Tobacco-Specific Carcinogens Induce Hypermethylation, DNA Adducts, and DNA Damage in Bladder Cancer

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Abstract

Smoking is a major risk factor for the development of bladder cancer; however, the functional consequences of the carcinogens in tobacco smoke and bladder cancer–associated metabolic alterations remain poorly defined. We assessed the metabolic profiles in bladder cancer smokers and non-smokers and identified the key alterations in their metabolism. LC/MS and bioinformatic analysis were performed to determine the metabolome associated with bladder cancer smokers and were further validated in cell line models. Smokers with bladder cancer were found to have elevated levels of methylated metabolites, polycyclic aromatic hydrocarbons, DNA adducts, and DNA damage. DNA methyltransferase 1 (DNMT1) expression and extensive DNA damage in bladder cancer cells. Cotreatment of bladder cancer cells with these carcinogens and the methylation inhibitor 5-aza-2′-deoxycytidine rewired the methylated metabolites, DNA adducts, and DNA damage. These findings were confirmed through the isotopic-labeled metabolic flux analysis. Screens using smoke-associated metabolites and DNA adducts could provide robust biomarkers and improve individual risk prediction in bladder cancer smokers. Noninvasive predictive biomarkers that can stratify the risk of developing bladder cancer in smokers could aid in early detection and treatment.

Introduction

Bladder cancer is a leading cause of cancer-related deaths globally (1–4), and approximately 50% of bladder cancer patients are cigarette smokers (5–7). In addition, patients with a current or past history of smoking have a 3-fold higher chance of developing bladder cancer (8–11). Moreover, the intensity and duration of smoking has been shown to affect the grade and stage of the bladder cancer, and high-dose smokers have more aggressive bladder cancer phenotypes (8, 12). Smokers with bladder cancer are also more likely to have resistance to chemotherapy (13). At
least 70 tobacco smoke compounds, including 4-(methyl nitro-
saminio)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene
(BaP) are carcinogens (14, 15). NNK has been identified as a
potent carcinogen that induces DNA adducts, mutations, and,
and promotes tumor growth through receptor-mediated effects
(14, 16). Thus, prognostic and predictive biomarkers that can
stratify the risk of developing bladder cancer based on smoking
habits are urgently needed. Recently, we found that hyper-
methylation of xenobiotic enzymes was associated with blad-
ner cancer progression (17). However, the metabolic signature
of smoke-induced bladder cancer and its downstream effects
remain to be elucidated.

Using a metabolomics approach, we compared the metabolism
in smoking and nonsmoking patients to identify metabolic sig-
natures and determined the underlying mechanism associated
with smoking-induced bladder cancer. In addition, we detected
methylated metabolites in the urine of patients, which could be
used to develop a noninvasive, urine-based assay to detect bladder
cancer in smokers.

Materials and Methods

A total of 119 pathologically verified bladder cancer tissues
(78 smokers and 41 non-smokers), normal bladder (n = 10
smokers) or benign tissues (n = 14 smokers), and 108 bladder
cancer urine samples (71 smokers and 37 non-smokers) were
obtained from different tumor banks (Supplementary Table
S1). We procured sample cohorts of smokers and non-smokers,
which include early to late stages (1a, 1b, 2, 3, and 4), distant
lymph nodes (N0, N1, N2, N3, and Ns) and lymphovascular
invasion status of bladder cancer (Supplementary Table S1).
Metabolites and DNA adducts were examined using a 6490
QQQ equipped with a 1290 LC/MS (Agilent Technologies).
ROC curves and logistic regression modeling were used to
assess bladder cancer in urine samples. An integrative approach
was used to identify transcriptomic signatures differentiating
high-grade from low-grade bladder cancer in public datasets
and evaluated the correlation between the bladder cancer grade
and smoking status of the patients. In vitro models were estab-
lished to study the tobacco smoke compounds effects. Com-
prehensive information of sample preparation protocols, meta-
bolites, DNA adducts, quality control, data acquisition and
processing, and statistical analysis is described in the Supple-
mentary Methods.

Results

Smoker-associated bladder cancer metabolic profiles

Metabolic profiling by LC/MS was used to characterize
metabolites. A summary of the identified metabolites, DNA
adducts, their experimental masses, and retention times are
shown in Supplementary Tables S2 and S3. The experimental
strategy used for profiling is shown in Fig. 1A. We measured
the metabolites using different chromatographic methods (Sup-
plementary Methods). In comparison with non-smokers, bladder
cancer smokers have 90 altered metabolites out of 300 iden-
tified metabolites (Fig. 1B), and the former are different from
the 66 altered metabolites found in smokers of normal bladder
and bladder cancer tissues (Supplementary Fig. S1B). Specifi-
cally, smokers with bladder cancer had elevated levels of
methylated metabolites, hexosamine biosynthetic pathway
intermediates, acetylated metabolites, polycyclic aromatic
hydrocarbons (PAH)/their aromatic counterparts and hydrox-
ylated derivatives compared with non-smokers with bladder
cancer (Fig. 1B).

Furthermore, expression of intermediates of the methionine
cycle was altered, in which S-(5’-adenosyl)-L-methionine (SAM)
and S-adenosyl-L-homocysteine (SAH) were decreased and
increased, respectively, in smokers compared with non-
smokers with bladder cancer. In addition to changes in the
primary metabolites, aniline, a xenobiotic compound that is
known to be involved in bladder carcinogenesis (18–20), was
high in smokers compared with non-smokers with bladder
cancer.

Pathway analysis

To address the deregulated metabolic pathways in smokers
with bladder cancer, we used the online enrichment analysis
platform ConsensusPathDB (21) and mapped the differential
metabolites between smokers and non-smokers using KEGG,
Reactome, and GOBP (Gene Ontology Biological Process) data-
base pathway analysis. We observed alterations in the levels of
metabolites associated with methionine metabolism, DNA meth-
ylation, nicotine metabolism, glutathione metabolism, nucleo-
tides, and methionine salvage pathways in smokers with bladder
cancer (Fig. 1C).

Evaluation of tissue-derived PAHs and methylated
metabolites in urine

Given the ability to discriminate between smokers and non-
smokers based on the expression of bladder cancer–associated
metabolites, we investigated the potential metabolites that can be
used as urine biomarkers. We used urine specimens from bladder
cancer patients (smokers and non-smokers) to measure tissue-
derived bladder cancer–associated metabolites using a single
reaction monitoring–based approach (Supplementary Tables
S1 and S2). Out of the 90 tissue-specific metabolic signatures
in smokers with bladder cancer, 52 were detected in the urine
samples (Supplementary Fig. S1A), out of which 40 were differ-
entially expressed in smokers and non-smokers and were further
used to run individually as ROC classifiers. Briefly, to run an ROC
curve, a logistic regression model was used to build a classifier
that was trained on a randomly selected subset of two thirds of
the urine specimens (n = 72, training set) and its predictive
performance was assessed on the remaining one third of the samples
(n = 36, test set; Fig. 1D). Logistic regression–based classification
model using all 52 metabolites was used to derive the activity
score (which has an AUC of 0.70 with a P value of 0.03; Supple-
mental Fig. S1C). On the basis of the performance of the
individual metabolites, 23 (of the 40 differential compounds)
were found to have significant ROC (P < 0.05; Supplementary
Fig. S1D). Similar logistic regression model, which was built on a
training dataset (n = 72) using activity score combining these 23
metabolites, yields an ROC in randomly selected test data (n = 36)
with an AUC = 0.87 (P < 0.0001; Fig. 1D).

Analysis of gene expression data and the clinical association
between methylation and smokers with bladder cancer

We used transcriptomic profiles of patients with bladder cancer
from publicly available datasets: Riester (22, 23); Vallot (24); Kim
(25); Lindgren (26); and The Cancer Genome Atlas (TCGA;
Supplementary Fig. S2). We generated transcriptomic signatures
of bladder cancer in smoker and non-smoker patients using the
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Riester and TCGA datasets. Next, we generated gene signatures of high-grade versus low-grade bladder cancer patients using the Vallot, Riester, Lindgren, and Kim datasets. Using the TCGA cohort, we computed the activity scores of the Riester smoking versus nonsmoking signatures and the Vallot high-grade versus low-grade signatures and then determined their Pearson

Figure 1.
Metabolic profiling of bladder cancer tissue and urine samples from smokers and non-smokers. A, An overview of the strategy used to profile and characterize the metabolome of bladder cancer tissues from non-smokers (n = 41) and smokers [(n = 78; 36 current and 42 former)]. B, Heatmap of hierarchical clustering of 90 differential metabolites across 119 bladder cancer tissues. Columns, individual tissue samples; rows, distinct metabolites. Shades of yellow and blue represent higher and lower levels of metabolites, relative to the median metabolite levels respectively (FDR < 0.1). C, Pathway analysis of the metabolic profiles in the “smoking-associated bladder cancer metabolic signature,” the node size is proportional to the number of metabolites in the pathway, and colored node represents a statistically significant enrichment. D, An ROC curve generated using 23 of the smoke-associated bladder cancer metabolites in urine to delineate smokers from non-smokers with bladder cancer (AUC = 0.87, P < 0.0001).
correlation coefficient. The two signatures were found to be highly correlated ($r = 0.8$, $P < 5 \times 10^{-5}$; Supplementary Fig. S2A), indicating a strong link between smoking and high-grade bladder cancer (Supplementary Fig. S2A and S2B). To avoid dataset-specific bias, we compared two smoking signatures (Riester and TCGA) with four high-grade versus low-grade signatures (Riester, Kim, Vallot, and Lindgren) across patient cohorts (TCGA, Kim, and Lindgren; Supplementary Fig. S2B). All of the comparisons revealed significant positive correlations ($P < 0.05$ and $r > 0$), supporting the strong association between the transcriptomic profiles of smoking and high-grade bladder cancer, suggesting that smoking could lead to an aggressive bladder cancer ($P < 0.05$ and $r > 0$). Finally, we evaluated a large patient cohort (TCGA) in which gene signatures and DNA methylations were collected. Following the methodology used in the TCGA consortium studies (28), we sorted the specimens by Riester high-grade versus low-grade signature scores and plotted the top 1000 variable CpGs (Supplementary Fig. S2C). The molecular profiles of specimens from smokers with high-grade bladder cancer exhibited higher levels of DNA hypermethylation than specimens from non-smoker with low-grade bladder cancer.

DNA adducts and detoxification enzymes in bladder cancer smokers

We analyzed DNA from 30 bladder cancer patient tissues (15 smokers and 15 non-smokers) for aminobiphenyl (ABP), methyl, NNK, BaP, hydroxy-hydroxymethyl propan-1,3-diy1 (HMMHP), and trihydroxybutyl (THB) adducts (Fig. 2A) and found higher levels of these adducts in the smokers than nonsmokers with bladder cancer (Fig. 2A; Supplementary Table S3). In addition, alterations in the xenobiotic metabolism are often considered as a causal factor for bladder cancer (29). To test this, we examined transcript levels of detoxification enzymes in bladder cancer tissues of smokers ($n = 6$) and non-smokers ($n = 6$) using qPCR (Supplementary Table S4). The mRNA levels of cytochrome P450 1B1 (CYP1B1), N-acetyltransferase 2 (NAT2), aldehyde oxidase 1 (AOX1), and epoxide hydrolase 1 (EPHX1) were lower in the tissues of smokers with bladder cancer, whereas aromatic hydrocarbon receptor (AhR), glutathione S-transferase kappa 1 (GSTK1), glutathione S-transferase T1 (GSTT1), and ATP-binding cassette subfamily B member 1 (ABCB1) were higher in the tissues of smokers with bladder cancer (Fig. 2B), suggesting that detoxification pathways associated with carcinogenic aromatic amines could modulate bladder cancer carcinogenesis.

Elevated levels of phosphorylated histone H2A variant (γ-H2AX) and phospho Chk2 in smokers with bladder cancer

DNA double-strand breaks are rapidly generated when cells are exposed to smoke or carcinogens, resulting in the phosphorylation of the histone H2A variant H2AX at Ser 139 (γ-H2AX) and Checkpoint kinase 2 (Chk2) at Thr68, which are considered to be the sensitive markers for DNA damage (30, 31). We used Western blot analysis to assess the level of γ-H2AX and phosphorylated Chk2 in bladder cancer tissues. We observed an increased expression of γ-H2AX and phospho-Chk2 in bladder cancer tissues compared with non-smokers with bladder cancer and expression levels were quantified (Fig. 2C). We further confirmed the elevated levels of γ-H2AX in tissues of smokers and non-smokers with bladder cancer by IHC (Fig. 2D).

In vitro exposure of bladder cancer cell lines to tobacco-specific carcinogens

We treated J82 bladder cancer cells with NNK and BaP and performed metabolic analysis and observed decreased levels of methionine, SAM, and increased levels of SAH, homocysteine, and glutathione compared with untreated cells. The levels of methyl DNA adducts, including N-methylguanine, methyladenine, methyladenosine, methylecysteine, methylguaninosine, and methylycystidine, were upregulated following NNK and BaP treatments (Fig. 3A).

Mitigation of carcinogenic effects using DNMT1 inhibitor

In an attempt to reverse the effects of exposure to carcinogen, we treated J82 cells with 5-aza-2’-deoxycytidine (AZA), which is a known inhibitor of DNMT1 (32). Interestingly, the altered levels of the metabolites and higher level of DNA adducts upon NNK and BaP treatment were reversed following the AZA treatment (Fig. 3A and B, respectively). We also measured DNA damage after treatment with NNK, or BaP alone, and NNK or BaP followed by AZA using confocal microscopy. The levels of γ-H2AX increased in bladder cancer cells after treatment with NNK or BaP and decreased after treatment with AZA. The expression of γ-H2AX was measured and quantified (Fig. 3C). We further confirmed these results in UMUC3 and TCCSUP cell lines (Supplementary Fig. S3A and S3B). These results strongly suggest that carcinogenic compounds of tobacco smoke activate DNMT1.

Aphidicolin in combination with NNK enhances the DNA damage

To determine the extent of DNA repair during DNA damage, J82 cells were treated with aphidicolin, an inhibitor of DNA repair, in the presence and absence of NNK. Western blot analysis showed that the γ-H2AX levels were high in aphidicolin treatment and were even higher in NNK and aphidicolin treatment. However, γ-H2AX expression was lower in cells treated with NNK followed by AZA (Fig. 3D).

DNMT1-dependent metabolomics and an altered methionine pathway in smokers with bladder cancer

We measured DNMT1 expression in tissues from smoker and non-smoker bladder cancer patients and found mRNA expression was higher in smokers than in non-smokers (Fig. 3F). A similar increase of DNMT1 protein levels was seen in smokers than non-smokers by Western blot analysis and IHC staining (Fig. 3E and G). We evaluated the effect of NNK exposure on DNMT1 levels in vitro and found that NNK treatment increased the DNMT1 mRNA and protein expression (Fig. 4A). To examine the role of DNMT1 in the methionine pathway, we successfully knocked down DNMT1 in J82 cells by shRNA (Supplementary Fig. S3C) and performed the metabolic analysis. Interestingly, the knockdown cells had significantly lower levels of SAH, homocysteine, glutathione, and methyl adducts, but had higher levels of methionine and SAM (Supplementary Fig. S3D).

We also evaluated methionine flux in J82 and its DNMT1 knockdown cells using [13C5]-labeled methionine and measured SAM and SAH levels after treatment with NNK alone and NNK followed by AZA (Fig. 4B and C). Isotopic tracing analysis showed that the 13C-labeled SAM (M+5) level decreased, and the 13C-labeled SAH (M+4) level increased after NNK treatment, indicating that NNK enhanced the DNMT1 activity.
(Fig. 4C), and subsequent AZA treatment reversed this trend (Fig. 4C). The methionine flux was repeated in DNMT1 knockdown cells and showed that the 13C-labeled SAM (M+5) level increased and the 13C-labeled SAH (M+4) level decreased (Supplementary Fig. S3E).

To understand the role of DNMT1 in cancer progression in bladder cancer patients, we correlated DNMT1 levels with epithelial–mesenchymal transition (EMT) scores using five publicly available bladder cancer datasets and found that DNMT1 expression correlated positively with the EMT scores in all datasets (Fig. 4D; Supplementary Table S5). Expression of mesenchymal genes positively correlated, whereas epithelial EMT genes negatively correlated with DNMT1 expression (P < 0.05; Supplementary Table S5). These results are consistent with cells treated with NNK and BaP, where mesenchymal EMT markers, N-cadherin, and vimentin increased, while epithelial marker E-cadherin decreased, and non-smokers (n = 6; P < 0.005). C, Western blots and quantification of g-H2AX and Chk2 protein levels in tissues of smokers (n = 15) and non-smokers (n = 15) with bladder cancer (P < 0.0008). b-Actin was used as the loading control. D, IHC staining of g-H2AX.
Figure 3.
Tobacco-specific carcinogens induce methylation, DNA adducts, DNA damage, and activation of DNMT1. A, Heatmap of metabolites from J82 cells that were treated with NNK (100 μmol/L), BaP (10 μmol/L), with NNK followed by AZA (5 μmol/L; (NNK + AZA) and BaP + AZA (FDR < 0.25). B, DNA adducts in untreated and treated cells with NNK, BaP, NNK + AZA, and BaP + AZA were measured by LC/MS-MS (P < 0.005). C, Confocal microscopy and quantification analysis of γ-H2AX (green) in J82 cells treated with NNK, NNK + AZA, BaP, and BaP + AZA (P < 0.0001). D, Western blots and quantification of γ-H2AX protein levels in J82 cells treated with aphidicolin, NNK, and AZA (P < 0.0001). β-Actin was used as the loading control. Comparison of DNMT1 protein (E) and mRNA (F) expression in tissues of smokers (n = 15) and non-smokers (n = 15) and their quantification (P < 0.05). G, IHC analysis of DNMT1 expression in bladder cancer tissues from smoker and non-smoker.
bladder cancer have higher levels of methylated metabolites, DNA adducts, and DNA damage (Fig. 4I). In addition, activation of DNMT1 by tobacco smoke may contribute to the EMT phenotype in smokers with bladder cancer.

**Discussion**

To understand the role of smoking in the development and progression of bladder cancer, and to gain insight into the bioprocesses associated with the metabolic defects in smokers, we conducted comprehensive LC/MS–based targeted metabolomic profiling of tissue and urine samples from smokers and non-smokers with bladder cancer. In this study, we found that the bladder cancer–associated metabolome was enriched with methylated metabolites, PAHs, and DNA adducts, which suggests that methylation is a hallmark of bladder cancer in smokers. The tissue-derived metabolic signature from bladder cancer smokers was validated in urine with high predictive performance (AUC = 0.70).

**Figure 4.**

NNK induced DNMT1 expression, methionine flux, and EMT markers. **A**, Box plots showing mRNA and protein expression of DNMT1, in untreated and treated J82 cells with NNK (P < 0.005) and their quantification. **B**, The role of DNMT1 in methionine pathway. **C**, Isotopic distributions of 13C-labeled methionine pathway metabolites showing downregulation of SAM and upregulation of SAH upon treatment with NNK and reverse with NNK followed by AZA in J82 cells. **D**, Correlation of DNMT1 expression with EMT scores from multiple public cohorts. **E** and **F**, mRNA and protein expression of N-cadherin, E-cadherin, and vimentin in J82 cells treated with NNK and BaP (P < 0.005) and their quantification. β-Actin was used as the loading control. **G**, Working model showing the alterations in bladder cancer associated with smoking.
0.87). Moreover, this signature is unique to the smokers and differs from normal versus bladder cancer urine signature, which was earlier published by our group (17). These urine-based smoke-specific metabolites can be further delineated to highly specific metabolites, which can be used as predictive/prognostic biomarkers in patients at risk of developing bladder cancer progression. Furthermore, analysis of publicly available bladder cancer transcriptomic and epigenomic data suggested a strong association between the smokers and high-grade bladder cancer. Using TCGA, which contains DNA methylation profiles, the top 1,000 variable CpGs associated with smoking and high-grade cancer were identified. In addition, specimens associated with high-grade bladder cancer and smoking exhibited DNA hypermethylation. The association of DNA hypermethylation observed in smokers with bladder cancer is consistent with our metabolomic data showing upregulation of methylated metabolites in smokers with bladder cancer.

Tobacco-specific carcinogens, known to cause lung, larynx, oral cavity, esophagus, pancreas, cervix, stomach, and acute myeloid leukemia (33–35), get converted into reactive intermediates that interact with DNA (35–37) with potential mutagenic consequences. We identified NNK, methyl, and other DNA adducts in smokers with bladder cancer, indicating that these carcinogens exert their effects by forming their reactive intermediates, which interact with DNA. Formation of DNA adducts in critical genes may alter protein function, leading to carcinogenic progression of cellular division (36). Our novel findings demonstrated elevated levels of methylated DNA adducts and metabolites in smokers as well as NNK- and BaP-treated cell lines, which indicates that NNK and BaP-induced hypermethylation is not only involved in the epigenetic modification of tumor suppressor genes (37, 38), but also plays a role in DNA damage and alteration of cellular metabolism. In addition, studies of elevated levels of γ-H2AX and Chk2 in tissues from smokers with bladder cancer and in cell lines treated with NNK and BaP support the hypothesis that an increased DNA damage may cause bladder cancer. Furthermore, elevated levels of tissue-derived methylated metabolites secreted into urine and their signature can potentially predict the risk of developing bladder cancer in smokers.

Previous studies have demonstrated DNMT1 induction by NNK in lung and liver cancer patients and mice models (39, 40), indicating its role in cancer development by suppressing the tumor suppressor genes through hypermethylation (37, 41, 42). The overexpression of DNMT1 in bladder cancer (43) has already been shown in previous studies. However, its association with smoking has not been demonstrated. In this study, we showed the activation of DNMT1 in smokers with bladder cancer and in cells treated with NNK, which confirms the role of DNMT1 in bladder cancer development. DNMT1 inhibitors are currently being investigated as epigenetic therapeutics for the treatment of cancer (44–46), and our findings further support the investigation of DNMT1 inhibitors as potential therapeutics for smokers with bladder cancer.

Methylation of DNA occurs at CpG+ and the methyl groups are ultimately derived from methionine (47). Isotopic labeling flux studies revealed that (i) methionine and SAM levels decreased, whereas (ii) SAH increased upon NNK and BaP treatment, suggesting that SAM synthesized from methionine is converted to SAH in NNK and BaP-induced methylation reactions. Furthermore, levels of methionine, SAM, and SAH were rewired upon treatment with AZA, confirming that tobacco smoke carcinogens alter the methionine pathway and lead to the formation of methylated metabolites and DNA adducts, resulting in the development of bladder cancer. Moreover, higher levels of methionine, SAM and lower levels of DNA adducts, SAH in DNMT1 knockdown in bladder cancer cells further strengthen the role of DNMT1 in bladder cancer development. These results are further consistent with the correlation of DNMT1 with EMT phenotype in multiple patient cohorts. These findings are in line with the earlier studies in lung and breast cancer (48, 49) where smoke induced EMT phenotype and causes more aggressive cancer. In lung cancer, smoke induces EMT by recruiting histone deacetylases via the transcriptional repressors LEF-1 and Slug, which are responsible for E-cadherin suppression (50). The mechanism of the smoke-induced EMT phenotype in bladder cancer requires further investigation.

In summary, our study provides the first clinical and in vitro evidence that tobacco smoke carcinogens, such as NNK and BaP, induce DNA adducts and damage in bladder cancer. We have identified a highly significant metabolic signature in urine that has the potential to noninvasively predict bladder cancer in smokers. The relevant findings were that smoking induced methylation by DNMT1, which plays a critical role in the accumulation of methylated metabolites with resultant DNA damage and altered metabolism in bladder cancer. Currently, there are no clinical markers for bladder cancer in smokers, and the dysregulated metabolites that we identified may serve as biomarkers that can predict the risk of developing bladder cancer in smokers in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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