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Genotoxicity of tobacco smoke-derived aromatic amines and bladder cancer: current state of knowledge and future research directions

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ABSTRACT **Bladder cancer is a significant public health problem, worldwide. In the United States, bladder cancer is the fourth most common cancer in men, and its recurrence rate is the highest among all malignancies. Tobacco smoking is the leading risk factor for bladder cancer. The risk of bladder cancer is directly related to the intensity and duration of smoking, while quitting smoking reduces this risk. The increased risk of smokers for developing bladder cancer is attributable to their exposure to aromatic amines, which constitute a family of known bladder carcinogens present in tobacco smoke. The underlying mechanism of action of aromatic amines in the genesis of bladder cancer is not, however, fully delineated. Research has identified a genotoxic mode of action, specifically DNA adduction and mutagenicity, for aromatic amines, which may account for their carcinogenicity. The present review summarizes our current knowledge on the DNA adduction and mutagenicity of aromatic amines in relation to smoking-associated bladder cancer. For illustrative purposes, representative results from published research on aromatic amine-induced DNA adduction and mutagenesis are discussed. The direction of future research on the underlying mechanisms of tobacco smoke-associated bladder carcinogenesis is also outlined. Understanding the molecular mechanisms of bladder carcinogenesis is essential for improving future strategies for prevention, early detection, treatment, and prognosis of this malignancy.—Besaratinia, A., Tommasi, S. Genotoxicity of tobacco smoke-derived aromatic amines and bladder cancer: current state of knowledge and future research directions. *FASEB J.* 27, 2090–2100 (2013). www.fasebj.org**

Key Words: 4-aminobiphenyl · 4-ABP · carcinogenesis · DNA adducts · mutation · tumorigenesis

BLADDER CANCER IS THE FOURTH most common cancer in men in the United States, and its recurrence rate is the highest among all malignancies (1–3). Tobacco smoking is the most important risk factor for bladder cancer (4, 5). The risk of bladder cancer is directly related to the intensity and duration of smoking, while quitting smoking reduces this risk (6–8). The elevated risk of bladder cancer in smokers is ascribed to their exposure to a specific class of chemical carcinogens, *i.e.*, aromatic amines, which are formed during tobacco pyrolysis (7–10). The excess risk of bladder cancer in smokers of black (air-cured) tobacco relative to blond (flue-cured) tobacco is attributable to the richer content of aromatic amines in the former tobacco products (7, 8, 11). To date, however, the underlying mechanism of aromatic amine-induced bladder carcinogenesis is not fully delineated. Elucidating the mode of action of aromatic amines in the genesis of bladder cancer can significantly increase our understanding of the pathology of this disease, which will, in turn, help improve future strategies for prevention, early detection, and treatment of this malignancy.

Exposure to tobacco smoke or its constituents is known to trigger a cascade of events in the multistage process of carcinogenesis (12–15). Of these, genotoxic effects are of significance because they occur frequently and in the early stages of carcinogenesis (16, 17). Specifically, formation of promutagenic repair-resistant DNA damage (DNA adduct) in key cancer-related genes is a common event in smoking-associated cancers (14–16). A working hypothesis is that tobacco smoke-derived aromatic amines cause bladder cancer through induction of DNA adducts and mutation in crucial cancer-related genes. This hypothesis has been extensively tested by many research investigators in the hope that a tenable theory will be produced (7–10). The research has focused on establishing the connection between aromatic amine-induced DNA adducts, mutagenesis, and bladder tumorigenesis. The present re-

Abbreviations: 4-ABP, 4-aminobiphenyl; LM-PCR, ligation-mediated polymerase chain reaction; *N*-OH-AABP, *N*-hydroxy-4-acetylaminobiphenyl; PAH, polycyclic aromatic hydrocarbon; SHS, secondhand smoke; TD-PCR, terminal transferase-dependent polymerase chain reaction; UVB, ultraviolet B

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view summarizes our current knowledge on the DNA adduction and mutagenicity of aromatic amines of relevance to bladder carcinogenesis. Although smoking is the main source of exposure to aromatic amines in the general population (4, 5), occupational exposure to these chemicals also occurs in a wide range of industries, including rubber, cable, and textile manufacturing; aluminum transformation; and gas, coal, pesticide, and cosmetics production (18–20). In addition, other sources of exposure to aromatic amines exist, including dietary (*e.g.*, pesticides in food), life style (*e.g.*, hair dye use), and environmental (*e.g.*, engine exhaust) sources (18–20). The present review, however, exclusively focuses on the DNA adduction and mutagenesis induced by aromatic amines in connection to smoking-associated bladder cancer. For illustrative purposes, representative results from published research on aromatic amine-induced DNA adducts and mutation, which has been conducted in our laboratory over the past decade, are discussed. The direction of future research on the mechanisms of tobacco smoke-associated bladder carcinogenesis is also outlined.

GENOTOXICITY OF TOBACCO-SMOKE CARCINOGENS

Many chemicals present in tobacco smoke, such as aromatic amines, polycyclic aromatic hydrocarbons (PAHs), and tobacco-specific nitrosamines, are known human and/or animal carcinogens (17, 21–24). A genotoxic mode of action for these chemicals has been delineated, which relates to their ability to directly or after biotransformation cause DNA damage, *e.g.*, by producing electrophilic species that are capable of forming covalently bound DNA lesions, known as DNA adducts (12, 14–16). Formation of DNA adducts is an event of potential significance in carcinogenesis because repair-resistant DNA adducts can be misinstructional during DNA replication, thereby giving rise to mutations (12, 16). Specific mutations in key cancer-related genes that control crucial cellular functions, such as growth and survival, may lead to tumorigenesis (12, 16, 17). Thus, formation of persistent DNA adducts in protooncogenes or tumor suppressor genes can be considered as an early event that occurs during carcinogenesis (12, 16).

INVESTIGATING CANCER ETIOLOGY BY MAPPING OF CARCINOGEN-INDUCED DNA ADDUCTS AND MUTATION

Many genotoxic carcinogens are known to leave a unique mutational signature on the human genome (25–28). These mutational signatures manifest as specific types of mutation, *e.g.*, base substitutions or insertion/deletion, occurring at distinctive nucleotide positions in protooncogenes and/or tumor suppressor genes in the tumor genome from carcinogen-exposed individuals (25, 27, 29, 30). The mutational signature of carcinogens often accords with the mutagenic potentials of their respective DNA adducts (15, 25, 26). Specifically, the signature mutation of carcinogens is preceded by the formation of carcinogen-induced DNA adducts at the same genomic loci in target organs of tumorigenesis (25). This phenomenon is best illustrated by the exemplary case of tobacco smoke-derived PAH compounds (25, 26). The *RAS* oncogenes and *TP53* tumor suppressor gene in lung tumors of smokers harbor G→T transversions at specific methylated CpG-containing codons, which colocalize to hotspots of persistent DNA adduction in PAH-exposed human lung cells *in vitro* (**Fig. 1** and refs. 13, 25, 31, 32).

Today, investigations of human cancer etiology often employ *in vitro*/*in vivo* model systems to recapitulate the mutational signature of carcinogens specific for certain types of human cancer (25, 28, 30, 33). These investigations determine whether DNA adduction and mutagenesis in carcinogen-exposed animals or cells

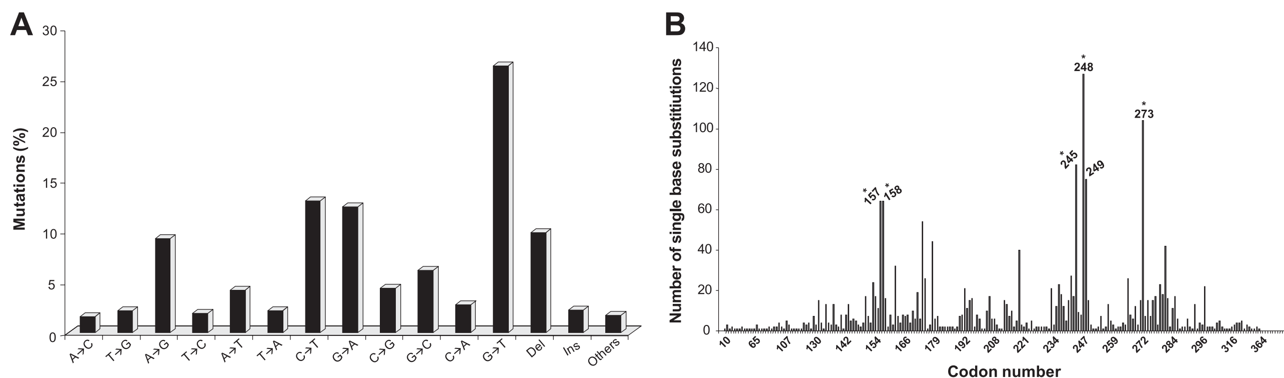


Figure 1. Mutation spectrum (*A*) and codon distribution (*B*) of the *TP53* tumor suppressor gene in tobacco smoking-associated lung cancer. Data were obtained from the *TP53* mutation database of the International Agency for Research on Cancer (http://p53.iarc.fr/P53main.html; R12 version). Entries with confounding exposure to asbestos, mustard gas, or radon were excluded. Codons containing methylated CpG sequences are indicated by asterisks. Of the major mutation hotspots found in the *TP53* gene in lung tumors of smokers, 5, including codons 157, 158, 245, 248, and 273, are preferential sites of PAH-DNA adduction (13, 31).

accord with one another, and with the mutational signature found in a cancer genome with a history of exposure to the same carcinogen (25, 26). A colocalization of the experimentally induced DNA adducts and mutations in the genome of carcinogen-treated animals or cells, which further shows consistency with tumor-specific mutations found in carcinogen-exposed individuals, indicates the etiologic relevance of the tested carcinogen (25–28, 30). We have extensively used two versatile DNA-footprinting techniques, namely ligation-mediated polymerase chain reaction (LM-PCR) and terminal transferase-dependent (TD)-PCR, which enable sensitive and specific detection of DNA adducts, at the level of nucleotide resolution, in any gene of interest in the mammalian genome (25, 34–37). Recently, we have also developed a novel high-throughput next-generation sequencing-based method to detect the mutational signature of environmental carcinogens (38). The new method is devised to establish the pattern and frequency distribution of carcinogen-induced mutations in a highly validated mouse model system (38). Work in our laboratory is also under way to develop a new DNA footprinting technique to globally map carcinogen-induced DNA adducts in mammalian cells. These novel techniques will provide a unique opportunity for investigating the underlying mechanisms of action of carcinogens, in particular, the global formation and repair of DNA adducts and induction of mutations by tobacco smoke-derived carcinogens, such as aromatic amines.

TOBACCO SMOKE-DERIVED AROMATIC AMINES AND BLADDER CANCER

As a representative compound of the class of aromatic amines, 4-aminobiphenyl (4-ABP) has been extensively studied to elucidate the underlying mechanism of bladder carcinogenesis (18, 39). A genotoxic mode of action for 4-ABP has been demonstrated both *in vitro* and *in vivo* that involves the induction of DNA adducts and mutation (40, 41). 4-ABP requires metabolic activation to exert its genotoxic effects (39). The biotransformation of 4-ABP consists of *N*-oxidation catalyzed primarily by the cytochrome *P*-450 1A2 (42) and, to a much lesser extent, *N*-methylation or peroxidation (43, 44). The resulting hydroxyarylamine may undergo detoxification through *N*-acetylation, or conjugation with acetate, sulfate, or glucuronate (45–47). The acetate and sulfate *O*-conjugates can readily interact with DNA or proteins, whereas the glucuronate *O*-conjugate can circulate in the body and reach the urinary tract, wherein it undergoes hydrolysis at the acidic pH of urine (46, 47). The resultant electrophilic nitrenium cation can bind directly the DNA of the uroepithelial cells and form covalent adducts, predominantly at the *C8* position of guanine, *N*-(deoxyguanosine-8-yl)-4-ABP (known as 4-ABP-DNA adduct; refs. 41, 48, 49). It is widely believed that persistent 4-ABP-DNA adducts and similar adducts from the family of aromatic amines are etiologically involved in the genesis of bladder cancer (39–41).

FOOTPRINTING OF DNA ADDUCTS AND DETECTION OF MUTATIONS INDUCED BY AN ACTIVATED METABOLITE OF 4-ABP IN MOUSE FIBROBLAST CELLS

We investigated the genotoxic effects of 4-ABP in the Big Blue mouse model system *in vitro* (50). The transgenic Big Blue mouse is an extensively validated model system for the study of DNA adduction and mutagenesis both *in vitro* and *in vivo* (25, 51). The genome of these transgenic animals contains multiple copies of a chromosomally integrated λ*LIZ* shuttle vector, which carries two mutational reporter genes, *i.e.*, the *cII* and *lacI*, which can be used for simultaneous analysis of DNA adducts and mutation in any organ of interest (25). We mapped the formation and kinetics of repair of DNA adducts in relation to mutagenesis in the *cII* transgene in Big Blue mouse embryonic fibroblasts treated *in vitro* with *N*-hydroxy-4-acetylaminobiphenyl (*N*-OH-AABP), which is an activated metabolite of 4-ABP (50). We used the TD-PCR technique (25, 36, 37) for footprinting of DNA adducts in the *cII* transgene of *N*-OH-AABP-treated mouse fibroblasts. We also used the *cII* mutation detection assay (52) and direct DNA sequencing to establish the mutant frequency and mutation spectrum, respectively, in the *cII* transgene of carcinogen-treated cells. Our TD-PCR footprinting analysis showed a repair-resistant formation of DNA adducts in the *cII* transgene of *N*-OH-AABP-treated cells. Five major hotspots of persistent DNA adduction were found, including nucleotide positions 97, 125, 132, 179–184, and 205–207 (see **Fig. 2**), of which all but one (*i.e.*, 97 nt) were frequent sites of mutations in the *cII* gene of carcinogen-treated cells (see below).

Our mutagenicity analysis revealed that *N*-OH-AABP treatment of the cells caused a dose-dependent increase in *cII* mutant frequency, which reached 12.8-fold over the background at the highest concentration of chemical tested (*i.e.*, 320 μM). Mutation spectrometry analysis showed a distinct pattern of mutations in the *cII* transgene in carcinogen-treated cells, as characterized by high occurrence of mutations at G:C base pairs, with the majority being G:C→T:A transversions (**Fig. 3*A***). Many of these induced mutations, particularly the G:C→T:A transversions, clustered at 4 of the 5 preferential sites of DNA adduction in the *cII* transgene in *N*-OH-AABP-treated cells (Figs. 2–**4**). Analysis of the mutation spectrum in relation to mutant frequency confirmed that G:C→T:A transversions accounted for 47.8% of all the increase in *cII* mutant frequency in carcinogen-treated cells (Fig. 3*B*). Other major types of base substitution contributing to the increase in *cII* mutant frequency in *N*-OH-AABP-treated cells were G:C→A:T transitions and G:C→C:G transversions, which comprised 20.8 and 8.5%, respectively, of all the induced *cII* mutations (Fig. 3*B*). The above targeting of

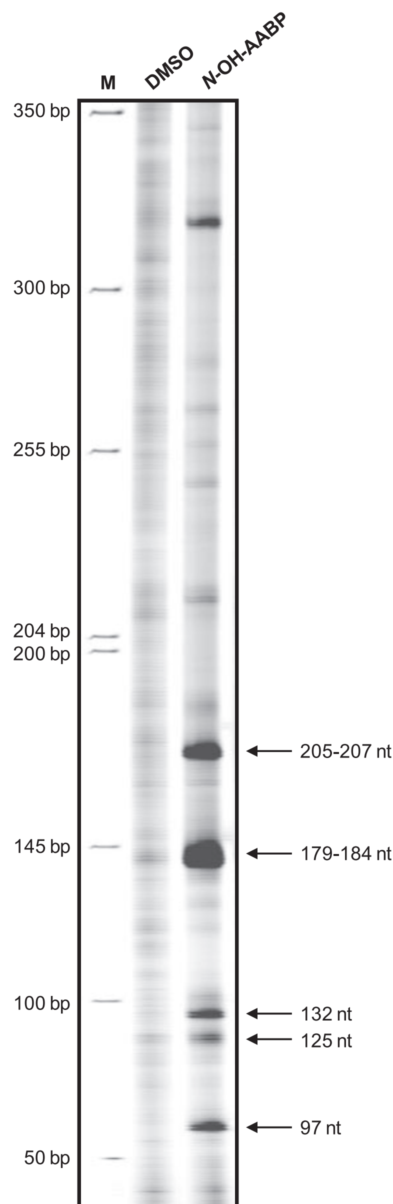


Figure 2. TD-PCR mapping of 4-ABP-DNA adducts in the *cII* gene in mouse embryonic fibroblasts treated with *N*-OH-AABP *in vitro*. Four of the 5 hotspots of 4-ABP-DNA adduction, including nucleotide positions 125, 132, 179–184, and 205–207, were also frequent sites of 4-ABP-induced mutations (see Fig. 3). Adapted from ref. 50.

mutations at G:C base pairs is consistent with the high affinity of 4-ABP to bind guanine residues in the DNA (41, 48, 49). 4-ABP or its derivatives react preferentially with the *C8* position of guanine, thereby forming a major covalent adduct, 4-ABP-DNA adduct (41, 48, 49). Theoretical and spectroscopic analyses have shown that 4-ABP-DNA adduct readily adopts a *syn* conformation around the guanine-deoxyribose linkage, and this conformational change intensifies in destabilized or unwound DNA helices, *e.g.*, during DNA replication (53, 54). The *syn* conformation places the *O6* and *N7* atoms of the modified guanine in a position to mispair with *N6* and *N1* atoms of an adenine or with *N1* and *N2* atoms of a guanine in the complementary strand, thus resulting in G→T or G→C transversion mutations, respectively (55, 56). *In vitro* and/or *in vivo* studies in various model systems have shown that 4-ABP or its metabolites induce both G:C→T:A and G:C→C:G transversion mutations, with the former being the most frequent type of mutation (57–61).

In summary, we have demonstrated a DNA adduct-driven mutagenicity of the activated metabolite of 4-ABP (*N*-OH-AABP) in mouse cells *in vitro*. The preferential formation of repair-resistant DNA adducts, colocalizing to hotspots of induced mutations, in the *cII* transgene of *N*-OH-AABP-treated cells provides mechanistic clues on the underlying mechanism of mutagenesis in this model system. Specifically, the type and frequency distribution of induced mutations in the *cII* transgene of *N*-OH-AABP cells, which correspond to the known mutagenic potentials of 4-ABP-DNA adducts (57–61), allude to the origin of mutagenicity of aromatic amines in this model system. Altogether, our findings indicate that aromatic amine-induced DNA adduction may initiate mutagenesis, which might play a role in the carcinogenicity of these chemicals.

ORGAN SPECIFICITY OF 4-ABP IN INDUCING DNA ADDUCTS AND MUTATION IN MICE

We investigated the genotoxic effects of 4-ABP in transgenic Big Blue mice *in vivo* (62). We determined the formation and kinetics of repair of 4-ABP-DNA adducts in relation to *cII* mutagenesis in the bladder and various surrogate organs of transgenic Big Blue mice treated weekly with intraperitoneal injections of 4-ABP for 6 wk, followed by a 6-wk recovery period. We used an immunodot blot assay with a specific antibody raised against 4-ABP-DNA adduct (63) to evaluate DNA damage and repair in the bladder and various surrogate organs of carcinogen-treated mice. We and others have previously used this antibody for immuno-based detection of 4-ABP-DNA adducts in various tissues of smoking populations (63–67). In addition, we used the *cII* mutation detection assay (52) to assess the organ specificity of *cII* mutagenesis and DNA sequencing to establish the type and frequency distribution of induced *cII* mutations in 4-ABP-treated mice. Of note, in our preliminary experiments, we optimized the treatment protocol to efficiently deliver a progressively increasing dose of 4-ABP $\geq$ 420 mg/kg body weight, which is tumorigenic in adult mice (68), and to induce DNA adducts and mutation, without causing any adverse health effects (including no tumor formation within the study period).

Our immunodot blot analysis of 4-ABP-DNA adducts in various organs of carcinogen-treated mice before and after the recovery period showed a repair-resistant formation of DNA adducts in the bladder, kidney, and liver of chemically treated mice. The highest levels of 4-ABP-DNA adducts were found in the bladder, liver, kidney, in the order of decrease, in carcinogen-treated mice both after the treatment and 6 wk afterward (**Fig. 5A,**

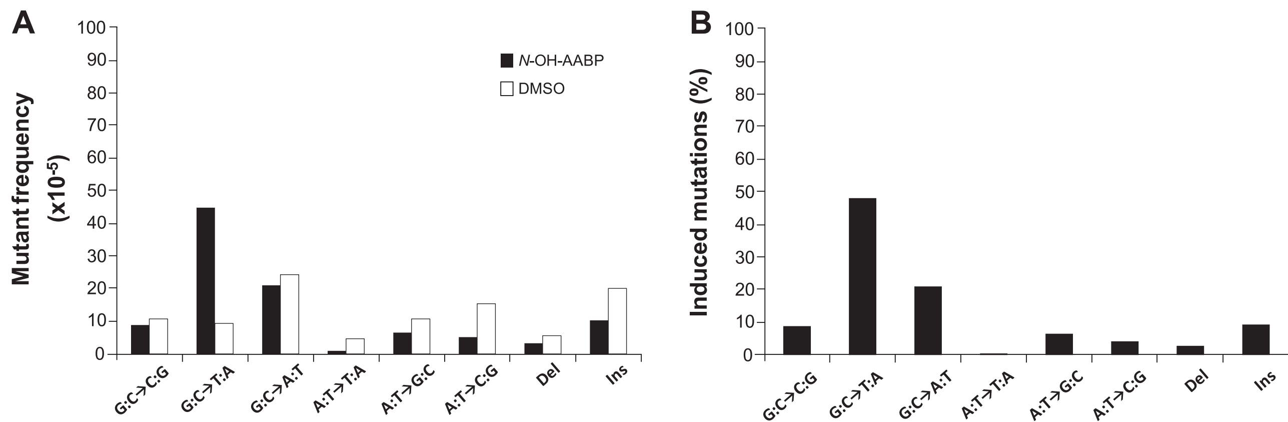


Figure 3. Spectra of mutations in the *cII* gene in mouse embryonic fibroblasts treated with *N*-OH-AABP *in vitro*. *A*) Absolute mutant frequency of each specific type of mutation in the *cII* transgene of *N*-OH-AABP-treated cells and controls was established by direct DNA sequencing. *B*) Percentage increase [induced mutations (%)] in the frequency of each specific type of mutation in the *cII* transgene of *N*-OH-AABP-treated cells relative to control. Induced mutations percentage is calculated as [(mutant frequency of each type of mutation in treatment group − mutant frequency of the respective type of mutation in control group × 100) ÷ (overall induced mutant frequency in treatment group − overall spontaneous mutant frequency in control group)].

B). This organ-specificity of 4-ABP-DNA adduction accords with its biotransformation inasmuch as reactive metabolites of 4-ABP, which are initially produced in the liver, are transported to the urinary tract, where they come in contact with the kidney and bladder urothelial cells (42, 45–47).

Our mutagenicity analysis was consistent with the above organ specificity of 4-ABP-DNA adduction in carcinogen-treated mice (68, 69). As shown in Fig. 5*C*, there was a predominant mutagenicity of 4-ABP to the bladder of carcinogen-treated mice, which remained sustained after 6 wk of recovery. In addition, much weaker but significant mutagenic responses were found in the liver and kidney of 4-ABP-treated mice. The pronounced persistence of 4-ABP-DNA adducts in the bladder relative to liver and kidney (Fig. 5*A*, *B*) can partially explain its highest mutagenicity to this organ found in carcinogen-treated mice (Fig. 5*C*). It is also likely that varying cellular proliferation rates, specific for each of these three organs, might be additionally responsible for the different mutagenic responses found in the respective organs in 4-ABP-treated mice. More specifically, the high proliferation capacity of the bladder urothelial cells may have contributed to the predominant mutagenic response found in this organ in 4-ABP-treated animals. In this study, the mutagenic effect of 4-ABP to the liver of carcinogen-treated mice manifested immediately posttreatment, and remained unchanged after an ensuing 6-wk recovery period; however, the mutagenic response in the kidney of 4-ABP-treated mice was delayed and became detectable only after 6 wk of recovery (Fig. 5*C*). Although the exact mechanism of this delayed mutagenic effect is currently unknown, the slowly proliferating renal cells may require prolonged time for fixation of mutations.

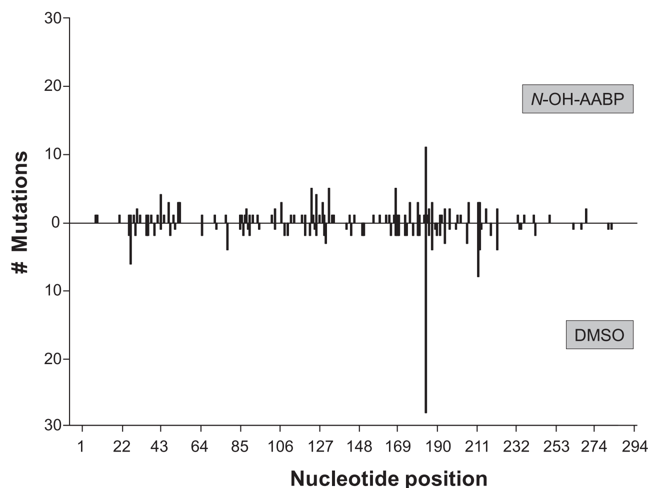
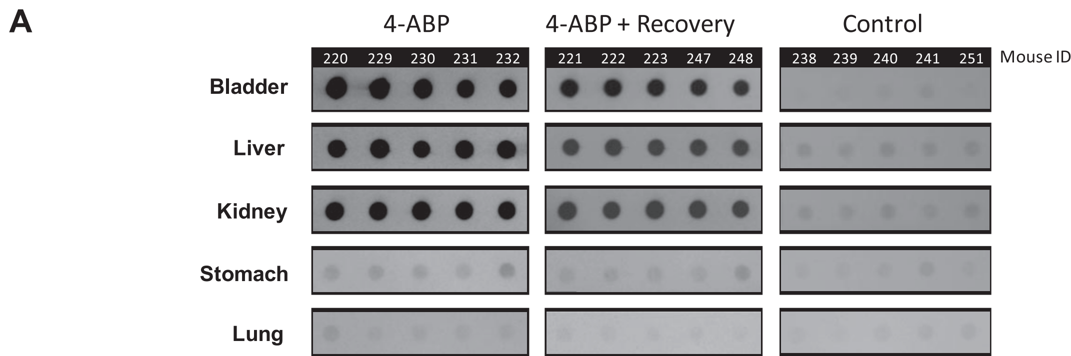
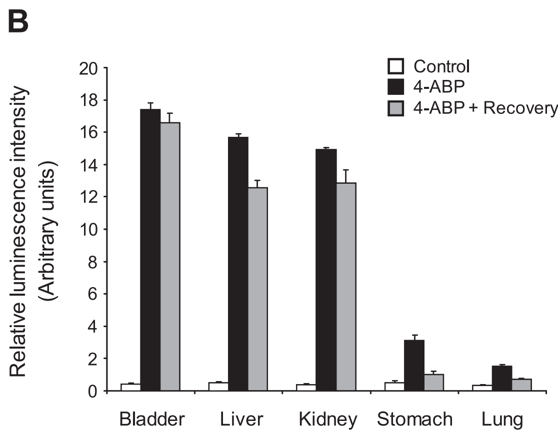


Figure 4. Distribution of mutations in the *cII* gene in mouse embryonic fibroblasts treated with *N*-OH-AABP *in vitro*. The spectra of mutations in the *cII* transgene in *N*-OH-AABP-treated cells and controls were established by direct DNA sequencing. The *N*-OH-AABP-induced mutations are indicated above the reference *cII* sequence, whereas the control mutations are shown below the reference *cII* sequence.

Our mutation spectrometry analysis was in accordance with the known mutagenic potentials of 4-ABP-DNA adducts (50, 57, 61). We found a characteristic spectrum of mutations in the bladder of 4-ABP-treated mice, which included a preponderance of mutations occurring at G:C base pairs, with the majority being G:C→T:A transversion mutations (**Fig. 6*A***). Analysis of the mutation spectrum in relation to mutant frequency revealed that G:C→T:A transversion mutations accounted for approximately half of all the increase in *cII* mutant frequency found in the bladder of carcinogen-treated mice (Fig. 6*B*). Other major types of mutation contributing to the elevation of *cII* mutant frequency in the bladder of 4-ABP-treated mice were G:C→A:T transitions and G:C→C:G transversions, which comprised 23.1 and 11.9%, respectively, of all the induced *cII* mutations (Fig. 6*B*). The above targeting of mutations at G:C base pairs and the type-specificity of the induced mutations correspond to the propensity of





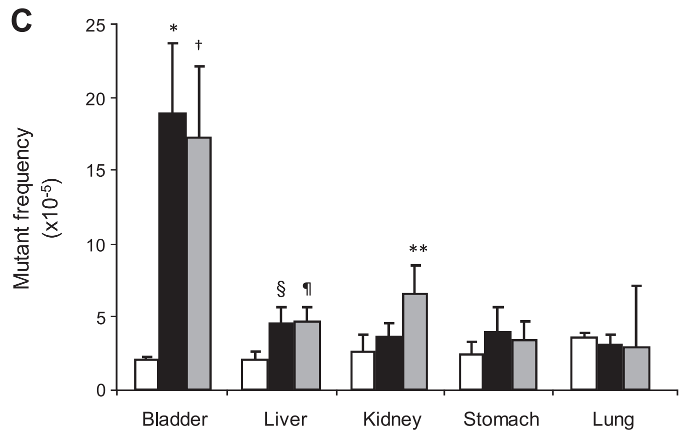


Figure 5. DNA adducts and mutation in various organs of 4-ABP-treated mice and controls. *A*, *B*) The formation and repair of 4-ABP-DNA adducts in the bladder, lung, stomach, kidney, and liver of mice treated with 4-ABP (6 weekly doses), before and after a 6-wk recovery period, were determined using an immunodot blot assay with the mouse monoclonal 4C11 antibody. *A*) Qualitative results; numbers indicate mouse IDs. *B*) Quantitative results, expressed as medians (bars). Error bars = 95% confidence intervals (CIs). *C*) The mutant frequencies of *cII* transgene in the bladder, lung, stomach, kidney, and liver of mice chronically treated with 4-ABP (6 weekly doses), before and after a 6-wk recovery period, were determined using the *cII* mutagenesis assay. $^{*}P = 0.0079$; $^{\dagger}P < 0.008$; $^{\S}P = 0.0317$; $^{\P}P < 0.008$; $^{**}P = 0.0465$ *vs.* control. Results are expressed as medians (bars). Error bars = 95% CIs. Adapted from ref. 62. Note that the background immunostaining and frequency of *cII* mutants in each organ in 2 control groups, including 6 wk sham exposure and 6 wk sham exposure plus 6 wk recovery, were not significantly different from one another (data not shown). Thus, we used the data only from the second control group for all comparison purposes.

4-ABP to form covalent adducts with guanine (41, 48, 49) and the known mutagenic potentials of the respective DNA adducts (50, 57–61), respectively.

In summary, we have demonstrated a unique organ specificity of 4-ABP in inducing persistent DNA adduction and mutagenesis in mice *in vivo*. Whereas repair-

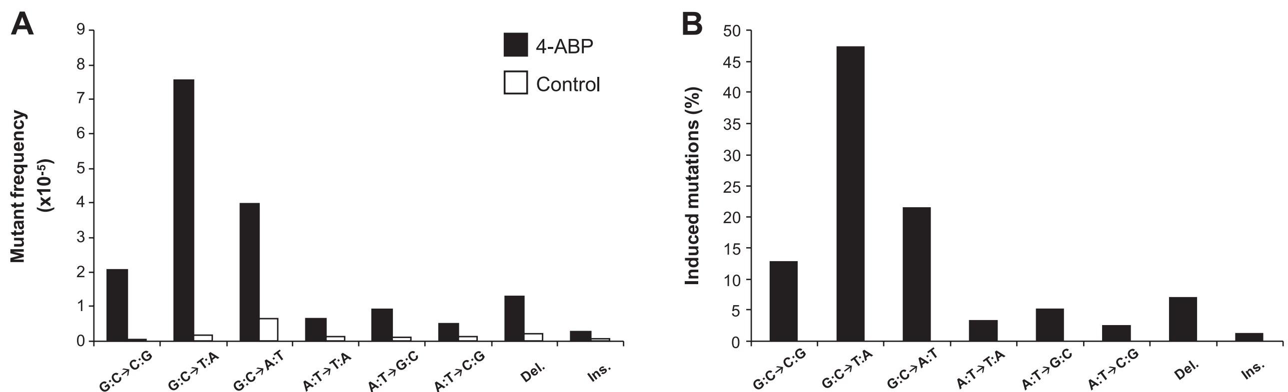


Figure 6. Spectra of *cII* mutations in bladder of 4-ABP-treated mice and controls. *A*) Absolute mutant frequency of each specific type of mutation in the *cII* transgene of bladder DNA from 4-ABP-treated mice and controls. Horizontal lines within bars represent the contribution of mutations occurring specifically at 5′-CpG dinucleotides. *B*) Percentage increase [induced mutations (%)] in the frequency of each specific type of mutation in the *cII* transgene of bladder DNA from 4-ABP-treated mice relative to control (see Fig. 3). Adapted from ref. 62.

resistant 4-ABP-DNA adducts are formed in the bladder, kidney, and liver of carcinogen-treated mice, which accords with the bioactivation pathway of this chemical in the respective organs (42, 45–47), a predominant and sustained mutagenic effect is found in the bladder, consistent with bladder specificity of tumorigenesis known for this chemical (68, 69). In addition,, 4-ABP is weakly but significantly mutagenic in the kidney and liver of carcinogen-treated mice. Of significance, the spectrum of mutations produced in the bladder of 4-ABP-treated mice perfectly reflects the known mutagenic potentials of 4-ABP-DNA adducts (50, 57–61). Altogether, our findings support a potential involvement of 4-ABP in the genesis of bladder cancer and provide a perspective on how DNA adduction leading to mutagenesis, specifically targeting the bladder urothelial cells, may account for bladder tumorigenicity of this carcinogen.

DEVELOPMENT OF A HIGH-THROUGHPUT NEXT-GENERATION SEQUENCING-BASED METHOD FOR DETECTING THE MUTATIONAL SIGNATURE OF CARCINOGENS

To develop a novel high-throughput method for detecting the mutational signature of carcinogens, we devised a cost-, time-, and labor-effective strategy in which a widely used transgenic mutagenesis assay is made compatible with a next-generation sequencing platform (38). Accordingly, we modified the Big Blue mouse mutation detection assay (25, 51) and incorporated it into the Roche/454 Genome Sequencer FLX Titanium next-generation sequencing technology (454 Life Sciences, Branford, CT, USA). In addition, we set up a detailed bioinformatics approach to process and analyze the high-volume sequencing data generated. As proof of principle, we used this novel method to detect the mutational signature of 3 prominent environmental carcinogens with varying mutagenic potencies, including sunlight ultraviolet B (UVB; $\lambda=280-320$ nm), 4-ABP, and second-hand smoke (SHS) that are known to be strong, moderate, and weak mutagens, respectively (62, 70, 71). For verification purposes, we compared the mutational signatures of these carcinogens obtained by our newly developed method to those obtained by parallel analyses using the conventional low-throughput approach, *i.e.*, standard mutation detection assay followed by direct DNA sequencing using a capillary DNA sequencer.

Applying this new method, we successfully established the mutational signatures of sunlight UVB, 4-ABP, and SHS by detecting 3 distinct *cII* mutation spectra in the genomes of Big Blue mice/cells treated with the respective carcinogens. The mutational signature of sunlight UVB was characterized by the preponderance of dipyrimidine-targeted mutations, which were predominantly G:C→A:T transitions, and clustered at several codon positions in the *cII* gene in the genome of UVB-irradiated cells (**Figs. 7*A*, *B*** and **8*A***).

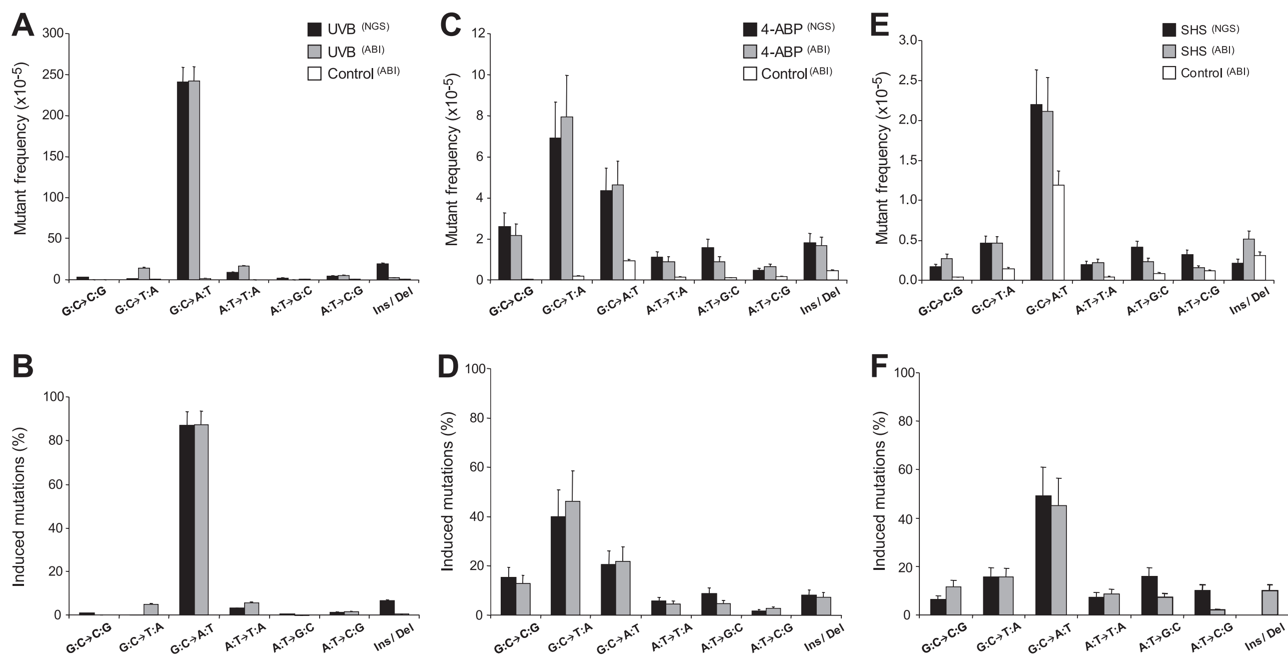


Figure 7. cII Mutant frequency and mutation spectrum in carcinogen-treated cells or mice *vs.* control. *A*, *C*, *E*) Absolute mutant frequency of each specific type of mutation in the *cII* gene of carcinogen-treated mice or cells or control, as determined by both the new method [next-generation sequencing (NGS)] and the conventional method [Applied Biosystems Inc. (ABI; Foster City, CA, USA) capillary sequencing] in UVB-irradiated cells (*A*), 4-ABP-treated mice (*C*), and SHS-exposed mice (*E*). Average results (bars) from multiple analyses plus 95% CI (error bars) are shown. *B*, *D*, *F*) Percentage increase in the frequency of each specific type of mutation in the *cII* gene of carcinogen-treated mice or cells or control, as determined by both NGS and ABI, in UVB-irradiated cells (*B*). 4-ABP-treated mice (*D*), and SHS-exposed mice (*F*) (see Fig. 3). Adapted from ref. 38.

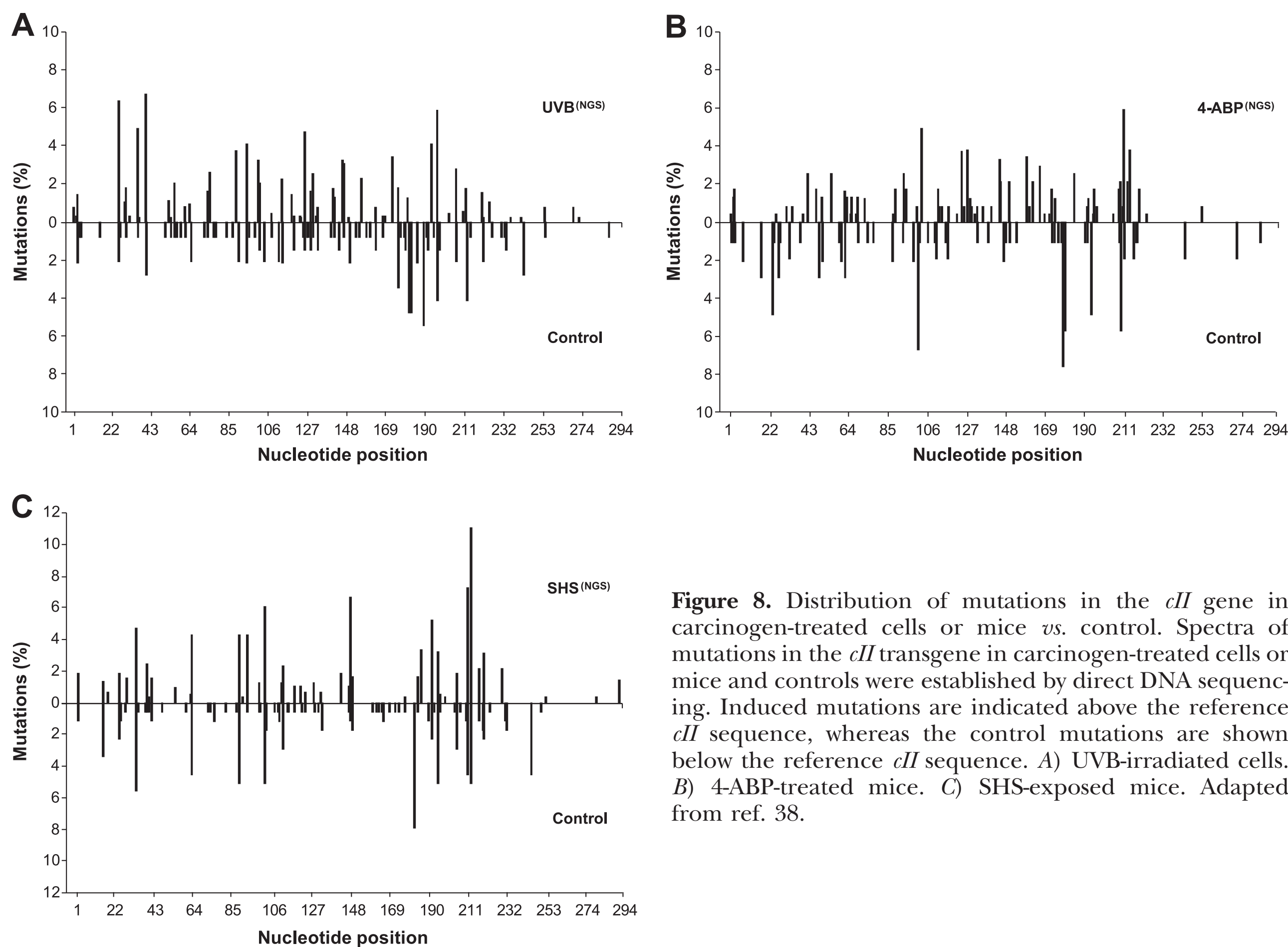


Figure 8. Distribution of mutations in the *cII* gene in carcinogen-treated cells or mice *vs.* control. Spectra of mutations in the *cII* transgene in carcinogen-treated cells or mice and controls were established by direct DNA sequencing. Induced mutations are indicated above the reference *cII* sequence, whereas the control mutations are shown below the reference *cII* sequence. *A*) UVB-irradiated cells. *B*) 4-ABP-treated mice. *C*) SHS-exposed mice. Adapted from ref. 38.

The 4-ABP-induced mutational signature was discernible as the prevailing G:C base pair-localized mutations, which were mostly G:C→T:A transversions, and occurred frequently at several codons in the *cII* gene in the genome of 4-ABP-treated mice (Figs. 7*C*, *D* and 8*B*). In the case of SHS, a subtle, yet distinguishable, mutational signature was established as the induced mutations, mostly being G:C→A:T transitions, were localized to G:C base pairs at specific codons in the *cII* gene in the genome of SHS-treated mice (Figs. 7*E*, *F* and 8*C*). The above-specified mutational signatures of these 3 carcinogens are comparable to those found previously in the same model system using the conventional low-throughput method (62, 70, 71).

In summary, we have demonstrated that this new high-throughput next-generation sequencing-based method is highly specific and sensitive to detect the mutational signature of 3 prevalent environmental carcinogens. The method is reproducible, and its accuracy is comparable to that of the currently available low-throughput method. This novel method has the potential to move the field of carcinogenesis forward by allowing high-throughput analysis of mutations induced by endogenous and/or exogenous genotoxic agents.

CONCLUSIONS AND FUTURE DIRECTIONS

Bladder cancer is a major public health problem, causing significant mortality and morbidity worldwide (2, 3). In the United States, bladder cancer is the fourth most common cancer in men, and its recurrence rate is the highest among all malignancies (1–3). Tobacco smoking is the leading risk factor for bladder cancer (4, 5). The elevated risk of bladder cancer in smokers is ascribed to their exposure to a specific class of chemicals, *i.e.*, aromatic amines, which are formed during tobacco combustion (7–10). The mechanism of action of aromatic amines in the genesis of smoking-associated bladder cancer is, however, not fully delineated. Mechanistic research in the past decades has identified a genotoxic mode of action for aromatic amines that centers on their ability to induce DNA adducts leading to mutagenesis. *In vivo*, the DNA-adduct driven mutagenicity of aromatic amines is most pronounced in the target organ of tumorigenesis, which may (partly) explain the bladder carcinogenicity of these chemicals (62). Future research is expected to fill the gap of knowledge by identifying the global genetic changes that occur during the initiation and progression of bladder cancer. The availability of next-generation sequencing technologies will be instrumental in overcoming the challenges of future research on the underlying mechanisms of bladder carcinogenesis. Development of next-generation sequencing-based DNA footprinting techniques will enable global mapping of carcinogen-DNA adducts, thus helping elucidate the initiating events that occur during bladder carcinogenesis. In

addition, genome-wide detection of tumor-specific mutations in the bladder cancer genome is a requisite for understanding the alterations of molecular pathways that cause malignant transformation of the bladder urothelial cells. Although whole-genome sequencing of bladder tumors is the most comprehensive approach toward identifying the genetic alterations that occur during the initiation and progression of bladder cancer, targeted sequencing of the bladder cancer genome may also prove invaluable. For example, whole-exome sequencing is an efficient strategy to selectively sequence the protein-coding regions of the genome (exons) as a less expensive, yet effective, alternative to whole-genome sequencing (72, 73). For genetic research, whole-exome sequencing provides high coverage in sequence depth, which allows one to find the relationship between mutations and phenotypes (74), while requiring only a fraction of the costs and resources that are needed for whole-genome sequencing (75, 76). Although exons comprise a small portion of the genome (77), the vast majority of disease-causing mutations occurs in these short functionally important sequences of DNA (74, 76). For example, in the human genome, there are ~180,000 exons, which constitute ~1% of the whole genome (~30 Mb in length; ref. 77); however, 85% of the disease-causing mutations occur in the protein-coding regions of the human genome (74). Prospectively, whole-exome sequencing can be used for the detection of mutations in the tumor genome of bladder cancer patients with a history of smoking, as well as in experimentally induced tumors from animals treated *in vivo* with tobacco smoke-derived aromatic amines.

An important, yet understudied, area of research on aromatic amine-induced genotoxicity is how replication blocking of high-fidelity polymerases by aromatic amine-DNA adducts and subsequent bypassing of these lesions by low-fidelity Y-family polymerases, *i.e.*, error-prone translesion DNA synthesis, leads to mutagenesis. Also, another area of importance for future research on aromatic amine-induced genotoxicity is how impairment of DNA repair machinery, *e.g.*, by genetic variations or common polymorphisms in nucleotide excision repair enzymes, enables aromatic amine-DNA adducts to induce mutagenesis and cause genomic instability.

Furthermore, although all indications are that aromatic amines cause genotoxic effects through DNA adduct-driven mutagenesis, alternative/additional mechanisms of action for these chemicals may also exist, which might be of relevance to bladder carcinogenesis. For instance, epigenetic effects, such as aberrant DNA methylation, chromatin remodeling, or histone modifications, may represent an alternative/additional mode of action for aromatic amines (78–85). There is suggestive evidence that aberrant DNA methylation associated with dysregulation of gene expression may occur consequent to exposure to tobacco smoke carcinogens in bladder cancer (86–88). Or, some histone modifications, such as the polycomb mark H3K27me3, are rather labile, and may easily be perturbed at many gene loci after exposure to tobacco smoke carcinogens (89). Currently, work in many laboratories is under way to utilize chromatin immunoprecipitation (ChIP)-on-chip and next-generation sequencing technologies to investigate the above epigenetic alterations in bladder cancer. Altogether, genome-wide genetic- and epigenetic studies are poised to provide new insights into the underlying mechanisms of smoking-associated bladder carcinogenesis. Increasing the mechanistic knowledge of bladder carcinogenesis can help uncover the molecular pathways perturbed during the initiation and progression of this malignancy, which will, in turn, help improve future strategies for prevention, early detection, treatment, and prognosis of this malignancy (90–93). FJ

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