International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.4, pp 573-579, 2014-2015

# Evaluation of Mitochondrial DNA Content in Saliva of Oral Squamous Cell Carcinoma and Leukoplakia as Non-invasive Biomarker

**Lana sayall[1]*,Ammar mashlah[1], Issam Kassem[2]**

**[1]Damascus University, Faculty of Dentistry, Department of Oral Medicine.**
**[2]National Commission Of Biotechnology Center. Syria.**

**Abstract:** Mitochondria are key organelles in eukaryotic cells principally responsible for multiple cellular functions. The identification of reduced or increasedmtDNA copy number has been increasingly reported inhuman cancers.
Oral squamous cell carcinoma is the most common malignant epithelial neoplasm affecting the oral cavity. They may be preceded by oral potential malignant lesions(OPMLs) that may last for many years. Tumor progression in epithelia has been classified as normal, hyperplastic (non-dysplastic), dysplastic, carcinoma in situ and invasive carcinoma. Malignant transformation of cells into cancer arises due to long term accumulation of genetic and epigenetic events. Almost half of the patients with OSCC worldwide are diagnosed at advanced stages (III, IV). Thus, early diagnosis (at stages I/II) and referral are the key to a better prognosis, reducing the mortality from the disease.We study the present state of our knowledge regarding evaluation of mtDNA quantity levelsand whether we can utilize this measurement as a diagnosticbiomarker for identifying genetic predisposing in saliva of oral squamous cell carcinoma and potential malignant lesion, We describe here a rapid, simple and accurate quantitative real-time PCR method for direct synchronized analysis of mitochondrial (mtDNA) DNA in saliva samples by using tag man probe and primers.
**Keywords:** Mitochondrial DNA, Oral Squamous Cell Carcinoma, Leukoplakia, Non-invasive Biomarker.

## Introduction:

Mitochondria are unique cytoplasmic organelles of the eukaryotic system that play a central role in regulating metabolism, cellular energy production ,apoptosis[1], cell motility, transport, cell proliferation[2], and calcium homeostasisand are involved in free radical production[3]. Mitochondria have their own genome, namely mitochondrial DNA (mtDNA), which possesses hundreds to thousands of copies per mammalian cell. Human mtDNA isa16,569 bp, maternally inherited, closed circular double-stranded molecule[4] encoding,2 rRNAs, 22 tRNAs demanded for mitochondrial protein synthesis,13 proteins(total of 37gens) necessary for oxidative phosphorylation,[5,6]and displacement loop (D-Loop) which regulatesmtDNA replication and transcription[3].

mtDNA may independently duplicate and does not coincide with nDNA[5]MtDNA is highly susceptible to damage by environmental carcinogens because ofthe absence of protective histones, lack of introns, limited DNA repair capacity , and its closeness to high levels of endogenous reactive oxygen species (ROS) in the mitochondrial inner membrane as a by-product of the oxidative phosphorylation system [3,7,8].

The effect of mtDNA in carcinogenesis or progression to malignancy can be achieved through a number of changes in mtDNA.Point mutation or length instability of displacement loop and Coding region deletion or short deletion of mitochondria have been found involved in the breast, colorectal, gastric, thyroid

cancer and head and neck. Furthermore, specific changes in the mtDNA copy number[1,9]have been increasingly observed in human cancers and their premalignant counterparts and have been proposed as significant oncological biomarkers[10].

Oral cancers are malignant neoplasms that attack the structures or tissues of the mouth[11]. However, this term tends to be used alternately with oral squamous cell carcinoma (OSCC), which representsthe most frequent of all oral cancers. It is estimated at 96% of all cancers of the oral cavity[12,15].It is known that a significant ratio (up to 62%) of OSCCs come from clinically recognizable precursor oral potential malignant lesions (OPMLs), such as leukoplakia[18,19].

Oral leukoplakia is a lesion that presents as A white plaque in the oral mucosa[19,20] that cannot be characterized clinically or histopathological as any other disease[21,23].Leukoplakia lesions are classified clinically(homogeneous or non-homogeneous)[24]andhistologically,which may undergo sequential pathological changes fromhyperplasia, mild dysplasia,severe dysplasia or carcinoma in situ and finally invasive carcinoma[13,25,27]. The presence of epithelial dysplasia is the strongest predictive parameter [28]associated with an increased risk of malignant transformation of up to 31%[17]and the absence of dysplasia does not allow the clinician to consider the lesion at low risk[28], for that dysplasia grading is not an exact predictor. Most OPMD regress or remain unchanged and dysplasia grading has been criticized as subjective, inadequate for clinical management, and poorly reproducible [29]. However, clinical and pathological characteristics have limited prognostic value for predicting which leukoplakia will proceed to malignancy[17] There is a need for better predictive biomarkers of transformation to improve the management of patients with oral cancer[10]. Genetic biomarkers may be currently useful to identify lesions at a risk assessment for malignant transformation[17]. Our study explored the evaluation of mtDNA copy number change in bodily fluids (saliva) of OSCC and leukoplakia and whether we can utilize it as a diagnostic molecular biomarker that may have use in evaluating the malignant transformation ,To test these hypotheses, we did quantitative PCR for calculating the relative ratio of the quantity of a given mtDNA-encoded gene to that of a nDNA-encoded gene of saliva samples obtained from 17 OSCC and 21 leukoplakia and a control group of 20 participants without oral cancer.

## Materials and Methods:

### Sample Procurement:

Damascus university approval was obtained to perform studies at the Laboratory of National Commission for Biotechnology and to acquire a salivary sample of OSCC from Damascus Hospital and AlmohsaaHospital and to acquire aleukoplakia salivary sample from theFaculty of Dentistry.

### Sample selection

The study sample was defined clinically by suspicion of a risk lesion and, if no risk had been considered clinically, by pathologic diagnosis of dysplasia. The sample was designed to include all submitted biopsy specimens for which thePathology report would be expected to provide information on the possible risk of malignant transformation, The histologic diagnosis of Oral scc and $OPML_S$ was made and agreed upon by one senior pathologists at the Department of Pathology (Faculty of Dentistry) based on World Health Organization (WHO) guidelines.

### Subject:

The saliva samples used in this study were collected in a time period from 2013-2014. The patients involved in the study (n = 58) were divided into 3 groups: 1) OSCC group (n = 17); 2) leukoplakia lesion group (n = 21) and 3) a healthy control group (n = 20). Written consent forms were collected from all patients who were involved in this study. The institutional ethics committee approved the protocol for the study. Patients' data (age, tobacco and alcohol consumption, drug taking, systemic disease, tumor size, lymph node involvement, extent of metastasis, and histopathological report) were obtained from the pathological reports. We also identified allindividuals with a history of any other systemic disorder, individuals suffering from acute inflammatory conditions of the oral cavity (e.g. dental abscess, pericoronitis), patients receiving chemotherapy/radiotherapy, individuals taking drugs that induce hyposalivation (e.g. anticholinergics, antihistaminics, antihypertensives and beta adrenergic blockers) and individuals using secretogoguesand excluded them from the study. All saliva samples were taken before any surgical interventions or therapeutic treatments.

**Sample collection:**

Whole unstimulated saliva was collected from all the subjects. The subjects refrained from eating, drinking, using chewing gum, mints, etc., for at least 2 h prior to the evaluation. We instructed the subject to do two times of gargling for 15 seconds with 60 mL of de-ionized, purified water and to throw out The 60 ml gargle samples. Samples were obtained by requesting the subjects to swallow first, tilt their head forward and expel the saliva (500ml) into 2-ml sterile plastic vials for 10 min.

**Processing of saliva samples:**

The samples (500ml) were centrifuged at 1600 rpm for 10 minutes. The supernatant was discarded and the cell pellet (200ml) was retained and stored at −80 °C until the biochemical analysis.

**DNA extraction:**

Salivary DNA was extracted from cell pellets using a standard laboratory invisorb spin forensic kit (stratec molecular GmbH). Briefly, the saliva sample was incubated at 56°C for 20 min under continuous shaking in 100 ml lysis buffer M, 100 ml $ddH_2o$, carrier RNA and 10 ml proteinase K. The sample was then transferred to a new RTA spin filter tube to which 200 ml binding buffer B6 was added, centrifuged for 1min at 12,000 rpm. A washing buffer was added and centrifuged for 30sec at 12,000 rpm. Then we added elution buffer D and centrifuged the sample for 1 min at 8,000 rpm and stored the eluted DNA at – 80 °C.

**Standard preparation:**

Genomic DNA was extracted from whole-blood samples viaGeneJET,Genomic(DNA)Purification Kit (Thermoscientific,(EU)Lithuania), **Amplification** was carried out in 0.2 ml tubes containing: 12.5 Dream Taq PCR Master Mix(2x) (Thermo Scientific, (EU) Lithuania), 1 µl each primer (0.5µM) (VbCBiotech,Vienna) (Table 1), 2 µl purified DNA and 8.5ml PCR water to make a volume of 25 µl. Thermal cycling conditions were as follows:initial denaturation and Taq polymerase activation at 95 °C for 3 minutes,followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds,72°C for 30second and final extension 72°C for 7 minutes. The PCR products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide staining along with 50bp and 100bp ladders (Fermentase, Germany).We purified the PCR product via Exosape(Affymetrix, USA).

**DNA quantification:**

A nanodrop spectrophotometer was used, according to the manufacturer's instructions, to measure the total amount of extracted DNA.Theconversion ofthe unitng/µI tocopies/µl was done by exploiting the software application available at this site http://www.endmemo.com/bio/dnacopynum.php. We performed dilution standards containing 10-fold a dilution series($10^8$,$10^7$,$10^6$,$10^5$,$10^4$,$10^3$,$10^2$,$10^1$copies/µl). All dilution standards were kept at 4 °C for the duration of the study.

**Real time qPCR:**

MtDNA content was assessed by quantification of a uniquemitochondrial fragment relative to a single copy region of the nuclearb2M using a taqman assay. A 65-bp fragment of MtDNA was amplified using the primers: hmito-F,hmito-R, and hmitoP were used as the hybridization probe, containing the FAM (6-carboxy fluorescein) as a fluorescentreporter dye and NFQ as a quencher dye at the 3` end. Nuclear contentwas quantified by targeting a unique region of the B2 M gene using the JOE/TAMRA™ Probes, primer limited(VbCBiotech,Vienna). For each 20 µl reaction, 3 µl of DNA was amplified containing 2µl of eachmtDNA primer (0.4µm), 2 µl (1µm) for nDNA, 2µl(0.4µm) probe, 3 ml nuclease freewater and 10 master mix(Kapabiosystems, USA). The real-time PCR conditions consisted of initial denaturation and Taq polymerase activation at 95°C for 3 minutes followed by 40 cycles mtDNA-50cycles nDNA of 95°C for 3 seconds, 60°C mtDNA-55°C nDNA for 20 seconds, and 72°C for 10second.Somemeasurement was repeated in triplicate and a non-template control and a positive controlwere included in each experiment. mtDNA to nuclear DNA (nDNA) ratios were calculated by dividing themtDNA signal for each gene by the corresponding nuclear signal.

**Table1. Primers/probes used in the study.**

| Product size (bp) | Oligonucleotide sequence | Primer/probe | Gene accession no |
|---|---|---|---|
| 65 | CTTCTGGCCACAGCACTTAAAC<br>GCTGGTGTTAGGGTTCTTTGTTTT<br>FAM-ATCTCTGCCAAACCCC | hmito F<br>hmMito R<br>hmito P | Human mitochondrial genome NC_012920 |
| 95 | GCTGGGTAGCTCTAAACAATGTATTCA<br>CCATGTACTAACAAATGTCTAAAATGGT<br>JEO-CAGCAGCCTATTCTGC | hB2M F<br>hB2M R<br>hB2M P | Humanb2M Accession number M17987 |

**Statistical Analysis:**

The copy number of mtDNA amongoscc, leukoplakia, control subject saliva specimens were compared by the parametric test ANOVA,Differences in host characteristics(Table2) gender, age, oral health, smoking status, between patients and control subjects were assessed via spearman,Pearson correlation coefficientsand Student's *t*-test for continuous variables, Within oscc patients and leukoplakia, mtDNA content by tumor stage and histopathological characteristicsof leukoplakia was also compared, All statistical analyses were performed usingthe SPSS statistical package (version 11.5, Chicago, IL).P values < 0.05 were considered significant.

**Table2.Distribution of selected host characteristic:**

| variable | Cases oscc no=17(28.3%) | Leukoplakia=21(36.7%) | Control=20(35.0%) |
|---|---|---|---|
| **Sex** | | | |
| Male | 11(64.7) | 16(77.3) | 12(61.9) |
| femal | 6 (35.3) | 5 (22.7) | 8 (38.1) |
| **Age** | | | |
| Std± | 16.9 | 9.7 | 15.7 |
| Mean | 52.8 | 46.3 | 43.7 |
| Range | 30-82 | 25-69 | 24-77 |
| **Oral health** | | | |
| Good | 3(17.6) | 0(0) | 3(14.3) |
| Mild | 2(11.8) | 13(59.1) | 6(28.6) |
| Bad | 12(70.6) | 9(40.9) | 12(57.1) |
| **Smoking** | | | |
| Never | 5(29.4) | 6(27.3) | 9(42.9) |
| current | 12(70.6) | 16(72.7) | 12(57.1) |

## Results:

We determined the ratio of copy number of mtDNA to copy number of β2 microglobulin (β*2M*) gene nDNA in cells from the saliva of 58 individuals by including the sample in the real time quantitative PCR analysis. The patients and controls were similar in distributions with regard to age, sex, oral health and smoking status(match case- control). The relative mean mtDNAcopy number was significantly higherin leukoplakia patients (6900.40)than inoscc and controls (680.51, 382.16) respectively. However,there were no statistically significant differences between the cases(oscc,leukoplakia) and controls subjects(P = 0.435); also there were no statistically significant differences in terms of age(P = 0.985), gender (oscc P = 0.616, leukoplakia( P = 0.068), control (P = 0.724), oral health (P = 0.460),smoking status(osccP = 0.749), leukoplakia( P = 0.534), control (P = 0.395)as presented in (Table 3).

To assess the alteration of mtDNA content on tumor stage and histopathological change in leukoplakia, we examined the relative content of mtDNA/nDNA insaliva from the early stage (stages I and II) versus advanced stage (stages III and IV) OSCC and histopathological characteristics of leukoplakia. The results

showed thatthere were no statistically significant differences in tumor stage (P = 0.306), and histopathological characteristics of leukoplakia (P = 0.324) Table 4.

**Table3.**mtDNA copy number by host characteristics of cases and control subject**:**

| variable | oscc | leukoplakia | control | P value |
|---|---|---|---|---|
| **Group(mean)** | 680.51 | 6900.40 | 382.16 | 0.435 |
| **Sex(mean)** | | | | |
| Male | 842.03 | 303.85 | 413.02 | |
| female | 384.38 | 28009.37 | 324.86 | |
| **P value** | 0.616 | 0.068 | 0.724 | |
| **Age(P value)** | 0.157 | 0.947 | 0.072 | 0.985 |
| **Oral health(mean)** | 0.843 | 0.266 | 0.969 | 0.460 |
| **Smoking status(mean)** | | | | |
| Never | 463.56 | 292.72 | 504.88 | |
| current | 770.90 | 9543.48 | 300.35 | |
| **P value** | 0.749 | 0.534 | 0.395 | |

**Table4.Overall saliva mtDNA copy number by histopathological characteristics**

| Tumor(TNM stage) | Cases no% | P value |
|---|---|---|
| 0 | 3(17.6) | 0.306 |
| II | 1(5.9) | |
| III | 4(23.5) | |
| IVA | 4(23.5) | |
| IVB | 1(5.9) | |
| IVC | 4(23.5) | |
| leukoplakia | | |
| Dysplasia | 11(54.5) | 0.324 |
| Hyperplasia | 10(45.5) | |

## Discussion:

Mitochondria is essentially involved in many important physiological processes and cellular bioenergy including metabolism, apoptosis, signaling, cellcycle, and differentiation, Unlike nuclear DNA, mtDNA is present at a consistently high level in normal cells, and the mitochondrial genome lacks protective histones andintrons and is involved in free radical production. As a consequence, the mutation rate of mtDNA is substantially greater than that of nuclear genomic DNAand copy number variations have been frequently reported in various human cancers. Previous studies showed that the increased mtDNA in human cells have long been suspected as contributer to carcinogenesis including oscc cancer[9]. However, the prognostic value of copy number variations of mtDNA in oscc cancer patients remains to be explored. In this study, we investigated relative mtDNA/nDNA copy number (match case- control) in saliva of oscc, leukoplakia and normal subject (control subjects) by using real-time quantitative PCR method. Our data showed that relative mean mtDNA content showed no significant differences in oscc, leukoplakia,and control subjects. This does not correspond with the(Jiang et al, Mondal et al, Masayesva et al, Kim et al)[9,30,31,32]studies because they had significant difference between cancer and control subjects. Moreover, we did not find associations of mtDNA content with gender, age, oral health, smoking status, tumor stages and histological differentiation of leukoplakia, Our data corresponds with (Mondal et al)[30] studies that showed no relationship between mtDNA content and age of patient, but also our study does not correspond with the(Jiang et al, Kim et al)[9,32] studies because mtDNA content was identified to be altered by aging, tumor stage and histopathological differentiation of leukoplakia. Moreover our study does not correspond with the(Jiang et al, Mondal et al, Masayesva et al, Kim et al)[9,30,31,32] studies, which showed increased levels of mtDNA in smokers as compared with non-smokers. According to this study, diagnostic results require a surgical biopsy and this means a more accurate and golden standardin judging the nature of suspicious lesions clinically.

In conclusion, we investigated relative mtDNA/nDNA content in saliva of oscc, leukoplakia, and control subjects, demonstrated that no statistically significant differences of m DNA content exist between patient and control subjects ,and did not findan association between mtDNA and cigarette smoking, age, gender, oral health or tumor stage. Therefore, the mtDNA copy number content method is an inaccurate method for diagnosing these lesions.

## References


1. Kulawiec M.K., Owens M.,and Singh K.K., Cancer cell mitochondria confer apoptosis resistance and promote metastasis, Cancer Biol Ther., 2009,8, 1378-85.
2. Wei-Wen Jiang., E.R., ElizabethMambo., and Marianna Zahurak., DecreasedMitochondrial DNAContent in Posttreatment Salivary Rinses from Head and Neck Cancer Patients, Clin Cancer Res, 200612, 1564-1569.
3. Yu. M., Generation, function and diagnostic value of mitochondrial DNA copy number alterations in human cancers, Life Sci, 2011,89, 65-71.
4. Yu. M., Circulating cell-free mitochondrial DNA as a novel cancer biomarker: opportunities and challenges, Mitochondrial DNA, 2012,23, 329-32.
5. Radpour R., Current understanding of mitochondrial DNA in breast cancer, Breast J, 2009, 15 505-9.
6. Chatre L., and Ricchetti M., Prevalent coordination of mitochondrial DNA transcription and initiation of replication with the cell cycle, Nucleic Acids Res, 2013. 41, 3068-78.
7. Dasgupta S., Mitochondrial DNA mutation in normal margins and tumors of recurrent head and neck squamous cell carcinoma patients,Cancer Prev Res (Phila), 2010, 3, 1205-11.
8. Chatterjee A., Dasgupta S., and Sidransky D., Mitochondrial subversion in cancer, Cancer Prev Res (Phila), 2011,4, 638-54.
9. Jiang W.W., Increased mitochondrial DNA content in saliva associated with head and neck cancer, Clin Cancer Res, 2005,11, 2486-91.
10. Challen C., Mitochondrial DNA mutations in head and neck cancer are infrequent and lack prognostic utility, Br J Cancer, 2011,104, 1319-24.
11. Johnson N.W., Jayasekara P., and Amarasinghe A.A., Squamous cell carcinoma and precursor lesions of the oral cavity: epidemiology and aetiology, Periodontol 2000, 2011,57, 19-37.
12. Mehrotra R., and Yadav S., Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations, Indian J Cancer, 2006, 43, 60-6.
13. Messadi D.V., Diagnostic aids for detection of oral precancerous conditions, Int J Oral Sci, 2013,5, 59-65.
14. Omar E.A., The Outline of Prognosis and New Advances in Diagnosis of Oral Squamous Cell Carcinoma (OSCC): Review of the Literature, Journal of Oral Oncology, 2013,2013, 13.
15. Markopoulos A.K., Current aspects on oral squamous cell carcinoma, Open Dent J, 2012,6, 126-30.
16. Puneet Kumar R.D., Prince Kumar., and Harkanwal P., Imperative role of early diagnosis in precancerous lesions: Oldideas, new findings, yet more questions,Clinical Cancer Investigation Journal, 2012,1, 185.
17. Cervigne N.K., *R*ecurrent genomic alterations in sequential progressive leukoplakia and oral cancer: drivers of oral tumorigenesis, Hum Mol Genet, 2014,23, 2618-28.
18. ThomasV., Mucosal disorders with oral epithelial dysplasia risk--development of a simple screening tool for general health care setting, Oral Oncol, 2012,48,671-7.
19. van der Waal I., Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management ,Oral Oncol, 2009,45, 317-23.
20. Liu W., Oral cancer development in patients with leukoplakia--clinicopathological factors affecting outcome,PLoS One, 2012, 7, 34773.
21. Lemmer L.F., a.J., Oral Leukoplakia as It Relates to HPV Infection: A Review, International Journal of Dentistry, 2012,2012, 7.
22. Parlatescu Ioanina o.S., Mihai Lelia., TREATMENT APPROACH OF ORAL LEUKOPLAKIA, REVIEW OFLITERATURE, 2013, 8, 39-43.
23. Boy S.C., Leukoplakia and erythroplakia of the oral mucosa--a brief overview, SADJ, 2012,67 558-60.
24. Anand Kumar L.C., James A., Cyrus J., and Darryl Coombes D.G., How should we manage oral leukoplakia, British Journal of Oral and Maxillofacial Surgery, 2012,2013, 377-383.
25. Ramasubramanian A., Immunohistochemical evaluation of oral epithelial dysplasia using cyclin-D1, p27 and p63 expression as predictors of malignant transformation, J Nat Sci Biol Med, 2013,4, 349-58.

26. McCullough M.J., Prasad G., and Farah C.S., Oral mucosal malignancy and potentially malignant lesions: an update on the epidemiology, risk factors, diagnosis and management , Aust Dent J, 2010,55, 61-5.
27. Speight P.M., Update on oral epithelial dysplasia and progression to cancer, Head Neck Pathol, 2007,1 61-6.
28. Lucio M., Between-lesion discrepancies in terms of dysplasia, cell turnover and diagnosis in patients with multiple potentially malignant oral lesions, Open Dent J, 2013, 7, 169-74.
29. SperandioM., Predictive value of dysplasia grading and DNA ploidy in malignant transformation of oral potentially malignant disorders, Cancer Prev Res (Phila), 2013,6, 822-31.
30. Mondal R., Mitochondrial DNA Copy Number and Risk of Oral Cancer: A Report from Northeast India, plos, 2013, 8,1-8.
31. Masayesva B.G., Mitochondrial DNA Content Increase in Response to Cigarette Smoking, cancer Epidemiol Biomarkers Prevention, 2006, 15, 19-24.
32. Kim M.M., Mitochondrial DNA Quantity Increases with Histopathologic Grad in Premalgnant and Malignant Head and Neck lesions, Clinical Cancer Research, 2004,10, 8512-8515.

*****