Original Article

Peripheral blood leukocyte mitochondrial DNA content and risk of lung cancer

Gregory T. Kennedy[1], Nandita Mitra[2], Trevor M. Penning[3,4,5], Alexander S. Whitehead[3,5], Anil Vachani[1,3,6]

[1]Pulmonary, Allergy, & Critical Care Division, University of Pennsylvania, Philadelphia, PA, USA; [2]Department of Biostatics & Epidemiology, University of Pennsylvania, Philadelphia, PA, USA; [3]Center of Excellence in Environmental Toxicology, University of Pennsylvania, Philadelphia, PA, USA; [4]Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, USA; [5]Department of Systems Pharmacology and Translational Therapeutics, University of Pennsylvania, Philadelphia, PA, USA; [6]Philadelphia VA Medical Center, University of Pennsylvania, Philadelphia, PA, USA

Contributions: (I) Conception and design: GT Kennedy, AS Whitehead, A Vachani; (II) Administrative support: GT Kennedy; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: GT Kennedy, AS Whitehead, A Vachani; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Anil Vachani, MD, MS. University of Pennsylvania, Philadelphia, PA 19104, USA. Email: avachani@pennmedicine.upenn.edu.

Background: Previous studies of peripheral blood leukocyte mitochondrial DNA (mtDNA) content and risk of lung cancer have yielded inconsistent results, and no studies have evaluated the association between mtDNA content and post-resection lung cancer outcomes.

Methods: Using a case-control study design, we evaluated the association between mtDNA content and risk of lung cancer in 465 cases and 378 controls. We also evaluated the association between mtDNA content and survival in 189 cases with surgically resected non-small cell lung cancer (NSCLC). Relative mtDNA content was measured using a quantitative real-time polymerase chain reaction (PCR) assay in peripheral blood genomic DNA. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) using multivariable logistic regression, adjusting for age, gender, race, and smoking history.

Results: mtDNA content was lower in cases compared to controls, with medians of 1.26 [interquartile range (IQR), 0.98–1.70)] and 1.79 (IQR, 1.34–2.10; P<0.001), respectively. Compared to the quartile of subjects with the highest mtDNA content, there was significantly higher likelihood of lung cancer in the second lowest quartile (OR 3.44; 95% CI: 2.06–5.75) and the lowest quartile (OR 6.36; 95% CI: 3.86–10.47). In patients with resected NSCLC, there was no association between lower mtDNA content and recurrence-free survival (RFS) [hazard ratio (HR) 0.89; 95% CI: 0.47–1.66] or overall survival (OS) (HR 0.71; 95% CI: 0.35–1.46).

Conclusions: Thus, our results counter previous studies and find that lower mtDNA content is associated with lung cancer risk. Our results suggest that mtDNA content could potentially serve as a risk biomarker, but is not associated with survival outcomes in NSCLC.

Keywords: Lung cancer; mitochondrial DNA (mtDNA); risk biomarker

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Introduction

Mitochondria are subcellular organelles that produce the majority of cellular ATP through oxidative phosphorylation. Mitochondrial DNA (mtDNA) is a circular, double-stranded molecule comprising 16,569 base pairs that encode 37 genes, including 13 peptides involved in oxidative phosphorylation, two ribosomal RNAs (12S and 16S), and 22 tRNAs (1,2). The mitochondrial genome has several characteristics that differ from the nuclear genome: it is maternally inherited, it lacks introns and protective histones, and has limited DNA repair capacity. The increased susceptibility of mtDNA to damage results in a mutation frequency much higher than that of nuclear DNA (3,4).

Mutations of mtDNA are suspected to be due to the close spatial proximity of the mtDNA genome to the oxidative phosphorylation system, which is located on the inner mitochondrial membrane. mtDNA is thus susceptible to damage through leakage of reactive oxygen species (ROS) during oxidative phosphorylation (5). Some have hypothesized that, in order to maintain the cell's viability, mitochondria compensate for mutations by increasing mtDNA content (6,7). It has also been theorized that there is a level of ROS exposure beyond which this compensatory mechanism is unable to function, leading to decreases in mtDNA content at very high levels of carcinogen exposure (8). This would suggest that alterations in mtDNA content may be a marker for heightened exposure to various exogenous or endogenous carcinogens (e.g., tobacco smoke) resulting in an increased risk of cancer development.

Changes in mtDNA content have been reported in a wide variety of different cancers, with both increases and decreases described in tumor tissue, body fluids, or peripheral white blood cells (7,9-14). Given the conflicting findings from these previous studies, we evaluated mtDNA content in peripheral blood leukocytes in patients enrolled in a lung cancer case-control study to further evaluate the effect of mtDNA content on the risk of developing lung cancer, and to examine the effect across various levels of tobacco use and different races. We further assessed whether mtDNA content is a prognostic factor in patients with lung cancer who have undergone curative intent resection. We present the following article in accordance with the REMARK reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-21-979/rc).

Methods

Study population

All study subjects were selected from within a case-control study of genetic risk factors for lung cancer at the University of Pennsylvania Health System (Penn). Power calculations were not performed specifically for this study, but sample size was deemed to be adequate in comparison to existing studies of mtDNA content and cancer risk. All study subjects completed a detailed questionnaire and provided a peripheral blood sample prior to initiation of therapy. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All subjects provided informed consent for participation in this study under a protocol approved by the University of Pennsylvania Institutional Review Board (IRB Approval Number: 806390).

Cases were patients with incident lung cancer recruited through medical oncology, thoracic surgery, and pulmonary clinics between 2007 and 2012. Case status was confirmed by reviewing medical records using a standardized form and included only patients with histologically confirmed non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC). Patients who had a prior diagnosis of cancer at any site other than non-melanoma skin cancer were excluded.

Control subjects were ascertained concurrently with lung cancer cases. Controls were recruited from general medicine and pulmonary clinics. Control subjects were excluded if they had a history of any pulmonary malignancy. A standardized questionnaire was used to collect demographic information, tobacco use history, prior medical history, and other risk factors from cases and controls.

Clinical outcome data were collected for the subset of patients with stage I–II NSCLC that underwent curative intent surgery. In order to better standardize the patient sample for survival analysis, patients undergoing neoadjuvant chemotherapy or radiation were excluded from the study, as were patients who died within 30 days of surgery or had positive resection margins. Outcome data, including information on time to recurrence, recurrence site, and date of death, were obtained via chart review, direct patient contact, local cancer registry data, and from the Social Security Death Index (SSDI).

Measurement of mtDNA content

Whole blood samples were collected from study participants at the time of enrollment. Genomic DNA

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was extracted from whole blood by use of QIAamp DNA Mini kits (Qiagen). For the determination of mtDNA content relative to nuclear DNA, a quantitative real-time polymerase chain reaction (PCR)-based method was used as previously described, with some modifications (15). One primer pair was used for the amplification of a mitochondrial gene, MT-ND1: forward primer (ND1-F), 5'-CCCTAAAACCCGCCACATCT-3'; reverse primer (ND1-R), 5'-GAGCGATGGTGAGAGCTAAGGT-3'. Another primer pair was used for the amplification of human globulin (HGB), a single-copy nuclear gene: forward primer (HGB-1), 5'-GTGCACCTGACTCCTGAGGAGA-3'; reverse primer (HGB-2), 5'-CCTTGATACCAAC CTGCCCAG-3'. The ratio of ND1 copy number to HGB copy number, which is proportional to the mtDNA copy number in each cell, was determined for each sample from standard curves. The ratio for each sample was then normalized to a calibrator DNA sequence in order to standardize between different plates. The PCR reaction was performed in a total volume of 20 µL containing 10 µL Fast 2× SYBR Green Mastermix (Applied Biosystems), 2.5 µL ND1-F (or HGB-1) primer, 2.5 µL ND1-R (or HGB-2) primer, and 5 µL of genomic DNA. The thermal cycling conditions for the mtDNA (MT-ND1 gene) amplification were 95 ℃ for 10 minutes, followed by 40 cycles of 95 ℃ for 15 seconds, and 60 ℃ for 1 minute. The cycling conditions for HGB amplification were 95 ℃ for 10 minutes, followed by 40 cycles of 95 ℃ for 15 seconds, and 56 ℃ for 1 minute. All samples were assayed in duplicate on a 384-well plate with an Applied Biosystems 7900 Sequence Detection System. The PCR runs for ND1 and HGB were always performed on separate plates, but samples from a specific subject were assayed in the same well positions to avoid possible position effect.

A standard curve of a diluted reference DNA was included in each PCR batch to confirm linear assay response and determine copy number, and consisted of a reference DNA sample that was serially diluted to produce a six-point standard curve between 0.3125 and 10 ng of DNA. The R2 for each standard curve was 0.98 or greater. Any sample runs with standard deviations for the cycle of threshold value >0.25 were repeated, and all repeat assays passed this quality control cutoff.

Statistical analysis

The distribution of continuous variables (mtDNA content, age, pack-years of smoking, cigarettes per day, years smoked) was assessed by histograms, calculation of skewness and kurtosis, and Q-Q plots. The distribution of mtDNA among all study participants was unimodal but with moderate deviation from a normal distribution. Hence, mtDNA was natural log-transformed to meet underlying modeling assumptions where mtDNA was modeled as a continuous variable.

We compared distributions of selected case and control variables using the Wilcoxon rank-sum test, the Student's *t*-test for continuous variables, and the Pearson chi-squared test for categorical variables. Among case and control subjects separately, we compared mtDNA content (log-transformed) by selected characteristics. We assessed the correlation between mtDNA content and other continuous predictor variables (e.g., age, tobacco pack years) using Spearman correlation coefficients. The relationship between mtDNA content (log-transformed) and tobacco use (categorized as never, previous, and current) was assessed with one-way analysis of variance (ANOVA) followed by Bonferonni corrected pair-wise post-hoc comparisons.

To assess the association between mtDNA content and risk of lung cancer, we used multivariable logistic regression models to determine odds ratios (ORs) by quartile of mtDNA, and with mtDNA considered as a continuous variable. The models were adjusted for age at diagnosis (continuous variable), gender, tobacco use (never, previous, current), and race (White, Black, other). In alternate models, we adjusted for tobacco use by considering cigarettes per day, years smoked, and tobacco pack years. Pre-specified interactions were evaluated between mtDNA content and tobacco use, race, or gender. Stratified models were constructed to specifically examine effects of mtDNA on lung cancer risk by race. The associations between mtDNA content and recurrence-free survival (RFS) or overall survival (OS) were estimated using the method of Kaplan and Meier and assessed using the log-rank test. Cox regression models were used to adjust for potential confounders, with mtDNA content fitted as a two-level variable defined by the median (high *vs*. low). All analyses were conducted in Stata version 12.

Results

The characteristics of the case and control subjects differed significantly with respect to age, tobacco use, gender, and racial distribution (*Table 1*). As expected, cases smoked more cigarettes per day, more total years, and had a higher pack year total when compared to controls. The median mtDNA

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Table 1 Baseline characteristics of case and control subjects

Characteristics	Cases (n=465)	Controls (n=378)	P value
Age (years), median [IQR]	66 [60–73]	58 [52–64]	<0.001[2]
Gender, n (%)			0.2[1]
Male	259 (55.7)	194 (51.3)	
Female	206 (44.3)	184 (48.7)	
Race, n (%)			<0.001[1]
White	378 (81.3)	241 (63.8)	
Black	78 (16.8)	120 (31.8)	
Other	9 (1.9)	17 (4.5)	
Tobacco use, n (%)			<0.001[1]
Never	45 (9.7)	92 (24.3)	
Previous	337 (72.5)	160 (42.3)	
Current	83 (17.9)	126 (33.3)	
Cigarettes per day**, median [IQR]	20 [20–30]	20 [10–30]	<0.001[2]
Years smoked, median [IQR]	38 [30–45]	30 [18–40]	<0.001[2]
Pack years*, median [IQR]	41 [25–63]	27 [13–44]	<0.001[2]
mtDNA content, median [IQR]	1.26 [0.98–1.70]	1.79 [1.34–2.10]	<0.001[2]
mtDNA content, mean (±SD)	1.38 (±0.59)	1.83 (±0.74)	<0.001[3]

Specific cutpoints cigarettes per day, years smoked, and pack years established based on distribution among control subjects. *, data available for 669 of 706 ever smokers; **, data available for 684 of 706 ever smokers; [1], chi-square; [2], rank-sum; [3], Student's *t*-test (using natural log transformed data). IQR, interquartile range; mtDNA, mitochondrial DNA; SD, standard deviation.

content level for cases was 1.26 [interquartile range (IQR), 0.98–1.70] and 1.79 (IQR, 1.34–2.10) for controls (P<0.001).

Effect of tobacco use and race on mtDNA content

Table 2 describes the mtDNA content based on various characteristics of the case and control subjects. mtDNA content did not vary by tobacco history in case subjects but among control participants, mtDNA content among lifelong non-smokers was higher compared to current smokers, a trend which closely approached statistical significance (P=0.05). Among current or previous smokers, mtDNA content had a weak negative correlation with overall tobacco pack years (spearman rho −0.13; P<0.001) and total years smoked (spearman rho −0.09; P<0.001). Although mtDNA content did not vary by race among cases, there was significantly lower mtDNA content among White control subjects compared to Black control subjects (P<0.001).

mtDNA content and risk of lung cancer

Lower mtDNA content was significantly associated with lung cancer risk (*Table 3*). The results were similar in unadjusted models and after controlling for age, race, gender, and tobacco use. Compared to the highest quartile of mtDNA content, there was a significantly increased risk of lung cancer in the second lowest quartile [OR 3.44; 95% confidence interval (CI): 2.06–5.75] and the lowest quartile (OR 6.36; 95% CI: 3.86–10.47).

Because tobacco use is the most important risk factor for lung cancer, we performed additional adjustment for this variable. These analyses were performed in a subset of the data where lifetime non-smokers were excluded. Models with additional adjustments for tobacco pack years (*Table 3*), cigarettes per day (not shown) and years smoked (not shown) did not change the risk estimates appreciably.

Race did not significantly alter the relationship between mtDNA copy number and lung cancer risk (P=0.19). Even

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Table 2 mtDNA content by selected characteristics of study population

Variables	Cases (n=465)		Controls (n=378)	
	No. of subjects	mtDNA content, mean (±SD)	No. of subjects	mtDNA content, mean (±SD)
Age (years)				
<58	87	1.42 (±0.65)	180	1.90 (±0.80)
≥58	378	1.38 (±0.57)	198	1.76 (±0.68)
P value[1]		0.64		0.10
Gender				
Male	259	1.39 (±0.58)	194	1.80 (±0.70)
Female	206	1.37 (±0.60)	184	1.85 (±0.79)
P value[2]		0.76		0.60
Race				
White	378	1.37 (±0.59)	241	1.67 (±0.67)
Black	78	1.43 (±0.56)	120	2.12 (±0.78)
Other	9	1.61 (±0.78)	17	1.87 (±0.83)
P value[1]		0.32		<0.001*
Tobacco use				
Never	45	1.39 (±0.45)	92	1.92 (±0.65)
Previous	337	1.38 (±0.59)	160	1.83 (±0.69)
Current	83	1.39 (±0.64)	126	1.75 (±0.86)
P value[1]		0.89		0.05
Pack years				
<27 pack years	100	1.41 (±0.58)	136	1.86 (±0.82)
≥27 pack years	365	1.38 (±0.59)	242	1.80 (±0.70)
P value[2]		0.57		0.61
Years smoked				
<30 years	91	1.39 (±0.67)	129	1.80 (±0.72)
≥30 years	374	1.38 (±0.57)	249	1.84 (±0.76)
P value[2]		0.92		0.66
Cigarettes per day				
≤20 cigarettes	239	1.40 (±0.58)	199	1.78 (±0.78)
>20 cigarettes	226	1.37 (±0.60)	179	1.88 (±0.70)
P value[2]		0.57		0.08

All analyses performed on log transformed data; raw mean and SD presented. [1], ANOVA; [2], Student's *t*-test; *, *post-hoc* tests resulted in significant difference between Black *vs*. White (P<0.001). mtDNA, mitochondrial DNA; SD, standard deviation; ANOVA, analysis of variance.

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Table 3 Risk of lung cancer as estimated by mtDNA content

mtDNA content	Case patients, n	Control subjects, n	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
mtDNA content, by quartiles[1]				
Q4 (2.10–5.65)	37	97	Reference	Reference
Q3 (1.67–2.10)	57	92	1.49 (0.90–2.47)	1.57 (0.91–2.73)
Q2 (1.24–1.66)	105	96	2.87 (1.79–4.60)	2.85 (1.69–4.78)
Q1 (0.32–1.23)	266	93	6.89 (4.41–10.77)	6.44 (3.94–10.54)
mtDNA content, by quartiles[2]				
Q4 (2.10–5.65)	27	68	Reference	Reference
Q3 (1.67–2.10)	50	65	1.94 (1.09–3.46)	2.17 (1.15–4.06)
Q2 (1.24–1.66)	86	65	3.33 (1.92–5.78)	3.27 (1.81–5.93)
Q1 (0.32–1.23)	232	76	7.69 (4.59–12.87)	7.14 (4.07–12.52)

[1], adjusted for age (continuous), race (White, Black, other), gender, and tobacco use (never, previous, current); [2], adjusted for age (continuous), race (White, Black, other), gender, and tobacco pack years (dichotomous). Analysis restricted to current or previous smokers. mtDNA, mitochondrial DNA; OR, odds ratio; CI, confidence interval.

Table 4 mtDNA content and risk of lung cancer, stratified by race

mtDNA content, by median[1]	Case subjects, n	Control subjects, n	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
White and Black patients (n=817)				
High	90	176	Reference	Reference
Low	366	185	3.87 (2.84–5.27)	3.89 (2.81–5.38)
White patients only (n=619)				
High	71	98	Reference	Reference
Low	307	143	2.96 (2.06–4.27)	3.28 (2.20–4.90)
Black patients only (n=198)				
High	19	78	Reference	Reference
Low	59	42	5.77 (3.04–10.92)	4.83 (2.45–9.51)

[1], adjusted for age (continuous), gender, and tobacco use (never, previous, current). mtDNA, mitochondrial DNA; OR, odds ratio; CI, confidence interval.

though we had a relatively small number of Black subjects, we performed separate analyses by race to further examine any potential differences in lung cancer risk (*Table 4*). For these analyses, we also dichotomized mtDNA content at the median based on the distribution among control subjects. The risk of lung cancer among Black subjects with low mtDNA copy number was similar when compared to White subjects (OR 4.83 among Blacks *vs*. OR 3.28 for Whites) after adjustment for age, gender, and tobacco use (*Table 4*).

OS and RFS

Of the cases included in this study, 189 patients with early-stage NSCLC treated with surgical resection were included to study the effect of mtDNA on RFS and OS. There were a total of 42 cancer recurrences and 34 deaths during the follow-up period. Age, and gender were significantly associated with OS, whereas none of the evaluated variables were associated with RFS. mtDNA copy number was not associated with either RFS (P=0.61) or OS (P=0.32)

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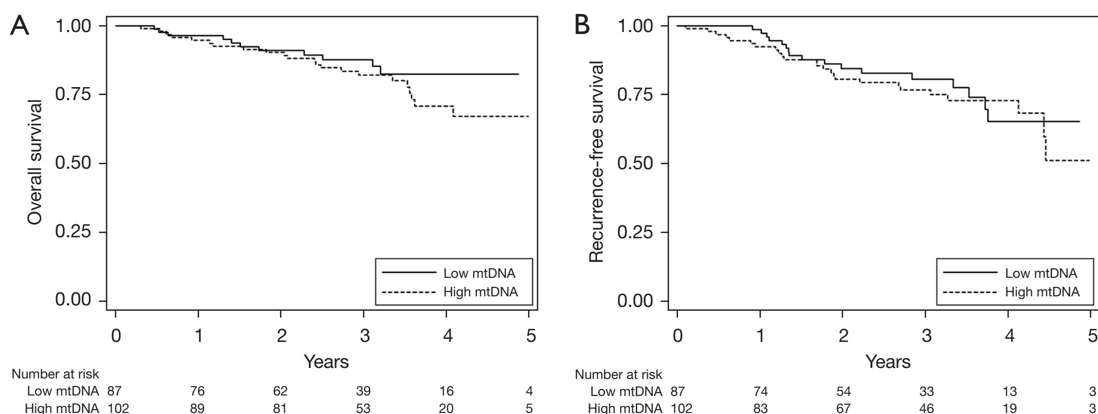

Figure 1 OS and RFS. (A) OS by mtDNA content. (B) RFS by mtDNA content. mtDNA, mitochondrial DNA; OS, overall survival; RFS, recurrence-free survival.

Table 5 Summary of prior studies of mtDNA content and risk of lung cancer

Study	Year	Study type	Number of cases	Number of controls	Method of quantifying mtDNA content	Key findings
Bonner *et al*. (9)	2009	Case-control	122	122	Sputum mtDNA content determined via qPCR	Higher mtDNA content (>157 copies/cell) was associated with lung cancer risk compared with those with ≤157 copies/cell
Hosgood *et al.* (10)	2010	Prospective cohort	227	227	Whole blood mtDNA content determined via qPCR	Highest quartile of mtDNA content associated with risk of lung cancer compared to lowest quartile
Kim *et al.* (17)	2014	Pooled case-control	880	885	Whole blood mtDNA content determined via qPCR	No consistent evidence of an association across populations by sex or smoking status/intensity
Meng *et al.* (16)	2016	Pooled case-control	463	463	Peripheral blood leukocyte mtDNA content determined via qPCR	Among current smokers, the median level of mtDNA content was associated with a higher risk of lung cancer than the high level of mtDNA content
Chen *et al.* (11)	2018	Case-control	128	107	Plasma mtDNA content determined via qPCR	Lower mtDNA content associated with cases compared to controls

mtDNA, mitochondrial DNA; qPCR, quantitative polymerase chain reaction.

in univariate analysis (*Figure 1*). After adjusting for age, gender, and stage, there remained no association between lower mtDNA and either RFS [hazard ratio (HR) 0.89; 95% CI: 0.47–1.66] or OS (HR 0.71; 95% CI: 0.35–1.46).

Discussion

In this case-control study, we show a strong, dose-dependent association between lower levels of mtDNA content in peripheral blood cells and lung cancer. The likelihood of lung cancer was most pronounced in those with the lowest amount of mtDNA content. We also found a lower mtDNA content among current smokers, when compared to either previous or life-long non-smokers, and lower mtDNA content among white control subjects compared to other races. There were no differences in mtDNA content between men and women. Finally, there was no effect of mtDNA on either risk of RFS or OS among patients with early-stage NSCLC treated with surgical resection.

Our results help clarify the existing body of literatures on mtDNA content and risk of lung cancer, which has yielded inconsistent results (*Table 5*). Our findings are concordant with a recent case-control study nested within two large prospective cohort studies: the Health Professionals Follow-Up Study and the Nurses' Health Study. In this study, the

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authors found that among current smokers, the median level of mtDNA content was associated with a higher risk of lung cancer than the high level of mtDNA content (17). These results contrast with a nested case-control study conducted within the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Cohort Study which showed that higher mtDNA copy number was associated with higher risk of lung cancer (10). However, in a pooled analysis where the ATBC study was combined with two additional prospective investigations nested in the Shanghai Women's Health Study (SWHS) and the Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial, mtDNA content was not consistently associated with lung cancer risk. Furthermore, an isolated analysis of the PLCO screening trial showed that mtDNA content was inversely associated with lung cancer risk among male smokers (16).

Cancer sites other than the lung have been studied, including renal cell (15), gastric (18), breast (13), head and neck (14), and non-Hodgkin's lymphoma (12). These studies also yield inconsistent results for the association between mtDNA content and disease risk. Although there is no clear explanation for the heterogeneity of these results, there are several potential reasons for these inconsistencies. It is possible that some of the differences seen across the various tumor types may be due to differences in study design. Several of these studies, including our own, utilized a case-control design, which raises the possibility that the findings may be due to reverse causation. Some studies suggest that the lower mtDNA content (or mtDNA depletion) may be a consequence of cancer, although these findings are commonly from studies comparing tumor tissues to corresponding normal tissue (19). Previous research in a number of different cancers has provided evidence for two potential mechanisms of mtDNA depletion. The first mechanism involves somatic point mutations in the D-loop region, a 1,124 base-pair stretch of mtDNA that contains essential promotion and replication sequences for mtDNA (20). Mutations in the D-loop region may significantly alter the replicative pattern of mtDNA resulting in reduced tumor mtDNA content (2,7,21,22). mtDNA depletion may also be a consequence of mutations in the p53 pathway. Aberrant expression of p53 may sensitize mtDNA to ROS and alter mtDNA replication (23-27). Although these pathways for mtDNA depletion elucidated in tumor cells have been described, it remains unclear whether solid tumors, such as lung cancer, use these mechanisms to lower mtDNA content in peripheral white blood cells.

In the control arm of our study, we noted significantly lower mtDNA content among current smokers compared to previous or never smokers. This finding is consistent with two recent multi-institutional studies that found a significant decrease in mtDNA content in heavy smokers as compared to former smokers or never smokers (8,17). However, some studies have shown a positive association between tobacco use and mtDNA content (15,28), and others have shown no significant effect (29).

The lack of consistent results in the literature has raised concerns regarding the methodology used to determine mtDNA content (5,30). In most of the analyses performed to date, the methods used to measure the mtDNA content result in data that cannot be directly compared across studies. Studies that have evaluated the variation in mtDNA content measurements across different laboratories suggest some consensus but also significant variability (31,32). Other measurement issues include the potential for duplication of the mitochondrial genome in the nuclear genome, use of inappropriate primers, and template preparation issues (5). It is likely that more robust and reproducible assays are needed to assess mtDNA content. Furthermore, there are a number of issues and uncertainties that prevent mtDNA content from being implemented as a screening biomarker given the current state of knowledge in the field. These issues are summarized in *Table 6*, with suggested directions for future work in the field.

Although our study includes a large, racially diverse population of subjects with and without lung cancer, there are several important limitations. The issue of case-control study design was previously discussed, and raises the possibility of reverse causation. In addition, a case-control design does not allow for repeated measures of mtDNA content, as a single measurement may not accurately reflect mtDNA content over the relevant period of disease risk. Second, our study population consists primarily of current or previous smokers, and includes only a small number of never smokers. Because new lung cancers occur only 10–15% of the time in never smokers, this is a difficult population to recruit. Given the potentially important confounding effects of tobacco use on mtDNA variation and content, further studies in never smokers may help determine the effect of other important variables, such as environmental factors, comorbidities, and age on mtDNA content and subsequent disease risk.

The mechanisms involved in the initiation and

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Table 6 Summary of key issues surrounding the current state of knowledge and barriers to implementing mtDNA testing as a biomarker for lung cancer risk

Barrier to implementing mtDNA testing as a screening biomarker	Putative root causes	Direction for future work
Inconsistency in associations found between mtDNA content and risk of lung cancer	Variability in study design and mtDNA assays used in different laboratories	Detailed, side-by-side comparison of mtDNA content levels determined by different tests using samples from the same individuals. Ultimately this will help standardize methods and work toward creation of CLIA-certified tests
Possibility of reverse causation	Case-control study design	Large prospective cohort studies of mtDNA content and lung cancer risk
Uncertainty regarding reproducibility of mtDNA content measurements over time	Case-control study design with only a single measurement of mtDNA content	Longitudinal studies of mtDNA content in the same individuals over time
Uncertainty regarding isolated effect of nicotine on leukocyte mtDNA content due to high affinity nicotinic acetylcholine receptors in leukocytes	Difficult to isolate effect of nicotine from other elements of cigarette smoke	Studies on the effect of nicotine from cigarettes compared to nicotine from other sources (nicotine replacement therapy, vaping) on mtDNA content
Potential for baseline mtDNA content variation between populations with different environmental exposures, racial/ethnic background, comorbidities, or age	Few existing studies of mtDNA content and lung cancer risk with diverse populations	Further prospective longitudinal studies (particularly in never smokers) in racially and geographically diverse populations

mtDNA, mitochondrial DNA; CLIA, Clinical Laboratory Improvement Amendments.

progression of cancer due to alterations in mtDNA are not completely understood. The risk of lung cancer is greatest among long term current smokers, and an increased risk can persist for decades after smoking cessation. The finding of greater mtDNA depletion among patients with lung cancer and among current smokers supports the possibility that this represents a marker of ongoing "injury" and may identify individuals at highest risk of lung cancer. Further longitudinal, prospective studies are needed to confirm these findings of mtDNA content and lung cancer risk, to elucidate the effect of variation in the mitochondrial genome on mtDNA content, and to study how this variation is modified by smoking and other important risk factors for lung cancer.

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Footnote

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Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-21-979/dss

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the University

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of Pennsylvania Institutional Review Board (IRB Approval Number: 806390) and all patients gave written informed consent.

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