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# MITOCHONDRIAL DNA 4977 BP DELETION AMONG SUDANESE ORAL LESIONS

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## ABSTRACT

**Background/Objectives**: Recently, oral cancer mortality and morbidity is very high in Sudan, particularly among men due to environmental factors and habits of life style. The study aimed to investigate the frequency of mitochondrial DNA (D- loop) mutations (mtDNA4977) in oral lesions**. Materials and Methods**: This is a retrospective study on oral lesions biopsies in Khartoum state - Sudan. The data were collected from examination of biopsies and patients records. All oral biopsies were examined and classified into histopathological pattern by using hematoxylin & eosin standard method. The detection of mtDNA4977 bp deletion was performed using a PCR-based technique. Data was analyzed by using the statistical package for social sciences (SPSS) for Windows computing program version 20. **Results:** One hundred and fifteen oral biopsies were taken from patients during surgical operation (61 male; 54 female), their mean age was 47±18. Of the study samples, 81 (70.4%) samples were malignant, 18 (15.7%) were benign and 12 (10.4%) were inflamed. Squamous cell carcinoma was the most frequent 55 (67.9%) of malignant cases, followed by adenocarcinoma 15 (18.5%), mucoepidermoid carcinoma 5 (6.2%), ameblastoma 4 (4.9%), and lymphoma 2 (2.5%). Four of the malignant cases (1 male and 3 female) were having the 4,977 -bp deletion.

**Conclusions:** It could be concluded that there was no obvious correlation between 4.977 bp deletion and prognostic indicators among Sudanese with oral lesions, which limits it`s utility as prognostic biomarker in oral disease. The study suggested further large scale studies in order to increase the rate of mutation detection.

**KEYWORDS:** mitochondrial DNA, 4.977 bp deletion, Oral lesions, Sudanese patients.

## 1. INTRODUCTION

Oral cavity extends from skin-vermilion junction of lips to junction of hard and soft palate above and to line of circumvallate papillae below. It is composed of buccal mucosa, tongue, gingiva, with the presence of the two lips at its entrance. The major and minor salivary glands open by different ducts into the oral cavity. In the cavity the food is masticated, mixed with saliva and formed into bolus.[1] Clinically, benign tumors and tumor-like conditions include eosinophilic granuloma, fibroma, granular cell tumor, keratoacanthoma, leiomyoma, osteochondroma, lipoma, schwannoma, neurofibroma, papilloma and rhabdomyoma. Benign epithelial tumors include: Squamous cell papilloma, mainly affects adults and consists of stratified squamous epithelium supported by a vascular connective tissue core, Adenoma arises from minor salivary glands. They form smooth, round, swellings and are most frequently found on the palate.

Oral cancer is a common cancer and constitutes a major health problem in developing countries, representing the leading cause of death.[2] Some 95% of malignant neoplasms affecting the oral cavity are squamous cell carcinomas. Squamous cell carcinoma (SCC) is an epithelial malignancy with morphologic features of squamous cell differentiation.[3] Oral squamous cell carcinoma (OSCC) poses a major health risk and is one of the leading causes of mortality. Distribution of the incidence of OSCC varies across the world with south-central Asia and Africa leading, followed by eastern and central Europe, and to a lesser extent Australia, Japan, and the United States. In the United States alone, about 17 new cases/100,000.

Oral cancer is the fifth most common and sixth leading cause of cancer-related mortality per year globally.[4] A key factor in the lack of improvement in prognosis over the years is the fact that a significant proportion of oral squamous cell carcinomas (OSCCs) are not diagnosed or treated until they reach an advanced stage. This diagnostic delay may be caused by either patients (who may not report unusual oral features) or by health care workers (who

may not investigate observed lesions thoroughly) and it is presumed that such delays are longer for asymptomatic lesions. The prognosis for patients with OSCC that is treated early is much better, with 5-year survival rates as high as 80%. In addition, the quality of life improves after early treatment, because cure can be achieved with less complications and less aggressive treatment than is necessary for advanced lesions.[2] A significant proportion of oral squamous cell carcinomas (OSCCs) develop from premalignant lesions such as leucoplakia and oral sub mucous fibrosis. Histological examination of tissue remains the gold standard for diagnosis and identification of oral lesions. Generally oral diseases are major health problems in developing countries including Sudan, many studies explain due to the local life style habits such as use of smokeless tobacco "Toombak", which is known to contain high level of the potent carcinogenic component of the tobacco (TSN).[5]

Toombak, was introduced approximately 400 years ago. It is always processed into a loose moist form and its use is widespread in the country. Tobacco used for manufacture of the Toombak is of the species Nicotiana rustica and the fermented ground powder is mixed with an aqueous solution of sodium bicarbonate. The resultant product is moist, with a strong aroma, highly addictive and its use is widespread particularly among males. Its pH range is 8-11, moisture content ranges 6-60% and nicotine content is from 8 to 102 mg/g dry wt and tobacco specific nitrosamines TSNAs contents in micrograms (N'-nitrosonornicotine NNN 420-1 550; 4-(methyl-nitrosamino)-l-(3-pyridyl)-l-butanone NN 620-7 870; N'-nitrosoanatabine NAT 20-290).[6]

Toombak dippers develop a clinically and histologically characteristic lesion at the site of dipping. The risk for cancer of the oral cavity among Toombak users was high (RR 7.3-73.0-fold)[7] tobacco specific nitrosamines present in Toombak possibly acting as principal carcinogens, So the use of Toombak plays a significant role in etiology of oral squamous cell carcinomas (OSCCs)[8], and suspected to be associated with neoplasm of salivary glands.[9- 10]

Human mitochondrial DNA (mtDNA) is a 16,569 bp double-stranded circular DNA molecule, and several hundred to several thousand copies of mtDNA are present in each cell. The human mtDNA encodes 13 polypeptides, which are essential constituents of respiratory enzyme complexes, and 22 transfer RNAs and two ribosomal RNAs that are required for protein synthesis in mitochondria.[11]

Mutations in mtDNA have been reported in almost all forms of primary tumors examined. As recently classified by Carew and Huang the majority of the mutations are base substitutions; [12] mutations occur in all protein-coding mitochondrial genes; the D-loop region is the most frequent site of somatic mutations across most tumor types.[13]

The D-loop is a triple stranded non-coding region with regulatory elements required for replication and transcription of the mtDNA. Hence mtDNA mutations in this region might responsible for the changes on copy number and gene expression of the mitochondrial genome.

Based on the published data, Carew and his colleagues addressed four common features of mtDNA mutations in all tumor types including that the base substitutions are the most common mutations; mutations occur in all protein coding mitochondrial genes; the D-loop region is the hot spot of somatic mutations among most of tumor types; and the presence of homoplasmic mutant mtDNA in tumors suggests that they may play an important role in the development of tumors.[14]

Majority of local studies focused on environmental risk factors and life style of patients such as "Toombak" use and correlated that with oral squamous cell carcinoma, but there were no traced studies investigated the genetic alteration as risk factor and its role in oral lesions among Sudanese, therefore the aim of this study was to investigate the 4977 bp deletions of mitochondrial DNA in oral lesions and its potential roles in the development of cancer.

## 2. MATERIALS AND METHODS

This is a molecular based retrospective study on oral lesions biopsies in Khartoum, Sudan. The study was approved by the Faculty Research Board of Sudan University for Science and Technology in collaboration with Ministry of Health ethical approval committee. One hundred and fifteen (115) paraffin embedded biopsies of oral lesions taken from patients underwent surgical operations, aged between 10 – 90 years with a mean age of 47±18 years. The sample size was total coverage of population referred to the two major center in Khartoum (National laboratory, STAK) and Khartoum hospital- Sudan), during the period from 2012 to 2013. The data were collected from examination of collected biopsies and from patients' records. All oral biopsies were examined and classified histopathologically as malignant, benign, and inflammatory by well professional pathologist using Hematoxylin & Eosin adopting Mayer’s procedure.[11]

Serial sections from the same formalin fixed paraffin embedded (FFPE) biopsies were taken for DNA extraction using QIAGEN QIAamp DNA FFPE Tissue Kits. [15]

MtDNA4977 deletion was detected by using three primers PCR procedure. The Primers sequences as in (Table 1) contain two control regions as positive and negative controls and one deletion region.

| Primer | PCR Product Size (bp) | Sequences of Primer Pairs (5'>3') |
|---|---|---|
| Deletion region | 470 (8164-13611) | Forward: CGG TCA ATG CTC TGA AAT CTG TG<br>Reverse: TCG AGT GCT ATA GGC GCT TGT C |
| Positive control | 1029 (6251-7280) | Forward: TAT AGT GGA GGC CGG AGC AG<br>Reverse: GAA TGA GCC TAC AGA TGA TA |
| Negative control | 564 (9981-10545) | Forward: TGA GGG TCT TAC TCT TTT AGT<br>Reverse: GGT GTG AGC GAT ATA CTA GT |

**Figure (1): polymerase chain reaction primers used in the study.**

PCR reactions were carried out using Maxime PCR PreMix (i-Taq; for 20µl rxn) - 96 tubes.[20]

The PCR reaction mixture (20µl total volume) contains 1 ng total cellular DNA, 2.5mM dNTPs, 10 pmoles of each primer, 2.5 U Tag polymerase (5U/µl).

After denaturation at 94°C for 2 min, the reaction mixture was cycled 35 times at 94°C for 20 s, 55°C for 10 s and 72°C for 20 s, finally extended at 72°C for 5 min. PCR products were analyzed by 1.2 % agarose gel electrophoresis at 100 V (the buffer fluid was 1 × TAE buffer). The electrophoresis gels were observed and photographed by gel documentation system under ultraviolet light.

Statistical analysis was performed by the SPSS version 20.0. The results were tested by chi-square. P value lower than 0.05 was considered statistically significant. We used frequency and percentage to show descriptive data of mutation and cross tabulation for correlating the mutation with the factor of sex, age and type of tumor.

## 3. RESULTS

**Table (1): Distribution of age, gender, oral lesions among study cases.**

| **Parameters** | | | **Numbers** | |
|---|---|---|---|---|
| Age (years) | Minimum | | 10 | |
| | Maximum | | 90 | |
| | Mean±SD | | 47±18 | |
| **Frequencies** | | | **N (%)** | |
| Age groups (Years) | <40 | | 46 (40.0) | |
| | 40-60 | | 42 (36.5) | |
| | >60 | | 27 (23.5) | |
| Gender | Male | | 61 (53.0) | |
| | Female | | 54 (47.0) | |
| Diagnosis | Malignancy | Squamous cell carcinoma | 55 (67.9) | 81 (70.4) |
| | | Adencarcinoma | 15 (18.5) | |
| | | Mucoepidermoid carcinoma | 5 (6.2) | |
| | | Ameblastoma | 4 (4.9) | |
| | | Lymphoma | 2 (2.5) | |
| | Benign tumor | | 18 (15.7) | |
| | Inflammation | | 12 (10.4) | |
| | Others | | 4 (3.5) | |
| mtDNA4977 deletion | Male | | 1 (0.9) | |
| | Female | | 3 (2.6) | |

The study investigated 115 subjects, their ages range from 10 to 90 with a mean age of 47±18 years. Among these 115 participants, 61 (53.0%) male and 54 (47.0%) female, as shown in Table (1).When evidence of malignancy correlated with gender variable, the study showed 42 (68.9%) of male and 39 (72.2%) of female having malignant tumor, The distribution of malignancy regarding age groups, among 46 individuals at age group < 40 years there were 27 cases constitute 58.7%. of 42 individuals at age group 40 – < 60 years there were 29 malignant cases constitute 69%. and 92.6% of population at 60 years or more with malignancy.

Of the 115 oral lesion patients, 4 (3.5%) showed 4,977 -bp deletion. When characteristics and risk factors including age, sex, tumor cell differentiation status analyzed cross tabulating to 4,977 -bp deletion, the study showed 3(75%) were female and 1 (25%) was male as in table.[1]

When the 4,977 -bp deletion (figure 2) correlated to Histopathological feature, all the 4,977 -bp deletion {4 cases constitute (100%)} occurred among population having malignant

lesions, and when classified as sub malignancy, 3 (75%) of mutation among cases of squamous cell carcinoma, table-2.

The 4,977 bp deletion cases distributed within population age group as 1 case at age group < 40 years, 2 cases at age group 40-<60 Years and 1 case at age group 60 years or more, as shown in table-3.

**Table (2): Relation between Mutation and type of lesions Count**

| | | Mutation Result | | Total |
|---|---|---|---|---|
| | | No mutation | Mutation | |
| Type of Lesion | Malignancy | 77 | 4 | 81 |
| | Benign tumor | 18 | 0 | 18 |
| | Inflammation | 12 | 0 | 12 |
| | Others | 4 | 0 | 4 |
| Total | | 111 | 4 | 115 |

**Table (3): Distribution of Mutation among study cases regarding Age groups**

| Age groups | Mutation Result | | Total |
|---|---|---|---|
| | No mutation | Mutation | |
| < 40 years | 45 | 1 | 46 |
| 40-<60 Years | 40 | 2 | 42 |
| 60 years or more | 26 | 1 | 27 |
| Total | 111 | 4 | 115 |

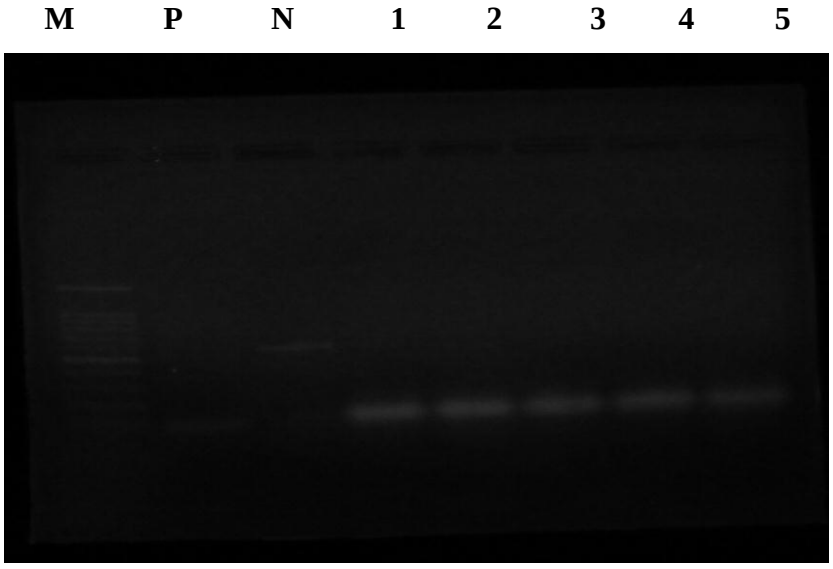


**Figure (2) Agarose gel electrophoresis of PCR shows (P) positive control and (N) negative control. M= Ladder. 1,2,3,4 and 5 samples with no mutation**

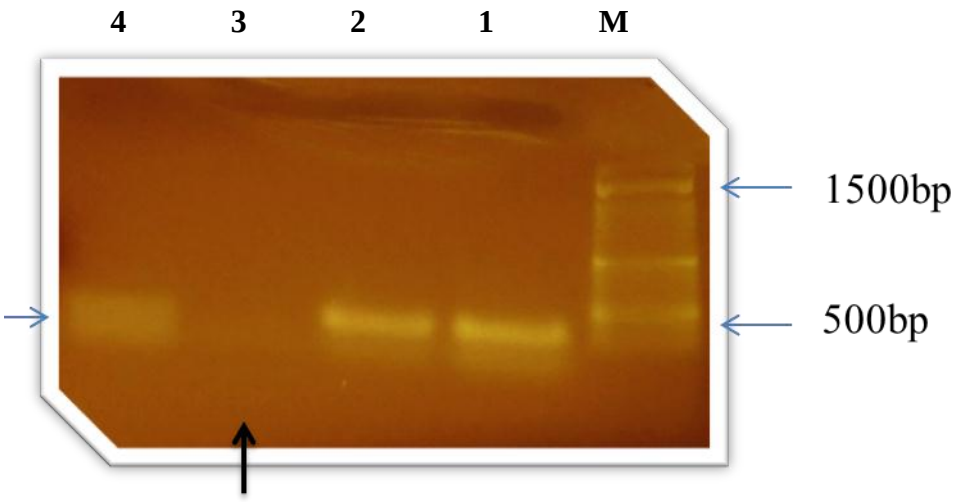


**Figure (3) Agarose gel electrophoresis of PCR products of mitochondrial DNA. Lane M, 50 bp Plus DNA Ladder; Sample 1 , 2 and 4 did not show mutation. Mentioned sample 3 show mutation**

## 4. DISCUSSION

This study is a hospital-based retrospective design. It was conducted in Khartoum, Sudan during the period from 2013 to 2014. The aim of the study was to investigate the frequency of mitochondrial DNA (D- loop) mutations in oral lesions and to determine whether the genetic alteration has a role in oral carcinogenesis. The study investigated 115 subjects, their ages range from 10 to 90 with a mean age of 47 years. Among those 115 participants, 61 (53.0%) were male and 54(47.0%) were female. Based on the laboratory diagnosis the study showed different patterns of lesions including normal, inflammatory, pre malignant and malignant lesions. Of 115 subjects, 81 (70.4%) classified as having malignant lesions, 18 (15.7%) benign lesions, 12 (10.4%) inflammation and 4 (3.5%) of cases as other normal conditions. These prevalence and distribution figures indicate that not all oral lesions are malignant, that may due to early diagnosis of some cases.

In this study majority of the (81) malignant cases was Squamous cell carcinoma constitute 67.9%, followed by Adenocarcinoma 18.5%, Mucoepidermoid carcinoma 6.2%, Ameblastoma 4.9% and Lymphoma 2.5%. These findings agreed with the reports by Amit and David, they found that oral squamous cell carcinoma (OSCC) poses a major health risk and is one of the leading causes of mortality. Distribution of the incidence of OSCC varies across the world with south-central Asia and Africa leading, followed by eastern and central Europe, and to a lesser extent Australia, Japan, and the United States.[4]

Referring to age, in this study the median age distribution of OC was not different from what was reported in the literature, there was significant correlation (P-value =0.032) between the percent of malignancy and age, there was positive relationship between poor prognosis or tumor aggressiveness and age.

MtDNA alterations within the highly variable D-loop control region have been reported as a frequent event in cervical cancer, breast cancer, gastric carcinoma, colorectal cancer, hepatocellular cancer, lung cancer, and renal cell carcinoma in the forms of point mutations, insertions, deletions, and mitochondrial microsatellite instability (mtMSI).[16]

Referring to the previous studies there were a lot of studies demonstrated significant correlation between mitochondrial DNA (D-loop) mutation and cancer, but not included the lesions of oral cavity.

Regarding oral lesions, the percentage of D loop mutation in the present study was comparatively low and could not indicate strong correlation between lesions and mutation, of the 115 oral lesion patients, there were 4 (3.5%) showed 4,977 -bp deletion.

Previous study by Chen lee said: Although the 4,977 bp deletion of mtDNA has been frequently detected in various types of cancers, the incidence and amount of the 4,977 bp-deleted mtDNA are significantly lower in the malignant tissues as compared with the paired normal tissues of cancer patients.[15]

H Brown reported about of 109 patients in HNSCC by[17], they found no correlation between D-loop mutations and prognosis or response to chemotherapy.[18]

It has been stated that, the majority of mtDNA mutations observed in tumors might not be involved in carcinogenesis[19] while others mentioned that mtDNA mutations might have a role in the development of cancer but not in the progression.[20]

These findings support a report indicating that the 4977 bp deletion is present at similar frequency in both normal and tumor tissues. Therefore, mtDNA4977 deletion, which is not specific to cancer, may reflect the environmental and aging-process influences operative during cancer progression.[21]

In the present study, when the 4,977 -bp deletion correlated to histopathological feature, all the 4,977 -bp deletion {4 cases constitute (100%)} occurred among population having malignant lesions, and when classified as sub malignancy, 3 (75%) of mutation occurred among cases of squamous cell carcinoma this may not confirm the relation between 4977 bp deletion and type of malignancy particularly (SCC), because the majority of the malignant cases was squamous cell carcinoma which constitute 67.9%. These findings may agree with a report of SA Liu et al[22] indictated no significant difference between the mutation group and non-mutation group in age, gender, primary site, histological features, pathological stage, smoking, betel quid chewing, alcohol consumption, and postoperative radiotherapy.

Our study concluded that, not all oral lesions undergo surgical operations with poor prognosis and there was no obvious correlation between 4.977 bp deletion and prognostic indicators among Sudanese with oral lesions, therefore other factors such as environmental risk factors, habitual, biological factors and nuclear genetic alterations may play major roles in oral carcinogenesis more than mitochondrial DNA mutations.

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