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Research Article

GENETIC DIVERSITY OF MITOCHONDRIAL DNA GENES IN ORAL CAVITY CANCERS IN SENEGAL

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ABSTRACT

Oral cavity cancers (OCC) represent approximately 25 to 30% of cancers of the upper aero digestive tract (UAT) i.e. head and neck carcinomas. OCC is the 4th most common cancer in men and the14th most frequent among women worldwide. Here, we aimed to determine the variability and genetic diversity of two mitochondrial markers, namely MT-CYB and D-Loop, in cancerous tissues and control subjects with OCC in Senegal. A total of 45 patients with OCC were enrolled as subjects. Blood sample and biopsy of the tumor were obtained from each patient and total DNA was extracted from the samples. Our study highlighted the feminization and rejuvenation of the pathology with 62% women compared with 38% men. The mean age of our patients was 53 ± 16.64 years. The most frequent location of the tumor was the gum with 42% of cases. Nine amino acids showed a significant mean difference between healthy and cancerous tissues. For the molecular characterization of OCC, D-Loop and MT-CYB genes were amplified and sequenced. Notably, cancerous tissues were more polymorphic than healthy tissues for both D-Loop and MT-CYB. Furthermore, MT-CYB was more polymorphic than *D-Loop* in patients with OCC in Senegal. Our study also confirmed the major role of tobacco in the occurrence of OCC. Together, our results are in agreement with previous studies, and further implicate D-Loop and MT-CYB polymorphism in OCC tumor progression and development.

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INTRODUCTION

Each year, 650,000 new cases of oral cavity cancers (OCCs) are diagnosed worldwide and there are approximately 350,000 deaths related to this disease [1]. 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. This type of cancer ranks 6th in the world because of its prevalence; over 80% of these cancers are so-called squamous cell carcinomas [2], which only affect the superficial cells of the mucous membranes of the ENT sphere. The neoplastic process develops in squamous epithelium, progressing through hyperplasia and dysplasia to in situ carcinoma and eventually invasive carcinoma. OCCs are common in men; they are positioned at fourth most common cancers after prostate, bronchial, colon, and rectal cancers, and the 14th most common in women worldwide [3]. Nevertheless, rejuvenation and feminization of the affected population has been noted in the past 30 years [4]. The cancer of the lips and the oral cavity is the 15th most widespread cancer in Africa [5]. The highest incidence rates of OCCs are observed in Pakistan, India, and France [6]. Thus, they are an important public health issue in many countries. In Senegal, the epidemiological profile is that of a relatively young woman (the age group most represented is that of the 45-54 age groups) who does not smoke or drink and often with mediocre oral hygiene [7].

The majority of human cancer types are characterized by genetic instabilities. Apart from nuclear DNA, mitochondrial DNA (mtDNA) is the only other genetic material of the human genome [8-9].The displacement-loop (D-loop) region, the only non-coding region in mtDNA, is considered to be important, as it is the major control site for mtDNA expression and is involved in mtDNA replication [10]. Genetic variability in the D-loop region has been suggested to affect the function of the respiratory chain, there by leading to high ROS levels and possibly contributing to cancer initiation [11].

MT-CYB is a region of the mitochondrial genome, located in positions 14747 and 15887 [12]. The MT-CYB gene encodes

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for the respiratory complex III subunit. MT-CYB is a component of the respiratory chain, and is also known as the bc1 complex or c reductase ubiquinol-cytochrome complex III. It is involved in the binding of substrate to quinone and is responsible for the transfer of electrons by which the transmembrane redox energy is converted into a proton motive power. Thus, the complex III plays a key role in cells [13].

The present study aimed at determining the variability and genetic diversity of the two mitochondrial markers MT-CYB and D-Loop in cancerous tissues and control subjects in order to examine the role of these genes in oral squamous cell carcinoma (OSCC) tumorigenesis. In addition, we investigated whether there is a correlation between risk factors and OCC in Senegal.

MATERIALS AND METHODS

Tissue Specimens and DNA Extraction

Tissue samples from oral cavity tumors were collected from 45 patients treated in the Department of Oral and Maxillofacial Surgery, Affiliated Hospital Aristide Le Dantec Hospital (Dakar, Senegal). All patients gave their written informed consent, and the study was approved by the University Cheikh Anta Diop Research Ethics committee (Reference: /2018/CER/UCAD). Personal details of each patient were recorded in a questionnaire during their enrolment. Information regarding age, gender, medical and family history, result of the histopathological examination, and nature of consuming tobacco habit (smoking or smokeless) and alcohol were also recorded. A portion of each biopsy was sent for histopathological examination, and the remaining was used for DNA extraction. Blood samples were collected from healthy patients into an EDTA tube as control. Human DNA was extracted using a Quick-DNA ™MiniprepPlus Kit (Zymo Research), in accordance with the manufacturer's protocol. The extracted DNA was stored at 4°C.

Mitochondrial DNA Amplification and Sequencing

PCR amplification was carried out using an Eppendorf thermal cycler. In order to amplify the mtDNA D-Loop, specific oligonucleotide primers were designed based on Genbank database (NC_012920.1). The sequence for the forward primer was H408 (TGTTAAAAGTGCATACCGCCA) and for the reverse primer L16340 (AGCCATTTACCGTACATAGCA CA). The total reaction volume was 50µl containing 2µl of genomic DNA, 5 µl of 10 X buffer with 1µl of $MgCl_2$, 2µl of dNTPs, 0.1µl DNA polymerase, 2.5µl of each primer, and 34.9µl of nuclease-free water. The PCR conditions were as follows: initial denaturation at 95°C for 15 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 35 s, and extension at 72°C for 2 min; and a final extension at 72°C for 10 min.The cytochrome b gene was amplified by PCR using two primers, H15915 (TCTCCATTTCTGGTTTA CAAGAC) and L14723 (ACCAATGACATGAAAAATCATG GTT). A 50-µl PCR was setup as described above, except that 0.2 µl of DNA polymerase was added. The reactions were carried out with an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 s; 1 min of annealing at 52°C, 1 min 30 s of elongation at 72°C, and a final extension step at 72°C for 10min, followed by holding at 10°C. At the end, 5 µl of the reaction mix was analyzed using 1.5% agarose gel electrophoresis. All the PCR products were purified and sequenced with an ABI Big Dye Terminator cycle sequencing ready reaction kit and sequenced using an ABIPRISM 3730xl sequencer (Applied Biosystems, Foster City, CA).

Genetic Analyses

Sequences were carefully cleaned and corrected using BioEdit version 7.1.9 [14]. These sequences were then aligned using the Clustal W multiple alignment editor [15]. To estimate the variability and genetic diversity of each gene (MT-CYB and D-Loop) in the cancerous tissues and control subjects, we determined the sample size (n), the size of the sequences (N), the number of variable and invariable sites, the number of informative and non-informative variable sites, the total number of mutations (Eta), the number of haplotypes, the haplotype diversity (Hd), the nucleotide diversity Pi (π), and the average number of nucleotide differences (k) using DnaSP software version 5.10.01 [16]. The nucleotide frequencies, the nature of the mutations (% transitions and % transversions), and the mutation rate (R) were determined using MEGA version 7.0.14 program [17].We then proceeded to highlight amino acid variations. MT-CYB being a coding gene, the nucleotide sequences were converted into amino acid sequences using the best reading frame under MEGA version 7.0.14 [17].

Next, we analyzed the genetic structure of concatenated D-Loop and MT-CYB to form one single matrix. The determination of intra-group and inter group genetic distances of Nei (1987) was done with the MEGA version 7.0.14 [17] using the Kimura 2 parameter model [18]. Here the groups are related to the nature of the tissue and risk factors such as age, sex and location. Genetic differentiation between control and cancerous tissues was demonstrated with the fixation index (Fst), which was estimated using Arlequin software version 3.5.2.2 [19]. The *P* value allows checking the significance of the Fst value; the level of significance was set at 5%.

Statistical Analyses

The Shapiro-Wilk test was performed usingR studio version 1.1.447 to test the normality of our data (amino acid variability). The initial hypothesis (H0) that the sample follows a normal distribution was chosen. The Wilcoxon test was performed for amino acids that do not follow normality. Differences were considered to be statistically significant at $P< 0.05$.

RESULTS

General Characteristics of the Patient Population

The study population comprised of 45 patients. Of these,62% were women and 38% were men, with a sex ratio of 0.60 (Figure 1.A). The average age of patients was 53 ± 16.64 years, with extremes from 14 to 86 years (Figure 1.B). Our study showed that 51.72% of patients were non-alcoholic-smokers. Smokers comprised of only a small number of patients (17.24%), whereas the tobacco-alcohol combination accounted for 31.03% (Figure 1.C). Squamous cell carcinoma accounted for 91% of the OCCs. The other histological types were divided into: 3% papillomatosis, 3% sarcoma, and 3% chondromyxoid fibroma (Figure 1.D). It is important to note

that the most frequent location of the tumor (Figure 2) was the gum with 42% of the cases, followed by the inner side of the cheeks (21%), the tongue (21%), the facial (7%), lips (7%), and palate (2%).

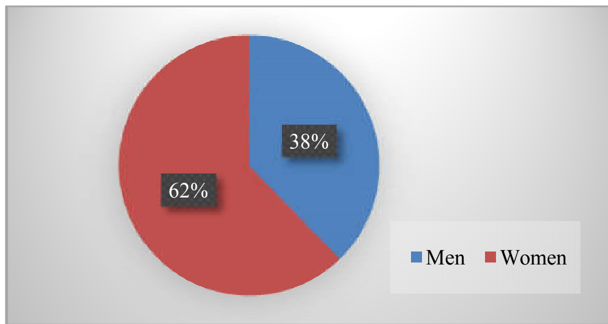

Figure 1 A Distribution of patients by sex

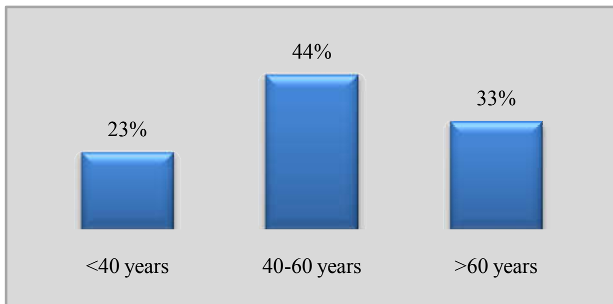

Figure 1 B Distribution of patients by age group

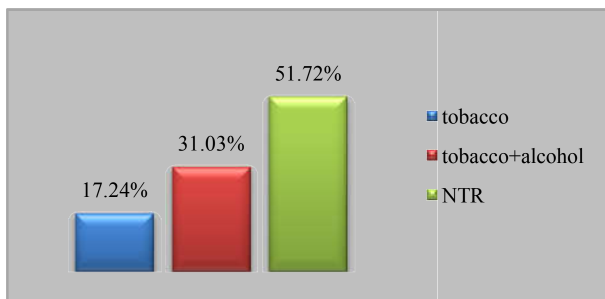

Figure 1 C Distribution of patients by history

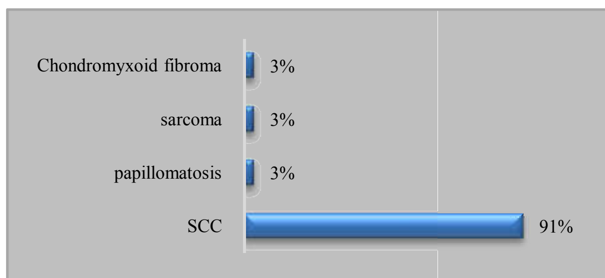

Figure 1 D Distribution of patients by histological type

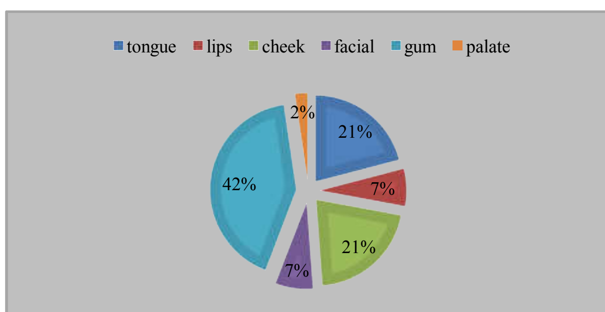

Figure 2 Topographic distribution of oral cell carcinoma

Sequences Obtained

Sequence alignment and correction resulted in 31 sequences with a length of 629 bp for the D-Loop and 44 sequences with a length of 358 bp for MT-CYB. Other sequences not present in the data set were deleted, because they were too diverse.

Polymorphism and Genetic Diversity of MT-CYB and D-Loop

The results of the polymorphism and genetic diversity parameters are shown in Table 1

Table 1 Parameters of polymorphism and genetic diversity of MT-CYB and D-Loop

	MT-CYB		D-LOOP	
	TT	NT	TT	NT
Sample size	44	23	31	23
Number of sites	358 (no gap)	358 (no gap)	629 (no gaps)	629 (no gaps)
Invariable sites	63.40%	74.86%	83.14%	91.41%
Polymorphic sites	36.60%	25.13%	16.85%	8.59%
parsimonious sites	108 (82.44%)	61 (67.77%)	36 (33.96%)	25 (46.29%)
with 2 variants	96 (88.88%)	51 (83.60%)	29 (80.55%)	22 (88%)
with 3 variants	10 (9.25%)	10 (16.39)	6 (16.66%)	2 (8%)
with 4 variants	2 (1,85%)	-	1 (2.77%)	1 (4%)
Singletons sites	23 (17.55%)	29 (32.22%)	70 (66.03%)	29 (53.70)
with 2 variants	21 (91.30)	28 (96.55%)	61 (87.14%)	28 (96.55)
with 3 variants	2 (8.69%)	1 (3.44%)	8 (11.42%)	1 (3.44%)
with 4 variants	-	-	1 (1.42%)	-
Total number of mutations, Eta	41.06%	28.21%	19.71%	9.37%
Number of haplotypes, h	23	17	30	22
Average number of nucleotide difference K	12.995	26.257	15.856	10.040
Mutation rate	1.38	1.89	1.50	1.98
% transitions	58.09	66.12	67.07	66.08
%transversions	42.91	33.88	32.93	33.92
Nucleotide frequencies %	A (25.2) T (31.3) C (9.7) G (33.7)	A (25.8) T (31.4) C (10.7) G (32.1)	A (25.1) T (27.8) C (17.1) G (30.1)	A (25.0) T (27.7) C (17.0) G (30.3)
Molecular distance	0.044 ±0.004	0.079 ±0.010	0.026 ±0.003	0.016 ±0.003
1st position	0.030 ±0.006	0.035 ±0.010		
2nd position	0.031 ±0.006	0.058 ±0.014		
3rd position	0.084 ±0.015	0.155 ±0.026		
Ks substitution rate	0.027 ±0.005	0.135 ±0.024		
Kns substitution rate	0.026 ±0.002	0.038 ±0.007		

NT: Normal Tissue; TT: Tumor Tissue

In case of MT-CYB, cancerous tissues showed the highest number of variable sites (36.60%) compared with that in normal tissue (25.13%). In sick individuals, there were more parsimonious sites (82.44%) and fewer singletons (17.55%), compared with that in the healthy individuals (67.77% and 32.22%, respectively). The total number of mutations (Eta) confirmed the polymorphism of MT-CYB (41.06%) of cancerous tissue. The results of the nucleotide frequencies revealed a predominance of A and T (56.5%) compared with that of C + G (43.4%) in sick individuals, as well as in healthy individuals (A + T = 57.2%; C + G = 42.8%). The results also showed a mutational bias in C and G at the second position of the cod on. The substitutions of MT-CYB evolved more rapidly at the 3rd position of the codon with a molecular distance of 0.084 +/- 0.015 compared to the molecular distances at the 1st (0.030 +/- 0.006) and the 2nd position of the codon (0.031 +/- 0.006).

D-Loop showed 91.41% invariable sites in normal tissue and 83.14% in cancerous tissue. Normal tissues showed the smallest number of variable sites (8.59%) compared with the sick individuals (16.85%). Sick individuals had fewer parsimonious sites (33.96%) and more singletons (66.03%) compared with healthy individuals (46.29% parsimonious sites and 53.70% singletons sites). The total number of mutations

Eta in cancerous tissue (19.71%) was much higher than that in healthy tissue (9.37%). Transitions were superior to transversions in both cancerous and healthy tissues. Frequencies of A and T (52.9%) were higher than those of C + G (47.1%) in patients; the same was observed in healthy individuals (A + T = 52.7%, C + G = 47.3%).

Based on all the parameters tested, we consider that MT-CYB is more polymorphic than D-Loop in oral cavity cancers.

Cytochromeb Amino Acid Variability

A total of 9 amino acids, of which 3 were essential (lysine, threonine, valine) and 6 non-essentials (alanine, cysteine, glycine, as paragine, glutamine, tyrosine), showed a significant average difference between healthy and cancerous tissues.

Differentiation and Genetic Structure Analysis

Genetic distances and genetic differentiation values are shown in Tables2 and 3. Analysis of the genetic structure of the concatenated sequences revealed intra-tissue genetic distances of 0.038 and 0.035 in healthy and cancerous tissues, respectively. The value of the inter-tissue genetic distance was0.046. The genetic differentiation between the two types of tissues was0.203 with a significant p value ($p \leq 0.001$).

Table 2 Intra and inter-population genetic distances

	Genetic distances	
	Intra-tissue distance	Inter-tissue distance
Normal tissue	0.038+/-0.004	0.046+/-0.004
Cancerous tissue	0.035+/-0.002	

Table 3 AMOVA test and genetic differentiation index (Fst)

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Fixation index
Among populations	1	129.598	4.32579	20.32%	Fst= 0.2032 P≤0.001
Within populations	51	865.194	16.96459	79.68%	

Our resultsalso revealed the existence of a genetic structuringbased onsex (Fst = 0.085, $P = 0.002$) and consumption of tobacco (Fst = 0.04679, $P = 0.04888$) (Table 4).

Table 4 Analysis of genetic structuring by sex, age, and tobacco consumption

Variables	Fst	P-value
Sex		
Men		
Women	0.08535	0.00293
Age		
Young-intermediate	0.04615	0.08504
Young-old	0.00796	0.22776
Intermediate-old	0.02957	0.42424
Tobacco consumption		
Smoker	0.04679	0.04888
Non-smoker		

DISCUSSION

In this study, the male to female sex ratio was 0.60, which is comparable to that reported by Touré *et al*. [7] who observed 60% female cases in their study on the epidemiological profile of OCC in Senegal. However, according to the epidemiological study by Piette [20], males are 1.3 to 10 times more affected than females. In Zimbabwe, Chidzonga[21] found 65% of male cases with OCC. Many other studies have also shown that men are more likely to have OCC, although the feminization of the disease is becoming more common [4].

The average age of our patients was 53 ± 16.64 years. Siriwardena *et al*. [22] showed that OCC incidence increases at age 40 and reachesits peak at age 60. The mean age of OSCC is between 50 and 60 years according to Zwetyenga *et al*. [23]. In Senegal, non-smokers account for 94.6% of the adult population aged 15 and over. Of these, 5.3% were former daily smokers and 89.4% were never daily smokers [24]. In our study, 51.72% said they never smoke or drankalcohol. Furthermore, 91% of carcinomas treated in this study were epidermoids. This result follows the trend described by Dieng *et al*.[25], wherein 98% of OCC observed in Senegal were OSCC cases.

The most common tumor location was the gum, with 42% of cases, followed by the tongue and the cheek with 21% of cases. In Zimbabwe, Chidzonga [21] reported predominance of OCCs at the tongue (38% of cases). Toure *et al*. [7] reported a predominance of OCCs at the tongueand at the palate respectively. According to Piette [20], the preferential location of oral tumors would be, in 50% of cases, the mobile tongue and the floor of the mouth.

Our results reveal that the frequency of certain amino acids derived from cytochrome b sequences were different between normal and cancerous tissues. The cellular amino acid content appears to be essential for tumor growth. It has been postulated that tumor cells use more amino acids than normal cells [26]. Higher frequencies of threonine, tyrosine, asparagine, and glycine were detected in cancer tissue samples compared with normal tissue. Our results may indicate either that tumor cells have higher amino acids biosynthesis rate [27], or that tumor cells use more amino acids as energy sources [28]. However, considering the anaerobic conditions of the tumor cells, it seems obvious that the oxidation of the amino acids in these cells is minimal. We also noted a significant decrease in lysine and valine in cancerous tissue. Being essential amino acids, this suggests that the nutrition of the tumor cells would be influenced by the nutrition of their host. Furthermore, we noted a decrease in glutamine, alanine, and cysteine. Although it is thought that tumor cells consume large amounts of glutamine to reconstitute the trichloroacetic acidcycle [9], an in vitro study on cell lines of squamous cell carcinoma of the head and neck revealed that glutamine is not the primary source of energy for these cells [29].

Examination of the genetic structure of MT-CYB and D-Loop concatenated by the analysis of their genetic distance (0.046) and the genetic differentiation index (Fst = 0.20318, $P \leq 0.001$), showed an onset of genetic differentiation between the two groups of tissues. It should be noted that the intra-tissue genetic distance of cancerous tissue (d = 0.035) was slightly less than that of healthy tissue (d = 0.038). The essential molecular variance may be explained by an intra-tissue differentiation (Fst = 79.68%).

The study also showed significant genetic structuring based on sex ($P = 0.00293$). However, the reasons for this need to be investigated, as apart from the identified risk factors (tobacco, alcohol, poor oral hygiene), there are no rational explanations.

We also noted genetic structuring based on tobacco consumption. Tobacco is a recognized cause of oral cavity cancers, pharynx, and larynx [30]. The risk of OCCs increases with the number of cigarettes smoked and with the duration of smoking. For lung cancer, smoking duration has been shown to be the most important determinant. This does not seem to be the case for upper aerodigestive tract carcinomas. The risk decreases when smoking stops. Analysis of a UK physician cohort after a 50-yearfollow-up [31] showed that upper aerodigestive tract and esophagus cancer mortality was seven times higher in cigarette smokers than in non-smokers and was three times higher in ex-smokers than in non-smokers. Compared with non-smokers, pipe or cigar smokers also showan increased risk of the oral cavity cancers, oropharynx, hypopharynx, and larynx [30].

CONCLUSION

This study aimed to determine the variability and genetic diversity of two mitochondrial markers namely MT-CYB and D-Loop in cancerous tissues and control subjects of oral cavity cancers in Senegal. The major role of tobacco and to a lesser extent alcohol in the occurrence of oral cavity cancers revealed in previous studies was confirmed in the current study. In addition, our observations indicate that the joint effect of the consumption of these two is more than multiplicative. Our study also highlighted the feminization and rejuvenation of the pathology.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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