Research Article

Cancer Prevention Research

Mitochondrial DNA Content as Risk Factor for Bladder Cancer and Its Association with Mitochondrial DNA Polymorphisms

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Abstract

Mitochondrial DNA (mtDNA) content has been shown to be associated with cancer susceptibility. We identified 926 bladder cancer patients and compared these with 926 healthy controls frequency matched on age, gender, and ethnicity. Patients diagnosed with bladder cancer had significantly decreased mtDNA content when compared with control subjects (median, 0.98 vs. 1.04, $P < 0.001$). Low mtDNA content (i.e., less than the median in control subjects) was associated with a statistically significant increased risk of bladder cancer, when compared with high mtDNA content [Odds ratio (OR), 1.37; 95% confidence interval (CI), 1.13–1.66; $P < 0.001$). In a trend analysis, a statistically significant dose–response relationship was detected between lower mtDNA content and increasing risk of bladder cancer (P_{trend} <0.001). When stratified by host characteristics, advanced age (>65 years), male sex and positive smoking history were significantly associated with low mtDNA content and increased risk of bladder cancer. We identified two unique mtDNA polymorphisms significantly associated with risk of bladder cancer: mitot10464c (OR, 1.39; 95% CI, 1.00–1.93; $P = 0.048$) and mitoa4918g (OR, 1.40; 95% CI, 1.00–1.95; $P = 0.049$). Analysis of the joint effect of low mtDNA content and unfavorable mtDNA polymorphisms revealed a 2.5-fold increased risk of bladder cancer (OR, 2.50; 95% CI, 1.60–3.94; $P < 0.001$). Significant interaction was observed between mitoa4918g and mtDNA content ($P_{interaction} = 0.028$). Low mtDNA content was associated with increased risk of bladder cancer and we identified new susceptibility mtDNA alleles associated with increased risk that require further investigation into the biologic underpinnings of bladder carcinogenesis. *Cancer Prev Res; 8(7); 607–13. ©2015 AACR.*

Introduction

There are an estimated 74,690 new cases and 15,580 deaths from bladder cancer in the United States projected in 2014 (1). Despite multimodality treatment strategies, oncologic outcomes have not improved resulting in an increased demand for enhanced early detection, patient selection, and targeted therapeutic strategies (2). Although the mortality/incidence ratio is higher for bladder cancer than for prostate cancer, the comparatively low incidence in the general population has been an obstacle to the development of effective screening initiatives (3). Screening of well-defined high-risk populations with bladder cancer prevalence comparable with tumor entities accepted for screening (e.g., breast cancer or colorectal cancers) may offer a solution to this problem (3, 4).

Gender-specific differences for bladder cancer diagnosis and prognosis have been well described with men being diagnosed more frequently (5). However, women tend to be diagnosed with more advanced disease which may translate into inferior oncologic outcomes (5, 6). Human mitochondrial DNA (mtDNA) is a maternally inherited genome consisting of a 16 569–base-pair circular double-stranded DNA molecule that encodes 13 polypeptides of the respiratory chain, 22 transfer RNAs, and 2 ribosomal RNAs (7). Each mitochondrion contains 2 to 10 mtDNA molecules with the number of mtDNA copies in a cell ranging from several hundred to more than 10,000 copies (7, 8). The mtDNA content varies per cell type from peripheral blood mononuclear cells having 223 to 854 copies (9) to neurons having 1,200 to 10,800 mtDNA content per cell (10). Moreover, prior studies have reported a non-normal mtDNA content distribution of mtDNAs extracted from human peripheral leukocytes or whole blood (only leukocytes in whole blood contain mtDNA; refs. 11, 12).

Normally, mtDNA content exists in a steady state related to tissue-specific energy demand of the host cells (13). Variations of mtDNA content in cells among the general population have been reported (14). Moreover, mtDNA content appears to have high heritability (8) with a decrease in mtDNA content being associated with many types of cancers. We have previously published on the heritability and reliability of mtDNA content derived from peripheral blood lymphocytes as well as the association between decreased mtDNA content and increased risk of renal cell carcinoma (8), esophageal (15), and sarcoma (16) Other studies also

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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doi: 10.1158/1940-6207.CAPR-14-0414

reported similar association in ovarian (17), gastric (18), hepatocellular (19), and breast (20) cancers. There are also several epidemiologic studies that demonstrated a statistically significant association between increasing mtDNA content in peripheral blood and increased risk of breast cancer (21) as well as other malignancies, including non-Hodgkin lymphoma (22), lung cancer (23), pancreatic cancer (24), and colorectal cancer (25). One study has even found no association between mtDNA content and gastric cancer (26). Furthermore, gender seems to play an important role in mtDNA as males have been confirmed to exhibit lower levels of mtDNA than women (8, 15, 16). No study to date has evaluated the association between constitutive mtDNA content in peripheral blood lymphocytes and risk of bladder cancer. Thus, we performed a study to test our hypothesis that individuals with lower mtDNA content are at increased risk of bladder cancer.

In addition to mtDNA content, the displacement loop (D-loop) region in mtDNA is highly polymorphic and several polymorphisms have been investigated and associated with the development of a variety of solid tumors (27–29). Numerous genetic defects are involved in the initiation and progression of bladder cancer. Moreover, Wada and colleagues (30) identified mutations in mtDNA D-loop region with the most frequent mutations noted in the poly(C) mononucleotide repeat located at positions 303 to 309. Unfavorable polymorphisms in mtDNA may contribute to increased susceptability in mtDNA damage, leading to decreased mtDNA content, which may lead to increased bladder carcinogenesis. The association between mtDNA polymorphisms and mtDNA content in modulating bladder cancer risk has yet to be determined. Therefore, the second objective of our study was to provide proof-of-principle evidence within the context of the current case–control study of bladder cancer for the hypothesis that unfavorable mtDNA polymorphisms are jointly associated with low mtDNA content, which are significantly associated with bladder cancer risk.

Materials and Methods

Patient population and data collection

In total, 926 bladder cancer patients with histologically confirmed nonvariant urothelial carcinoma were selected for inclusion in the study. Patients with a long history (>1 year) of bladder cancer before referral to MD Anderson or Baylor College of Medicine or with a prior medical history of recurrence were excluded. There were no age, gender, ethnicity, or cancer stage restrictions on recruitment. Nine hundred and twenty six healthy control subjects without a history of cancer except nonmelanoma skin cancer and frequency matched to cases by age, gender, and ethnicity were identified and recruited from the Kelsey Seybold clinic (8). After written informed consent was obtained, all study participants completed a 45-minute in-person interview that was administered by MD Anderson Cancer Center staff interviewers. The interview elicited information on demographics, smoking history, family history of cancer, occupational history and exposures, and medical history. At the conclusion of the interview, a 40-mL blood sample was drawn into coded heparinized tubes and delivered to the laboratory for analysis. This analysis was restricted to white subjects because of small sample sizes of subjects of other ethnicities. All patients provided written informed consent and the study protocol has been approved by the MD Anderson Cancer Center, Baylor College of Medicine, and Kelsey Seybold Clinic institutional review boards.

Smoking status and pack-years of smoking were defined as such. A never smoker was defined as a person who had never smoked or smoked fewer than 100 cigarettes in his or her lifetime. A former smoker was defined as a person who had stopped smoking at least 1 year before the diagnosis of cancer (for case patients) or 1 year before the interview (for control subjects). A current smoker was someone who continued smoking or who had stopped smoking less than 1 year before the diagnosis of cancer (case patients) or before the interview (control subjects). The number of pack-years was calculated as the average number of cigarettes smoked per day divided by 20 cigarettes and then multiplied by smoking years.

Determination of mtDNA content by real-time quantitative PCR

Genomic DNA was extracted from whole blood for all the samples by use of QIAamp DNA Mini kits (Qiagen). Relative mtDNA content was measured by a quantitative real-time PCR–based method as previously described (8, 31, 32). In brief, two pairs of primers were designed and used in the two steps of relative quantification for mtDNA content. One primer pair was used for the amplification of the *MT-ND1* gene in mtDNA. The primer sequences were as follows: forward primer (ND1-F), 5′-CCCTAAAACCCGCCACATCT-3′; reverse primer (ND1-R), 5′-GAGCGATGGTGAGAGCTAAGGT-3′. Another primer pair was used for the amplification of the single-copy nuclear gene human globulin (HGB). The primer sequences were as follows: forward primer (HGB-1), 5′-GTGCACCTGACTCCTGAGGAGA-3′; reverse primer (HGB-2), 5′-CCTTGATACCAACCTGCCCAG-3′. In the first step, the ratio of mtDNA content to HGB content was determined for each sample from standard curves. This ratio is proportional to the mtDNA content in each cell. The ratio for each sample was then normalized to a calibrator DNA to standardize between different runs. The calibrator DNA is a genomic DNA sample from a healthy control subject to be used for comparison of results of different independent assays. The PCR mixture in a total volume of 14 μL contained 1× SYBR Green Mastermix (Applied Biosystems), 215 nmol/L ND1-R (or HGB-1) primer, 215 nmol/L ND1-F (or HGB-2) primer, and 4 ng of genomic DNA. The thermal cycling conditions for the mtDNA (*MT-ND1* gene) amplification were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute; and for the HGB amplification were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 56°C for 1 minute. All samples were assayed in duplicate on a 384-well plate with an Applied Biosystems 7900 Sequence Detection System. The PCRs for mtDNA and HGB were always performed on separate 384-well plates with the same samples in the same well positions to avoid possible position effect. A standard curve of a diluted reference DNA, one negative control, and one calibrator DNA were included in each run. For each standard curve, one reference DNA sample was serially diluted 1:2 to produce a seven-point standard curve between 0.3125 and 20 ng of DNA. The R^2 for each standard curve was 0.99 or greater. SDs for the cycle of threshold (C_t) value were accepted at 0.25. Otherwise, the test was repeated. To further assess intraassay variation, we assayed nine blood DNA samples from healthy

control subjects three times on the same day. To further evaluate interassay variation, we evaluated the same blood DNA samples from the nine control subjects on different days. All the laboratory personnel performing the experiments described above were blinded to the case–control status of the DNA samples.

mtDNA genotyping

Genomic DNA was extracted from peripheral blood samples from 803 control subjects and 803 patients diagnosed with bladder cancer using the method described above. Sixty-three mtDNA SNPs were selected for genotyping. These mtDNA SNPSs were genotyped previously from the iSelect platform. All of the patients' genotypes were called and exported using BeadStudio software (Illumina). The average call rate for the SNP array was 99.7%. All genotyping experiments were carried out according to the standard protocol provided by Illumina Inc. with BeadStudio software (20).

Statistical analysis

The Pearson χ^2 test was used to examine the differences in the distribution of case patients and control subjects in terms of sex and smoking status. The rank sum test was used to test for differences in age, pack-years of smoking, and mtDNA content as continuous variables. To assess the association between mtDNA content and the risk of bladder cancer, data were dichotomized by the use of the median from the distribution of the mtDNA content in control subjects. To assess the dose–response trend, we categorized the mtDNA content using the quartile distribution of the mtDNA content in control subjects. A trend test was performed to test for a linear trend in the ORs by the quartile cutoff points in control subjects. Unconditional multivariable logistic regression analysis was performed to estimate the OR and 95% confidence interval (CI), adjusting for age, sex, and smoking status.

The effects of genotypes of SNPs on bladder cancer risks were estimated as odds ratios (ORs) and 95% CIs using unconditional multivariate logistic regression under the dominant models of inheritance adjusted for age, gender, and smoking status, where appropriate. The correlation was tested between mtDNA content and each mtDNA SNP in the case and control subjects using the Spearman and Kendall correlations. The cumulative effects of mtDNA SNPs that showed significant main effects with risk of bladder cancer were assessed by summing the number of unfavorable genotypes (i.e., genotypes associated with increased risk) in each subject and dividing the subjects into two groups according to the median distribution of their risks. The joint effect and interaction analysis were used for the cumulative effects of mtDNA SNPs and mtDNA content. All statistical analyses were two-sided. All statistical analyses were performed with the Stata 10.0 statistical software package (Stata Corp.).

Table 1. Distribution of selected characteristics of case patients with bladder cancer and healthy control subjects

Variables	Cases ($n = 926$)	Controls ($n = 926$)	P^b
Age (y), mean (SD)	65.2 (11.4)	63.3 (11.1)	0.7328
Sex, *N* (%)			
Male	738 (79.7)	738 (79.7)	1.000
Female	188 (20.3)	188 (20.3)	
Smoking, *N* (%)			
Never	248 (26.8)	405 (43.7)	<0.001
Former	436 (47.1)	442 (47.7)	
Current	242 (26.1)	79 (8.5)	
Ever	678 (73.2)	521 (56.3)	<0.001
Mean pack-years of smoking (SD)[a]	42.2 (30.0)	31.2 (28.3)	<0.001

NOTE: The Student *t* test was used to test for differences in age and pack-years of smoking. All statistical tests were two-sided.
[a]Pack-year was assessed for ever smokers only.
[b]Differences in the distribution of sex and smoking status were tested with the Pearson χ^2 test.

Results

Risk estimates for bladder cancer

The characteristics of the 926 cases and 926 controls are shown in Table 1. Although there was no significant difference in age between the cohorts ($P = 0.7328$) and gender, bladder cancer patients were significantly more likely to ever smokers (73.2% vs. 56.3%, $P < 0.001$) with increased mean pack-years of smoking (42.2 vs. 31.2 pack-years, $P < 0.001$).

mtDNA content were significantly different between cases and controls across age and sex subgroups (Table 2). The median mtDNA content was significantly lower among cases versus controls (0.98 vs. 1.04, $P < 0.001$), respectively. Although never smokers were found to not have significantly different mtDNA content between cases and controls (1.02 vs. 1.01, $P = 0.840$), ever smokers with bladder cancer had significantly reduced mtDNA content than ever smokers in the control group (0.97 vs. 1.05, $P < 0.001$).

Table 2. mtDNA copy number by host characteristics of case patients with bladder cancer and control subjects

Variables	Cases		Controls		
	Patients, *n*	mtDNA, median (range)	Patients, *n*	mtDNA, median (range)	*P*
mtDNA copy number					
Median (range)	926	0.98 (0.38–3.26)	926	1.04 (0.47–5.09)	<0.001
Age, y					
≤65	463	0.99 (0.38–3.26)	496	1.03 (0.48–5.09)	0.005
>65	463	0.97 (0.40–3.24)	430	1.04 (0.47–3.86)	0.002
Sex					
Male	738	0.97 (0.38–3.26)	738	1.02 (0.47–5.09)	<0.001
Female	188	1.04 (0.62–2.70)	188	1.08 (0.56–2.67)	0.058
Smoking status[a]					
Never	248	1.02 (0.44–2.54)	405	1.01 (0.47–3.91)	0.840
Ever	678	0.97 (0.38–3.26)	521	1.05 (0.47–5.09)	<0.001

NOTE: Log-rank sum was used to test for differences in mtDNA copy number, age, sex, and smoking status. All statistical tests were two-sided.
[a]Smoking status: individuals who had smoked 100 cigarettes in their lifetime were defined as ever-smokers; others were never-smokers. Smokers included current smokers and former smokers. Individuals who had quit smoking at least 1 year before diagnosis were categorized as former smokers.

Table 3. Risk of bladder cancer as estimated by mtDNA copy number

mtDNA copy number	Cases, *N* (%)	Control, *N* (%)	Adjusted OR[a] (95% CI)	*P*
By median[b]				
>1.04	385 (41.6)	463 (50.0)	1 (reference)	
≤1.04	541 (58.4)	463 (50.0)	1.37 (1.13–1.66)	0.001
By quartile[c]				
>1.24	167 (18.0)	231 (25.0)	1 (reference)	
1.04–1.24	218 (23.5)	232 (25.0)	1.23 (0.93–1.63)	0.153
0.88–1.04	252 (27.2)	232 (25.0)	1.42 (1.08–1.87)	0.013
≤0.88	289 (31.2)	231 (25.0)	1.63 (1.24–2.15)	<0.001
P_{trend}				<0.001

[a]ORs were adjusted by age, sex, and smoking status. The trend in ORs was tested by use of the test for linear trend. All statistical tests were two-sided.
[b]Median is based on levels in control subjects.
[c]Quartile is based on levels in control subjects.

The risk of bladder cancer according to mtDNA content is depicted in Table 3. After dichotomization at the 50th percentile value (or median) of mtDNA content among the control group, individuals with low mtDNA content were at a statistically significant increased risk of bladder cancer (adjusted OR, 1.37; 95% CI, 1.13–1.66). This was observed for mtDNA content according to quartiles among the control group where individuals with low mtDNA content (1st quartile) had a statistically significant increased risk of bladder cancer (OR, 1.63; 95% CI, 1.24–2.15) with a significant dose–response trend ($P_{trend} < 0.001$).

We also performed stratified analyses by host characteristics and found that age >65 years (OR, 1.56; 95% CI, 1.20–2.02; $P < 0.001$), male sex (OR, 1.37; 95% CI, 1.12–1.69; $P < 0.002$), and ever smoking history (OR, 1.62; 95% CI, 1.29–2.04; $P < 0.001$) were all significantly associated with low mtDNA content and increased risk of bladder cancer (Table 4). Moreover, we did not observe a significant interaction between lower mtDNA content and smoking history with increased risk of bladder cancer. We observed similar effect sizes and trends for male and female (Supplementary Table).

Table 4. Association of bladder cancer with mtDNA copy number stratified by selected host characteristics

Host characteristics	Cases, *N* (%)	Control, *N* (%)	Adjusted OR[a] (95%CI)	*P*
Age, y				
Age ≤65				
>1.04	192 (40.51)	260 (49.15)	1 (reference)	
≤1.04	282 (59.49)	269 (50.85)	1.27 (0.98–1.66)	0.073
Age >65				
>1.04	224 (40.58)	230 (51.00)	1 (reference)	
≤1.04	328 (59.42)	221 (49.00)	1.56 (1.20–2.02)	<0.001
Sex				
Male				
>1.04	320 (38.69)	356 (47.21)	1 (reference)	
≤1.04	507 (61.31)	398 (52.79)	1.37 (1.12–1.69)	0.002
Female				
>1.04	96 (48.24)	134 (59.29)	1 (reference)	
≤1.04	103 (51.76)	92 (40.71)	1.48 (0.99–2.21)	0.058
Smoking status[b]				
Never smoking				
>1.04	123 (45.22)	211 (48.17)	1 (reference)	
≤1.04	149 (54.78)	227 (51.83)	1.09 (0.80–1.48)	0.580
Ever smoking				
>1.04	293 (38.86)	279 (51.48)	1 (reference)	
≤1.04	461 (61.14)	263 (48.52)	1.62 (1.29–2.04)	<0.001

[a]Age, adjusted for sex and smoking status; sex, adjusted for age and smoking status; smoking status, adjusted for age and sex.
[b]Smoking status, individuals who had smoked 100 cigarettes in their lifetime were defined as ever-smokers; others were never-smokers. Smokers included current smokers and former smokers. Individuals who had quit smoking at least 1 year before diagnosis were categorized as former smokers.

Detemination of mtDNA alleles associated with bladder cancer risk

In the case group, only variant genotype of mitoc15834t was significantly associated with increased mtDNA content ($P = 0.0372$; Table 5). There were several variant genotypes (mitog15929a, mitoa11813g, mitoa14906g, mitoa4918g, and mitot10464c) significantly associated with increased mtDNA content in the control group. There were two notable mtDNA alleles, which achieved significance for risk of bladder cancer: mitot10464c (OR, 1.39; 95% CI, 1.00–1.93; $P = 0.048$) and mitoa4918g (OR, 1.40; 95% CI, 1.00–1.95; $P = 0.049$). mitot10464c was significantly correlated with increased mtDNA content in the control group ($r = 0.1023$; $P = 0.0152$), had borderline significance for decreased mtDNA content in the case group ($r = -0.0534$; $P = 0.0941$) and significantly associated with increased risk of bladder cancer. There was a significant interaction between mitoa4918g and low mtDNA content ($P_{interaction} = 0.028$). No other significant interactions were observed.

When we assessed the joint effect of mtDNA content and unfavorable mtDNA polymorphisms on risk, we observed a significant increase of risk for a low mtDNA content with 0 to 1 unfavorable genotypes (OR, 1.34; 95% CI, 1.06–1.68) and low mtDNA content with most number of unfavorable genotypes (OR, 2.50; 95% CI, 1.60–3.94), compared with 0 to 1 unfavorable mtDNA polymorphisms and a high mtDNA content (Table 6).

Discussion

Despite multimodality treatments for bladder cancer, there remains a need for improved early detection and patient selection to optimize outcomes. Several clinicopathologic characteristics influence the course of treatments for bladder cancer; however, there is a need for the development of improved biomarkers (33). Mitochondrial genome deletion may serve as a surrogate predictive biomarker for bladder cancer biopsy. There is evidence supporting that notion as, recently, a mutation in mtDNA has been used to help diagnose prostate cancer in patients with negative prostate biopsy (34).Our study identified low mtDNA content to be significantly associated with increased risk of bladder cancer and mtDNA content may aid in the detection of individuals at risk for bladder cancer. Furthermore, we identified several mtDNA SNPs associated with mtDNA content, two new susceptibility mtDNA SNPs associated with bladder cancer risk and a significant interaction noted between mitoa4918g and low mtDNA content.

Given the essential involvement of mitochondria in many important physiologic processes, including metabolism, signaling, apoptosis, cell cycle, and differentiation, it is not surprising that alterations in mtDNA content can contribute to the development of various cancers (35). The mitochondrial genome encompasses thousands of copies of mtDNA, which retains the 13 most important genes that control oxidative phophorylation (35). A shift from oxidative to more glycolytic-like metabolism is a hallmark finding in many cancer cells (35). Moreover, a

Table 5. Determination of mtDNA genotypes and copy number among subset of case patients with bladder cancer and healthy control subjects

	Association of mtDNA SNPs with mtDNA content						Association of mtDNA SNPs with bladder cancer risk	
Genotypes	Nonvariant mtDNA, *n*	Nonvariant mtDNA, median (range)	Variant mtDNA, *n*	Variant mtDNA, median (range)	Linear coefficient	*P*[a]	Adjusted OR[b] (95% CI)	*P*
Cases ($n = 806$)								
mitoc15834t	767	0.96 (0.40–3.26)	16	1.16 (0.53–2.44)	0.1533	0.0372	1.12 (0.54–2.34)	0.7621
mitog16392a	739	0.97 (0.40–3.26)	15	1.02 (0.65–2.75)	0.1302	0.0872	1.00 (0.47–2.15)	0.9916
mitot10464c[c]	694	0.97 (0.40–3.26)	97	0.95 (0.60–1.65)	−0.0534	0.0941	1.39 (1.00–1.93)	0.0482
Controls ($n = 787$)								
mitog15929a	669	1.01 (0.47–3.91)	76	1.12 (0.65–3.54)	0.1273	0.0022	1.38 (0.99–1.92)	0.0571
mitoa11813g	733	1.01 (0.47–3.91)	62	1.12 (0.65–3.54)	0.1275	0.0054	1.11 (0.77–1.61)	0.5706
mitoa14906g	707	1.01 (0.47–3.91)	74	1.12 (0.65–3.54)	0.1068	0.0119	1.36 (0.98–1.90)	0.0662
mitoa4918g[c]	722	1.01 (0.47–3.91)	72	1.12 (0.65–3.54)	0.1055	0.0140	1.40 (1.00–1.95)	0.0492
mitot10464c[c]	718	1.01 (0.47–3.91)	75	1.11 (0.65–3.54)	0.1023	0.0152	1.39 (1.00–1.93)	0.0482

[a]Derived from linear regression analysis adjusted by age, sex, and smoking status.
[b]ORs were adjusted by age, sex, and smoking status. The trend in ORs was tested by use of the test for linear trend. All statistical tests were two-sided.
[c]Joint analyses, mitot10464c and mtDNA content ($P_{interaction} = 0.0570$); mitoa4918g and mtDNA content ($P_{interaction} = 0.028$).

decrease in mtDNA content may also promote cancer cells to become resistant to apoptosis, which may further lead to the induction of many carcinogenic pathways (36). However, different types of cancer undergo different bioenergetic alterations, leading some to be more glycolytic and others to become more oxidative, which may explain increased mtDNA content associated with certain malignancies as well (23, 24).

In the present study of bladder cancer, we found lower mtDNA content was associated with a 1.37-fold increased risk of bladder cancer when mtDNA content was dichotomized by median cutoff in the controls. To the best of our knowledge, this is the first molecular epidemiologic study to evaluate mtDNA content in peripheral blood lymphocytes as a susceptibility biomarker for bladder cancer. These findings corroborate prior studies in other cancers, which identified similar decreases in mtDNA content associated with cancer risk (8, 15–20). Furthermore, our study demonstrated a significant dose-incremental increased risk of bladder cancer according to mtDNA depletion. As cancer cells shift from an oxidative to more glycolytic metabolism they may induce several carcinogenic pathways, including the PI3K–PTEN–AKT signal transduction pathway limiting apoptosis and increase cancer cell survival (37). Thus, a decrease in mtDNA content suggests a shift to a more glycolytic state and increased susceptibility to bladder cancer initiation and propagation. The use of mtDNAs extracted from human peripheral leukocytes or whole blood (only leukocytes in whole blood contain mtDNA) has been previously proven as a robust method for demonstrating the heritability and associated risks for other malignancies (8, 15).

Table 6. Joint effect analyses of mtDNA copy number and unfavorable mtDNA polymorphisms associated with bladder cancer risk

Unfavorable polymorphisms, mtDNA copy number based on median[a], *n*	Cases, *N* (%)	Control, *N* (%)	Adjusted OR[a] (95% CI)	*P*
0–1, high	252 (34.6)	319 (42.8)	1 (reference)	
0–1, low	357 (49.0)	336 (45.1)	1.34 (1.06–1.68)	0.0129
2–4, high	48 (6.6)	55 (7.4)	1.16 (0.80–1.78)	0.5119
2–4, low	72 (9.9)	35 (4.7)	2.50 (1.60–3.94)	<0.001
$P_{interaction}$				0.1280

[a]Unfavorable genotypes, mitot9900c, mitog5047a, mitog752a, mitot12415c, mitog5461a, and mitoa4918g.

Furthermore, the use of mtDNA extracted from lymphocytes uses a reproducible method, which moving forward will be helpful to compare data for this and other malignancies.

Smoking history is a well-known risk factor for many malignancies with current smokers having a 3-fold increased risk of bladder cancer than nonsmokers (38). After controlling for age and sex, we identified smoking history to be significantly associated with decreased mtDNA content and increased risk of bladder cancer. However, further analyses failed to identify a significant interaction between smoking history and low mtDNA content with risk of bladder cancer. Prior studies in renal cell carcinoma indicated that an increased mtDNA content was associated with smoking history (8) leading to the assumption that smokers have an increased need for more copies of mtDNA, which are damaged by smoking. Our data suggest the possibility that the decreased ability to produce extra copies of mtDNA due to damage from smoking may further increase the risk of bladder cancer. Thus, although we failed to observe a significant interaction, the present study makes a compelling assumption that decreased mtDNA content is an important biomarker for bladder cancer, which may be exacerbated by the carcinogenic risks associated with smoking.

Men had significantly lower mtDNA content than women among both case patients and control subjects. Bladder cancer is the fourth most common cancer among men and is no longer among the top 10 cancers diagnosed among women in the US (1). However, female gender has been associated with inferior outcomes and poor response to treatments when disease develops (39). Although the impact of gender on the staging and prognosis has attempted to explain this discordance through differences in access to care versus tumor biology (40), our findings support that genetic determinants may predispose to gender-specific risk of bladder cancer. The role of sex-steroids and bladder cancer has suggested increased risk to bladder cancer among men with polymorphisms leading to a more aggressive phenotype (41). Our findings suggest that mtDNA may also be an important driver for bladder cancer; however, the precise mechanisms that determine mtDNA content and how gender influences mtDNA content remain to be elucidated.

We report herein two new susceptibility mtDNA SNPs associated with bladder cancer risk. The biologic implications of these SNPs remain to be defined and warrant further laboratory investigation. mtDNA SNPs have been previously implicated

in other malignancies, including malignant melanoma and renal cell carcinoma where alleles in the D-loop mtDNA were associated with an increased cancer risk (42, 43). Moreover, examination of cancers of the bladder, head and neck, and lung primary tumors has previously revealed a high frequency of mtDNA mutations (27). The authors found mtDNA variant alleles to be dominant in tumor cells, readily detectable and 19 to 220 times as abundant as mutated nuclear p53 DNA. Taking our study results with mtDNA clonal nature and mtDNA content, mtDNA mutations may provide a powerful molecular marker for noninvasive detection of bladder cancer.

We observed an increased risk of bladder cancer according to low mtDNA content and unfavorable mtDNA genotypes with a significant interaction noted between mitoa4918g and low mtDNA content. We are the first to report these findings according to underlying risk of bladder cancer. A prior study identified variability in the mtDNA D-loop control region (303 polyC or 16184 polyC) in 76.9% of patients suspected of having bladder cancer (44). The noncoding mtDNA D-loop control region may effect mtDNA production and represents a hot spot for somatic mutations (45). We were unable to identify the functional significance of mitoa4918g or any of the other unfavorable genotypes; however, this represents an area of research needed to discern the precise role of these interactions in the early recognition of bladder carcinogenesis.

Our findings must be interpreted in the context of our study design and had a few limitations. First, although we found low mtDNA content in peripheral blood leukocytes to be associated with increased risk of bladder cancer, the cause–effect relationship between mtDNA content as a surrogate marker and cancer remains to be determined. In our study, we recruited newly diagnosed bladder cancer cases and collected blood samples before treatment, which should minimize the potential impact of treatment on mtDNA content. Second, the study is restricted to Caucasion populations. Validation of the finding in other ethnicity is warranted. Third, we previously published the utility and heritability of mtDNA extracted from peripheral blood leukocytes (8, 15, 16, 18); however, differences of mtDNA content among leukocyte subpopulations exists, which may warrant further studies among different subpopulations of leukocytes to discern this variability. Finally, although we provide further evidence to suggest the importance of mtDNA content and identify significant mtDNA polymorphisms with an interaction noted between mitoa4918g and low mtDNA content, future prospective validation is warranted to confirm our findings.

In summary, we found that low mtDNA content was significantly associated with increased risk of bladder cancer. Furthermore, we identified two new susceptibility mtDNA alleles associated with bladder cancer risk and a significant interaction noted between unfavorable genotype mitoa4918g and low mtDNA content. These findings warrant further validation to better identify patients most at risk for bladder cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.B. Williams, X. Wu
Development of methodology: S.B. Williams
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.B. Williams, D.W. Chang, C.P. Dinney
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.B. Williams, Y. Ye, M. Huang, D.W. Chang, X. Pu, X. Wu
Writing, review, and/or revision of the manuscript: S.B. Williams, Y. Ye, D.W. Chang, A.M. Kamat, X. Pu, C.P. Dinney, X. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Wu
Study supervision: A.M. Kamat, X. Wu

Grant Support

This work is supported by U01 CA 127615 (to X. Wu), P50 CA 91846 (to X. Wu and C.P. Dinney), and Center for Translational and Public Health Genomics at MD Anderson Cancer Center (to X. Wu)

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 20, 2014; revised March 17, 2015; accepted April 1, 2015; published OnlineFirst April 20, 2015.

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