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Research Article

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Increased mitochondrial DNA content in salivary leukoplakia patients associated with aging

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ABSTRACT

Mitochondria are key organelles in eukaryotic cells principally responsible for multiple cellular functions. Mitochondria play an important role in programmed cell death and regulate a multitude of different metabolic and signaling pathways. The primary function of mitochondria is to produce ATP through the process of oxidative phosphorylation, which is conducted by the four RC complexes (complexes I–IV) and the ATP synthase (complex V), all located in the inner mitochondrial membrane. Leukoplakia was most recently defined as a potentially malignant disorder with recognizable white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer. Aging is thought to be a degenerative process caused by accumulated damage that leads to cellular dysfunction, tissue failure, and death. A decline in mitochondrial function plays a key role in the aging process and increases the incidence of age-related disorders. We study the present state of our knowledge regarding the role of mtDNA copy number change in bodily fluids (saliva) of leukoplakia subjects in aging, 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: mDNA, Age, leukoplakia, Saliva, q PCR, MFRTA.

INTRODUCTION

Mitochondria are unique cytoplasmic organelles of the eukaryotic system that play a central role in regulating metabolism, cellular energy production ,apoptosis[1, 2,3], cell motility, transport, cell proliferation[4], and calcium homeostasis and are involved in free radical production[5, 6]. Mitochondria have their own genome, namely mitochondrial DNA (mtDNA), which possesses hundreds to thousands of copies per mammalian cell. Human mtDNA is a16,569 bp, maternally inherited, closed circular double-stranded molecule[6] encoding,2 rRNAs, 22 tRNAs demanded for mitochondrial protein synthesis,13proteins(total of 37gens) necessary for oxidative phosphorylation,[7, 8]and displacement loop (D-Loop)which regulates mtDNA replication and transcription[5].

mtDNA may independently duplicate and does not coincide with nDNA[7]. mtDNA is highly susceptible to damage by environmental carcinogens because of the 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[5, 9, 10].

Oral leukoplakia is a lesion that presents as A white plaque in the oral mucosa that cannot be characterized clinically or histopathological as any other disease[11, 12]

Age-related changes in mitochondria are associated with decline in mitochondrial function. With advanced age, mitochondrial DNA volume, integrity and functionality decrease due to oxidative damage induced and accumulation of mutations by reactive oxygen species (ROS)[13]. In aged subjects, mitochondria are characterized by impaired function such as lowered oxidative capacity, decreased ATP production, reduced oxidative phosphorylation, increase in ROS generation, and diminished antioxidant defense.[14, 15]

A number of aging theories have been proposed, and the mitochondrial free radical theory of aging (MFRTA) has taken center stage for several decades. According to this theory, mitochondria play a crucial role in mediating and amplifying the oxidative stress that drives the aging process According to this theory, with age, mitochondria accumulate ROS-induced damage and become dysfunctional. The increased ROS production with age because of a decline in mitochondrial function, activity of several ROS-scavenging enzymes declines with age, and mutations of mitochondrial DNA(mtDNA) accumulate during aging, can subsequently lead to a vicious cycle of exponentially increasing levels of mtDNA damage and oxidative stress[16] in the cell which in turn results in a further increase in ROS production.[14, 15]ROS are considered to be unwanted toxic byproducts of aerobic metabolism that induce oxidative damage to various cellular macromolecules due to their high chemical reactivity[17, 18]. The respiratory chain (RC), located in the inner mitochondrial membrane[19], is a main production site of superoxide, an abundant ROS in the cell formed at the level of complexes I and III during electron transport The superoxide anion is converted to hydrogen peroxide by SOD. Although hydrogen peroxide itself is not a free radical, it can be converted to the highly reactive hydroxyl radical in the presence of transition metals through the Fenton reaction[20]. The hydroxyl radical is considered to be the most damaging form of ROS, as it is highly reactive and causes oxidative damage to virtually every molecule type in the cell, including lipids, proteins, and nucleic acids[21, 22].Our study evaluate the relationship betweenmt DNA copy number in bodily fluids (saliva) and aging, To test these hypotheses, we did quantitative PCR for calculating the ratio of the quantity of a given mtDNA-encoded gene to that of a nDNA-encoded gene in saliva samples obtained from 21 subject.

EXPERIMETAL SECTION

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 21 leukoplakia subject from the Faculty of Dentistry.

Subject:
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 all individuals 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 secretogogues and excluded them from the study.

Processing of saliva samples:
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.

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.

Real time qPCR:
MtDNA content was assessed by quantification of a unique mitochondrial fragment relative to a single copy region of the nuclear b2M using a taqman assay. A 65-bp fragment of MtDNA was amplified using the primers Table1: hmito-F, hmito-R, and hmitoP were used as the hybridization probe, containing the FAM (6-carboxy fluorescein) as a fluorescent reporter dye and NFQ as a quencher dye at the 3` end. Nuclear content was 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 2 µl of each mtDNA primer (0.4µm), 2 µl (1µm) fornDNA, 2µl (0.4µm) probe, 3 ml nuclease free water and 10 master mix (Kapabio systems, 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-50 cycles nDNA of 95°C for 3 seconds, 60°C mtDNA-55°C nDNA for 20 seconds, and 72°C for 10 second. Some measurement was repeated in triplicate and a non-template control and a positive control were included in each experiment. mtDNA to nuclear DNA (nDNA) ratios were calculated by dividing the mtDNA signal for each gene by the corresponding nuclear signal.

Table1. Primers/probes used in the study

Gene accession no	Primer/probe	Oligonucleotide sequence	Product size (bp)
Human mitochondrial genome NC_012920	hmito F	CTTCTGGCCACAGCACTTAAAC	65
	hmMito R	GCTGGTGTTAGGGTTCTTTGTTTT	
	hmito P	FAM-ATCTCTGCCAAACCCC	
Human b2M Accession number M17987	hB2M F	GCTGGGTAGCTCTAAACAATGTATTCA	95
	hB2M R	CCATGTACTAACAAATGTCTAAAATGGT	
	hB2M P	JEO-CAGCAGCCTATTCTGC	

Statistical Analysis:
The copy number of mtDNA among 21 leukoplakia salivary specimens(more than 45 years, less than 45 years) were compared by the parametric test T-student, Differences in host characteristics (Table2) such as related of copy number with age subjects were assessed via Pearson correlation coefficients. All statistical analyses were performed using the SPSS statistical package (version 11.5, Chicago, IL).P values < 0.05 were considered significant.

Table2. Distribution of selected host characteristic

variable	More than 45 year	Less than 45 year
Sex	**57.10%**	42.90%
Male	8(66.7)	8(88.9)
female	4 (33.3)	1 (11.1)
Age		
Std±	7.4	6.8
Mean	52.1	38.2
Oral health		
Good	0(0)	0(0)
Mild	6(50.0)	6(66.7)
Bad	6(50.0)	3(33.3)
Smoking		
Never	4(33.3)	2(22.2)
current	8(66.7)	7(77.8)

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 21 leukoplakia subject by including the sample in the real time quantitative PCR analysis.

However, there were statistically significant differences between theAge related with m DNA / nDNA(p =0.043) and m DNA/ n DNA cases in advance age and early age (P = 0.017)as presented in (Table 3,4).

Table3. Show that Age related with m DNA / nDNA: was significantly elevated in mtDNA / nDNA In the saliva with advance age (Pearson correlation coefficients)

Subject	p-value
mtDNA	0.037
nDNA	0.328
mtDNA / nDNA	0.0.43

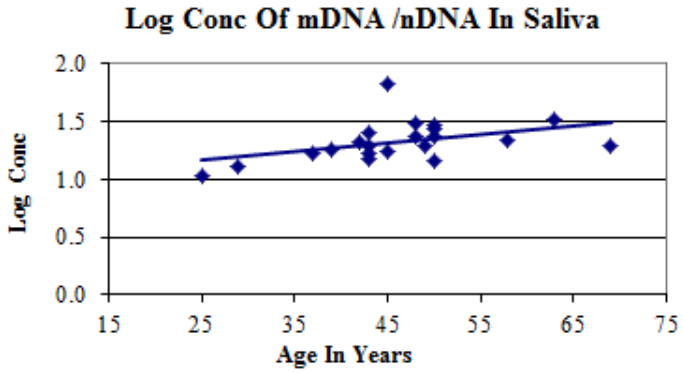

Fig1: Scatter plots of mtDNA / nDNA content in saliva shows that advance Age subject have significantly higher mtDNA content compared with early Age

Table4.shows there were statistically significant differences between theAge related with m DNA / nDNA

subject	mtDNA	nDNA	mtDNA/ nDNA
More than 45 years			
Mean	8.89	6.35	1.4
Sd±	1.35	0.44	0.17
Less than 45 years			
Mean	7.51	6.13	1.23
Sd±	0.86	0.41	0.11
P value	0.015	0.259	0.017

DISCUSSION

Mitochondria is essentially involved in many important physiological processes and cellular bioenergy including metabolism, apoptosis, signaling, cell cycle, and differentiation, Unlike nuclear DNA, mtDNA is present at a consistently high level in normal cells, and the mitochondrial genome lacks protective histones and introns and is involved in free radical production[4, 23].

As a consequence, the mutation rate of mtDNA is substantially greater than that of nuclear genomic DNA and copy number variations have been frequently reported in various ages. It is clear that a role of mitochondria in aging, Age-related changes in mitochondria are associated with decline in mitochondrial function[15]. With advanced age, mitochondrial DNA volume, functionality decrease due to accumulation of mutations and oxidative damage induced by reactive oxygen species (ROS), decreased ATP production, and diminished antioxidant. Previous studies showed that the increased mtDNA in human cells have long been suspected as contribute rages[4, 13, 14]. However, the prognostic value of copy number variations of mtDNA in age subjects remains to be explored. In this study, we investigated relative mtDNA/nDNA copy number in saliva of (more than 45 years, less than 45 years) by using real-time quantitative PCR method. Our data showed that relative mean mtDNA content showed significant differences in more than 45 years and less than 45 years subjects. This does not correspond with the(Mondal et al)[24]studies that showed no relationship between mtDNA content and age of patient, but also our study correspond with the (Jiang et al, Kim et al) studies because mtDNA content was identified to be altered by aging[4, 25].

CONCLUSION

we investigated relative mtDNA/nDNA content in saliva of advance and early age in leukoplakia subjects, demonstrated that statistically significant differences of mtDNA / nDNA content exist between (more than45 and less than45 subjects).Therefore, the mtDNA copy number content method is an accurate method for diagnosing the relationship between the age of leukoplakia subject and mtDNA copy number.

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