

Measurement of 4-Aminobiphenyl-Hemoglobin Adducts in Lung Cancer Cases and Controls

Ainsley Weston, Neil E. Caporaso, Koli Taghizadeh, Robert N. Hoover, Steven R. Tannenbaum, Paul L. Skipper, James H. Resau, Benjamin F. Trump, and Curtis C. Harris¹

Laboratory of Human Carcinogenesis [A. W., C. C. H.] and Environmental Epidemiology Branch [N. E. C., R. N. H.], National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; Whitaker College of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [K. T., S. R. T., P. L. S.]; and Department of Pathology, University of Maryland, Baltimore, Maryland 21201 [J. H. R., B. F. T.]

ABSTRACT

Hemoglobin adducts of the activated carcinogenic aromatic amine 4-aminobiphenyl have been measured in a case-control study of lung cancer. Data obtained for lung cancer cases are compared to those obtained for controls that consisted of patients with either chronic obstructive pulmonary disease or non-pulmonary cancers. Both simple and multivariate analysis found a positive association of 4-aminobiphenyl-hemoglobin adducts with the quantity of tobacco smoked as determined by either urine cotinine or questionnaire data. No association was found between 4-aminobiphenyl-hemoglobin adducts and cancer diagnosis, and adduct levels were not related to remote tobacco use, *i.e.*, total pack years of smoking. There was no association between the levels of adducts detected and the ability of an individual to metabolize debrisoquine (debrisoquine metabolic phenotype, CYP2D6). Whereas 4-aminobiphenyl-hemoglobin adduct levels reflected recent tobacco smoking, they were not correlated with lung cancer risk.

INTRODUCTION

More than half a million cases of lung cancer are diagnosed worldwide each year, and this figure is projected to increase to 2 million by the year 2000 (1, 2). Despite the clear involvement of tobacco smoking in the etiology of lung cancer, this disease is now thought to be the leading cause of cancer deaths in the United States for both men and women (3). Aromatic amines are among a number of components of tobacco smoke that have been found to be carcinogenic in laboratory animals (4), and their role in the etiology of human bladder cancer was recognized in the last century (5). These chemicals, however, require metabolic activation before they can exert their biological effects (6). Their metabolism is complex and they can either undergo *N*-oxidation or acetylation, although an initial activation step for both the parent compound and an aromatic amide (product of metabolism by acetyl CoA-dependent acetylation) is P450-mediated *N*-oxidation (CYP1A2) (6, 7). Adducts of 4-ABP² may have a role in lung carcinogenesis since it has been reported that 4-ABP-DNA adducts can be found in human peripheral lung (8).

Tobacco consumption is implicated as an etiological agent in lung cancer, chronic obstructive pulmonary disease, and heart disease (3). Tobacco smoke is widely accepted as the major etiological factor in lung cancer; however, differences in individual susceptibility have been inferred from the observation that only a minority of cigarette smokers develop lung cancer (9, 10). This may be due to inheritance of specific risk factors or competing causes of death that are smoking related. Wide

interindividual variations in the ability to metabolize drugs, carcinogens, and other xenobiotics have been found in the human population (7, 11). These pharmacogenetic differences have been considered as a possible explanation for differences in individual cancer susceptibility, and they are also consistent with data that implicate other heritable genetic risk factors in lung cancer etiology (12-24). The ability to metabolize the antihypertensive drug debrisoquine is under autosomal genetic control (25, 26), and inheritance of the extensive metabolizer phenotype of this drug has been suggested as a host risk factor for lung cancer (19, 27, 28). It is not yet clear exactly which carcinogens, if any, may be activated by the debrisoquine hydroxylase (CYP2D6), but a recent link has been suggested between activation of tobacco specific *N*-nitrosamines [4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone] and CYP2D6 (29). In bladder cancer an association between risk following exposure to aromatic amines and the slow acetylator phenotype has been demonstrated (30). Similarly, in smokers it appears that individuals who consume black tobacco products, which contain higher levels of aromatic amines, are at greater risk of bladder cancer and have higher 4-ABP-hemoglobin adducts than smokers of blond tobacco (31).

Human molecular dosimetry, *e.g.*, the measurement of covalent binding of activated carcinogens to macromolecules, provides complementary information, regarding carcinogen exposure, to data obtained concerning metabolizer phenotype. This report describes a study designed to examine the hypothesis of an association between the formation of 4-ABP-hemoglobin adducts and lung cancer risk and whether formation of these adducts is related to other markers of tobacco smoking. Therefore, 4-ABP-hemoglobin adducts were measured in subjects from a lung cancer case-control study. This study used two separate comparison groups (chronic obstructive pulmonary disease patients and patients with cancers at anatomical sites other than the lung and bladder) and controlled for recognized lung cancer risk factors and other potential confounding factors (tobacco consumption and ethnic background of subjects).

MATERIALS AND METHODS

Case-Control Study Subjects. The design of this case-control study of lung cancer is described in detail elsewhere (19). Briefly, patients with histologically confirmed lung cancer who had not yet received radiation or chemotherapy were recruited at the University of Maryland and Baltimore Veterans Administration Hospitals between 1985 and 1989. Histological diagnosis of lung cancer was confirmed by pathological review. Two control groups were recruited, patients with chronic obstructive pulmonary disease and patients with cancers at anatomical sites other than the lung, bladder, and liver. The chronic obstructive pulmonary disease patients were diagnosed clinically and had either abnormalities in pulmonary function tests (forced expiratory volume in 1 s of <75% of the predicted normal and/or forced expiratory volume in 1 s/forced vital capacity <75% of predicted) and/or a smoking

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¹To whom requests for reprints should be addressed, at Room 2C01, Building 37, Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

²The abbreviations used are: 4-ABP, 4-aminobiphenyl; CI, confidence interval.

history of 40 pack-years cigarette smoking or more. A second control group was recruited from cancer patients with a variety of malignancies including colon, esophagus, stomach, breast, and melanoma.

Study recruits were screened for eligibility or study exclusion. Exclusion criteria included presence in the intensive care unit, blood pressure <100/60 mm Hg, inability to take oral medication or be interviewed, general anesthesia within the last 5 days, severe renal or liver disease (creatinine >4.0 mg/ml or total bilirubin >3 IU, or serum glutamic-oxaloacetic transaminase or serum glutamic-pyruvic transaminase >300 IU), previous diagnosis of separate primary malignancy other than basal cell carcinoma, inability or refusal to give informed consent and physician refusal. An in-person interview of approximately 45 min was administered to eligible subjects by a trained study nurse/phlebotomist. Data were collected concerning sociodemographic and anthropomorphic characteristics, recent and remote tobacco use, personal medical history, usual and recent diet, current medications, family history of cancer, alcohol use, and occupational and residential history. Medical records were reviewed to abstract selected information including histological diagnoses from pathology reports, results of clinical staging, medications administered, and results of routine clinical laboratory studies. Details of diagnoses, male:female ratio, age (mean years), and smoking histories (pack years) are given in Table 2 and "Results."

Laboratory Assays. Hemoglobin was isolated from RBCs and purified by dialysis. An internal standard consisting of 4'-fluoro-4-aminobiphenyl (1 ng) was added, and free amines were liberated from the protein by alkaline hydrolysis. The hydrolysates were extracted with hexane, and materials partitioning into the organic phase were treated with pentafluoropropionic anhydride. The resulting pentafluoropropionamides were quantitated by capillary gas chromatography combined with negative ion chemical ionization mass spectrometry (32). Quality control measures consisted of measuring levels of 4-ABP in replicate samples (15%) that had been recoded to prevent bias.

Urine (2 ml) and blood-derived plasma samples (500 μ l) were analyzed for the presence of cotinine by radioimmunoassay [American Health Foundation, Valhalla, NY (33)]. Debrisoquine metabolites were measured by gas chromatography and nickel-63 electron-capture detection in samples of urine collected 8 h after drug administration. The debrisoquine metabolic ratio was calculated from the ratio of the percentage of dose excreted as unchanged debrisoquine:4-hydroxydebrisoquine metabolite, using 7-methoxy-guanoxan as an internal standard (19).

Statistical Analysis. Arithmetic means, standard errors, correlation coefficients (Pearson), Student's *t* test, and stepwise and general linear regression were performed using the SAS statistical package (34). Log-transformed, simple means, and Wilcoxon ranking statistics were determined for analysis of continuous variables. Categorical variables were created from continuous variables (e.g., age, recent or remote smoking) for stratified and certain multivariate analyses. A stepwise regression model was used to consider combinations of the following variables in multivariate analysis: current cigarette smoking (cigarettes smoked during the last 24 h, cigarettes smoked during the last week, urine and plasma cotinine), age (years), race (black, white), gender, alcohol (drinks/day; beer, wine, and hard liquor separately and together), anthropomorphic indices (weight, height, Quetelet's body mass index), history of occupational exposure (chemical, textile, rubber, and dye workers), and measures of genetically determined ability to metabolize debrisoquine (19). A logistic regression model was used to determine crude and adjusted odds ratios, using a mainframe computer (34).

RESULTS

Analysis of hemoglobin to determine 4-ABP adduct levels was performed in 109 study subjects (53 lung cancer cases and 56 controls). The levels of adducts were highly correlated with measures of recent smoking (both biochemical and questionnaire data). These data were corroborated by biochemical measures of urine and plasma cotinine levels (Fig. 1 and Table 1). Therefore, the levels of 4-ABP-hemoglobin adducts (pg 4-ABP/

g hemoglobin) were higher in smokers (126 ± 12 ; mean \pm SE) than in nonsmokers (86 ± 7) (Table 1). 4-Aminobiphenyl-hemoglobin adducts exhibited a positive correlation (Pearson) with all measures of recent smoking. Urine cotinine exhibited the best correlation ($r = 0.39$, $P = 0.0001$). Tobacco use (cigarettes/week derived from the questionnaire data) was also significantly correlated ($r = 0.18$, $P = 0.05$), but measures of remote smoking (pack years) did not exhibit a significant correlation ($r = -0.07$, $P = 0.45$). The hypothesis in this study addresses the possibility of a positive association between 4-ABP-hemoglobin adduct levels and lung cancer. In fact, the mean adduct level determined for the control groups was higher in each case than that for the lung cancer group, but these differences were not statistically significant (Fig. 1, Table 1). Neither was there an association between adduct levels and specific histological type of lung cancer compared to controls (Table 2).

Possibilities of associations between other potential lung cancer risk factors and 4-ABP-hemoglobin adduct levels were also examined. In a previous report the extensive metabolizer phenotype of debrisoquine (an antihypertensive drug) was found to confer a relative risk for lung cancer of 6.1 (95% CI, 2.2–17.1) (19). However, no association was found between the level of 4-ABP-hemoglobin adducts and the debrisoquine metabolizer phenotype (Table 1). More detailed analysis of metabolizer phenotype was performed by seeking a correlation between 4-ABP-hemoglobin adduct levels and the log of the metabolic ratio (ln debrisoquine/4-hydroxydebrisoquine), but no association was found ($r = 0.05$, $P = 0.63$, $n = 103$). Similarly, no association was found between adduct levels and age, race, or remote smoking (pack years).

An association was found between the level of 4-ABP-hemoglobin adduct levels and weight. Individuals who weighed >170 lbs had higher levels of adducts (117 ± 14) than those individuals who weighed <170 lbs (93 ± 7) (Table 1). The association of weight and 4-ABP-hemoglobin adduct levels persisted when study subjects weighing >250 lbs were excluded. Questionnaire data were examined to seek an explanation for this observation, but none was forthcoming. In particular, adduct levels were unrelated to any dietary items consumed (servings in the 24 h or servings in the week prior to study). The tendency for heavier individuals to have higher adduct levels, which was significant in univariate analysis, became less important after adjustment for smoking. In a representative analysis, the odds ratio for elevated adduct level was 4.1 (95% CI, 1.7–10.0) in current smokers but only 1.9 (95% CI, 0.8–4.7) in

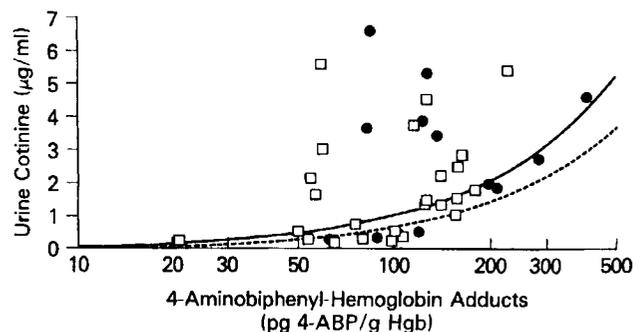


Fig. 1. Association between urine cotinine level and 4-aminobiphenyl-hemoglobin adduct levels in lung cancer cases (—●—) ($n = 53$) and controls (—□—) ($n = 56$). Points with a urine cotinine level of <0.2 μ g/ml are not plotted (44 cases, 29 controls) but the regression lines are based on the total sample set.

Table 1 Relationship of study variables with levels of 4-aminobiphenyl-hemoglobin adducts

Study variable	No. of subjects	Level of adducts
All study subjects	109	100 ± 6 ^a
Measure of smoking		
Questionnaire		
No cigarettes last 24 h	68	86 ± 7
≥cigarette last 24 h	41	126 ± 12
Urine cotinine ^b		
≤200 ng/ml	65	84 ± 7
≥200 ng/ml	39	126 ± 12
Plasma cotinine		
≤5 ng/ml	42	74 ± 7
≥5 ng/ml	41	118 ± 10
Diagnosis		
Cases (lung cancer)	53	95 ± 8
Controls		
Chronic obstructive pulmonary disease	33	104 ± 14
Non-pulmonary cancer	23	112 ± 13
Debrisoquine metabolic phenotype ^c		
Poor metabolizer	6	111 ± 37
Intermediate metabolizer	12	129 ± 18
Extensive metabolizer	85	99 ± 7
Race		
Black	53	99 ± 10
White	56	103 ± 8
Weight		
≥170 lbs	35	117 ± 14
≤170 lbs	74	93 ± 7

^a pg 4-aminobiphenyl/g hemoglobin, mean ± SE.

^b Pearson correlation coefficient between adduct level and urine cotinine; $r = 0.39$, $P = 0.0001$, $n = 109$.

^c Debrisoquine metabolic phenotype is defined by a maximum-likelihood three-mix model (19).

individuals weighing >170 lbs. These analyses included adjustment for gender, race, case status, pack years smoking, and recent alcohol intake.

Occupational history data collected using the questionnaire instrument were analyzed. Subjects were specifically questioned regarding certain exposures potentially associated with aromatic amines. In addition, employment in 12 industries was considered; these included chemical, textile, rubber, dye, and paper industries. None of the 12 occupations examined was significantly associated with elevated 4-ABP-hemoglobin adduct levels. However, a history of employment in one nonspecific occupational group (non-petrochemical chemical workers) had elevated adduct levels. Five of 92 subjects had 4-ABP-hemoglobin levels of 178 ± 58 compared to 96 ± 7 for the other 87 subjects; this elevation, however, was likely accounted for by higher recent smoking in the 5 former workers (suggested by urine cotinine levels of 1584 ± 797 compared to 796 ± 143 ng/ml).

DISCUSSION

The results of this case-control study demonstrate that lung cancer diagnosis is unrelated to formation of 4-ABP-hemoglobin adducts. Furthermore, adjustment for smoking and other study variables does not succeed in finding any association. There is also no association between adduct levels and histological type of lung cancer. Curiously, subjects with non-pulmonary cancers exhibit some relatively elevated levels of adducts among the nonsmokers. Unfortunately, the number of current smokers among the breast cancer and colon cancer groups was insufficient to allow any firm conclusions. Further medical review of records and questionnaires did not reveal any likely reason to account for this. In the case of urinary bladder cancer, which has been associated with the slow acetylator phenotype (30), increased 4-ABP-hemoglobin adduct levels have been ob-

served in slow acetylators after adjustment for cigarette smoking (31). Therefore, an association with the disease is plausible. These observations are consistent with activation of aromatic amines by the competing pathway of *N*-hydroxylation (CYP1A2), when protonated (by the acidic conditions in the urinary bladder) hydroxylation products form reactive electrophiles (7, 35). The data presented in this report suggest that this mechanism does not appear to be involved in the pathogenesis of lung cancer.

The 10 nonsmoking women have relatively elevated adduct levels, and most of this is accounted for by the 5 women with breast cancer. The comparisons presented in this study were all repeated excluding these women with no changes observed. In light of the gender difference in bladder cancer incidence, further studies of these markers in groups of smoking and nonsmoking women without cancer is warranted.

No association was found between the formation of 4-ABP-hemoglobin adducts and propensity for metabolism of the anti-hypertensive drug debrisoquine. Taken together with the observation that 4-ABP-hemoglobin adducts are not a risk factor for lung cancer, the data are consistent with previous studies that identified the extensive metabolizer phenotype of debrisoquine

Table 2 4-Aminobiphenyl hemoglobin adducts and current smoking status

Study variable	Smokers ^a		Nonsmokers	
	No.	Level of adducts	No.	Level of adducts
All study subjects	41	126 ± 12 ^b	68	86 ± 7
Diagnostic group				
Cases ^c				
Lung cancer	23	122 ± 13	30	74 ± 9
Squamous cell	11	108 ± 21	15	69 ± 17
Small cell	2	214 ± 17	3	115 ± 34
Large cell	1	128	2	80 ± 15
Adenocarcinoma	9	118 ± 13	10	67 ± 8
Controls ^{c,d}				
Chronic obstructive lung disease	11	152 ± 34	22	81 ± 10
Non-pulmonary cancer	7	98 ± 14	16	115 ± 17
Breast	1	138	5	143 ± 35
Colorectal	1	71	4	132 ± 46
Esophagus	3	119 ± 8	4	101 ± 18
Melanoma	1	83	2	47 ± 13
Other	1 ^e	37	1 ^f	93
Race				
Black	18	110 ± 22	35	93 ± 11
White	23	139 ± 13	33	78 ± 8
Gender				
Men	39	128 ± 12	58	79 ± 7
Women	2	87 ± 50	10	123 ± 23
Lifetime pack-years of smoking				
>50	20	137 ± 20	32	67 ± 7
<50	21	116 ± 14	36	102 ± 11
Debrisoquine metabolic phenotype ^g				
Extensive metabolizer	33	123 ± 13	52	84 ± 8
Intermediate metabolizer	4	154 ± 29	8	117 ± 22
Poor metabolizer	3	155 ± 68	3	67 ± 12
Weight				
>170 lbs	12	150 ± 29	23	99 ± 15
<170 lbs	29	116 ± 12	45	79 ± 7
Alcohol				
>Median ^h	20	123 ± 15	32	65 ± 5
<Median	19	129 ± 20	33	104 ± 13

^a Smoker defined as subjects who have smoked one cigarette or more during the week prior to study.

^b pg/g hemoglobin week prior to study; mean ± SE.

^c Age (mean ± SE): cases, 64 ± 7 years; controls, 61 ± 9 years.

^d Pooled controls consist of subjects with chronic obstructive pulmonary disease and subjects with a variety of non-pulmonary cancers.

^e Gastric.

^f Gallbladder.

^g Metabolic phenotype is defined by a maximum-likelihood three-mix model (19).

^h 3 drinks/day (beer, wine, hard liquor).

by CYP2D6 to be a risk factor for human lung carcinogenesis (19, 27, 28, 36). These data are further consistent with studies that show that P450 isozymes other than CYP2D6 are involved in the activation of 4-ABP (6, 7).

Previous studies involving the measurement of 4-ABP-hemoglobin adducts by gas chromatography/mass spectrometry have shown that smokers have higher levels of adducts when compared with nonsmokers (31, 32, 37, 38). Furthermore, the type of tobacco consumed influences the levels of adducts formed through a dose-response mechanism. Thus, smokers of black tobacco (that contains higher levels of aromatic amines) generate higher adduct levels than smokers of blond tobacco (31, 32). The results of the current study are consistent with these previous findings, although black tobacco smoking is rare in the United States. The data presented here confirm that measurement of 4-ABP-hemoglobin adducts are representative of recent smoking history, even though exposure to 4-ABP could result from certain dietary sources (39). Questionnaire data including recent dietary consumption of approximately 40 items failed to identify any specific item in the recent diet that could be an obvious source of 4-ABP exposure. One explanation for failure to detect an association between adduct levels and occupation may be that questionnaire data provide information concerning remote rather than recent exposure. In order to address this question, workers whose current occupation provides potential exposure should be studied.

In the framework of multistage carcinogenesis, the formation of chemical-DNA adducts are thought to be necessary for the action of certain types of chemical carcinogens, such as 4-ABP, but not sufficient for cancer development caused by most chemical agents studied (40, 41). It has been demonstrated that 4-ABP-DNA adducts are formed in human peripheral lung tissues (8), indicating the presence of CYP1A2 or transportation of the hydroxylamine metabolite of 4-ABP after activation of the free amine in another tissue. Therefore, it may be concluded that either lung cancer is not mediated simply through 4-ABP-DNA adduct formation or 4-ABP-hemoglobin adducts are not reflective of 4-ABP activation and DNA adduct formation in the lung. Another argument is that until all of the study subjects have died we cannot eliminate the possibility that some of the controls will develop lung cancer (e.g., chronic obstructive pulmonary disease is a risk factor for lung cancer). This possibility suggests a prospective study. In summary, 4-ABP-hemoglobin adduct levels are indicative of tobacco smoking but do not appear to constitute an independent risk factor for lung cancer.

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