ACCELERATED PAPER

Immunohistochemical quantitation of 4-aminobiphenyl-DNA adducts and p53 nuclear overexpression in T1 bladder cancer of smokers and nonsmokers

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An immunoperoxidase method, using a monoclonal antibody which recognizes 4-aminobiphenyl (4-ABP)-DNA adducts, was developed for the detection and quantitation of DNA damage in bladder tissue and applied to stored paraffin blocks of transurethral resection specimens of 46 patients with T1 bladder cancer. Mean relative staining intensity for 4-ABP-DNA adducts was significantly higher in current smokers (275 ± 81, n = 24) compared to nonsmokers (113 ± 71, n = 22) (P < 0.0001). There was a linear relationship between mean levels of relative staining and number of cigarettes smoked with lower levels in the 1–19 cig./day group (205 ± 30, n = 5), compared to the 20–40 (289 ± 40, n = 7) and the >40 cig./day group (351 ± 57, n = 3)(P < 0.001). Nuclear overexpression of p53, analyzed by immunoperoxidase staining, was observed in 27 (59%) of the 45 stage T1 tumors analyzed. There was a significant correlation between p53 overexpression and recurrence of disease (odds ratio = 12.3, P < 0.01). Nuclear staining of p53 was also correlated with smoking status, cig./day and 4-ABP-DNA adducts. This work demonstrates that the immunohistochemical method has sufficient sensitivity for detection of 4-ABP-DNA adducts in human bladder samples. The method has several advantages including small sample size, the possibility of retrospective analysis of stored paraffin blocks, the ability to analyze binding in specific cell types, and a relatively low cost.

Introduction

Bladder cancer is strongly linked to occupational and environmental exposures to chemical carcinogens with tobacco smoking considered an important cause (1). In western countries ~50% of male and 25% of female cases may be attributable to smoking (2). Carcinogens in cigarette smoke include polycyclic aromatic hydrocarbons, nitrosamines, benzene and aromatic amines (3). In contrast to cancer of the upper respiratory tract,

*Abbreviations: 4-ABP, 4-aminobiphenyl; GC/MS, gas chromatography/mass spectroscopy; IHC, immunohistochemical; TUR, transurethral resection; OR, odds ratio; CI, 95% confidence interval.

for which tobacco specific N-nitrosamines and polycyclic aromatic hydrocarbons have been implicated as causative agents, bladder cancer is more likely caused by aromatic amines (4).

4-Aminobiphenyl (4-ABP*), a known bladder carcinogen in both humans and experimental animals, is metabolically activated to several electrophilic intermediates which covalently bind to DNA (4). The predominant adduct is N-(2'-deoxyguanosin-8-yl)-ABP, which accounts for ~70% of the adducts formed (5). The covalent modification of DNA by carcinogens such as 4-ABP appears to be a critical event in chemical carcinogenesis. As a result, the analysis of carcinogen-DNA adducts is considered a useful means of assessing exposure to chemical carcinogens. Evidence for human exposure to 4-ABP and its metabolic activation to a reactive intermediate was initially obtained from the detection of 4-ABP-hemoglobin adducts, with higher levels being observed in cigarette smokers versus nonsmokers (6–7). More recently, a number of methods have been developed for quantitation of DNA damage resulting from environmental or occupational exposure to 4-ABP including immunoassays, ^{32}P-postlabeling-HPLC and gas chromatography-mass spectroscopy (GC-MS) (8). These methods require the isolation of bulk DNA from tissues and thus do not allow the detection of adducts in specific cell types. In addition, some methods require relatively large amounts of DNA for analysis, limiting their application.

We previously developed a monoclonal antibody, 3C8, recognizing 4-ABP-DNA adducts (9). The antibody is highly specific for 4-ABP-DNA and, at the highest concentrations tested, did not recognize the DNA adducts of several other aromatic amines including 1-aminopyrene, 8-nitro-1-aminopyrene and 6-nitro-1-aminopyrene. A quantitative immunofluorescence method for detecting adducts was developed and tested in 4-ABP-treated mice. There was a dose-related increase in specific nuclear staining in liver and bladder. DNAs extracted from liver tissues were also analyzed for 4-ABP adducts after alkaline hydrolysis by GC-MS. A good correlation (r = 0.98, P < 0.0001) was found between DNA damage levels determined by the two methods.

p53 is a common target for carcinogenic agents and frequently mutated in bladder cancers. The product of the gene is a nuclear phosphoprotein involved in cell cycle regulation. Levels of p53 increase in response to DNA damage, arresting the cell cycle in G1 phase and allowing time for DNA repair (10). Compared with the wild-type protein, most mutant p53 products have a prolonged half-life and can be detected using immunohistochemical (IHC) assays. For patients with superficial bladder cancer, a correlation between staining for p53 and invasive behaviour of the tumor with a poor clinical outcome has been proposed (11–13). p53 overexpression is also associated with cigarette smoking (14–16).

In this study, we report on the development of an immunoperoxidase method for direct quantitation of 4-ABP-DNA adducts in paraffin embedded bladder samples of patients with T1 bladder cancer. We assessed the relationship between the levels of 4-ABP-DNA adducts and cigarette smoking status. We also investigated the relationship between p53 overexpression and cigarette smoking or 4-ABP-DNA adduct levels. One additional aim of the study was to confirm the usefulness of IHC staining of p53 overexpression as a prognostic marker in superficial bladder cancer.

Materials and methods

The medical charts of consecutive patients with bladder cancer, seen at the Department of Urology of the Catholic University in Rome from May 1985 to May 1989, were reviewed. We studied those patients with transurethral resection (TUR) specimens diagnosed as transitional cell carcinoma at the T1 stage according to the TNM system, with no previous adjuvant therapy or history of bladder cancer. We obtained a total of 46 consecutive cases (37 males and 9 females). All tumors were pathologically graded as grade 1 ($n =$ 11), 2 ($n = 23$) and 3 ($n = 12$). The median age of the patients was 64 years (range 47 to 74 years) median follow-up after TUR was 92.5 months (range 72 to 118 months). Of the 46 patients, 24 (52%) developed a recurrence and 6 (13%) of these progressed to a more advanced stage and higher pathological grade. Patients developing muscle-invasive tumors underwent a radical cystectomy. Information on ever/never smoking was abstracted from the medical charts for all 46 patients. Patients were classified as smokers if they smoked for a defined period during their lifetime and were currently smoking at the time of diagnosis ($n = 24$). Patients smoking <200 cigarettes during their lifetime and who never used any other tobacco-related products were classified as nonsmokers ($n = 19$). Patients who stopped smoking at least two years before cancer diagnosis were classified as exsmokers ($n = 3$). Information about the type of tobacco (black air cured or blond flue cured) was not avalaible. Information on number of cigarettes per day (cig./day) was avalaible for only 15 patients. In this group, smoking exposure was classified into three categories: 1–19 ($n=$ 5), 20–40 ($n = 7$) and >40 ($n = 3$) cig./day.

Immunoperoxidase detection of 4-ABP-DNA adducts

Five micrometer sections of archival formalin-fixed, paraffin-embedded bladder tissue were placed on slides coated with poly-L-Lysine (Sigma, Chemical Co., St Louis Md). 4-ABP-DNA adducts were analyzed essentially as described previously (9). After deparaffinization, slides were washed with PBS, treated with RNase (100 mg/ml Sigma) at 37°C for 1 h and washed with PBS. They were treated with proteinase K (10 mg/ml, Sigma) at room temperature for 10 min to remove histone and non-histone proteins from DNA and increase antibody accessibility. To denature DNA, slides, after washing in PBS, were incubated with 4N HCl for 10 min and then with 50mM Tris Base for 5 min, both at room temperature. After washing with PBS, slides were incubated with 0.3% H_2O_2 in methyl alcohol at room temperature for 30 min to quench endogenous peroxidase activity. Nonspecific binding was blocked with 1.5% normal horse serum and slides incubated with the anti-4-ABP-DNA monoclonal antibody 3C8 (9) (1:5 dilution in 1.5% horse serum) overnight at 4°C. Tissues were then incubated with a biotinylated horse anti-mouse secondary antiserum (Vector Laboratories, Burlingame, CA), and reactivity was visualized with ABC and diaminobenzidine (DAB) kits (Vector) used as directed by the manufacturer. Slides were dehydrated and cleaned in serial ethyl alcohol and xylene and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Liver tissue from a Balb/c femal mouse treated with 80 mg/kg 4-ABP and sacrificed 24 h later was used as a positive control. Normal bladder from a TUR specimen of a nonsmoking patient was used as a negative control. To demonstrate staining specificity, a subset of slides from smokers and non-smokers were pretreated with DNase (100 mg/ml for 1 h at 37°C) before staining, stained with a nonspecific antiserum (8G1; 1:10 dilution of hybridoma supernatant) recognizing DNA damage produced by the photoactivated drug 8-methoxypsoralen (17) or with antiserum preabsorbed with 4-ABP-DNA (1 μg/μl for 20 min at room temperature) before use. Because of the limited number of slides from each individual these controls could not be run for all samples. A Cell Analysis System 200 microscope (Becton Dickinson, San Jose, CA) was used to measure the relative intensity of nuclear staining in 100 randomly selected cells of bladder epithelium using the Cell Measurement Program software package. Data presented are the object average optical density multiplied by 1000.

Immunoperoxidase detection of p53 overexpression

Immunohistochemical staining for p53 was performed essentially as for 4-ABP except that pretreatment with RNase, proteinase K and HCl was omitted.
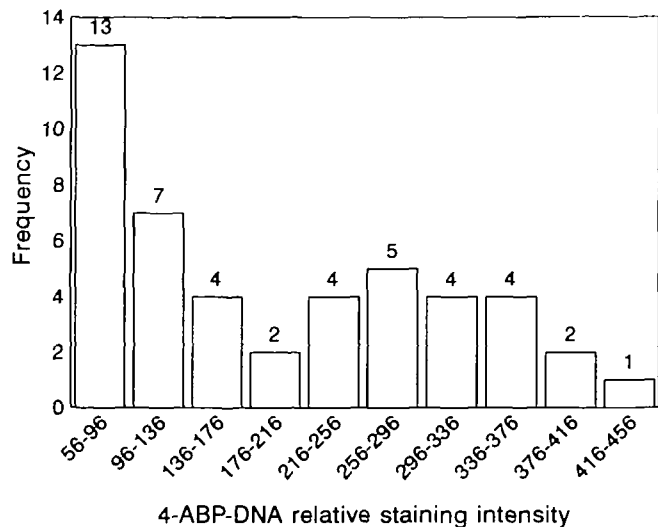
Fig. 1. Distribution of relative intensity of peroxidase staining for 4-ABP-DNA adducts in human urinary bladder cancer mucosa of smokers and nonsmokers.

Tissue sections were incubated overnight at 4°C with the mouse monoclonal antibodies PAb 1801 (Ab2 Oncogene Science Inc. Cambridge MA) at 1:100 dilution and DO-7 (gift of Dr D.P.Lane, Dept. of Biochemistry, University of Dundee, Scotland, UK) at 1:1400 dilution. A counterstain of 1% modified Harris hematoxilin was used. A colon carcinoma with known *p53* mutation and documented accumulation of p53 protein by immunohistochemical analysis was used as a positive control. Normal urothelium, used as a negative control, demonstrated no immunoreactivity.

Three investigators, without prior knowledge of tumor stage or clinical follow-up, separately reviewed the slides to determine p53 staining. Depending on the percentage of nuclei exhibiting positive staining, tumors were categorized as follows: no nuclear reactivity, patchy (1–24% positive nuclei), heterogeneous nuclear reactivity (25–49% of positive nuclei) and intense homogeneous nuclear reactivity (50–100% of nuclei).

Data analysis

Differences between two means was determined by *t*-test and one-way analysis of variance was used to compare the distribution of 4-ABP relative staining intensity between different categories. Data were analyzed both as a continous variable and dicotomized into high and low relative staining intensity. As the distribution of 4-ABP relative staining was clearly bimodal (Figure 1), we arbitrarily chose a relative staining intensity of 179 as the cut-off point between low and high staining. Odds ratios for p53 overexpression and recurrence and for p53 and smoking status and 4-ABP staining intensity were estimated and tested by χ^2 test with or without Yates' correction for continuity depending on the number in each entry of 2×2 tables. All statistics were carried out by SAS and EGRET software packages.

Results

Immunoperoxidase quantitation of 4-ABP-DNA adducts

Higher levels of specific nuclear staining were observed in bladder tissue of smokers compared to nonsmokers. Representative staining of a smoker and a nonsmoker are illustrated in Figure 2A and B, respectively. Staining was quantitated in 100 randomly selected cells in the bladder epithelium. To demonstrate the staining specificity, several samples were stained under various control conditions. Because limited slides were available for each individual, these controls were run on different subjects. Preabsorption of primary antibody with 4-ABP-DNA before use decreased relative staining intensity in a smoker from 271 $\pm$ 30 to 52 $\pm$ 15 (Figure 2C) and in a nonsmoker from 132 $\pm$ 13 to 33 $\pm$ 2 (data not shown). Staining with a nonspecific antibody recognizing DNA damage produced by 8-methoxypsoralen decreased staining from 326 $\pm$ 37 to 44 $\pm$ 4 in a smoker (Figure 2D) and from 79 $\pm$ 5 to
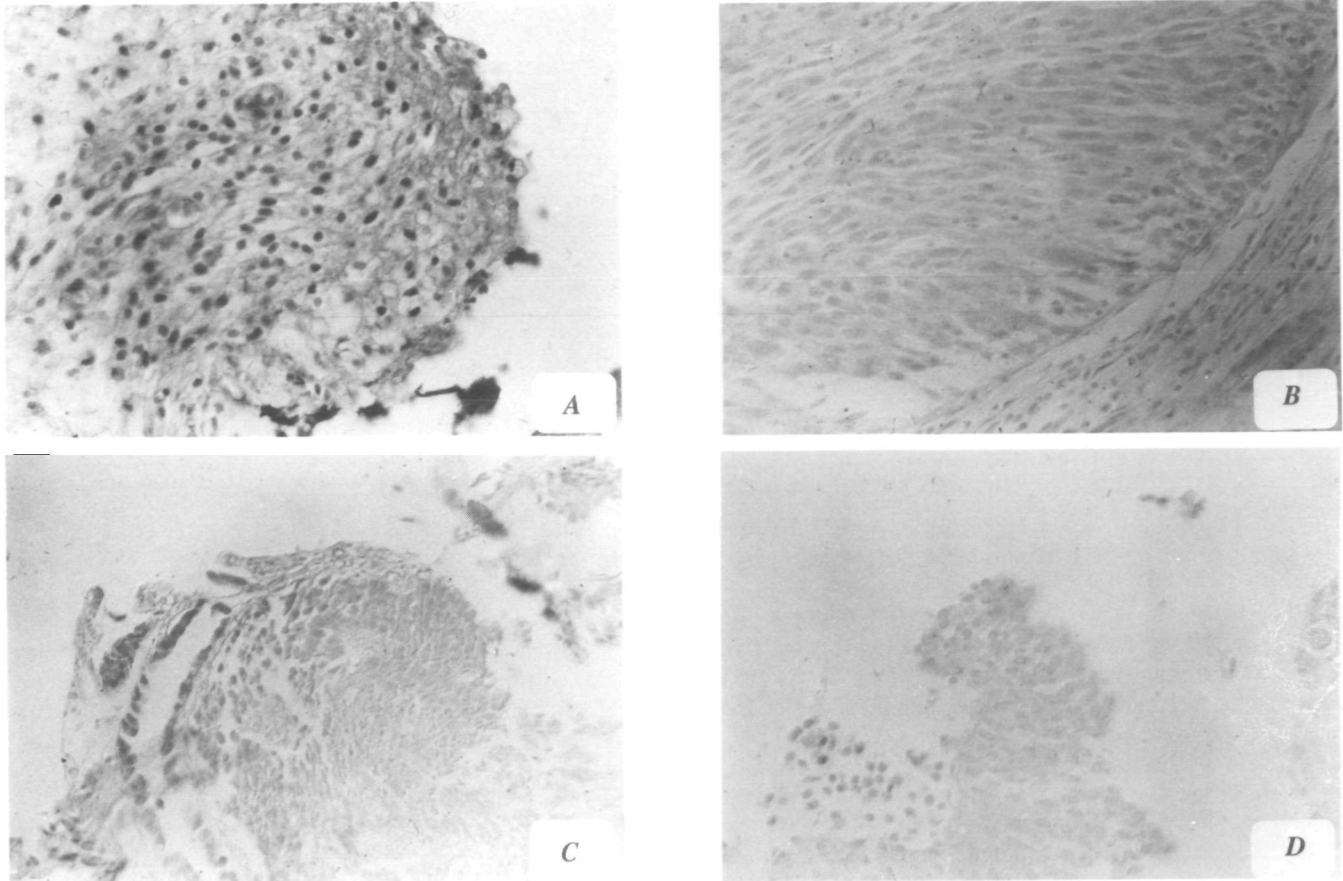
Fig. 2. Immunoperoxidase staining for 4-ABP-DNA of tumor bladder tissue from a smoker (**A**) (mean relative staining intensity=133 ± 35) and a nonsmoker (**B**) (mean = 86 ± 8). As controls, tumor mucosa from two different smokers were stained with antibody 3C8 preabsorbed with 4-ABP-DNA before use (**C**) (mean = 52 ± 15) and with a nonspecific antibody recognizing 8-methoxypsoralen-DNA (**D**) (mean = 48 ± 6) (× 400).

48 ± 6 in a nonsmoker (data not shown). Pretreatment of slides with DNase also decreased mean relative staining from 300 ± 33 to 33 ± 4 in a smoker and from 56 ± 3 to 34 ± 3 in a nonsmoker as did omission of primary antibody, performed only in a smoker (from 179 ± 15 to 44 ± 4) (data not shown). There was an ~3-fold range in relative staining in both smokers (179 ± 15 to 427 ± 98) and the combined non and exsmokers (56 ± 3 to 170 ± 40). Because levels of 4-ABP-DNA adducts in urothelial DNA of exsmokers (123 ± 26, n = 3) were not significantly different from those of nonsmokers (111 ± 76, n = 19), they were combined for further analysis. Mean relative staining in current smokers (275 ± 81, n = 24) was significantly (P < 0.0001) elevated compared to nonsmokers (113 ± 71, n = 22) (Table I, Figure 3A), mean level of relative staining increased with dose with lower levels in the 1–19 cig./day group (205 ± 30, n = 5), compared to the 20–40 (289 ± 40, n = 7) and the >40 cig./day (351 ± 57, n = 3) (P < 0.001) (Figure 3B).

4-ABP-DNA adducts were not associated with age (P = 0.559) or sex (P = 0.961). A grading-related increase in mean relative staining was observed, with lower levels of 4-ABP-DNA adducts in the G1 (159 ± 102) compared to the G2 (206 ± 103) or G3 grade (217 ± 134) (Table I). This relationship was not statistically significant (P = 0.559).

Immunoperoxidase quantitation of p53 overexpression

Nuclear overexpression of p53 was observed in 27 (58.6%) of the 45 stage T1 tumors analyzed. Typical staining was localized

Table I. Immunohistochemical staining for 4-ABP-DNA adducts in bladder tumor tissue and clinico-pathological variables, smoking status and p53 nuclear overexpression

Variables	Group	No.	Mean relative staining intensity (± SD)
Sex	male	37	197 (114)
	female	9	199 (104)
Age	<65	23	217 (125)
	≥65	23	186 (100)
Grading	G1	11	159 (102)
	G2	23	206 (103)
	G3	12	217 (134)
Recurrence	No	22	194 (122)
	Yes	24	201 (102)
Smoking	nonsmoker[a]	22	113 (71)
	smoker	24	275 (81)[b]
p53 staining	Negative	18	160 (91)
	Positive	27	227 (116)[c]

[a]Includes three exsmokers.
[b]t-test with P < 0.0001 compared to smokers.
[c]t-test with P < 0.05 compared to p53 negative tumors.

in the nuclei with variable but not appreciable cytoplasmic staining (data not shown). Identical results were obtained with both antisera. Depending on the percentage of positively stained cells, three groups of tumors were identified. In 5 of 46 tumors (10%), <25% of cells exhibited a positive staining reaction for p53. In 12 samples (26%), the number of cells
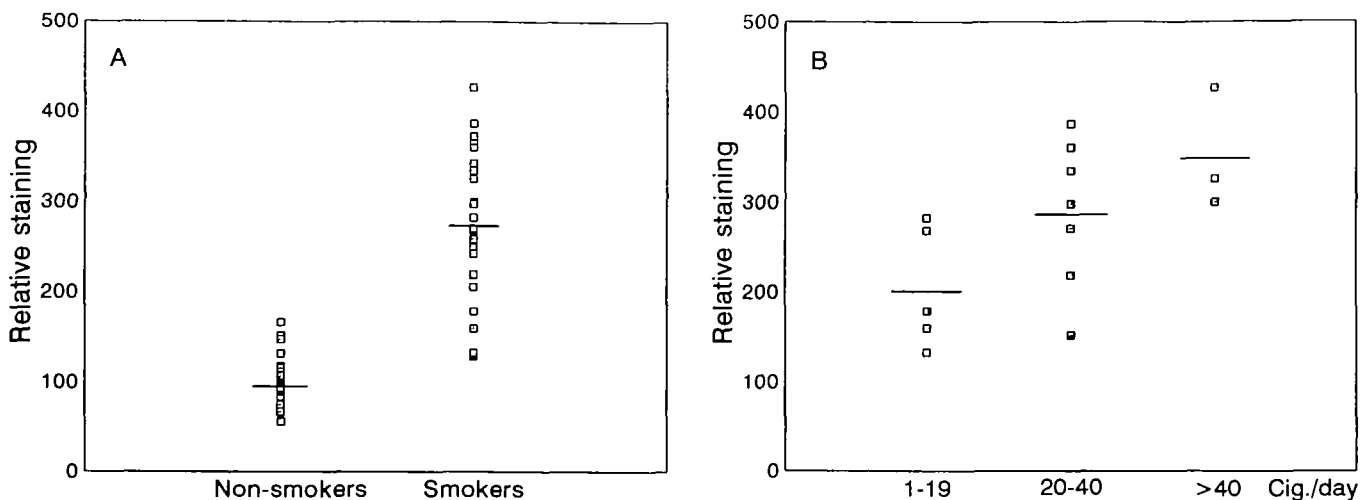
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Fig. 3. Comparison of relative intensity of staining for 4-ABP-DNA damage of tumor bladder mucosa of smokers and nonsmokers (A) and comparison of relative intensity in relation to cig./day (B). Median values are shown by the line within the points.

stained positively was >25% and <50%. In the third group of 10 tumors (21%), nuclear reactivity was present in >50% of cells. With a median follow-up of 92.5 months, 24 (52%) of 46 patients presented a recurrence and 6 (13%) of these progressed to a more advanced stage. Twenty (83%) of the 24 patients with recurrences were positive for p53 staining. A strong correlation was found between p53 nuclear overexpression and recurrence of disease (OR=12.3, $P < 0.01$). p53 was not associated with other clinico-pathological variables analyzed (age, sex, and grade). Nuclear staining of p53 protein was observed in 17 of 24 (66%) current smokers and in 10 of 21 (45%) nonsmokers. There was a nonsignificant association between p53 overexpression and smoking status with an OR of 2.7 (CI 0.7–13.7, $P = 0.099$). We found a dose–response relationship between cigarettes smoked per day and p53 overexpression; corrected odds ratios were 3.0 ($P > 0.1$) and 19.0 ($P < 0.05$) for 1–19 cig./day and $\geq$20 cig./day, respectively, compared with nonsmokers. A relationship was also found by univariate ($P < 0.05$) and stratified (OR=2.9, $P = 0.099$) analysis between 4-ABP relative staining intensity and p53.

Discussion

Previously, we used an indirect immunofluorescence method to detect 4-ABP-DNA adducts in liver and bladder of treated mice. A good correlation between relative fluorescence intensity and DNA adduct levels in liver tissue determined by GC/MS was observed (9). Here, a quantitative immunoperoxidase method for direct monitoring of smoking related 4-ABP-DNA adducts in paraffin embedded specimens of human tumor bladder tissue was developed. This method is an improvement over the previous immunofluorescence method since it allows direct quantitation of staining on the slides. As for the immunofluorescence method, we obtained a dose–response relationship between immunoperoxidase relative staining intensity of frozen and paraffin sections of liver tissue and adduct levels measured by GC/MS (not shown).

Our results indicated that the prevalence of 4-ABP-DNA adducts in human bladder is significantly associated with smoking status (Figure 3A). A linear dose–response relationship was observed between levels of staining for 4-ABP-DNA and number of cigarettes smoked per day (Figure 3B). Quitting

smoking is associated with a dramatic drop in the levels of 4-ABP-DNA adducts. The exsmokers who had quit a minimum of two years prior to diagnosis had mean relative staining levels similar to those of nonsmokers. The weak staining observed in nonsmokers may be due to passive smoking exposure. Low levels of 4-ABP-hemoglobin adducts have been observed in nonsmokers (6–8) and suggested to be due to exposure to environmental tobacco smoke (8,18). 4-Nitrobiphenyl in diesel exhaust has also been suggested as a source of 4-ABP adducts (19). However, low levels of nonspecific binding of primary or secondary antisera may also be responsible for the weak staining in nonsmokers. We previously demonstrated no cross-reactivity of 3C8 with several other aromatic amine-DNA adducts but there may still be recognition of other untested adducts (9). The controls run here also confirmed the specificity of the method on human samples.

It is difficult to convert relative staining intensity of human bladder tissue to absolute adduct levels. However, the standard curve of immunoperoxidase staining of paraffin liver tissue of 4-ABP-treated-mice versus absolute adduct levels determined by GC/MS could be used to obtain an estimate. These data suggest that the human samples contain ~1–7 adducts/10^6 nucleotides. The limitations of this estimate are the species and tissues extrapolations and the possibility of multiple exposures in humans compared to the single 4-ABP exposure of mice. It would be necessary to derive a standard curve of human bladder tissues to overcome these limitations. Lower levels of adducts have been reported using other methods of analysis. An immunoassay using a polyclonal antiserum to the 4-ABG-guanosine monoadduct of DNA isolated from normal urinary bladder found levels of adduct up to 5/10^7 (20) while a GC/MS method found levels up to 4/10^8 (21). [^{32}P] Postlabeling with the butanol extraction procedure of bladder biopsy samples indicated a mean level of N-(2'-deoxyguanosinyl)-ABP of 1.5/10^8 in smokers (5). Adduct levels have also been monitored in exfoliated bladder cells by postlabeling. Using an excess ATP method, levels in exfoliated cells of black and blond tobacco smokers were 8.8 and 7.7/10^9, respectively (22).

The grading-related increase in mean relative staining shows that 4-ABP-DNA adducts are related to histologic changes in the epithelium of urinary bladder. A previous report (18)

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showed that smoking habit is associated with an increase of cell rows and number of cells with atypical nuclei. In our study, poorly differentiated T1 bladder cancer had higher mean staining than moderately or well differentiated tumors.

There was an ~3-fold range in relative staining in both smokers and nonsmokers, suggesting the importance of interindividual differences in metabolism of 4-ABP and/or repair of DNA damage. Furthermore, the total distribution of 4-ABP-DNA adduct mean staining intensity in current smokers and nonsmokers was clearly bimodal, with two convex curves (Figure 1). Liver N-acetyltransferase (NAT2) and bladder NAT1, because of their major role in the biotransformation of aromatic amines and their polymorphisms affecting metabolism, would be expected to modulate the levels of 4-ABP-DNA adducts in the target organ (19).

T1 bladder cancer is an interesting model to study the impact of the *p53* gene product on the development and progression of cancer. First, the high incidence permits a thorough investigaton of this tumor. Furthermore, in contrast to other tumors, all stages of the disease from precursor lesions and Tis (*in situ*) to metastatic disease can be examined, allowing the analysis of molecular alterations in the course of malignant disease. The present study confirms earlier reports (11–13) which demonstrated that p53 nuclear overexpression occurs with high frequency in T1 bladder tumors. The multivariate analysis demonstrated p53 to be an important and independent prognostic factor for disease recurrence and progression. Thus, loss of p53 function is an important step in the development of bladder cancer.

Increased prevalence of p53 nuclear staining was found in smokers compared to nonsmokers. Spruck *et al.* (14) first reported a relationship between smoking status and p53 genotype abnormalities, consistent with other successive findings (15–16). Our results also showed an association between p53 overexpression and smoking status or cigarettes per day. In addition, we explored the possible dose–response relationship between 4-ABP-DNA adduct staining and p53. The association by stratified analysis was significant, supporting the hypothesis that 4-ABP derived from cigarette smoking may induce *p53* mutations as a result of direct interaction with DNA.

We and others have used IHC to analyze DNA damage in humans resulting from exposure to several chemical carcinogens. Aflatoxin B1-DNA adducts have been detected by immunofluorescence staining in liver tissue obtained at the time of surgery (20) as well as in biopsies obtained for diagnosis of liver cancer (21). Polycyclic aromatic hydrocarbon adducts in oral mucosa cells of smokers (22), lymphocytes of coke oven workers and controls (23) and lung, cervix and placental tissues (24) have been monitored by immunofluorescence or immunoperoxidase techniques. O^6-ethyl guanine has also been monitored in lymphocytes of patients treated with alkylating chemotherapeutic agents (25).

The immunoperoxidase method for 4-ABP-DNA developed here will be useful to further investigate interindividual differences in damage and, potentially, the risk for cancer development. This method provides both good selectivity and sensitivity which are required for the detection and quantification of 4-ABP-DNA adducts at the levels found in human samples and can be applied to stored paraffin samples (our specimens were 6–10 years old). Immunohistochemical detection of 4-ABP-DNA adducts as a potential marker of exposure in case-series studies has several advantages: retrospective analysis on paraffin embedded specimens, requirement for

small numbers of cells, making the method applicable to biopsy samples and exfoliated cells, possibility to easily collect many samples, high cell-specificity in the detection of the adducts and relatively low cost of the methodology. The results of this pilot study indicate that this analysis is a promising addition to biomonitoring studies.

Acknowledgements

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