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High Level of Tobacco Carcinogen–Derived DNA Damage in Oral Cells Is an Independent Predictor of Oral/Head and Neck Cancer Risk in Smokers

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Abstract

Exposure to tobacco-specific nitrosamines (TSNA) and polycyclic aromatic hydrocarbons (PAH) is recognized to play an important role in the development of oral/head and neck squamous cell cancer (HNSCC). We recently reported higher levels of TSNA-associated DNA adducts in the oral cells of smokers with HNSCC as compared with cancer-free smokers. In this study, we further investigated the tobacco constituent exposures in the same smokers to better understand the potential causes for the elevated oral DNA damage in smokers with HNSCC. Subjects included cigarette smokers with HNSCC (cases, $n = 30$) and cancer-free smokers (controls, $n = 35$). At recruitment, tobacco/alcohol use questionnaires were completed, and urine and oral cell samples were obtained. Analysis of urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and *N*′-Nitrosonornicotine (NNN; TSNA biomarkers), 1-hydroxypyrene (1-HOP, a PAH), cotinine, 3′-hydroxycotinine, and the nicotine metabolite ratio (NMR) were performed. Cases and controls differed in mean age, male preponderance, and frequency of alcohol consumption (but not total alcoholic drinks). Univariate analysis revealed similar levels of NNN, 1-HOP, and cotinine between groups but, as reported previously, significantly higher DNA adduct formation in the

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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cases. Multiple regression adjusting for potential confounders showed persistent significant difference in DNA adduct levels between cases and controls [ratio of geometric means, 20.0; 95% CI, 2.7–148.6). Our cohort of smokers with HNSCC demonstrates higher levels of TSNA-derived oral DNA damage in the setting of similar exposure to nicotine and tobacco carcinogens. Among smokers, DNA adduct formation may act as a predictor of eventual development of HNSCC that is independent of carcinogen exposure indicators.

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is one of many cancers that are strongly associated with tobacco use. Although HNSCC is often seen in tobacco users, many tobacco users do not develop carcinoma, and the differences pertaining to carcinogenesis between smokers with and without HNSCC are poorly studied to date. Some smokers may be inherently more susceptible to developing carcinoma due to patterns of tobacco use, innate metabolism of carcinogens, altered excretion, or variation in DNA damage and repair. One approach to better understand the extent of exposure to, and metabolism of, tobacco carcinogens is through the study of their biomarkers, such as urinary metabolites or DNA adducts (1–3). A greater appreciation for the role of specific carcinogens in HNSCC is important for determining mechanisms of tobacco-induced cancer. In addition, studying tobacco carcinogen uptake can provide information about eventual risk of carcinoma among smokers (3, 4). Identification of those smokers who are at greatest risk for HNSCC, but have failed quit attempts, would have great benefit through targeted smoking cessation efforts and enhanced surveillance (5).

Tobacco-specific nitrosamines (TSNA) include 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN). Urinary levels of NNN and NNK biomarkers in smokers have demonstrated association with the eventual risk of esophageal and lung carcinoma, respectively, in agreement with their organ specificity in laboratory animals (3, 4, 6). The results of our previous work also indicate that there may be a relationship between NNN uptake and the risk of HNSCC in smokers (1).

Although urinary biomarker levels are a measure of carcinogen exposure, measurement of DNA adducts represents an opportunity to directly assess the extent of DNA damage caused by tobacco carcinogens. DNA adduct formation is a common mechanism of chemical carcinogenesis and results from carcinogens or their electrophilic derivatives reacting with nucleophilic sites within DNA. DNA adducts disrupt DNA double helical structure and require repair. The extent to which DNA adduct formation occurs can be correlated with the magnitude of mutagenesis (7). Not surprisingly, DNA adduct formation at susceptible sites, such as proto-oncogenes, if unrepaired, can lead to tumorigenesis (8).

Metabolic activation of NNK and NNN leads to the formation of pyridyloxobutyl (POB)–DNA adducts (9, 10). Under strong acid hydrolysis conditions, POB–DNA adducts can decompose to release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB; Fig. 1; refs. 11–13). Studies in laboratory animals indicate the importance of HPB-releasing DNA adducts in NNK- and NNN-induced carcinogenesis (10, 14).

The presence of HPB-releasing DNA adducts in various human samples has been reported in prior publications (15–19), yet overall, the research in this field has been somewhat limited. In our efforts to provide a robust analytic methodology for the analysis of HPB-releasing adducts in studies of tobacco-induced cancer risk, we recently developed a highly sensitive liquid chromatography nanoelectrospray–high-resolution tandem mass spectrometry method for the analysis of these adducts using small amounts of DNA extracted from human oral cells (20). Application of this method to the analysis of HPB-releasing adducts in buccal cell DNA from smokers with and without HNSCC showed significantly higher adduct levels in HNSCC cases compared with cancer-free smokers. In the current study, we conducted additional analyses to determine whether the identified increase in HPB-releasing DNA adduct levels among smokers with HNSCC is driven by differences in TSNA exposure or whether they are reflective of other interindividual differences, such as TSNA metabolic activation and/or DNA repair. The additional analyses included urinary biomarkers of tobacco constituent exposure, such as total NNAL and total NNN (biomarkers of NNK and NNN, respectively), 1-hydroxypyrene (1-HOP, a biomarker of exposure to polycyclic aromatic hydrocarbons), cotinine, the ratio of 3′-hydroxycotinine to cotinine (nicotine metabolite ratio, NMR), as well as demographics, history of smoking, and alcohol use.

Materials and Methods

Subject enrollment and specimen collection

Subject recruitment and collection of biological samples was approved by the University of Minnesota Research Subjects' Protection Programs Institutional Review Board: Human Subjects Committee (IRB Study # 0903M62203). After obtaining informed consent, enrollment of 30 smokers with head and neck squamous cell carcinoma (HNSCC) and 35 smokers without HNSCC occurred in the Department of Otolaryngology-Head and Neck Surgery at the University of Minnesota (Minneapolis, MN). Study participants were included if they were self-described as daily smokers and smoked at least 5 cigarettes per day for at least 5 years. HNSCC patients were identified upon presentation to the Otolaryngology-Head and Neck Surgery clinic with a new diagnosis of squamous cell carcinoma of the upper aerodigestive tract. This included tumors of the oral cavity, oropharynx, larynx, and hypopharynx. In some cases, cancers were first identified in our clinic, whereas in others, cancers were diagnosed at outside institutions and referred to our clinic for definitive management. Cancer-free controls were recruited in the same outpatient clinic. The control subjects were visiting the clinic for clinical evaluation of problems other than cancer (i.e., sinusitis, hearing loss) and were approached for enrollment upon identification as daily cigarette smokers. All enrolled subjects were smoking cigarettes daily at the time of enrollment. Demographic data collected included cigarettes per day, duration of use, alcoholic drinks per day, alcoholic frequency, and tumor-related variables, such as subsite and stage.

Urine was collected by standard "clean-catch" technique at the time of clinic visit. The urine samples were kept in a −20°C freezer until assays were performed. The details of oral sample collection and processing have been described previously (20).

Analysis of urinary biomarkers

Total NNAL—This biomarker was analyzed by LC/MS-MS as described previously (21). Briefly, 96-well plate technology was used for sample enrichment by supported liquid extraction plate, mixed mode reverse-phase/cation exchange solid-phase extraction and LC/MS-MS analysis. The detection limit for NNAL was 0.4 fmol, and calibration curves were linear in the range measured (R^2 = 0.99). The intraday precision of the assay was 1.2% and interday precision was 2.7% relative SD (RSD).

Total NNN—Analysis of total NNN was performed as described previously (22). Intraday precision ranged from 1.9% to 10% for NNN, while intraday deviations from the nominal added amounts ranged from −3.4% to 7.4%. Interday precision ranged from 14% to 16%, and deviations from nominal ranged from −3.1% to 9.8%.

1-HOP—The levels of 1-HOP were determined by high-performance liquid chromatography (HPLC) with fluorescence detection, using [D_9]1-HOP as the internal standard. Intraday and interday precision were less than 5% RSD (23, 24).

Cotinine and 3′-hydroxycotinine—Urinary total cotinine (cotinine plus cotinine glucuronide) and 3′-hydroxycotinine levels were assayed by gas chromatography/mass spectrometry as described previously (25, 26).

Creatinine—Urinary creatinine was assayed in all subjects by Fairview-University Medical Center Diagnostic Laboratories with a Kodak Ektachem 500 chemistry analyzer. Total NNAL, total 1-HOP, total NNN, and total cotinine were expressed per milligram creatinine to normalize for urinary dilution.

Analysis of HPB-releasing DNA adducts in oral cells

The data on HPB-releasing adduct levels have been generated previously (20). Briefly, DNA was isolated from the collected samples by using the commercial DNA Purification Kit (Qiagen). The isolated DNA samples were subjected to acid hydrolysis to release HPB and purified on 25-mg HyperSep Hypercarb cartridges (Thermo Fisher Scientific). The analysis of HPB in the purified samples was carried out on an LTQ Orbitrap Velos instrument (Thermo Fisher Scientific) interfaced with a Nano2D-LC HPLC (Eksigent) system with nanoelectrospray ionization, as described previously (20)

Statistical analyses

Statistical analyses were performed using SAS version 9.3 (SAS Institute Inc.) software. Demographic data and data gleaned from tobacco and alcohol use questionnaires were examined using the Wilcoxon rank sum test and Fisher exact test. Because of skewed distributions to high values, comparison of urinary tobacco biomarkers between cases and controls were analyzed in the natural logarithmic scale with the two-sample *t* test. Results were reported as geometric means. For the samples that had undetectable levels of a particular biomarker, a value equal to 50% of the corresponding limit of detection was assigned for calculation purposes. Finally, a multiple regression model was created to adjust for possible confounding variables.

Results

Our study population consisted of 35 cigarette smokers (controls) and 30 smokers who had developed tumors of the upper aerodigestive tract (cases). Tumor site distribution was oropharynx (50%), larynx (27%), oral cavity (17%), and other (5%). Univariate analysis of their demographic data revealed no difference in cigarettes per day and number of alcoholic drinks per day. Comparison of mean duration of tobacco use between groups (cases: 28.2 years, controls: 24.1 years) revealed they were not significantly different. However, the cases had a higher frequency of alcohol consumption ($P = 0.042$). Cases reported drinking 2 to 4 times per month, while controls reported once per month or less. Also, among cases, mean age was 58 years compared with 48 years among controls; this difference was statistically significant ($P = 0.001$). Finally, cases were more likely to be male (83% male) compared with controls (57% male; $P = 0.031$). Demographic data are summarized in Table 1.

Table 2 summarizes levels of urinary tobacco carcinogen biomarkers measured in this study, and the previously reported (20) levels of HPB-releasing DNA adducts in oral cells from the same subjects. HPB-releasing DNA adduct levels were significantly elevated in cases compared with controls with a dramatically higher geometric mean [ratio of geometric means, 30.82;95%confidence interval (CI), 5.27–180.37; Fig. 2]. Because of insufficient urine volume and/or technical issues with the analytic procedures, some urine samples could not be analyzed for all biomarkers. As a result, the number of data points for each biomarker varies. Mean creatinine-corrected urinary total NNAL values were 1.558 pmol/mg in cases and 0.744 pmol/mg in controls. Despite a higher mean value in the cases, this trend did not reach statistical significance ($P = 0.122$). The creatinine-corrected total NNN ranged from undetectable to 0.023 pmol/mg in cases and undetectable to 0.031 pmol/mg in controls, and the difference in the mean levels between cases and controls was not statistically significant (0.012 pmol/mg vs. 0.016 pmol/mg, respectively, $P = 0.518$). 1-HOP levels were similar between cases and controls, ranging from 0.554 to 1.240 pmol/mg creatinine and undetectable to 1.105 pmol/mg creatinine ($P = 0.681$), respectively. Cotinine range was 1,074 to 2,657 ng/mg creatinine in cases and 1,033 to 2,051 ng/mg creatinine in controls ($P = 0.583$). The calculated NMR was 3.83 in cases and 2.96 in controls; however, this difference did not reach statistical significance. Similar exposure to nitrosamines and 1-HOP in both groups suggests comparable carcinogen exposure among subjects in both groups. On the basis of the similar cotinine levels between groups, cases and controls experienced similar levels of tobacco exposure.

A multiple regression analysis was performed to allow adjustment for demographic variables that were dissimilar in univariate analysis of cases and controls. The regression model adjusted for gender, age, and self-reported alcohol frequency. This analysis resulted in a ratio of HPB-releasing adduct levels in cases over controls of 20.0 (95% CI, 2.7–148.6) with an adjusted P value of 0.005. In this analysis, none of the other covariates demonstrated statistically significant differences. We also repeated the multiple regression calculation with inclusion of NNAL, as this nitrosamine, although not statistically significant, was approximately two times higher in smokers with HNSCC. The result showed that NNAL was not significant in the model ($P = 0.773$), and the ratio of HPB-releasing adduct levels changed only minimally from the prior calculation (ratio = 19.1, $P = -0.027$).

Discussion

Tobacco carcinogen–derived DNA adducts represent a direct measure of DNA damage in smokers, and their levels may be the result of a variety of factors, such as the amount of the carcinogen taken in, its metabolic activation, DNA repair capacity, and other individual characteristics. This, together with the central role of DNA adduct formation in the chemical carcinogenesis paradigm, suggests that the levels of specific DNA adducts in smokers may serve as a direct measure of their cancer risk. Therefore, our recent finding of the dramatically higher levels of HPB-releasing DNA adducts in the oral cells of smokers with HNSCC than in cancer-free smokers (20) called for a closer examination of the potential factors that may have driven that difference.

Although HPB-releasing adducts have only recently been studied in HNSCC, DNA adducts have been previously examined in the upper aerodigestive tract in a broader sense. The presence of various DNA adducts has previously been documented in both blood and tissue sites. For example, formaldehyde-derived DNA adducts were found to be elevated in leukocytes of smokers compared with nonsmokers (27). HPB-releasing adducts have been measured in the lungs (16) and in buccal cells (19) of smokers. Adducts created from aldehyde exposure have been studied in gingival tissue and found to be elevated in smokers compared with nonsmokers (28). The current analysis allows for interpretation of the DNA adduct data in the context of tobacco exposure data.

The results of urinary analyses in both cases and controls show that levels of total NNAL, total NNN, and other tobacco constituent biomarkers were not significantly different between HNSCC cases and controls. Therefore, the differences in the levels of HPB-releasing DNA adducts in these subjects cannot be explained by differences in exposure to NNK and NNN, or to cigarette smoke in general. The results may be due to greater rates of adduct formation or decreased DNA repair in the study group (cases). It should be noted that in our previous study performed on a different group of smokers, participants with HNSCC had higher levels of urinary total NNN and 1-HOP compared with cancer-free smokers (1). The discrepancy in the findings of the two studies is most likely related to the inherent variability that is often demonstrated in individual subjects when quantifying their tobacco biomarkers in urine. Both studies had similar samples sizes, and the effects of inherent variability are certainly lessened by increasingly larger sample sizes, such as that utilized in large cohort studies of cigarette smokers in the general population (3). However, large samples sizes are difficult to obtain when studying smokers with HNSCC in the United States, where HNSCC is less prevalent than other highly affected countries, such as China and India. Furthermore, the sample sizes we have studied represent the largest groups of smokers with HNSCC examined in relation to tobacco biomarkers and DNA adducts thus far. An additional contrast between the current study and our prior publication of carcinogen biomarker levels in cases and controls relates to the use of self-reported cigarettes per day in the prior study as opposed to cotinine (the gold standard for assessing tobacco exposure) in the current study. The former has since been shown to be a less accurate measure of tobacco exposure in this population (29).

CYP2A6 is also responsible for the metabolism of nicotine: It mediates the conversion of nicotine to cotinine and the subsequent conversion of cotinine to 3′-hydroxycotinine (30). Thus, the 3′-hydroxycotinine to cotinine ratio, or NMR, serves as an *in vivo* phenotype of CYP2A6 activity (31). Therefore, we calculated NMR for our study subjects, to provide insights into the potential effect of CYP2A6 activity on the observed differences in HPB-releasing adduct levels between HNSCC cases and cancer-free controls. Although not reaching statistical significance, the mean NMR was higher in cases than in controls, suggesting higher CYP2A6 activity in cases (Table 2). This observation calls for a more thorough investigation of the potential contribution of CYP2A6 genotype to the differences in HPB-releasing adduct levels in smokers. This is particularly relevant to our finding of high levels of HPB-releasing adducts in oral cells of smokers with HNSCC, because CYP2A6 is thought to be primarily responsible for the metabolic activation of NNN, a potent oral and esophageal carcinogen (32–35). Thus, at similar levels of NNN exposure, smokers with higher CYP2A6 activity may be forming higher levels of NNN-derived HPB-releasing DNA adducts in the oral cavity, which could contribute to the higher risk for the development of HNSCC. To further understand the significance of nitrosamine metabolism on risk of HNSCC, we plan to perform CYP2A6 genotyping as the next step in analyzing these subjects.

We also analyzed demographic characteristics and tobacco and alcohol use history as the potential factors responsible for the differences in oral cell adduct levels in our subjects. Although the smoking history and the number of alcoholic drinks per day were similar in cases and controls, the frequency of alcohol use was higher in cases than in controls. This finding potentially indicates that simultaneous presence of TSNA and alcohol in the oral cavity is leading to elevated DNA damage in oral cells, possibly through alcohol induction of CYP2A6 enzyme (36), thus resulting in higher cancer risk. Also, variables of gender and age were dissimilar between groups. There were a greater number of males in the smokers with HNSCC. This finding is expected as HNSCC affects men disproportionately (37). Existing data suggest that the male preponderance in cases compared with controls in our study does not explain the presence of elevated DNA adduct formation in cases (38, 39).

The finding of greater average age of patients in cases compared with controls is contrasted with the above difference in gender in that it represents a potential confounder. On the basis of this difference, it may be theorized that the increased age of smokers with carcinoma may have predisposed them to a greater risk of developing tobacco-induced cancer because of age-related reduction in DNA repair. This, in turn, might explain a finding of higher DNA adducts in this group. However, the chance of actual confounding is highly unlikely based on several considerations. First, the differences in DNA repair occurring with advancing age are seen only when cells or individuals with dramatically different ages are compared. For example, when comparing fibroblasts from donors aged 21 to 34 years old with those from donors aged 63 to 88 years old, a decrease in DNA repair capacity was identified (40). Additional data suggest that mutation accumulation tends to occur to a greater degree after crossing the age of 65 (41). By way of contrast, the difference in mean ages in our study was under 10 years (57 years in the study group and 48 years in the control group). Therefore, although there is a difference in mean age between the groups that we studied, prior data suggest that the difference in mean age is not sufficient to explain a greater than 32-fold (or

20-fold in multivariate analysis) difference in DNA adduct formation among those smokers who develop carcinoma. Second, further statistical analysis of our sample through multiple regression did not validate age difference as an explanation for our results. Finally, a multivariate regression approach to adjust for age and other variables, including gender and alcohol frequency, did not demonstrate statistical significance for these variables. Indeed, only HPB adduct levels remained significant following multivariate analysis. In summary, for reasons stated above, the demographic differences in cases and controls do not explain the dramatic divergence in DNA adduct levels between groups.

HNSCC is a life-altering diagnosis that threatens those afflicted first and foremost with significant risk of mortality. For those who survive, difficulties with eating, speaking, and breathing following treatment are common, resulting in significant quality of life degradation. Demonstration of the type, and distribution, of DNA adducts seen in oral cells of smokers with HNSCC has the potential to create greater understanding of carcinogenic pathways of this devastating disease. Our data expand significantly upon that which is present in the literature by examining differences in carcinogenic activity between smokers with HNSCC and smokers without HNSCC. The salient finding that TSNA-related DNA adduct formation in the oral tissues of smokers with head and neck cancer is significantly elevated compared with TSNA-related DNA adduct formation in smokers without carcinoma has important clinical implications for future screening efforts relating to tobacco-induced HNSCC. Prior work has demonstrated a high degree of correlation between smoking-related DNA adduct levels in the oral cavity regardless of whether the tissue is obtained by biopsy or by brushing/scraping (as was done in our study; ref. 42). Thus, a noninvasive collection of DNA from the oral cavity can be used to provide significant information about tobacco damage at the tissue level. This raises the possibility of using this technique in screening initiatives as well as the assessment of premalignant lesions. It is possible to envision a scenario in the future where an assessment of DNA adducts in the oral cavity may create the opportunity to target administration of DNA repair agents, in addition to routine tobacco cessation counseling. Finally, this work adds additional important data to the existing literature surrounding tobacco products. The findings pertaining to nitrosamine-related DNA damage offer additional support for FDA regulation of specific tobacco constituents. Such authority was given to the FDA following the 2009 enactment of the Family Smoking Prevention and Tobacco Control Act.

The main limitation of our study is sample size with a total of 65 subjects examined. This leaves our analysis more vulnerable to the variability in data, which is somewhat inherent to the study of DNA adducts and tobacco biomarkers. However, the differences we have identified are significant and, given the large effect size, unlikely to be related to sample size alone. As we continue to add patients to our cohort, we anticipate analyzing larger sample sizes. Furthermore, the use of multiple regression has helped to decrease the likelihood of a type II error in our results, especially relating to the possibility that NNAL could play a role in determining levels of HPB-releasing adducts.

In summary, we have examined tobacco carcinogen and exposure biomarker levels in smokers with HNSCC that have higher DNA adduct formation when compared with cancer-free smokers. We have identified similar levels of carcinogen exposure in smokers with

HNSCC compared with smokers without HNSCC while demonstrating that both groups experienced similar exposure to nicotine. We therefore conclude that the differences in DNA adduct levels in both groups are most likely related to differences in adduct formation and/or DNA repair occurring between individuals in each group. The data provide strong evidence supporting further work to examine genetic variations that govern DNA repair and the nature of DNA repair itself following adduct generation in patients who have developed cancer of the head and neck.

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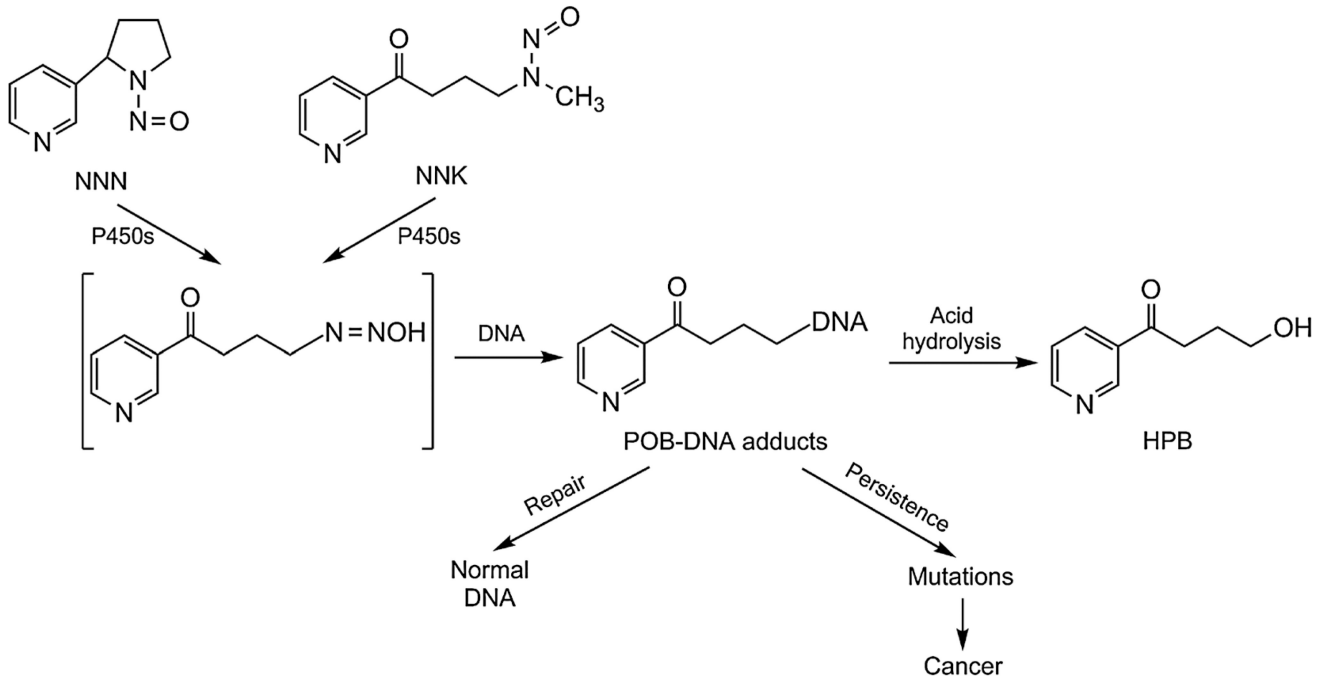

Figure 1.

Formation of HPB-releasing DNA adducts from NNK and NNN. The metabolic pathway by which nitrosamines are converted to reactive intermediates, which then bind DNA and release HPB.

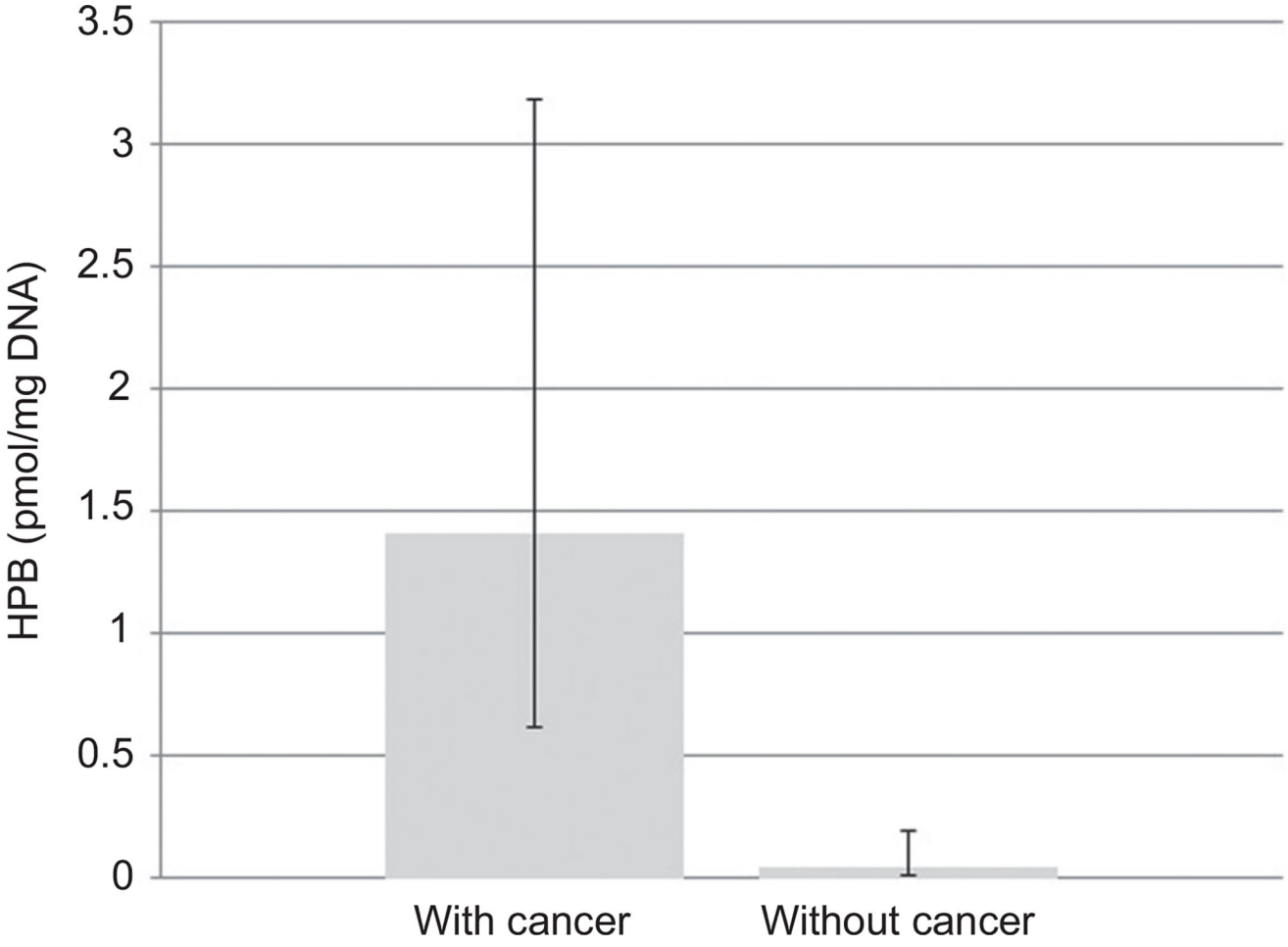

Figure 2.
Geometric mean HPB levels by univariate analysis. Comparison of mean HPB levels in smokers with head and neck cancer and smokers without cancer.

Table 1

Subject characteristics of smokers by presence or absence of cancer

Characteristics	Without cancer *n* = 35 Mean, (SD), median [min/max] *N*=	With cancer *n* = 30 Mean, (SD), median [min/max] *N*=	*P*[a]
Age (years)	48.1 (11.5), 48.0 [24/70], *n* = 35	57.8 (9.8), 58.0 [34/75], *n* = 30	**0.001**
Cigarettes per day	14.4 (6.7), 15.0 [1/30], *n* = 35	12.6 (11.8), 10.0 [0/45], *n* = 30	0.104
Smoking years	24.1 (13.3), 25.0 [1/50], *n* = 34	28.2 (12.8), 30.0 [2/45], *n* = 15	0.367
Alcohol frequency	2.3 (1.4), 2.0 [1/5], *n* = 35	3.2 (1.6), 3.5 [1/5], *n* = 28	**0.042**
# Alcoholic drinks	3.9 (2.5), 3.0 [1/12], *n* = 21	3.8 (2.5), 3.0 [0/10], *n* = 21	0.980
	Frequency (%)	Frequency (%)	*P*[b]
Gender			
Female	15 (42.9%)	5 (16.7%)	**0.031**
Male	20 (57.1%)	25 (83.3%)	
T stage[c]	NA		NA
1		4 (15.4%)	
2		11 (42.3%)	
3		4 (15.4%)	
4		7 (26.9%)	

NOTE: *P* values < 0.05 are considered significant.

[a]The *P* value was from the nonparametric Wilcoxon rank sum test.

[b]The *P* value is from Fisher exact test.

[c]Four subjects with cancer were missing T stage.

Table 2

Univariate comparison of biomarkers from smokers with and without cancer

Biomarker[a]	**Without cancer Geometric mean (95% CI) *n* =**	**With cancer Geometric mean (95% CI) *n* =**	**Cancer/no cancer Ratio of Geo means (95% CI)**	***P*[b]**
HPB (pmol/mg DNA)	0.046, (0.010–0.210), *n* = 34	1.406, (0.622–3.179), *n* = 30	30.82 (5.27–180.37)	<**0.001**
NNAL (pmol/mg creatinine)	0.744, (0.373–1.485), *n* = 27	1.558, (0.818–2.969), *n* = 21	2.09 (0.81–5.38)	0.122
NNN (pmol/mg creatinine)	0.016, (0.008–0.031), *n* = 27	0.012, (0.006–0.023), *n* = 21	0.74 (0.29–1.88)	0.518
1-HOP (pmol/mg creatinine)	0.739, (0.494–1.105), *n* = 27	0.829, (0.554–1.240), *n* = 22	1.12 (0.64–1.97)	0.681
Cotinine (ng/mg creatinine)	1,456, (1,033–2,051), *n* = 27	1,689, (1,074–2,657), *n* = 20	1.16 (0.68–1.99)	0.583
3OH cotinine (ng/mL)	3,575, (2,322–5,503), *n* = 30	4,233, (2,834–6,321), *n* = 25	1.18 (0.66–2.12)	0.564
Biomarker ratio	**Mean (SD) (95% CI) *n* =**	**Mean (SD) (95% CI) *n* =**	**Difference in means cancer-no cancer (SD; 95% CI)**	***P*[c]**
3OH cotinine/total cotinine ratio	2.96 (1.77), (2.29–3.64), *n* = 29	3.83 (1.89) (3.02–4.65) *n* = 23	0.87 (1.82; −0.15–1.89)	0.094

NOTE: *P* values < 0.05 are considered significant.

[a] Biomarkers were analyzed in the natural logarithmic scale.

[b] The *P* value is based on the two-sample *t* test for the difference of means in the log scale.

[c] The *P* value is based on the two-sample *t* test for the difference of mean ratios.