Associations between cigarette smoking and mitochondrial DNA abnormalities in buccal cells

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DNA alterations in mitochondria are believed to play a role in carcinogenesis and are found in smoking-related cancers. We sought to replicate earlier findings for the association of smoking with increased mitochondrial DNA (mtDNA) content in buccal cells and further hypothesized that there would be an increased number of somatic mtDNA mutations in smokers. Buccal cells and blood lymphocytes were studied from 42 healthy smokers and 30 non-smokers. Temporal temperature gradient electrophoresis screening and sequencing was used to identify mtDNA mutations. The relative mtDNA content was determined by real-time polymerase chain reaction. Assuming that mtDNA in lymphocytes represents the inherited sequence, it was found that 31% of smokers harbored at least one somatic mtDNA mutation in buccal cells with a total of 39 point mutations and 8 short deletions/insertions. In contrast, only 23% of non-smokers possessed mutations with a total of 10 point mutations and no insertions/deletions detected. mtDNA somatic mutation density was higher in smokers (0.68/10 000 bp per person) than in non-smokers (0.2/10 000 bp per person). There was a statistically significant difference in the pattern of homoplasmy and heteroplasmy mutation changes between smokers and non-smokers. Whereas non-smokers had the most mutations in D-loop region (70%), smokers had mutations in both messenger RNA encoding gene (36%) and D-loop region (49%). The mean ratio of buccal cells to lymphocytes of mtDNA content in smokers was increased (2.81) when compared with non-smokers (0.46). These results indicate that cigarette smoke exposure affects mtDNA in buccal cells of smokers. Additional studies are needed to determine if mitochondrial mutation assays provide new or complementary information for estimating cigarette smoke exposure at the cellular level or as a cancer risk biomarker.

Introduction

Mitochondria are intracellular organelles that produce adenosine triphosphate by the coupling of oxidative phosphorylation to respiration, providing a major source of energy to the cell. They are the major source of endogenous reactive oxygen species (ROS) and play an important role in apoptosis. In each cell, several hundreds to thousands of mitochondrial DNA (mtDNA) copies are present (1,2).

Abbreviations: Co, cytochrome c oxidase; HT, heteroplasmy; HM, homoplasmy; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; ROS, reactive oxygen species; TTGE, temporal temperature gradient electrophoresis.

mtDNA is a circular, double-stranded, 16 569 bp DNA molecule that encodes 13 polypeptides that constitute the respiratory enzyme complexes and the 22 transfer RNA and 2 ribosomal RNA genes. It is intronless, lacks a protective histone backbone or other specific DNA-binding proteins, replicates faster than nuclear DNA and does not have proofreading or an efficient DNA repair (3,4). mtDNA is typically located near the inner membrane of the mitochondrion and is continually exposed to high levels of ROS and free radicals from the electron transport chain of mitochondria. Owing to these characteristics, mtDNA is particularly susceptible to damage by mutagens and ROS (5,6). As a result, the mitochondrial genome exhibits 17 times higher rates of mutation than does the nuclear genome (7), and DNA damage persists longer in the mitochondrial genome (8,9). Thus, we hypothesize that mtDNA damage would be a good biomarker of tobacco smoke exposure.

Cigarette smoke is a complex mixture of >3800 compounds, including high concentrations of both free radicals and chemical compounds that readily react to form other reactive substances (10,11). mtDNA mutations are found in tumors typically associated with smoking, such as oropharyngeal and lung cancer (12–14). Smoking-related damage to respiratory chain function in lymphocytes has been correlated with measures of oxidative damage (15). Oxidative damage caused by smoking has also been shown to inhibit mitochondrial enzyme activity in platelets and cause mitochondrial dysfunction in alveolar macrophages (16). In addition, an increase in mtDNA content and decline in mitochondrial function also occurs in response to DNA-damaging agents, including tobacco (12,17). Studies of lung tissue from smokers have shown elevated measures of DNA damage and increased DNA mutations when compared with lung tissue from non-smokers (10,18). Taken together, these studies suggest that comprehensive somatic mutation screening and DNA content investigation of the mitochondrial genome may be a marker for tobacco smoke exposure and consequently for tobacco-related DNA damage and smoking-associated cancer risk. To the best of our knowledge, there are no published data characterizing both somatic mutations of the entire mitochondrial genome and also variations in mtDNA content in the buccal cells of smokers.

Methods

Subjects and biospecimen collection

Smokers (n = 42) were recruited via newspaper advertising for a study of smoking behavior and health effects. Smokers must have been 18 years and older, have smoked >10 cigarettes per day with a stable smoking pattern for 6 months, must not have had a prior history of cancer or concurrent infection and not been on medications that would affect the immune system response. Non-smokers (n = 30), defined as having not smoked >100 cigarettes in their lifetime, were recruited from prostate and breast cancer screening clinics at the Lombardi Comprehensive Cancer Center. Recruitment was done following Institutional Review Board Approval.

Buccal cells were collected via a mouthwash protocol as published previously (19). Briefly, blood was collected via standard phlebotomy procedures in BD Vacutainer cell preparation tubes with sodium heparin (BD Vacutainer Systems, Franklin Lakes, NJ), which allow for the separation of lymphocytes from other blood components, according to the manufacturer's instructions (20).

Isolation of DNA from lymphocytes and buccal cells

DNA was isolated from buccal cells and lymphocytes using proteinase K (Invitrogen Corp., Carlsbad, CA) digestion and MagAttract DNA mini M48 kit in combination with the RioRobot M48 workstation (Qiagen Inc, Valencia,

Table I. mtDNA mutation frequency and density by age and smoking status

Analyzed variables	Total mutation number	Mutation frequency n (%)[a]	P[b]	Mutation density (mean ± SD)[c]	P[d]	Frequency n (%)				P[e]
						0 mutation	1 mutation	2 mutations	≥3 mutations	
Age (years)										
<45	23	9/33 (27)	0.93	0.42 ± 0.98	0.82	24 (73)	4 (12)	2 (6)	3 (9)	0.90
≥45	34	11/39 (28)		0.52 ± 1.07		28 (72)	3 (8)	3 (8)	5 (13)	
Gender										
Female	20	7/36 (19)	0.11	0.35 ± 0.88	0.20	29 (81)	2 (6)	3 (8)	2 (6)	0.25
Male	37	13/36 (36)		0.62 ± 1.14		23 (64)	5 (14)	2 (6)	6 (17)	
Race										
Caucasian	29	12/46 (26)	0.67	0.38 ± 0.88	0.54	34 (78)	5 (11)	3 (7)	4 (9)	0.83
AA[f] and Asian	28	8/28 (28)		0.65 ± 1.23		18 (64)	2 (7)	2 (7)	4 (14)	
Smoking status										
Non-smoker	10	7/30 (23)	0.48	0.20 ± 0.40	0.05[g]	23 (77)	4 (13)	3 (10)	0 (0)	0.04
Smoker	47	13/42 (31)		0.68 ± 1.27		29 (69)	3 (7)	2 (5)	8 (19)	
Pack-years										
5–18.9	15	5/14 (36)	0.76	0.65 ± 1.15	0.60	9 (64)	2 (14)	0 (0)	3 (21)	0.62
19–36.9	11	3/13 (23)		0.51 ± 1.35		10 (77)	1 (8)	1 (8)	1 (8)	
>37	21	5/15 (33)		0.85 ± 1.37		10 (67)	0 (0)	1 (7)	4 (27)	
Age at smoking initiation										
<17	24	8/22 (36)	0.51	0.66 ± 1.28	0.66	14 (64)	3 (14)	2 (9)	3 (14)	0.16
≥17	23	5/20 (25)		0.69 ± 1.29		15 (75)	0 (0)	0 (0)	5 (25)	
Years smoked										
5–19	10	3/13 (23)	0.54	0.46 ± 1.33	0.36	10 (77)	2 (15)	0 (0)	1 (8)	0.55
20–37	25	7/17 (41)		0.88 ± 1.30		10 (59)	1 (6)	1 (6)	5 (29)	
>38	12	3/12 (25)		0.60 ± 1.21		9 (75)	0 (0)	1 (8)	2 (17)	
Cigarettes smoked per day										
10–15	20	6/15 (40)	0.49	0.81 ± 1.20	0.27	9 (60)	1 (7)	1 (7)	4 (27)	0.4
16–24	10	4/19 (21)		0.32 ± 0.86		15 (79)	2 (11)	1 (5)	1 (5)	
>25	17	3/8 (38)		1.28 ± 1.95		5 (63)	0 (0)	0 (0)	3 (38)	

[a]Mutation frequency: number of subjects with mutations/total number of subjects (%).
[b]Chi-square test.
[c]Mutation density (somatic mutation number/10000 bp per person).
[d]Wilcoxon rank test.
[e]Fisher's exact test.
[f]AA, African-American.
[g]Mean difference in mutation density between smokers and non-smokers 0.47 ± 0.40 (mean ± SD).

CA). The concentration of DNA was determined using a Spectra Max Plus 384 Microplate Spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

Comprehensive mutational analysis of the entire mitochondrial genome
The presence of mtDNA mutations was screened by temporal temperature gradient electrophoresis (TTGE). TTGE is based on the difference in the sequence-specific melting behavior of normal and mutant DNA fragments that results in different electrophoretic mobility of partially denatured mismatched mtDNA strands. The entire 16.5 kb mtDNA genome was subjected to polymerase chain reaction (PCR) using 32 pairs of overlapping primers that produce amplicons varying in size from 306 to 805 bp (21,22). Each fragment has an average of 74 bp on each end overlapping the neighboring fragments. PCR products were denatured at 95°C for 30 s and slowly cooled to 45°C over a period of 45 min at a rate of 1.1°C/min decrease. TTGE was performed on a Bio-Rad D-Code mutation detection apparatus, using two back-to-back 20 cm × 20 cm × 1 mm 4.5–6% polyacrylamide (acrylamide:Bis = 37.5:1) gels in 1.25× TAE buffer containing 6 mol/l urea. Electrophoresis was carried out at 145 V for 4–5 h at a constant temperature increment of 1–2°C/h. The beginning and ending temperatures were determined by computer simulation from the melting curve (50% denatured) of the DNA fragment (MacMelt software, Bio-Rad Laboratories, Hercules, CA) (21–23). For TTGE analysis, a single band shift represents a homoplasmic DNA alteration and a multiple banding pattern represents a heteroplasmic mutation. The 32 mtDNA fragments from buccal cells and lymphocytes of the same subject were analyzed side by side.

mtDNA with TTGE banding pattern abnormalities then underwent direct DNA sequencing using the same PCR fragment; detected mutations underwent repeat PCR and sequencing for confirmation. The PCR products were purified prior to sequencing using ExoSap-IT (USB Corp., Cleveland, OH), which removes primers and unincorporated nucleotides. DNA sequencing was performed using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) and a MegaBACE 1000 DNA analysis system (Amersham Biosciences). The results of DNA sequence analysis were compared with the Human Mitochondrial DNA Revised Cambridge Reference Sequence deposited in GenBank (accession number NC_001807) and MitoMap (http://www.mitomap.org) using Vector NTI Advance 9 software (Invitrogen Corp, Carlsbad, CA). Any DNA sequence differences between buccal cells and matched lymphocytes mtDNA were scored as somatic mutations.

Detection of mtDNA deletions
The detection of two deletions of 4977 bp (np8470–8482/np13447–13459) and 4839 bp (np8703–8711/np13541–13549) was performed using PCR with four sets of primers. During PCR, the forward primer mtF8416 (5′-CCTTACAC-TATTCCTCATC-3′) was used in the presence of two reverse primers mtR9169 (5′-TGAAAACGTAGGCTTGGAT-3′) (within the region of 4977 and 4839 bp deletions) and mtR13738 (5′-TGAGAAATCCTGCGAATAG-3′) (outside of the 4977 and 4839 bp deletions). mtDNA without a deletion would serve as a template for only the mtR8416 and mtR9196 primer sets and yield a PCR product of 754 bp. mtDNA with the 4977 or 4839 bp deletion would serve as a template only for the mtR8416 and mtR13738 primer sets and yield a 346 or 485 bp PCR product, respectively.

Measurement of mtDNA content (relative copy number)
An Applied Biosystems 7900 real-time PCR system was used to perform quantitative PCR amplification for mtDNA gene cytochrome c oxidase I (CoI). β-actin was used as a 'housekeeping gene' to normalize all of the threshold cycle (C_t) values. Primers were custom designed and obtained from Invitrogen. CoI gene forward primer (mtCoxI-F5908), 5′-TCGCCGACCGTTGAC-TATTCTCT-3′ and reverse primer (mtCoxI-R6103), 5′-AAGATTATTAC AAATGCATGGGC-3′ (196 bp product) and β-actin forward primer, 5′-AC-CCACACTGTGCCCATCTAC-3′ and reverse primer, 5′-TCGGTGAG-GATCTTCATGAGGTA-3′ (103 bp product). Taqman probes (Applied Biosystems, Foster City, CA) included (CoI) probe 6-FAM-AACGACCAC-ATCTACAACGTTATCGTCAC-TAMRA and β-actin probe 6-FAM-ATG CCCTCCCCCATGCCATCC-TAMRA (6). Real-time PCRs were performed

in triplicate for each sample. Amplifications were carried out in buffer containing $1\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer, 200 nM of eachTaqMan probe and 5 ng of total genomic DNA. Thermal cycling conditions were an initial 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s of denaturation at 95°C and 60 s of annealing/extension at 60°C. Fluorescent signal intensity was recorded and analyzed using SDS version 2.1 software (Applied Biosystems). The C_t value within the linear exponential increase phase was used to measure the original copy number of the DNA template. An average β-actin C_t value was chosen as a normalization value. The correction C_t value for β-actin and *CoI* as well as the relative number of mtDNA copies were calculated using the following equations, respectively: β-actin correction value = mean sample C_t β-actin value − 20 and the corrected *CoI* C_t value = mean *CoI* C_t − β-actin correction value. The maximum normalized C_t value of all samples was determined to equal one copy of *CoI*, and the relative *Cox* copy number = $2^{(\text{maximum } C_t - \text{corrected CoI } C_t \text{ value})}$. The mtDNA content was expressed as the ratio of *CoI I* copy number to β-actin gene copy number.

Statistical analysis

Pack-years were calculated as usual packs of cigarettes smoked per day multiplied by years of smoking. Subjects were classified into smoking and age categories by the median and tertiles, as indicated in Tables I and IV. Fisher's exact test, chi-square test and Wilcoxon rank test were used to analyze the association between mtDNA mutations and cigarette smoking status. The *t*-test, paired *t*-test and analysis of variance test were used to analyze the difference in mutation density and mtDNA content between smokers and non-smokers. The probability of the trend for increased mutation frequency in relation to smoking status was tested by the Cochran–Armitage test. The correlation between mtDNA mutation density, relative content and smoking status was analyzed by the non-parametric Spearman's rho correlation test and the Pearson's correlation test after log transformation of the data. *P* values <0.05 are considered statistically significant. All statistical computations were done using the SPSS v14.0 (SPSS, Chicago, IL) and SAS v9.12 (SAS Institute, Cary, NC). All reported *P* values are two sided.

Results

Subjects' age ranged from 19 to 87 years with a mean age of 45.4 ± 13.4 for smokers and 45.8 ± 14.5 for non-smokers. Among smokers,

there were 22 men and 20 women, 29 were Caucasians and 13 were African-Americans. Among non-smokers, there were 14 men and 16 women. Seventeen were Caucasians and 13 were African-Americans or Asians.

mtDNA somatic mutations analysis

Somatic mtDNA mutations in buccal cells from 42 smokers and 30 non-smokers were detected by parallel analysis using TTGE and direct sequencing. Sequence patterns that were different comparing buccal cells with lymphocytes were considered to represent acquired mutations in the buccal cells (Figure 1). The mtDNA mutation frequency and density by age and smoking status is shown in Table I. There was no correlation found between mutation density and age among all subjects ($P = 0.58$), smokers ($P = 0.79$) or non-smokers ($P = 0.47$). The frequency of mutations, mutation density and type of mutation also did not differ by gender or race.

Table II provides summary data for mtDNA mutations and Table III lists all mutations found in all subjects. Among smokers, 31% (13/42) harbored at least one mutation with a total of 39 point mutations and 8 short deletions/insertions detected. In contrast, only 23% (7/30) of non-smokers possessed mutations with a total of 10 point mutations and no deletions/insertions. Although there was no significant difference in mutation incidence detected between these two groups ($P = 0.48$), mtDNA somatic mutation density, defined as somatic mutation number/10000 bp per person, was statistically higher in smokers (0.68) than in non-smokers (0.20) on average. The mean difference was 0.47 ± 0.40 ($P = 0.05$, Table I). In smokers, most mutations were spread across the entire mtDNA genome, whereas non-smokers had most of their mutations in the D-loop region. For example, only 10% of non-smokers had mutations in the messenger RNA-encoding region, whereas 36% smokers had mutations in that region. About 49% of smokers had mutations in the D-loop region, whereas 70% of non-smokers had mutations in the D-loop region ($P = 0.10$, Fisher's exact test, Table II). In addition, eight short deletions/insertions were found in 19% of smokers, which represents 17% of all somatic mutations detected. We found four deletion/insertions of C or CC at nucleotide position 303–309 mononucleotide repeat (poly-C) region, three single nucleotide deletion/insertions (7447–7452 del A, 7466–7471 ins C and 15940–15944 del T) in transfer RNA serine and threonine and one deletion/insertion in the cytochrome c oxidase subunit I (7397–7402 ins C) that results in a frameshift and a truncated polypeptide of 510 amino acid residues. There were 17 point mutations located in the messenger RNA region. Among them, 44% were missense mutations. Twelve of 39 (31%) distinct somatic mutations found in smokers have not been reported previously in the Mitomap database; 27 (69%) of these occur in germ line polymorphic sites.

The incidence rate of heteroplasmy (HT) and homoplasmy (HM) of mtDNA somatic mutations, comparing buccal cells with lymphocytes, were different between smokers and non-smokers. The most prevalent mutation pattern found in smokers was HM → HM. The frequency of each of four patterns HM → HM, HM → HT, HT → HM and HT → HT was 55, 30, 13 and 2% in smokers, whereas it was 20, 70, 0 and 10% in non-smokers, respectively ($P = 0.04$, Table II).

Among smokers, there was no significant difference in mtDNA mutation frequency for pack-years ($P = 0.76$), number of cigarettes smoked per day ($P = 0.49$), years smoked ($P = 0.54$) and age at smoking initiation ($P = 0.51$). The relationship between mtDNA mutation status and smoking is summarized in Table I. The incidence of multiple mutations is significantly higher in heavy smokers than in non-smokers ($P = 0.045$). Heavy smokers had greater numbers of mutations than lighter smokers, but the trend was not statistically significant ($P = 0.10$).

mtDNA content and large-scale deletions

mtDNA content was measured by calculating the ratio of relative copy number of the *CoI* gene to β-actin. There was no relationship between mtDNA content and age, gender or race (Table IV).

Table II. Summary of mtDNA mutations in smokers and non-smokers

Analyzed variable	Non-smoker	Smoker	*P* value[a]
Total number of subjects	30	42	0.48
Numbers of subjects with mutations, *n* (%)	7 (23)	13 (31)	
Total number of mutation, *n*	10	47	
Distribution, *n* (%)			
mRNA	1 (10)	17 (36)	0.10
tRNA	0 (0)	5 (11)	
rRNA	2 (20)	2 (4)	
D-loop and non-coding area	7 (70)	23 (49)	
Homoplasmic/heteroplasmic patterns, *n* (%)			
HM → HM	2 (20)	26 (55)	0.04
HM → HT	7 (70)	14 (30)	
HT → HM	0 (0)	6 (13)	
HT → HT	1 (10)	1 (2)	
Mutation type, *n* (%)			
Short deletion or insertion	0 (0)	8 (17)	0.33
Point mutation	10 (100)	39 (83)	
Transition	9 (90)	37 (95)	0.50
Tranversion	1 (10)	2 (5)	
Type of mRNA mutations			
Missense mutation	0 (0)	7 (44)	1.00
Silent mutation	1 (100)	9 (56)	

mRNA, messenger RNA; tRNA, transfer RNA; rRNA, ribosomal RNA.
[a]Two-sided Fisher's exact test.

Table III. Somatic mtDNA mutations in smokers and non-smokers

NP	Frequency	Cambridge sequence	Sequence PF/IR[a] (%)	Lym	Buc	Pattern	Mutation[b]	Function location, codon and AA change[b]	Previous reported status
Non-smoker									
2444	1	A	100	A/C	C/A	HT → HT	A2444C	rRNA 16S	Novel
2706	1	A	19.5	A	G	HM → HM	A2706G	rRNA 16S	SNP site, oral cancer
10115	1	T	97.7	C	T	HM → HM	C10115T	mRNA ND3, ATC → ATT, I19I	SNP site, lung cancer
16223	3	C	53.0	T	T/C	HM → HT	T16223C	D-loop HVS1, 7S DNA	SNP site, oral cancer
16278	4	C	93.1	C	T/C	HM → HT	C16278T	D-loop HVS1, 7S DNA	Oral cancer
Smoker									
303	4	7C	91.9	7C	8C	HM/HT → HM/HT	303–309 ins C	D-loop TFY, CSB2, OH, HVS2	SNP site, esophageal, ovarian and oral cancer
322	1	G	99.8	G/A	G	HT → HM	A322G	D-loop OH, HVS2	Novel
930	1	G	97.8	G/A	A	HT → HM	G930A	rRNA 12S	SNP site
936	1	G	100	G/A	A	HT → HM	G936A	rRNA 12S	Novel
4529	1	A	98.7	T	A	HM → HM	T4529A	mRNA ND2, ACT → ACA, T20T	SNP site
7315	1	T	100/55.6	T/C	T/C	HT → HT	T7315C	mRNA CoI, ATG → ACT, **M471T**	Novel
7397	1	6C	100	5C	6C	HM → HM	**7397–7402 ins C**	mRNA CoI	Novel
7409	1	C	100/66.7	C	C/A	HM → HT	C7409A	mRNA CoI, TAC → TAA, **Y502T**	Novel
7447	1	6A	100	6A	5A	HM → HM	**7447–7452 del A**	tRNA serine 1	Novel
7466	1	6C	100	6C/5C	6C	HT → HM	**7466–7471 ins C**	tRNA serine 1	Novel
7951	1	A	99.9	A	G	HM → HM	A7951G	mRNA CoII, ATA → ATG, M122M	Novel
9947	1	G	99.5	G	A	HM → HM	G9947A	mRNA CoIII, GTG → GTA, V247V	SNP site
10034	2	T	98.6	T	C	HM → HM	T10034C	tRNA glycine	SNP site
10086	1	A	99/<20	A/G	G	HT → HM	A10086G	mRNA ND3, AAC → GAC, **N10D**	SNP site
10115	1	T	97.7	T	C	HM → HM	T10115C	mRNA ND3, ATT → ATC, I19I	SNP site, lung cancer
10238	1	T	96.9	C	T	HM → HM	C10238T	mRNA ND3, ATC → ATT, I60I	SNP site
10373	1	G	99	G	A	HM → HM	G10373A	mRNA ND3, GAG → GAA, E105E	SNP site
10398	1	A	54.2/44.4	A	G	HM → HM	A10398G	mRNA ND3, ACC → GCC, **T114A**	SNP site
10530	1	G	99.9/22.2	G	A	HM → HM	G10530A	mRNA ND4L, GTA → ATA, **V21M**	Novel
13105	1	A	94.9/55.6	G	A	HM → HM	G13105A	mRNA ND5, GTC → ATC, **V257I**	SNP site
13563	1	A	98.6	A	G	HM → HM	A13563G	mRNA ND5, CTA → CTG, L409L	SNP site
13590	1	G	95.9	G	A	HM → HM	G13590A	mRNA ND5, CTG → CTA, L418L	SNP site
13650	1	C	94.3	C	T	HM → HM	C13650T	mRNA ND5, CCC → CCT, P438P	SNP site
15824	1	A	99.3/<20	A	G	HM → HM	A15824G	mRNA Cytb, ACA → GCA, **T360A**	Novel
15940	1	5T	100	5T	4T	HM → HM	**15940–15944 del T**	tRNA threonine	Novel
16153	1	G	99.4	G	A	HM → HM	G16153A	mRNA HVS1, 7S DNA	SNP site
16179	1	C	99.7	T	C	HM → HM	T16179C	D-loop HVS1, 7S DNA	SNP site
16220	1	A	99.9	A	C	HM → HM	A16220C	D-loop HVS1, 7S DNA	SNP site
16223	2	C	53.0	T	C/T	HM → HT	T16560C	D-loop HVS1, 7S DNA	Novel
16255	1	G	99.6	A	G/A	HM → HT	A16255G	D-loop HVS1, 7S DNA	SNP site
16256	1	C	98.5	T	C/T	HM → HT	T16256C	D-loop HVS1, 7S DNA	SNP site, oral cancer
16265	1	A	98.6	A	G/A	HM → HT	A16265G	D-loop HVS1, 7S DNA	SNP site
16270	1	C	96.4	T	C/T	HM → HT	T16270C	D-loop HVS1, 7S DNA	SNP site, oral cancer
16278	3	C	93.1	C	T/C	HM → HT/HM	C16278T	D-loop HVS1, 7S DNA	SNP site, oral cancer
16294	1	C	93.9	C	T	HM → HM	C16294T	D-loop HVS1, 7S DNA	SNP site, oral cancer
16298	1	T	90.4	T	C/T	HM → HT	T26298C	D-loop HVS1, 7S DNA	SNP site, oral cancer
16362	2	T	75.2	T	C/T	HM → HT	T16362C	D-loop HVS1, 7S DNA	SNP site, oral cancer
16384	1	G	100	G	G/A	HM → HT	G16384A	D-loop 7S DNA	SNP site
16390	1	G	97	A	G/A	HM → HT	A16390G	D-loop 7S DNA	SNP site, oral cancer

NP, nucleotide position; Lym, lymphocytes; Buc, buccal cells; AA, amino acid; SNP, single-nucleotide polymorphism; TFY, mtTF1-binding site; OH, H-strand origin; HVS, hypervariable segment; Co, cytochrome c oxidase subunit; ND, nicotinamide adenine dinucleotide(H) dehydrogenase subunit.
[a]PF, polymorphism frequency of Cambridge sequence at this position in Human Mitochondrial Genome Database (mtDB, http://www.genpat.uu.se/mtDB/); IR, identical rate in evolution conservation: identical 100%. The sequences from multiple phyla including human, gorilla, bovine, mouse, chicken, frog, fly, and urchin were used for evolution conservation analysis. conserved >70%, similar 20–70%, non-similar <20%; evolution conserved analysis (consensus calculation) was made by Vector NTI 9.0 (Invitrogen).
[b]Missense mutation, deletion and insertion are in bold.

Table IV. The ratio of *CoI*/β-actin and ratio of buccal cells/lymphocytes (mean ± SD)

	Total numbers	*CoI*/β-actin in buccal cells	*P* value	*CoI*/β-actin in lymphocytes	*P* value	Ratio of buccal/lymph	*P* value
Smoking status							
Non-smoker	30	247.88 ± 239.59	0.28	613.00 ± 362.54	0.01	0.46 ± 0.39	0.004
Smoker	42	398.70 ± 586.01		505.72 ± 693.13		2.81 ± 6.22	
Pack-years							
5–18.9	14	447.69 ± 722.73	0.59	318.21 ± 192.77	0.73	2.69 ± 4.39	0.81
19–36.9	13	259.80 ± 296.83		687.85 ± 980.40		4.09 ± 9.99	
≥37	15	473.35 ± 647.19		522.87 ± 521.17		1.80 ± 2.73	
Age at smoking initiation							
<17	22	390.66 ± 606.19	0.46	340.48 ± 416.56	0.01	3.98 ± 8.17	0.15
≥17	20	407.54 ± 578.56		687.49 ± 789.48		1.60 ± 2.47	
Years smoked							
5–19	13	435.62 ± 737.06	0.96	322.71 ± 226.70	0.19	4.92 ± 9.69	0.47
20–37	17	415.29 ± 629.88		619.70 ± 571.57		1.45 ± 3.79	
≥38	12	335.20 ± 322.21		542.51 ± 964.43		1.86 ± 2.26	
Cigarettes smoked per day							
10–15	15	280.25 ± 279.87	0.27	542.42 ± 842.46	0.9	4.92 ± 9.69	0.35
16–24	19	487.91 ± 830.50		479.88 ± 546.17		1.45 ± 2.79	
≥25	8	408.93 ± 171.33		498.27 ± 448.60		1.86 ± 2.26	

All *P* values are from *t*-test or analysis of variance performed on log-transformed observations.
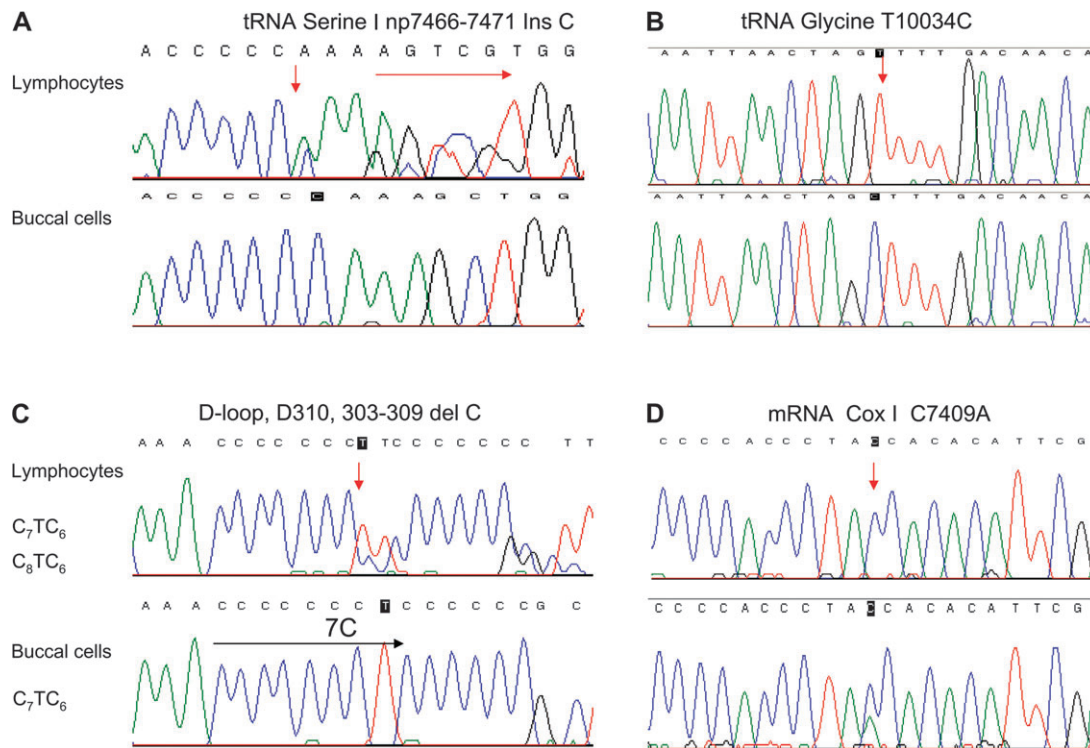
Fig. 1. Four representative somatic mutation sequences at different mtDNA regions in smokers. (**A**) Heteroplasmic to homoplasmic insertion of cytidine at np7466–7471 transfer RNA (tRNA) serine 1; (**B**) HM to HM change of T10034C at tRNA glycine; (**C**) Heteroplasmic to homoplasmic deletion C at poly-(C)*n* tract D310 of D-loop region and (**D**) HM to HM transversion change of C7409A at cytochrome c oxidase subunit I of messenger RNA coding region.

The ratio of mtDNA content of buccal cells to lymphocytes is significantly higher in smokers (2.81) than in non-smokers (0.46, *P* = 0.004, Table IV). The mean mtDNA content in the buccal cells of smokers increased 1.6 times relative to non-smokers. Although the relative mean mtDNA content in lymphocytes was significantly higher than in buccal cells for non-smokers (*P* < 0.001, paired *t*-test), no significant difference was detected in smokers. We also investigated the relationship of mutation frequency and density to mtDNA content. There was no significant correlation between mutation density and content of mtDNA (*P* = 0.76 for buccal cells and *P* = 0.75 for lymphocytes). In addition, a low positive correlation between ratio of buccal cells/lymphocytes mtDNA content and smoking status has
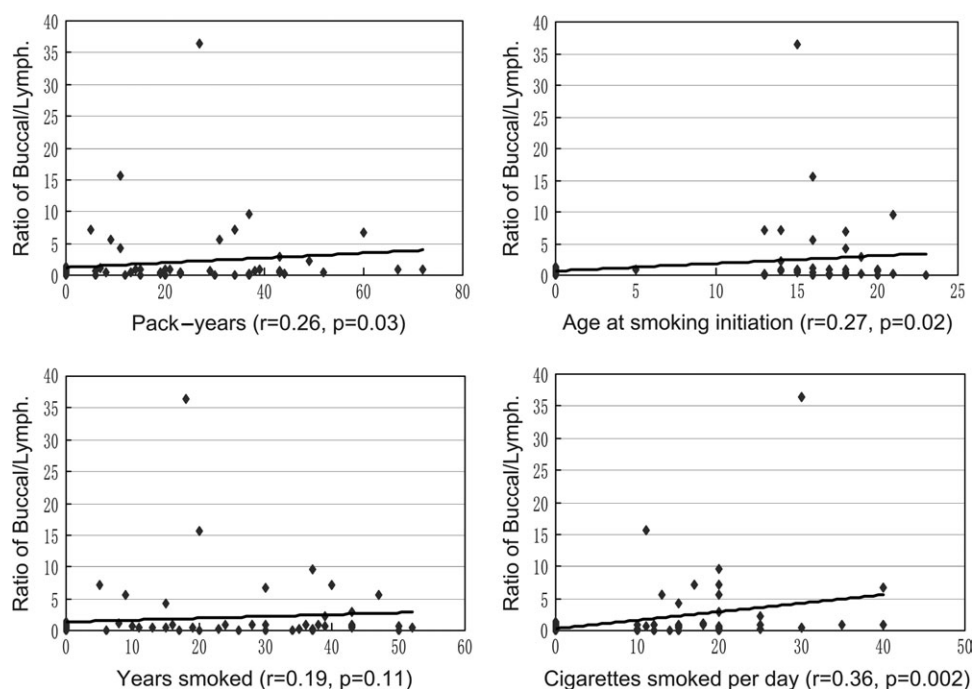
Fig. 2. Correlations between ratio of buccal cells/lymphocytes mtDNA content and smoking status. Pearson's correlation test (two tailed) after data log transformation.

been observed (Figure 2) ($r = 0.26$, $P = 0.03$ for pack-years; $r = 0.27$, $P = 0.02$ for age at smoking initiation and $r = 0.36$, $P = 0.02$ for cigarettes smoked per day).

Large 4977 bp mutations are frequently reported as ageing-associated molecular events (24). A high frequency of 4839 bp deletion of mtDNA was found in smokers with lung cancer (10). In our study, the large 4977 bp common deletion was detected in 4.8% of smokers and 3.3% of non-smokers. No 4839 bp deletions were found in either smokers or non-smokers. There was no difference in frequencies by age, gender race or smoking status (data not shown). There also was no difference by pack-years, age at initiation, number of years of smoking or cigarettes per day (data not shown).

Discussion

There are no validated smoking-related biomarkers for cancer risk, although some exist for smoke exposure (25). To date, there has been little research directed toward the study of mtDNA as a biomarker for smoke exposure or tobacco-related disease risk. In this study, we found that buccal cells of healthy smokers harbored both a higher mtDNA mutation frequency and mutation density, relative to non-smokers. The type of mitochondrial mutations was different for smokers and non-smokers.

There are several reasons why mtDNA analyses could be a useful biomarker for tobacco smoke exposure. It is known that mtDNA is highly prone to oxidative damage as it is in close contact with the ROS produced in the mitochondria (3,26,27). Also, lipids within the mitochondrial membrane and lipid peroxidation have been shown to be one potential source of continued DNA damage. Secondary ROS reactions have the potential of overwhelming the repair capacity of the mitochondrion, which already functions with limited capacity, and can lead to persistent DNA damage (28). The mtDNA mutation rate is 10–100 times higher than that of nuclear DNA. The vulnerability of the mitochondrial genome to oxidative damage could be due to many factors including: (i) the absence of histones or DNA-binding proteins; (ii) a limited basic repair mechanism; (iii) genes consisting only of exons without introns and (iv) replicating rapidly without a significant proofreading system.

In this study, we consider buccal cells as an easily accessible tissue for biomarker studies in smokers and a target organ for smoking-related cancer. Previous studies have shown that smoking is associated with increased mtDNA content in a dose-dependent fashion (6). mtDNA alterations in response to smoking persist for several decades after smoking cessation, consistent with long-term smoking-related damage (6). However, we are not aware of published data for mtDNA mutations in buccal cells by smoking status. Studies have shown that normal lung tissue of smokers have more mtDNA mutations by age and smoking history (10,18,29). Smoking also increases the frequency of mtDNA deletions in lung tissues, and the incidence and proportion in current smokers were significantly higher than in those of non-smokers (10). Tobacco smoke exposure has been shown to induce mitochondrial damage and depolarization, as well as mtDNA damage, in cardiovascular tissue (30). In this report, we present a comprehensive study of both mtDNA somatic mutations and copy number in buccal cells of smokers and non-smokers. The results show that 31% (13/42) of smokers harbored somatic mtDNA mutations compared with 23% of non-smokers. While this difference was not statistically significant, a larger study might have revealed a statistical difference. This percentage of mutations is higher than prior reports for betel quid chewers, who have a mutation rate of 10% (31). About 1.5% of human bronchial epithelium colonies contained hundreds or even thousands of homoplasmic mutant cells (32). Our results show that the mutation density in smoker increases with smoking. The results herein reflect, to a certain extent, the relationship between smoking and cancer. Lung cancers have a mutation rate of 47% (33) and oral cancers have a rate of 78% (14). In this study, nine (33%) of the identified somatic mutations have been reported previously as somatic mutations in smoking-related oral or lung cancer, but have not been found in other mitochondrial diseases or other tumors (21,33–37).

In this study, 17% of smokers had a mtDNA length alteration in their buccal cells and the insertion or deletion (C7->8/9 or C8/9->7) of poly-(C)n tract 303–309 has been found in four (9%). This has not been reported previously for normal tissues, but can occur in 21–72% of breast, oral and prostate cancers (12,14,21,35,36,38). The mononucleotide repeats between nucleotides 303 and 309 are among the

most polymorphic of mitochondrial microsatellites. This homopolymeric C stretch is part of the conserved sequence block II located within the regulatory D-loop region and is involved in the formation of a persistent RNA–DNA hybrid that leads to the initiation of mtDNA heavy-strand replication (39). The alteration rate in this area is sequence dependent (40). That is, if the sequence has 7 C repeats, it will be stable and so is less prone to frameshift mutations. The remaining four single-base deletions in this study occurred in the transfer RNA and messenger RNA regions, which can lead to mitochondrial dysfunction, increase ROS production and promote aberrant cell proliferation. It is also interesting to note that both homoplasmic and heteroplasmic mutations occur. Unlike in non-smokers, more than half of the mutations found in smokers are homoplasmic, indicating that cigarette smoke may accelerate the shifting process from heteroplasmic to homoplasmic mutation or these mutation sites are sensitive to the attack of mutagens.

Although it is difficult to predict the pathogenic role of specific mutations in smokers, some of the mutations we detected were located in structurally and/or functionally important regions. Several would alter protein sequence and structure, e.g. a missense mutation G10530A that would replace a valine with methionione in the nicotinamide adenine dinucleotide (H) dehydrogenase subunit 4L or others at C7409A (Y502T), T7315C (M471T), G13105A (V257I), G10530A (V21M) and A15824G (T360A). Whether mtDNA mutations are markers for morphologically normal cells that will transform to cancer can only be determined from prospective studies. Tumor cells containing a mtDNA mutation grow faster than cells with identical nuclei and unmutated mitochondria (36), so mtDNA mutations in buccal cells of smokers without cancer might reflect an early carcinogenic process.

While DNA damage to nuclear DNA actively promotes the carcinogenic process, the role of mtDNA mutations might also be important. A recent study in 202 non-small cell lung cancer patients found that 70 patients (35%) had various mutations in the D-loop region of mtDNA in cancer cells with mutations at 16117A → T and 16368 A → T being the most prevalent. The D-loop region mutation rate of non-small cell lung cancer patients in stage IIIB or higher as well as T3 stage of tumor-node-metastasis factors was significantly greater than in other patients (13). In addition, three mutation hot spots were observed in the D-loop at nucleotides 146, 152 and 186 in oral squamous cell carcinomas (12). Other studies also have shown that homoplasmic and heteroplasmic mutations occur in 47–78% cases of lung and oral cancers (12,14,33,41). These somatic mutations range from severe insertion–deletion and chain termination mutations to mild missense mutations. Mutations anywhere in the mtDNA might have an adverse effect because the entire mtDNA genome is expressed and continuously replicated. Thus, mutations might cause a decreased capacity for apoptosis and increased production of free radicals. For example, severe mutations may inhibit oxidative phosphorylation, increase ROS production and promote tumor cell proliferation, whereas milder mutations may permit tumors to adapt to new environments. Hence, mitochondrial dysfunction does appear to be a factor in cancer etiology, an insight that may suggest new approaches for diagnosis and treatment.

Some studies have shown that an increase in mtDNA content is associated with DNA damage and reduced respiratory chain function secondary to oxidative damage (34). As described above, smoking is associated with increased mtDNA copy number among 42 smokers, dose dependently. In our study, mtDNA copy number increased up to 1.6-fold (61% increase), whereas Masayesva *et al.* (6) reported a 31% increase. The reasons for increased mtDNA copy number are unclear, but one hypothesis is that increasing copy number may be a compensatory mechanism in response to oxidative damage induced by cigarette smoke (6). This hypothesis is consistent with observations that decreased energy production from damaged mitochondria induces nuclear signals leading to compensatory replication (1,6,36). We also found that mtDNA content of lymphocytes is higher than that of buccal cells of both smokers and non-smokers. This might be due to differences in cell proliferation rates or cellular energy requirements and metabolism.

Our results demonstrate that cigarette smoking increases mtDNA mutations and copy number in the buccal cells of smokers. Although some of these mutations are located in structurally/functionally important regions and may affect the biogenesis of mitochondria, no single mutational hot spot or signature was found. This study did not investigate the biopersistence of biomarkers, and so it is unknown if these findings reflect recent or long-term exposure, although the data showing correlations with cigarettes per day and not years smoking suggests the former. This study did not address the half-life of the markers, which can only be done with a smoking cessation study. In addition, we did not evaluate the possibility of the presence of leukocyte in the mouthwash. Although it is possible that oral cells might be a mix of epithelial cell and other cells, we were able to detect the somatic changes in mitochondrial genome between oral cells and blood lymphocytes. Further research will be needed to determine if mtDNA analysis has the specificity for detecting changes in smoke exposure or if the estimation of an individual's risk could be improved by coupling of mitochondrial mutations to other markers for tobacco smoke-associated disease risk or smoking-related harm. Also, the data presented here provide justification for the development of economically feasible high-throughput methods for detecting mtDNA mutations.

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