Mitochondrial DNA mutations in oral squamous cell carcinoma


1School of Biological Sciences, University of Wales Swansea, Swansea, SA2 8PP, UK, 2Department of Histopathology and 3Department of Oral and Maxillofacial Surgery, Morriston Hospital, Swansea SA6 6NL, UK and 4South West Wales Cancer Institute, Singleton Hospital, Swansea SA2 8QA, UK


Carcinogenesis vol.27 no.5 pp.945–950, 2006

Abbreviations: mtDNA, mitochondrial DNA; SCC, squamous cell carcinoma

It has previously been demonstrated that mitochondrial DNA (mtDNA) mutations within the ND2 gene of histologically normal parotid salivary gland tissue of smokers may be molecular biomarkers for smoking-induced mtDNA damage. Oral squamous cell carcinoma (SCC) is strongly related to cigarette smoking; therefore, we used PCR and direct sequencing to establish whether mtDNA mutations were also present in oral SCC which could be used as additional biomarkers for smoking-associated DNA damage. In addition to searching for mutations in the ND2 gene, the mitochondrial D-Loop was also analysed. Three mutation hotspots were observed in the D-Loop at nt 146, 152 and 186, two of which (nt 146 and 152) have also been implicated in oesophageal SCC, another smoking-related cancer. The mutation hotspot observed at nt 186 has not previously been reported in other tumours. Furthermore, we show that the mutations previously reported within the ND2 gene in normal parotid tissue of smokers were not evident in these samples, but that a mutation hotspot occurs at nucleotide 4917 in oral SCC. We also show that D-Loop mutations occur predominantly in male smokers and female non-smokers and that this association with gender is statistically significant (P = 0.003). We conclude that the mtDNA mutation hotspots found in this study, in particular nt 186, are potential biomarkers for oral SCC. However, owing to gender-specific differences in occurrence in smokers and non-smokers, and a lack of environmental smoking history, in general, it is difficult to associate these mutations with mtDNA damage induced by smoking. If the mutations observed in the subset of male patients are smoking induced, given our previous findings, mutation hotspots in the ND2 gene may be tissue specific suggesting the causative mutagens for mtDNA damage within these tissues are likely to be different.

Introduction

Oral cancer kills just over 50% of individuals diagnosed with this condition (http://www.cancerresearchuk.org/statistics), a case mortality rate which exceeds that of cutaneous melanoma, breast and cervical cancer, primarily owing to its late detection (http://www.rdoc.org.uk). In the UK in 2002, nearly 3000 deaths were caused by oral squamous cell carcinoma (SCC) and, although still relatively rare, the incidence of oral SCC is rising, particularly in women for whom the incidence rate is ∼25% (http://www.baoms.org.uk).

The term ‘oral cancer’ includes malignant neoplasms of the mucous surface of the lip, tongue, gums and floor of the mouth, and excludes the major salivary glands, oropharynx, nasopharynx and hypopharynx. Over 80% of oral cancers are SCCs (1), where the neoplastic process develops in squamous epithelium, progressing through hyperplasia and dysplasia to carcinoma in situ and eventually invasive carcinoma.

Tobacco consumption by smoking, chewing or inhalation as snuff is thought to be the major aetiologic risk factor for the subsequent development of oral cancer, with those smoking >20 cigarettes a day having a 6-fold increased risk of developing the disease compared with non-smokers (http://www.baoms.org.uk). Cigarette smoke contains >4000 chemical constituents including at least 40 carcinogens (2) such as nitrosamines, free radical forming compounds such as hydroquinone and bulky DNA adduct forming compounds such as polycyclic aromatic hydrocarbons (PAHs) (3). Free radicals contained in the particulates of cigarette smoke include relatively stable semi-quinones (4,5), whereas the gas phase includes short-lived carbon and oxygen-centred organic radicals which, despite their short life span, are maintained for substantial periods by redox reactions and the presence of nitrogen oxides, aldehyde species and carbon monoxide (6,7).

Cigarette smoke causes extensive damage not only to DNA in the form of single-strand breakages, but also results in oxidation of protein thiols and lipid peroxidation via the actions of these organic radicals and toxic compounds (8). Mitochondrial DNA (mtDNA) is particularly susceptible to damage by reactive oxygen species (ROS), such as superoxide radical (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (OH•), owing to the lack of the protective histone backbone and complex DNA repair mechanisms associated with nuclear DNA (9). It is well documented that a variety of mitochondrial mutations are caused by oxidative damage via ROS that are generated either endogenously during oxidative phosphorylation or by exogenous sources and other mutagens (10). Mitochondrial function can be impaired by oxidative damage, which can further enhance the production of ROS as a result of electron leakage within the aerobic respiration apparatus. Function is particularly impaired at complex I (NADH dehydrogenase) which contains the nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit 2 (ND2) protein, and complex III (succinate-CoQ reductase) which contains the majority of the cytochromes (11). Such functional changes result in increased subcellular damage as antioxidant defences are overwhelmed (12). Additionally, mitochondria that display respiratory deficiencies as a result of mtDNA mutation.
may thus release abnormally high levels of ROS into the cytosol, exposing the nucleus and other organelles of the cell to these cytotoxic compounds, potentially contributing to carcinogenesis (13).

Many cancer-related mitochondrial defects have been recognized at the organelle level, including abnormal activity of aerobic respiratory chain subunits, decreased oxidation of NADH-linked substrates and altered expression of mtDNA and mutations of mtDNA (14). Mutations in mtDNA have been observed in a number of cancers including breast (15,16), colon and rectum (17,18), stomach (19), prostate (20), bladder, head and neck, and lung (21,22). Their role in neoplastic pathways is still unclear. However, as mtDNA does not contain any intron sequences, mutations that do occur would lie in coding or regulatory regions and are potentially biologically significant (23).

Lewis et al. (24) have previously demonstrated that oncocytes within Warthin’s tumour (a benign head and neck neoplasm of the parotid salivary gland) contain mtDNA mutations. In addition, Warthin’s tumours contain morphologically abnormal and respiratory deficient mitochondria (25,26). It has also been demonstrated that over 90% of people who develop Warthin’s tumour smoke cigarettes (27). Lewis et al. (28) investigated whether mtDNA mutations were present in the normal parotid salivary gland of smokers. They concluded that certain mtDNA mutations within the ND2 gene were elevated in smokers and were a potential biomarker for smoking induced mtDNA damage prior to histological changes. The discovery of A:T to G:C and G:C to A:T transition mutations at nt 4767 and 4853 can be considered indicative of oxidative damage to the mitochondrial genome (28).

The present study aimed at investigating whether the previously reported ND2 gene mutations were present in oral SCC—another head and neck tumour strongly associated with cigarette smoking. We also investigated whether mutations were present in the D-Loop (or control region) of the mitochondrial genome, as this region is known to contain a number of published tumour-associated mutations, particularly within the homopolymeric C-tract positioned in a conserved segment of the hypervariable D-Loop (29). We report, for the first time, using matched histologically normal tissue, the presence of mitochondrial DNA mutations in oral SCC which may be useful smoking-related cancer biomarkers.

Materials and methods

mtDNA was extracted from 30 paired samples of tumour and non-tumour tissue (tumour-negative lymph node) from neck dissections performed at Morriston Hospital, Swansea. Samples were anonymized before processing and approval for the study was obtained from the local research ethics committee.

DNA extraction

For each sample DNA was extracted from preselected regions of tumour and control tissue using two unstained 20 μm sections of formalin fixed, paraffin wax embedded tissue, using the process described by Kim et al. (30). Briefly, tissue sections were dewaxed in two xylene washes and subsequently dehydrated in 100% ethanol. The tissue was collected by centrifugation, the resultant pellet was dissolved in 140 μl PCR–TE buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA), 20 μl 10% SDS (10 g/100 ml lauryl sulfate) and 80 μl of 1% proteinase K (100 μg/ml), and incubated overnight at 55°C to digest the tissue. DNA was subsequently isolated by phenol/chloroform and re-suspended in 100 μl filter sterilized H2O.

PCR primers were designed using the mitochondrial genome sequence obtained from MitoMap (http://www.mitoimap.org), and synthesized by Operon (Germany). All oligonucleotides were shipped desalted and dehydrated, and on delivery were rehydrated in Tris–EDTA (pH 7.0) and diluted to 20 pmol/μl and stored at −20°C.

ND2 PCR primers

Nest PCR was undertaken to amplify the DNA from archival sections, outer primers that flank the designated ND2 region were used to amplify an initial 624 bp PCR product using the forward primer OUTERF1 (nt 4429–4449, 5'-GCCCATACCCCGAAAATGTTG-3') and reverse primer OUTERR1 (nt 5031–5053, 5'-GGTGTAGAATCTCATATTCATCCAC-3'). All the PCRs contained 1.25 mM of each dNTP, 20 pmol of each primer, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2 and Pfu DNA polymerase (3 U/μl) (Promega, UK) in a total volume of 50 μl. PCR conditions were as follows: initial denaturation step at 94°C for 1 min; 28 cycles of denaturation at 94°C for 30 s; annealing at 56°C for 30 s; elongation at 30°C for 45 s. To determine the presence of ND2 gene mutations, a 473 bp PCR product was amplified from within the 624 bp product using the primers specified by Lewis et al. (28): forward primer L2 (nt 4504–4526, 5'-CCATCTTTGGCAAGCACTCATC-3') and reverse primer H2 (nt 4955–4977, 5'-ATCCACCTGCACTTGATG-3'). PCR conditions were as follows: initial denaturation step at 94°C for 5 min; 20 cycles of denaturation at 94°C for 45 s; annealing at 58°C for 1 min; elongation at 72°C for 30 s. PCR products were purified using PCR Purification Kit (Qiagen, UK) and separated on a 6% polyacrylamide gel, and visualized by silver staining.

D-Loop PCR primers

Outer primers that flank the desired area of the D-Loop, including the C-tract, were used to amplify an initial 649 bp PCR product using the forward primer OUTERF2 (nt 16514–16535, 5'-CAGGGTCTAAGCCCTAAATAG-3') and reverse primer OUTERR2 (nt 573–594, 5'-GAGTCAAAGCTCATAAACTG-3'); initial denaturation step at 94°C for 1 min; 28 cycles of 94°C for 30 s; 56°C for 30 s; 72°C for 45 s. To determine the presence of any mutations associated with the C-tract, a 421 bp PCR product was amplified from within the 649 bp product using the forward primer D1 (nt 8–29, 5'-GGTCTATACCTTATACAC-3') and reverse primer D2 (nt 408–429, 5'-CTGTTAAAGGTGCTACCCAGGG-3'), using 25 cycles of 93°C for 45 s; 61°C for 1 min; 74°C for 1 min. PCR products were purified and visualized on 6% polyacrylamide gels using silver staining.

DNA sequencing

All PCR products were purified using PCR Purification Kit (Qiagen, UK), and sequenced using an ABI capillary sequencer by MWG Biotech (UK).

Statistical analysis of mutation hotspots

Statistical analysis using Fisher’s exact test and identification of significant mutational hotspots were performed using iMARS software (www.clinicalbioinformatics.net).

Results

Study population

Using PCR and DNA sequencing we investigated the occurrence of somatic point mutations in regions of both the ND2 gene and D-Loop of the mitochondrial genome in oral SCC from a total of 30 patients in both tumours and matched non-tumour lymph node tissue. The age range of patients was 44–77 where 24 (80%) patients were male and 6 (20%) were female. Patients had been asked their smoking status and were classified as smokers or non-smokers according to their response. Of the males 20 were classified as smokers and 1 was a non-smoker, whereas 1 female was a smoker and 5 were non-smokers (we were unable to obtain smoking status for 3 patients). There was a statistically significant association between gender and smoking heavily biased towards males ($P = 0.0004$).

Mutation detection in the ND2 gene

We surveyed the same region of the ND2 gene between nt 4504 and 4977 as we had done previously in parotid tissue of
smokers and non-smokers (28). Two different types of point mutation were observed within this region in oral SCC mtDNA. We found six point mutations within the ND2 gene all of which were base substitutions. Of these base substitutions, four (66.7%) were A:T to G:C transitions: two at A4767, one at A4769, one at A4580 (Table I). Furthermore, two (33.3%) G:C to A:T transitions were found at position G4917 (Table I). The mutations observed at nt 4769 and 4580 were silent and thus would not change the resulting amino acid sequence. Nucleotide position 4917 was found to be polymorphic within this set of patients. The transition mutations observed in this study at nt 4917 would result in an amino acid change, either an Asn to Asp or the reverse, although that this nucleotide is polymorphic within the population it is likely that an A to G (or the reciprocal) mutation is very slightly, if at all, deleterious. Nucleotide 4917 was, however, a significant mutation hotspot ($P = 0.027$) and thus a potential smoking-associated biomarker in oral SCC. All patients having a mutation were males and classed as smokers with the exception of patient 5 whose smoking status was not known. Of the six patients harbouring an mtDNA mutation in the ND2 gene, four were heteroplasmic. There appeared to be no association between gender and mutation frequency, or between age and mutation frequency for this region, although the number of mutations in this particular study of the ND2 gene was small.

Although we had previously identified mutations at nt 4767 and 4853 in parotid tissue, neither mutation was observed in oral SCC in this study.

**Mutation detection in the D-Loop**

Seven different types of mutation were discovered in the region of the D-Loop between nt 8 and 429. The frequency of mutations was much higher in the non-coding D-Loop relative to the ND2 gene where, in total, 40 base substitutions, 10 insertions and 1 deletion were found (Table II). Base substitutions were observed in 16 (53.3%) different patients where 15 of these patients had a classified smoking status. Of these, the 10 male patients with mutations were all self-classified smokers whereas, conversely, 4 of the 5 females with mutations were self-classified as non-smokers. This association of sex (males) and smoking status was statistically significant ($P = 0.003$) for patients with mutations.

Of the base substitutions 14 (35.0%) were A:T to G:C; 4 (10.0%) were A:T to C:G; 6 (15.0%) were G:C to T:A; 15 (37.5%) were G:C to A:T and 1 (2.5%) was A:T to T:A. A total of 29 base substitutions (72.5%) were observed at just three nucleotides (nt 146, 152 and 186). Nucleotides 152 and 186 were also significant mutation hotspots ($P = 0.0148$ and $P = 0.0012$, respectively), strongly suggesting that these to be biomarkers in oral SCC. Furthermore, mutations at these three nucleotide positions almost always occurred simultaneously in patients.

Many of the mutations occur at known polymorphic and/or somatic mutation sites. Although nt 186 has previously been shown to be polymorphic, the C to A substitution, to our knowledge, has not previously been reported. Perhaps the most striking observation was that, in addition to being a significant mutation hotspot, this nucleotide was mutated in tumours derived from 12 (40.0%) different patients. In similar fashion to the mutations in the ND2 gene, most mutations observed in the D-Loop were heteroplasmic although a small number of mutations within this region were homoplasmic (Table II). An example of the differing homoplasmic and heteroplasmic states of mutations at nt 186 within different patients is displayed in online supplementary Figure 1 (http://www.clinical-bioinformatics.net/ossc/figure_1.htm). All single nucleotide insertions occurred within the hypermutable C-tract between nt 303 and 309 inclusive. Interestingly, the one single nucleotide deletion involved loss of the thymine at position 310 which occurs in the middle of the C-tract.

**Discussion**

We present, for the first time, a comprehensive study of mutation screening in selected regions of the mitochondrial genome in oral SCC. This study is an extension of work conducted by Lewis et al. (28) on normal parotid salivary gland tissue in smokers where the primary objective was to screen mtDNA for potential smoking-related biomarkers. Our previous study revealed the presence of smoker-specific A:T to G:C transition at A4767 and a G:C to A:T transition at G4853. The present study investigated regions of the ND2 gene and D-Loop in 30 oral SCCs and matched normal tissue to search for previously detected and novel mutations in mtDNA which might be related to cigarette smoking. We were interested to see if the mutations reported in parotid tissue in our previous study are also present in the ND2 gene in oral SCC. We
were unable to demonstrate the presence of the previously reported mutations but revealed the occurrence of mutations in six patients at three different nucleotide positions that were either G:C to A:T or A:T to G:C. Nucleotide positions 4580 and 4769 are known polymorphic sites (http://www.mitomap.org), whereas mutations at nt 4917 have not been reported as a mutation hotspot in other cancer types. A simple explanation may be that the mutations at this position may be caused by specific mutagen(s) in cigarette smoke in the mouth but other tissue may not be exposed to such mutagens. An insertion of an extra cytosine prior to the thymine at nt 310 was observed in 10 of our tumour samples. Whereas the most common number of cytosines prior to nt 310 is 7, numerical changes in the homopolymeric C tract have often been reported in tumours (http://www.mitomap.org), including lung cancer (35) and oesophageal SCC (34). Although we would not be confident to propose mutations within this particular C-tract as biomarkers for oral SCC, we feel the presence of insertions and deletions here may often be due to sequencing errors. In addition, this stretch of mtDNA is commonly reported as being polymorphic not just in tumours, but also in normal tissue.

Both A:T to G:C (39.1%) and G:C to A:T (37.0%) accounted for over half of all the base substitutions (ND2 and D-Loop) found in this study. These mutation types are characteristic signatures for a number of potent mutagens known to occur in cigarette smoking and also for oxidative damage that could occur owing to cellular stress (36). However, our results indicate that mutations in the D-Loop occur in both smokers and non-smokers with a significant association with gender ($P = 0.003$). Given that only 1 male out of 24 was classified as a non-smoker it is, perhaps, not surprising that all 10 males having mtDNA mutations with known smoking status were smokers. Of the six females with known smoking status,
five were classified as non-smokers. This raises the important question as to whether there is indeed a higher frequency of mtDNA mutations in non-smoking females in oral SCC or whether the smoking status of these females is inaccurate. The significant association between smoking and gender might suggest a different aetiology between male smokers and female non-smokers for oral SCC. Whether smoking or exposure to environmental cigarette smoke directly causes oral SCC in smokers and non-smokers remains to be established. Similarly, whether the cause of mtDNA mutations in smokers and non-smokers is due to the same mechanisms also needs confirmation. The occurrence of the D-Loop mutation hotspots in both males and female (smokers and non-smokers) in this study, however, suggests a similar mechanism. The association between mtDNA mutations in oral SCC in female patients may be a consequence of females, especially within this age group, being more likely to misclassify themselves as never smokers, masking a previous smoking history or that they have been passive smokers (37). However, there is much evidence suggesting that oral SCC in non-smokers occurs predominantly in females (38–41). In addition, female non-smokers presenting oral SCC tend to be more elderly (39). It has also been found that head and neck SCC in non-smokers occurs predominantly in white patients associated with rare alcohol abuse but with a significant association with environmental tobacco smoke exposure (40). Thus, we cannot conclude with confidence whether smoking or non-smoking females in oral SCC. Whether smoking or non-smokers is due to the same mechanisms also needs confirmation. The occurrence of the D-Loop mutation hotspots within both the ND2 gene and D-Loop, two of which have been identified in another smoking-related cancer, oesophageal SCC. Our findings may be further evidence for smoking-related damage to mtDNA, at the very least in male subjects, which could directly compromise respiration within a cell and indirectly lead to a higher level of oxidative damage with the potential to cause further mutations in nuclear DNA. Given that we only surveyed less than one-eighth of the mitochondrial genome, the level of mutations may far exceed the number we have observed. Future studies should aim to establish the actual causes of smoking-associated mitochondrial mutations by means of in vitro cell culture studies, and whether these are present in other smoking-related cancers.

Acknowledgement

This work was made possible by the generous support of the British Association of Oral and Maxillofacial Surgeons (BAOMS) from a grant awarded to P.W.B.

Conflict of Interest Statement: None declared.

References


Received May 20, 2005; revised November 9, 2005; accepted December 20, 2005.