtain more substantial data about the relationship between *p53* overexpression/mutation and adduct levels in larynx tissue.

In conclusion, the data obtained in our patients with proliferative processes of the larynx, indicate that 4-ABP–DNA adducts correlate in a significant fashion with the smoking habit. They also suggest that larynx tissue is able to metabolically activate 4-APB to reactive intermediates.

Lastly, the utilized immunohistochemical method has shown a fine selectivity and sensitivity for evaluating 4-ABP–DNA adducts in human samples, and the 3C8 anti-4-ABP–DNA monoclonal antibody has demonstrated to work satisfactorily in different tissues (18) and to be thus suitable for extensive applications in large series of patients.

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