1.1. INTRODUCTION

Oral cancer is a common cancer and constitutes a major health problem in developing countries, representing the leading cause of death. (Jemal et al, 2007).With a global significant portion of head and neck malignancies (~90%), oral squamous cell carcinoma (OSCC) poses a major health risk and is one of the leading cause 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 (Amit and David,2009).

Oral cancer is tremendously increasing in Sudan, particularly among men (Ahmed et al,2007). Several studies have linked the etiology of oral cancer in the Sudan to the habit of Toombak use (Tobacco Specific Nitrose amine (TSN)) rich tobacco (Idris et al 2009). No recent study has investigated the association between mitochondrial DNA mutation and OSCC among Sudanese patients.

It is well established that virtually all oral squamous cell carcinomas (OSCCs) are preceded by visible changes in the oral mucosa, usually by way of white (leukoplakia) and red patches (erythroplakia). In addition, there are other inflammatory disorders of the oral mucosa such as lichen planus, submucous fibrosis and perhaps oral fibrosis due to systemic sclerosis that have been associated with an increased risk of OSCC development(Stefano Fedele 2009).

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 (Jemal et al, 2007).

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 malignant oral lesions. Biopsy is an invasive technique with surgical implications, technique limitations for professionals and psychological implications for most patients. In recent decades, in addition to histopathology, cytological and molecular methods have gained importance as rapid and simple methods. Changes occur at the cellular and molecular level before they are seen within the tissue and before clinical changes occur (John et al, 2002).

The cellular changes that characterize cancers are initiated by various degrees of interaction between host factors and exogenous agents. Many factors have been involved in the initiation of cancerous changes. These factors may be direct or indirect intact to the individual life- style. Principal environmental factors implicated in the genesis of oral cancer are tobacco and alcohol (Agarwal et al, 2005).

Identification of high-risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys to reducing the mortality, morbidity and cost of treatment associated with oral cancer.

Mitochondria are intracellular organelles that produce adenosine triphosphate by the coupling of oxidative phosphorylation to respiration, providing a major source of energy to the cell. They are the major source of endogenous reactive oxygen species (ROS) and play an important role in apoptosis. In each cell, several hundreds to thousands of mitochondrial DNA (mtDNA) copies are present. MtDNA is a circular, double-stranded, 16 569 bp DNA molecule that encodes 13 polypeptides that constitute the respiratory enzyme complexes and the 22 transfer RNA and 2 ribosomal RNA genes. It is intronless, lacks a protective histone backbone or other specific DNA-binding proteins, replicates faster than nuclear DNA and does not have proofreading or an efficient DNA repair. mtDNA is typically located near the inner membrane of the mitochondrion and is continually exposed to high levels of ROS and free radicals from the electron transport chain of mitochondria. Owing to these characteristics, mtDNA is particularly susceptible to damage by mutagens and ROS. As a result, the mitochondrial genome exhibits 17 times higher rates of mutation than does the nuclear genome, and DNA damage persists longer in the mitochondrial genome (Duanjun Tan etal, 2008).

Mitochondrial DNA has a limited ability to repair itself. As a result, reactive oxygen species easily damage mitochondrial DNA, causing a buildup of additional somatic mutations. As it does not contain any intron sequences, mutations that do occur would lie in coding or regulatory regions and are potentially biologically significant (Hussain et al, 2010).

LITERATURE REVIEW

2.1 Scientific Background

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 in to the oral cavity. In the cavity the food is masticated, mixed with saliva and formed in to bolus (Gartner, 2001).

Lips and cheeks: The lips are composed of three parts: a cutaneous area, a red area, and sebaceous and sweat glands. The outer surface of the lips is stratified squamous keratinized epithelium (the epithelium of skin). In the red area; the superficial cells of the stratified squamous epithelium contain eleiden, rendering them translucent, and the blood provides the red color. The inner epithelial surface of the lips and cheeks is composed of stratified squamous non- keratinizing type (Bergman, 1996).

Tongue: It is a mass of skeletal muscle covered by mucous membrane; the dorsal tongue is villous, normally exposed surface, Ventral tongue has no villous undersurface; Anterior 2/3 of tongue (oral tongue) is freely mobile portion of tongue that extends anteriorly from line of circumvallate papillae to undersurface of tongue at junction of floor of mouth (Sinnatamby1999).

Floor of mouth: myelohoid muscles, extending from inner surface of lower alveolar ridge to undersurface of tongue.

Gingiva: soft tissue in area of teeth and palate; extends from labial sulcus and buccal sulcus to a cuff of tissue around each tooth.

The palate: is the roof of the mouth. The hard plate is lined by an epithelium of stratified squamous variably keratinizing type, has along

vascular papillae give it a pink color. The soft plate covered by a non-keratinized stratified squamous epithelium.

The teeth: each tooth is formed of (1) a crown, which is the projection part above the gum. (2) A root, which is embedded part of the tooth in the socket of the bone. (3) A neck, which is the junctional zone between the crown and the root.

Gums: the gums have stratified squamous epithelium that is variably keratinized (Bergman, 1996).

The entire oral cavity is lined by stratified squamous epithelium, which varies in thickness and Keratinization according to anatomic and functional sites. The degree of normal surface Keratinization (genetically predetermined) varies, the hard palate, gingiva and dorsal of the tongue are Keratinized. Oropharynx, soft palate, lateral and ventral tongue and floor of mouth are non-keratinized. The buccal and labial mucosa is intermediate between these two extremes (Bibbo Marluce, 1991).

2.2. Oral Disorders

2.2. 1. Congenital and inherited disorders

Hare- lip: This results from failure of fusion of the lower part of the median nasal process with the maxillary process. In more than 80% of cases, this clefting is unilateral and In two-thirds of conditions is left sided, the male : female ratio is 3:2 this may be due to the fact that some cases of hare –lip are inherited in an X-linked recessive pattern. Hare-lip may be isolated defect, but in 50% of cases it is associated with a cleft palate.

Cleft Palate: Cleft palate as an isolated defect is less common than hare-lip, occurring in about 1per 2500 live births; with predominantly in females. Cleft palate is seen also as part the gamut of abnormalities

associated with certain major chromosomal abnormalities, such as trisomies 13 and 18.

Congenital Epulis: This is a mass, present at birth, occurring in the gum. It consists of aggregates of large granular cells covered by well-differentiated squamous epithelium. In haematoxylin and eosin –stained material, these cells cannot be distinguished from those of granular tumor. Unlike the cells of granular tumor, binding of the antibody to S-100 protein does not occur. The lesion occurs almost exclusively in females and is benign.

White Sponge Naevus: This is an autosomal, dominantly inherited disorder. The lesions appear as shaggy with patches on oral mucosa, with frequents involvement of buccal areas. Microscopic examination shows the patches to be composed of well-differentiated but hyperplastic squamous epithelium in which there is marked intercellular oedema.

Hemi facial hypertrophy: In this condition, both hard and soft tissues on one side of the face grow more rapidly than those on the other side. The teeth on the affected side and also half of the tongue may be affected. The latter showing the presence of hypertrophied papillae.

Fordyce's granules: These are small yellowish – white granules situated either on the lateral part of the vermilion of the upper lip or in the buccal mucosa. They represent ectopic development of sebaceous glands. They occur in more than 50% of normal adults, without clinical significance.

Congenital lip Pits: These are small fistulae connecting the surface squamous epithelium with underlying mucous glands. They are usually bilateral and occur on the lower lip. The pits may be associated with hare-lip, cleft palate and genesis of the second premolars, or may be an isolated phenomenon.

Endogenous pigmentation: Melanin pigmentation of the lips, oral mucosa and circumoral skin occurs in several conditions. These include neurofibromatosis, fibrous dysplasia of bone, Addison's disease and haemochromatosis.

2.2.2. Bacterial infections

Acute Ulcerative Gingivitis: This is a painful condition characterized by ulceration of the gum between the teeth and usually caused by amixture of spirochaetes and fusiform bacteria. These organisms flourish in anaerobic environment and thus tissue breakdown promotes their growth.

Actinomycosis: Actinomyces species form a part of the normal mouth flora, their presence alone is not sufficient to cause disease. It is suggested that invasion of the oral tissues by these organisms occurs as the result of abrasions. The lesions present as firm mucosal swelling from which pus containing typical (sulphur granules) exudes.

Tuberculosis: Oral tuberculosis is uncommon. It is usually due to spread from pulmonary lesion. The tongue is most frequently involved, showing irregular ulcers.

Syphilis: Although Syphilis affecting the oral cavity is now rare, but when occurs it may involve the three stages of the disease:

{1} chancres may occur on the lips or tongue.

{2} in the secondary stage the mucosa may be involved.

{3} the gummatous involve all three stages of the disease. Chancres may occur on the lips or tongue. In secondary stage, the mucosa may be involved. The gummatous lesions characteristic of tertiary syphilis may affect the tongue or palate (Woolf, 1998).

2.2. 3. Viral infections

Herpes Simplex virus (HSV): the pathogen is transmitted from person to person, most often by kissing. In adult the primary infection is asymptomatic *(Kumar, 2002).* In the oral cavity HSV type - 1 is usually responsible for inflammatory lesions. These may occur in two forms: Acute Herpetic Gingivostomatitis, commonly seen in children aged 1-3 years. The oral mucosa shows blisters and ulcers, associated with fever and enlargement of draining lymph nodes. Lesions usually heal within 2-3 weeks and Herpes Labialis (Cold Sore), this is the commonest recurrent disease produced by HSV-1. Localized clusters of vesicles occur usually at the junction between the skin and the lip vermilion. Vesicles rupture and painful ulcers form, which heal without scaring. Such lesions may occur repeatedly in the same location.

Herpes Zoster: usually affects the skin, but may involve the oral mucosa. The lesions are usually unilateral and initially take the form of painful vesicles on a reddened mucosa.

Human Papilloma Virus (HPV): The most obvious evidence of HPV infection is the presence of warts. Some lesions show the typical microscopic features of koilocytosis.

Human Immunodeficiency Virus (HIV): The oral manifestations of HIV are due chiefly to decline in cell- mediated immunity, leading to infections and neoplasms. Infection may be bacterial, viral, or fungal. The most common neoplasm to be associated with HIV is Kaposi's sarcoma. In patients with Acquired Immune Deficiency Syndrome (AIDS) who have Kaposi's sarcoma, lesions are present in the oral cavity in about 50%, the palate being the site affected most frequently (Woolf, 1998).

2. 2.4. Fungal infections:

The most important fungus affecting the oral cavity is C.albicans.

Oral candidiasis (thrush): commonly affects neonates and very young infants, although it may also occur in immunosuppressed adults. About 5-10% of neonates are affected, and the infection being acquired during delivery. Discrete white patches involving any part of the oral mucosa, including the tongue, and there may be extensive pseudo- membrane formation consisting of keratinous debris and many yeasts.

Acute Atrophic Candidiasis: painful reddened lesions develop on the tongue, associated with atrophy of the filiform papillae. They may occur as a consequence of prolonged broad –spectrum antibiotic treatment, and also in individuals using steroid containing inhalers for the treatment of asthma. Successful treatment requires removal of any such predisposing factor.

Candida cheilities (inflammation of the lips): This may affect the angles of the mouth or the lip vermilion. It is most likely in association with excessive licking of the lips, or with the use of topical steroids.

Candida leucoplakia: In this condition there are discrete areas of epithelial hyperplasia associated with Candida in the superficial layers. Lesions may occur anywhere in the oral or lingual mucosa. Patients complain either of pain or of a burning sensation in the affected areas. Some degree of cellular atypia may be seen in the hyperplastic areas and carcinoma has been reported as a late complication in some instances (Cawson and Odell, 1998).

However, a more recently proposed classification of oral candidiasis distinguishes primary oral candidiasis, where the condition is confined to the mouth and perioral tissues, and secondary oral candidiasis, where there is involvement of other parts of the body in addition to the mouth.

The global acquired immunodeficiency (HIV/AIDS) pandemic has been an important factor in the move away from the traditional classification since it has led to the formation of a new group of patients who present with atypical forms of oral candidiasis(Scully et al, 2008) Oropharyngeal candidiasis is common during cancer care, and it is a very common oral sign in individuals with HIV. (Li et al, 2013) Oral candidiasis occurs in about two thirds of people with concomitant AIDS and esophageal candidiasis (T Yamada et al, 2009). The incidence of all forms of candidiasis have increased in recent decades. This is due to developments in medicine, with more invasive medical procedures and surgeries, more widespread use of broad spectrum antibiotics and immunosuppression therapies.

The diagnosis of oral candidiasis can typically be made from the clinical appearance (Park et al, 2011) but not always. As candidiasis can be variable in appearance, and present with white, red or combined white and red lesions, the differential diagnosis can be extensive. In pseudomembraneous candidiasis, the membranous slough can be wiped away to reveal an erythematous surface underneath. This is helpful in distinguishing pseudomembraneous candidiasis from other white lesions in the mouth that cannot be wiped away, such as lichen planus, oral hairy leukoplakia. Erythematous candidiasis usually has a diffuse border that helps distinguish it from erythroplakia, which normally has a sharply defined border (MS Greenberg et al, 2008). Special investigations to detect the presence of candida species include oral swabs, oral rinse or oral smears. (Kumaraswamy, 2012) Smears are collected by gentle scraping of the lesion with a spatula or tongue blade and the resulting debris directly applied to a glass slide. Oral swabs are taken if culture is required. Some recommend that swabs be taken from 3 different oral

sites (Kerawala et al,2010) Oral rinse involves rinsing the mouth with phosphate-buffered saline for 1 minute and then spitting the solution into a vessel that examined in a pathology laboratory. Oral rinse technique can distinguish between commensal candidal carriage and candidiasis. If candidal leukoplakia is suspected, a biopsy may be indicated (Kumaraswamy, 2012) Smears and biopsies are usually stained with Periodic acid-Schiff, which stains carbohydrate in fungal cell walls magenta. Gram staining is also used as Candida stains strongly Gram +ve (Odell, 2010).

Sometimes an underlying medical condition is sought, and this may include blood tests for full blood count and hematinics.

If a biopsy is taken, the histopathologic appearance can be variable depending upon the clinical type of candidiasis. Pseudomembranous candidiasis shows hyperplastic epithelium with a superficial parakeratotic desquamating (i.e., separating) layer. (Purkait, 2011). Hyphae penetrate to the depth of the stratum spinosum,(Samaranayake et al, 2009) and appear as weakly basophilic structures.Polymorphonuclear cells also infiltrate the epithelium, and chronic inflammatory cells infiltrate the lamina propria. Atrophic candidiasis appears as thin, atrophic epithelium, which is non-keratinized. Hyphae are sparse, and inflammatory cell infiltration of the epithelium and the lamina propria. In essence, atrophic candidiasis appears like pseudomembranous candidiasis without the superficial desquamating layer (Purkait , 2011).

2.2.5. Benign tumors of oral cavity

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. Verruca vulgaris are infective warts, seen particularly in children and result from autoinoculation from warts on the hands. Adenoma arises from minor salivary glands. They form smooth, round, swellings and are most frequently found on the palate (Cawson and Odell, 1998).

2.2.6. Precursor malignant oral lesions

The terms "precancer," "precursor lesions," "premalignant," "intraepithelial neoplasia," and "potentially malignant" have been used in the international literature to broadly describe clinical presentations that may have the potential to become cancer. They all convey the a priori assumption that there is uniformity in how individual patients and tissues behave. The terminology ought to reflect our best understanding of carcinogenesis in the oral mucosa, and to aspire to engender consistency in use. The latest WHO monograph on head and neck tumors uses the term "epithelial precursor lesions" (Barnes, 2005).

Certain cellular and tissue alterations are associated with malignancy and premalignancy. Altered cells appear more primitive than normal, and these changes are presumed to exemplify immature or inappropriate differentiation, although pathologists typically refer to them as dysplasia or atypia. A key alteration of dysplastic epithelial cells is variation in the shape of the cells and nuclei. This pleomorphism is unusual outside of cancers and precancers. Premature production of keratin below the surface layer is another important alteration, but is much more commonly seen in oral carcinomas than in oral premalignancies. The detected risk of malignant transformation varies depending on whether one considers the presence of epithelial dysplasia, and there is need for

molecular markers to identify lesions with definite potential for malignant transformation. Oral epithelial dysplasia is subdivided into three prognostically significant categories: mild (grade I), moderate (grade II), and severe (grade III). Investigation has found that 20–35% of severely dysplastic lesions develop to carcinoma (Research TMCfD, 2010). Over 90% of OSCCs are preceded by a preexisting potentially malignant lesions (Noonan et al, 2005). Although some primary tumors can be treated, many patients will develop second primary tumors, suggesting multifocal tumor development. Two major etiological factors in oral cavity SCC are social habits of tobacco use and alcohol consumption (Proia et al, 2006).

2.2.7. Malignant neoplasm

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. The features of squamous differentiation, observable on routine stained tissues under light microscopy, include one or more of the following: Flattened polyhedral, round, or ovoid epithelial cells; intracellular or extra cellular keratinization and intercellular bridge (Woolf, 1998).

Oral SCC may take various clinical forms. It may resemble a leukoplakia, a verrucous leukoplakia, an erythroleukoplakia, or an erythroplakia, any of which may eventually develop into a necrotic looking ulcer with irregular, raised indurated borders (Bagan et al, 2010), sometimes the lesions are raised above the surface appearing as smooth or papillary or warty projections. Sometimes the lesions are small non-indurated ulcers. The tongue, especially its ventro lateral surface, and the floor of mouth are the sites most frequently affected, accounting for over 50% of cases.

Oral squamous cell carcinoma arises within the epithelium of the oral mucous membrane, Changes within cells cause alterations in color and texture. There is no standard clinical appearance of SCC, it depends upon the stage in which it is discovered and from what part of the mucosa it arises (Calenoff et al, 1995). Oral SCC is usually painless unless it is secondarily infected. Large lesions may interfere with normal speech, mastication or swallowing (Bagan et al, 2010).

Prognosis of OSCC

Prognosis of oral SCC depends on its stage when treated. It depends on the size of the primary tumor (T), whether lymph nodes are involved (N), and whether distant metastasis (M) has occurred. Staging of this disease is accomplished by a combination of the TNM and I–IV systems.

The prognosis for patients with oral SCC that is treated early is much better, with 5-year survival rates as high as 80%. The 5-years survival rate for oral SCC has remained at approximately 50% for the past several decades (Epstein et al, 2002). For all stages combined, about 84% of persons with oral cavity and Pharynx cancers survive 1 year after diagnosis. The 5 year and 10year relative survival rates are 60% and 48%, respectively (Jemal et al, 2007).

About two-thirds of oral SCC are already of substantial size, and will have clinically detectable metastases to cervical lymphnodes at the time of diagnosis (Brandizzi et al,2008).Importantly, in about 20% - 40% of cases with no clinical or imaging evidence of metastatic spread to lymphnodes at the time of diagnosis of oral SCC, histopathological examination of the regional lymphnodes will show metastatic growth (Massano et al, 2006).

Squamous cell carcinoma of the lip, hard palate and maxillary gingiva infrequently metastasize to regional lymphnodes, usually run a relative

indolent course and have a relatively favorable prognosis, while SCC of the tongue, floor of the mouth and of the mandibular often metastasize to regional lymphnodes and are more aggressive with a less favorable prognosis. In general, SCCs of the posterior part of the oral cavity are much more likely to metastasize to regional lymphnodes than are comparable SCCs of the anterior part of the oral cavity (Shah et al, 2009) Small well-differentiated, low-grade oral SCCs usually metastasize to regional lymphnodes only after invading connective tissue, muscle or bone. On the other hand, poorly-differentiated, high-grade oral SCCs are biologically more aggressive and tend to metastasize to regional lymphnodes early in the course of the disease (Shah et al, 2009).The depth of the infiltration of the tumor as determined histopathologically correlates significantly with the prognosis. Oral SCCs that have infiltrated more than 5 mm into the underlying tissues, are more likely to metastasize to lymphnodes with a poorer prognosis (Massano et al, 2006).

Oral SCC can arise from pre-existing potentially malignant disorders including oral leukoplakia, erythroplakia, submucous fibrosis and lichenoid dysplastic lesions, or can arise de novo (Van der Waal, 2009). It has been suggested that oral SCC evolving from leukoplakic lesions have a better prognosis than those emerging de novo, but a recent study has shown that the prognosis is not significantly different in these two groups of oral SCC (Weijers et al, 2008)   A key factor in the lack of improvement in prognosis over the years is the fact that a significant proportion of oral SCCs 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 health care workers (who may not investigate observed lesions thoroughly). Identification of high-

risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys to reducing the mortality, morbidity and cost of treatment associated with SCC. In addition, certain patients are known to be at high risk for head and neck cancer, specifically those who use tobacco or alcohol and those over 45 years of age (John et al, 2002).

2.2. 8. Oral cancer, world wide

It is estimated that each year there are over 484,000 people diagnosed with oral cancer in the world and approximately 261,000 people die of this disease (Jeng et al, 2010). The coming decades will bring dramatic increases in morbidity and mortality from cancer in the developing world. The burden of cancer is increasing globally, with an expected 20 million new cases per year in 2020, half of which will be in low- and middle-income countries (Mellstedt, 2006).

The incidence of oral cancer has significant local variation. In India and other Asian countries, oral and pharyngeal carcinomas comprise up to half of all malignancies, with this particularly high prevalence being attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco and betel quid chewing (Takuji et al, 2001)

Worldwide, there is a great variation in the incidence of cancer of the oral cavity. It accounts for less than 5% of all cancers in United States, Western Europe and Australia. India, few pockets in France, Brazil, central and eastern Europe have few of the highest rates of cancer of the oral cavity in the world. The differing social customs are likely to be responsible for regional variations in the disease incidence (Jayanta et al, 2011).

2.2.9. Oral Cancer, Etiology and Risk Factors

2.2.9.1. Tobacco:

Oral cancer (OC) affects anterior tongue, cheek, floor of mouth, gingiva or any other part of the oral cavity (Jayanta et al, 2011). There are ample evidences suggesting that tobacco in various forms, including smoking, chewing and in betel quid etc., have carcinogenic impact in oral cavity. Nicotine stomatitis is a thickened, hyperkeratotic alteration of the palatal mucosa that is most frequently related to pipe smoking, but milder examples can also develop secondary to cigar smoking or, rarely, from cigarette smoking (Petersen, 2009).The palatal mucosa becomes thickened and hyperkeratotic, sometimes developing a fissured surface. The surface often develops numerous elevations with red centers, which represent the inflamed openings of the minor salivary gland ducts.

Another specific tobacco-related oral mucosal alteration occurs in association with smokeless tobacco use, such as either snuff or chewing tobacco (Meurman, 2010). Such lesions typically occur in the buccal or labial vestibule where the tobacco is held, but they can also extend onto the adjacent gingiva and buccal mucosa. Early lesions show slight wrinkling that disappears when the tissues are stretched. Other lesions may appear as hyperkeratotic, granular patches. Advanced lesions exhibit greatly thickened zones of grayish white mucosa with well-developed folds and fissures. The degree of clinical alteration depends on the type and quantity of tobacco, the duration of tobacco usage, and host susceptibility. Smokeless tobacco keratosis shows microscopic hyperkeratosis and acanthosis of the mucosal epithelium. True epithelial dysplasia is uncommon, and when dysplasia is found, it tends to be mild (Takuji et al, 2001).

In Sudan, smokeless tobacco (ST) is usually used in a form of oral dipping tobacco, locally called toombak, that was introduced over 400 years ago (Ahmed et al, 2007). Toombak is not chewed but dipped and retained between the gums and the lips, cheeks, or floor of the mouth, and sucked slowly for approximately 10–15 min. The tobacco used for manufacturing toombak is Nicotiana rustica, and the fermented ground powder is mixed with an aqueous sodium bicarbonate solution. The resulting product is processed into a loose moist form with a strong aroma, and its use is widespread in the country; popular brands of toombak are *Saute', Wad Amari*, and *Sultan El-kaif* . Sudanese snuff or toombak differs from the types of snuff used in Scandinavia and the USA in terms of tobacco species, fermentation, aging, manufacturing methods, pH, moisture, and nitrosamine content. The factors of toombak believed to have significant adverse health consequences, particularly in terms of addiction and oral cancer development, are its pH and high levels of tobacco-specific nitrosamines (TSNAs) (Idris et al, 1998). Toombak has a pH range of 8–11, with a moisture content of 6–60%, nicotine content of 8–102 mg/g dry weight, and TSNA contents in micrograms, i.e., Ń-nitrosonornicotine (NNN), 420–1550 μg/g; 4-(methyl-nitrosamine)-1- (3-pyridyl)-1-butanone, 620–7870 μg/g; N-nitrosoanatabine (NAT) 20–290 μg/g (Ahmed et al, 2007). TSNAs, particularly NNN and NNK are found in the saliva and body fluids of toombak dippers (Idris et al,1998). Compared with snuff from Sweden and the USA, toombak contains 100-fold higher levels of TSNAs. Toombak dippers develop a clinically and histologically characteristic lesion at the site of dipping. Researchers have demonstrated that the use of toombak plays a significant role in the etiology of oral SCCs, the tobaccospecific nitrosamines present in toombak possibly acting as

principal carcinogens (Ahmed et al, 2007)., 145). In addition to play a major role in the etiology of oral cancer toombak is suspected to be associated with neoplasm of salivary glands (. Idris et al, 1994).

In Sudan, (Ahmed et al, 2007) found that of the 82 subjects, there were 57 (70%) Toombak users, among the 39 patients with OC, 24 (61.5%) were Toombak users. Among 43 with atypical changes, 33 (76.7%) were Toombak users.

Several studies from Sudan have proved that Toombak use is a major risk factor that is responsible of high frequencies of potential malignant oral lesions and oral cancers and in particular OSCCs in the Sudan. Most of tumors were observed at the site of dip application (lower lip). Oral cancer seems to be gender-specific, as the majority of cases were males.

However, all of the preceded discussed literatures support the criminal role of toombak in the etiology of oral cancer in the Sudan. Probably toombak has a major role but it is not alone responsible of oral cancer in the Sudan, particularly in the recent years with dramatic increase in overall cancer risk (Ahmed, 2013).

The evidence that smokeless tobacco causes oral cancer was confirmed recently by the International Agency for Research on Cancer (Petersen, 2009).

2.2.9.2. Alcohol

Numerous studies have suggested alcohol to be a major risk factor for OC. There is a certain degree of controversy whether alcohol alone may have carcinogenic impact. This is due to simultaneous tobacco and alcohol intake of study subjects in various epidemiological studies. Studies have shown that individuals consuming more than 170 g of whisky daily have ten times higher risk of OC than the light drinkers

(Calenoof et al, 1995). Alcohol may have additive effect and it has been suggested that it facilitates the entry of carcinogens into the exposed cells, altering the metabolism of oral mucosal cells. However, the current evidences do not suggest that pure ethanol alone is carcinogen for the development of OC (Epstein et al, 2002).

2.2.9.3. Viral infections- risk factor

In addition to the classic risk factors of oral cancer, namely alcohol and tobacco, other factors both infectious and environmental are thought to be associated with the development of oral malignancy. Infections in the oral cavity may be an important preventable cause of cancer. Poor oral hygiene, periodontal disease, chronic candidiasis, human papilloma virus (HPV) and herpes virus infections link statistically with cancer. Infections may trigger cell proliferation, inhibit apoptosis, interfere with cellular signaling mechanisms and up-regulate tumor promoters (Meurman, 2010).

Viruses are capable of hijacking host cellular apparatus and modifying DNA and the chromosomal structures and inducing proliferative changes in the cells (Hapk et al, 2004).

Studies carried out during the recent years have etiologically linked viruses with human cancers. The current estimate is that about 20% of human cancers worldwide are virally related. Viruses are implicated in oncogenesis based upon the consistency of association with specific cancer types and upon the ability to produce cancer-like transformation in animal models or cell cultures. Detection of viral genomes within tumor cells strengthens a virus–tumor relationship. Viruses can be connected to a single or to a limited number of tumor types (e.g. hepatitis B virus) or to multiple tumor types (e.g. Epstein–Barr virus), a difference that probably reflects the extent of tissue tropism(s) of the viruses. Some

viruses may contribute to tumorigenesis only in a subset of a given type of cancer, or may merely accelerate tumor formation of an already established cancer. Also, the genomes of some viruses, such as Epstein–Barr virus and cytomegalovirus, show regions with substantial polymorphisms, and only certain genotypes may be oncogenic (Puchhammer et al, 2006). Viruses differ from other cancer-causing agents, such as chemicals and radiation, by their ability to induce oncogenic changes through interaction between the infecting virus and the related host response. Viruses may cause cell transformation and proliferation by directly expressing oncogenic genes in infected cells, or by acting as a necessary or a noncompulsory cofactor in the development of malignancy. However, even though most individuals harbor oncogenic viruses in the oral cavity, cancer occurring as a result of infection with such viruses is relatively rare. Risk factors apart from the viral infection are obviously important for cancer development, including family history, age, tobacco smoking and alcohol consumption.

The most important oncoviruses of the human mouth are Epstein–Barr virus, herpesvirus-8 and papillomaviruses (Samonis et al, 2000); HPV and Herpes simplex virus (HSV) have been established in recent years as causative agents of OC.

2.2.9.3.1. Human papilloma viruses (HPV) infection:

 The HPV are a large family of non-enveloped DNA viruses, mainly associated with cervical cancers. Recent epidemiologic evidence has suggested that HPV may be an independent risk factor for oropharyngeal cancers (Turner et al, 2011). HPV has a wide disease spectrum affecting the cutaneous and mucosal areas of the body, ranging from benign common warts to invasive carcinoma. HPV infections have been

reported in a number of body sites, including the anogenital tract, urethra, skin, larynx, tracheobronchial mucosa, nasal cavity, Para nasal sinus, and oral cavity.

A recent study from Sudan reported the presence of HPV in head and neck cancers (HNCs) in general and in oral in particular. The study was performed on 150 samples of patients diagnosed with HNSCs. Six of the 150 (4%) HNSCCs were HPV positive. HPV16 was the most prevalent type, with single infections present in 3/6 (50%) cases, whereas HPV18 and HPV33 were detected in 2/6 (33%) and 1/6 (17%), respectively. HPV infections were detected in 3 (50%) cases of oral cavity and 3 (50%) cases of pharynx (Ahmed, 2013)

 (Ginawi et al, 2010) reported that there is association between HPV16 and 18 infections and OSCC in different regions of Sudan.

HPV has been identified in approximately 23.5% of OC cases (Warnakulasuriya et al, 2007). The most commonly detected HPV in head and neck squamous cell carcinoma (HNSCC) is HPV-16, which has been demonstrated in 90–95% of all HPV positive HNSCC cases, followed by HPV-18, HPV-31, and HPV-33(Hani et al, 2011).

High-risk oncogenic types such as HPV-16 and HPV-18 are associated with squamous cell carcinoma and they are capable of transforming epithelial cells. This transforming potential is largely a result of the function of two viral oncoproteins, E6 and E7, which inactivate two tumor suppressor proteins, p53 and pRb, respectively. Expression of E6 and E7 results in cellular proliferation, loss of cell cycle regulation, impaired cellular differentiation, increased frequency of mutations, and chromosomal instability (Mine Tezal, 2012).

Ang et al reported that tumor HPV status is a strong and independent prognostic factor for survival among patients with oropharyngeal cancer.

They also noted that the risk of death significantly increased with each additional pack-year of tobacco smoking (Ang et al, 2010).

2.2.9.3.2. Epstein–Barr virus

Epstein Barr Virus (EBV) and cytomegalovirus have also been reported as risk factors of OSCC in different studies.

Epstein Barr Virus is involved in a great variety of cancers. The virus possesses factors capable of immortalizing B lymphocytes and epithelial cells, contains several potentially oncogenic antigens can induce several oncogenic gene products (bcl-2, bcl-10, c-fgr and jun ∕ fos). In addition, the nuclear antigen EBNA-LP of the Epstein–Barr virus can interfere with the function of the tumor-suppressor proteins p53 and pRb, thereby dysregulating the cell cycle (Goldenberg et al, 2001).

2.2.9.3.3. Human immune deficiency virus (HIV)

Immunocompromised hosts show increased susceptibility to malignant diseases. Oral tumors in HIV-infected individuals are virtually all virus related. Lymphoid neoplasms in the oral cavity of HIVinfected individuals are typically aggressive B-cell neoplasms (Grogg et al, 2007). Tonsillar cancer shows a particularly close relationship with papillomaviruses (Hammarstedt et al, 2006). Oral squamous cell carcinoma accounts for 2–3% of all malignancies and 300,000 new cases occur worldwide every year (Kademani, 2007). Papillomaviruses have been related to oral squamous cell carcinoma and focal epithelial hyperplasia.Some squamous cell carcinoma lesions contain both papillomavirus and Epstein–Barr virus (Hermann et al, 2004). The oncogenic papillomavirus type 16 is present in about two-thirds of papillomavirus-positive oral tumors, but rarely infects normal oral mucosa (Bouda et al,2000). Papillomavirus-16 DNA was detected in

72% of paraffin-embedded tumor specimens, and the papillomavirus-16 oncoprotein E6 or E7, or both, were serologically detected in 64% of patients with oral cancer. Papillomavirus-16-positive head and neck squamous cell carcinoma was recently found to be independently associated with several measures of sexual behavior and exposure to marijuana but, in contrast to squamous cell carcinomas free of papillomavirus-16, was not linked to cumulative measures of tobacco smoking, alcohol drinking or poor oral hygiene (Gillison et al, 2008). (Rintala et al, 2006) showed the importance of the oral route for papillomavirus transmission between partners; a spouse had a 10-fold higher risk of acquiring persistent oral papillomavirus infection if the other spouse had a persistent oral papillomavirus infection. Papillomavirus-positive oral tumors also show a strong association with multiple oral sex partners.Fortunately, current papillomavirus vaccines, designed to prevent cervical cancer, will probably also decrease the incidence of papillomavirus-related oral cancers (Ferraccioli et al,2007).

2.2.9.4. Candidiasis: Candida has been suggested to play a role in initiation of OC. Clinical studies have reported that nodular leukoplakia infected with Candida has a tendency for higher rate of dysplasia and malignant transformation. It has also been shown that epithelium of the chick embryo, when infected with Candida albicans show squamous metaplasia and higher proliferative phenotype (Mine Tezal, 2012).

2.2.9.5. Dental Hygiene

There is inverse association between oral hygiene and incidence of OC. Poor oral hygiene and prolonged irritation from sharp teeth have been viewed for their possible role in the development of OC. Poor oral

hygiene and dental sepsis is thought to promote carcinogenic action of tobacco (Mine tezal, 2012).

2.2.9.6. Inflammation

Cytokines, including interleukins (ILs), tumor necrosis factors (TNFs), and certain growth factors, are an important group of proteins that regulate and mediate inflammation and angiogenesis. Tumor growth, invasion and metastasis are facilitated when there is a deregulation in their production. Genetic association studies suggest a putative correlation between functional DNA polymorphisms in cytokine genes and oral cancer (Serefoglou et al, 2008).

Increased serum levels of proinflammatory cytokines, interleukin (IL)-1β, IL-6, IL-8, and TNF-αas well as the anti-inflammatory cytokine, IL-10, are seen in patients with oral cancer in comparison to healthy controls. The anti-inflammatory cytokine IL-4 inhibits oral cancer invasion by the downregulation of matrix metalloproteinase-9.

2.2.9.7. Genetic factors:

Genetic mutations often produce early phenotypic changes that may present as clinically apparent, recognizable lesions. An oral premalignant lesion is an area of morphologically or genetically altered tissue that is more likely than normal tissue to develop cancer. The reported rates of malignant transformation of leukoplakia range from less than 1% to 18% (Rebeil, 2003). Various factors, such as the location within the oral cavity, clinical appearance (homogeneous versus heterogeneous), and the presence of dysplasia are correlated with the risk of progression. The histological finding of dysplasia is strongly associated with an increased rate of invasive cancer development (Takuji et al, 2011).A reddish mucosal erythroplakia, is associated with a higher rate of cancer

development, occurs much less frequently, Virtually all erythroplakic lesions contain severe dysplasia, carcinoma in situ, or early invasive carcinoma at the time of presentation *(*Reichart et al, 2005).

2.2.9.8. Age and Sex factors

The incidence of OC is directly correlated with age of subjects. Rates rise dramatically after the age of 40–49 years, and reach a plateau around the age of 70–79 years. OC is more frequent in men than women, and depending on its location within the oral cavity, males are two to six times more likely to be affected than females, largely owing to their higher intake of alcohol and tobacco (Jayanta et al, 2011).

2.3. Mitochondrion - structure and function

2.3.1 Structure of Mitochondrion:

Mitochondrion contains outer and inner membranes composed of phospholipid bilayers and proteins (Albert et al 1994). The two membranes have different properties. Because of this double-membraned organization, there are five distinct parts to a mitochondrion. They are:

- Outer mitochondrial membrane
- Intermembrane space (the space between the outer and inner membranes)
- Inner mitochondrial membrane
- Cristae space (formed by infoldings of the inner membrane)
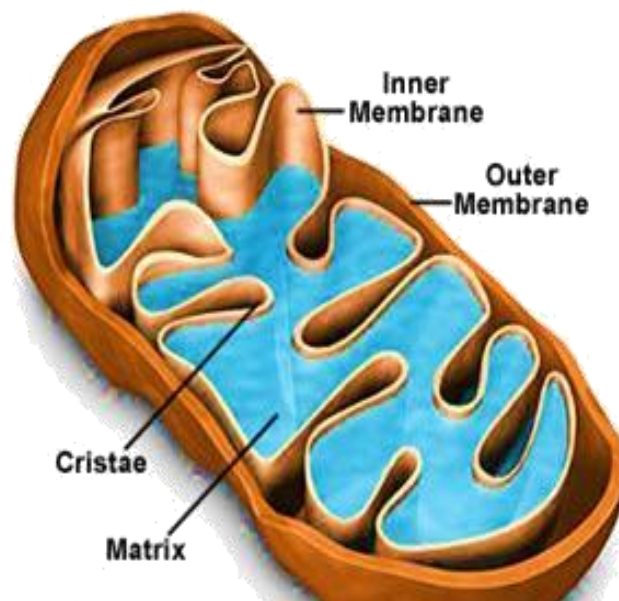- The matrix (space within the inner membrane).

Figure (2.1) mitochondrion inner structure

Outer membrane

The outer mitochondrial membrane, which encloses the entire organelle, has a protein-to-phospholipid ratio similar to that of the eukaryotic plasma membrane (about 1:1 by weight). It contains large numbers of integral proteins called porins. These porins form channels that allow molecules 5000 Daltons or less in molecular weight to freely diffuse from one side of the membrane to the other(Albert et al 1994). Larger proteins can enter the mitochondrion if a signaling sequence at their N-terminus binds to a large multisubunit protein called translocase of the outer membrane, which then actively moves them across the membrane (Herrmann et al, 2000). Disruption of the outer membrane permits proteins in the intermembrane space to leak into the cytosol, leading to certain cell death (Chipuk et al, 2006). The mitochondrial outer membrane can associate with the endoplasmic reticulum (ER) membrane, in a structure called MAM (mitochondria-associated ER-membrane). This is important in the ER-mitochondria calcium signaling and involved in the transfer of lipids between the ER and mitochondria (Hayashi et al 2009).

Intermembrane space

The intermembrane space is the space between the outer membrane and the inner membrane. It is also known as Perimitochondrial space. Because the outer membrane is freely permeable to small molecules, the concentrations of small molecules such as ions and sugars in the intermembrane space is the same as the cytosol (Albert et al 1994). However, large proteins must have a specific signaling sequence to be transported across the outer membrane, so the protein composition of this space is different from the protein composition of the cytosol.

One protein that is localized to the intermembrane space in this way is cytochrome c (Chipuk et al, 2006).

Inner membrane

The inner mitochondrial membrane contains proteins with many types of functions (Albert et al 1994) those that perform the redox reactions of oxidative phosphorylation

1. ATP synthase, which generates ATP in the matrix
2. Specific transport proteins that regulate metabolite passage into and out of the matrix
3. Protein import machinery.
4. Mitochondria fusion and fission protein.

It contains more than 151 different polypeptides, and has a very high protein-to-phospholipid ratio (more than 3:1 by weight, which is about 1 protein for 15 phospholipids). The inner membrane is home to around 1/5 of the total protein in a mitochondrion. In addition, the inner membrane is rich in an unusual phospholipid, cardiolipin. This phospholipid was originally discovered in cow hearts in 1942, and is usually characteristic of mitochondrial and bacterial plasma membranes *(*McMillin, 2002). Cardiolipin contains four fatty acids rather than two, and may help to make the inner membrane impermeable (Albert et al 1994). Unlike the outer membrane, the inner membrane doesn't contain porins, and is highly impermeable to all molecules. Almost all ions and molecules require special membrane transporters to enter or exit the matrix. Proteins are ferried into the matrix via the translocase of the inner membrane (TIM) complex or via Oxa1 (Herrmann et al, 2000). In

addition, there is a membrane potential across the inner membrane, formed by the action of the enzymes of the electron transport chain.

Cristae

The inner mitochondrial membrane is compartmentalized into numerous cristae, which expand the surface area of the inner mitochondrial membrane, enhancing its ability to produce ATP. For typical liver mitochondria, the area of the inner membrane is about five times as great as the outer membrane. This ratio is variable and mitochondria from cells that have a greater demand for ATP, such as muscle cells, contain even more cristae. These folds are studded with small round bodies known as F1 particles or oxysomes. These are not simple random folds but rather invaginations of the inner membrane, which can affect overall chemiosmotic function (Mannella, 2006).

One recent mathematical modeling study has suggested that the optical properties of the cristae in filamentous mitochondria may affect the generation and propagation of light within the tissue (Thar et al, 2004).

Matrix

The matrix is the space enclosed by the inner membrane. It contains about 2/3 of the total protein in a mitochondrion. The matrix is important in the production of ATP with the aid of the ATP synthase contained in the inner membrane. The matrix contains a highly concentrated mixture of hundreds of enzymes, special mitochondrial ribosomes, tRNA, and several copies of the mitochondrial DNA genome. Of the enzymes, the major functions include oxidation of pyruvate and fatty acids, and the citric acid cycle (Albert et al 1994).

Mitochondria have their own genetic material and the machinery to manufacture their own RNAs and proteins. A published human mitochondrial DNA sequence revealed 16,569 base pairs encoding 37 total genes: 22 tRNA, 2 rRNA, and 13 peptide genes. The 13 mitochondrial peptides in humans are integrated into the inner mitochondrial membrane, along with proteins encoded by genes that reside in the host cell's nucleus.

Mitochondria-associated ER membrane (MAM)

The mitochondria-associated ER membrane (MAM) is another structural element that is increasingly recognized for its critical role in cellular physiology and homeostasis. Once considered a technical snag in cell fractionation techniques, the alleged ER vesicle contaminants that invariably appeared in the mitochondrial fraction have been re-identified as membranous structures derived from the MAM—the interface between mitochondria and the ER. Physical coupling between these two organelles had previously been observed in electron micrographs and has more recently been probed with fluorescence microscopy (Rizzuto et al, 2009). Such studies estimate that at the MAM, which may comprise up to 20% of the mitochondrial outer membrane, the ER and mitochondria are separated by a mere 10-25 nm and held together by protein tethering complexe.

Purified MAM from subcellular fractionation has shown to be enriched in enzymes involved in phospholipid exchange, in addition to channels associated with Ca^{2+} signaling (Brito et al, 2010). These hints of a prominent role for the MAM in the regulation of cellular lipid stores and signal transduction have been borne out, with significant implications for mitochondrial-associated cellular phenomena, as discussed below. Not

only has the MAM provided insight into the mechanistic basis underlying such physiological processes as intrinsic apoptosis and the propagation of calcium signaling, but it also favors a more refined view of the mitochondria. Though often seen as static, isolated 'powerhouses' hijacked for cellular metabolism through an ancient endosymbiotic event, the evolution of the MAM underscores the extent to which mitochondria have been integrated into overall cellular physiology, with intimate physical and functional coupling to the endomembrane system.

Phospholipid transfer

The MAM is enriched in enzymes involved in lipid biosynthesis, such as phosphatidylserine synthase on the ER face and phosphatidylserine decarboxylase on the mitochondrial face (Lebiedzinska et al, 2009). Because mitochondria are dynamic organelles constantly undergoing fission and fusion events, they require a constant and well-regulated supply of phospholipids for membrane integrity. But mitochondria are not only a destination for the phospholipids they finish synthesis of; rather, this organelle also plays a role in inter-organelle trafficking of the intermediates and products of phospholipid biosynthetic pathways, ceramide and cholesterol metabolism, and glycosphingolipid anabolism (Osman et al, 2011).

 In contrast to the standard vesicular mechanism of lipid transfer, evidence indicates that the physical proximity of the ER and mitochondrial membranes at the MAM allows for lipid flipping between opposed bilayers. Despite this unusual and seemingly energetically unfavorable mechanism, such transport does not require ATP (Osman et al, 2011). Instead, in yeast, it has been shown to be dependent on a multiprotein tethering structure termed the ER-mitochondria encounter

structure, or ERMES, although it remains unclear whether this structure directly mediates lipid transfer or is required to keep the membranes in sufficiently close proximity to lower the energy barrier for lipid flipping (Kormann et al, 2009).

The MAM may also be part of the secretory pathway, in addition to its role in intracellular lipid trafficking. In particular, the MAM appears to be an intermediate destination between the rough ER and the Golgi in the pathway that leads to very-low-density lipoprotein, or VLDL, assembly and secretion The MAM thus serves as a critical metabolic and trafficking hub in lipid metabolism (Lebiedzinska et al, 2009).

A critical role for the ER in calcium signaling was acknowledged before such a role for the mitochondria was widely accepted, in part because the low affinity of Ca^{2+} channels localized to the outer mitochondrial membrane seemed to fly in the face of this organelle's purported responsiveness to changes in intracellular Ca^{2+} flux (Rizzuto et al, 2009). But the presence of the MAM resolves this apparent contradiction: the close physical association between the two organelles results in Ca^{2+} microdomains at contact points that facilitate efficient Ca^{2+} transmission from the ER to the mitochondria. Transmission occurs in response to so-called "Ca^{2+} puffs" generated by spontaneous clustering and activation of IP3R, a canonical ER membrane Ca^{2+} channel (Hayashi et al, 2009).

The fate of these puffs in particular, whether they remain restricted to isolated locales or integrated into Ca^{2+} waves for propagation throughout the cell is determined in large part by MAM dynamics. Although reuptake of Ca^{2+} by the ER (concomitant with its release) modulates the intensity of the puffs, thus insulating mitochondria to a certain degree

from high Ca^{2+} exposure, the MAM often serves as a firewall that essentially buffers Ca^{2+} puffs by acting as a sink into which free ions released into the cytosol can be funneled(Rizzuto et al, 2009). This Ca^{2+} tunneling occurs through the low-affinity Ca^{2+} receptor VDAC1, which recently has been shown to be physically tethered to the IP3R clusters on the ER membrane and enriched at the MAM. The ability of mitochondria to serve as a Ca^{2+} sink is a result of the electrochemical gradient generated during oxidative phosphorylation, which makes tunneling of the cation an exergonic process (Decuypere et al, 2011).

But transmission of Ca^{2+} is not unidirectional; rather, it is a two-way street. The properties of the Ca^{2+} pump SERCA and the channel IP3R present on the ER membrane facilitate feedback regulation coordinated by MAM function. In particular, clearance of Ca^{2+} by the MAM allows for spatio-temporal patterning of Ca^{2+} signaling because Ca^{2+} alters IP3R activity in a biphasic manner (Rizzuto et al, 2009). SERCA is likewise affected by mitochondrial feedback: uptake of Ca^{2+} by the MAM stimulates ATP production, thus providing energy that enables SERCA to reload the ER with $Ca^{2+}$for continued Ca^{2+} efflux at the MAM.Thus, the MAM is not a passive buffer for Ca^{2+} puffs; rather it helps modulate further Ca^{2+} signaling through feedback loops that affect ER dynamics (Decuypere et al, 2011).

Regulating ER release of Ca^{2+} at the MAM is especially critical because only a certain window of Ca^{2+} uptake sustains the mitochondria, and consequently the cell, at homeostasis. Sufficient intraorganelle Ca^{2+} signaling is required to stimulate metabolism by activating dehydrogenase enzymes critical to flux through the citric acid cycle (Hajinoezky et al, 2011). However, once Ca^{2+} signaling in the

mitochondria passes a certain threshold, it stimulates the intrinsic pathway of apoptosis in part by collapsing the mitochondrial membrane potential required for metabolism. Studies examining the role of pro- and anti-apoptotic factors support this model; for example, the anti-apoptotic factor Bcl-2 has been shown to interact with IP3Rs to reduce Ca^{2+} filling of the ER, leading to reduced efflux at the MAM and preventing collapse of the mitochondrial membrane potential post-apoptotic stimuli (Rizzuto et al, 2009).   Given the need for such fine regulation of Ca^{2+} signaling, it is perhaps unsurprising that dysregulated mitochondrial Ca^{2+} has been implicated in several neurodegenerative diseases, while the catalogue of tumor suppressors includes a few that are enriched at the MAM (Decuypere et al, 2011).

Organization and distribution

Mitochondria are found in nearly all eukaryotes. They vary in number and location according to cell type. A single mitochondrion is often found in unicellular organisms. Conversely, numerous mitochondria are found in human liver cells, with about 1000–2000 mitochondria per cell, making up 1/5 of the cell volume. The mitochondrial content of otherwise similar cells can vary substantially in size and membrane potential, (Das et al, 2011). with differences arising from sources including uneven partitioning at cell divisions, leading to extrinsic differences in ATP levels and downstream cellular processes.The mitochondria can be found nestled between myofibrils of muscle or wrapped around the sperm flagellum.

Often they form a complex 3D branching network inside the cell with the cytoskeleton. The association with the cytoskeleton determines mitochondrial shape, which can affect the function as well.[39] Recent

evidence suggests that vimentin, one of the components of the cytoskeleton, is critical to the association with the cytoskeleton (Tang et al, 2007).

2.3.2. Mitochondrial Function

The most prominent roles of mitochondria are to produce the energy of the cell, ATP (i.e., phosphorylation of ADP), through respiration, and to regulate cellular metabolism.The central set of reactions involved in ATP production is collectively known as the citric acid cycle, or the Krebs cycle. However, the mitochondrion has many other functions in addition to the production of ATP (Voet et al, 2006).

Energy conversion

A dominant role for the mitochondria is the production of ATP, as reflected by the large number of proteins in the inner membrane for this task. This is done by oxidizing the major products of glucose, pyruvate, and NADH, which are produced in the cytosol (Voet et al, 2006). This process of cellular respiration, also known as aerobic respiration, is dependent on the presence of oxygen. When oxygen is limited, the glycolytic products will be metabolized by anaerobic fermentation, a process that is independent of the mitochondri. The production of ATP from glucose has an approximately 13-times higher yield during aerobic respiration compared to fermentation (Rich, 2003). Recently it has been shown that plant mitochondria can produce a limited amount of ATP without oxygen by using the alternate substrate nitrite (Stoimenova et al, 2007).

Pyruvate and the citric acid cycle

Each pyruvate molecule produced by glycolysis is actively transported across the inner mitochondrial membrane, and into the matrix where it is oxidized and combined with coenzyme A to form CO_2, acetyl-CoA, and NADH (Rich, 2003).

The acetyl-CoA is the primary substrate to enter the citric acid cycle, also known as the tricarboxylic acid (TCA) cycle or Krebs cycle. The enzymes of the citric acid cycle are located in the mitochondrial matrix, with the exception of succinate dehydrogenase, which is bound to the inner mitochondrial membrane as part of Complex II (King et al, 2006). The citric acid cycle oxidizes the acetyl-CoA to carbon dioxide, and, in the process, produces reduced cofactors (three molecules of NADH and one molecule of $FADH_2$) that are a source of electrons for the electron transport chain, and a molecule of GTP (that is readily converted to an ATP) (Rich,2003).

NADH and FADH$_2$: the electron transport chain

The redox energy from NADH and FADH2 is transferred to oxygen (O2) in several steps via the electron transport chain. These energy-rich molecules are produced within the matrix via the citric acid cycle but are also produced in the cytoplasm by glycolysis. Reducing equivalents from the cytoplasm can be imported via the malate-aspartate shuttle system of antiporter proteins or feed into the electron transport chain using a glycerol phosphate shuttle. Protein complexes in the inner membrane (NADH dehydrogenase (ubiquinone),cytochrome c reductase, and cytochrome c oxidase) perform the transfer and the incremental release of energy is used to pump protons(H+) into the intermembrane

space. This process is efficient, but a small percentage of electrons may prematurely reduce oxygen, formingreactive oxygen species such as superoxide (Rich, 2003). This can cause oxidative stress in the mitochondria and may contribute to the decline in mitochondrial function associated with the aging process (Huang et al, 2004).

As the proton concentration increases in the intermembrane space, a strong electrochemical gradient is established across the inner membrane. The protons can return to the matrix through the ATP synthase complex, and their potential energy is used to synthesize ATPfrom ADP and inorganic phosphate (Voet et al, 2006). This process is called chemiosmosis, and was first described by Peter Mitchell (Nobel Foundation, 2007) who was awarded the 1978 Nobel Prize in Chemistry for his work. Later, part of the 1997 Nobel Prize in Chemistry was awarded to Paul D. Boyerand John E. Walker for their clarification of the working mechanism of ATP synthase (Nobel Foundation Retrieved, 2007)

Heat production

Under certain conditions, protons can re-enter the mitochondrial matrix without contributing to ATP synthesis. This process is known as proton leak or mitochondrial uncoupling and is due to the facilitated diffusion of protons into the matrix. The process results in the unharnessed potential energy of the proton electrochemical gradient being released as heat (Voet et al, 2006). The process is mediated by a proton channel called thermogenin, or UCP1. Thermogenin is a 33kDa protein first discovered in 1973. Thermogenin is primarily found in brown adipose tissue, or brown fat, and is responsible for non-shivering thermogenesis.

Brown adipose tissue is found in mammals, and is at its highest levels in early life and in hibernating animals. In humans, brown adipose tissue is present at birth and decreases with age (Mozo, et al 2005).

Storage of calcium ions

The concentrations of free calcium in the cell can regulate an array of reactions and is important for signal transduction in the cell. Mitochondria can transiently store calcium, a contributing process for the cell's homeostasis of calcium. In fact, their ability to rapidly take in calcium for later release makes them very good "cytosolic buffers" for calcium. (Rossier, 2006). The endoplasmic reticulum (ER) is the most significant storage site of calcium, and there is a significant interplay between the mitochondrion and ER with regard to calcium (Pizzo et al, 2007). The calcium is taken up into the matrix by a calcium uniporter on the inner mitochondrial membrane. It is primarily driven by the mitochondrial membrane potential. Release of this calcium back into the cell's interior can occur via a sodium-calcium exchange protein or via "calcium-induced-calcium-release" pathways. This can initiate calcium spikes or calcium waves with large changes in themembrane potential. These can activate a series of second messenger system proteins that can coordinate processes such asneurotransmitter release in nerve cells and release of hormones in endocrine cells.

Ca2+ influx to the mitochondrial matrix has recently been implicated as a mechanism to regulate respiratory bioenergetics by allowing the electrochemical potential across the membrane to transiently "pulse" from $\Delta\Psi$-dominated to pH-dominated, facilitating a reduction ofoxidative stress (Schwarzlander et al, 2012).

Cellular proliferation regulation

The relationship between cellular proliferation and mitochondria has been investigated using cervical cancer Hela cells. Tumor cells require an ample amount of ATP (Adenosine triphosphate) in order to synthesize bioactive compounds such as lipids, proteins, and nucleotides for rapid cell proliferation (Weinberg et al, 2009). The majority of ATP in tumor cells is generated via the Oxidative Phosphorylationpathway (OxPhos). Interference with OxPhos have shown to cause cell cycle arrest suggesting that mitochondria plays a role in cell proliferation (Moreno et al, 2007). Mitochondrial ATP production is also vital for cell division in addition to other basic functions in the cell including the regulation of cell volume, solute concentration, and cellular architecture (Pattappa et al, 2011). ATP levels differ at various stages of the cell cycle suggesting that there is a relationship between the abundance of ATP and the cell's ability to enter a new cell cycle. ATP's role in the basic functions of the cell make the cell cycle sensitive to changes in the availability of mitochondrial derived ATP. The variation in ATP levels at different stages of the cell cycle support the hypothesis that mitochondria play an important role in cell cycle regulation. Although the specific mechanisms between mitochondria and the cell cycle regulation is not well understood, studies have shown that low energy cell cycle checkpoints monitor the energy capability before committing to another round of cell division (McBride et al, 2006).

2.4. Mitochondrial DNA

The human mitochondrial genome is a circular DNA molecule of about 16 kilobases. It encodes 37 genes: 13 for subunits of respiratory complexes I, III, IV and V, 22 for mitochondrial tRNA (for the 20 standard amino acids, plus an extra gene for leucine and serine), and 2 for rRNA (Chan, 2006)

In animals, mitochondrial genome is typically a single circular chromosome that is approximately 16 kb long and has 37 genes. The genes, while highly conserved, may vary in location. Curiously, this pattern is not found in the human body louse (Pediculus humanus). Instead this mitochondrial genome is arranged in 18 minicircular chromosomes, each of which is 3–4 kb long and has one to three genes (Shao et al, 2009).

2.4. 1. Mitochondrial DNA Structure

In most multicellular organisms, the mtDNA is organized as a circular, covalently closed, double-stranded DNA,but in many unicellular (e.g. the ciliate Tetrahymena or the green algaChlamydomonas reinhardtii) and in rare cases also in multicellular organisms (e.g. in some species of Cnidaria) the mtDNA is found as linearly organized DNA. Most of these linear mtDNAs possess telomerase independent telomeres (i.e. the ends of the linear DNA) with different modes of replication, which have made them interesting objects of research, as many of these unicellular organisms with linear mtDNA are known pathogens (McMillin et al,2002)

For human mitochondrial DNA (and probably for that of metazoans in general), 100-10,000 separate copies of mtDNA are usually present per cell (egg and sperm cells are exceptions). In mammals, each double-

stranded circular mtDNA molecule consists of 15,000-17,000 base pairs (Mannella,2006).
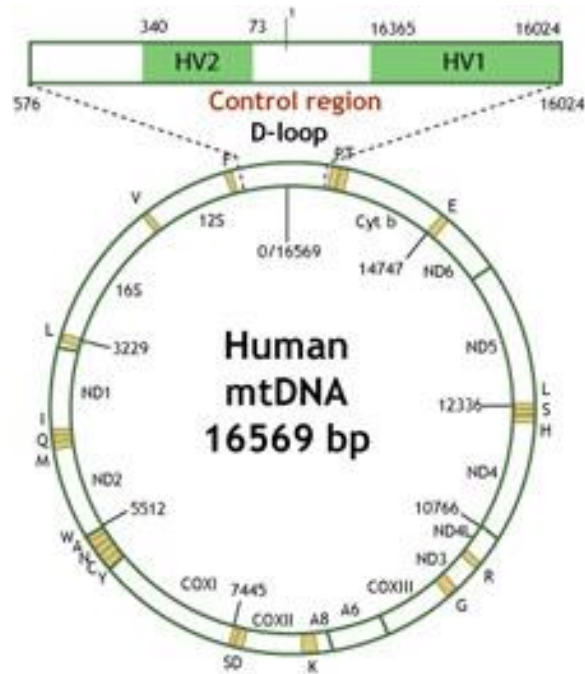

Figure (2. 2) structure of mtDNA

The two strands of mtDNA are differentiated by their nucleotide content with the guanine-rich strand referred to as the heavy strand (or H-strand), and the cytosine-rich strand referred to as the light strand (or L-strand). The heavy strand encodes 28 genes, and the light strand encodes 9 genes for a total of 37 genes. Of the 37 genes, 13 are for proteins (polypeptides), 22 are for transfer RNA (tRNA) and two are for the small and large subunits of ribosomal RNA (rRNA). This pattern is also seen among most metazoans, although in some cases one or more of the 37 genes is absent and the mtDNA size range is greater. Even greater variation in mtDNA gene content and size exists among fungi and plants, although there appears to be a core subset of genes that are present in all eukaryotes (except for the few that have no mitochondria at all) (Thar et al, 2004).

2.4. 2. Mitochondrial DNA - Replication and inheritance

It has recently been revealed that mitochondria actually divide by budding similar to the reproduction of many of alpha-proteobacteria, On the other hand, mitochondria can fuse with other mitochondria (Johns, 2003). The regulation of this division differs between eukaryotes. In many single-celled eukaryotes, their growth and division is linked to the cell cycle. For example, a single mitochondrion may divide synchronously with the nucleus. This division and segregation process must be tightly controlled so that each daughter cell receives at least one mitochondrion. In other eukaryotes (in mammals for example), mitochondria may replicate their DNA and divide mainly in response to the energy needs of the cell, rather than in phase with the cell cycle. When the energy needs of a cell are high, mitochondria grow and divide. When the energy use is low, mitochondria are destroyed or become inactive. In such examples, and in contrast to the situation in many single celled eukaryotes, mitochondria are apparently randomly distributed to the daughter cells during the division of the cytoplasm. Understanding of mitochondrial dynamics, which is described as the balance between mitochondrial fusion and fission, has revealed that functional and structural alterations in mitochondrial morphology are important factors in pathologies associated with several disease conditions (Garrigan et al. 2006).

An individual's mitochondrial genes are not inherited by the same mechanism as nuclear genes. Typically, the mitochondria are inherited from one parent only. In humans, when an egg cell is fertilized by a

sperm, the egg nucleus and sperm nucleus each contribute equally to the genetic makeup of the zygote nucleus. In contrast, the mitochondria, and therefore the mitochondrial DNA, usually come from the egg only. The sperm's mitochondria enter the egg but do not contribute genetic information to the embryo (Garrigan and Hammer, 2006). The egg cell contains relatively few mitochondria, but it is these mitochondria that survive and divide to populate the cells of the adult organism. Mitochondria are, therefore, in most cases inherited only from mothers, a pattern known as maternal inheritance. This mode is seen in most organisms including all animals. However, mitochondria in some species can sometimes be inherited paternally. This is the norm among certain coniferous plants (Zeviani et al, 2004).

Table (2.1): Comparison between the human nuclear and mitochondrial genome.

Characteristic	Nuclear genome	Mitochondrial genome
Size	~3.3 x 109 bp	16,569 bp
Number of DNA molecules per cell	23 in haploid cells; 46 in diploid cells	Several thousand copies per cell (polyploidy)
Number of genes encoded	~20,000–30,000	37 (13 polypeptides, 22 tRNAs and 2 rRNAs)
Introns	Frequently found in most genes	Absent
Gene density	~1 per 40,000 bp	1 per 450 bp
Associated proteins	Nucleosome-associated histone proteins and non-histone proteins	No histones; but associated with several proteins that form nucleoids
Mode of inheritance	Mendelian inheritance for autosomes and the X chromosome; paternal inheritance for the Y chromosome	Exclusively maternal
Replication	Strand-coupled mechanism that uses DNA polymerases α and δ	Strand-coupled and strand-displacement models; only uses DNA polymerase γ
Transcription	Most genes are transcribed individually	All genes on both strands are transcribed as large polycistrons
Recombination	Each pair of homologues recombines during the prophase of meiosis	Recombination occurs at a cellular level but little evidence that it occurs at a population level

Modified from (Taylor et al, 2005

2.4. 3. Mitochondrial Dysfunction and diseases

Damage and subsequent dysfunction in mitochondria is an important factor in a range of human diseases due to their influence in cell metabolism. Mitochondrial disorders often present themselves as neurological disorders, but can manifest as myopathy, diabetes, multiple endocrinopathy, or a variety of other systemic manifestations (Lim et al, 2010). Diseases caused by mutation in the mtDNA include Kearns-Sayre syndrome, MELAS syndrome and Leber's hereditary optic neuropathy (Schapira,2006). In the vast majority of cases, these diseases are transmitted by a female to her children, as the zygote derives its mitochondria and hence its mtDNA from the ovum. Diseases such as Kearns-Sayre syndrome, Pearson's syndrome, and progressive external ophthalmoplegia are thought to be due to large-scale mtDNA rearrangements, whereas other diseases such as MELAS syndrome, Leber's hereditary optic neuropathy, myoclonic epilepsy with ragged red fibers (MERRF), and others are due to point mutations in mtDNA .These diseases are inherited in a dominance relationship, as applies to most other genetic diseases. A variety of disorders can be caused by nuclear mutations of oxidative phosphorylation enzymes, such as coenzyme Q10 deficiency and Barth syndrome (Lim et al, 2010). Environmental influences may interact with hereditary predispositions and cause mitochondrial disease (Bugger et al, 2010).

Other pathologies with etiology involving mitochondrial dysfunction include schizophrenia, bipolar disorder, dementia, Alzheimer's disease, Parkinson's disease, epilepsy, stroke,cardiovascular disease, retinitis pigmentosa, and diabetes mellitus (Bender et al,2006)

MtDNA mutation, Homoplasmy and heteroplasmy

Mitochondrial DNA mutations are most commonly single nucleotide polymorphisms (SNPs), in which one of the four bases or basic units of DNA, cytosine (C), guanine (G), adenine (A), or thymine (T), is replaced by one of the others. The most common replacements (greater than 95%), C to T and vice versa, and A to G and vice versa, are called transitions; all other replacements are called transversions. Mutations may also consist of a base being inserted or deleted (indels). Those types of de novo mutations are similar to those in nuclear DNA, including Y-chromosome DNA; in fact, SNP mutations are exactly the same in mtDNA as in YDNA (Chung et al,2005). Only one copy of Y-DNA is transmitted from father to son, since there is only one copy of the Y chromosome in each cell. For mtDNA, each cell may contain hundreds or even thousands of mitochondria and therefore hundreds or thousands of copies of mtDNA, so that multiple copies of mtDNA are transmitted from mother to child. However, the number of copies transmitted is limited by bottlenecks in egg development (Shoubridge et al, 2007).

The polyploid nature of the mitochondrial genome up to several thousand copies per cell gives rise to an important feature of mitochondrial genetics, homoplasmy and heteroplasmy. In simple terms, homoplasmy is when all copies of the mitochondrial genome are identical; heteroplasmy is when there is a mixture of two or more mitochondrial genotypes. The value of these terms is apparent when we consider mtDNA mutations that lead to disease. Some mutations affect all copies of the mitochondrial genome (homoplasmic mutation), whereas others are only present in some copies of the mitochondrial genome (heteroplasmic mutation). In the presence of heteroplasmy, there is a threshold level of mutation that is important for both the clinical

expression of the disease and for biochemical defects, as routinely demonstrated by the cytochemical assessment of cytochrome c oxidase (COX; complex IV) activity in an individual cell. Numerous single-cell and TRANSMITOCHONDRIAL CYBRID CELL studies have shown that the mutated form is functionally recessive and that a biochemical phenotype is associated with high levels of mutation above a crucial threshold (Sciacco et al, 1994).

The concept of homoplasmy is more apparent than real. In most individuals there is no evidence of heteroplasmy, but all available evidence indicates that mtDNA is constantly undergoing mutation, with clonal expansion or loss of either point mutations or deletions(Coller,2001), Because these acquired mutations occur at random, all acquired mutations will be present at a low level and therefore might not be detected in a tissue homogenate or blood sample. Perhaps we should only use 'homoplasmy' to describe a state in which we cannot detect these acquired mutations, although it would be naive to think that all mitochondrial genomes within an individual, tissue or even a cell are identical (Taylor et al, 2005)

Computer models show that random drift can also be a sufficient mechanism to explain homoplasmic nature of cancer cells (Carew et al, 2002). Relaxed replication phenomenon analysis in normal cells showed that replicative or metabolic advantage is not indispensable for homoplasmy to arise (Carew et al, 2002). Although a role for other mechanisms is not excluded, random processes are sufficient to explain the incidence of homoplasmic mtDNA mutations in human tumors. Depending on how the mutant copies are distributed between stem and transition cells, the mutants are depleted or enriched and the number of mutants in the lineage fluctuates as a random walk. On average, only

approximately 70 generations are required for a mutation that is destined to become homoplasmic. The number of 70 generations is small if compared with the number of cell division that a tumor progenitor cell is expected to undergo (Anna et al, 2007).

The functional implications of the build-up of mtDNA mutations, both in homoplasmy and in heteroplasmy, have not been defined yet, but they may lead to functional alterations of the mtDNA-encoded proteins. Most of the mtDNAencoded proteins are involved in the electron transport chain, and hence these may lead to electron flow disruption and increased generation of ROS (Carew et al, 2002).

2.4.4. Mutation of D-loop region of mtDNA - previous studies

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; (Cenk et al, 2007) mutations occur in all protein-coding mitochondrial genes; the D-loop region is the most frequent site of somatic mutations across most tumor types (Kanki et al, 2004). The presence of homoplasic mutant mtDNA in tumors suggests that they may play an important role in the development of tumors (Cenk et al, 2007).

In addition to mutations in the coding region of mtDNA, a high frequency of somatic mutation was located in the non-coding displacement loop (D-loop) region of mtDNA. The D-loop region has been described as the most frequent host for mtDNA mutations in variety of human cancers.

Several studies of somatic mutation in the D-loop region of mtDNA has revealed that insertions or deletions at nucleotide position (np) 303-309, a polycytidine stretch (C-tract) termed D310, are the most common mutations of mtDNA in human cancers including colorectal cancer

(Lievre et al, 2005), gastric cancer (Wu et al. 2005), hepatocellular carcinoma (Tamori et al. 2004), melanoma (Takeuchi et al,2004), ovarian cancer (Liu et al,2001), uterine serous carcinoma (Pejovic et al, 2004). 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 (Carew et al, 2002).

Large-scale deletions of mtDNA have been detected in various types of cancers, For example, it was reported that a 4,977 bp deletion was largely accumulated in sun24 exposed skin tissues, the squamous cell carcinomas and precancerous skin tissues (Pang et al, 1994). The 4,977 bp deletion of mtDNA was later detected in oral cancers and paired nonmalignant oral tissues of patients with betel quid chewing history (Lee et al, 2001). Moreover, an increase of mtDNA large-scale deletions was reported in radiation-associated thyroid tumors (Rogounovitch et al. 2002). However, even 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. It has been suggested that during cancer progression the mtDNA with a deletion is decreased (diluted) as a result of clonal expansion of cell lineages that

contain less or no mtDNA deletion. The study with micro-dissected tumor tissues further confirmed the lower incidence of 4977 bp mtDNA deletion in most tumors (Dani et al. 2004).

A comprehensive study on clinical features and mtDNA of 104 colorectal cancer patients in the Wenzhou area of China. In particular, using a quantitative real time PCR method analyzed the 4,977 bp deletion and mtDNA content in tumor tissues and paired non-tumor areas from these patients. The study showed that the 4,977 bp deletion was more likely to be present in patients of younger age ($\leq$65 years). In patients with the 4,977 bp deletion, the deletion level decreased as the cancer stage advanced (Tao et al, 2011).

In the assessment of mtDNA obtained from material of 35 patients with chronic cervicitis, heteroplasmy found in 28 patients (80.0%). Seven patients (20.0%) were normal, whereas none of the patients with chronic cervicitis had the 4977 bp homoplasmic deletion. The mtDNA assessment of 21 patients with cervical cancer showed that 17 patients (81.0%) were normal, two patients (9.5%) had heteroplasmy, and two patients (9.5%) had the 4977 bp homoplasmic deletion. Study of the mtDNA tissue samples from 16 patients in the control group showed that five patients had heteroplasmy (31.2%), 11 individuals were normal (68.8%) ($p = 0.0001$). None of the control subjects had a 4977 bp homoplasmic deletion (Kara et al, 2012).

3.1. THE STUDY RATIONALE

Oral cancer is a health and economic problem, in addition to the high mortality it associated also with loss of eating and speech function, disfigurement and psychological distress. The study focused on mitochondrial DNA (D-loop) region because of it's a mutational "hot spot" in some human carcinogenesis. The study findings may provide a new method that may improve the diagnostic, therapeutic targets or screening program in Sudan.

3.2. THE STUDY OBJECTIVES

3.2.1. General objective

To study the mitochondrial DNA mutations in oral lesions among Sudanese patients

3.2.2. Specific objectives:

The specific objectives of this study are:

1. To detect the mitochondrial DNA mutations in oral lesions by demonstrating the 4977 bp deletion.

2. To correlate the 4977 bp deletion to aggressiveness of oral lesions.

3. To correlate the histopathologic patterns to the 4977 bp deletion in oral lesions.

4. To determine the patterns of oral lesions and to compare that with the international published series.

4. Materials and Methods

4.1. Study Design: Retrospective descriptive cross- sectional study, investigated the relationship between histopathologic patterns and molecular changes of mitochondrial DNA in oral lesions among Sudanese patients.

4.2 Study area: the study was conducted in Khartoum state from 2011 to 2014.

4.3. Study population & sampling:

The study included One hundred and fifteen (115) formalin fixed paraffin embedded biopsies of oral lesions taken from patients underwent surgical operation treatment. Clinical and demographic data were collected from hospital registration records.

4.3.1 Samples processing:

Serial sections from (FFPE) biopsies were taken using rotary microtome for confirming the histopathology diagnosis using hematoxylin & eosin staining method, and other serial sections from the same biopsies were taken in to tubes for DNA extraction using QIAGEN QIAamp DNA FFPE Tissue Kits for molecular purpose.

4.3.1.1: Isolation of DNA from FFPE Tissue Sections:

Principle of extraction

The QIAamp DNA FFPE Tissue procedure consists of 6 steps:

■ Remove paraffin: paraffin is dissolved in xylene and removed

■ Lyse: sample is lysed under denaturing conditions with proteinase K

■ Heat: incubation at 90°C reverses formalin crosslinking

■ Bind: DNA binds to the membrane and contaminants flow through

■ Wash: residual contaminants are washed away

■ Elute: pure, concentrated DNA is eluted from the membrane

Preparing Buffer AW1: 25 ml ethanol (96–100%) added to the bottle containing 19 ml Buffer AW1 concentrate.

Preparing Buffer AW2: 30 ml ethanol (96–100%) added to the bottle containing 13 ml Buffer AW2 concentrate.

The check box on the bottle label was ticked to indicate that ethanol has been added. Reconstituted Buffers stored at room temperature (15–25°C) Note: Before starting the procedure, reconstituted Buffers are mixed by shaking.

Extraction Procedure: the following steps applied to all specimens

1. Using a scalpel, excess paraffin off the sample block trimmed.

2. 8 sections 5–10 μm thick were cut.

3. Immediately the sections placed in a microcentrifuge tube and 2 ml xylene was added to the sample. The lid closed and vortexed vigorously for 10s. Incubated at room temperature (R.T) for 5mins

4. Centrifuge at full speed for 2 min at room temperature.

5. The supernatant was carefully removed by pipetting.

6. 2 ml ethanol (96–100%) was added to the pellet, and mixed by vortexing. Then Incubated at room temperature (R.T) for 5mins (The ethanol extracts residual xylene from the sample).

7. Centrifugation at full speed for 2 min at room temperature.

8. Carefully ethanol removed using a fine pipet tip.

9. The tube opened and incubated at room temperature (15–25°C) until all residual ethanol had evaporated.

10. The pellet suspended in 180 μl Buffer ATL. And 20 μl proteinase K added and mixed by vortexing.

11. Incubation at 56°C for 1 h in water bath until the sample had been completely lysed.

12. The 1.5 ml tube briefly centrifuged to remove drops from the inside of the lid.

13. 200 µl Buffer AL added to the sample, and mixed thoroughly by vortexing. Then 200 µl ethanol (96–100%), and mixed again thoroughly by vortexing.

(The sample, Buffer AL, and ethanol were mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution).

14. The 1.5 ml tube briefly centrifuged to remove drops from the inside of the lid.

15. The entire lysate carefully transferred to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, the lid closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. the QIAamp MinElute column placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discarded.

16. The QIAamp Min Elute column carefully opened and 500 µl Buffer AW1added without wetting the rim. Centrifugation at 6000 x g (8000 rpm) for 1 min. the QIAamp Min Elute column placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discarded.

18. Carefully the QIAamp MinElute column opened and 500 µl Buffer AW2 was added without wetting the rim. Centrifugation at 6000 x g (8000 rpm) for 1 min. the QIAamp MinElute column placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discarded.

19. Centrifugation at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. This step was necessary, since ethanol carryover in to the elute may interfere with some downstream applications.

20. The QIAamp MinElute column was placed in a clean 1.5 ml micro centrifuge tube and the collection tube containing the flow-through discarded. Carefully the lid of the QIAamp MinElute column opened and 20–100 μl Buffer ATE applied to the center of the membrane.

21. The lid closed and incubated at room temperature (15–25°C) for 1 min, centrifuged at full speed (20,000 x g; 14,000 rpm) for 1 min. Incubating the QIAamp MinElute column loaded with Buffer ATE for 5 min at room temperature before centrifugation was performed to increase DNA yield.

4.3.1.2. PCR Primers

The preparation of primers based on gene sequences from previous studies and confirmed from mtDNA map. Nucleotide sequences of the primers are shown in the table (4.1) below:

Table (4.1): PCR primers

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 Reverse: TCG AGT GCT ATA GGC GCT TGT C
Positive control	1029 (6251-7280)	Forward: TAT AGT GGA GGC CGG AGC AG Reverse: GAA TGA GCC TAC AGA TGA TA
Negative control	564 (9981-10545)	Forward: TGA GGG TCT TAC TCT TTT AGT Reverse: GGT GTG AGC GAT ATA CTA GT

4.3.1.3. Polymerase chain reaction (PCR):

Maxime PCR PreMix (i-Taq; for 20µl rxn) component in 20 µl reaction:

I-TaqTM DNA Polymerase (5U/µl) …………………….. 2.5U

dNTPs …..…………………………... 2.5mM each

 Reaction Buffer (10x) ……………………….…… 1x

Gel loading buffer …………………………………. 1x

Table (4.2): PCR cycling parameters

PCR cycle	Temp		Time
Initial denaturation		94℃	2min
35 Cycles	Denaturation	94℃	20sec
	Annealing	55℃	10sec
	Extension	65-72℃	20sec
Final extension		72 ℃	2-5min
Final old		10 ℃	

PCR Protocol

1. Template DNA and primers were added into Maxime PCR PreMix tubes (i-Taq).

2. Distilled water was added into the tubes to a total volume of 20µl.

Table (4.3): PCR reaction mixture:

Reagent	Volume
Template DNA	2µl
Primer (F: 10pmol/µl)	1µl
Primer (R: 10pmol/µl)	1µl
Distilled Water	16 µl
Total reaction volume	20 µl

3. The pellet was easily dissolved by standing at RT for 1-2min after adding water.

 4. PCR Performed.

4.3.1.4. Electrophoresis of Amplified DNA

Agarose Gel Electrophoresis Protocol

1. Agarose Gel Prepared as (1.2%).

2. Pour the gel when the agarose has cooled to about 55° C. proper comb was inserted for the particular gel rig.

3. The comb carefully removed and gel placed in the gel rig with the wells closest to the cathode (black) end, covered with 1X TAE buffer.

4. Total PCR product volume Loaded on agarose gel without adding a loading-dye, 6 µl of 1 Kb ladder was placed at end of the series of samples.

5. The gel electrophoresis performed at 100 volts for 1 h.

7. The gel Visualized with U.V. light and photographed with a Polaroid Photo documentation camera.

4.3.2. Mutation detection

During visualization, a 470 bp fragment for the deletion region, a 1029 bp fragment for the positive control site outside the deletion region and a 564 bp fragment for the negative control site in the deletion region were observed.

4.4. Statistical analysis

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.

5. RESULTS

The study investigated 115 subjects, their ages ranged from 10 to 90 with a mean age of 47 years. Among these 115 participants, 61(53.0%) were males and 54(47.0%) females, as shown in tables (5.1) and (5.2) respectively.

The population distribution by age group as in table (5.3) was 46 (40%) were less than 40 years, 42 (36.5%) were ranged from 40 - < 60 years and 27 (23.5%) were at 60 years or more. According to the anatomical site, the population lesions were classified as 18 (15.7%) buccal mucosal lesions, 13 (11.3%) lower lip, 18 (15.7%) tongue, 26 (22.6%) salivary glands, 8 (7%) gingival, 7 (6.1%) palate, 22(19.1%) mandibular and 3 (2.6%) glottis lesions as shown in table (5.4). The laboratory diagnosis of subjects gave the results of malignancy 81 (70.4%), benign lesion 18 (15.7%), inflammation 12 (10.4%) and 4 (3.5%) of cases were other conditions as demonstrated in table (5.5). The sub classification distribution of the 81 malignant lesions as shown in table (5.6) was squamous cell carcinoma 55 (67.9%), lymphoma 2 (2.5%), mucoepidermoid carcinoma 5(6.2%), adenocarcinoma 15(18.5%) and ameblastoma 4(4.9%). As demonstrated in table (5.7) and according to the type of benign lesions, the 18 subjects classified as having adenogenic cyst 4 (22.2%), pleomorphic adenoma 10 (55.6%), fibroma 3 (16.7%) and Hemangioma 1 (5.6%).

When evidence of malignancy was correlated with gender variable, the study showed 42 (68.9%) of males and 39 (72.2%) of females having malignant tumor, table (5.8). The distribution of malignancy regarding age groups, among 46 individuals at age group < 40 years there were 27

cases with malignancy constitute 58.7%. Of 42 individuals at age group 40 – < 60 years there were 29 malignant cases constituting 69% and 92.6% of population at 60 years or more with malignancy as shown in table (5.9).

Of the 115 oral lesion patients, 4 (3.5%) showed 4,977 -bp deletion (p. value = 0.4). When the 4,977 -bp deletion was correlated to Histopathological features, all the 4,977 -bp deletion 4 cases constituting (100%) occurred among population having malignant lesions, and when classified as sub malignancy, 3 (75%) of mutation were among cases of squamous cell carcinoma as shown in table (5.10). When characteristics and risk factors including age, sex, and tumor cell differentiation status analyzed cross tabulating to 4,977 -bp deletion, the study showed 3(75%) were females and only one (25%) was male as in table (5.11). 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, Table (5.12).

Table (5.1): Distribution of the Study Population by Age

Age	Parameters
Mean	47
SD	18
Minimum	10
Maximum	90

Table (5.2): Distribution of the Study Population by Gender

Gender	**Frequency**	%
Male	61	53.0
Female	54	47.0
Total	115	100.0

Table (5.3): Distribution of the Study Population by Age group

Age group	Frequency	%
< 40 years	46	40.0
40-<60 Years	42	36.5
60 years or more	27	23.5
Total	115	100.0

Table (5.4): Distribution of lesions by anatomical sites

Site of lesion	Frequency	%
Salivary glands	26	22.6
Mandible	22	19.1
Tongue	18	15.7
Buccal mucosa	18	15.7
Lower lip	13	11.3
Gingiva	8	7.0
Palate	7	6.1
Glottis	3	2.6
Total	115	100.0

Table (5.5): Distribution of the Study Population by diagnosis

Lab. Diagnosis	Frequency	%
Malignancy	81	70.4
Benign tumor	18	15.7
Inflammation	12	10.4
Others (normal)	4	3.5
Total	115	100.0

Table (5.6): Distribution of the Study Population by class of malignancy

Class of malignancy	Frequency	%
Squamous cell carcinoma	55	67.9
Adenocarcinoma	15	18.5
Mucoepidermoid carcinoma	5	6.2
Ameblastoma	4	4.9
Lymphoma	2	2.5
Total	81	100.0

Table (5.7): Distribution of the Study Population by class of benign lesion

Class of lesion	Frequency	%
Adenogenic cyst	4	22.2
Pleomorphic adenoma	10	55.6
Fibroma	3	16.7
Hemangioma	1	5.6
Total	18	100.0

Table (5.8): Correlation of gender with diagnosis

Lab. diagnosis \\ Gender of patients	Malignancy	Benign tumor	Inflammation	Others	Total
Male	42(68.9%)	11(18.0%)	4 (6.6%)	4(6.6%)	61(100%)
Female	39(72.2%)	7(13.0%)	8 (14.8%)	0(.0%)	54(100%)
Total	81(70.4%)	18(15.7%)	12 (10.4%)	4(3.5%)	115(100%)

Chi square = 5.9

P-value =0.11

Table (5.9):   Correlation of age groups with diagnosis

Diagnosis / Age group	Malignant	Benign tumor	Normal lesion	Total
<40 years	27 (58.7%)	12 (26.1%)	7 (15.2%)	46 (100.0%)
40 < 60 years	29 (69.0%)	4 (9.5%)	9 (21.5%)	42 (100.0%)
60 +	25 (92.6%)	2 (7.4%)	0(0.0%)	27(100.0%)
Total	81 (70.4%)	18 (15.7%)	16 (13.9%)	115 (100.0%)

Chi square = 13.8

P-value =0.032

Table (5.10): Relation between mutation and malignancy

Mutation Result	Classification of malignant lesions					Total
	Squamous cell carcinoma	Lymphoma	Mucoepidermoid carcinoma	Adeno-carcinoma	Ameblastoma	
No mutation	52 (67.5%)	2 (2.6%)	4 (5.2%)	15 (19.5%)	4 (5.2%)	77 (100.0%)
Mutation	3 (75.0%)	0 (0%)	1 (25.0%)	0 (0%)	0 (0%)	4 (100.0%)
Total	55	2	5	15	4	81 (100.0%)

P- Value = 0.4

Table (5.11): Relation between mutation and gender

Gender of patients	Mutation Result		Total
	No mutation	Mutation	
Male	60	1	61
Female	51	3	54
Total	111	4	115

P value: 0.25

Table (5.12): Relation between Mutation and Age

Age groups	Mutation Result		Total
	No mutation	Mutation	
< 40 years	45 (97.8%)	**1 (2.2%)**	46
40-<60 Years	40 (95.2%)	**2 (4.8%)**	42
60 years or more	26 (96.3%)	**1 (3.7%)**	27
Total	111(96.5%)	**4 (3.5%)**	115

P value: 0.8

6.1. DISCUSSION

Oral diseases are major health problems in Sudan; many studies relate this to the local life style and habits such as use of smokeless tobacco "Toombak", which is known to contain high level of the potent carcinogenic component of the tobacco (Ahmed et al, 2007).

This study was a cross sectional descriptive type, conducted in Khartoum, Sudan during the period 2011 to 2014. The aim of the study was to study the mitochondrial DNA mutation in oral lesions among Sudanese patients and relationship between histopathologic patterns and molecular changes of mitochondrial DNA. The study investigated 115 subjects, their ages ranged from 10 to 90 with a mean age of 47 years. Among those 115 participants, 61 (53.0%) were males and 54(47.0%) were females.

Based on the laboratory diagnosis our study showed different lesion patterns including normal, inflammatory, benign and malignant lesions. Of the 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 indicated that not all oral lesions were malignant, that may be due to early diagnosis of some cases before reaching advanced stage. The risk of malignant transformation varies from site to site within the mouth and from population to population. Pindborg et al 1975 reported in a classic study of over 30,000 Indian villagers, performed with follow-up over 7 years, that transformation rates between 10 and 24 / 100,000 per year.

According to the anatomical site of lesion, the study showed that the commonest site was salivary glands constituting 26 (22.6%) followed by mandible 22(19.1%). These findings agreed with a study by (Babiker, 2013) that found high frequency of oral lesions seen among Sudanese patients in salivary glands lesions.

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 (Amit and David2009). In this study majority of malignant cases was squamous cell carcinoma constituting (67.9%), followed by adenocarcinoma (18.5%), mucoepidermoid carcinoma (6.2%), Ameblastoma (4.9%) and lymphoma (2.5%).

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 presence of malignancy and age, there was positive relationship between tumor aggressiveness and age. Among the 46 individuals at age group < 40 years there were 27 cases constituting (58.7%). Of 42 individuals at age group 40 − < 60 years there were 29 malignant cases constituting (69%) and 92.6% of population at 60 years or more with malignancy.

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.

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

In the present study, of the 115 oral lesion patients, there were 4 (3.5%) showing 4,977 -bp deletion. This percentage of mutation was comparatively low (at level of p. value = 0.4) and could not indicate strong correlation between lesions and mutation. This finding matched to some extent with previous study by Chen lee reported that: 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 (Chen et al, 2009). Several authors stated that the majority of mtDNA mutations observed in tumors might not be involved in carcinogenesis (Salas et al,2005) while others mentioned that mtDNA mutations might have a role in the development of cancer but not in the progression (Kose et al,2005).

Other findings reported that the 4977 bp deletion is present at similar frequency in both normal and tumor tissue. Therefore, mtDNA4977 deletion, which is not specific to cancer, may reflect the environmental and aging-process influences operative during cancer progression (Dai et al, 2006).

In the present study, when the 4,977 -bp deletion correlated to Histopathological feature, all the 4,977 -bp deletion {4 cases constituting (100%)}were occurred among population having malignant lesions, and when classified as sub malignancy, 3 (75%) of mutation were occurred among cases of squamous cell carcinoma this may not confirm the really relation between 4977 bp deletion and type of malignancy particularly (SCC) because the majority of the malignant cases was Squamous cell

carcinoma which constituted 67.9%, This findings may agree with a report of (Liu et al,2011) that found there was 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.

6.2. CONCLUSION

The study provided that not all oral lesions underwent surgical operations were malignant. Although, the majority of malignant oral lesions were squamous cell carcinoma, there was a probability of other types of malignancy. The deletion of 4.977 bp observed in previous studies associated with many types of tumor worldwide such as colorectal and breast tumor, but there was no obvious significant correlation between 4.977 bp deletion and tumor among Sudanese with oral lesions in the present study, therefore other factors such as environmental risk factors, habitual, biological factors and nuclear genetic alterations may play major roles in oral carcinogenesis rather than 4.977 bp deletion of mitochondrial DNA.

6.3. RECOMMENDATIONS

A further study with wide scope in this topic is recommended among Sudanese suffering of oral lesions, considering other factors such as environmental, habitual, biological factors and nuclear genetic alterations which may contribute as risk factors with major roles in oral carcinogenesis in addition to mitochondrial DNA insertion and base substitution mutations.

Generally the noticed limitation of this study was missed data of the population in records of hospitals and diagnostic centers so cancer registry is recommended as an essential part for resolving cancer problem in Sudan. The high incidence of cancer invites wide popular awareness about risk factors through health education that importance for early diagnosis and treatment of oral lesions.

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APPENDICES

Appendix A: Hematoxylin & Eosin procedure

1. Take sections to water.

2. Place sections in haematoxylin for 8 minutes.

3. Wash in tap water.

4. Blue sections in lithium carbonate or tap water.

5. Wash in tap water.

6. Place sections in 1% acid alcohol for a few seconds.

7. Wash in tap water.

8. Stain with eosin for 1 minute.

9. Wash in tap water.

10. Dehydrate, clear.

11. Mount sections in DPX

Appendix B: DNA Extraction procedure

Principle of extraction

The QIAamp DNA FFPE Tissue procedure consists of 6 steps:

1. Remove paraffin: paraffin is dissolved in xylene and removed
2. Lyse: sample is lysed under denaturing conditions with proteinase K
3. Heat: incubation at 90°C reverses formalin crosslinking
4. Bind: DNA binds to the membrane and contaminants flow through
5. Wash: residual contaminants are washed away
6. Elute: pure, concentrated DNA is eluted from the membrane

Handling of QIAamp MinElute columns

Due to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling QIAamp MinElute columns to avoid cross contamination between sample preparations:

- Carefully apply the sample or solution to the QIAamp MinElute column. Pipet the sample into the QIAamp MinElute column without wetting the rim of the column.
- Always change pipet tips between liquid transfers. We recommend the use of aerosol-barrier pipet tips.
- Avoid touching the QIAamp MinElute column membrane with the pipet tip.
- After all pulse-vortexing steps, briefly centrifuge the microcentrifuge tubes to remove drops from the inside of the lids.
- Open only one QIAamp MinElute column at a time, and take care to avoid generating aerosols.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

Centrifugation

QIAamp MinElute columns will fit into most standard 1.5–2 ml microcentrifuge tubes. Additional 2 ml collection tubes are available separately. Centrifugation of QIAamp MinElute columns is performed at 6000 x g (8000 rpm) to reduce centrifuge noise. Centrifugation at full speed will not improve DNA yields. However, centrifugation of QIAamp MinElute columns at full speed is required in 2 steps of the procedure: the dry centrifugation step after the membranes are washed and the elution step. Centrifugation at full speed is also required to bring down the sample after the xylene treatment and the ethanol wash step.

All centrifugation steps should be carried out at room temperature (15 25°C).

Processing QIAamp MinElute columns in a microcentrifuge

- Always close QIAamp MinElute columns before placing them in the microcentrifuge. Centrifuge as described in the protocol.

- Flow-through fractions may contain hazardous waste and should be disposed of appropriately.

- For efficient parallel processing of multiple samples, we recommend filling a rack with collection tubes into which QIAamp MinElute columns can be transferred after centrifugation.

- Used collection tubes containing flow-through can be discarded, and the new collection tubes containing the QIAamp MinElute columns can be placed directly in the microcentrifuge.

Preparing Buffer ATL: Before starting the procedure, check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70°C with gentle agitation.

Preparing Buffer AW1: Add 25 ml ethanol (96–100%) to the bottle containing 19 ml Buffer AW1 concentrate. Tick the check box on the

bottle label to indicate that ethanol has been added. Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to 1 year.

Note: Before starting the procedure, mix reconstituted Buffer AW1 by shaking.

Preparing Buffer AW2: Add 30 ml ethanol (96–100%) to the bottle containing 13 ml Buffer AW2 concentrate. Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 1 year.

Note: Before starting the procedure, mix reconstituted Buffer AW2 by shaking.

Extraction Procedure:

1. Using a scalpel, trim excess paraffin off the sample block.

2. Cut up to 8 sections 5–10 μm thick (see "Starting material").

If the sample surface has been exposed to air, discard the first 2–3 sections.

3. Immediately place the sections in a 1.5 or 2 ml microcentrifuge tube (not supplied), and add 1 ml xylene to the sample. Close the lid and vortex vigorously for 10 s.

4. Centrifuge at full speed for 2 min at room temperature.

5. Remove the supernatant by pipetting. Do not remove any of the pellet.

6. Add 1 ml ethanol (96–100%) to the pellet, and mix by vortexing.

The ethanol extracts residual xylene from the sample.

7. Centrifuge at full speed for 2 min at room temperature.

8. Remove the supernatant by pipetting. Do not remove any of the pellet.

Carefully remove any residual ethanol using a fine pipet tip.

9. Open the tube and incubate at room temperature (15–25°C) or up to 37°C. Incubate for 10 min or until all residual ethanol has evaporated.

10. Resuspend the pellet in 180 μl Buffer ATL. Add 20 μl proteinase K, and mix by vortexing.

11. Incubate at 56°C for 1 h (or until the sample has been completely lysed).

12. Incubate at 90°C for 1h.The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA.

If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 90°C.

13. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the

14. Add 200 μl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200μl ethanol (96–100%), and mix again thoroughly by vortexing.

It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the QIAamp procedure.

15. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.

16. Carefully transfer the entire lysate to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp Min Elute column is empty.

17. Carefully open the QIAamp Min Elute column and add 500 μl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Min Elute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.

18. Carefully open the QIAamp MinElute column and add 500 μl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp Min Elute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

19. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely. This step is necessary, since ethanol carryover into the elute may interfere with some downstream applications.

20. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column and apply 20–100 μl Buffer ATE to the center of the membrane.

Important: Ensure that Buffer ATE is equilibrated to room temperature. If using small elution volumes (<50 μl), dispense Buffer ATE onto the center of the membrane to ensure complete elution of bound DNA.

QIAamp MinElute columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. The volume of eluate will be up to 5 μl less than the volume of elution solution applied to the column.

21. Close the lid and incubate at room temperature (15–25°C) for 1 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 1 min.

Incubating the QIAamp MinElute column loaded with Buffer ATE for 5 min at room temperature before centrifugation generally increases DNA yield.

Appendix C: Agarose Electrophoresis

Agarose electrophoresis is performed to visualize PCR products. This step determine whether PCR was successful, whether the resulting product is the correct size, whether other products were amplified as well, and whether the concentration of the resulting product is suitable for cycle sequencing.

 Precautions:

1. Always wear gloves! This is essential to limit the spread of PCR products around the lab as well as protecting against exposure to Ethidium Bromide.

 2. Treat Ethidium Bromide with respect (it is a powerful mutagen) and ensure that it does not get spread around the lab.

3. Wash hands when finished.

Materials

1. Agarose

2. 0.5X TBE

3. Weighing paper

 Equipment

1. Balance

 2. Erlenmeyer flask

3. Microwave

 4. Gel box with comb

Step 1: Mixing Gel

1. On the scale, weigh 1 gram of agarose onto a piece of weighing paper.

2. Add agarose to Erlenmeyer flask.

3. Add 100mL of 0.5X TBE to Erlenmeyer flask.

 4. Swirl vigorously to thoroughly mix agarose. Slurry will by opaque.

Step 2: Melting Gel

1. Put agarose and 0.5x TBE slurry into microwave.

 2. Heat on HIGH for 30 seconds at a time.

3. After 30 seconds, remove from microwave and swirl.

4. After 1 minute of heating, repeat heating and swirling procedure every 10 seconds until mixture is clear.

5. If the mixture begins to boil, stop and IMMEDIATELY remove the agarose. Swirl until mixture is clear. NOTE: Watch carefully to ensure that agarose mixture DOES NOT boil over in the microwave.

5. When the gel became cool enough add 2- 3 drops of ethdium promide.

Step 3: Pouring the Gel

1. Before pouring, gel must be cool enough for you to hold the Erlenmeyer flask in your hand. Pouring boiling hot gel will warp the gel boxes.

2. Place gel tray into casting chamber.

3. Add casting comb(s) into the appropriate slot(s).

4. Pour agarose into gel tray to about 5-7mm.

5. Let sit for at least 30 min, until gel is cool to touch and is opaque in appearance.

6. Once set, place gel and tray into gel rig, with wells on the left (cathode) side. Then, fill gel rig with 0.5X TBE sufficient to cover the entire gel.

7. Carefully remove combs by pulling them upwards firmly and smoothly in a continuous motion. The remaining depressions are the wells into which your samples will be loaded. NOTE: When pouring the gel, avoid creating bubbles as this will prevent current from flowing through the gel. NOTE: Do not pull comb out too quickly as wells will form holes, resulting in the loss of your sample.

Step 4: Loading the Gel

1. cut a piece of parafilm and place it flat on the bench top. You may have to rub or scratch it to stick it down.

2. Using a pipette, place small dots of 6X loading dye (about 1-2uL) onto parafilm, in rows of 8, 1 dot for each PCR sample that will loaded on the gel. It is not necessary to be exact, and it is not necessary to change the tip.

3. Using a pipette take 3 μl of product and pipette it onto its corresponding dye dot, then mix the sample and dye by pipetting up and down. Then, pipette up the dye/sample solution and pipette it into the proper well. NOTE: By convention, samples are loaded from left to right starting with well number 2, leaving the first well in each row of wells for the DNA ladder. NOTE: Insert pipette tip into well at an angle to avoid putting a hole in the bottom of the well.

4. After loading the first sample into the well, wash out your pipette tip by pipetting up and down several times in the buffer, then blot tip dry with a Kimwipe. In this way, all of your samples may be loaded using ONE tip.

5. Once all samples have been loaded into the gel, using a fresh tip, pipette 3uL of the DNA ladder into well number 1 of each row of wells.

6. Put gel box cover into place (this step is essential for your gel to run and to minimize the risk of electric shock). Turn on power supply. Run the gel for 75 minutes at 80 volts. 7. Check for bubbles at the cathode side to ensure that gel rig is running. Clean up, deglove, and wash hands. Tips to improve gels A. Do not shake the gel chamber while loading as this will facilitate diffusion of samples out of the wells B. Insert pipette tips into wells at an angle to avoid puncturing the well, which will result in loss of sample. C. Always make sure that your gel is running before leaving. D. Work as fast as you can without sacrificing accuracy and safety. The longer your samples sit in the buffer, the more diffuse your bands will be when you visualize the gel. E. Until you are comfortable, only load 1 row of samples at a time, run the gel for 5 minutes, then load the next row of samples. This will minimize sample diffusion.

Step 5: Visualizing the Gel

1. Turn off power supply. Using gloved hands, remove the cover from the gel box.

2. Remove the gel and casting tray from rig, avoiding dripping buffer all over the benchtop.

3. Carefully slide the gel off of the casting tray and into ethidium bromide solution. Caution: ethidium bromide is a mutagen and should be treated with respect. If you get ethidium bromide on you gloves, immediately change your gloves, placing the contaminated gloves into

the ethidium bromide trash. Do not spread ethidium bromide around the lab. .

4. Using the spatula, carefully remove the gel from the destaining buffer and place the gel on UV light box, avoiding creating bubbles underneath the gel.

5. Put on UV protecting face shield and turn on UV light box to see stained sample bands and DNA ladder. Ensure that all around you are protected for UV exposure.

6. If bands are strong, take a picture using the Polaroid camera (below). If bands are weak, return gel to ethidium bromide stain bath as the gel has been insufficiently stained. If the ladder is strong, but the PCR bands are weak, this indicates that the PCR reaction did not work well. Consult professor or TF on how to improve your PCR.

Step 6: Taking a picture.

Remember that the gel has been soaking in Ethidium Bromide. All objects and surfaces that the gel comes into contact with will be contaminated with Ethidium Bromide. Always wear gloves and limit the spread of Ethidium Bromide to protect yourself and your lab mates.

1. Ensure that you are still wearing your gloves and UV face shield.

2. With the gel flat on the UV light box, position the hood of Polaroid camera over the gel so that the hood is centered over the gel.

3. Turn on the UV light box.

4. Keeping the camera steady, pull the trigger and HOLD the trigger until there is silence (i.e. the shutter has opened and closed. This may take 0.5-2.0 seconds depending on the cameras exposure settings).

5. Turn off the UV light box.

6. Pull WHITE film tab on camera to remove film. Wait 1 minute

7. After one minute has passed, pull backing off of film to check whether the gel imaged properly.

8. Once picture is developed and you have a good picture of your gel, remove your gel and put in gel disposal box.

9. Wipe down the UV light box.

Remember that all of these surfaces and wipes are contaminated with Ethidium Bromide.

10. Remove the gloves and dispose of in the Ethidium Bromide trash.

11. Wash hands thoroughly.

12. Label the picture.

Appendix D: Agarose gel electrophoresis of PCR Product
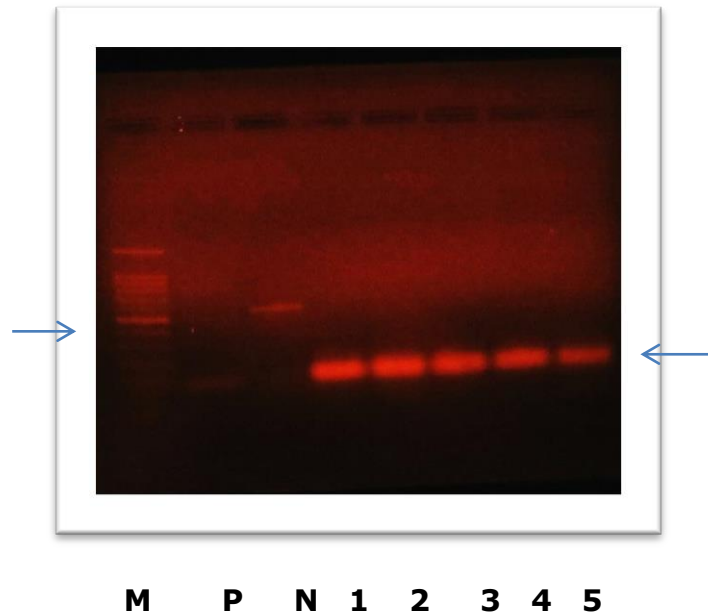

M   P   N   1   2   3   4   5

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

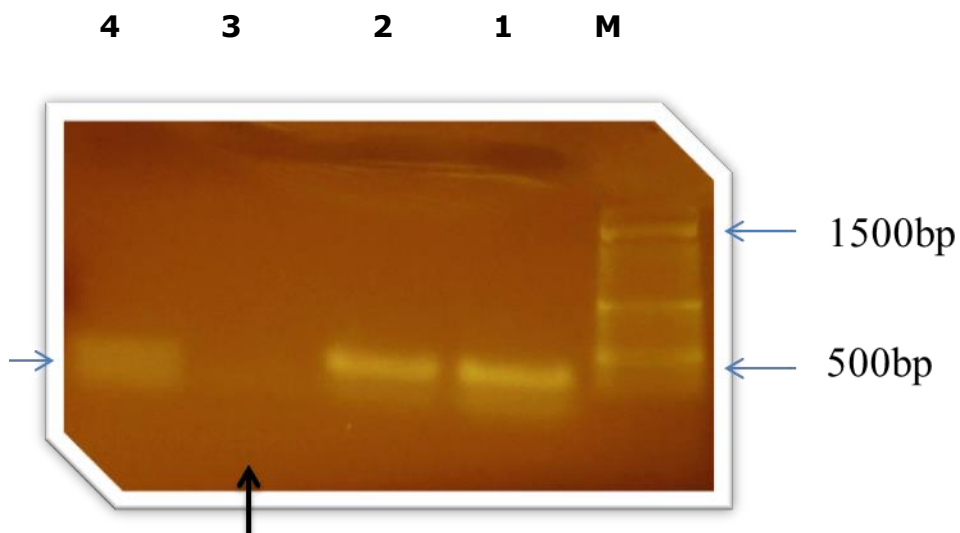**4   3   2   1   M**

1500bp

500bp

Photo (2) 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.

Appendix E: Maxime PCR Premix Kit (i-Taq)

Appendix F: Manuscripts