FACULTY OF MEDICINE AND DENTISTRY

Department of Clinical Dentistry

Department of Biomedicine

The Gade Institute

Centre for International Health

CELL CYCLE REGULATORY PROTEINS IN ORAL EPITHELIAL DYSPLASIA AND ORAL SQUAMOUS CELL CARCINOMAS FROM SUDAN; ASSOCIATION WITH *TOOMBAK*-USE

Thesis submitted to the Faculty of Medicine and Dentistry, University of Bergen in partial fulfilment of the requirements for the degree of Master of Philosophy in Dentistry

Munna Jaffar M. Ibrahim

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To my Father Jaafar Ibrahim and to the memories of my mother Siham Elmoghrabi, my brother Ahmed Ibrahim and my aunt Lila Elmoghrabi, whose foundation in my life has made me who I am today.

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Bergen 28th February 2008

Munna J. Ibrahim

Summary

In Sudan, oral squamous cell carcinoma (OSCC) has been found to be associated with the use of the Sudanese snuff, locally known as *toombak*. Expression of the cell cycle regulatory proteins, $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 have been found to be changed in OSCCs compared to normal oral mucosa (NOM). In this work, we aimed to investigate the expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1, in potentially malignant and malignant oral lesions related to *toombak*- use. Formalin fixed, paraffin embedded tissue specimens from 104 subjects with OSCCs and 20 with oral epithelial dysplasia (OED) from users and non users of *toombak* were studied by immunohistochemistry (IHC). Ten NOM from non-users were included as controls. A significant difference in the expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 was found between NOM, OED and OSCC ($p<0.001$). In the OSCCs, expression of $p21^{WAF1}$ was significantly higher in *toombak*- users compared to non- users ($p<0.001$). Compared to NOM, the expression of $p16^{INK4a}$ was found to be lower in both OSCCs and OED ($p<0.001$), ($p<0.006$) respectively. Expression of $p16^{INK4a}$ was found to be significant ($p = 0.031$) when related to the histological differentiation of the OSCCs and particularly in the *toombak*– users ($p = 0.040$). There was a high expression of Cyclin D1 in the OSCCs compared to both NOM and OED ($p<0.001$), but there was no significant difference between *toombak*- users and non- users. In conclusion, the expression of the cell cycle regulatory proteins $p21^{WAF1}$ and Cyclin D1 was found to be increasing from NOM to OED to OSCCs. Patients with OSCCs who were *toombak* users had high expression of $p21^{WAF1}$. Our findings suggest that the difference in expression of these proteins may be important in potentially malignant and malignant oral lesions. In particular, the findings related to $p21^{WAF1}$ indicate its possible role in the transformation process leading to malignancy in the habitual *toombak* users.

Abbreviations

Cdk	Cycline dependent kinase
Cdki	Cycline dependent kinase inhibitor
G0	Gap 0 phase of the cell cycle
G1	Gap 1 phase of the cell cycle
G2	Gap 2 phase of the cell cycle
H&E	Haematoxylin and eosin stain
IARC	International Agency for Research on Cancer
IHC:	Immunohistochemistry
MD	Moderately differentiated squamous cell carcinoma
NOM	Normal oral mucosa
OED	Oral epithelial dysphasia
OSCC	Oral squamous cell carcinoma
OSCCs	Oral squamous cell carcinomas
PAH	Polycyclic aromatic hydrocarbons
PD	Poorly differentiated squamous cell carcinoma
Rb	Retinoblastoma
S	Syntheses phase of the cell cycle
SCC	Squamous cell carcinoma
ST	Smokeless tobacco
TSNAs	Tobacco-specific nitrosamines
UICC	International Union Against Cancer
WD	Well differentiated squamous cell carcinoma
WHO	World Health Organisation

Introduction

Oral squamous cell carcinomas

Epidemiology

Oral squamous cell carcinoma (OSCC) is a global health problem. Of the 615,000 new cases of head and neck SCCs reported worldwide in year 2000, 300,000 were primary found to occur in the oral cavity (Parkin et al., 2005; Kademani, 2007). The overall mortality rate for intra-oral SCC is reported to be about 50% (Noonan and Kabani, 2005). In Africa, Sudan has a particularly high incidence rate of OSCC (11.60 for males and 6.91 for females) (Idris et al., 1995b). OSCC has been found to demonstrate a non-uniform pattern of distribution, both geographically and demographically, with an alarming number of cases occurring in the developing countries (Boyle and Ferlay, 2005; Ferlay et al., 2007). This observation might reflect an environmental, cultural and/or habitual influence in the prevalence of this disease (Day et al., 2003; Copper et al., 2003). For example, the high incidence rate of OSCC reported in Australia and New Zealand, mainly lip SCC, has been suggested to be related to solar irradiation (Parkin et al., 1999; Walker et al., 2003). In Sudan, OSCC is strongly attributed to the habit of extensive use of *toombak* – a form of snuff commonly used in Sudan (Elbeshir et al., 1989; Idris et al., 1991; Idris et al., 1992; Idris et al., 1994; Idris et al., 1995b).

OSCC affects predominately individuals with low socio-economical status. This distribution is usually associated with use of tobacco, heavy alcohol consumption and deficient diet (Johnson, 1991; Hashibe et al., 2003; Hobdell et al., 2003). The International Agency for Research on Cancer (IARC) confirmed that smoking of the various forms of tobacco (cigarettes, cigars, pipes, etc) is carcinogenic to humans (IARC, 1986). Other forms of tobacco, known as smokeless tobacco (ST), are also reported to be associated with an increased risk of developing OSCC (IARC, 1986). A recent report from the Sudan Federal

Ministry of Health in 2000, illustrated that tobacco-related cancers are evident in the list of the 10 most common cancers (Hamad, 2006).

A low intake of fresh fruits and vegetables has been linked to an increased risk of OSCC (McLaughlin et al., 1988; De et al., 1999). Although the individual micronutrients responsible for that, have not been formally identified, vegetables and fruits that protect against oral cancer are rich in β-carotene, vitamin C and vitamin E with anti-oxidant properties (Walker et al., 2003).

Clinical presentation and diagnosis

Despite the accessibility of the oral cavity to direct clinical examination, OSCCs are not easily detectable (Pereira et al., 2007). Patients with OSCCs usually seek consultation after developing severe and persistent pain with the most frequent complaint "irritation/ burning sensation" in the mouth. Early OSCC may be painless or associated with only a mild degree of irritation. Pain usually occurs when the lesion becomes ulcerated, but sometimes, although rarely, a patient might seek consultation because of a "lump in the neck" that represents a metastasis from an oral lesion of which the patient may be completely unaware (Silverman et al., 2003). The symptoms and signs however, may reflect an identifiable pathological process. The common signs and symptoms attributed to OSCCs include;

- ***Ulceration or erosion:*** This sign of the disease reflects the destruction of the epithelial integrity due to discrepancy in cell maturation, loss of intercellular attachments, and disruption of basemen membrane as seen in (Fig.1)
- ***Erythema:*** Illustrates redness reflecting inflammation, thinness and irregularity of epithelium.
- ***Induration:*** Demonstrates hardness due to an increase number of cells and an

inflammatory infiltrate.

- ***Fixation:*** Fixation to underlying tissues occurs when abnormally dividing cells invade to deeper areas of muscle and bone.
- ***Chronicity:*** Indicating failure to heal.
- ***Lymphadenopathy***: Hardening and enlargement of regional nodes due to engorgement with neoplastic cells that spread by lymphatic vessels. Lymph nodes are usually painless and often become fixed because of capsular erosion and local infiltration. Tumours that involve marked induration, fixation, and lymphadenopathy are signs of advanced cancer.

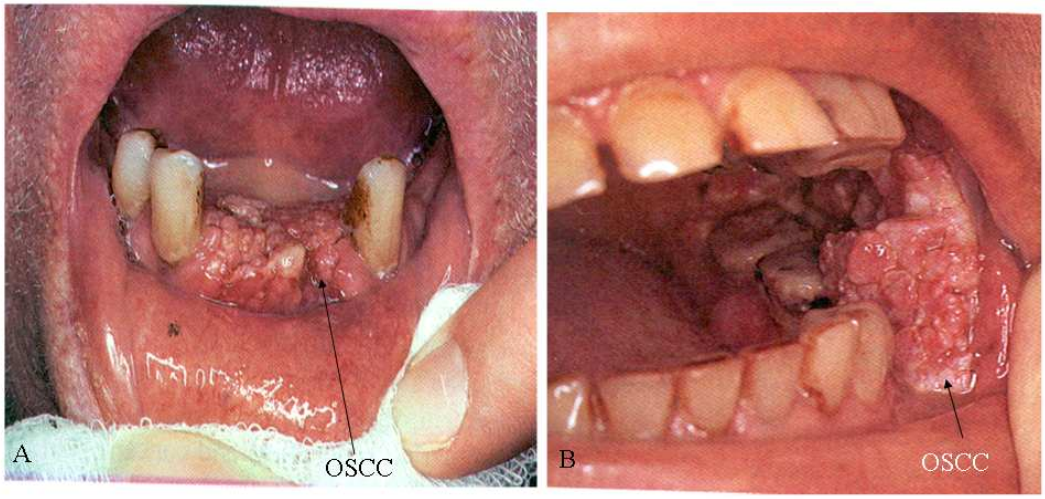

Fig.1. Examples of oral squamous cell carcinoma lesions illustrating destruction of the oral mucosa at two different sites (A: Labio-sulcus; B: Buccal pouch) of *toombak* dipping.

Histological classification/ criteria

OSCCs are usually characterized by ulcerated appearance, high degree of local invasion and a high rate of metastasis to the regional cervical lymph nodes. The histopathological features (Fig.2) show variable degree of keratinisation, pleomorphism and mitotic activity. Therefore the histopathological classification of OSCCs is based on a method which takes

into account an assessment of the degree of keratinisation, cellular and nuclear pleomorphism and mitotic activity (Barnes L et al., 2005). Accordingly, three grades, based on the degree of differentiation of the lesion, have been commonly in use:

Grade 1	Well differentiated
Grade 2	Moderately differentiated
Grade 3	Poorly differentiated

The well and moderately differentiated OSCCs can be grouped together as low grade and poorly differentiated and undifferentiated ones as high grade. Generally, the histopathological grading of malignancy has limited impact on the survival rate due to the subjective nature of the assessment of the various features involved. Moreover, the cancer cells frequently show local recurrence after initial treatment, probably due to microinvasion and/or micrometastasis of the tumour cells around the primary site. Therefore, the average five-year survival rate of OSCC has not improved over the last few decades and remains as poor; 20% to 50%, depending on the clinical stage of the tumour (Casiglia and Woo, 2001).

The TNM (Tumour, Node and Metastases) staging system which was developed and maintained by the International Union Against Cancer (UICC) (Carinci et al., 1999) is a universally accepted tool for staging of OSCCs. However, it does not take into account the histopathology and the biology of the tumour (Rapidis et al., 1977; Partridge, 1999). Therefore, the terms Site (S) and histopathology (P) were added in a staging system denoted as STNMP that correlated with survival (Rapidis et al., 1977). However, due to the limited prognostic value(s) of the STNMP staging in OSCCs, many patients are still over-or under-treated, resulting in significant personal and socioeconomic impact (Bankfalvi and Piffko, 2000). Intriguingly however, two well-established histological predictive factors — tumour

thickness and extracapsular spread of nodal metastases — have not become part of the routine TNM classification (Woolgar, 2006).

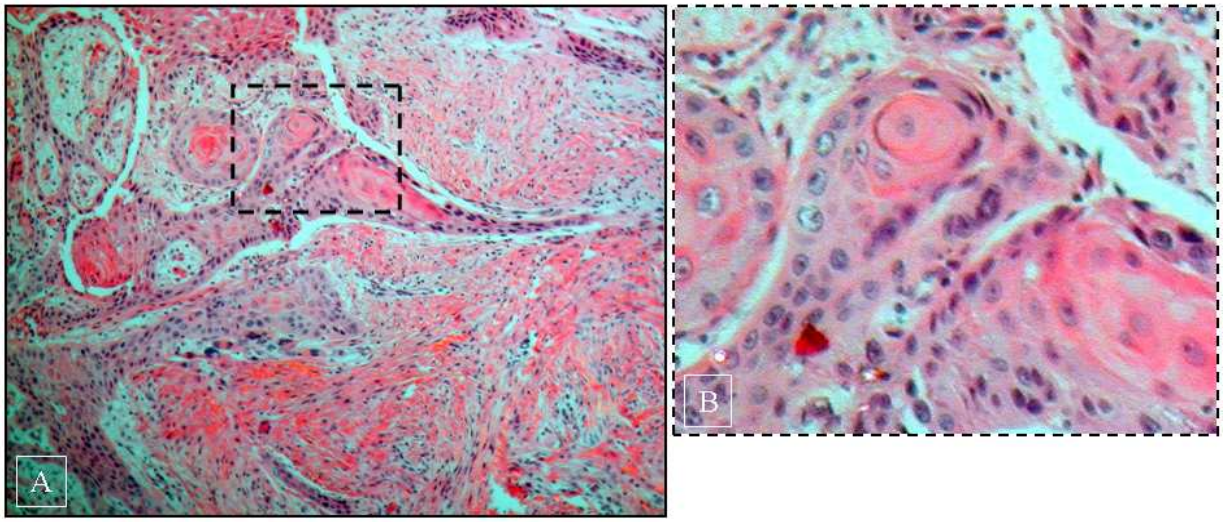

Fig.2. Histopathological appearance of an oral squamous cell carcinoma lesion (panel A, original magnification x150) and (panel B, original magnification x320) showing strands of malignant epithelial cells forming keratin pearls in a moderately well differentiated tumour.

Treatment and prognosis

Despite an intensive research efforts to develop new therapeutic strategies for OSCCs, the five-year survival rate of patients with this pathology has not been improved over the past 3 decades and remains at 20- 50% (Noonan and Kabani, 2005; Ayllon et al., 2008). The standard treatment for patients with potentially malignant oral lesions or with an aggressive malignant oral lesion varies from observations and follow up of patients to complete surgical removal of the lesion (Lodi et al., 2004). The combination of surgery/chemotherapy or radiotherapy is commonly in use for treatment of OSCCs. These lesions are good candidates for gene therapy because primary and recurrent lesions are readily accessible for injection or application of the therapy (Xi and Grandis, 2003).

Potentially malignant oral lesions

Over 90% of the OSCCs are often preceded by a pre-existing potentially malignant lesion (Noonan and Kabani, 2005).The terms precursor lesions, premalignant, intra epithelial neoplasia and potentially malignant lesions have been used in the literature to broadly describe the clinical presentations of these lesions that may have the potential to become cancer. All these terms convey the concept of a two-step or multi-step process of cancer development. The term "potentially malignant disorders", is recently recommended to refer to these lesions as it conveys that, not all lesions described under this term may transform into cancer, but that there is a family of morphological alterations amongst which some may have an increased potential for malignant transformation (Warnakulasuriya et al., 2007). Although most of the patients with OSCC present without prior diagnosis of a potentially malignant lesion, two main types of potentially malignant lesions are well documented: leukoplakias (white patches) (Fig.3) and erythroplakias (red patches) (Fig.4), which both are much more likely to show histological features of dysplasia and possibility to progress to OSCC (Reichart and Philipsen, 2005). It is common to state that OSCCs are often clinically preceded by potentially malignant lesions, mainly leukoplakia, although the proportion of the cases that develop through clinically recognizable potentially malignant stages is not known (Speight and Morgan, 1993). The presence and degree of epithelial dysplasia is generally accepted as the best indicator of malignant potential of leukoplakia (Mincer et al., 1972). Although less common than leukoplakia, erythroplakia poses a greater threat of transformation into malignancy, and the lesions usually demonstrate significant epithelial dysplasia, carcinoma *in situ* or invasive SCC (Barnes L et al., 2005) (Fig.4) A novel binary grading system (high/low risk) for OED was compared with the WHO classification system from 2005 (Kujan et al., 2006). This showed a satisfactory agreement on the distinction of the mild dysplasia from

severe dysplasia and from carcinoma *in situ*, but assessment of moderate dysplasia remained a problem. The sensitivity and specificity of the new binary grading system for predicting malignant transformation in OED were 85% and 80%, respectively and the accuracy was 82% (Kujan et al., 2006).

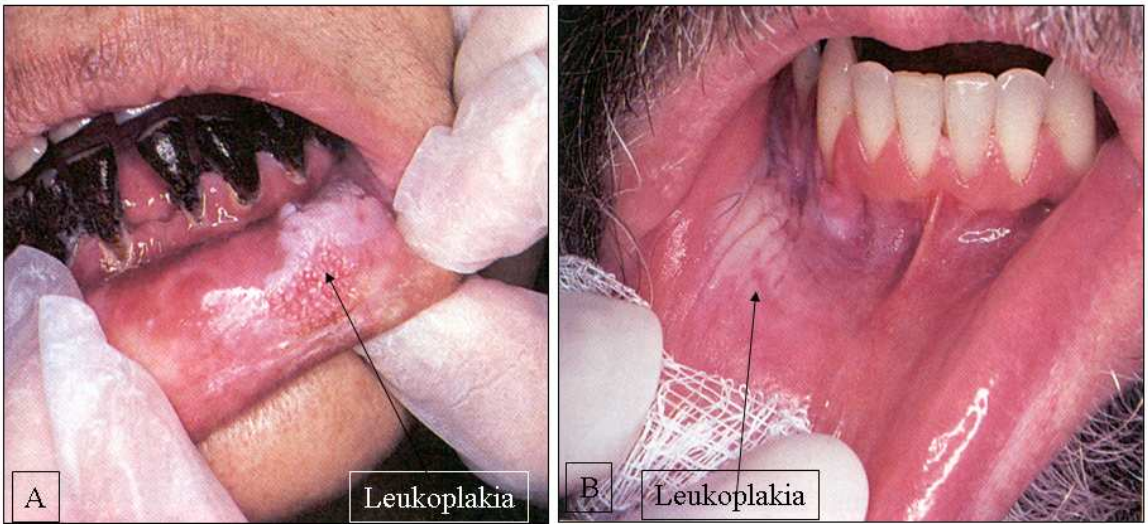

Fig.3. This figure illustrates the clinical appearance of two white lesions (leukoplakias) developing at two different sites of *toombak* dipping (A & B).

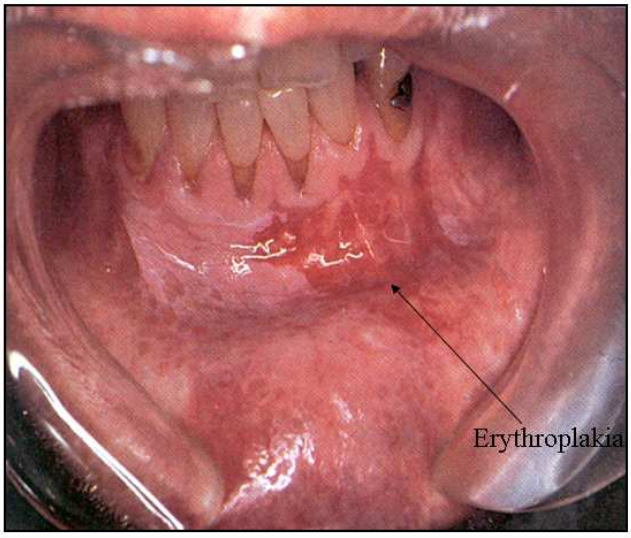

Fig.4. This figure demonstrates the clinical appearance of a serious red lesion (erythroplakia) developing at the site of *toombak* dipping.

The Sudanese toombak

Smokeless tobacco (ST) constitutes a wide range of habits and products used in many parts of the world (Johnson, 1991). Although the WHO reported that habits of snuff dipping/tobacco chewing are practiced by millions of people in Africa, most of the information on these habits and their relation to oral cancer is either from western Europe, North America and/or South East Asia (Johnson, 1991; Boffetta and Parkin, 1994). In Sudan, ST is commonly used in a form of oral dipping tobacco locally called *toombak* which was introduced approximately over 400 years ago (Ahmed and Mahgoob, 2007). The tobacco used for the manufacture of *toombak* is of the species *Nicotiana rustica* (Fig.5), and the fermented ground powder is mixed with an aqueous solution of sodium bicarbonate. The resultant product is processed into a loose moist form with a strong aroma and its use is widely spread in the country. *Toombak*, or Sudanese snuff, differs from other types of snuff used in Scandinavia and the USA when it comes to tobacco species, fermentation and aging, methods of manufacturing, pH value, moisture, nicotine, and nitrosamines content (Idris et al., 1998a).

Fig.5. Tobacco plant of the species *Nicotiana rustica*

Over one-third of the adult males in Sudan are regular users of *toombak*, and the

prevalence of the habit among the older men in rural areas is close to 50% (Idris et al., 1998b). *Toombak* use is uncommon among the females, as it is considered to be a social stigma. Albeit, there are some female users in particular older females.

There are approximately 5 million users of *toombak* in Sudan (Elbeshir et al., 1989; Idris et al., 1994). It has been reported that more than 81% of the *toombak* users in Sudan do not smoke cigarettes (Elbeshir et al., 1989; Idris et al., 1991; Idris et al., 1992; Idris et al., 1994; Idris et al., 1995b).

The factors which are believed to have significant adverse health consequences in *toombak*, particularly addiction and oral cancer development, are the pH value and the high levels of the tobacco-specific nitrosamines (TSNAs) (Idris et al., 1998a). The *toombak* pH range is 8-11, with a moisture content in the range of 6-60% and nicotine content is from 8 to 102 mg/g dry weight and TSNAs contents in micrograms (N'-nitrosonornicotine NNN 420-1 550; 4-(methyl-nitrosamino)-l-(3- *pyridyl*)-l- *butanone* NNK 620-7 870; N'-nitrosoanatabine NAT 20-290) (Ahmed and Mahgoob, 2007).

Compared with snuff from Sweden and USA, *toombak* contains a 100-fold higher levels of the TSNAs (Cogliano et al., 2004), which – together with their metabolites- are considered to be the major groups of carcinogens (Idris et al., 1991; Ibrahim et al., 1999; Loro et al., 2000; Ibrahim et al., 2002). The metabolites of TSNAs, particularly NNN and NNK are found to be present in the saliva of *toombak* dippers, as well as in their body fluids (Idris et al., 1991; Idris et al., 1992; Idris et al., 1998a).

The second highest relative frequency (17%) of OSCCs in Africa, is reported from Sudan, which is strongly attributed to extensive use of *toombak* (Elbeshir et al., 1989; Idris et al., 1991; Idris et al., 1992; Idris et al., 1994; Idris et al., 1995b). .This high relative frequency has been documented by epidemiological studies that suggested a strong causal association

between *toombak* use and development of oral cancer and the adverse oral health effects including increased gingival recession and teeth discoloration (Elbeshir et al., 1989; Idris et al., 1991; Idris et al., 1992; Idris et al., 1994; Idris et al., 1995b) (Fig.3). Moreover, it has been shown that 80% of the patients with OSCCs are *toombak* users (Elbeshir et al., 1989; Idris et al., 1991; Idris et al., 1992; Idris et al., 1994; Idris et al., 1995a; Idris et al., 1995b). *Toombak* dipping has also been suggested to be associated with neoplasm of salivary glands (Elbeshir et al., 1989; Idris et al., 1991; Idris et al., 1992; Idris et al., 1994; Idris et al., 1995b) and is a potential risk factor for development of oesophageal and lung cancer (Idris et al., 1994).

During *toombak* dipping, and when a Sudanese individual consumes 1 gram of *toombak* 10 times a day (this is a conservative estimates since 1 gram of *toombak* lasts only 15- 30 minutes of average in the oral vestibule), more than 80% of the TSNAs are extracted from *toombak* by saliva and the negative pressure created by the sucking effect of the *toombak* quid placed in the labio-sulcus region. The user in this case will be exposed to an average of 11.3 mg of NNN and 23.1 mg of NNK per day (Idris et al., 1991; Idris et al., 1994). It is possible therefore to claim that in over one year an average *toombak* user in Sudan will be exposed to high levels of these carcinogens, which are high enough to induce OSCCs if administered to experimental animals (Hecht et al., 1986; Hoffmann et al., 1996). *Toombak*- users usually develop clinically and histopathologically characteristic lesions (leukoplakia) at the site of dipping. Oral leukoplakia caused by ST has been reported in Sweden, USA and Sudan (Roed-Petersen and Pindborg, 1973; Jungell and Malmstrom, 1985; Greer et al., 1986; Andersson et al., 1989; Daniels et al., 1992; Idris et al., 1996).Chronic exposure of the oral cavity to tobacco, often results in the development of potentially malignant lesions, mainly leukoplakia, 5–10% of which might undergo malignant transformation to OSCC, over a period of 2–10 years (Ralhan et al., 2006).

The Cell cycle

The cell reproduces through an orderly sequence of events which culminates in the generation of two new cells (Blomen and Boonstra, 2007). This cycle of events is known as "the cell cycle". The principle function of the cell cycle is to replicate DNA and to segregate the replicated chromosomal DNA into two daughter cells. The cell cycle is divided into 4 major phases. The first phase is defined as the gap1 (G1) phase, during which the cell makes the decision to either proceed to DNA syntheses or to exit the cell cycle into a quiescent state called (G0). Cells in the G0 phase account for the major part of the non-growing, non-proliferating cells in the human body. Once the decision to start DNA replication has been made, the cell becomes irreversibly committed to complete the cell cycle. The time in the late G1 phase at which this decision is made is designated the "restriction point" (Pardee, 1989). The cell then proceeds to the so called the synthesis (S) phase in which a copy of a cell's DNA is replicated. The cell then, in the gap 2 (G2) phase, is ready to segregate its replicated DNA. G1 and G2 phases together provide additional time for the cell to grow and duplicate its cytoplasmic organelles. G1, S and G2 phases constitute the inter-phase of cell division, which is the period between one mitosis phase and the next one. Segregation of the DNA takes place in the mitotic (M) phase, which is divided into four stages (prophase, metaphase, anaphase and telophase) depending on the stage of DNA segregation and degree of cytokinesis. The progression from the G1→S→G2→M→G1 is controlled by a number of proteins (Blomen and Boonstra, 2007). This control is essential to ascertain genomic replication without fault and to assure the equal segregation of the DNA into two daughter cells.

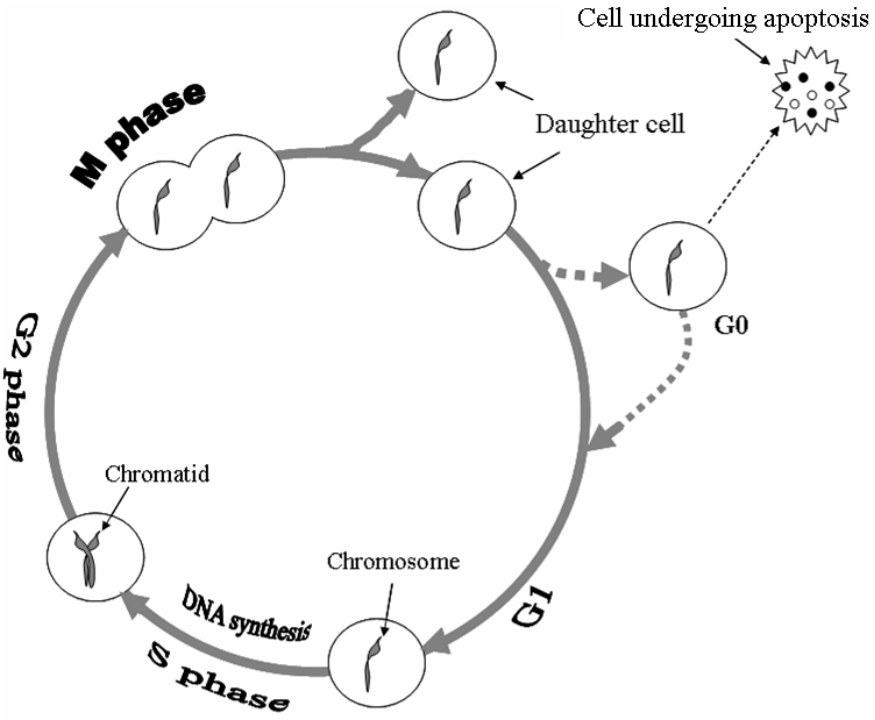

Fig.6. A schematic overview illustrating the different cell cycle phases

Cell cycle checkpoints

The cell has developed a process through which it can monitor the cell cycle to ensure proper replication and segregation of the DNA. This process consists of 4 checkpoints in the cycle as shown in (Fig.6) A checkpoint is defined as a biochemical pathway that ensures dependence of one process upon another process that is otherwise biochemically unrelated (Elledge, 1996). Checkpoints refer usually to points in the cell transitions that ensure the fidelity of cell division (Elledge, 1996). Control mechanisms at these checkpoints ensure that chromosomes are intact and that each phase of the cell cycle is completed before the succeeding one starts.

Apoptosis

The process of apoptosis is the genetically regulated form of cell death that permits the safe disposal of cells at the time when they have fulfilled their intended biological functions

(Afford and Randhawa, 2000; Loro et al., 2003). The term apoptosis was introduced by John Kerr in 1972 (Kerr et al., 1972). Apoptosis is known to be directly stimulated by the p53 tumour suppressor protein (Balint and Vousden, 2001; Ryan et al., 2001). Stimuli, such as DNA damage, activate the p53 protein to induce cell cycle arrest or apoptosis (Bargonetti and Manfredi, 2002) and selectively, eliminates stressed or damaged cells and thereby protecting the organism from development of cancer. In order to facilitate this process, apoptosis should be tightly coupled to the cell cycle checkpoints (King and Cidlowski, 1995). In almost all instances of human cancer development, dysregulation of the cell death (apoptosis) and the cell proliferation have been found to play a major role in the process of carcinogenesis (Carson and Ribeiro, 1993; Wyllie, 1997).

Cell cycle regulatory proteins

The progression of the cell cycle through the different phases is controlled by proteins called cyclines (Sherr, 1993; Hartwell and Kastan, 1994). There are different types of cyclines (A- J) each of which acts at a certain phase of the cell cycle. In order for the cyclines to function, they have to form complexes with cycline dependent kinases (cdk); cdk1, cdk2….cdk8. (Nurse, 1990; Reed, 1992; Sherr, 2000). These complexes are denoted (cdkc). There are three subtypes of Cyclin D (D1, D2 and D3) which are expressed through the cell cycle in response to mitogenic signals. Together with its catalytic partners cdk4 and cdk6, Cyclin D1 accelerates G1 progression by phosphorylating the retinoblastoma protein (RB) (Todd et al., 2002) (Fig.7).

Cdk activity can be counteracted by the cell cycle inhibitory proteins, called cdk inhibitors (cdki) which bind to the complexes of the cycline-cdk and inactivate the cdkc (Ekholm and Reed, 2000; Hall et al., 2007). Two distinct families of the cdk inhibitors have

been identified, the INK4 family and Cip/Kip family (Vermeulen et al., 2003). The INK family is composed of four members: $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$, which specifically inactivate the G1 cdk (cdk4 and cdk6). The second family of inhibitors, the Cip/Kip family, includes $p21^{WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$ (Ekholm and Reed, 2000).

Cell cycle regulatory proteins and oral carcinogenesis

The multistep process of oral carcinogenesis involves functional alterations of the cell cycle regulatory proteins combined with the escape from the cellular quiescence and apoptotic signalling pathways (Todd et al., 2002; Jayasurya et al., 2005). Uncontrolled cell proliferation is the hallmark of cancer development, and tumour cells have typically acquired damage to genes that directly regulate their cell cycles (Pavelic et al., 1996; Lundberg and Weinberg, 1999). Dysregulation of the cell cycle, apoptosis and the cell-cell/cell-matrix adhesions are the pathways mainly influencing this multistage event (Lo et al., 2007). Several studies have shown altered patterns of expression of the cell cycle regulators in OSCCs (Pavelic et al., 1996; Pande et al., 1998; Pande et al., 2002). The two well-defined pathways that are shown to be prominently altered in a variety of cancers including OSCCs are the cell cycle regulatory pathways led by either *p*53 or Rb genes (Jayasurya et al., 2005). Therefore, these two pathways are considered as important interconnected biochemical pathways that are frequently found to be changed in OSCCs and were suggested to be a near universal target in oral carcinogenesis (Okami et al., 1999).

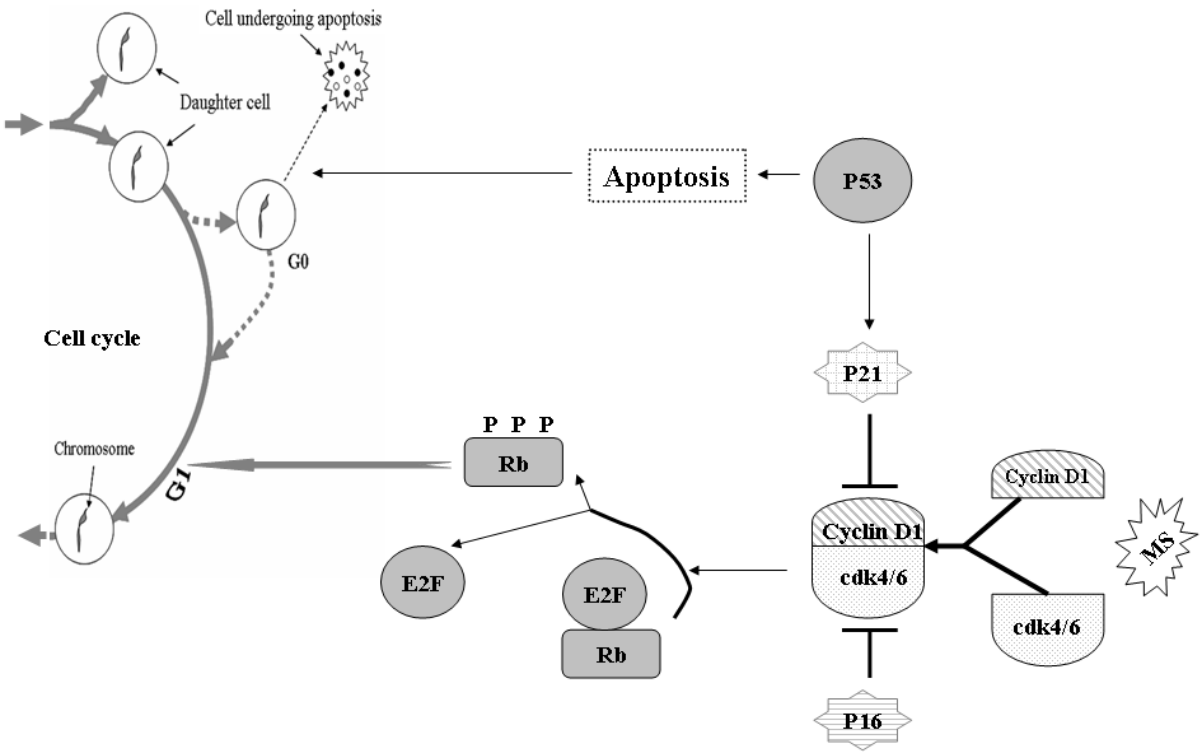

Fig.7. A schematic overview showing part of the cell cycle and the essential steps involved in the cell cycle regulation. The role of the major components of the *p*53 and the retinoblastoma (Rb) pathways and the site of the activity of the regulatory cdk/cyclin complexes is also indicated. (→: activation, ⊥: inhibition, MS: mitogenic signal)

p21^{WAF1}

p21^{WAF1}, a cell cycle regulatory protein encoded by the *p21*^{WAF1}gene, is the first identified inhibitor of the cyclin/cyclin dependent kinase (cdk) complex (Harada and Ogden, 2000). p21^{WAF1}attracted a great deal of attention because it sheds further light on the function of the p53 as well as it is involved in some interactions and biological functions, going far beyond the control of the cell cycle (Dotto, 2000). It is believed that the inhibitory effects on the cell division by p21^{WAF1} are closely related to the cell differentiation, senescence, cancerous change of normal cells, regulation and induction of apoptosis involving p53 (Harada and Ogden, 2000). Several *p21*^{WAF1} mutations have been reported in human cancers, and some of these mutations were shown to abrogate the p21^{WAF1} activity as cdk inhibitor

(Roninson, 2002).

The cdk inhibitor $p21^{WAF1}$ is a downstream component of the p53 pathway and it mediates the cell cycle arrest after genotoxic stress (el-Deiry et al., 1993; Yeudall and Jakus, 1995; Harada and Ogden, 2000; Dotto, 2000). Therefore, $p21^{WAF1}$ has a tumour suppressor ability, demonstrated in OSCC model experiments, in which overexpression of the $p21^{WAF1}$ suppresses tumour growth (Cardinali et al., 1998). However, several functions of the $p21^{WAF1}$ are likely to promote carcinogenesis and tumour progression, such as its ability to inhibit apoptosis and the expression of genes involved in the quality control of mitosis, and to promote the assembly of active cyclin-cdk complexes (Roninson, 2002). Furthermore, it has been reported that $p21^{WAF1}$ expression increases in OSCC (Erber et al., 1997; Agarwal et al., 1998; Tatemoto et al., 1998; Nemes et al., 2005), suggesting that this protein alone may not be sufficient to arrest the cell cycle and inhibit tumour cell growth (Ng et al., 1999; Regezi et al., 1999; Yuen et al., 2001; Yanamoto et al., 2002). In addition, $p21^{WAF1}$ overexpression has been reported in lower lip SCCs (Horta et al., 2007).

$p16^{INK4a}$

Since its discovery as an inhibitor of the cdk 4/ 6, the tumour suppressor $p16^{INK4a}$ continued to gain attention in cancer research (Kato et al., 2000). $p16^{INK4a}$ is one of the direct links between cell-cycle control and cancer development (Serrano, 1997). The mechanisms that regulate $p16^{INK4a}$ have been characterized (Serrano, 1997). Changes that are commonly observed in human cancers are related to mutation, homozygous deletions and/or hypermethylations of the promoter region of the tumour suppressor gene $p16^{INK4a}$. Inactivation of the $p16^{INK4a}$ gene through these mechanisms may lead to loss of the gene product $p16^{INK4a}$ (Reed et al., 1996). The high frequency of deletions of $p16^{INK4a}$ found in

tumour cell lines first suggested an important role for $p16^{INK4}$ in human carcinogenesis. This initial genetic evidence was subsequently strengthened by numerous studies demonstrating inactivation of the $p16^{INK4a}$ in several human cancers, and it is now believed that loss of the $p16^{INK4a}$ is an early and often critical event in tumour progression (Rocco and Sidransky, 2001).

Inactivation of the $p16^{INK4a}$ was found to occur at the early stage of the multistep process of oral tumourigenesis (Vairaktaris et al., 2007) and is a frequent event in squamous cell carcinomas of the head and neck (Reed et al., 1996; Ogawa et al., 2006). Aberrant methylation of $p16^{INK4a}$ is relatively frequently observed in OSCC. This also suggests that loss of $p16^{INK4a}$ expression is a true oncogenic event (Auerkari, 2006). It has been found that head and neck squamous cell carcinomas were $p16^{INK4a}$ immunostaining- negative and that the absence of $p16^{INK4a}$ immunostaining was correlated with a homozygous deletion of the *$p16^{INK4a}$* gene or hypermethylation of the *$p16^{INK4a}$* gene promoter (Reed et al., 1996).

Cyclin D1

Cyclin D1 is expressed through the cell cycle in response to mitogenic stimulation and it interacts with cdk4 and cdk6 (Todd et al., 2002). Ultimately, it contributes to the phosphorylation of the retinoblastoma gene product (pRb) and is also necessary for the G0/G1 transition (Todd et al., 2002) (Fig.7). In the early G1 phase, pRb is present in a hypophosphorylated form that binds to and inactivates some of the E2F transcription factors that have a fundamental role in preparing the nucleus for DNA replication. Hence, until pRb is fully phosphorylated, E2F cannot be released and DNA synthesis cannot take place (Weinberg, 1995).

Cyclin D1 protein which is involved in the cell cycle regulation and proliferation, is

reported to be overexpressed in OSCCs (Sathyan et al., 2006; Wang et al., 2006). Overexpression of Cyclin D1 has been shown to occur early during development of OSCCs (Schoelch et al., 1999; Koontongkaew et al., 2000; Fabbrocini et al., 2000; Jayasurya et al., 2005). Experimental evidence has also suggested that Cyclin D1 functions as an oncogene and that increased expression of Cyclin D1 accelerates the G 1 to S phase transition and likely provides a proliferative advantage to tumour cells (Jiang et al., 1993; Sherr, 1994; Izzo et al., 1998).

Aims of the study

The objective of the present study is to evaluate the expression of the cell cycle regulatory proteins $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1, in oral epithelial dysplasias and oral squamous cell carcinoma lesions, and the possible association with *toombak* use.

Materials and Methods

Study subjects

One hundred and twenty four consecutive patients (85 males and 39 females, mean age: 52.95, SD: 17.70 and range 22- 96 years) with potentially malignant or malignant oral mucosal lesions were included in this study. All the patients attended the Department of Oral and Maxillofacial Surgery, Khartoum Dental Teaching Hospital, University of Khartoum, Sudan. The patients had not been previously treated. Specimens of normal oral mucosa (NOM) were obtained from 10 healthy volunteers, who had no evidence of any oral mucosal lesions and who attended the same hospital for removal of their lower wisdom teeth. The patients were interviewed on oral habits such as *toombak* dipping, cigarette smoking and alcohol usage. The majority of the patients (*n=89*) (71%) were habitual consumers *toombak* and 6 (30%) in the OED group used alcohol, while no *toombak*/ or alcohol user was found among the ten patients with NOM, but only 2 of them (20%) were smokers. Among the OSCC group only 17.3% (18/104) used alcohol. All participants signed an informed written consent. The protocol was approved by the local ethics committee at the University of Khartoum, Sudan.

Tissue specimens

From each patient, a surgical tissue sample of the lesion was taken, fixed in 10% buffered formalin and embedded in paraffin. The diagnosis of the study samples was based on clinical examination and histopathological analysis of the tissue specimens at The Department of histopathology, National Health Laboratory, Khartoum, Sudan. All the OED and OSCC samples were examined and reconfirmed by an oral pathologist, one of the authors, Anne C. Johannessen.

From all the formalin-fixed, paraffin-embedded tissue specimens *(n=134)*, serial sections (4-5µm thick) were cut and processed for routine histopathological and subsequent immunohistochemical (IHC) studies. Haematoxylin and eosin (H&E)-stained sections were examined under a light microscope and diagnosed according to the WHO criteria for histological classification of tumours of the oral cavity (Barnes et al., 2005). OED *(n=20)*, were obtained from the following sites: gingiva *(n=10)*, floor of the mouth *(n=2)*, and buccal mucosa *(n=8)*, and were graded as mild [15% *(n=3)*], moderate/ severe [85% (*n=17))*] based on the WHO criteria (Barnes et al., 2005). Biopsies of OSCC *(n=104),* were obtained during surgery from the primary intra-oral tumours from the following sites: Mucosa of the lip *(n=22),* gingiva *(n=26)*, tongue *(n=7),* floor of the mouth *(n=20),* buccal mucosa *(n=19),* and palate *(n=11)*. The biopsies (approximately 10 mm in diameter) were taken from the deeper part of the tissue routinely excised, half way between the periphery and the centre of the tumour mass. Eighty nine of the OSCCs were well differentiated (WD) (85.6%), 11 were moderately differentiated (MD) (10.6%), and 4 were poorly differentiated (PD) (3.8%).

Immunohistochemistry

The samples obtained from the subjects were used to assess the expressions of $p21^{WAF1}$, $p16^{INK4a}$, and Cyclin D1 using IHC. Sections (on silane- coated slides) were deparaffinised in xylene and dehydrated through graded alcohol and washed in Tris-buffered saline (TBS; pH 7.6). Cyclin D1 and $p21^{WAF1}$ antigen retrieval procedure was performed with TRIS/EDTA buffer (pH 9.0) using a microwave oven at high power setting (900W, for a period of 5-7 min) followed by a low power setting (300 W for 15 min). $p16^{INK4a}$ antigen retrieval was carried out in a water bath at 95°C for 60 min, using an epitope retrieval solution supplied in the $p16^{INK4a}$ Research Kit (Dako A/s, Copenhagen, Denmark). After cooling for

20 min at room temperature and washing in TBS (10 min), the sections were incubated with the Dako peroxidase block, 0.03% hydrogen peroxide containing sodium azide (Code K4007) for 5 min to eliminate endogenous peroxidase activity. After washing in TBS for 10 min, the sections were then incubated with the relevant primary antibodies for 60 min on the DAKO Autostainer Universal Staining En VisionTM+ System (Dako A/s, Copenhagen, Denmark) using antibodies against $p21^{WAF1}$ (Clone DCS-60.2 dilution 1:100, Oncogene Research Products, Cambridge. GB), Cyclin D1 (NCL-CYCLIN D1, Clone DCS-6, dilution 1:100 in Dako Antibody Diluent; Novocastra Laboratories Ltd., Newcastle-Upon-Tyne, UK). The $p16^{INK4a}$ monoclonal antibody E6H4, (dilution 1:25 Dako Cytomation, Copenhagen, Denmark) was used as the primary antibody to detect $p16^{INK4a}$ protein expression.

The secondary antibody and visualization were provided by a $p16^{INK4a}$ research kit (OA315 Dako Cytomation Denmark A/S). Briefly, a polymer agent (dextran conjugated polyclonal goat anti-mouse immunoglobulin) was used as the second antibody and 3,3'-diaminobenzidine tetra-hydrochloride was used as a chromogen.

After cooling for 20 min at room temperature and washing in TBS (10 min), the sections were incubated with the En Vision Horseradish peroxidase (DAB) for 30 min, washed twice in TBS for 5 min each, and further developed twice with the DAB + chromogen for 5 min each. The sections were then counterstained with haematoxylin, rinsed in tap water for 10 min, rehydrated and mounted using the Eukitt mounting medium. Cases in which the primary antibody was omitted and substituted with TBS served as negative controls. Tissue samples of endometrium known to show high expression of the proteins were used as positive controls.

Evaluation of the immunohistochemistry

Sections were examined for $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 positive nuclear staining using a light microscope. Staining of $p21^{WAF1}$ and Cyclin Dl was present exclusively in the nucleus, but $p16^{INK4a}$ immunoreactivity was seen in both the nucleus and the cytoplasm. The mixed nuclear and cytoplasmic staining pattern of the $p16^{INK4a}$ protein has been described as a mosaic pattern i.e. made up of many small segments (Saito et al., 1999) (Fig.8).

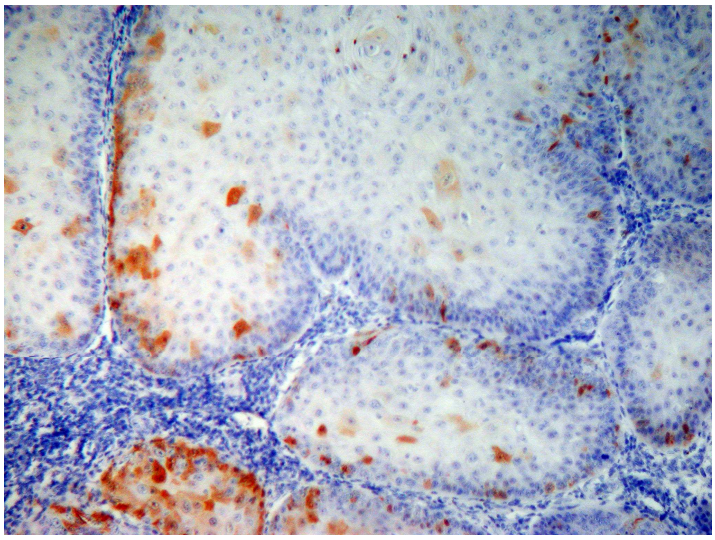

Fig.8. Immunohistochemical demonstration of $p16^{INK4a}$ stained cells present in a mosaic pattern among cancer cells in squamous cell carcinoma (original magnification x250).

Epithelium from NOM was divided into two parts: one – to two cell layers close to the connective tissue was referred to as the basal cell layer. The rest of the epithelium overlying the basal cell layer was designated as the suprabasal cell layer. OED were evaluated in two regions of the epithelium, namely the basal part and the superficial part. Specimens of well differentiated (WD) and moderately differentiated (MD) OSCC were divided into a one – to two cell layers compartment in contact with or close to the surrounding connective tissue stroma, designated as the peripheral part, made up of undifferentiated tumour cells; the central

part comprising more differentiated cells in the centre of tumour islands. This subdivision did not apply to poorly differentiated (PD) OSCC because of lack of differentiation and anaplastic nature of these tumours. The stained cells were counted at 400X magnification, in 5-10 randomly selected fields, using a Leica GMBH microscope with a fitted ocular grid. A minimum of 1000 cells were counted per slide. The stained cells were scored as a proportion of the total cells counted and results were presented as a percentage of positive cells. The immunoreactivities were also graded as absent when there was complete lack of staining in the tumour cell nuclei, (+) (when there are <10% of the cells stained) and (+ +) (when there are ≥ 10% of the cells stained). Sections that showed + + immunoreactivities for any of the three proteins examined, were considered to over express the corresponding protein.

Statistical analyses

The statistical analysis was performed using SPSS for Windows computer program version 15.0.1 (SPSS Inc., Chicago, IL, USA) and the graphics were made using commercially available computer software (Graph Pad Prism; Graph Pad Software Inc, San Diego, Calif., USA). Data are presented as mean (SD). Comparison between two groups was made by using two sample t- test and comparison between three groups was made by using a one-way analysis of variance (ANOVA), followed up by a post-hoc test where Bonferroni's correction was applied. The chi-square test was used to analyse categorical data. The level of significance was set at 0.05.

Results

Demographical features

One hundred and four subjects had OSCC [(males/females =68/36, mean age: 59.0 (13.7), range 16-96 years)]; 20 had OED [(males/females =17/3; mean age: 51.3 (14.8), range 24-73 years)] and 10 NOM [(males/females =5/5; mean age: 26.3 (6.5), range 22-40)]. Males with OSCC were represented in all age groups; however, no female under the age of 20 had OSCC. In the OED group, no male was younger than 20 and no female less than 60 years was registered.

Toombak-use

There were no *toombak*/or alcohol users among the ten patients with NOM, while 90% (18/20) were *toombak*- users and 6 (30%) used alcohol in the OED group. In the OSCC group, 79.4 % (54/68) of the males, and 47.2 % (17/36) of the females were *toombak* users, (p=0.001) and only 17.3% (18/104) used alcohol. In the primary sites of *toombak* application (lip, buccal, and floor of mouth), we found that, there is higher incidence of OSCC. This has previously been shown by Idris et al. (1995a) documenting 375/646 squamous cell carcinomas at the primary site of *toombak* application and its use was more common in people with cancers of lip, buccal or floor of mouth compared with other oral sites (58% vs. 19%).

Analysis of **p21**^{WAF1}

In the NOM, the nuclear staining of p21^{WAF1} was found in 30% of the cases examined and the mean percentage of expression of the protein was found to be 8.6% (Fig.9). The pattern of expression was largely confined to the immediate suprabasal region, being frequently detected in the differentiating layers of normal oral epithelium. Twelve (60%) of the OED cases,

showed ≥10% (+ +) of expression of $p21^{WAF1}$ protein and the mean percentage of expression was 17.7% (Fig.9). The staining was largely confined to the superficial part, the upper more differentiated layers of epithelium, while a low number of $p21^{WAF1}$expressing cells were observed in the basal part. Interestingly, nuclear expression of $p21^{WAF1}$ was found in the entire layer of the OED cases, even the basal cell layer, but most predominantly in the suprabasal differentiating cell layer.

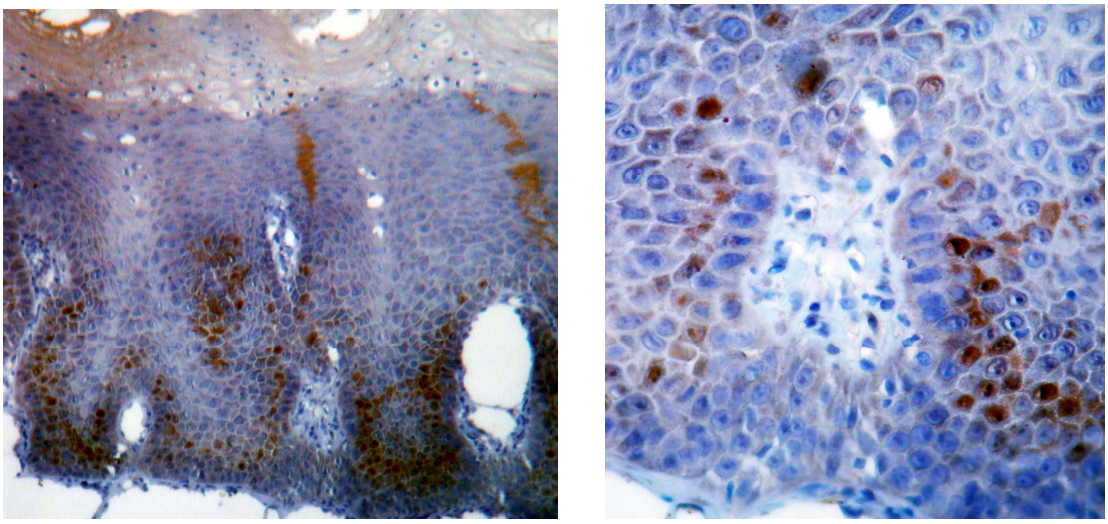

Fig.9. Immunohistochemical demonstration of $p21^{WAF1}$ in oral epithelial dysplasia, stained cells were scattered mainly in the suprabasal cell layer, (left panel, original magnification x120) (right panel, original magnification x320)

In the OSCCs, the nuclear staining of the $p21^{WAF1}$was found more pronounced toward the peripheral part compared to the central part of the tumour. The $p21^{WAF1}$ protein nuclear staining was found in (96.6%) of the OSCC cases examined and the mean percentage of expression was found to be 38.7% (Fig.11). The localization of stained cells was found most frequent in the peripheral part and less in the central part of the tumour nests (Fig.10).

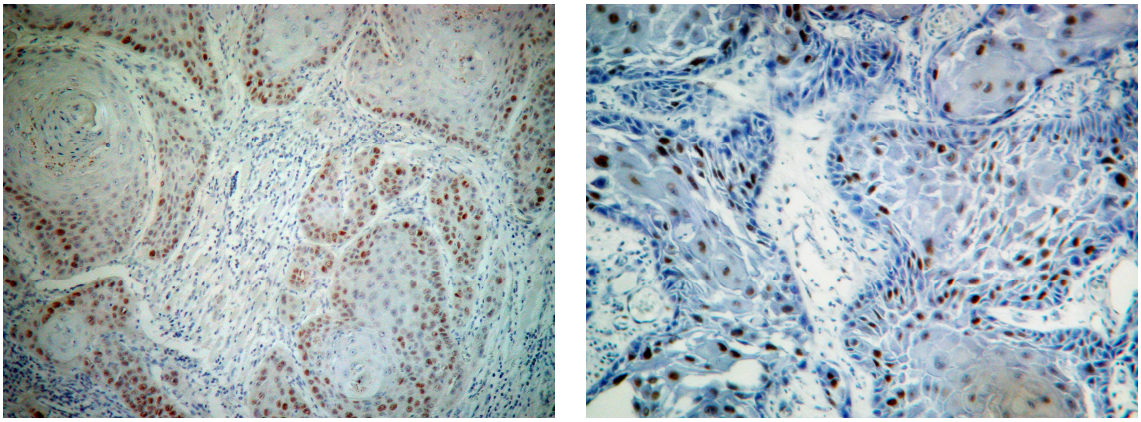

Fig.10. Immunohistochemical demonstration of $p21^{WAF1}$ in oralsquamous cell carcinoma, nuclear stained cells were found more frequent in the peripheral part and less in the central part of the tumour nests, (left panel, original magnification x120) (right panel, original magnification x250)

The expression of $p21^{WAF1}$ in the sample group showed a significant difference in the mean percentage of $p21^{WAF1}$ between NOM, OED and OSCC (8.6 (3.4), 17.7 (15.2) and 38.7 (15.8), respectively, (p<0.001). Fig.11. shows the significant difference between NOM and OSCC, (p<0.001) and between OED and OSCC, (p<0.001), and a clear trend of increasing expression of the protein from NOM to OED to OSCC.

A chi-square test revealed a significant association between $p21^{WAF1}$ expression and the histological differentiation in OED, (p=0.049) (Table 1), particularly in the *toombak* users (p=0.025) (Table 3). No significant association was found between $p21^{WAF1}$ expression and histological differentiation of the OSCC group (p=0.767) (Table 1). In the OSCC group, a significant association was also found between $p21^{WAF1}$expression and *toombak* users, (p=0.004) (Table 2). In the OSCC group, $p21^{WAF1}$ expression was significantly higher in the *toombak* users compared with non-users: 43.0 (12.8) vs. 29.5 (17.8), respectively, (p<0.001) (Fig.11).

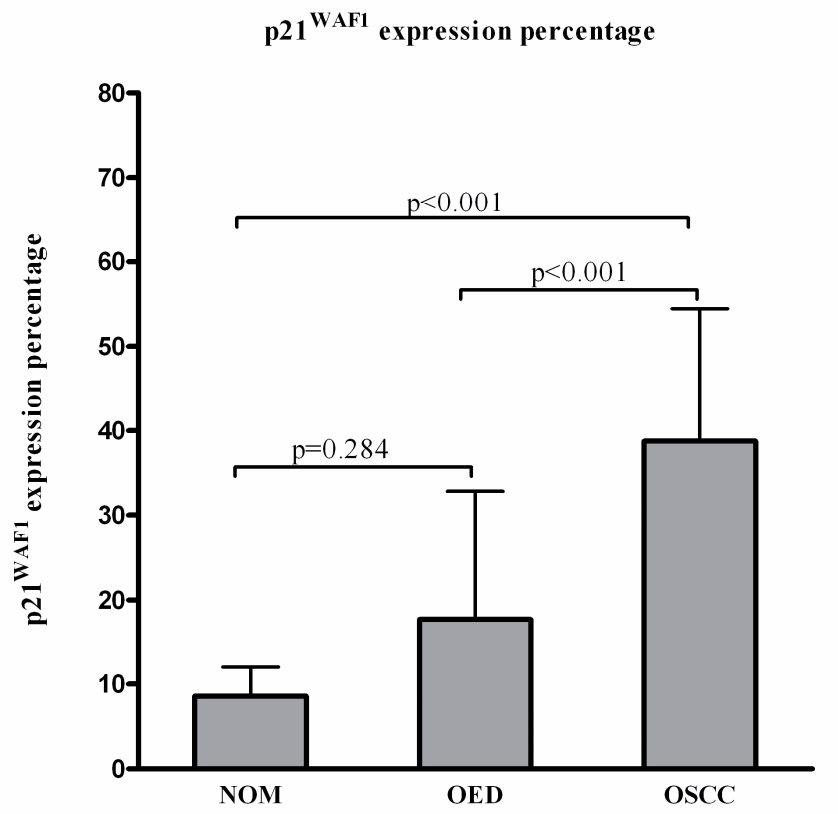

Fig.11. The expression of $p21^{WAF1}$ is shown as mean percentage (Error bar: SD) in Normal (NOM), Dysplasia (OED) and Oral Cancer groups (OSCC).

***Analysis of* p16^{INK4a}**

In the NOM group, 9/10 of the cases exhibited nuclear/ cytoplasmic immunostaining of $p16^{INK4a}$ protein. The mean percentage of expression of $p16^{INK4a}$ was found to be 25.1% (Fig.15). The immunoreactivity for $p16^{INK4a}$ was observed in the basal cell layer.

In the OED, the expression of 16^{INK4a} was found to be absent in 7 (35%) of the cases. The epithelia with moderate and severe dysplasia showed clear stained cells being scattered among the many negative cells within the basal part of 11/ 17of the cases (64.7%) (Fig.12).

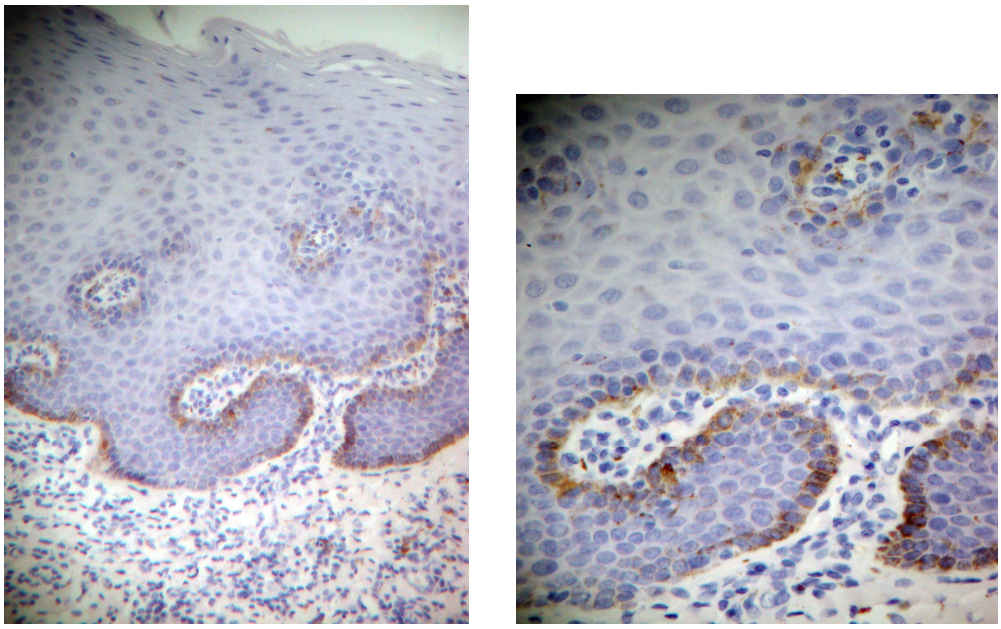

Fig.12. Immunohistochemical demonstration of $p16^{INK4a}$ stained cells in dysplastic epithelium, stained cell are scattered in the basal part of the epithelium (left panel, original magnification x120) (right panel, original magnification x250)

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In both OED and OSCCs, cells with $p16^{INK4a}$ protein expression were found present only in the mosaic pattern among dysplastic cells and cancer cells. In the OSCCs, staining was confined predominantly to the central keratinized cell pearls. Some isolated neoplastic cells were also found to be strongly stained (Fig.13).

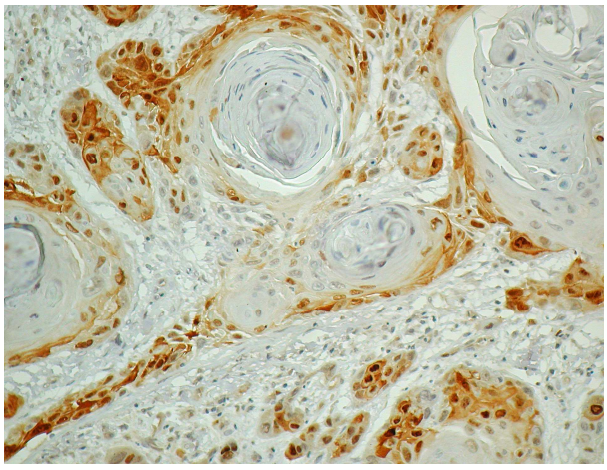

Fig.13. Demonstration of $p16^{INK4a}$ stained cells present in a mosaic pattern among cancer cells in squamous cell carcinoma staining was confined predominantly to the central keratinized cell pearls (original magnification x320).

There was a lack of $p16^{INK4a}$ protein expression in 52 of the 104 (50%) cases of OSCC examined. The majority of these sections demonstrated both nuclear and cytoplasmic staining in the tumour cells, scattered nuclear positivity for $p16^{INK4a}$ protein was also observed at the tumour margins (Fig.14).

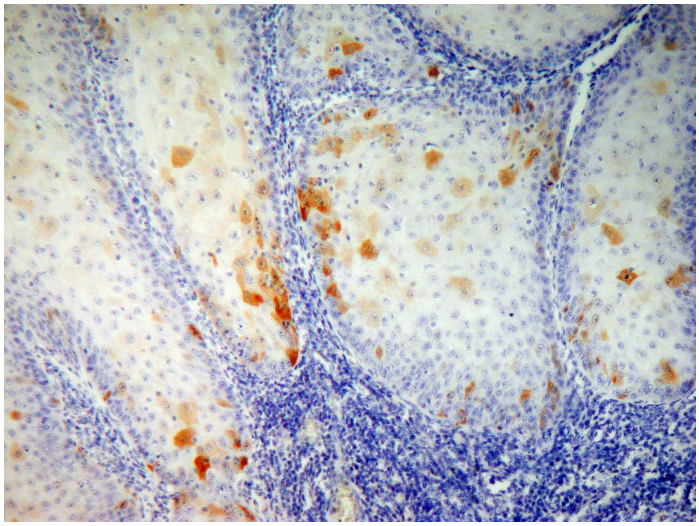

Fig.14. Scattered nuclear and cytoplasmic stained cells of $p16^{INK4a}$ in squamous cell carcinoma (original magnification x250).

The statistical test showed an overall difference in $p16^{INK4a}$ expression percentage between pairs of groups, with a trend of decreasing expression of the protein from NOM to OED to OSCC 25.1 (15.0), 8.64 (10.4) and7.60 (13.2), (p=0.001). The statistical significant differences are reflected between NOM and OSCC (p=0.001) and between NOM and OED (p=0.006) (Fig.15).

In the OSCC group, a significant association was found between $p16^{INK4a}$ expression and histological differentiation (p=0.031) (Table 1). No significant association was found between $p16^{INK4a}$ expression and histological differentiation in the OED group (p=0.270) or between $p16^{INK4a}$ expression and *toombak*- use (p=0.521). However in relation to the histological differentiation in the OSCC group, a significant association was found between $p16^{INK4a}$ expression and *toombak*- users (p=0.040) (Table 3).

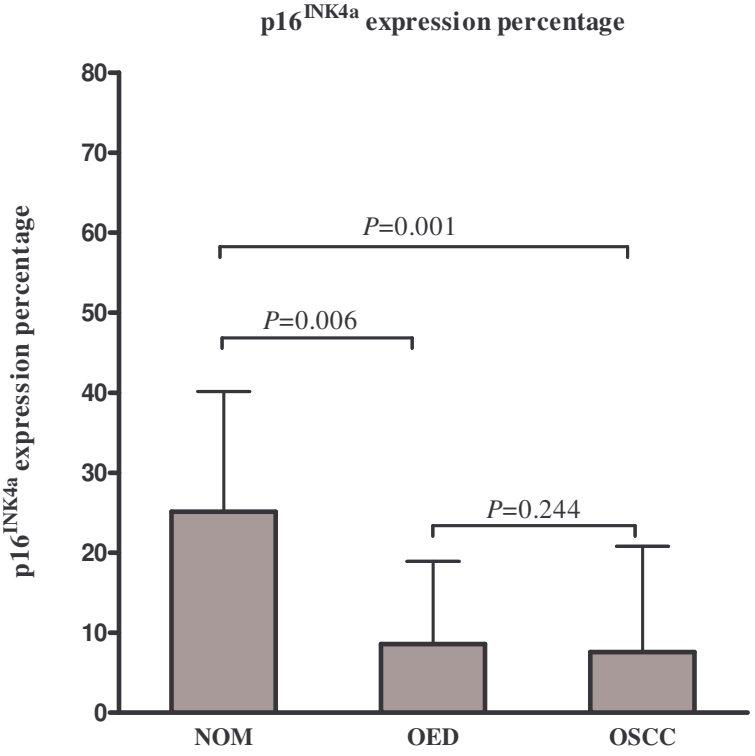

Fig.15. The expression of $p16^{INK4a}$ is shown as mean percentage (Error bar: SD) in Normal (NOM), Dysplasia (OED) and Oral Cancer groups (OSCC).

***Analysis of* Cyclin D1**

In normal oral mucosa, the mean percentage of expression of Cyclin D1 was found to be 7.4% being detectable in all NOM samples and was observed in the suprabasal cell layers.

Out of 20 OED cases, 19 (95%), showed abundant of Cyclin Dl expression, in which various numbers of nuclear stained cells were identified more frequently than in normal epithelia. They clearly appeared in the supra basal cell layer as shown in (Fig.16).

Different from $p21^{WAF1}$, Cyclin Dl nuclear staining was found in the majority of the tumour cells of OSCCs, and most abundantly in the peripheral part of the tumour. Strongly-stained tumour cells were found distributed in the periphery of tumour nests of the well differentiated OSCCs as shown in (Fig.17). Expression of Cyclin Dl was found in all of the OSCCs examined (100%) and all showed clear distinct nuclear staining of the infiltrating tumour cells.

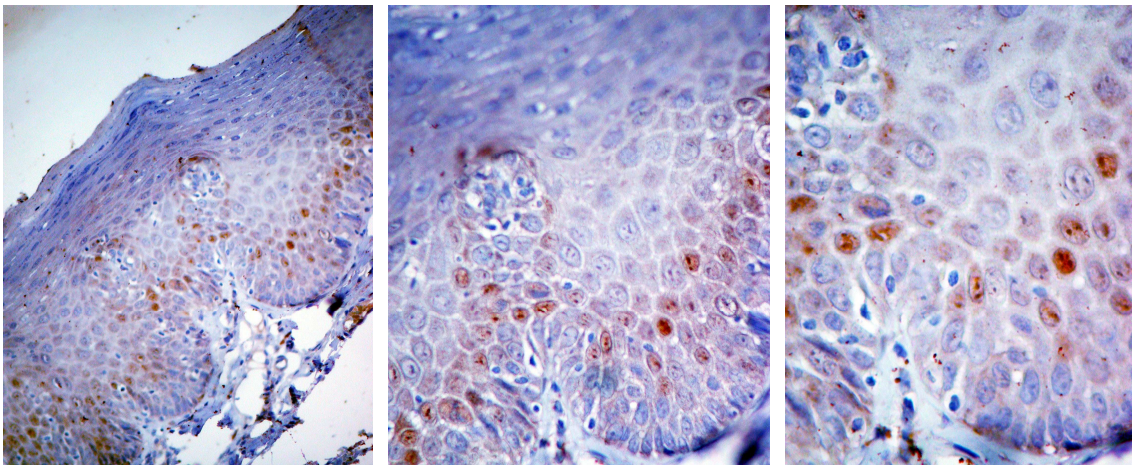

Fig.16. Immunohistochemical demonstration of Cyclin Dl nuclear stained cells in the basal cell layer of oral epithelial dysplasia (left panel, original magnification x120) (middle panel, original magnification x250) (right panel, original magnification x320)

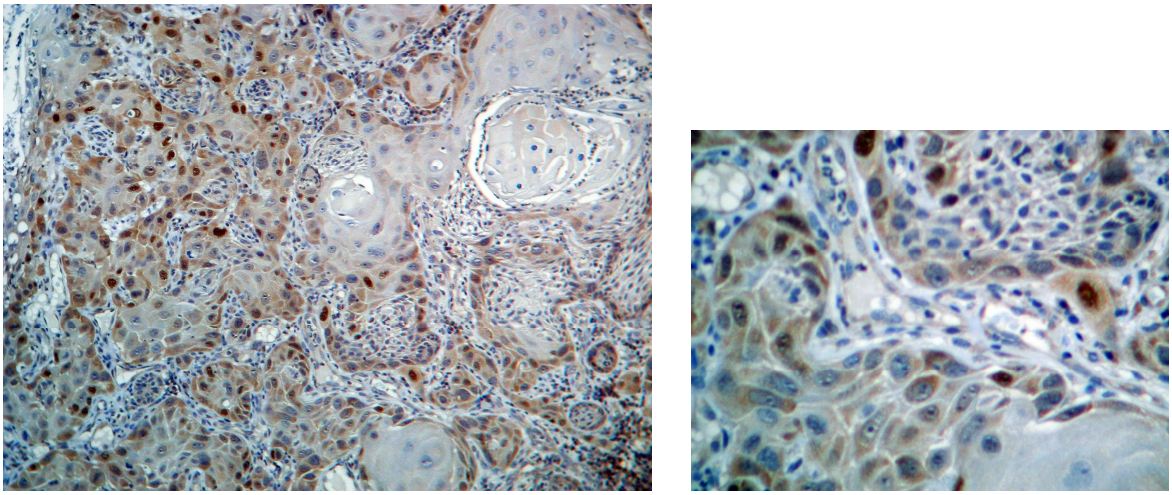

Fig.17. Immunohistochemical demonstration of Cyclin Dl nuclear stained cells distributed in the periphery of tumour nests of oral squamous cell carcinoma (left panel, original magnification x120) (right panel, original magnification x320).

There was a significant difference in the mean percentage of Cyclin Dl expression all over the pairs of groups to be compared, i.e. NOM, OED and OSCCs (7.4 (2.9), 22.5 (10.3)

and 67.0 (11.0)) (p<0.001), (Fig.18). Similar to $p21^{WAF1}$, Cyclin Dl showed a trend of increasing expression from NOM to OED to OSCC, but with even higher value of percentage of expression of the protein in the OSCCs (67.0%).

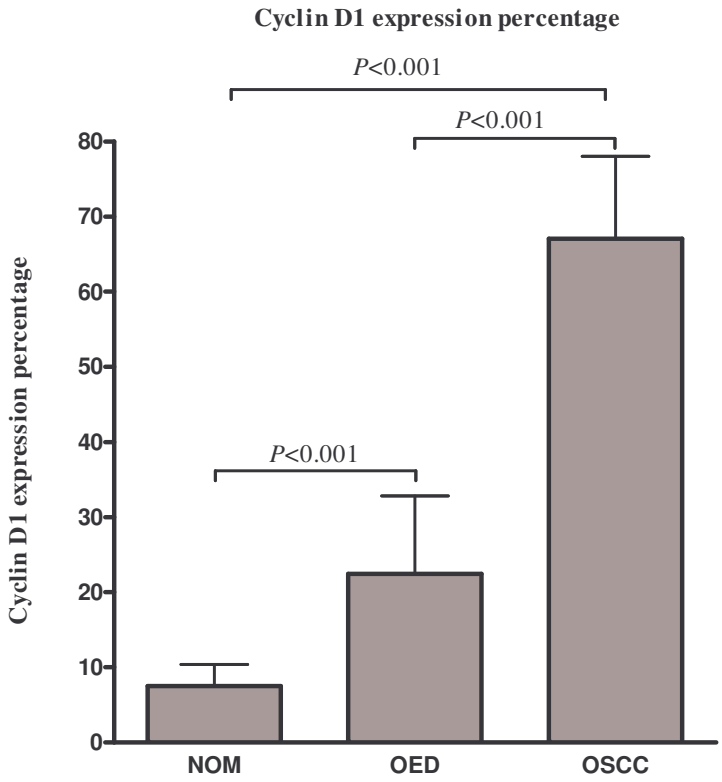

Fig.18. The mean expression percentage (Error bar: SD) of expression of Cyclin Dl in Normal (NOM), Dysplasia (OED) dysplasia and in the Oral cancer (OSCC) groups.

The results of the immunohistochemical expression of the proteins $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin Dl, in OSCC in relation to *toombak* users and non- users are presented in (Fig.19).

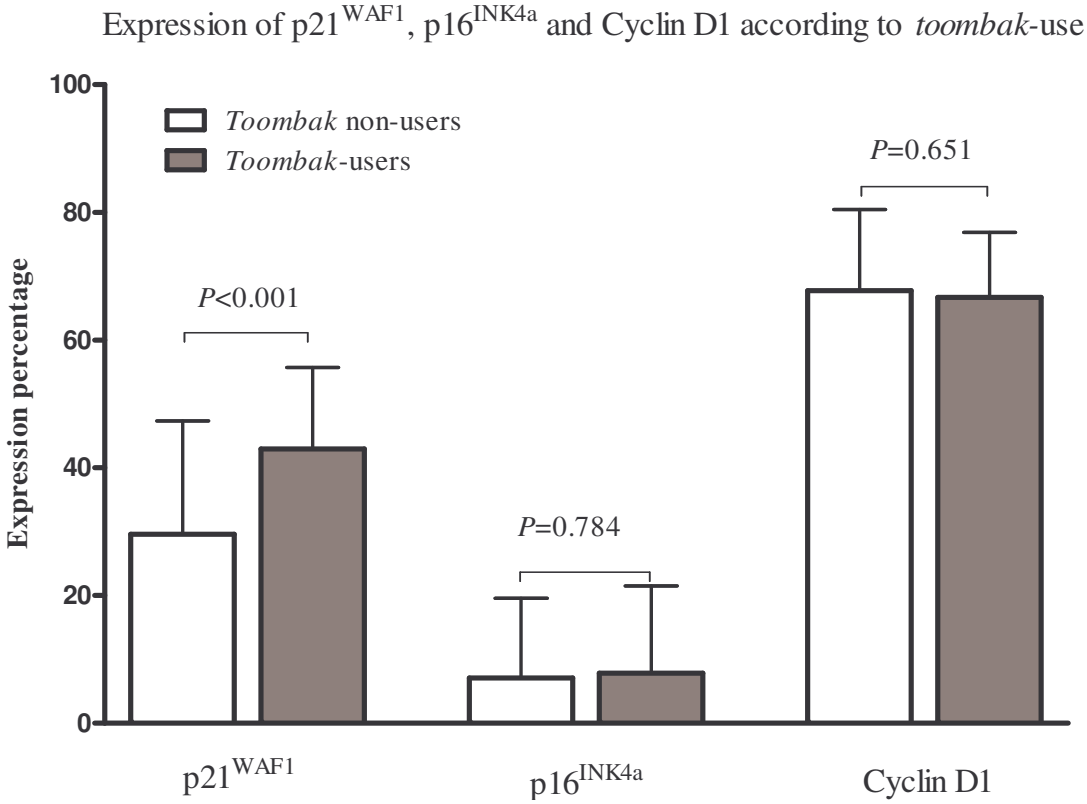

Fig.19. Shows mean percentage (Error bar: SD) of expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 in OSCCs *toombak*- users and non-users from Sudan.

Discussion

In this work, the expression of some of the G1-S cell cycle regulatory proteins was examined in normal, potentially malignant, and malignant oral lesion in association with *toombak*- use. We found that there was significant difference in $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 expressions between NOM, OED and OSCC, with high expression of $p21^{WAF1}$ and Cyclin D1, but a low expression of $p16^{INK4a}$ in the OSCCs examined, $p21^{WAF1}$ expression was found to be significantly higher in the *toombak* users compared with non-users (p=0.004), and a significant association was also found between $p21^{WAF1}$ expression and the histological differentiation of OED (p=0.049). However, no difference was found between expressions of Cyclin D1 in association with *toombak*- use.

Referring to the gender and *toombak* use, 19/39 females were *toombak*- users, this means that only 21% (19/89) of the users are actually females. As *toombak* use is considered as a social stigma in Sudan, its use is uncommon among females. This is why oral cancer due to *toombak* use is gender-specific mostly restricted to men.

The harmful effects of *toombak* on the oral mucosa have been suggested by various epidemiological studies (Elbeshir et al., 1989; Idris et al., 1994; Idris et al., 1995a; Idris et al., 1995b). To the best of our knowledge, this is the first study that shows the possible effect of *toombak* on the expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 in OSCCs from *toombak* users. Expression of p53 protein has been examined in snuff induced oral lesions by immunohistochemical analysis (Ibrahim et al., 1997). Moreover, p53 mutations has been reported in oral squamous cell carcinomas from ST consumers (snuff dippers from USA, Sudan, Norway, Sweden and tobacco chewers from South Asia including India) (Warnakulasuriya and Ralhan, 2007).

In Sudan, the majority of the OSCCs are associated with long term use of *toombak*,

wherein exposure of the entire oral cavity to *toombak* carcinogens may result in field cancerization (Strong et al., 1984; Braakhuis et al., 2003). Absence of $p16^{INK4a}$ expression was seen in 35% of the oral tissues obtained from patients with potentially malignant lesions and in 50% of the OSCCs. These findings might suggest that lack of $p16^{INK4a}$ expression may precede histological changes in the oral mucosa. Our data support the previous report that $p16^{INK4a}$ is the earliest gene known to be inactivated in head and neck SCCs (Papadimitrakopoulou et al., 1997). On the other hand, accumulation of the $p21^{WAF1}$ was observed in 60% and 96.6 % of the oral tissues obtained from the OED and the OSCC lesions respectively, suggesting that alterations in of gene/gene product might occur in later stages of oral carcinogenesis.

An interesting finding of the present study was the sequential increase in Cyclin D1 and $p21^{WAF1}$ expression from histological NOM, to, OED and OSCC. Low expression of $p16^{INK4a}$ and increased expression of Cyclin D1 in dysplastic lesions OED may lead to increased cell proliferation. These observations corroborate other studies in preinvasive lesions of the upper aero digestive tract, wherein it has been shown that Cyclin D1 deregulation was associated with an increased risk for the development of cancer and with the histological progression of this pathology (Uhlman et al., 1996). Experimental evidence have also suggested that Cyclin D1 can function as an oncogene and that increased expression of Cyclin D1 accelerates the G 1 to S phase transition and likely provides a proliferative advantage to tumour cells (Jiang et al., 1993; Sherr, 1994; Izzo et al., 1998). Izzo et al. showed that *Cyclin D1* gene was amplified in 7 of 10 cases, which progressed from potentially malignant oral lesions to invasive carcinoma (Izzo et al., 1998). Over expression of Cyclin D1 has been shown to occur early in the development of head and neck carcinogenesis (Izzo et al., 1998; Schoelch et al., 1999; Koontongkaew et al., 2000).

It has been previously shown that $p21^{WAF1}$ was over expressed in OSCC (Agarwal et al., 1998) and in lower lip SCCs (Horta et al., 2007), although down regulation of $p21^{WAF1}$ was seen in patients with tongue SCC (Sathyan et al., 2006). In spite of its over expression, $P21^{WAF1}$ seems to be unable to arrest tumour progression in OSCC (Neves et al., 2004). However, the mechanisms allowing proliferation despite the presence of accumulated $p21^{WAF1}$ remains unknown (Affolter et al., 2005). Other studies suggest additional roles for $p21^{WAF1}$ in apoptosis, independent of its role as a cell cycle inhibitor. Thus $p21^{WAF1}$ is probably involved in other functions including inhibition of apoptosis rather than inhibition of cell cycle progression (Kudo et al., 1999). It has been reported that there is a lower apoptotic rat in OSCC from Sudan compared with those from Norway (Loro et al., 2000). Our finding of over expression of $p21^{WAF1}$in OSCC patients who are *toombak* users further demonstrates that $p21^{WAF1}$ may play an important role during the pathogenesis of OSCC by affecting apoptosis and hence favouring uncontrolled cell proliferation leading to cancer. Components of tobacco, including the polycyclic aromatic hydrocarbons (PAH), nicotine and the TSNAs have been found to have specific effects on cell cycle arrest at the G0/G1 phase, probably by increasing the expression of $p21^{WAF1}$ (Lee et al., 2005). Considering the unusually high levels of the TSNAs found in *toombak*, high incidence of p53 gene mutations in OSCC from *toombak* users has been reported (Ibrahim et al., 1999). Thus $p21^{WAF1}$ together with *p53*, may be target genes of oral carcinogenesis in OSCC from *toombak* users, with the TSNAs possibly acting as a principal carcinogen in these types OSCC (Ibrahim et al., 2002).

The tumour suppressor protein $p16^{INK4a}$ is one of the most direct links between cell-cycle control and cancer (Serrano, 1997). The $p16^{INK4a}$ gene is frequently inactivated in human tumours, and inheritance of mutant alleles results in susceptibility to several types of cancers (Serrano, 1997). A lower expression percentage of $p16^{INK4a}$ in both dysplastic and

tumour samples has been shown in our study, suggesting that abnormalities of the $p16^{INK4a}$ protein may be closely associated with the pathogenesis of OSCC. Down regulation of the $p16^{INK4a}$ protein has been found to play an important role in the generation of cancer (Kato et al., 2000). It has been found that head and neck SCC were $p16^{INK4a}$ immunostaining- negative and that the absence of $p16^{INK4a}$ immunostaining was correlated with a homozygous deletion or hypermethylation of the *p16*^{INK4a} gene promoter (Reed et al., 1996). Reed et al. reported that twenty-four out of 29 (83%) head and neck squamous cell carcinoma tumours displayed a complete absence of $p16^{INK4a}$ nuclear staining (Reed et al., 1996). He also stated that immunohistochemical analysis for expression of the *p16*^{INK4a} gene product is an accurate and relatively simple method for evaluating *p16*^{INK4a} gene inactivation. In addition, Geradtes et al reported that immunohistochemical analysis is a straightforward method to detect *p16*^{INK4a} inactivation regardless of the precise genetic mechanism involved (Geradts et al., 1995). Inactivation of *p16*^{INK4a} occurs at the early stage of oral mucosal dysplasia in the multistep process of oral tumourgenesis. The high frequency of deletions of *p16*^{INK4a} in tumour cell lines first suggested an important role for *p16*^{INK4a} in carcinogenesis. This initial genetic evidence was subsequently strengthened by numerous studies documenting *p16*^{INK4a} inactivation in several human cancers. It is now believed that loss of $p16^{INK4a}$ is an early and often critical event in tumour progression (Pande et al., 1998). Although $p16^{INK4a}$ may be involved in cell senescence, the physiologic role of $p16^{INK4a}$ is still unclear.

This study has found that Cyclin D1 protein is over-expressed in OSCC examined, with no difference in regards to *toombak*- use. This suggests that Cyclin D1 could play a role in the development of OSCCs by providing a growth advantage for severe epithelial dysplasia, thus supporting its role in proliferative activity.

Among the potentially malignant lesions, we observed absence of $p16^{INK4a}$ was seen in

(26.7%) *toombak* users 4out of 15 moderate / severe dysplasias. One of the interesting findings of this study is the observation that 18 out of the 20 potentially malignant lesions examined (90%) were from *toombak* users with altered expression of all the three markers examined. This suggests that altered expression of these proteins may lead to malignant transformation of these lesions. It is worthwhile noting that the percentage of dysplasia that progresses to malignant lesion has been shown to vary from 5 to 43% related to the severity of dysplasia (Rocco and Sidransky, 2001). Considering these observations, our findings might suggest that chronic *toombak* users may be at risk for developing malignancy when changes of the proteins examined can be detected in potentially malignant lesion obtained from these users. Based on these observations, we propose that dysplastic lesions harbouring concomitant alterations in expression of these markers may be at high risk for transition to malignancy in habitual *toombak* -users from Sudan.

In conclusion, the results of the present study shows that the $p21^{WAF1}$ protein is significantly over expressed in OSCCs and especially in those from *toombak* users. However, $p16^{INK4a}$ expression was considerably low and Cyclin D1 is over expressed in all the OSCCs examined. Alternations in these proteins may play a role in oral carcinogenesis and may be used as biomarkers in potentially malignant and malignant oral lesions from habitual users of *toombak*. The findings of low expression of $p16^{INK4a}$ might indicate that it is one of the earliest events in the process of development of oral cancer, and that Cyclin D1 over expression is an important event in the acquisition of the potentially malignant lesions.

There is very little data related to the biological markers in oral cancer lesions from African populations, in particular Sudanese *toombak* - dippers and non - dippers. However, there is now an opportunity in Sudan to study oral cancer associated with *toombak,* and thus to have data potentially important for understanding the molecular biology of this problem.

The magnitude of the problem of oral cancer in Sudan obliges identification of early signs and symptoms, so that preventive and curative measures can be followed. This involves education of oral health workers on signs and symptoms of potentially malignant and malignant oral lesions and methods that can be used for early detection to characterise the damage induced by *toombak* either in short or long- term use of *toombak*. This may help in determining the internal dose of exposure and disease risk, or may predict early biological changes associated with the development of oral cavity lesions.

Research oriented methods for detection and characterisation of these lesions would eventually increase the public awareness and knowledge of long standing *toombak*- induced lesions and oral cancer in the area.

The major challenge to treatment of cancer in Sudan, as in most developing countries, is that most patients first present with advanced stage disease. A total of 78% of the Sudanese patients have stage III or IV disease (TNM classification) when they first seek medical treatment (Hamad, 2006). In these stages, treatment may often involve multiple modalities, including surgery, radiotherapy, chemotherapy and hormone therapy, and has a markedly diminished chance of success. In addition, cancers like oral cancer are largely curable if detected early. Therefore, there is an urgent need for applying methods that might help in early detection of oral cancer in Sudan, especially in those at high risk to develop the disease (*toombak*- users), to make treatment more effective, less costly, less invasive, and more accessible and acceptable to patients.

Table 1. Relationship between expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 and histological differentiation in oral epithelial dysplasia (OED) and oral squamous cell carcinomas (OSCCs)

	$P21^{WAF1}$ expression n (%)		$p16^{INK4}$ expression n (%)		Cyclin D1 expression n (%)	
OED	<10% (+)	≥10% (++)	<10% (+)	≥10% (++)	<10% (+)	≥10% (++)
Dysplasia (Histology) (n =20)						
Mild (n=3)	3 (100)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	3 (100)
Moderate/ severe (n=17)	5 (29.4)	12 (70.6)	12 (70.6)	5 (29.4)	1 (5.9)	16 (94.1)
p-value		**0.049***		**0.270**		
OSCC (Histology) (n=104)						
Well differentiated (n=89)	8 (9.0)	81 (91.0)	72(80.9)	17 (19.1)	0 (0)	89 (100)
Moderately/ Poorly differentiated (n=15)	1 (6.7)	14 (93.3)	8(53.3)	7 (46.7)	0 (0)	15 (100)
p-value		**0.767**		**0.031***		

* Statistically significant; < 10% (+), less than 10% cell stained; ≥10% (++), more than 10% cells stained.

Table 2. Relationship between expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 and *toombak*- use in oral epithelial dysplasia (OED) and oral squamous cell carcinomas (OSCCs)

	$p21^{WAF1}$ expression n (%)		$p16^{INK4}$ expression n (%)		Cyclin D1 expression n (%)	
OED	<10% (+)	≥10% (++)	<10% (+)	≥10% (++)	<10% (+)	≥10% (++)
Dysplasia (*toombak*- use)						
Users (n=18)	6 (33.3)	12 (66.7)	11 (61.1)	7 (38.9)	1 (5.6)	17 (94.4)
Non-users (n=2)	2 (100)	0 (0)	2(100)	0 (0)	0 (0)	2 (100)
p-value		**0.147**		**0.521**		
OSCC (*toombak*- use)						
Users (n=71)	2(2.8)	69 (97.2)	53 (74.6)	18 (25.4)	0 (0)	71 (100)
Non-users (n=33)	7 (21.2)	26 (78.8)	27 (81.8)	6 (18.2)	0 (0)	33 (100)
p-value		**0.004***		**0.465**		

* Statistically significant; 10% (+), less than 10% cell stained; ≥10% (++), more than 10% cells stained.

Table 3 Relationship between expression of $p21^{WAF1}$, $p16^{INK4a}$ and Cyclin D1 in *toombak* users according to histological differentiation of oral epithelial dysplasia (OED) and oral squamous cell carcinomas (OSCCs).

Toombak users	$p21^{WAF1}$ expression n (%)		$p16^{INK4}$ expression n (%)		Cyclin D1 expression n (%)	
OED	<10% (+)	≥10% (++)	<10% (+)	≥10% (++)	<10% (+)	≥10% (++)
Dysplasia (n=18)						
Mild (n=3)	3 (100)	0 (0)	1 (33.3)	2 (66.7)	0 (0)	3 (100)
Moderate/ severe (n=15)	3 (20)	12 (80)	10 (66.7)	5 (33.3)	1 (6.7)	14 (93.3)
p-value		**0.025***		**0.528**		
OSCCs (n=71)						
Well differentiated (n=62)	2 (3.2)	60 (96.8)	49(79)	13 (21)	0 (0)	62(100)
Moderately/ Poorly differentiated (n=9)	0 (0)	9(100)	4 (44.4)	5 (55.6)	0 (0)	9 (100)
p-value		**> 0.8**		**0.040***		

* Statistically significant; < 10% (+), less than 10% cell stained; ≥10% (++), more than 10% cells stained.

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