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Mitochondrial DNA mutations in exhaled breath condensate of patients with lung cancer

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Breath test

Summary
Introduction: Lung cancer is a leading cause of cancer mortality worldwide. Non-invasively collected biofluids such as exhaled breath condensate (EBC) present a potential sampling medium to detect and study pathological changes implicated in tumourigenesis. Mitochondrial DNA changes have been implicated in the carcinogenesis process. Consequently, the detection of mitochondrial changes in EBC could expand our understanding of lung carcinogenesis as well as identifying specific markers for future studies.
Methods: EBC and saliva was collected from newly diagnosed subjects with lung cancer and control subjects in a cross-sectional study. The EBC and saliva was analysed for mitochondrial DNA D-loop changes using a PCR sequencing approach. The sequences obtained were compared to paired salivary DNA and the revised Cambridge Reference Sequence (rCRS) to identify somatic mutations, and quantitative and qualitative differences in mutations were analysed between groups.
Results: A total of 25 subjects (9 NSCLC patients, 10 smokers/ex-smokers and 6 non-smokers) were recruited. A significantly elevated D-loop mutation rate in the lung cancer group compared to the control groups was present (7 vs 3.5 for smokers/ex-smokers, and 7 vs. 4 for non-smokers, $p = 0.034$). The recognised mutation T16217C showed specificity for lung cancer.
Conclusions: Mitochondrial DNA mutations are more common in the EBC of patients with lung cancer. This suggests that these processes are associated with the carcinogenesis of lung cancer and may be a marker of the disease.
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Introduction

Lung cancer remains the leading cause of cancer death globally, accounting for 13% of all deaths.[1] The low 5-year survival rate of 15% can be attributed to late diagnosis in the vast majority of cases,[2] for which there are suboptimal therapies. Exhaled breath condensate (EBC) is a non-invasive method of sampling the respiratory epithelial lining fluid.[3] Genetic[4–7] and epigenetic[8] changes in EBC of patients with lung cancer have demonstrated mixed results, but these changes may be informative markers of disease[9] and recently tissue studies have highlighted the potential role of mitochondrial DNA changes in carcinogenesis and as biomarkers of lung cancer.

Mitochondrial DNA (mtDNA) is a 16,569 base pair (bp) circular double-stranded molecule coding for 37 genes including 13 polypeptides involved in respiration and oxidative phosphorylation.[10] mtDNA is more susceptible to oxidative damage than nuclear DNA due to its proximity to the electron transport chain, inefficient repair mechanisms and lack of protective histones.[11–13] Somatic mtDNA mutations have been hypothesised to play a part in cancer through pathways such as mitochondrial respiratory chain dysfunction which increases reactive oxygen species (ROS) production,[14] interference with cellular apoptosis[15,16] and translocation causing activation of nuclear oncogenes, although recent studies have identified anti-tumorigenic mtDNA mutations as well.[17]

mtDNA mutations accumulate in the D-loop region, a 1,124bp non-coding region containing the major control elements for mtDNA expression, replication and transcription.[18] Studies of lung tissue, BAL and sputum have demonstrated that D-loop mutations are prevalent in lung tumours[19–27] and correlate weakly with clinical characteristics such as tumour grade and lymph node metastasis.[27]

EBC may be a non-invasive method of detecting mitochondrial markers of lung cancer. Due to its high copy number and high mutation rate, mutations in the D-loop region of mtDNA are ideal to study in low-cellularity samples like EBC, and may play a role in contributing to the genetic instability involved in carcinogenesis.[28] Specifically, EBC mtDNA may reflect changes observed in studies of lung tissue and it was hypothesised that a higher somatic mutation rate in patients with lung cancer would be observed when compared with healthy control subjects.

Materials and methods

Subject recruitment

This cross-sectional observational study was granted ethics approval (SESAHS HREC04/179). Subjects were recruited from the Prince of Wales Hospital (POWH), and gave written, informed consent. The study population had a confirmed histological diagnosis of non small cell lung carcinoma (NSCLC) by bronchoscopic biopsy or transthoracic cytological aspiration and were enrolled prior to treatment. Control subjects were recruited from the inpatient, outpatient and community populations. Exclusion criteria: history of chronic respiratory diseases, $FEV_1/FVC < 70\%$, known history of lung cancer and recent or current respiratory illness ($<$2 weeks). Demographic and medical details were obtained via a questionnaire. Ex-smokers were those who had ceased smoking for $>$1 year.

EBC and saliva collection

Subjects refrained from smoking, eating or drinking 2 h prior to EBC collection. After rinsing their mouths with water, subjects provided a saliva sample (average volume 0.5 mL) which was stored at −80 °C.

EBC was collected using a custom-made glass condenser as previously described[29,30]

Subjects breathed through a unidirectional valve, at a normal frequency and tidal volume, for 20 min. EBC was collected on ice at 4 °C, aliquoted and deaerated with argon degassing at 0.4L/min for 1 min, and immediately stored at −80 °C. Unpublished studies demonstrated that this method does not allow salivary DNA contamination or cross-over DNA contamination.

Nucleic acid isolation

EBC (minimum volume 400 μL; average 1000 μL) was concentrated to 200 μL using a SpeedVac SC110A (Thermo Scientific, Victoria, Australia). DNA was extracted from the concentrated EBC using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with modifications to increase DNA yield and concentration; DNA was eluted in 55 μL of pre-heated buffer AE and the eluate was passed through the column again in a second elution step. DNA was extracted from saliva using the same kit using a 100 μL elution volume. The resulting EBC-DNA and saliva-DNA either immediately underwent PCR amplification or was stored at −20 °C.

Polymerase chain reaction amplification

A 1450 base-pair (bp) segment of the mitochondrial genome containing the D-loop region was amplified using the forward primer 5′-TACTCAAATGGGCCTGTCCT-3′ and reverse primer 5′-AGGGTGAACTCACTGGAACG-3′. These primers were custom-designed, based on the revised Cambridge Reference Sequence (rCRS, accession number: NC_012920), using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) and submitting to BLAST search (Basic Local Alignment Search Tool, www.ncbi.nlm.nih.gov/BLAST/). Primers were purchased from Invitrogen (Carlsbad, CA, USA).

PCR was performed in 50 μL reactions with 15 μL of EBC-DNA, 2.5U of Fast Start High Fidelity Taq (Roche Applied Science, Mannheim, Germany) in supplied PCR buffer, 0.4 mM of each primer, 1.5 mM Mg^{2+}, 200 μM of deoxyribonucleoside triphosphate (dNTPs) and 5% DMSO. Thermal cycling was performed on a GeneAmp PCR System 9700 (Applied Biosystems, Victoria, Australia) with an initial denaturing step at 95 °C for 2 min, followed by 42 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min 15 s, and a final extension at 72 °C for 7 min. Salivary DNA PCR was performed on 30 ng of sample in a final volume of 25 μL using the same reaction

components and PCR protocol as for EBC-DNA, except that 25 cycles of PCR were used.

A negative control (elution buffer and enzyme without DNA template) was included in every PCR series.

PCR purification and DNA sequencing

PCR amplicons were run on a 1.5% agarose gel, and confirmed by comparison to reference bands on a 500-5000 bp DNA ladder (Bioline, London, UK). PCR products were purified using a PCR purification kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In every sample, the first 855 bp of the D-loop (nt 16024-nt 310) was sequenced in three overlapping segments using nested sequencing primers (Fig. 1). Sequencing reactions were performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Victoria, Australia), according to the manufacturer's instructions, except that 5% DMSO was added to the reaction mixture to aid annealing where secondary structures existed. Cycle conditions were 30 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, followed by 7 min at 4 °C, performed on a GeneAmp PCR System 9700 (Applied Biosystems, Victoria, Australia). Samples were then cleaned by EDTA/ethanol precipitation according to the recommended protocol and dried by evaporation at 90 °C. These products were analysed using capillary sequencing.Duplicate amplifications were performed on all patient EBC samples, consisting of an independent PCR and sequencing reaction to exclude PCR artefacts and sequencing errors.

Sequence analysis

A consensus sequence was constructed for each sample and aligned to the rCRS using the commercial software SeqScape (Applied Biosystems, Victoria, Australia). The chromatographs were then manually screened for somatic mutations, which were defined as any base change from both the rCRS and paired saliva from the same patient. This definition determined which deviations from the rCRS were germline polymorphisms (appearing in both saliva and EBC) and which were somatic mutations (appearing only in EBC). A heteroplasmic mutation was only defined if it occurred in the sequences obtained from both independent PCR amplifications. The analysis was carried out in a blinded manner, and sequence analysis was repeated a second time for all sequences.

The sample sequences were compared to nuclear mitochondrial sequences (NumtS) downloaded from the UCSC[31] to enable the likelihood of co-amplification of nuclear DNA to be assessed.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism v.5 software (Graph Pad, La Jolla, CA). Non-parametric tests were used for comparison of rate of D-loop mutations as these were discrete data; Kruskal—Wallis with Dunn's multiple comparison test for data where there were multiple groups and Mann—Whitney *U* test for data where there were two groups. Fisher's exact test was used to analyse categorical data (prevalence of individual mutations between groups). Correlations were performed using Spearman's test. A two-tailed *p*-value $p < 0.05$ was considered to be statistically significant.

Results

Subject characteristics

Twenty-five subjects were recruited and their characteristics are displayed in Table 1. The lung cancer group was slightly older than the smokers/ex-smokers and non-smokers ($p < 0.05$). No difference in the gender distribution or smoking history was found.

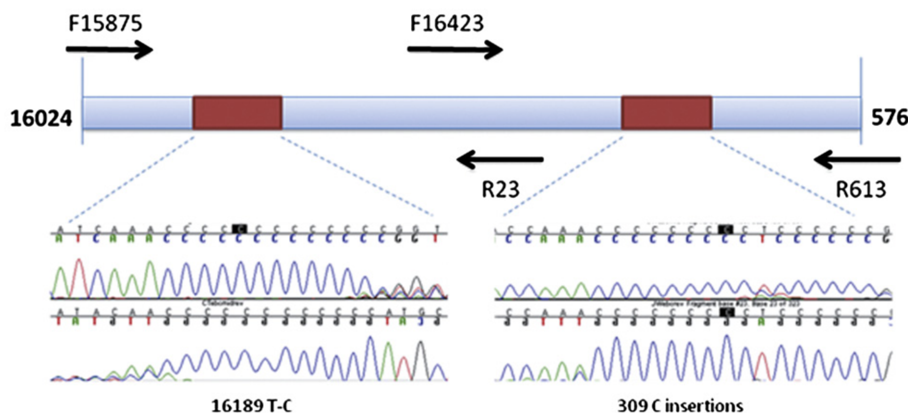

Figure 1 Sequencing strategy. Sequencing primers were placed to allow entire D-loop to be sequenced even when C-stretch length heteroplasmy is present (regions shown in red). Primers are numbered according to the position of the 3′ of the primer on the revised Cambridge reference sequence; F denotes a forward primer and R denotes a reverse primer. The top sequence represents the data obtained with the forward primer; the bottom represents the sequence from the reverse primer. As can be seen, the sequence becomes unreadable in each direction after the homopolymer C-stretch. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1 Subject characteristics.

	Patients with NSCLC ($n = 9$)	Control subjects	
		Smokers/ex-smoker ($n = 10$)	Non-smokers ($n = 6$)
Age (yrs)	71 ± 3	58 ± 3	58 ± 4
Gender (male/female)	6/3	5/5	3/3
Lung cancer histology			
Squamous cell carcinoma	2	N/A	N/A
Adenocarcinoma	3	N/A	N/A
Large cell carcinoma	4	N/A	N/A
Lung cancer stage			
Stage I	1	N/A	N/A
Stage 2	0	N/A	N/A
Stage 3	1	N/A	N/A
Stage 4	7	N/A	N/A
Pack-years (smokers and ex-smokers)	51 ± 11	32 ± 7	0

Rate of D-loop changes in patients with NSCLC vs. control subjects

PCR amplification yielded a single product for sequencing.Analysis of the median number of D-loop mutations between the lung cancer, smokers/ex-smokers and non-smokers groups demonstrated a significant difference between groups ($p = 0.034$), with a higher median number of mutations in the lung cancer group compared to the smokers/ex-smokers (7 vs. 3.5, $p < 0.05$ Fig. 2).

No differences in the prevalence of heteroplasmic mutations between groups was found ($p = 0.85$ Fig. 3A) however a significantly different rate of homoplasmic mutations between groups was present ($p = 0.035$), with the lung cancer group having the highest median number of homoplasmic mutations compared to both the smokers/ex-smokers and non-smokers groups (2 vs. 0 and 2 vs. 1 respectively, $p < 0.05$ Fig. 3B).

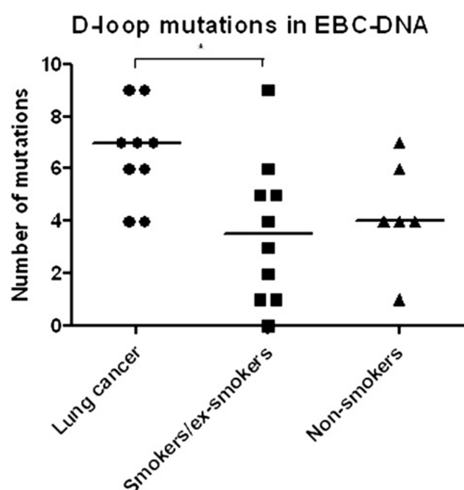

Figure 2 Number of D-loop mutations in EBC-DNA of lung cancer patients ($n = 9$), smokers/ex-smokers ($n = 10$) and non-smokers ($n = 6$). There was a significant difference between groups ($p = 0.034$, Kruskal–Wallis test) with the median number of mutations in the lung cancer group being higher than the smokers/ex-smokers group ($p < 0.05$, Dunn's multiple comparisons test). Bars represent medians. *$p < 0.05$.

Mutations by clinicopathological features

No significant difference in mutation rates between the histological lung cancer subtypes or by stage was found. There was no correlation between mutation incidence and age when all subjects (lung cancer subjects, smokers/ex-smokers and non-smokers) were assessed. The frequency of mutations also did not differ by gender or race. Among smokers, there was no significant difference in mtDNA mutation frequency for pack-years, number of cigarettes smoked per day, year smoked and age at smoking initiation. Heavy smokers (>40 pack-years) had greater numbers of mutations than lighter smokers, but the trend was not statistically significant ($p = 0.16$Fig. 4).

Analysis of prevalence of specific mutations

Analysis of the prevalence of specific point mutations showed that one mutation (T16217C) was associated with lung cancer ($p = 0.036$). The prevalence of the mutations observed are summarised in Table 2.

A polymorphism was detected in an EBC sample obtained from a patient with lung cancer. The data was confirmed by an independent PCR and sequencing analysis, which demonstrated a heteroplasmic T insertion at nt 60 (www.hmtdb.uniba.it).

Discussion

mtDNA mutations have been reported in biofluids such as nipple aspirate,[32] BAL, urine[19] and others. This is the first study to demonstrate the feasibility of detecting mtDNA changes in breath condensate, and as such represents an original contribution to the field of breath research. This study highlights the possibility of using this non-invasive method to detect biomarkers of lung cancer.

In this study population, the D-loop mutation prevalence in the EBC-DNA of patients with lung cancer was higher than

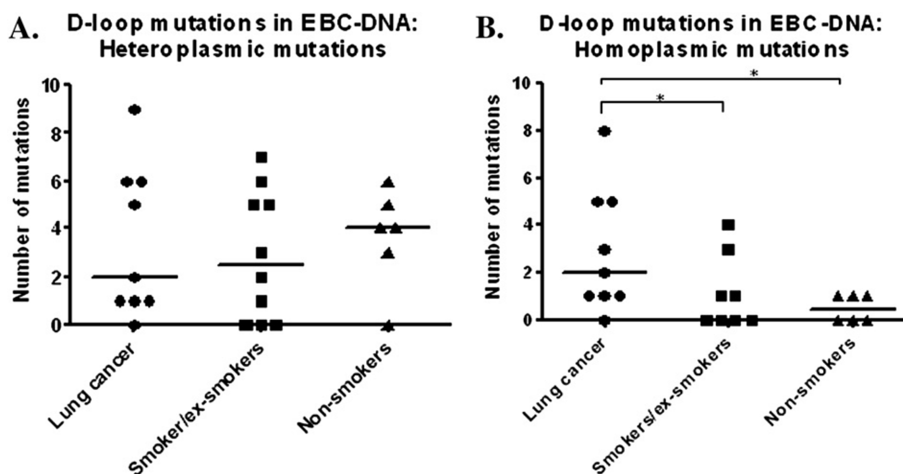

Figure 3 Number of heteroplasmic (A) and homoplasmic (B) D-loop changes in EBC in those with lung cancer ($n = 9$), smoker/ex-smoker ($n = 10$) and non-smoker ($n = 6$) control subjects. There was no significant difference in the number of heteroplasmic mutations between groups ($p = 0.85$, Kruskal—Wallis test), however a significant difference in homoplasmic mutations between groups was seen ($p = 0.035$, Kruskal—Wallis test), and the lung cancer group had a significantly higher median number of homoplasmic mutations compared to the smoker/ex-smokers ($p < 0.05$) and non-smokers ($p < 0.05$, Dunn's multiple comparisons test). Bars represent medians. *$p < 0.05$.

the control group, perhaps indicating a greater degree of mtDNA damage in the cells of the respiratory tract, possibly caused by increased oxidative damage due to the oxidative burden induced by neoplasia,[33—36] or it may be an reflection of widespread mitochondrial DNA damage that preceded and contributed to the carcinogenic process.

It is biologically plausible that these mutations are caused by exposure to carcinogens and oxidants such as those in cigarette smoke. Therefore the EBC mtDNA mutations in those with lung cancer may be an amalgamation of cigarette smoke-induced damage preceding neoplastic changes and oxidative damage exerted by cancer cells. One limitation is that the control group excluded diseases such as COPD which are common in patients with lung cancer. mtDNA mutations occur at a higher incidence in the lung tissues of smokers, and there is variability in the mutation rates between smokers,[35] however, all of these studies focused on large-scale deletions, in particular the 4977-bp 'common deletion' and this is the first to study point mutations in smokers and demonstrate similar inter-individual variability. The degree of variability may reflect differing susceptibilities of lung tissue to cigarette smoke-induced damage, as most smokers do not in fact develop lung cancer.[36] Also, some of the smokers with a higher number of mutations may already in fact have pre-cancerous changes in their lung tissue, or have a greater predisposition to developing lung cancer. One study[37] used multiple biopsies taken from patients with lung cancer to demonstrate that mtDNA mutations present in the tumour are also found in multiple sites of normal mucosa distinct from the tumour, suggesting that smoking exposure may lead to clonal patches of mtDNA dysfunction which could later undergo neoplastic change.

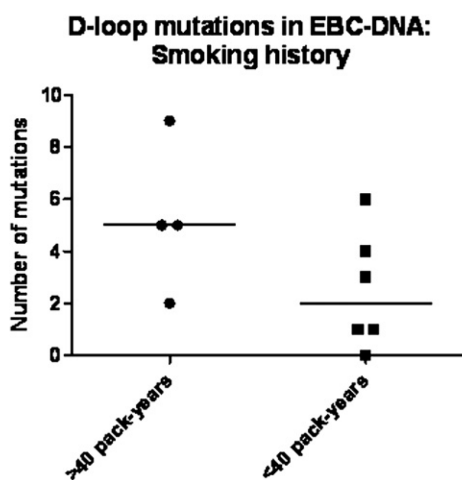

Figure 4 Number of D-loop mutations in EBC-DNA in heavy smokers (>40 pack-years, $n = 4$) and light smokers (<40 pack-years, $n = 6$). There was a trend towards a higher number of mutations in the heavy smoking group ($p = 0.16$, Mann—Whitney U test). Bars represent medians.

Interestingly, the majority of the mutations observed in our study were heteroplasmic in nature [Table 2]. There are several interpretations of this observation:age-associated mutations tend to be heteroplasmic[38,39] and hence this may not be a disease-associated phenomenon, and the degree of heteroplasmic mutation was similar between groups in this study. In addition EBC theoretically samples the entire respiratory tract, and as such represents a mixture of DNA from different parts of the lung. Therefore although different mutations may become dominant in certain parts of the lung, the consensus sequence shows these mutations as heteroplasmic.

Some mutations were found to be homoplasmic, and the median number of homoplasmic mutations was significantly higher in the lung cancer group compared to the control group. Several reasons could exist for this phenomenon: homoplasmic mutations tend to accumulate in tumours[19] perhaps indicating clonal proliferation of cancerous cells, or reflecting a greater replicative advantage that these mutations impart.

Table 2 Specific point mutations found in EBC and their prevalence in each group divided into homoplasmic/heteroplasmic nature, with previously reported associations with disease.

Mutation	Lung cancer ($n = 9$)	Control ($n = 16$)	Previous reported status
T16093C	0	1	SNP site, breast and prostate tumour
G16129A	3	2	SNP site
T16140C	2	1	SNP site
A16182C	4	4	SNP site, prostate tumour
A16183C	6	6	SNP site, lung and prostate tumour
T16189C	6	7	SNP site, prostate tumour
16189 T del	0	1	SNP site
T16217C	3	0	SNP site, prostate cancer
C16221T	0	1	SNP site
C16223T	2	3	SNP site
C16256T	1	1	SNP site
C16257A	1	4	SNP site
C16261T	1	4	SNP site
T16263C	1	0	SNP site
C16266A	2	1	SNP site
T16352C	1	0	SNP site
T16362C	4	1	SNP site
G16391A	1	0	SNP site, ovarian tumour
A16482G	1	0	SNP site
T16519C	3	5	SNP site, gastric, lung, ovarian and prostate tumour
C41T	0	1	SNP site
T72C	1	3	SNP site
A73G	3	4	SNP site, thyroid and prostate tumours
T146C	0	1	SNP site, ovarian carcinoma, prostate tumour
C150T	4	7	SNP site, lung, thyroid and prostate tumour. Longevity.
T152C	1	0	SNP site, ovarian carcinoma, breast tumour
T199C	1	0	SNP site, ovarian carcinoma
T204C	2	0	SNP site, various tumours
G207A	1	0	SNP site, oral, prostate and thyroid tumours
A210G	2	2	SNP site
T239C	1	1	SNP site, ovarian tumour
T250C	1	0	SNP site
A297C	0	1	SNP site

The significance of the nt 60 mutation in this study is not known, however one possibility is interference with mtDNA replication. A recent study[40] discovered a major replication origin at nt 57 of the D-loop, which is only four nucleotides downstream of the T insertion at nt 60. In addition, an increased incidence of mtDNA mutations and copy number are seen in epithelial neoplasia, suggesting that these contribute to the genetic instability required for neoplastic progression.[28]Unfortunately this patient died shortly after participating in the study from advanced lung cancer, and no additional samples could be obtained to assess mtDNA copy number in EBC. Saliva was not obtained from this patient and therefore it cannot be determined whether this was a germline polymorphism or somatic mutation, however its heteroplasmic nature suggests that somatic mutation is more likely. Nevertheless this sample was excluded from the main analysis of D-loop changes in lung cancer vs. control subjects, and included in this paper to demonstrate the utility of this method in detecting potentially pathological mtDNA changes for further study.

Confounding factors of age and smoking history which are associated with greater mtDNA damage may be important.[38–41] No correlation between age and mutation load was found, which may indicate that age is not solely responsible for the differences in mutation prevalence observed. A bias may have been introduced by amalgamating all the ex-smoker and smoker subjects into one group, as lung cancer risk decreases after smoking cessation,[42] however, a subgroup analysis reveals that the median number of mutations was similar between smokers and ex-smokers in this study ($p = 0.92$) and hence it was valid to combine them into one group.

The interpretation of mitochondrial DNA sequencing can be affected by the inadvertent co-amplification of nuclear DNA[43] particularly in low-cellularity samples. This was minimised by designing primers specific to mitochondrial DNA and comparing the primer sequences and sample sequences obtained with known NumtS sequences. In addition, the comparison of EBC sequences with paired sequences obtained from the more DNA-rich saliva obtained with the same primers demonstrated adequate similarity to rule out nuclear co-amplification. Ideally, nuclear co-amplification would be excluded by using the primers to amplify DNA obtained from, for example, RhoO cells[43]

however comparison of sample sequences with NumtS tracks demonstrated that the DNA sequence obtained was most likely mitochondrial in origin.

Low amounts of DNA in EBC resulted in a limited sample size. This may impact upon the significance of the results obtained, however this preliminary study suggests that the methods are feasible and identifies areas for future research. Undoubtedly, if the technique were to be used in a clinical situation, better yields would be required, although sputum cytology also has a variable yield of cells from the lower respiratory tract and requires multiple specimens- this may be the case for EBC also. Initially EBC samples were obtained from 58 subjects however 15 samples did not contain enough EBC for analysis (mainly lung cancer subjects, who were less able to breathe into the apparatus for sufficient time), and inadequate DNA was obtained from a further 15 samples (mainly control subjects). Although the exact origin of EBC DNA has yet to be determined, similar mechanisms may operate as those proposed in production of circulating cell-free DNA, including normal cell turnover, lysis of cancer cells, cell necrosis, apoptosis and spontaneous active release of DNA by tumours.[44] These mechanisms may be less active in disease-free individuals and may explain why DNA yield was lower in EBC obtained from control subjects.

As there is overlap in mutation rates between the lung cancer and control groups, the mutation prevalence may reflect non-specific oxidative damage and specific mutations may be better biomarkers of lung cancer. This mutation (T16217C) shows specificity for the lung cancer group and is worthy of future studies, as the D-loop contains the major control elements for transcription and replication of mtDNA and accumulation of specific mutations may indicate clonal expansion of neoplastic cells. As much of the D-loop is non-coding, and most mutations found in this study were located outside the functional regions of the D-loop, these mutations are not likely to have caused the lung cancer but are more likely to be a reflection of widespread mtDNA damage, which has been shown to promote tumour cell proliferation or permit tumours to adapt to new environments.[45] There are reports of some anti-tumorigenic mtDNA mutations,[17] however no mutations were associated with lower cancer risk in this study population. Probably such anti-tumorigenic mutations would be found in functional regions of the mtDNA, such as the respiratory complex regions,[17] which were not studied here. Functional studies such as the use of transmitochondrial cybrids[46] are required to investigate the pathogenicity of mutations such as the T16217C mutation. A larger study could be conducted to screen for particular mutations such as those identified in this study using techniques such as PCR/RFLP and random mutation capture assay.[47]

The chance of PCR-induced mutations was minimized by the use of a high fidelity enzyme as well as performing duplicate PCR and sequencing reactions. No discrepancies were found between the two independent analyses for each sample and therefore the chances that the mutations identified in this study were PCR artefacts is extremely low. To confirm reproducibility of our results, 3 EBC samples were obtained from one subject and the 3 DNA sequences obtained did not show any discrepancy.As this study aimed to sample the lower respiratory tract, exclusion of salivary contamination was vital. Salivary contamination was excluded by mouth rinsing, a unidirectional valve and saliva trap, confirmed by amylase assay, and that the salivary DNA sequence differed than that obtained from EBC.

This preliminary study has verified the feasibility of detecting D-loop mutations in EBC and demonstrated its potential as a non-invasive biomarker for lung cancer. In this study population, the D-loop mutation frequency was higher in the EBC of patients with lung cancer compared to control subjects, reflective perhaps of greater mtDNA damage. Furthermore, the T16217C mutation was associated with lung cancer, however its functional effect is yet to be determined. Despite the small study size, this pilot study has demonstrated the promise of EBC as a method of investigating pathophysiological mtDNA changes in the lungs and identifying biomarkers for further study, as well as a potential screening tool for lung cancer, after identification of suitable biomarkers.

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Sources of support

Nil.

Contributions

Sylvia Si Yang Ai Designed the study, recruited participants, performed the analyses and wrote the first draft of the ms.

Kenneth Hsu assisted in the DNA analysis and development of the techniques, and contributed to the draft ms.

Cristan Herbert assisted in the DNA analysis and development of the techniques, and contributed to the draft ms.

Zujian Cheng recruited participants, assisted in the DNA analysis and development of the techniques, and contributed to the draft ms.

John Hunt assisted in the DNA analysis design, development of the techniques, and contributed to the draft ms.

Craig R. Lewis assisted in the design of the study and contributed to the ms.

Paul S. Thomas assisted in the design of the study and development of the techniques, and contributed to the ms.

Conflict of interest statement

None declared.

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