MOLECULAR PREDICTIVE BIOMARKERS FOR ORAL SQUAMOUS CELL CARCINOMA IN HUMANS IN IBADAN, NIGERIA

BY

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CERTIFICATION

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DEDICATION

This research work is dedicated to the ever loving memory of my late parents; Mr. and Mrs. Fycountry George Onyegbula who sowed this undying seed right from my childhood, which now has germinated in spite of all odds.

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Most importantly, my eternal gratitude goes to my creator for enabling grace.

ABSTRACT

Squamous Cell Carcinoma is the most prevalent malignant tumour of the oral cavity in humans. Late hospital presentation and diagnosis often result in high mortality, recurrence and metastatic rates. Prognosis is poor with a low 5-year survival rate. There is a possibility that molecular events underlie the aetiology of oral squamous cell carcinoma (OSCC) and could prove useful in predicting OSCC-susceptible individuals, but this information is largely lacking in Nigerian cohorts. Thus, this study was designed to identify molecular predictive diagnostic biomarkers for OSCC from patients in Ibadan.

Using a retrospective-prospective study design, a total of 100 (58 males, 42 females) histologically-classified OSCC cases were identified from 1527 tumour cases recorded at the University College Hospital, Ibadan, Nigeria, between January 2004 and December 2015. Patients' demographic variables were extracted. Archived formalin-fixed, paraffin-embedded tissue samples were retrieved and processed immunohistochemically for Epithelial Membrane Antigen (EMA) and cytokeratin protein expression. The DNA from samples was also profiled for aberrant CpG island methylation and genotypes of rs7528484 polymorphism in *RUNX3* gene by methylation-specific and restriction fragment length polymorphism-PCR, respectively. Data were analysed using descriptive statistics, while association between patients' demographic variables, aberrant CpG island methylation and rs7528484 polymorphism in *RUNX3* were assessed by Pearson's χ^2 test at $P \leq 0.05$, Monte-Carlo exact test and Odds Ratios (OR) at Confidence Interval (CI) of 95%, respectively.

The prevalence of OSCC was 6.5%. The moderately differentiated class was the most prevalent (65.0%), with a general prevalence peak at the seventh decade age group and the palate being the most affected location. EMA was expressed by the well and moderately differentiated classes, while cytokeratin was expressed by the well, moderately and poorly differentiated classes. *RUNX3* promoter hypermethylation was detected in 45.0% of OSCC, suggesting that aberrant CpG island promoter hypermethylation in *RUNX3* was prevalent in the disease. The rs7528484 polymorphism in *RUNX3* was also detected with a genotype distribution of 52.7% (39) homozygote normal (CC), 28.4% (21) heterozygote mutant (CT), 18.9% (14) homozygote mutant (TT), and a C>T allelic ratio of 0.67:0.33. There was significant association between aberrant CpG island promoter hypermethylation in *RUNX3* and tumour location ($P<0.05$). Genotypes of rs7528484 polymorphism in *RUNX3* and their alleles were significantly associated with both male and female gender ($P<0.05$) and histologic class ($P<0.05$). Mutant genotypes (CT) and (TT) showed odds of predicting OSCC (OR 0.28, 95% CI: 0.1889 - 0.3711) and (OR 0.18, 95% CI: 0.1118 - 0.2482), respectively. Mutant allele (T) showed odds of predicting OSCC (OR 0.66, 95% CI: 0.52 - 0.80).

Aberrant CpG island promoter hypermethylation of *RUNX3* in combination with tumour location and rs7528484 polymorphism in *RUNX3* in combination with gender served as epigenetic and genetic predictors, respectively for oral squamous cell carcinoma, while epithelial membrane antigen expression pattern served as an immunohistochemical predictor for oral squamous cell carcinoma.

Keywords: Oral squamous cell carcinoma, *RUNX3* genotypes, Aberrant CpG methylation, Epithelial membrane antigen, Single nucleotide polymorphism

Word count: 462

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
AIDS	Acute immunodeficiency syndrome
APC	Adenomatous polyposis
ATM	Ataxia telangiectasia mutated
CCND1	Cyclin D1
CDC	Center for disease control
CDH1	Cadherin 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CI	Confidence interval
CK	Cytokeratin
COE	Conventional oral examination
COL-1C	Collagen type 1C
CpG	Cytosine-phosphate-guanine
DAB	Diamino benzene
DAPK-1	Death associated protein kinase 1
DCC	Deleted in colorectal carcinoma
DDAH-2	Dimethylarginine dimethylaminohydrolase 2
DKK-3	Dickkopf-related protein 3
DNA	Deoxyribonucleic acid
DUSP-1	Dual-specificity phosphatase 1
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
ERBB1	Erythroblastic leukemia viral oncogene homolog 2
FFPE	Formalin fixed paraffin embedded
GFAP	Glial fibrillary acidic protein
GWAS	Genome wide association study
HER2	Human epidermal growth factor receptor 2
HIF1A	Hypoxia inducible factor 1 alpha
HIV	Human immunodeficiency virus
HMB45	Homatropine bromide 45

HPV	Human pappiloma virus
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOC	Lab-on-chip
LOH	Loss of heterozygosity
MGMT	Methylguanine methyltransferase
MLH1	MutL homolog 1
MSP	Methylation specific polymerase chain reaction
MYF5	Mouse myogenic factor 5
MYF6	Mouse myogenic factor 6
MYOD1	Myoblast determination protein 1
NPV	Negative predictive value
NSAID	Non-steroidal anti-inflammatory drugs
NSE	Neurone specific enolase
OPL	Oral premalignant lesion
OPMD	Oral premalignant diseases
OR	Odds ratio
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
PPV	Positive predictive value
PTX3	Pentraxin-related protein
RARβ	Retinoic acid receptor-β
RASSF-1	Ras association domain family 1
RASSF-1A	Ras association domain family 1A
RASSF-2	Ras association domain family 2
RB	Retinoblastoma
RETN	Resistin
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RUNX	Runt-related transcription factor
SCC	Squamous cell carcinoma

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SFRP	Secreted frizzled related protein
SNPs	Single nucleotide polymorphisms
TNM	Tumor-node-metastasis
VEGF	Vascular endothelial growth factor
WIF-1	Wnt inhibitory factor 1
WNT-1	Wingless-related integration 1
XRCC1	X-ray repair cross-complementing protein 1

CHAPTER ONE

INTRODUCTION

1.1 Background

Oral carcinomas are malignant growths in the oral cavity (Al-Rawi and Talabani, 2008). The commonest is squamous cell carcinoma; a biologically heterogeneous cancerous tumour made up of several layers of squamous cells characterized by confined devastating growth along with remote metastases (Bavle *et al*., 2016). It consists of three histological classes depending on the degree of differentiation and clinical severity: the well, moderately and poorly differentiated squamous cell carcinoma (Bungett *et al*., 2018). Oral squamous cell carcinoma constitutes 94% of all oral carcinomas and 4% of all cancers irrespective of site (Khan and Khan, 2015; Del Corso *et al*., 2016; Dissayanake *et al*., 2017 and Gao *et al*., 2018).

Globally, there are approximately over five hundred thousand new cases every year, out of which over 271,000 deaths occur, representing 50% mortality (Hema *et al*., 2017). Approximately 4 - 6% of oral squamous cell carcinoma (OSCC) occurs in persons below forty years of age and less commonly in females than in males in a 3:1 proportion (Del Corso *et al*., 2016). It has been variously ranked fifth, sixth and eighth most commonly encountered cancer of all sites exhibiting variation in incidence among countries (Faggons *et al*., 2015; Del Corso *et al*., 2016). Post-management recurrence and metastatic phenomena have been observed in 60% of patients and 15 - 20% of cases respectively while 40 - 59% of patients stay alive for five years (Leemans *et al*., 2011; Cordeiro-Silva *et al*., 2012).

The disease rate is estimated to be about 38.2% in Europe, 28.5% in Asia, 24.9% in Africa and 21.5% in America (Jemal *et al*., 2011). In Nigeria as in other African countries, there

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is a male preponderance with increasing incidence in the younger population, high morbidity and mortality rates and poor treatment outcome (Lilly-Tariah *et al*., 2009).

The aetiology of OSCC is multifactorial, where no sole actuating element has been specifically fingered and where inherent factors such as epigenetic and genetic modifications in oncogenes and tumor suppressor genes plus extrinsic factors are often seen to play significant roles (Khan and Khan, 2015; Bavle *et al*., 2016; Del Corso *et al*., 2016). Although indulgence in cigarette smoking coupled with alcoholic liquor consumption are highly taunted as being likely risk factors of the disease, viruses such as the Human papilloma viruses (HPVs) and Human immunodeficiency virus (HIV) are also highly suspected as critical risk factors (Del Corso *et al*., 2016; Hema *et al*., 2017). Additional risk factors linked to OSCC include; alcohol in mouth-washes, marijuana smoking, socio-economic status, diet and nutrition, immunosuppression and trauma (Hema *et al*., 2017; Bungett *et al*., 2018).

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The burden emanating from cancer-related morbidity and mortality is increasing alarmingly in Sub-Saharan Africa, with deaths emanating from cancer in the region projected to increase by 85% between 2008 and 2030 (Faggons *et al*., 2015). Pathologically this condition is notorious for the severity of its illness with attendant high death rates; it has few therapeutic options aside from surgical intervention, use of cancer cell destroying medications and radiotherapy (Khan and Khan, 2015; Faggons *et al*., 2015). While early detection via screening exercises, better understanding of tumor behaviour coupled with its pathologic process and advanced chemotherapeutic with ancilliary care have added largely to improving prognosis of this disease in technologically advanced nations, the case has been the opposite in Sub-Saharan Africa mainly due to little or nonavailability of diagnostic facilities, treatment and possibly, differences in cancer aetiological factors in the region (Faggons *et al*., 2015).

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Pathology as a profession now includes increasing engagement with the care of patients with cancer. Pathology practitioners and pathology scientists often search for biomarkers not only serving the purpose of diagnosis but also for prognostication and therapeutics

using solid tumor samples obtained from patients. Practical example of such biomarker used immunohistochemically for the evaluation of prognosis and response to therapeutic intervention in subjects with cancer of the breast (Kermani *et al*., 2019), prostate (Carneiro *et al*., 2018) and ovaries (Mehner *et al*., 2017) is the epidermal growth factor receptor 2 (*HER2-neu*) derived from humans. Several other biological markers as well as proliferation pathways have been identified in recent times in several human cancers such as lung cancer using basic molecular tools (Kreeger and Lauffenburger, 2010).

The effect of incorporating methyl groups to the DNA along with the newly revealed epigenetic action of hydroxymethylation in humans is presently generating key interest in the life sciences (Greenberg and Bourhis, 2019). Advancement in this area of scientific interrogation has further been accentuated by the knowledge that these changes in the set of nucleic acid in an organism's genome are linked with major activities of the cell such as division, multiplication, growth, specialization, expression of the genetic material and the pathologic process including development of cancer (Ishiyama *et al*., 2017).

According to Feero *et al*., (2010), tiny modifications in the sequence of the genetic material being transferred from parents to children referred to as single nucleotide polymorphisms (SNPs) constitute the commonest form of genetic aberration or differences in humans, these aberrations (known as alleles) if present in at least 1% of a population are referred to as polymorphic alleles. Therefore, although the similarity in humans at the DNA sequence level is not in doubt, but because of the size of the genome there is wide latitude for genetic diversity in individuals. It is commonly observed that SNPs appear about once in every 800 base pairs. Therefore, not only has there been a renewed interest in the contribution of SNPs to the occurrence and progression of cancer, but also in their contribution to the detection and prediction of risk of disease development. These SNPs determine individual differences in our ability to succumb to or resist disease development including cancer (Ghagane *et al*., 2016).

Accumulation of polymorphisms (SNPs and mutations) and/or sporadic somatic polymorphisms (non-germline polymorphisms) from parents to progeny in genes that order events such as cell cycle, DNA revamp plus growth signaling, causes cancer development. Knowledge of these molecular changes can be of immense value in patient

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management (Kirk *et al*., 2002). Therefore, identifying specific changes in the genetic material enhances the ability to unravel novel biomarkers that enable prompt and error-free diagnosis of disease including cancer and also provide vulnerability stratification of subjects subsequent to diagnosis.

In relation to disease detection and identification, the idea of separating people into various groups with intention of administering tailor-made care to each group has come into prominence in recent times. Especially in the last two decades, an understanding has grown among scientific researchers that onset of malignancy is the outcome of genetic and/or epigenetic modifications in oncogenes that code for proteins and tumor suppressor genes (Ghagane *et al*., 2016).

Genome analyses using modern molecular methodologies have led to greater understanding of the pathogenesis of OSCC. In craniofacial and neck malignancies, transformation of normal cells into malignant tumour cells is often the effect of mutations of traditional genes located in somatic cells. Data from genetic research also confirms the contribution made by several genes responsible for modulating tumor behavior in processes like activation of tumorigenesis as well as its perpetuity. The use of animals in research and newer drug delivery designs is also expected to advance hitherto unknown therapeutic systems and better prognostication of disease (Khan and Khan, 2015).

The postulation that external disturbances on the DNA can likely be a pathway for modifying expression of gene and promote tumor formation have found support in the works carried out on breast cancer (Hernandez-Vargas *et al*., 2011), lung (Vaissiere *et al*., 2009), stomach (Tsunematsu *et al*., 2009) and nasopharyngeal cancer (Tan *et al*., 2006). According to Stebbing *et al*., (2006), promoter hypermethylation constitutes a significant epigenetic pathway through which many genes that prevent tumour development are transcriptionally silenced and also the commonest pathway for gene silencing other than genetic mutation. Promoter hypermethylation of CpG islands occurs in several human malignancies one of which is OSCC.

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Analysis of alterations in nucleic acids and the subsequent expression of the altered forms in long-established genes with focus on early tumor formation have also elucidated what function these genes play in cancer pathogenesis (Garnis *et al*., 2004). One of such genes is *RUNX3*; which is responsible for regulating developmental processes and found on chromosome 1p36.1 (Tsunematsu *et al*., 2009). Prevention of manifestation of this gene has been achieved in human malignancies of the stomach (Tsunematsu *et al*., 2009), liver (Mori *et al*., 2005), colorectum (Ku *et al*., 2004) as well as lung (Yanada *et al*., 2005) amongst others. However, overexpression of this gene is seen in squamous cell carcinoma associated with the craniofacial as well as neck region (Tsunematsu *et al*., 2009), and basal cell carcinoma associated with the integument. Mechanisms for reduced expression include gene silencing, protein mislocalization, CpG island hypermethylation and point mutations (Tsunematsu *et al*., 2009).

In craniofacial and neck malignancies, overexpression of this gene promotes tumorigenesis and was implicated in tumor invasiveness and metastases and in mouse models it was also implicated in the promotion of cell proliferation and tumorigenesis. It was also implicated in the inhibition of programmed cell death from both serum scarcity and cytotoxic drug administration (Tsunematsu *et al*., 2009; Shan *et al*., 2017).

1.2 Statement of the Problem

The most frequently encountered malignancy of the craniofacial and neck region is OSCC, responsible for about nine in ten of all cancers affecting this anatomic site (Hema *et al*., 2017).

With its relatively high death rate and poor prognosis, it undoubtedly poses a critical epidemiologic concern of serious personal social and economic consequences. Among the possible reasons for this include the non-objectivenesss of the histological diagnosis of the disease which may be prone to human error. Decisions on management of the disease and prognosis also depend on this subjective histological diagnosis. There is poor correlation between disease outcome and histological diagnosis (Majumdar *et al*., 2017; Bungett *et al*., 2018). OSCC is a multifactorial disease, with wide ranging modulating factors

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including cigarette smoking, drinking of alcoholic liquor, infection with different viral strains, chronic inflammations, genetic and epigenetic risk factors (Salahshourifar *et al*., 2014; Bray *et al*., 2015; Irimie *et al*., 2016; Hema *et al*., 2017; Zhu *et al*., 2017), OSCC cell populations are heterogenous and this heterogenicity governs its exhibition of varying propensities for invasion along with spread of disease to other anatomic locations (Bavle *et al*., 2016; Dissanayake *et al*., 2017).

Though the molecular pathogenesis of OSCC is yet to be understood, these predisposing factors may culminate in a broad spectrum of genetic and epigenetic activities that elicit nucleic acid perturbations along with subsequent tumor pathogenesis. The beginning and evolution of precancerous derangements within the oral cavity and OSCC are the results of not only irreversible alterations in the DNA sequences (gene deletions, amplifications and mutations) resulting in the initiation of oncogenic activity or silencing of genes that should normally suppress tumorigenesis, but also of modification in the expression of genes not encoded in the nucleic acid sequence and are designated as epigenetic changes (Hema *et al*., 2017). Thus, chemical changes in nucleic acids and related proteins can modify the behaviour of a gene while not affecting the nucleic acid chemical organization (Hema *et al*., 2017).

As knowledge of genetic events and epigenetic modifications grows, it is expected to impact positively on all aspects of cancer biology. Hence, identifying the molecular pathogenic pathway through which OSCC develops in combination with the risk factors may be important in providing prophylactic, diagnostic and therapeutic benefits.

1.3 Justification of the Study

It is an incontrovertible fact that development of OSCC as with other cancers has as its propelling power the mounting of genetic and epigenetic events which may have been triggered by other risk factors. Furthermore, each cancer patient and cancer type is also biologically distinct. In Nigeria, the incidence and morbidity of OSCC is on the increase especially in younger populations and the mortality rate is exceedingly high, with poor treatment outcome. Traditionally, OSCC has been linked to the chronic use of tobacco

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along with alcoholic liquor consumption. However, in an investigation carried out by Adeyemi *et al*., (2008) in Ibadan, South-West Nigeria and Oji and Chukwuneke, (2007), South-East Nigeria, no positive correlation was established between this malignancy and chronic cigarette smoking and alcoholic liquor consumption. Furthermore, there is lack of scientific investigations on genetic and epigenetic epidemiology of this malignancy. As such, the need to identify diagnostic biomarkers which will hopefully aid early diagnosis and lead to better patient management, with the reduction of morbidity and mortality.

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1.4 Broad Objective

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To explore the possibility of using pattern of molecular changes, patients demography, or a combination of both as predictive diagnostic biomarkers in solid archived OSCC tissues.

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1.5 Specific Objectives

i. To evaluate the expression pattern of epithelial membrane antigen (EMA) and cytokeratin (CK AE1/AE3) proteins in OSCC tissues
ii. To evaluate OSCC tissues for presence of CpG island methylation in the promoter region of *RUNX3* gene
iii. To evaluate OSCC tissues for presence of single-nucleotide polymorphisms in *RUNX3* gene
iv. To determine the association between patients demography and polymorphisms with methylation in the *RUNX3* gene.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Anatomy of the Oral Cavity

All cancerous growths originating from the mouth and associated sites are collectively called malignancies of the oral cavity (Neville and Day, 2002). The oral cavity consists of different anatomic locations shown in Figure 2.1 (Shimpi *et al.*, 2018). Carcinomas of squamous cells constitute close to 90% of the malignancies originating in epithelial mucosa lining the oral cavity, while malignancies not associated with the mucosal epithelial lining make up the remaining 10 percent of oral malignancies (Neville and Day, 2002).

The two layers making up the oral mucosa are the outer and inner strata made up of epithelial cells and connective tissue respectively. Histologically, the outer layer is further divided into several layers of keratin or non-keratin containing cells which functionally serve as a shielding wall to prevent disruptions from external agents, while the inner surface made up of dense connective tissue provides rigid backbone and nutritional factors for the outer epithelium. Underneath the lamina propria lays a sub-mucosa rich in blood vessels, fat and secretory glands (Chen *et al.*, 2015; Ahsan, 2018). Functional demands within the entire length of the oral cavity however results in structural changes in both the outer epithelial and inner connective tissue layers (Chen *et al.*, 2015).

On the basis of tissue function, distinct histologic variants of the mucus-secreting layer of the oral cavity have been characterized. The first is the masticatory mucosa located on the hardened roof of the mouth and gums possessing a mechanically tough and keratinized epithelium which play the role of protection against shear forces during mastication, while the second which is the lining mucosa located on the base of the mouth, buccal regions, lips, softened upper part of the mouth and inner coating of the tongue is characteristically

devoid of keratin having flexible epithelium and the third histologic type consist of a combination of the keratinized and non-keratinized covering layer of cells having papillous projections which perform special functions and occupying the upper part of the tongue (Brierley *et al*., 2019).

The oral epithelium lies above the basement membrane having a bridge linking them with the underlying layer of tissue network. The epithelium is a highly organized multiple strata of densely packed cells above the basement membrane with various patterns of differentiation. The first stratum of cells is attached to the basement membrane. They are cuboidal or columnar in shape and the cells are undifferentiated and mitotically active. They are the deepest layer of the oral epithelia and are responsible for cell production and division (Groeger and Meyle, 2019). Basal cells undergo division and migrate upwards to form a layer of spiny projections which increase in size and volume, take up a polyhedral shape and produce differentiation-specific cytokeratins (Groeger and Meyle, 2019).

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Further upwards, these cells transform into the intermediate layer and become flattened with or without filaments of keratin that are densely packed. The outermost layer of cells is known as the superficial epithelium. It is made up of keratinized epithelial cells which have plasma membranes that are characteristically thickened, keratin filaments that are compact and condensed, lacking organelles and an external layer of lipid matrix which forms a selectively permeable barrier (Groeger and Meyle, 2019).

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A similar organization is seen in non-keratinized epithelium of lining mucosa. The difference is that organelles are present and nuclei persist, with a less effective permeability barrier than that of a keratinized oral epithelium (Nguyen *et al*., 2019). On the final analysis, the oral cavity receives cells that are sloughed off from the surface epithelium and gets replenished with cells from the basal layer as a consequence of mitosis.

Various anatomical sites of the oral mucosa exhibit different cell production and turnover rates. This ranges from between one to three weeks as against the epidermal layer of the

integument which ranges from between one to four weeks depending on the site (Nguyen *et al*., 2019). Anatomic location within the oral cavity is also a determinant of how thick the oral epithelium would be. For instance, at the base of the mouth the diameter is between 150 µm and 230 µm whereas in the area of the buccal mucosa lining the cheek, it is between 490 µm and 670 µm thick (Di Stasio *et al*., 2019).

The oral mucosa also has other non-epithelial cell types such as melanocytes responsible for pigmentation, Merkel cells, Langerhans' cells, and cells that respond to inflammatory processess among which are lymphocytes, leukocytes and mast cells (Groeger and Meyle, 2019). Together these non-epithelial cells constitute about 10 percent of the overall population of cells in the oral epithelium (Di Stasio *et al*., 2019). Break in the integrity of the keratinized or non-keratinized stratified squamous epithelia could therefore be a trigger in the failure to confer protection against mechanical, microbial and chemical agents thereby inducing a transformation from normal to malignant squamous epithelial cells.

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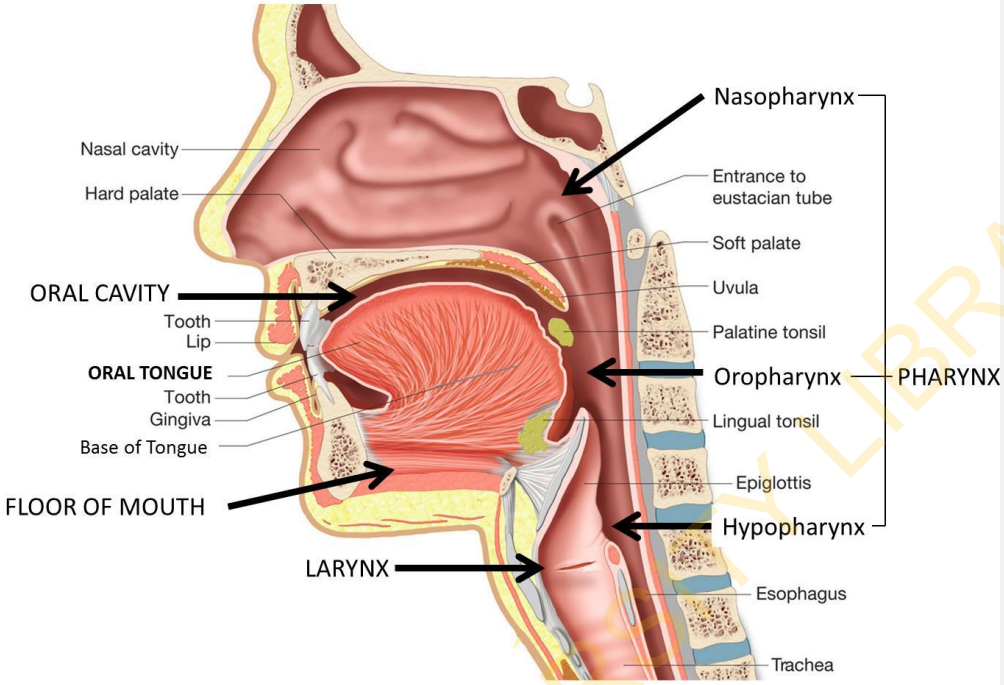

Figure 2.1: Major anatomical locations in the craniofacial and neck region (Lim, 2014).

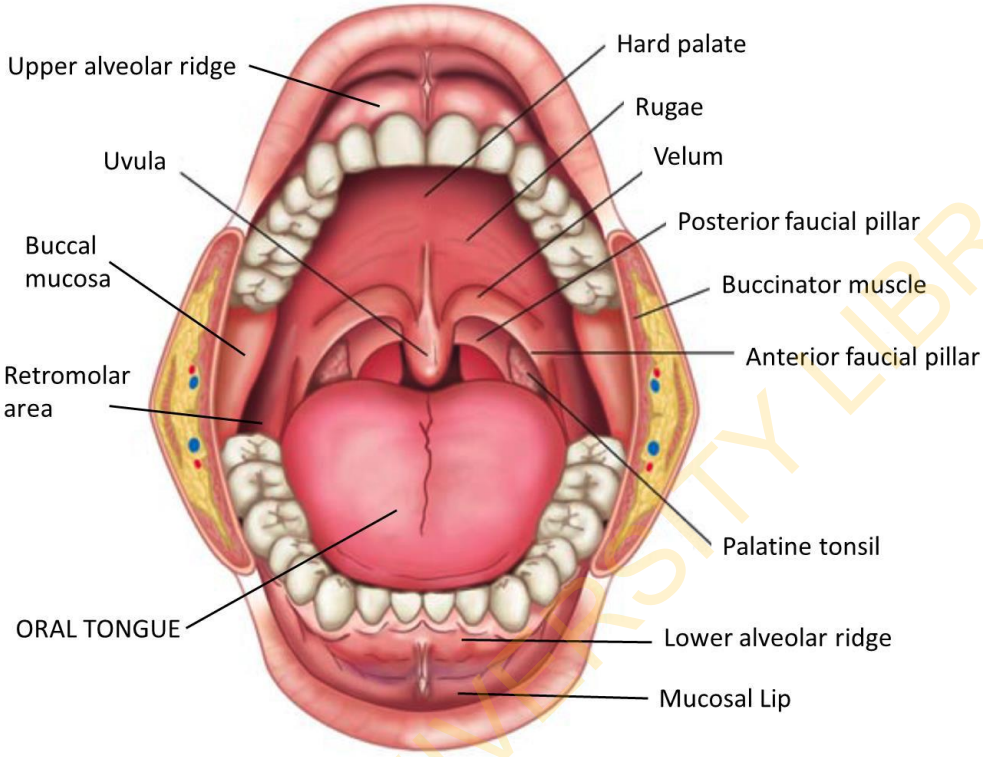

Figure 2.2: Front view of the oral cavity (Lim, 2014).

2.2 Oral Cancer Burden

Malignant tumors originating from sites such as the mucous membrane of the anterior aero-digestive canal, parotid, submandibular and sublingual glands, integumentary system, jaw, skull and facial osseous matter, non-osseous internal structures of craniofacial and neck regions including transportation channels, lymphatic channels, nervous system, fatty and fibrous cell layers, and cells of the neuroendocrine system are referred to as malignancies of the craniofacial and associated structures (Azhar *et al*., 2018). The most commonly encountered histologic form is the squamous cell carcinoma which affects not only the oral cavity but also the oropharyngeal, hypopharyngeal and laryngeal tracts and also as secondary tumors in the nodes of the lymphatics. It is on record that all over the world, about 644,000 fresh cases of cancers localized in the craniofacial and neck regions are detected annually, of which two-third occur in thirdworld countries (Del Corso *et al*., 2016). In 2007 according to Jemal *et al*., (2005), 45,600 fresh instances of malignancy of the craniofacial, neck and associated structures were identified in the United States alone representing 3.2 percent of the total identified cases with 2.2 percent of all mortalities linked to cancer generally. There is also a three-fold preponderance of this cancer in men than women. Notwithstanding the great advancement in cancer care techniques and procedures, the overall mortality rate is still as high as 50% (Walker *et al.*, 2003).

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Among the most frequently encountered cancers globally, oral cancer occupies the 8th position with differences in epidemiological parameters between geographic regions (Petersen, 2003; Hille and Johnson, 2017). The oral cavity harbours a wide array of histologic tumor types with OSCC making up the greater proportion of these cancers arising from mucous membrane surfaces inside the craniofacial and neck section (Ragin *et al*., 2007). In the East African nation of Kenya, the occurence of this disease was put at 2-3% of all neoplasms (Onyango *et al*., 2004). In the Southern and Central part of the Asian continent, the occurrence rate of this disease is perennially elevated, and ranks among three most commonly encountered malignancies, with report that sharp increases are also common in many European countries, Australia and the USA (Stewart and Kleihues, 2003).

About ninety in hundred of all cancerous tumours affecting the mouth and its ancilliary structures is squamous cell carcinoma (SCC), amounting to about five in hundred of all cancerous developments in men and two in hundred in women throughout the world (Parkin *et al.*, 2003).

At time of diagnosis, as many as 43% of patients are known to have the disease spread regionally, in addition to an additional nine in hundred distant metastatic forms, which culminates in overall poor disease outcome (CDC, 1998). According to Parkin *et al*., (2005), the global agency saddled with the responsibility of overseeing epidemiological investigations on cancer gave an estimated global gender frequency of occurrence for this particular cancer in a population of 100, 000 as 6.3 for the male and 3.2 for the female. Estimated data from this organization showed that the Asian continent has the highest frequency of cancer of the craniofacial and neck region for both male and female gender. An area in France known as Somme was reported to post the highest male frequency of occurrence for this disease of 46.2 in a population of 100,000 while Pakistanis had the highest feminine incidence of 23.9 per 100,000 in 2002. The National Cancer Institutes' program on end results of epidemiological survey covering a period of twelve years between 1992 and 2004 while reporting on male to female incidence rates among different racial/ethnic groups showed the following approximate figures per 100,000; non-Hispanic (7:3), black (9:3), Hispanic Latino (4:2) and 4:2 respectively for peoples of American-Indian and Asian/Pacific Islander origins (Brown *et al*., 2010).

Over nine in ten occurrence of this disease is tied to people who are 40 years and above, with a median of sixty (Silverman, 1998). Among Indians, highest age is at the minimum ten years younger in comparison to what is obtained in the Western hemisphere (Parkin *et al,*. 1993). Going by the report of Llewellyn *et al.*, (2003) and Schantz and Yu (2002) in recent years, the population of younger people having oral cancer has been on a steady rise both in the USA and United Kingdom. The highest age-adjusted frequency of occurence is seen in India and Thailand (Petersen, 2003).

Although males have a higher predilection for this cancer more than females, in recent times a progressive rise in women affected over the long term as a result of increased smoking has been observed. According to Barnes *et al.,* (2005), overly high rates have been noted amongst men in France, the Swiss Francophone region, North-Central European countries and some countries in Southern America. In America, a plunge in the male to female ratio in less than fifty years from 6:1 to 2:1 has been observed (Silverman, 1998). Heavy indulgence in chewing of tobacco has resulted in a higher female incidence over time in India (Barnes *et al.,* 2005).

A study emanating from South Africa covering a two year period by Hille *et al*., (1996) on gender incidence rate reported that African males have a yearly occurrence rate of 4.1 per 100,000 while their female counterpart have a yearly occurrence rate of 1.5 per 100,000. This same study reported that between 1988 and 1991 in South Africa of all cancer types, intra-oral cancer cases make up 5.0 percent in males and 1.8 percent in females, and that among the different population groups, male to female incidence rate was 9.05 and 1.75 in blacks; 5.24 and 6.66 in Asians; 13.13 and 3.5 in the mixed races; and 8.06 and 3.18 in whites. Thus females of Asian descent recorded the highest occurrence among women. A subsequent study by Hille and Johnson (2017) among the different population groups on the male to female frequency of occurence of this disease in South Africa, showed that it was 6.17 and 1.10 per 100,000 per annum in Negroes, 8.03 and 3.67 per 100,000 per annum in Caucasians, 4.49 and 8.88 per 100,000 per annum in Asians and 8.85 and 1.94 per 100, 000 per annum in the mixed races.

As reported by Marimo and Hille (2006), the male to female incidence rate for oral cancer (excluding cancer of the lip) between the year 1988 and 2000 in Zimbabwe was 1.09 and 0.51 per 100,000 per annum with an average of 0.79. The report of Kayembe and Kalengayi (1998) in a Congolese population between 1958 and 1994 puts the occurrence of OSCC as 2.1 percent of all cancers while, the male to female frequency of occurrence of this disease between 1988 and 1992 for Algeria was put at 1.1 and 0.3 and for the same period the male to female occurrence of this disease for Uganda was put at 1.2 and 1.9 in a population of 100,000 annually.

Ries *et al*., (2005) while reporting on occurrence of cancer of the oral cavity showed that Black-American men have a higher occurrence of malignancies associated with the oral cavity and oropharynx with 20.7 than their White counterpart with 6.2 per 100,000. They also reported that Black-American males have a death rate which is approximately double that for white males. In the United States, white patients are said to have a better five-year survival rate than for Black-American patients. This submission was buttressed by the report of Ries *et al*., (2005) who showed rates to be 61 to 64% for whites and 40 to 52% for blacks. Ragin *et al*., (2007), submitted that females have a tendency to survive oral cancer disease than males and that for over thirty years the global 5 year survivorship from this cancer disease which is generally lower than fifty percent has remained static.

Early oral cavity cancers could easily be cured however, this is not feasible due to the reason that most patients present for the first time with chronic illness. This makes the possibility of oral cancer resolution to be among the least of the critical malignant diseases. Other than prevention, successful treatment and survival of oral cancer would critically depend on early detection. Additionally, to preserve function of vital organ and physical aesthetics with a resultant improvement in the quality of living, management of these patients are executed mildly. However, this is not often achieved because most oral cancer patients exhibit chronic stage disease that has infiltrated surrounding nodules of the lymphatic system and/or distal sites when initially reporting to the medical facility thus suggesting that detection methods that are currently being practiced which is based on physical examination alone, are not sufficient to mark early stage tumor progression and molecular alterations (Basharat *et al*., 2019). It is therefore the opinion of Epstein *et al*., (2002) that research should be targeted at new diagnostic methods with the objective of developing tumor biomarkers which would ultimately promote early diagnosis by providing biochemical and cellular activities related to oral cancer development.

Consequently, research has prompted the description of several deoxyribonucleic acid, ribonucleic acid and protein biomarkers with significant demonstration of diagnostic relevance, prognostic ability and potential for therapeutics for the disease.

Epstein *et al*., (2002) reported that these biomarkers participate in modulating the life cycle of a cell prominent among them are the cyclins, growth enhancers and their respective receptors which initiate attachment along with migration of cells; angiogenic factors; immunological elements and apoptotic signaling factors which play important role in natural cell death. Because these series of cell transformation preceeds tissue derangements which heralds clinical manifestation of the disease, it is therefore imperative for research to be focused on description and development of diagnostic markers that will detect the disease at early stages to enable better management and raising the survival rate to 85% (Epstein *et al*., 2002).

Conclusively, OSCC is a pathological condition known for its elevated morbidity and death rates along with poor prognosis. Metastatic occurrence is also frequent with a stable 5-year survival rate despite available multiple therapeutic options. The male is more commonly affected perhaps due to socio-cultural peculiarities. Though a disease peculiar to the middle-aged and aged population, however, incidence in younger population has been on the rise (Rivera and Venegas, 2014; Hema *et al*., 2017). In Subsaharan Africa, incidence of this disease is under reported due to absence of focused regional cancer registries.

Comment [u51]: References included Ext

2.3 Risk Factors Associated With Oral Cancer

Aetiology of malignancies linked with the oro-cranial region is pluralistic with no one causative risk agent or factor identified as being responsible for its development. The risk factors associated with this type of cancer were however classified as known, evolving and possible but scientifically yet to be substantiated factors (Warnakulasuriya, 2009).

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2.3.1 Known risk factors

2.3.1.1 Cigarette use and alcoholic liquor consumption

In a report by Azhar *et al*., (2018), it was stated that long time indulgence in smoking of cigarettes and alcoholic liquor consumption are prominent social activities linked with the tendency of developing malignancy associated with the oro-cranial region. Additionally,

individuals who are tobacco addicts have a five to twenty-five fold increased possibility of having this cancer than individuals who have never ever used tobacco. In some areas in Africa and Asia, this cancer is the most commonly encountered cancer type with the commonly associated etiological factors being chronic abuse of tobacco and nuts of the betel palm (Gupta and Johnson, 2017; Azhar *et al*., 2018). In addition, statistical inferences from studies conducted in the Sudan showed a higher rate of this cancer in men than in women, as a result of high indulgence in a local mixture ground tobacco with bicarbonate of sodium known as "Toombak".

Even though several studies including that of Poddar *et al*., (2019) have reported that indulgence in tobacco and alcohol smoking and consumption respectively raises the tendency of developing this cancer in a quantity proportional fashion, yet Castellsague *et al*., (2004) reported that though there is no proven relationship between any commercially known alcoholic beverage and oral cancer development however, all tobacco types raises the risk of developing oral cancer. This report further showed that individuals who indulge in smoking black tobacco have a three times increased tendency of having this cancer than individuals who indulge in smoking other tobacco products. It further observed that simultaneous smoking of tobacco and consumption of alcoholic beverages has a thirteen-fold higher tendency of causing oral cancer. In addition, individuals who indulge in socio-cultural habits such as smoking of smokeless-tobacco as is done in America, chewing of nuts from the betel palm as is the case in India, South-Eastern Asia and Southern Africa; and chewing of khat as practiced by the Yemenese and some Africans raises the tendency for having this disease and other precancerous derangements.

It was reported by Johnson (2001) that Americans of Negroid ancestry has a higher occurrence rate for cancers associated with tobacco smoking than the Caucasian race. Also individuals with this condition have a nineteen-fold increased tendency of having OSCC.

2.3.1.2 Human papillomavirus (HPV) infection and trauma

There is proof linking infection with this virus to orofacial and neck malignancies as an aetiological risk factor; particularly the sub-types six and sixteen are linked with increased possibility of developing cancer affecting the back of the tongue and tonsil (Nabirye and

Kamulegeya, 2019). A positive association between the DNA of this virus and this disease was reported by Herrero *et al*., (2003) and that 4 percent of malignancies of the mouth cavity as well as 18 percent of malignancies of the pharynx tested positive for the viral DNA. It was also reported that outcome of molecular epidemiological studies showed that 25 percent of all oro-cranial carcinomas and 60 percent of oropharyngeal carcinomas are associated with HPV infection. As reported by Herrero *et al*., (2003), the most frequently affected sites for human papilloma virus related malignancies associated with the orofacial and neck region are the oropharynx and tonsil. They are also more frequently encountered in persons who do not smoke than in persons who smoke. As observed, these trends are related to the study population, specimen type and process of detection. Also chronic physical damage to the mucosal surface of the oral cavity in conjunction with other factors is assumed to play prominent part in the aetiology of this disease. It was therefore conclusively observed that there is an increased odd of having this cancer with long standing damage to the oral mucosal surface.

2.4 Evolving Risk Factors

2.4.1 Dietary and nutritional risk factors

According to Zhang *et al*., (2019), diet and nutrition constitute significant possible causes for this type of malignancy. Cancers of the mouth cavity is closely tied to Sideropenic dysphagia which is a medical syndrome characterized by deficiencies of vitamin A and iron.

A study by Pavia *et al*., (2006) on how eating of none animal food such as fruit and vegetable relates to this disease, showed that a regular diet that includes none animal food resulted in drastic decrease in the tendency of developing this kind of malignancy.

According to Llewellyn *et al*., (2004), children whose diet includes several portions of vegetarian food are less likely to develop this type of cancer at a younger age than children who consume two portions or less as they are more likely to develop resistance to oral carcinogenesis.

2.4.2 Immunological and socio-economic status

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Immunosuppression and low socio-economic status is suspected to have a correlation with genesis of malignancies of the mouth cavity. An amplified occurrence of lip cancer was reported in persons who had kidney transplant and this was shown to be related to immune-suppression. A strong association between ultraviolet rays and possibility to have cancers of the lip has been observed. To this end, Neville *et al*., (2009) reported that this phenomenon is prevalent in light-skinned individuals and in people whose occupations expose them to ultraviolet radiation from sunlight for long periods such as farmers and sailors. Also across the world people within the low socio-economic ladder (remuneration, education and occupation) are more vulnerable to this type of cancer.

2.5 Possible but Scientifically Unsubstantiated Factors

2.5.1 Human immunodeficiency virus (HIV) infection

Comment [u54]: Abbreviation added Int

It is anticipated that immunosuppression in Human Immunodeficiency Virus (HIV) infected individuals could lead to an increased tendency for occurrence of this type of cancer. Although HIV/AIDS is being linked with several malignant conditions, this is yet to be seen to have a positive correlation with cancers affecting this anatomic site in Kenya (Onyango *et al*., 2004). The authors are of the opinion that co-existing HIV and OSCC cases was yet to reach a level in terms of numbers as to inform any definite conclusion on HIV infection and oral cancer in the nation. The study did not produce information on other types of oral malignancies.

A latter study by Butt *et al*., (2008) on 200 HIV-infected Kenyan subjects showed that 68 percent of the patients had oral Kaposi's sarcoma while, 17 percent of the subjects had squamous cell carcinoma, 13 percent had non- Hodgkin's lymphoma and the remaining 2 percent had Burkitt's lymphoma of craniofacial and neck region. Another result emanating from this study showed that HIV patients developed the malignancy at a lower age than in their virus-free counterparts. Studies conducted thus far have not yielded any positive correlation between HIV/AIDS and malignancy of the mouth cavity. In addition other possible causative factors that are not yet scientifically substantiated include hereditary and familial risk factors, indulgence in smoking of marijuana and presence of ethanol in mouth-washes (Warnakulasuriya, 2009).

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2.6 Disease Progression in Cancer of the Oral Cavity

2.6.1 Clinical progression

According to van der Waal (2019), OSCC manifests as a secondary development to clinically visible premalignant lesions in the mucosal surfaces of the epithelia known as oral pre-malignant lesions (OPL). As described by Jackler and Kaplan (2004), these lesions characteristically appear as either white plaques or red plaques with the white plaques being the most frequently occurring variety of premalignant mouth lesions, accounting for 85 percent of all pre-malignant lesions.

Neville and Day (2002) intimated that anatomic sites frequently affected by leukoplakia include the mucosal surface of the buccal cavity, mucosal surface of the alveolar region and lower lip with a large proportion of the lesions presenting with non-malignant hyperkeratosis which is an inflammatory reaction to chronic irritation from artificial dentures, microorganisms such as lichen planus, use of tobacco or bitting of the cheek. Furthermore, it was observed by Pindborg *et al*., (1977); Neville and Day (2002) and Kaur (2019) that a fraction of leukoplakias could transform into full blown cancer at rates ranging from 3 percent to 17.5 percent.

On the other hand Neville and Day (2002); Ranganathan and Kavitha (2019) are of the opinion that erythroplakia is not commonly encountered as leukoplakia although it has a 90 to 100 percent correlation with either dysplastic changes or outright carcinoma at initial identification.

They are also of the opinion that clinical identification of such lesions having a high tendency of transforming into carcinoma cannot be overemphasized, as successful treatment and prevention of transformation into cancer hinges on it however, this becomes difficult as only a few of these lesions are known to actually progress to cancer. Factors considered in the assessment of malignant potential include clinical appearance, which could either be homogeneity or heterogeneity of the lesion, causative agent, anatomic site, occurence and extent of dysplastic changes. As opined by Reibel (2003), heterogeneous

white lesions have a 40 to 50 percent tendency of malignant transformation than the homogeneous lesions.

Anatomic sites that have been identified as been very susceptible to dysplastic and malignant transformation include the base of the oral cavity and the tip, side and under side of the tongue (Neville and Day, 2002). According to Reibel (2003), currently however, occurrence and extent of dysplastic changes observed on the lesion biopsy remains the gold standard for predicting malignant transformation.

2.6.2 Histological progression

Dysplasia is a histological term used in describing the occurrence and extent of abnormality in the epithelium of the oral acvity, often grouped into different types on the basis of degree of abnormality. Ahmed *et al*., (2019) highlighted diagnostic histological indicators of epithelial dysplasia as alterations in general tissue and individual cell morphology including aberrant nucleo-cytoplasmic ratio, presence of abnormal mitoses and variable nuclear and cytoplasmic shape and size.

In general, there is a direct proportionality between the tendency to develop cancer and the extent of abnormality observed in the epithelial cells covering the oral cavity (Reibel, 2003). Setbacks have been observed in the utility of extent of abnormalities in this anatomic site in predicting malignant transformation from oral precancerous lesions into cancer. Among them is the subjectivity which characterizes the diagnosis and grading of dysplasia which comes with high inter-observer variability (Karabulut *et al*., 1995). This is to a great extent due to the inexistence of a standardized method of characterizing these epithelial dysplastic changes (Pindborg *et al*., 1985); as well as the fact that not every one of these dysplastic lesions in the epithelium of the oral cavity will fully transform into cancer (Reibel, 2003).

As reported by Pindborg *et al*., (1977), withdrawal from tobacco use resulted in reversal of precancerous lesions exhibiting dysplastic changes in the oral cavity in about 15% of cases. Surprisingly oral precancerous lesions diagnosed as not exhibiting dysplastic changes have been seen to transform into cancer. As stated by Lee *et al*., (2000) and

Reibel (2003), this calls for a concerted clinical and laboratory effort particularly to develop diagnostic biomarkers for predicting oral premalignant lesions showing high tendency of progressing to cancer. According to Neville and Day (2002) and Rivera and Venegas (2014), OSCC may be viewed as developing from the transformation of premalignant lesions exhibiting dysplastic changes in the oral cavity into cancer. They further stated that presence of extreme dysplastic changes distinguished by the presence of abnormal cells localized within tissues lining the outer surface of the oral cavity progresses until the aberrant cells cross the basement membrane barrier and ultimately invade the underlying sub-mucosa to become invasive squamous cell carcinoma. Neville and Day (2002) and Reibel (2003) are of the opinion that about 40% of OSCC are traced to the lateral tongue while the base of the mouth is also a commonly affected anatomic location. This they said is likely due to the accumulation cancer-causing biomolecules in the saliva in these anatomic sites together with the loss of epithelial shielding which is characteristically thin and non-keratinized in this region. The border of the lip vermilion according to Neville and Day (2002) is highly susceptible to the occurence of several types of squamous cell carcinomas because of its excessive exposure to the sun although, it possesses a low tendency to metastasize to other sites.

At first diagnosis, more than 50 percent of OSCC exhibit advanced metastases to proximal oral cavity sites and distal sites including the cervical and other regional lymph nodes. Although distant metastases of OSCC can develop in any area of the body, they are however very commonly associated with the respiratory organs, osteous structures and liver (Neville and Day, 2002). At the point of diagnosis, about 9 to 14 percent of oral cancer patients often have an extra initial derangement in the anterior respiratory and digestive canal (Lipmann and Hong, 1989).

In oral cancer patients, the tendency to develop post-treatment second primary tumor annually is 3 to 7 percent higher than any other malignancy (Day and Blot, 1992). This trend is not unconnected to the effect of cancer-causing biochemical agents in tobacco and alcohol on the layer of mucus secreting epithelial cells in the oral enclosure through the process of carcinogenic alterations (Slaughter *et al*., 1953).

This theory which was originally proposed by Slaughter *et al*., (1953), suggests that as the cancer-causing biochemical agents in tobacco and alcohol comes in close contact with cells of the mucosa covering the oral cavity conditions this site for cancer development by conferring on it sub-clinical alterations pointing to carcinogenesis. The case in which a visually normal appearing mucosal epithelium lining the fringes of a lesion in the oral cavity or in a mirror-image surgical excision exhibit clear changes at the clinical, histopathological and molecular (Lydiatt *et al*., 1998) levels, lends credence to this theory.

2.6.3 Molecular progression

At the molecular level, OSCC develops through a multiple stage process involving several changes in the genetic, epigenetic and biochemical constitution in genes that suppress and promote cancer development resulting in clinically and histologically visible manifestations at the cellular and tissue morphology levels (Jain, 2019). These events at the molecular level takes place early in the series of events leading to cancer onset and may used to indicate the presence of disease and/or the measurement of how the disease is progressing (Califano *et al*., 1996; Lydiatt *et al*., 1998).

As with colorectal cancer, the picture of changes at the genetic level observed in each histopathological stage traversing precancerous lesions to invasive cancer has given insight into the step by step pattern of molecular progression for orofacial cancers (Califano *et al*., 1996). A typical example is the frequently encountered loss of the entire gene and the surrounding chromosomal region observed in the smaller segment of chromosome 9p21 which is believed to be an initial signature in the onset of cancer consequently causing loss of activity of *P16* gene encoding a cyclin-dependent kinase inhibitor fingered as mediating processes involving cell cycle (Califano *et al*., 1996). Other developments of this activity is the loss of heterozygosity at 17p13, 11q13 and 13q loci of *P53* , *CCND1* and *RB* genes respectively, whose proteins are important in the mediation of progression of several activities in the cell (Califano *et al*., 1996).

It should be emphasized therefore that the order at which these genetic events occur is not as significant as their accumulation in determining malignant progression. As was

observed by Rosin *et al*., (2000), in the exclusion of genetic changes the tendency of cancer development from oral precancerous lesions was minimal but witnessed a moderate rise with inclusion of these aberrations on the short arms of the third and ninth chromosomes and a tremendous rise when aberrations in these chromosomes occur simultaneously with losses in short arms of the eight and seventeenth chromosomes and long arms of the fourth, eleventh and thirteenth chromosomes.

As earlier said, continuous accumulation of genetic alterations resulting from exposure to cancer-causing agents in tobacco and alcohol products culminates in generalized genomic turbulence characteristic of advanced cancer progression and spread to distant sites. Inspite of providing a basic understanding of the genetic signatures seen in the initial stages of development of malignancy, it however fails to fully explain the process of tumor development. According to Sidransky (2002), onset of cancer is not just the outcome of genetic mutation only, but involves several other mechanisms such as the epigenetic and biochemical transformation of function of a cell which include promoter hypermethylation and post-translational protein rearrangements which results in structural, functional, enzymatic activity, localization and expressional changes.

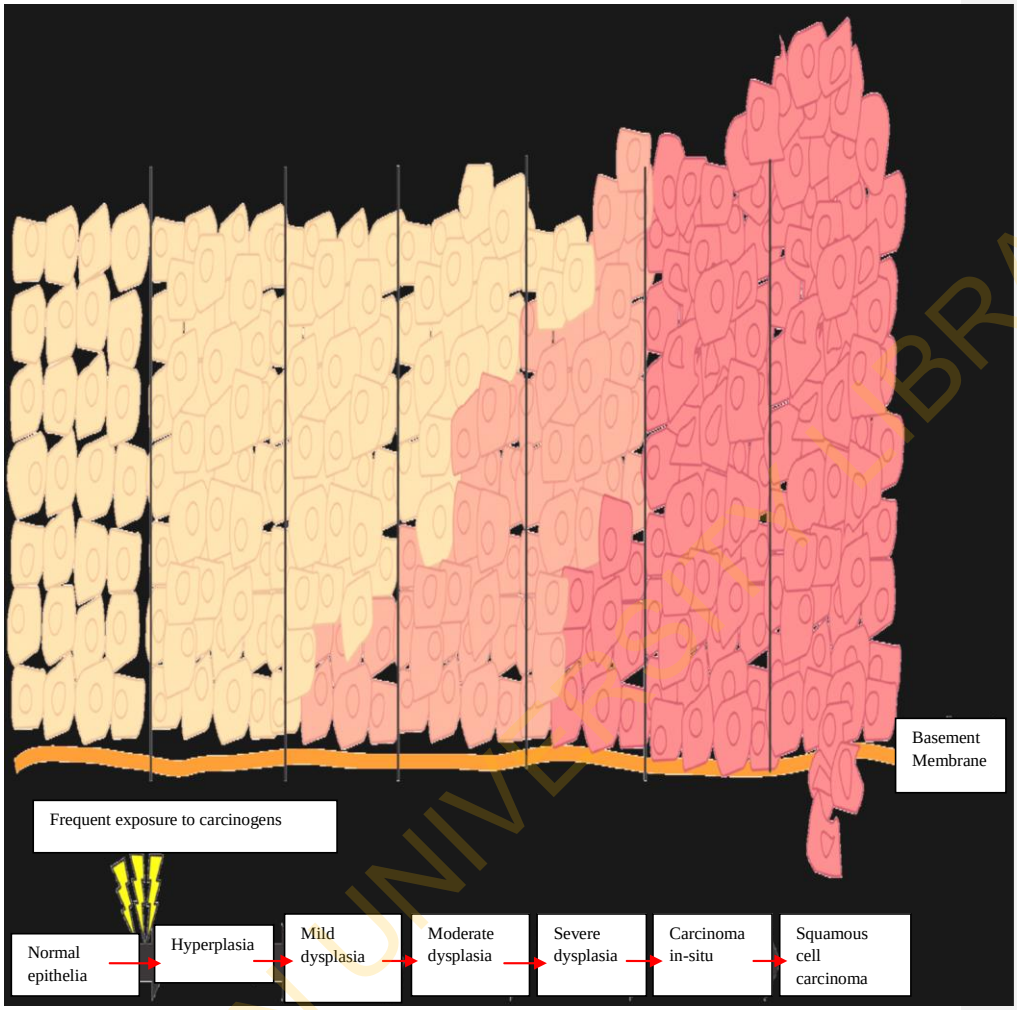

Figure 2.3: Microscopic changes in the development of oral cancer (Towle, 2016).

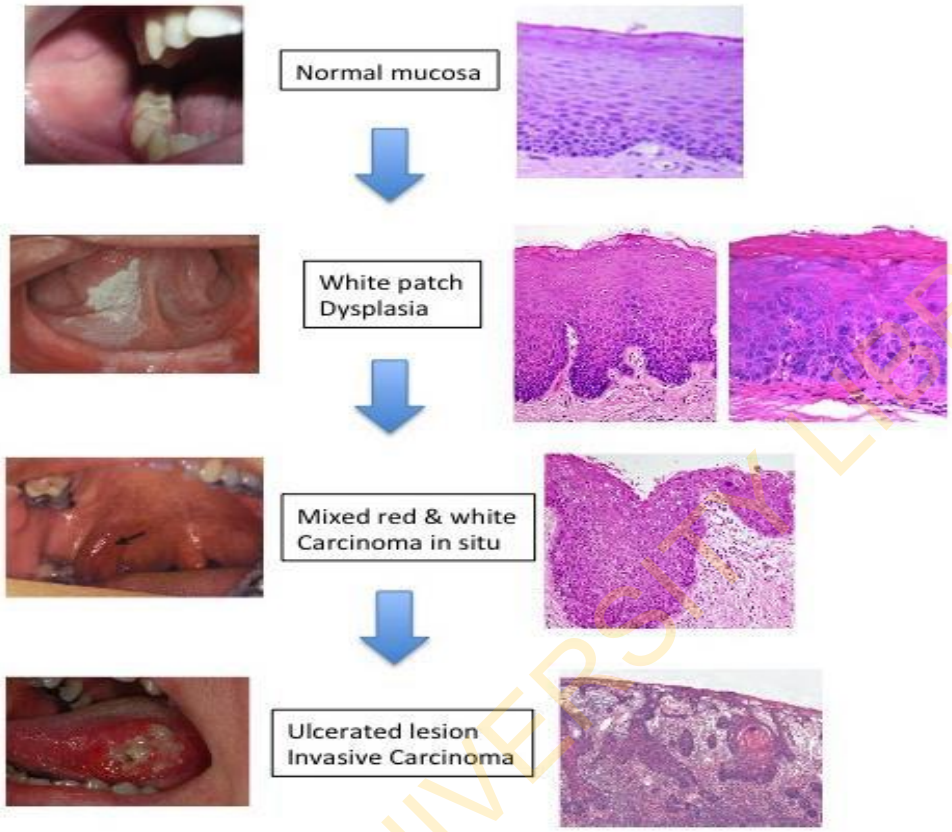

Figure 2.4: Anatomic and histologic model of progression of OSCC (Zaini, 2015).

2.7 Screening of Oral Cavity for Cancer

Conventional oral examination (COE) which involves physical interrogation of the oral cavity for abnormal developments in the mucosa of the epithelium is currently the universally accepted method of detecting pre-malignant and malignant derangements (Epstein *et al*., 2012). Using this protocol, Horowitz and Alfano (2001) was of the opinion that professional dental practitioners and primary care physicians who see patients on a regular basis are easily more able to recognize early-stage derangements but, only a meager 13 percent of the American population report being subjected to such examinations previously and the knowledge of oral cancer examination protocol seem to be inadequate among clinicians.

In Nigeria, Oyapero *et al*., (2016) in a survey embarked on to ascertain the extent of conversance about oral cancer examination in subjects visiting a dental facility observed that 66% of the participants attested to knowing about this cancer while, only 3% claimed to have received the information on oral cancer from their dental practitioner and 10 percent claimed to have received the information from their physician. They also stated that 82 percent of the participants have never received oral cancer examination while only 7 percent had received tongue examination. In another survey conducted to ascertain the knowledge of dental practioners on oral cancer examination practices, Gbotolorun *et al*., (2014) observed that 58.1 percent claimed to have adequate expertise in the evaluation of oral cancers while, 53.4 percent claimed to have adequate expertise in the evaluation of the oral cavity of all newly enrolled patients for cancer examination. While reporting on the outcome of seven different studies that evaluated this protocol as a means for the detection of initial malignant derangements, Downer *et al*., (2004) showed that its sensitivity ranged between 60 and 97% while specificity ranged between 75 and 99%, which are similar to values found in comparable protocols used for evaluation of malignancy thus suggesting that this protocol may be adequate for screening and also in the identification of disease derangements in the oral cavity. Among the protocol's shortcomings are its not been able to detect none severe and none symptomatous derangements or differentiate non-cancerous derangements from those with a serious tendency to transform into malignant lesions by using other detective measures (Lingen,

2007). Its success in reducing disease-related deaths is yet to be determined (Kujan *et al*., 2006).

2.8 Oral Cancer Detection and Staging

Following initial physical examination (with or without ancilliary tests) and identification of a suspected precancerous oral lesion, clinicians follow-up the suspected case typically by monitoring it for a two week period after which persistence of the lesion without an identifiable cause of local inflammation requires the performance of a surgical biopsy for definitive tissue pathology diagnosis (Epstein *et al*., 2007). Other important considerations for the performance of surgical biopsy include clinical picture of the lesion such as presence of induration, formation of erythematous tissue and ulceration of the mucosal epithelial cells, anatomic location; for example the base of the mouth or tongue, size of the lesion, patients history, such as previous cancer, involvement in tobacco and alcohol use and symptomatic manifestations for instance feelings of numbness or pain (Epstein *et al*., 2007).

Generally after the initial period of monitoring, moderately dysplastic oral precancerous lesions or worse conditions are removed surgically and further monitoring carried out subsequently (Neville and Day, 2002; Epstein *et al*., 2007). These procedures according to Holmstrup *et al*., (2006) do not prevent transformation of the precancerous lesion into a malignant lesion as. As explained by Holmstrup *et al*., (2006), this apparent shortcoming is partly due to failure to adequately define lesion margins during surgical excision and probably also due to the persistence of cancer cells that have migrated by metastases beyond the site of excision as contained in the field cancerization model. According to Green *et al*., (2002), confirmed cases of invasive OSCC are further subjected to a more comprehensive physical examination and other ancilliary imaging tests such as endoscopy among others in order to assess metastatic phenomenon, determine the stage of progression of the cancer, establish appropriate treatment modality and for prognostic evaluation.

In 2002, the TNM (tumour-node-metastasis) method for classification and grading of tumors where the acronym ‘TNM’ symbolizes primary size of tumour, degree of

involvement of lymph node and metastatic phenomenon was proposed (Green *et al*., 2002).

In this system of classification, according to Green *et al*., (2002) four clinical stages denoted by letters I to IV from which the five-year survival rate could be determined was established. They also stated that early stage disease denoted by letters I to II stands for disease survival rate of five-years that is close to 85 percent, whereas late stage disease denoted by letters III to IV has a similar survival rate that is however at or below 41percent.

2.9 Therapy and Prognosis of Oral Cancer

According to Palme *et al*., (2004), OSCC cases that fall within the early stage group (stage I-II) with tumour restricted to the oral cavity and not more than 4 cm in size are best managed by surgical excision and/or radiotherapy. Locally advanced disease (stage III-IVA/B) on the other hand requires a combination of treatment approaches which usually includes surgical excision, radiotherapy and chemotherapy (Seiwert and Cohen, 2005).

This combination therapy according to Seiwert and Cohen (2005) reduces death in most OSCC patients following loco-regional failure. They also stated that alternatively, neo-adjuvant chemo-radiotherapy with or without surgical excision are increasingly being used as a treatment modality to salvage and preserve organs. An area of concern in the management of this disease is the development of therapy-induced cytotoxicity. In the opinion of Seiwert and Cohen (2005) and Rao *et al*., (2015), this could be drastically reduced and treatment enhanced with the emergence of molecular targeted therapeutics one of which is the commercially available *EGFR* monoclonal antibody known as cetuximab (Erbitux®, Bristol MyersSquibb). According to Tralongo *et al*., (1999), metastasis of primary OSCC to loco-regional lymph nodes translates to reduction of survival rate by 50% thereby making lymph node status the most significant prognostic factor for OSCC.

It therefore means that in such patients the number of affected lymph nodes and spread to the extra-capsular region contribute to a poor prognosis. Other classic clinico-pathological characteristics such as tumor location, size, grade and widest thickness are not very significant in predicting and identifying patients who are likely to experience post-treatment recurrence or death (Tralongo *et al*., 1999).

2.10 Development of Oral Cancer Biomarkers

In the early 2000's, developments in high-throughput molecular technologies has simplified the rather hitherto complex cellular mechanisms associated with cancer development by identifying a number of oral cancer markers of initiation and progression at the molecular level (Gourin *et al*., 2006). In connection with oncology research, Kumar *et al*., (2006) defined biomarkers as any biochemical substance or mechanism pathway that indicates tissue malignancy. The medical usefulness attached to biomarkers is not only for detection of cancer but also include routine screening, definitive diagnosis, staging, prognostic prediction and monitoring of treatment and post-treatment relapse (Kumar *et al*., 2006). Efforts at reversing or inhibiting development of cancer have given rise to the unearthing of biomarkers with important role in tumourigenesis and have their origin in the tumour otherwise known as tumour-derived markers which acts as targets for therapeutic intervention (Brinkman and Wong, 2006).

Four important factors are taken into consideration when assessing biomarker efficiency; these are its sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). According to Kumar *et al*., (2006), sensitivity of a biomarker is taken to be the number of diseased persons whose test result returns as positive using the biomarker assay, whereas specificity of a biomarker is taken as the number of non-diseased individuals whose test result returns as negative. Therefore ideally for biomarkers, these parameters would be in the realm of 100% respectively with individuals having cancer testing positive and those not having cancer testing negative.

Kumar *et al*., (2006) further stated that sensitivity and specificity rates depend on the purpose for which the biomarker is being used where emphasis is laid on sensitivity for

those intended for diagnostic or prognostic tests and specificity for those intended for screening tests. Positive and negative predictive values are the other two factors used in assessing the performance of a biomarker. They are values estimated from either the sensitivity or specificity test result of a biomarker. While the positive predictive value gives the proportion of persons exhibiting an affirmative test result, the negative predictive valus gives the percentage of individuals with a negative test result (Kumar *et al*., 2006).

Advances have been made in describing the nature of some biomarkers that demonstrate significant potential in the detection, outcome and therapy in oral cavity associated malignancies. Their classification is based on their normal activities in the different processes which takes place in the cell (Epstein *et al*., 2002). Other genomic markers that have been described include those that are responsible for aberrations in cancer suppressor and activator genes resulting in cell cycle malfunctioning and those that give rise to genetic/chromosomal instability such as aneuploidy which is a change in the content of the DNA, micronuclei, microsatellite repeats, and loss of heterozygosity such as allelic imbalance (Brinkman and Wong, 2006). An important finding is the epidermal growth factor receptor (*EGFR*) which happens to be a biomarker that is not only upregulated in 90 percent of all OSCC using the lab-on-chip technology (LOC) but, also demonstrates significant relationship with early cancer development and aggressive cancer types (Pomerantz and Grandis, 2003).

During cancer development, several cellular activities are disrupted therefore a single biomarker alone will be unable to meet the high sensitivity and specificity rates needed for definitive cancer diagnosis. To this end according to Lee *et al*., (2000), a spectrum of biomarkers are needed for early diagnosis and examination even though the adjunctive use of single biomarkers in diagnostic procedures has proved to be important in not only enhancing prompt detection of this type of cancer but also in eliminating errors of non-objectivity linked with conventional tissue pathology diagnosis and giving molecular-based information of risk evaluation in primary or recurrent derangements. Aside from using malignant solid oral tissues or cells as diagnostic samples, several biomarkers that are of DNA, RNA and protein in origin have been described using extracellular fluids as

diagnostic samples for OSCC (Li *et al*., 2004). This is principally so for saliva not only as a result of its closeness to the primary growth site but also because of the preponderance of cells shed from the oral cavity and ease of access to the tumour.

Li *et al*., (2004) were able to describe seven biomarkers including several proteins exhibiting inflammatory activity and cellular enzymes with high degrees of sensitivity and specificity that have the ability to distinguish OSCC from non-malignant controls while examining the salivary mRNA transcript landscape. The shortcoming in this is the non-inclusion of controls from pre-cancerous lesions and other disease conditions such as infection of the gum in the research which puts the routine administration of such methods in clinical detection in the realm of uncertainty. Other approaches such as use of naturally exfoliated epithelial cells into oral rinse solutions or mechanically exfoliated cells have consistently failed to yield results needed for oral cancer detection because of the limited sampling of the cells of the basal epithelium where most dysplastic alterations are first observed but lost as the cells migrate upwards towards the exterior and become more developed and keratinized with little diagnostic relevance (Epstein *et al*., 2002).

A full mechanical sampling of the entire epithelial landscape using a cyto-brush for conventional cyto-morphological analysis has been described (Epstein *et al*., 2002). In a bid to improve the detection and characterization of OSCC, exfoliated cells from the oral cavity have also been subjected to a wide array of molecular analytical techniques such as loss of heterozygosity, simple sequence length polymorphisms, quantitative PCR for gene expression (Schwartz *et al*., 2008). In addition, non-mechanical sampling of epithelial cells shed into the saliva have also been shown to yield important information related to oral cancer diagnosis. They were able to identify more than 1000 proteins and 30 species of bacteria in the cells obtained from the saliva of OSCC subjects although, the effectiveness of this method for early cancer diagnosis is still doubtful because it is yet to be substantiated.

In spite of these advances leading to the identification of several OSCC biomarkers with potential for diagnossis and prognosis, the translation of these breakthroughs into clinical practice has not been successful.

According to Bast *et al*., (2005) a major hinderance in the application of biomarkers in clinical practice include the high complexity associated with many of the biomarker assay procedure such as the large instrumentation requirements, elaborate quality control and high level of technical skill involved in many of the genetic and proteomic techniques which exceed the capacity of health care centers. Also, the parochial focus of efforts at identifying OSCC biomarkers to the neglect of pre-cancerous lesions because of unavailability or inability to recruit this category of human subjects and their solid tumors have limited the discovery and utilization of biomarkers for early diagnosis. They also pointed to the astronomical financial implication linked with the discovery, accuracy checking and administrative sanctioning procedure as a setback. These are therefore important issues that should be considered if the tests would be adopted for routine use in clinical practice.

2.11 Cancer of the Oral Cavity in Young People

As stated by Cicciu *et al*., (2019), the occurrence of malignancies of the craniofacial and neck region depends on patient's age with about 98% of subjects being over 40 years in Europe. Although this type of cancer is uncommon in persons between 0 and 39 years old with about 4 to 6% of persons in this age bracket thought to be involved, in recent times however, the incidence has been observed to be on the increase. An increased occurrence of this type of malignancy in persons below forty-five in age have been observed by Schantz and Yu (2002); Llewellyn *et al*., (2003). It was reported by Llewellyn *et al*., (2003) that between 1989 and 1996, a rise of about 31% was observed in the occurrence of this malignancy in young people in Scotland and that although a history of consumption of alcohol and smoking of cigarette was reported in a high percentage of these young people, these risk factors did not seem to exert significant part in the onset of this type of malignancy in this age group. It was therefore concluded that genetics might have a part to play in the occurrence of this malignancy in these patients. Another point of view expressed by Johnson (2001) is that increased smoking of varieties of tobacco could lead to a high occurrence of this type of malignancy prior to the age of 35 years.

While assessing the pattern of OSCC with respect to age between 1971 and 2006 at Emory University, USA, it was reported by Müller *et al*., (2008) that 5 percent of the 1919 patients recruited for the research were young people between 0 and 39 years. They also reported that the average age at onset was $32^{1/2}$ years irrespective of gender which was at variance with what was reported in older people showing that females were about 5 years older than males. The same report also showed the existence of significant explosion in young people exhibiting cancer of the tongue as against older people. In their own report, Schantz and Yu (2002) showed that the existence of a 62% rise in the occurrence of craniofacial and neck cancer in young American individuals between two different time lines. This increase was partially linked to the rise in the occurrence of tongue cancer which is in concordance with the opinion of Müller *et al*., (2008) who noted an amplification of 62.1 percent in the occurence of this type of malignancy in the tongue in people below 40 years as compared to 27.4 percent in subjects above forty years old. It is yet to be definitively proved why this type of malignant aberration has predilection for the tongue in young people but it has been suggested by Llewellyn *et al*., (2003) that there may be a genetic undertone.

2.12 Mechanisms of Carcinogenesis in Oral Cancer

2.12.1 Genetic changes associated with oral carcinogenesis

Analogous to the progression model that is now accepted for colorectal carcinoma (Fearon and Vogelstein, 1990), the genetic alterations characteristic of OSCC are understood to occur in a multistep, sequential pattern (Califano *et al*., 1996; Leemans *et al*., 2011). The variety of potentially malignant disorders contains common chromosomal, genetic, and molecular aberrations that are also present in OSCC (Mithani *et al*., 2007). A working model for the formation of OSCC has been widely accepted. As initially propounded by Califano *et al*., (1996), this scheme describes sequential loss of heterozygosity (LOH) in chromosomal regions that hold key cancer-associated genes, and maps these genomic changes to clinical and histological precursor lesions. The model draws attention to alterations in the genes governing growth promotion (proto-oncogenes) and growth inhibition (Califano *et al*., 1996).

Mutations affecting proto-oncogenes result in abnormally activated oncogenes that confer growth self-sufficiency to cells, thus enabling them to escape normal growth control mechanisms (Kumar *et al*., 2005). Early research into proto-oncogenes in OSCC focused largely on *c-myc* (Eversole and Sapp, 1993), *K-ras* (Caulin *et al*., 2004; Vitale-Cross *et al*., 2004a) and *ErbB-1* genes (Wong, 1987).

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Ki67 is a biomarker of cell multiplication and growth employed in the detection and prognostication of malignancies of the mammary gland and prostate, in which the amount of *Ki67* cells exhibiting positivity correlates with the sequence of clinical disease manifestation (Scholzen and Gerdes, 2000). *Ki67* alterations have also been documented in OPMD (oral pre-malignant diseases). However, *Ki67* has yet to be validated as a prognostic marker for detecting the subset of OPMD that are destined to undergo malignant transformation (Kovesi and Szende, 2003).

More recent work on OSCC oncogenes has focused on the complex protein kinase-B (*Akt*) pathway. According to Wang *et al*., (2014), there lies a strong reason to believe in the potential therapeutic significance of this pathway. The tumour-suppressor genes of greatest significance in OSCC formation are *P53* and *CDKN2A* (Kumar *et al*., 2005). *CDKN2A* encodes *P16INK4a,* which controls the life cycle of a cell through inactivation of cyclin-dependent kinases. *CDKN2A* may be inactivated by promoter hyper-methylation in combination with deletions in chromosomal regions 3p and 9p21 while, (Califano *et al*., 1996). *P53* encodes a significant tumor-suppressor protein which is transformed in more than fifty percent of human malignancies (Yuen *et al*., 2001). *P53* mutations are a consistent feature of OSCC (Gasco and Crook, 2003). It is situated on the short arm of chromosome 17 and encrypts a transcription factor responsible for detecting disruptions in the DNA and other indicators of stress. *P53* acts as a 'gatekeeper' at critical stages of the cell cycle, permitting repair of damaged DNA prior to DNA synthesis and triggering apoptosis if irreparable DNA damage is detected (Liu and Gelmann, 2002). *P53* mutations therefore result in loss of this critical tumor-suppressor function.

This confers a significant survival advantage and is frequently an early step in oral carcinogenesis (Leemans *et al*., 2011). *P53* is upregulated in human OPMD and its

manifestation in suprabasal keratinocytes has been associated with cancerous changes (Kovesi and Szende, 2003). The carcinogens present in tobacco smoke (which represents a major determinant of OSCC development) are linked with a rise in both the number and type of *P53* mutations (Brennan *et al*., 1995).

While this genetic progression model continues to frame the current molecular understanding of OSCC formation, it remains incomplete. For example, the model does not account for the over-expression of epidermal growth factor receptor (*EGFR*) that features prominently in a high proportion of OSCC (Grandis and Tweardy, 1993a). The most reliable factors for predicting patients' risk of developing OSCC continue to be their cancer history and the presence/grade of epithelial dysplasia.

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Markers for LOH, chromosomal aneuploidy, and aberrant *P16INK4a/p53* expression remain adjuncts to these clinical and histopathological parameters (Leemans *et al*., 2011). Advances in the field of ploidy analysis are compromised by the questionable probity of some studies; initial hopes that reliability the detection of aneuploidy might identify which epithelium is susceptible to developing OSCC have yet to be widely accepted (Torres-Rendon *et al*., 2009). Doubts have also emerged as to the veracity of *P53* as a stand alone prognostic biomarker (Takeda *et al*., 2006). Although it is the gold standard of detection and prognostication however, reliability of histopathological examination is itself compromised by well-documented intra-and inter-observer variation (Kujan *et al*., 2007).

There is a continuing need to delineate the genetic alterations and molecular events of OSCC formation with a view to explicate the development of reliable diagnostic and prognostic biomarkers. A perfect biomarker should be measurable without ambiguity in tiny biopsies and changed in suspicious tissues in the initial phases of oral malignant transformation (Wu *et al*., 2010).

Stable tissue structure is promoted by cell adhesion molecules of which loss of their expression enhances proliferation of tumor cells and metastasis. E-cadherin is a substance made up of glycoprotein spanning the entire membrane saddled with the function of

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regulating cell to cell adhesion which when unable to do this results in dissemination of malignant cells to distant organs in OSCC. DNA and non-DNA aberrations involving *E-cadherin* gene is observed in tumor locations such as the mammary gland and stomach (Tanaka *et al*., 2003). In malignancies affecting the craniofacial and neck region, promoter hypermethylation of this gene is more important as it has been demonstrated in 46 percent of the samples examined where it is associated with the poorly differentiated histological grade (Nakayama *et al*., 2001) in addition to poor survival (Chang *et al*., 2003). Yeh *et al*., (2002) on their part did not observe this kind of relationship but reported no relationship between up-regulation and promoter hypermethylation of *E-cadherin*, hence there is likelihood that other genetic alterations were responsible for the pattern in this Taiwanese population.

Epigenetic aberration in *DAPK* has also been observed in 27 percent of tissues from the craniofacial and neck region examined however, only in a single report was it significantly associated with nodal stage and according to Kulkarni and Saranath (2004), no significant correlation has been made between *DAP-kinase* promoter hypermethylation and oral cavity and nasopharyngeal specimens respectively.

Although this epigenetic mechanism is a frequently occurring phenomenon in OSCC and has been extensively investigated in several genes that regulate cell cycle events including *P15* and *P16* genes however, no significant correlation with clinical, pathological and prognostic characteristics have been observed. In addition, this is also the case for benign as well as pre-cancerous oral lesions (Chang *et al*., 2004), and may therefore be important developmental signatures in the initial phases of alcohol and tobacco related carcinogenesis Chang *et al*., 2004; Kulkarni and Saranath, 2004).

Hypermethylation of *P14* gene has been correlated with favourable outcome in this type of malignancy while promoter hypermethylation of Deleted in Colorectal Carcinoma (*DCC*) gene has been significantly correlated with invasiveness in mandibular oral cancer which is interpreted as unfavorable outcome (Shaw *et al*., 2004). Promoter hypermethylated CpG islands have been identified at different sites in tumors however, because of not been

located close to known genes their functions have not been ascertained. In malignancies of the oral cavity, Ogi *et al*., (2002) have shown significant correlation with poor disease survival where promoter hypermethylation exist in *MINT1*and *MINT31*, but not *MINT2* and *MINT27* positions.

2.12.2 Epigenetic changes associated with oral cancer development

Much has been said about the genotoxic risk posed by tobacco smoke and alcohol which are genotoxic agents closely tied to the genesis of malignancies of the mouth and associated anatomic structures, and that the combined consequence of non-genetic influences and chemical agents that damage the genetic information may interactively raise the possibility for its development (Bavle *et al*., 2016; Del Corso *et al*., 2016).

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Non-genetic disturbance in parent cells could therefore be a frequent activity in human cancer and the chemical constituents of the DNA being a sensible target for early signatures in cancer development (Radhakrishnan *et al*., 2011). An important sign post in the chronicle of epigenetics was the discovery of bisulphite conversion of the genetic material which allows not only the interrogation of the whole set of haploid chromosomes in man but also the identification of various forms of methylation in the DNA and according to Uhlmann *et al*., (2002), the highly sensitive nature of this technology, the minute amounts of DNA samples needed to execute it and the possibility of partial automation by pyrosequencing enables reliable detection of as small as 0.1 percent methylated alleles within a certain spectrum of nucleic acids in the sequence of DNA that promotes transcription in human genes thereby suppressing the formation of their gene product because of the effect of 5-methylcytosine, which meddles with the fusing of sequence-specific DNA molecules or other DNA-fusing factors undermining transcription events.

DNA methylation events have played chief function in the identification of several significant gene and gene products however; OSCC development is yet to be tied to a single biological pathway. The *P16* gene was among the earliest genes used in looking at the significance of this mechanism in the development of orafacial malignancies and

associated structures and according to Gasche and Goel (2012); its expression was reported to be on account of hypermethylation in its promoter region together with heterozygous deletion of its chromosomal locus. Other reports have associated hypermethylation of the *P16* gene with amplified size of tumor and advanced cancer stage, metastasis to the lymph nodes, increased post-treatment resurgence along with an unwholesome end result of disease (Gasche and Goel, 2012). In an investigation involving methylation of *P16* in cancerous and benign oral cavity biopsies obtained from animal subjects, it was noted that atypical hypermethylation of the CpG islands of *P16* occurred in 26% of all the animals that were induced with a chemical initiator of tumorigenesis (Gasche and Goel, 2012).

According to Gasche and Goel (2012) who investigated the link connecting *P16* methylation and dysplasia in the oral cavity of rats induced with 4- nitroquinoline 1-oxide, *P16* methylation was seen to increase with the degree of dysplasia with an inverse relationship of the gene's expression in dysplastic tissues thus, suggesting that methylation of the initiator nucleotides of *P16* may function as a good biological marker for predicting outcome of OSCC. Shi *et al*., (2015) had also mentioned that the inactivation of this gene in craniofacial and neck cancers was via epigenetic mechanisms. This was considered to be an initial event which is common during early events of oral cancer development and especially so in the later stages (Radhakrishnan *et al*., 2011). Promoter methylation in CpG Islands of *P16* gene in pre-cancerous along with cancerous conditions of the mouth cavity and associated structures resulted in suppression and sometimes heterogeneous manifestation of the gene in biopsies from this anatomical location (Radhakrishnan *et al*., 2011).

Another important tumor-suppressor gene (*P14* gene), plays significant role in mediating multiplication and growth of the cell, division coupled with regulation of tumor-induced angiogenesis (Gasche and Goel, 2012).

Hypermethylation of this gene is said to result in the silencing of *P53* activity and deactivation of *P21*-induced cell multiplication and growth and is linked with increase in

size of the malignant swelling, staging, and spread to the lymph nodes (Gasche and Goel, 2012). According to Sailasree *et al*., (2008), 14 to 44 percent of OSCC tumors exhibited hypermethylation in the sequence of DNA in *P14* gene to which RNA polymerase binds for the initiation of transcription. Methylation of *P14* gene is a frequent occurrence in betel quid-related OSCC and oral pre-malignant lesions hence it was suggested that it could potentially be an early stage biomarker for detecting betel quid-associated OSCC (Gasche and Goel, 2012).

The *P15* gene is another common tumor suppressor gene which actively exhibits significant function in preventing the progression of cell cycle in the intermediate phase with the resultant inhibition of cell growth thereby functioning as a checkpoint control gene (Gasche and Goel, 2012). According to Takeshima *et al*., (2008), in oral precancerous lesions and OSCC this gene is commonly hypermethylated and inactivated while surrounding non-malignant tumor tissues are not. Consequently, it was observed that between 9 and 28% of OSCC tissues examined exhibited promoter hypermethylation of this gene. Chang *et al*., (2004) had observed that methylation of this gene is frequently encountered in surgical epithelial non-malignant margin biopsies of OSCC subjects with the background of concurrent cigarette and alcoholic liquor abuse than in nonsmokers and teetotalers. Radhakrishnan *et al*., (2011) reported that extent of methylation of *P16* together with *P15* genes found in the plasma of OSCC subjects were observed to be comparatively more than was found for non-malignant head and neck tissues thereby making the screening for aberrant methylation of these genes useful as a biomarker in identifying individuals in a population who may be predisposed to OSCC.

In human cancer cells, another tumor suppressor gene observed to function through the *WNT-1* (wingless) signaling pathway by indirectly inhibiting cell division, growth and multiplication is the *APC* (adenomatous polyposis coli) gene (Gasche and Goel, 2012). According to Uesugi *et al*., (2005), methylation of this gene and its subsequent inactivation results in the over-expression of *β*-catenin with a resultant increase in cell division.

Uesugi *et al*., (2005) and Gao *et al*., (2005) have both detected 15 percent and 25 percent hypermethylation of this gene in OSCC tumor biopsies and OSCC cell cultures respectively thereby suggesting that this phenomenon in this gene may be useful as a biomarker in identifying persons with tendency of developing OSCC.

Aside from genes that act by suppressing tumours, other types of genes known as oncogenes such as Survivin function by promoting division, expansion and multiplication of malignant cells on the one hand and by preventing natural cell death otherwise called apoptosis on the other hand (Gasche and Goel, 2012), and according to Tanaka *et al*., (2003), hypermethylation of transcription initiating sequences of survivin has not been identified in OSCC tissues.

It was shown by Gasche and Goel (2012) that in non-malignant tissue samples this gene is methylated and thus its action becomes suppressed and silenced but in OSCC tissue samples it is hypomethylated and thus it becomes activated resulting in increased division, growth and multiplication of malignant cells and concomitant decrease in apoptosis and promotion of cancer development. Lo *et al*., (2001) had earlier showed that upregulation of this gene was correlated with aggressiveness and invasiveness of cancer tumour. Empirical experiments done by Hsue *et al*., (2008) had also noted that buccal pouch mucosa samples from hamster treated previously with mineral oil were normally methylated for this gene while, the buccal pouch mucosa samples from hamster previously treated with a chemical carcinogen were hypomethylated for this gene leading to the development of buccal pouch carcinoma thereby increasing the evidence that hypomethylation of the gene may be used as a biological marker in the identification of individuals in a population susceptible to OSCC development.

An enhanced relationship between OSCC and hypermethylation of *CDH1* (cadherin 1) gene which is known to encode for the adhesion protein (E-cadherin) has been established and may thus be a very important early molecular signature in the onset of oral cancers. According to Gasche and Goel (2012), promoter hypermethylation of this gene in OSCC

tumors has been correlated with invasive tumor characteristics and a poor disease outcome.

Molecular events such as promoter hypermethylation in addition to loss of heterozygosity in *APC* gene have what's more been observed to cause differential cytoplasmic behavior of adhesion protein. In OSCC tissues the frequency of *CDH1* methylation ranges from 17% to 85% (Supic *et al*., 2011; Gasche and Goel, 2012). According to Alvarado *et al*., (2011), there is increased promoter hypermethylation in the *CDH1* gene of dysplastic lesions of basal epithelial cells meanwhile, Shaw *et al*., (2006) failed to observe notable variation in the state of methylation of malignant and benign biopsies obtained from the oral cavity thereby indicating that methylation in the *CDH1* gene may not be a candidate biomarker for OSCC.

Another gene worthy of mention according to Gasche and Goel (2012) is the *MGMT* (O-6-methylguanine-DNA methyltransferase) gene which prevents cancer development by removing mutagenic components from nucleic acids in the genome thereby initiating DNA repair. According to Huang *et al*., (2010), it was observed that this gene is silenced in 75% of OSCC tissues through the mechanism of hypermethylation of its promoter region thereby suggesting that this might be a possible pathway through which OSCC develops. Aside from hypermethylation other pathways are thought to also exercise important functions in modulating the expression of this gene (Gasche and Goel, 2012). Chronic indulgence in the chewing of betel quid is implicated as a contributory agent in the genesis of OSCC however, Huang *et al*., (2010) showed that *MGMT* was not expressed in these patients with a record of betel nut chewing. Silencing of the genetic material through hypermethylation was however shown to be correlated with a worsened outcome in OSCC patients (Taioli *et al*., 2009). Several researches have revealed that between 12% and 74% of OSCC tissues exhibited hypermethylation of the gene (Taioli *et al*., 2009; Gasche and Goel, 2012). This therefore suggests that methylation of *MGMT* is an important development not only for the detection of OSCC but also as a prognostic indicator.

Another gene in which promoter hypermethylation play important role in regulating protein expression pattern as a result of gene silencing in many human cancers is the DNA mismatch mending gene *MLH1* (mutL homolog 1) whose function is principally to prevent the buildup of mutations in the DNA (Gasche and Goel, 2012).

In addition, the methylation of this gene is suspected of being linked with higher potential for the occurrence of cancers linked with the oral region as had been earlier observed that about 76% of OSCC tissues exhibited promoter methylation of the *MLH1* gene with a large chunk corresponding to early stages of the disease and only a few corresponding to late stages of the diseases with 0% in benign oral cavity tissues. The non-occurrence of promoter methylation of this gene in benign oral cavity tissues has also been observed; it is therefore very likely according to Gasche and Goel (2012) that this might be a significant early fingerprint during OSCC development.

Another gene that has generated much interest because of the therapeutic use of retinoids for OSCC is the *RARβ* (retinoic acid receptor β) which regulate cell proliferation and specialization although its part in OSCC development remains uncertain according to Shaw *et al*., (2006). While conducting a pyrosequencing study, they observed that 73% and 62% aberrant promoter hypermethylation in both OSCC tissues and adjacent benign tissues respectively. The status of this gene in oral premalignant lesions was also investigated by Youssef *et al*., (2004). They noted promoter methylation in about 53% of the lesions. It is therefore uncertain if methylation of this gene is a definite initial process in OSCC development or a generalized alteration in all all oral mucosal tissues.

Imai *et al*., (2008) observed that 39% methylation of *RASSF2* gene occurred in OSCC tissues. Similarly, Huang *et al*., (2009) also observed 22% methylation of *RASSF1A* and 28% methylation of *RASSF2* genes in OSCC tissues which correlates with unwholesome disease end result. Tiaoli *et al*., (2009) also revealed that between 12 and 38 percent methylation of *RASSF1* gene were observed in OSCC biopsies. In addition, methylation of *RASSF1* gene was associated with chewing of betel nut in OSCC patients as a result of observation of 93% methylation of the gene in tissues of OSCC patients who indulge in

chewing of betel nut. Several members of the *RASSF* (Ras-association domain family) gene mediating important cellular activities such as suppression of tumor, cell cycle regulation, apoptosis, and formation of microtubule has been identified and silencing of the members of this gene by aberrant methylation is suspected to not only induce but also determine prognosis in OSCC patients (Volodko *et al*., 2014).

Epigenetic aberration of the DNA transcription region in *CDKN2A* (Cyclin-dependent kinase inhibitor 2A) gene are common occurrences in malignant conditions affecting the craniofacial and neck section (Zhou *et al*., 2018). According to Kulkarni and Saranath (2004, methylation of this gene are common occurrences in preinvasive phases of craniofacial and neck cancers especially in patients exposed to alcohol consumption and tobacco use thereby suggesting that this might be a pathway for the onset of craniofacial and neck malignancy including OSCC.

The *14-3-3σ* gene induced by the *P53* gene according to Radhakrishnan *et al*., (2011) participates in the mediation of normal behaviour in benign cells and abnormal cell behavior by epigenetic silencing in malignant cells. Epigenetic aberration of this gene frequently occurs in human malignant developments such as those of the head and neck section consisting of OSCC (Radhakrishnan *et al*., 2011). This gene is also frequently upregulated particularly epithelial cells especially in those that would differentiate into squamous cells in both benign and cancerous human cells (Radhakrishnan *et al*., 2011). This was also detected and reported by Gasco *et al*., (2002) in 35% and 50% of OSCC and oral precancerous lesions respectively.

Epigenetic aberration through promoter hypermethylation of *DAPK1* (Death associated protein kinase1) has been observed and used as a detective indicator in OSCC. From the work of Jayaprakash *et al*., (2017), promoter aberration in *DAPK1* correlates with spread to the lymph node and advanced disease stage in craniofacial and neck malignancies. Epigenetic regulation of *P73* gene as a consequence of methylation silencing was also connected with OSCC progression (Araki *et al*., 2002).

A large percentage of epigenetic aberration through methylation of *ATM* (Ataxia telangiectasia mutated) gene was also observed in OSCC biopsies (Rigi-Ladiz *et al*., 2011) thereby demonstrating that *ATM* is a prospective diagnostic indicator for the disease.

Pannone *et al*., (2010) suggests that malignancies around the oral cavity particularly OSCC may well be the resultant upshot of aberration in *catenin* gene following the upregulation of the *WNT* pathway through epigenetic changes in *SFRP*, *WIF-1* and *DKK-3* genes, while Khor *et al*., (2013) showed that *DDAH2* and *DUSP1* genes were differentially hypermethylated in OSCC and thereby signaling their prospect as diagnostic, prognostic and therapeutic target biomarkers in OSCC.

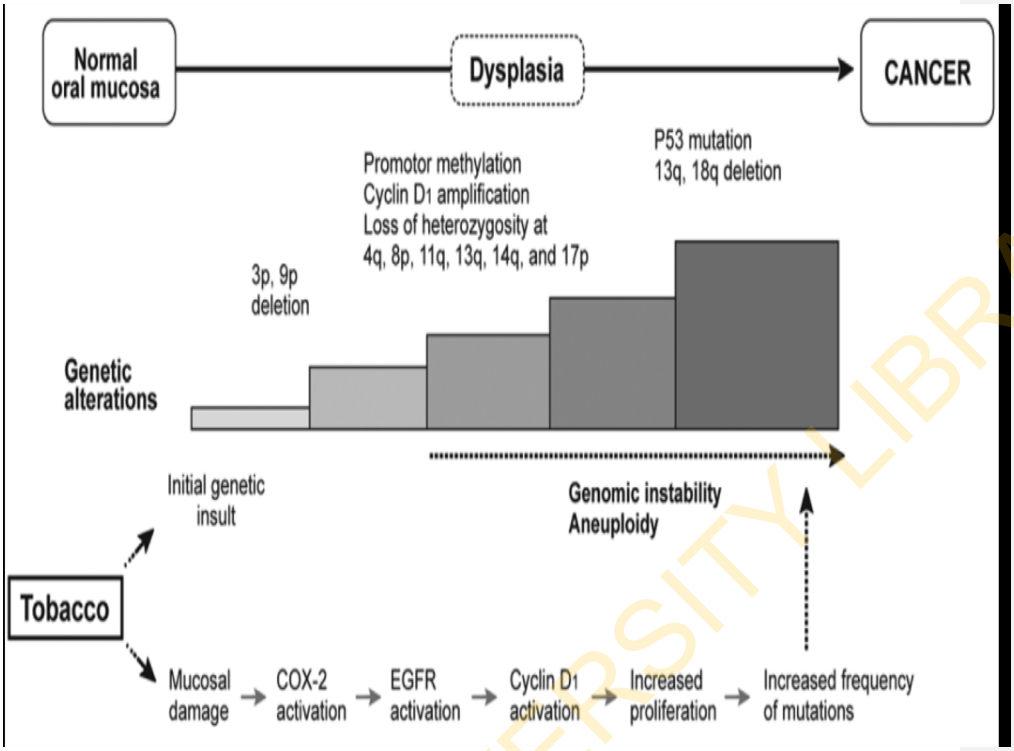

Figure 2.5: Multistep pattern of genetic progression in oral carcinogenesis (Choi and Myers, 2008).

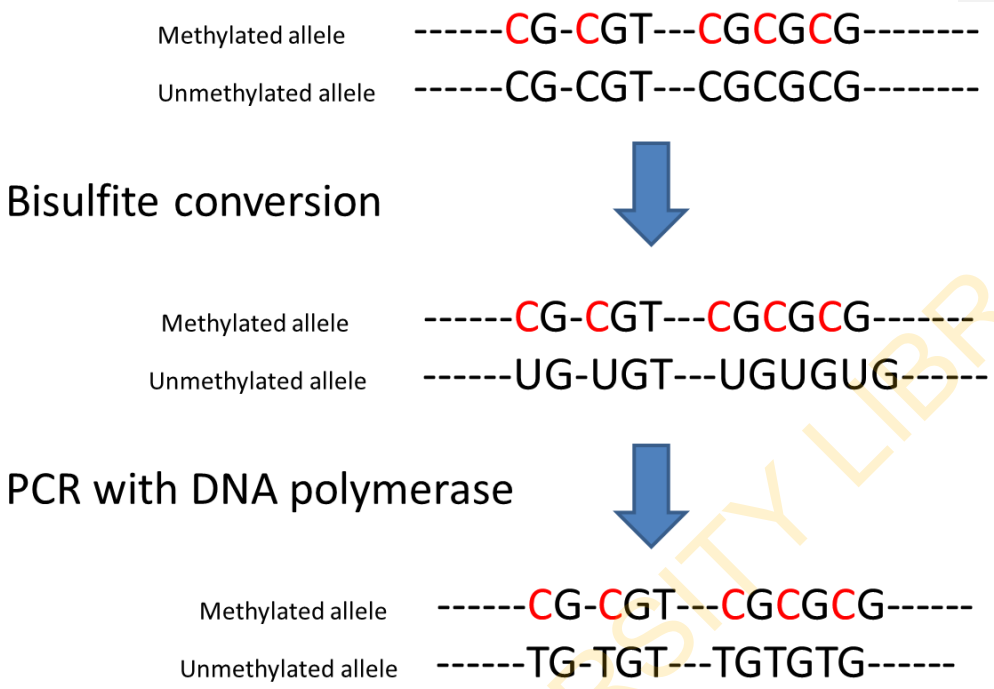

Figure 2.6: Effect of sodium bisulfite treatment on methylated and unmethylated alleles.

Methylated allelic nucleotides shown in red color are unaffected by sodium bisulfite conversion while unmethylated allelic nucleotides are converted to new nucleotides (Lim, 2014).

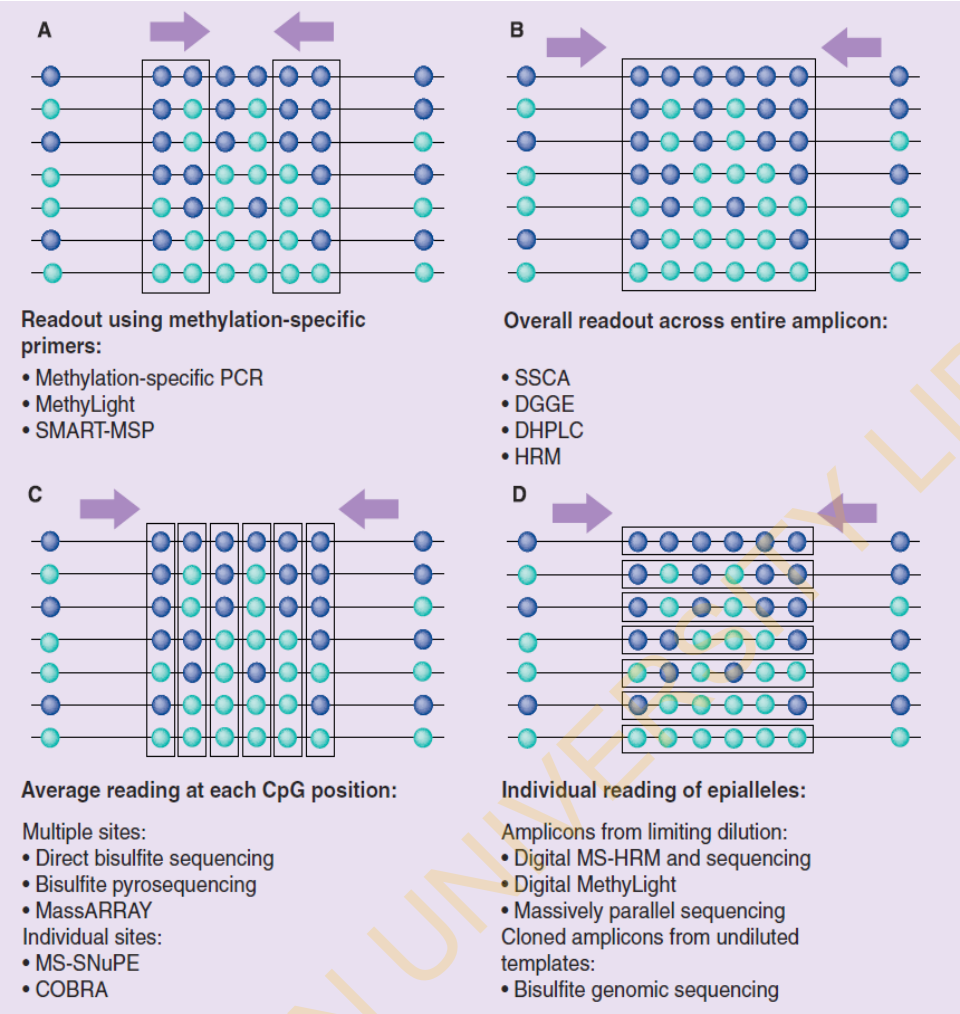

Figure 2.7: Techniques used in measuring DNA methylation.

○ Unmethylated CpG sites ● Methylated CpGs sites ➡ Primer locations (Mikeska *et al*., 2010).

2.13 The *RUNX3* Gene and its Role in Carcinogenesis

The *RUNX* family gene has remained essentially unchanged throughout the evolution process from lower to higher organisms, suggesting its important function as regulators of developmental processes and active participants in biological events such as involvement in human neoplasia (Manandhar and Lee, 2018). The *RUNX* family gene are made up of α-subunits (binding sites for DNA), and β-subunits (non-DNA-binding site). This gene family encode α-subunits and consists of *RUNX1*, *RUNX2* and *RUNX3* (Manandhar and Lee, 2018).

It is on record that *RUNX1* is implicated in the generation of blood stem cells, and therefore plays key function in leukemia (Ito, 2008), while *RUNX2* is said to be modulate bone development with oncogenic potential (Manandhar and Lee, 2018). *RUNX3* gene has been linked with multiple developmental activities in cells and specialization of several immunity moderating cells (Ito, 2008), nerves of the sympathetic ganglia (Levanon *et al*., 2002) and it additionally play the role of a major tumorigenic suppressor (Kim *et al*., 2015). Aside from functioning as a tumor suppressor, current developments portray *RUNX3* gene as an initiator of tumorigenesis in several malignant conditions (Lee *et al*., 2011; Park *et al*., 2017).

Several reports have explained that this gene is upregulated in several tissues with diversified functions. For example in several types of solid tumors especially gastric and colon cancer, this it suppresses tumor formation (Manandhar and Lee, 2018). The report of Lee *et al*., (2002) describes the silencing of *RUNX3* as being responsible for hyperplasia of the intestinal mucosa, while Inoue *et al*., (2002) credits severe limb ataxia to a *RUNX3* deletion. Several other reports have also emanated to strengthen the argument in support of the tumor suppressive role for *RUNX3*. These reports include the downregulation of this gene by the absence of one of the alleles, hypermethylation of promoter region of genes, epigenetic histone alterations and frequent accumulation of protein at a given site (Li *et al*., 2004; Chuang and Ito, 2010).

From emerging reports, it is becoming apparent that in addition to the known tumor suppressive roles of this gene, its function as an oncogene is being uncovered in a number of cancer types (Manandhar and Lee, 2018).

Activation of *RUNX* family genes by MYC proto-oncogene is associated with leukemogenesis (Cameron and Neil, 2004). Upregulation of *RUNX3* gene is also connected with the onset of an array of basal cell skin malignancy (Salto-Tellez *et al*., 2006), ovarian malignancy (Lee *et al*., 2011), OSCC (Tsunematsu *et al*., 2009) and malignancy of connective tissues (Bledsoe *et al*., 2014). Depending on the kind of cell under consideration, *RUNX3* gene could either function as an activator or deactivator of tumorigenesis (Manandhar and Lee, 2018).

DNA methylation is an important pathway through which *RUNX3* gene is silenced. Pattern by which this gene is expressed was evaluated in solid malignant biopsies from the endometrium while, the pattern by which the transcription initiating DNA sequences of this gene was methylated in cultivated cells from the endometrium was also evaluated and correlated with outcome of cancer of the endometrium. Non-expression of *RUNX3* gene was observed in 50.9 percent (27/53) of the solid endometrial cancer biopsies and in the cultivated cells. Methylation in the initiator transcription DNA sequences of *RUNX3* was also seen in 62.2 percent of the solid biopsies from the endometrium, 12.5 percent of the benign biopsies from the endometrium and in the cultivated cells. Grading and staging of tumor were positively associated with non-expression of this gene (Jeong *et al*., 2018).

In separate studies conducted to investigate the correlation between promoter hypermethylation of *RUNX3* and gastric cancer risk by meta-analytical methods. Lina *et al*., (2018) were successful in demonstrating a notable link between gastric carcinogenesis and this mechanism, thereby giving credence to the function of *RUNX3* as a gene responsible for suppressing tumorigenesis. In their study, the rate of promoter hypermethylation of this gene was greater in malignant biopsies than in benign biopsies from the intestinal tract of patients with gastric cancer, which is an indication of the close

relationship between gastric cancer and this mechanism rendering this pathway a probable biological marker for the detection of gastric carcinoma.

Mustafa and Rajaraman (2016) explored the pattern of expression of *RUNX3* in OSCC together with its association with clinical and pathological characteristics using immunohistochemical method. In the study, fifteen benign biopsies from the oral mucosa, thirty-seven OSCC biopsies and twenty-one biopsies from premalignant lesions were pooled together and subjected to immunohistochemical staining using rabbit anti-human polyclonal antibodies. It was observed that the expression of the gene in biopsies from the benign lesions, premalignant lesions and OSCC tissues were gradually downregulated and the rate of aberration of *RUNX3* was on the increase. It was therefore concluded that expression of the gene was closely connected with the extent of histological alterations showing that *RUNX3* could potentially be used in evaluating onset, progression along with prognostication in this cancer.

In summary, *RUNX* family genes are important due to their function as mediators of cancer cell development and specialization and their seemingly absurd dual role as tumor suppressors through mutations, chromosomal deletions and hypermethylation and initiators through chromosomal insertions in several cancers (Blyth *et al*., 2005).

2.14 Part Played by Immunohistochemistry in Diagnosis of Oral Malignancies

Identification of lesions for all intents and purposes relies on the infinitesimal study of their constituent cells and tissues where hematoxylin and eosin staining is the age old customary procedure for such diagnoses, however, hematoxylin and eosin sections have proved unquestionably not enough at the level of diagnoses when one engages in an etiologic, histogenetic or pathogenetic hunt leading to a consistent exploration for further techniques and one of such course of action is immunohistochemistry (IHC) which has positively altered the landscape of surgical pathology in the past 50 years (Gupta and Gupta, 2016). The immunohistochemical technique enables the identification of antigenic components of cells as well as tissues by way of antigen-antibody reactions and

localization of the reaction compound either by direct antibody marking or by employing a second tagging antibody.

Common uses of IHC in clinical practice include; the cellular differentiation of tumors for more accurate and precise diagnosis, clear delineation of benign lesions, *in-situ* lesions and invasive cancer, determination of specific therapeutic options, disease prognostication, cell type and antigen determination in non-cancerous lesions and confirmation and typing of viral infection (Kalebi and Dada, 2007).

2.14.1 Common immunohistochemical markers in oral pathology

Although conventional histologic examination of thinly cut tissue biopsies using hematoxylin and eosin stains is undisputably the standard in the routine pathological procedures involving the craniofacial and associated structures, IHC had progressively turned out to be an essential tool resulting in striking advancement in the microscopic identification of neoplasms (Patil *et al*., 2016). To this end, an array of tissue/organ specific indicators have been identified amongst which are the epithelial, lymphoid and vascular markers and these have been successfully used in the diagnosis and prognosis of diseases (Kalebi and Dada, 2007; Gupta and Gupta, 2016).

2.14.1.1 Markers associated with OSCC

Immunohistochemical markers of importance in OSCC are proteins of cytoskeletal origin associated with cells of the epithelium known as keratins, miscellaneous antigens and biochemical molecules produced by carcinomas of squamous cells employed in the assessment of the biologic behaviour of tumors; a typical example is the proliferation antigen. The keratins are intermediate filamentous cytoskeletal proteins of which in excess of 20 diverse types have been found in epithelial cells (Patil *et al*., 2016). They have been shown to be profoundly important in diagnostic immunohistochemistry for the classification of specific subtypes of carcinoma (Cerilli and Wick, 2002; Patil *et al*., 2016). In two independent pacesetting studies earlier conducted and reported by Patil *et al*., (2016), it was made known that the expression of Keratin-19 was amplified in both

oral premalignancy and hyperplastic lesions leading to the conclusion that it was not specific for premalignancy.

In a different study conducted by Cerilli and Wick (2002), it was observed that adenocarcinomas exhibited immunopositivity with keratin-20 while adenoid squamous cell carcinomas exhibited immunonegativity thereby forming a basis for immunohistochemical differentiation of the two.

Out of all tumors associated with the oral cavity, carcinomas of squamous cells have come to occupy the center stage in immunohistochemical studies where they are used in assessing the cell proliferation markers together with their likely prognostic outcome. It was shown that at various stages of the cell cycle, there is not only the production of different nuclear proteins but also DNA duplication as a result of cellular replication. This therefore underscores the great importance of cell cycle in the determination of cell propagation (Patil *et al*., 2016). Ki-67 antigen is acknowledged to be generally upregulated in all cells with the exception of cells in the resting phase (latent non-cycling cells) and therefore can be useful in differentiating proliferating from non-proliferating cells in human tissues (Cerilli and Wick, 2002).

2.14.1.2 Markers associated with oral salivary gland tumors

An extremely miscellaneous group of tumours consisting mostly of different proportions of ductal and myoepithelial cells and sometimes acinar cells are associated with the salivary gland with a consequential effect that a multiplicity of secondary morphological changes such as clear cell, cystic, oncocytic, sebaceous and prominent lymphoid stroma is common to quite a lot of diverse entities making interpretation of tissue materials difficult for diagnosis.

However, to a large degree immunohistochemistry has been able to resolve the mystery surrounding the subject of specific identification of salivary gland tumors as well as assist in accurate diagnosis and prognosis in a few numbers of cases (Patil *et al*., 2016). Pleomorphic adenoma (the most widespread nonmalignant salivary gland tumor) was

shown in a novel investigation carried out by Chenevert *et al*., (2012) to be immunopositive to glial fibrillary acidic protein (GFAP), while benign tissue from the salivary gland, chronic infection of the salivary gland, low grade non-cancerous salivary gland neoplasm, cancer arising in all salivary glands in addition to low-grade cancers of the mucous secreting/epithelial cells of both categories of salivary glands were immunonegative. GFAP therefore possesses usefulness in separating benign salivary gland tumours from the different forms of slow growing malignant salivary gland tumours.

2.14.1.3 Markers associated with oral melanoma

Cancers originating from the melanocytes may manifest as a main growth in the mucosa of the oral compartment and at other times as secondary tumour in adjoining non-bony structures in addition to bony structures of the jaw. The tissue microscopic picture may be complicated particularly in situations where the tissue specimen fails to reveal the crosslinks between one cell and another and with the epithelial surface and also in situations where the cells of the tumor lack melanin. There is inconsistency in the morphological appearance of these cancers which comprise epithelioid, spindle cell sarcomatous, or round cell formations that should be separated from carcinoma, sarcoma, and lymphoma correspondingly (Patil *et al*., 2016). These cancers have an emblematic antigenic feature, and its identification generally can be established without difficulty by means of immunohistochemical assay (Urso *et al*., 2005). It was shown by Cerilli and Wick (2002) that 95% of melanoma cases were immunopositive for the S-100 protein, neuron-specific enolase (NSE) and vimentin but negative for keratin. These markers can therefore be used to distinguish melanomas from histologically similar lesions. Furthermore, Cerilli and Wick (2002) also reported that melanoma-specific antigen revealed by means of monoclonal antibody homatropine methylbromide-45 (HMB-45) is consistently seen in between 70 to 80 percent of melanomas; particularly the desmoplastic (neurotropic) melanomas which often resemble soft tissue sarcomas and that 75% of these cancers are immunopositive for MELAN-A or anti-tyrosinase. In terms of specificity and sensitivity, Allen (2008) submitted that though S-100 protein is highly sensitive, its

specificity is questionable, whereas HMB-45 though highly specific exhibits moderate sensitivity when used as markers for oral melanomas.

2.14.1.4 Markers associated with benign mesenchymal tumors of the oral cavity

Due to the morphological complexity of tumors of mesenchymal origin, some members of this group of tumors have need of immunohistochemical assay to ensure accurate diagnostic recognition as well as discrimination from tumors that may appear similar. Some of these other tumors are the granular cell tumor, granular cell epulis, melanotic neuroectodermal tumor of infancy and schwannoma (Patil *et al*., 2016).

Granular cell tumor also known in the past as granular cell myoblastoma is an oral cavity lesion most commonly seen in the tongue of adult patients. It is a malignancy understood to exhibit common behavior with Schwann cell neoplasms as well as shown to be immunopositive for S-100 protein as well as vimentin, but unreactive to desmin and the actins which are muscle cell indicators just like in schwannoma and were also shown to be reactive to NSE, laminin and myelin basic proteins but immunonegative for neurofilament proteins and GFAP by Allen (2008).

Granular cell tumor of the gingiva of newborns also known as granular cell epulis which more often than not presents in their anterior alveolar ridge is different from granular cell tumor of adults and is widely thought to be of fibroblastic origin and according to Chenevert *et al*., (2012) exhibits immunopositivity for vimentin but in comparison to granular cell tumor of adults is unreactive to S-100 protein.

Melanotic neuroectodermal tumor of the infant otherwise called melanotic prognoma is an infrequent, typically non-malignant lump that manifests in the jawbones, most commonly in infants. It is morphologically made up of small neural-like cells and large, pigmented epithelial-like cells and exhibits a complex immunohistochemical feature showing its different differentiation characteristics (Patil *et al*., 2016). In one examination conducted by Chenevert *et al*., (2012), the large cell (cuboidal cell) component was shown to be reactive to the keratins and melanoma-specific antigen (HMB-45), although consistently

unreacive to S-100. In the same study, some small cell components were also shown to be reactive to vimentin, epithelial membrane antigen, GFAP, NSE and synaptophysin.

2.14.1.5 Markers associated with oral sarcomas

The oral cavity is also a habitat for varying forms of sarcomas affecting soft tissues and sarcomas of bony structures arising in the jaw. A prominent example of the former are the embryonal rhabdomyosarcomas which represent one of the most frequently encountered malignancies of soft tissues known to affect the maxillofacial section. These malignancies are most often seen in children who are in their first ten years of existence and very commonly manifests histologically as a "small round blue cell neoplasm" (Barnes *et al*., 2005).

This malignancy exhibits extensive morphological variation which is determined by different factors which include; functional specialization, density, along with the growth pattern of each cell. Some of the morphological variants include; neuroblastoma, Ewing's sarcoma, synovial sarcoma, melanoma, melanotic neuroectodermal tumor and malignant lymphoma. Additionally, it is often mixed up with the solid variant of alveolar rhabdomyosarcoma, with which it has prognostic implications (Patil *et al*., 2016).

Because of the mesenchymal origin of this malignancy, it is usually very consistently reactive to vimentin though its value is negligible however Rajendran (2006) demonstrated that desmin is the most frequently observed in paraffin-embedded biopsies exhibiting substantial reactivity in practically every single case of the malignancy in addition to the other morphologic variants, whereas other small round cell malignancies are none-reactive to desmin. In addition to desmin, another marker that has been conventionally used in the diagnostic identification of these tumors is myglobin. Regrettably notwithstanding its high specific accuracy, its sensitivity is poor and this renders it by and large not of great use in undifferentiated malignant cells. Related to this, is a group of myogenic compounds whose function is very essential in the channeling of primordial mesenchymal cells in the direction of a skeletal myogenic ancestry. The regulatory compounds which comprise MyoD1, myogenin, Myf-5, and Myf-6 are elicited

at a previous phase of differentiation than skeletal proteins, and have been of very great value in the diagnosis of poorly differentiated rhabdomyosarcomas (Patil *et al*., 2016).

In the case of osteosarcomas, several putatively osteoblast-specific markers have been developed and this include proteins responsible for differentiation, Type I collagen, COL-I-C peptide, decorin, osteocalcin, osteonectin, osteopontin, proteoglycans I and II, bone sialoprotein, as well as bone glycoprotein. No more than two of the listed proteins namely osteonectin and osteocalcin (OCN) have exhibited satisfactory detective value in biopsy sections as to worth being included in diagnostic immunohistologic investigations (Patil *et al*., 2016). Osteocalcin is among the most ubiquitous non collagenous intra-osseous proteins which are largely confined to osteoblasts.

Nagao *et al*., (2012) showed that OCN in the main has a fairly high level of close affinity for osteoblastic discrimination (in the region of seventy percent) and is, for matter-of-fact reasons, almost entirely unambiguous for bone-forming cells and malignancies. On the other hand every so often fibroblasts is shown to possess the ability to conjugate with the polyclonal anti-OCN reagents consequently monoclonal antibodies with discriminating peptide identification are favored for diagnostic investigations. As a consequence it can be employed on its own with good results in the detection of this family of malignancies.

2.15 Common Genetic Polymorphisms in Oral Cancer

Scully and Petti (2010) defined single nucleotide polymorphisms (SNPs) as positions in a genome containing sequences of DNA that although have undergone alteration but may not be able to modify an amino acid or they may be altered DNA sequences lack the ability to exarcerbate unwholesome effect in non-diseased individuals but may be targeted as biomarkers for risk of disease development. According to Matejcic *et al*., (2017), it was noted that polymorphisms in *ADH* (alcohol dehydrogenase) and *CYP450* (cytochrome p450) genes which regulates the activities of certain enzymes responsible for metabolizing systemic alcohol results in increased risk of developing OSCC. Increased possibility of developing oral cancer and cancers generally was also linked with 1722C>T and

1790G>A polymorphisms in *HIF1A* (hypoxia inducible factor-1 alpha) gene according to Anam *et al*., (2015) and Li *et al*., (2019).

Additionally, polymorphisms of the *VEGF* (vascular endothelial growth factor) have also been seen to contribute valuable impact in the onset as well as progression of malignancies of the mouth cavity. Specifically, Kammerer *et al*., (2010) discovered that +936 C>T, -2578 C>A polymorphisms in *VEGF* gene were identified more frequently in the blood of OSCC subjects than in the blood of non-diseased individuals. Arginine194Trptophan polymorphism of the *XRCC1* (x-ray fixing cross-complimenting group-1) gene was also strongly connected to the onset of OSCC (Avci *et al*., 2017).

Relationship between four SNPs of the *RETN* gene and OSCC vulnerability together with clinical prognosis in 935 OSCC patients and in 1200 healthy controls were examined. It was discovered that in persons with a record of cigarette smoking, *RETN* polymorphism individuals with the betel nut chewing practice have a six to ten times greater possibility to develop this malignancy in relation to the wild-type individuals without this practice. OSCC subjects who are A>A homozygous for *RETN* rs3219175 polymorphism exercised a greater tendency in favor of a superior tumor size (> T2) as opposed to the individuals with G>G homozygosity. Furthermore, A>T>G>G haplotype remarkably amplified the tendency for OSCC development with over one fold (Yang *et al*., 2018).

In a Saudi population the link between SNPs considered to be in charge of cell division phase along with DNA fixing genes plus the possibility of developing OSCC was investigated. The result of the investigation concluded that SNPs in such genes as *p21 C31A*, *Ku80 A2790G*, and *MDM2 T309G* increased the possibility of developing OSCC. These polymorphisms could therefore be assumed to be potential genetic biomarkers for screening of individuals suspected to be at elevated danger of developing OSCC (Al-Hadyan *et al*., 2012).

Influence of four SNPs in *PTX3* gene in addition to epigenetic factors on vulnerability to oral tumor formation was assessed in a Taiwanese population by Yeh *et al*., (2019). It was

observed that rs3816527 SNP correlated with the occurrence of late stage cancer in smokers with the conclusion that its part during oral tumor growth is unequivocal.

2.16 Oral Cancer Prevention

Alcohol consumption, cigarette abuse as well as chewing of betel nut are non-gender biased. Consequently, a large proportion of oral cancer attributed to these agents could be prevented by avoiding social vices such as smoking of cigarettes, consumption of alcohol and poor oral hygiene (Marron *et al*., 2010). They also observed that withdrawal within a period of one to four years from cigarette smoking resulted in about 35% lowered tendency of having malignancy development in the oral cavity and 80% lowered risk for a period of 20 years.

It was observed that individuals with poor oral health had a tendency to develop oral cancer twice as much as individuals who observed good oral hygiene. It was also observed that tobacco smokers had a tendency of developing oral cancer four times as much as non-smokers, while teetotalers had a tendency of developing oral cancer seven times as much as those who are not (Zeng *et al*., 2019).

The most commonly used procedure for examining this cancer is usually physical along with corporeal interrogation of the oral mucous membrane. Several investigations highlights that this protocol could at the acute stage recognize precancerous as well as cancerous derangements. However, evidence is still rudimentary and cannot be used toconclude if such interrogatory procedures will be effective in altering disease-specific mortality (Rethman *et al*., 2010).

Going by the submission of Wilson *et al*., (2011), therapeutic administration of NSAID (non-steroid anti-inflammatory) compounds have been linked to a lowered tendency for cancer development generally however, they observed that its use in lowering the tendency of developing this cancer specifically has not been substantiated.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Location, Design and Population

This research was executed at the Department of Oral Pathology, University College Hospital, Ibadan, in the Southwest of Nigeria. Ibadan is situated in the forest region of the tropics and bounded by both forest and derived savannah areas. It is about 150 m high in its lowest axis and about 275 m above sea level in its highest axis with a land mass spanning about 3,080 Km^2 in diameter (1,190 square miles). The city has four major rivers with several tributaries. These rivers include Ona River which traverses the Northern and Western parts of the city; on the Eastern part of the city is Ogbere River; flowing through the central part of the city is Ogunpa River and Kudeti River. Towards the Northwestern part of the city is the Eleyele Lake with the Osun River and Asejire Lake situated towards the Eastern part of the city (Egbinola *et al.*, 2017). Located in the city is The University College Hospital which offers tertiary health care services to inhabitants of the city. A retrospective-prospective approach was employed for the study. The study population was made up of subjects diagnosed with OSCC between January 2004 and December 2015. The Oyo State Health Ministry granted the ethical approval No. AD 13/479/4003[A] of 27/01/2021.

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3.2 Study Samples and Data Collection

10% buffered formalin-fixed paraffin-embedded (FFPE) solid blocks of biopsies and surgical specimen from OSCC (malignant) subjects and other oral lesions of epithelial origin (benign) designated as test and control samples respectively were obtained from the datasheet of the Department of Oral Pathology, University College Hospital, Ibadan, Nigeria.

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Demographic information of patient's consisting of gender, age; tumour site and histological class were also extracted from the database of the department, computed and presented as frequencies and percentages.

SPSS statistical package version 23.0 was employed to analyze the generated data. Association between histological classes of OSCC was tested against gender, age and tumor site using Pearson's χ^2 test. For all analysis, $\alpha_{0.05}$ was considered significant.

3.3 Laboratory Investigations

3.3.1 Immunohistochemical evaluation of cytokeratin (CK AE1/AE3) and epithelial membrane antigen (EMA) proteins in OSCC

3.3.1.1 Deparaffinization and rehydration of tissue sections

5µm thick sections were produced from the test and control samples using Histo-line laboratories rotary microtome (MR-2258) model and floated unto grease-free microscope slides. A hot plate was used to heat the slides to a temperature of 70^{o}C for 10 minutes, followed by subsequent introduction into two series of xylene for 5 minutes respectively. This was followed by incubation in two series of absolute ethanol for 2 minutes respectively, two series of 95% ethanol for 2 minutes respectively and finally in 70% ethanol for 2 minutes. Slides were then transferred into wash buffer containing Tris-base $NaCl_2$ Tween 20 (TBS Tween 20) for 2 minutes (Kabiraj *et al*., 2015).

3.3.1.2 Retrieval of tissue antigenic sites

Slides were then transferred into pre-heated retrieval solution containing Sodium citrate buffer (pH 6.0) and incubated at 95^{o}C for 20 minutes after which they were allowed to cool in the buffer for a further 20 minutes. Subsequently, slides were transferred into wash buffer (TBS Tween 20) and washed for 2 minutes. Excess buffer was allowed to drain off (Kabiraj *et al*., 2015).

3.3.1.3 Blocking for endogenous enzymes

Using a hydrophobic pen, tissue areas were marked on the slides and peroxidase block solution containing 3% Hydrogen peroxide (H_2O_2) was applied dropwise away from light to cover tissue completely and placed in a humidity chamber at room temperature for 5 minutes. Subsequently, slides were transferred into wash buffer (Tris Buffered Saline [TBS] Tween 20) for 2 minutes (Kabiraj *et al*., 2015).

3.3.1.4 Immunohistochemical staining for cytokeratin (CK AE1/AE3) and epithelial membrane antigen (EMA) proteins

The tissue slides were then placed in the humidity chamber after wiping off excess buffer. Diluted primary antibodies; epithelial membrane antigen (EMA) and cytokeratin (CK AE1/AE3) (DAKO A/S, Denmark) were applied onto tissue slides in triplicates each and incubated for 1 hour. Slides were subsequently transferred into three changes of wash buffer (TBS Tween 20) for 2 minutes respectively. Surplus buffer was wiped off from the slides and anti-mouse secondary antibody was added and left to react for 30 minutes and thereafter transferred into three changes of wash buffer (TBS Tween 20) for 2 minutes each. Diaminobenzidine (DAB) solution (DAKO A/S, Denmark) was then added to every one of the slides and incubated for 7 minutes and thereafter washed in running tap water. Slides were counter-stained with Gill's haematoxylin for 10 seconds, dehydrated in 70% ethanol, 95% ethanol for 1 minute each, two changes of 100% ethanol for 2 minutes each and finally cleared in xylene for 5 minutes. Thereafter slides were covered with mounting fluid, cover glass and air-dried for approximately 30 minutes (Kabiraj *et al*., 2015). Slides were thereafter viewed under a binocular microscope to determine pattern of immunoreactivity for each histological subtype of OSCC.

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3.3.2 Evaluation of OSCC for presence of CpG island methylation in *RUNX3* gene

3.3.2.1 Genomic DNA extraction

Four tissue sections (≤ 20 µm thick) were removed from each block of tissue and introduced into 1.5 mL microcentrifuge tubes for deparaffinization. 1 mL of xylene was added to each tissue sample, vortexed and incubated at room temperature for 1 hour with gentle rocking, centrifuge at 10,000 X g for 1 minute and discard supernatant. This step was carried out twice. This was followed up by washing twice for 5 minutes with 1 mL absolute ethanol, 5 minutes in 1 mL 95% ethanol and 5 minutes in 1 mL 75% ethanol with gentle shaking respectively. This was subsequently followed by washing for 5 minutes in 1 mL double distilled water once with gentle shaking. Excess water was removed from the samples at this stage in readiness for Protenaise K digestion.

3.3.2.2 Protenaise K digestion and DNA isolation

This was executed in accordance with manufacturer's instructions. To each of the deparaffinized tissues in microcentrifuge tubes was added 45µl H_2O, 45µl 2x digestion buffer and 10µl Protenaise-K to produce the digestion mix. The digestion mix was then incubated at 55^{o}C overnight.

Thereafter, the digestion mix was transferred to 94^{o}C and incubated for 20 minutes. 5µL of RNase-A was then added, mixed and allowed to incubate at room temperature for an extra 5 minutes. Thereafter, 350µL genomic lysis buffer (prepared in accordance with the manufacturers' instruction) was dropped into each tube, homogenized thoroughly using a vortex machine and centrifuged at 10,000 x g for 1 minute to eliminate insoluble debris. The supernatant from each tube were then transferred to a Zymo-spin column in a collection tube and centrifuged at 10,000 x g for 1 minute. 200µL of DNA pre-wash buffer was added into each spin-column in fresh collection tubes and centrifuged at 10,000 x g for 1 minute. Thereafter, 400µL of genomic DNA wash buffer was dropped into each spin-column and centrifuged at 10,000 x g for 1 minute. Each Zymo-spin column was then transferred to clean microcentrifuge tubes. 50µL DNA elution buffer was dropped into each spin-column at this stage and allowed to react for 5 minutes at room temperature followed by centrifugation at top speed for 30 minutes to elude the DNA. The eluted DNA were then collected in sterile PCR tubes and stored at -30^{o}C for subsequent applications.

3.3.2.3 Bisulfite-modification of isolated DNA

The protocol of Tsunematsu *et al*., (2009) was employed in the detection of methylation pattern of genomic DNA isolated from archival 10% buffered formalin-fixed, paraffin-embedded tissues of OSCC in Ibadan. 50µL of DNA from each sample was employed for sodium bisulfite treatment in order to change all unmethylated cytosines to uracils at the same time leaving methylated cytosines unchanged. For the denaturation of DNA, 50µL of genomic DNA from each sample was placed in sterile PCR tubes to which 0.2M NaOH was added and allowed to react for 10 minutes at 37^{o}C. Thereafter, 30µl of 10mM hydroquinone and 520µl of 3M sodium bisulfite at pH 5.0 both newly prepared was dropped into each tube, homogenized and allowed to react at 50^{o}C for 16 hours.

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Subsequently, impurities and contaminants were removed from the modified DNA using DNA purification resin (Promega) and eluted into 50µl of water. Modification was accomplished by adding NaOH (0.3M) and allowed to react for 5 minutes at room temperature, accompanied by ethanol precipitation.

3.3.2.4 Amplification of modified DNA by methylation-specific PCR

Each modified DNA was amplified in a 12.5µL total reaction volume containing 2µL of modified DNA, 2.25µL of Master mix, 2.0µL of *RUNX3* gene primer and 6.25µL of sterile water after an initial denaturation step at 95^{o}C for 3 minutes, subsequent denaturation at 95^{o}C for 15 seconds, annealing at 58^{o}C for 30 seconds, extension at 72^{o}C for 60 seconds and a final extension step at 72^{o}C for 5 minutes. This was carried out for 40 cycles. The PCR products were then loaded onto 2% agarose gel and viewed by ethidium bromide staining using 100bp ladder. Numerical data of C_pG islands methylation of *RUNX3* for gender, age, tumor location or anatomic site and histological class are presented as frequencies and percentages, while SPSS statistical package version 23.0 was employed for data analysis. Pearson's χ^2 test was employed in asessing association between gender, age, tumor location, histological class and promoter hypermethylation of *RUNX3*. For all analysis, $\alpha_{0.05}$ was considered significant. Table 3.1 contains information on primer sequences and methylation-specific polymerase chain reaction conditions.

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Table 3.1: Primer sequences and conditions for MSP

Gene of interest	Primer sequences	Product size	Annealing temperature
RUNX3 (M)	F 5'- ATAATAGCGGTCGTTAGGGCGTCG-3'	115 bp	58°C
	R 5'- GCTTCTACTTTCCCGCTTCTCGCG-3'		
(U)	F 5'- TTATGAGGGGTGGTTGTATGTGGG-3'	234 bp	58°C
	R 5'- AAAACAACCAACACAAACACCTCC-3'		

Source: Hu *et al*., (2010).

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MSP = methylation specific polymerase chain reaction; M = methylated primer; U = unmethylated primer; F = forward primer; R = reverse primer; bp = base pairs.

3.3.3 Evaluation of OSCC for presence of single-nucleotide polymorphisms in *RUNX3* gene

3.3.3.1 Genomic DNA extraction

Deparaffinization of tissue samples was done as explained before in section 3.3.2.1 while Protenaise K digestion and DNA isolation was done according to manufacturer's instruction and as explained before in section 3.3.2.2.

3.3.3.2 Amplification of DNA by restriction fragment length polymorphism-PCR

Isolated DNA was amplified in a polymerase chain reaction (PCR) using 2 pairs of primers specific for *RUNX3* gene at the following PCR thermal program; initial denaturation at 95^{o}C for 5 minutes, followed by 40 cycles for subsequent denaturation at 95^{o}C for 30 seconds, annealing at 60^{o}C for 30 seconds and elongation at 72^{o}C for 30 seconds. After the last cycle, a final extension at 72^{o}C for 5 minutes was performed. PCR products were digested separately by *Hinc*II and *BstZ171*-HF restriction endonucleases for rs7528484 and rs760805 polymorphism primers at 60^{o}C and 58^{o}C respectively for 16 hours. RFLP products were thereafter stained with ethidium bromide (EtBr) and subsequently viewed using 2.0% agarose gel. DNA bands were distinguished by use of 100bp DNA ladder (Zhang *et al*., 2019). Genotypes and alleles of *RUNX3* for gender, age, tumor site and histological class were presented as frequencies and percentages, while SPSS statistical package version 23.0 was used for statistical analysis. Pearson's χ^2 test, Monte Carlo exact test and odds ratios were employed in assessing association between gender, age, tumor site, histological class and single nucleotide polymorphism genotypes on the one hand and between gender, age, tumor site, histological class and single nucleotide polymorphism alleles on the other hand. For all analysis, $\alpha_{0.05}$ was considered significant. Table 3.2 contains information on SNP conditions.

Table 3.2: Primer sequences and conditions for RFLP-PCR on *RUNX3*

SNP ID	Primer (5-3)	Annealing Temperature	Restriction enzyme	Cuttable allele	Uncuttable allele
rs 760805	F 5′- TCTCCCACTCAGCAGTTCACAC-3′	58.7^{o}C	*BstZ171*-HF	A(152 and 22bp)	T(174bp)
rs 760805	R 5′- TACAGCTCTCAATATGCGCCAG-3′				
rs 7528484	F 5′- TGCGAGGCCCAGGGTGTTGA-3′	60^{O}C	*Hinc*II	C(107 and 18bp)	T(125bp)
rs 7528484	R 5′- CATGGAAGGGCACTCTGGTG-3′				

Source: Gao *et al*., (2016)

F = forward; R = reverse; bp = base pair

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CHAPTER FOUR

RESULTS

4.1 Frequency of OSCC by Gender, Age, Tumor Site and Histology Class

A total of 1527 tumor-associated cases were recorded at the dental facility of the University College Hospital, Ibadan, Southwest, Nigeria, between January 2004 and December 2015. From this total number, 100 representing 6.5% were histologically detected as OSCC consisting of 58 males (3.8%) and 42 females (2.7%) in a 1.4:1 male to female ratio. This shows that OSCC occurred preponderantly in men than in women in this study. Statistical analysis however showed no notable relationship between OSCC and gender ($p > 0.05$), and therefore gender on its own may not be a predictive marker for OSCC (Table 4.1).

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Out of the 100 OSCC cases recorded, 30 were of the well differentiated histology class while, 65 were of the moderately differentiated class and 5 were of the poorly differentiated class representing 30.0%, 65.0% and 5.0% respectively. Although the moderately differentiated class was the most preponderant, the variation in prevalence among the different histology classes had no statistical relevance ($p > 0.05$) (Table 4.2).

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It was observed that the moderately differentiated class was frequent in patients who were in their first, second and fourth decades of life, while other histology classes were not limited to any particular age group. Furthermore, the well differentiated and poorly differentiated histologic classes were prevalent in patients' within the 5th and 8th decade age groups, while the moderately differentiated histologic class was prevalent in patients' within the 7th and 8th decade age groups.

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Generally, irrespective of histologic class OSCC was observed to be prevalent in patients' within the 7th and 8th decade age groups. Statistical analysis failed to show the existence of any association between OSCC and age ($p>0.05$) thereby ruling out age as a predictive biological indicator for OSCC in this study (Table 4.3).

There was a selective predilection of the moderately differentiated class for the base of the mouth, parotid, buccal mucosa as well as commissure respectively, while the poorly differentiated class exhibited selective predilection for the oropharynx.

The palate was the most frequently affected anatomic location while the mandible, maxilla and tongue in decreasing sequence of preponderance were the other commonly affected sites. Inspite of these observations, a statistically relevant relationship was not established between OSCC and tumor anatomic site ($p > 0.05$). This goes to say that tumor anatomic site independently may not be a predictive biomarker for this cancer in the present study (Table 4.4).

Further observations on the relationship between the different histologic classes of OSCC, age groups and gender showed that a male to female ratio of 1:1 was observed in the well differentiated histologic class; 1.6:1 in the moderately differentiated histologic class and 1.5:1 in the poorly differentiated histologic class (Table 4.5).

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Table 4.1: Annual prevalence of OSCC in Ibadan

	Male			Female			Total	
Year	All cases	Sq.cell ca.	p-value	All cases	Sq.cell ca.	p-value	All cases	Sq.cell ca
2004	40	1	0.371	51	5	0.482	91	6
2005	45	2		39	0		84	2
2006	38	2		63	6		101	8
2007	50	5		57	3		107	8
2008	57	1		60	1		117	2
2009	61	6		51	2		112	8
2010	75	6		80	3		155	9
2011	97	6		89	4		186	10
2012	69	6		87	5		156	11
2013	73	2		75	3		148	5
2014	56	9		69	9		125	18
2015	68	12		77	1		145	13
Total (%)	729 (47.7)	58 (3.8)		798 (52.3)	42 (2.7)		1527	100 (6.5)

Sq = squamous; Ca = carcinoma

Table 4.2: Sharing of OSCC into histology classes

Year	Histology class						Total
	Well diff. sq. cell ca.	p-value	Mod.diff. Sq.cell ca.	p-value	Poorly diff.sq. cell ca	p-value	
2004	5	0.426	1	0.098	0	0.206	6
2005	0		2		0		2
2006	5		3		0		8
2007	4		3		1		8
2008	1		1		0		2
2009	2		6		0		8
2010	4		5		0		9
2011	2		6		2		10
2012	2		8		1		11
2013	0		4		1		5
2014	2		16		0		18
2015	3		10		0		13
Total (%)	30 (30)		65 (65)		5 (5)		100

Sq = squamous; Ca = carcinoma; Diff = differentiated; Mod = moderately

Table 4.3: Sharing of OSCC into age groups

Age group	Histology class						Total
	Well diff. sq. cell ca.	p-value	Mod.diff. Sq.cell ca.	p-value	Poorly diff.sq. cell ca	p-value	
0-10	0	0.291	1	0.253	0	0.341	1
11-20	0		2		0		2
21-30	2		4		0		6
31-40	0		8		0		8
41-50	6		6		2		14
51-60	4		10		1		15
61-70	5		15		0		20
71-80	6		12		2		20
81-90	1		3		0		4
91-100	1		1		0		2
Not indicated	5		3		0		8
Total	30		65		5		100

Sq = squamous; Ca = carcinoma; Diff = differentiated; Mod = moderately

Table 4.4: Sharing of OSCC into tumor site

Tumor site	Histology class						Total
	Well diff. sq.cell ca.	p-value	Mod.diff. Sq.cell ca.	p-value	Poorly diff.sq. cell ca	p-value	
Floor of mouth	0	0.336	2	0.282	0	0.353	2
Parotid	0		1		0		1
Oropharynx	0		0		1		1
Antrum	0		1		2		3
Facial mass	2		1		0		3
Buccal mucosa	0		4		0		4
Lip	2		5		0		7
Tongue	8		5		0		13
Maxilla	2		13		2		17
Mandible	7		12		0		19
Palate	7		16		0		23
Commissure	0		1		0		1
Not indicated	2		4		0		6
Total	30		65		5		100

Sq = squamous; Ca = carcinoma; Diff = differentiated; Mod = moderately

Table 4.5: Combined sharing of OSCC by histology class, age and gender

Age	Histology class/Age							
	Well diff. sq. cell ca		Mod. diff. sq. cell ca.		Poorly diff. sq. cell ca.		Total	
	M	F(M:F)	M	F(M:F)	M	F(M:F)	M	F(M:F)
0-10	0	0	0	1	0	0	0	1
11-20	0	0	2	0	0	0	2	0
21-30	1	1	3	1	0	0	4	2
31-40	0	0	5	3	0	0	5	3
41-50	3	3	5	3	0	0	8	6
51-60	2	2	7	3	1	1	8	6
61-70	2	3	10	5	0	0	10	5
71-80	3	3	7	5	1	1	12	8
81-90	1	0	1	2	0	0	2	2
91-100	1	0	0	1	0	0	1	1
Not indicated	2	3	1	2	0	0	3	5
Sub-total	15	15(1:1)	40	25(1.6:1)	3	2(1.5:1)	58	42(1.4:1)
Total	30		65		5		100	

Sq = squamous; Ca = carcinoma; Diff = differentiated; M = male; F = female

4.2 Pattern of Expression of Cytokeratin (CK AE1/AE3) and Epithelial Membrane Antigen (EMA) Proteins in OSCC

The well differentiated, moderately differentiated and poorly differentiated histologic classes of OSCC expressed cytokeratin (CK AE1/AE3) and epithelial membrane antigen (EMA) proteins in their epithelial and connective tissue components. However, the poorly differentiated histologic class did not express epithelial membrane antigen (EMA) protein in their connective tissue component (Table 4.6 and Figures 4.1a, 4.1b, 4.1c, 4.2a, 4.2b and 4.2c).

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4.3 Pattern of Methylation of *RUNX3* Gene in OSCC

4.3.1 Frequency of *RUNX3* methylation in OSCC

The frequency of *RUNX3* methylation was determined in 67 FFPE OSCC samples with 2 sets of primers (methylated and unmethylated) exclusively intended to be used for MSP procedures as earlier reported by Tsunematsu *et al.*, (2009). On the basis of the occurrence of C_pG island in the 5′ region (promoter) of *RUNX3* gene, 45.0% (30/67) of the OSCC tumor samples were found to be hypermethylated while, 55.0% (37/67) were unmethylated. MSP electrophoresis gel of OSCC tumor samples and cancer-free samples using methylated primers are presented in Figures 4.3a, 4.3b and 4.3c and MSP electrophoresis gel of randomly selected OSCC tumor samples using unmethylated primers is presented in Figure 4.3d.

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4.3.2 Association between *RUNX3* methylation with pattents' demographic variables

Out of a total of 43 male and 24 female OSCC samples available for use in this study, 21 male and 9 female samples respectively were hypermethylated while 22 male and 15 female samples were unmethylated. No association was established between promoter hypermethylation of *RUNX3* with gender (p = 0.157) (Table 4.7). Out of a total of 32 samples with ages in the range of 60 - 100 years, 13 were hypermethylated while 19 were unmethylated. Furthermore, out of the 32 samples with ages < 60 years, 16 were hypermethylated and 16 were unmethylated. Out of the 3 samples with undisclosed age, 1 sample was hypermethylated while 2 were unmethylated. No association was established between promoter hypermethylation of *RUNX3* with age (p = 0.223) (Table 4.7). Out of

the 17 well differentiated OSCC samples, 10 were hypermethylated while 7 were unmethylated. Furthermore, out of the 46 moderately differentiated OSCC samples, 19 were hypermethylated while 27 were unmethylated. And out of the 4 poorly differentiated OSCC samples used in the study, 1 was hypermethylated while 3 were unmethylated. No association was established between promoter hypermethylation of *RUNX3* with histological class (p = 0.199) (Table 4.7). Strong association was however established between promoter hypermethylation of *RUNX3* with tumor location (p = 0.010) (Table 4.8).

Table 4.6: Pattern of CK (AE1/AE3) and EMA proteins expression in OSCC

Histology class	EMA	CK
Well differentiated		
epithelium	+	+
connective tissue	+	+
Moderately differentiated		
epithelium	+	+
connective tissue	+	+
Poorly differentiated		
epithelium	-	+
connective tissue	-	+

EMA = Epithelial membrane antigen; CK = Cytokeratin (AE1/AE3)

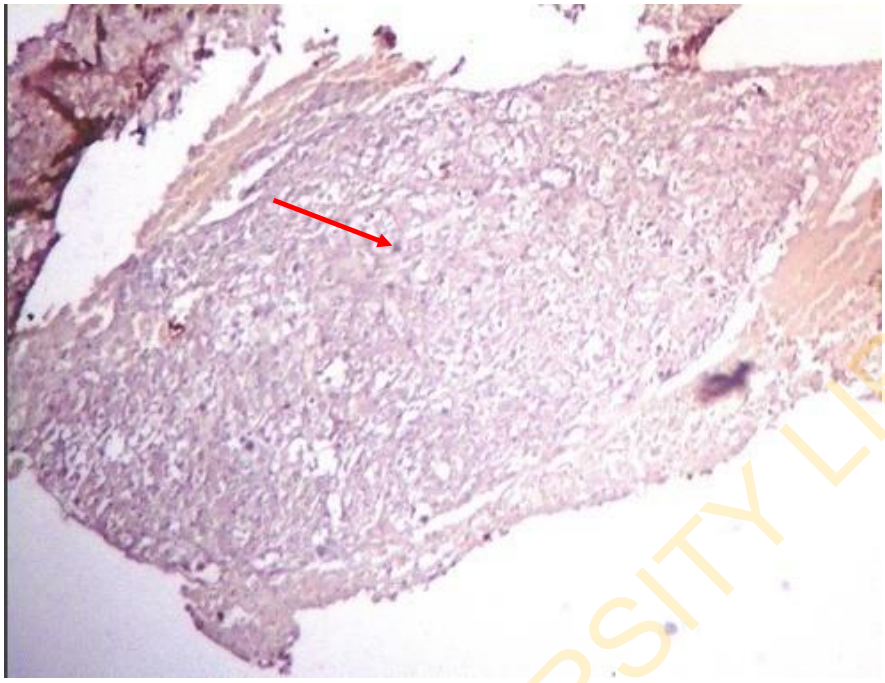

Figure 4.1a: Poorly differentiated squamous cell carcinoma showing bluish –ve EMA protein expression in arrow (100x Mag)

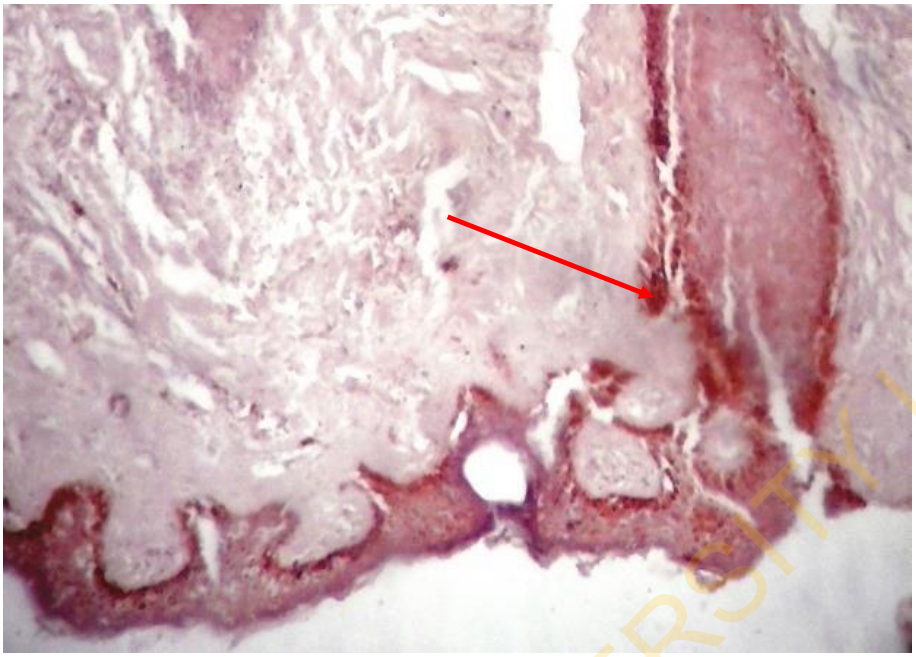

Figure 4.1b: Well differentiated squamous cell carcinoma showing brownish +ve EMA protein expression in arrow (100x Mag)

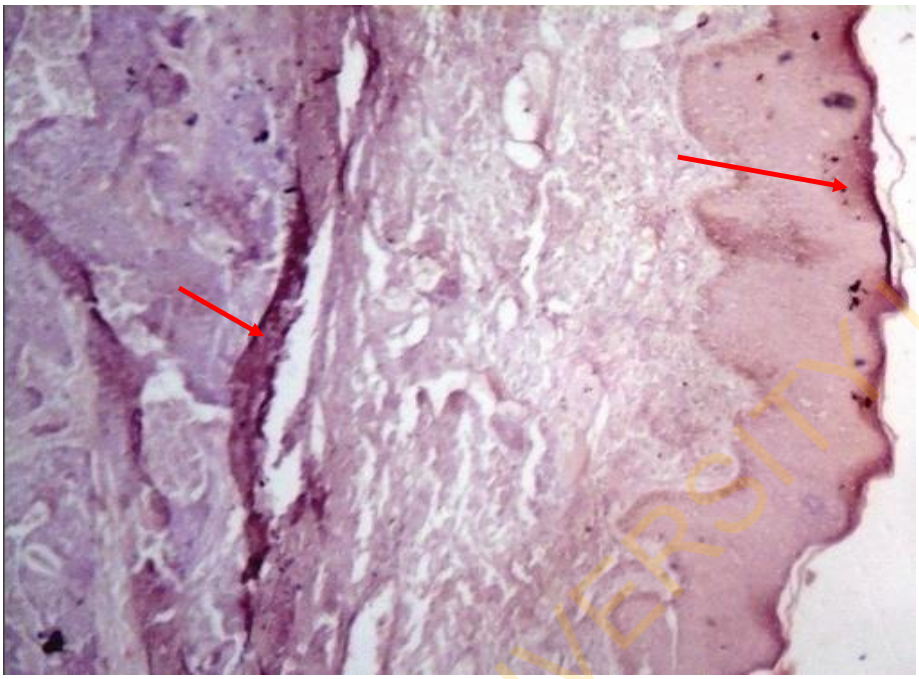

Figure 4.1c: Moderately differentiated squamous cell carcinoma showing brownish +ve EMA protein expression in arrow (100x Mag)

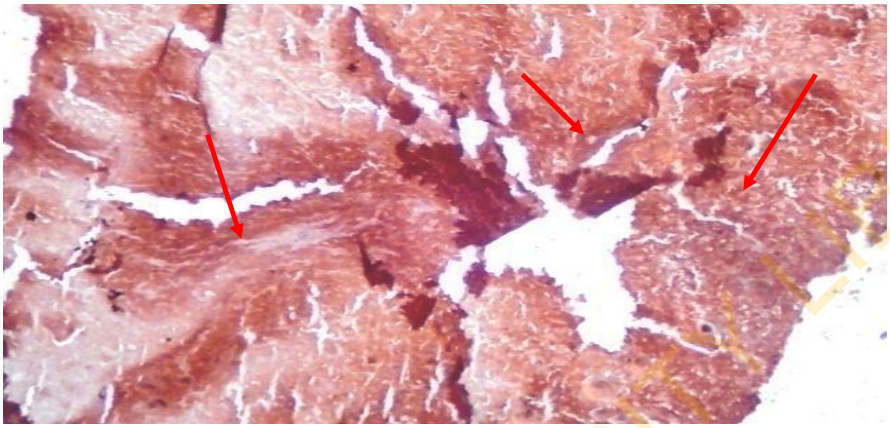

Figure 4.2a: Poorly differentiated squamous cell carcinoma showing brownish +ve CK AE1/AE3 protein expression in arrow (100x Mag)

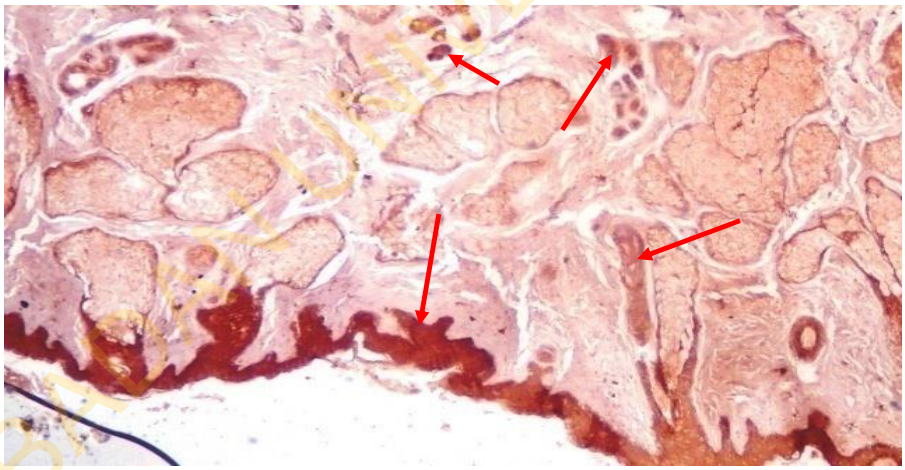

Figure 4.2b: Well differentiated squamous cell carcinoma showing brownish +ve CK AE1/AE protein expression in arrow (100x Mag)

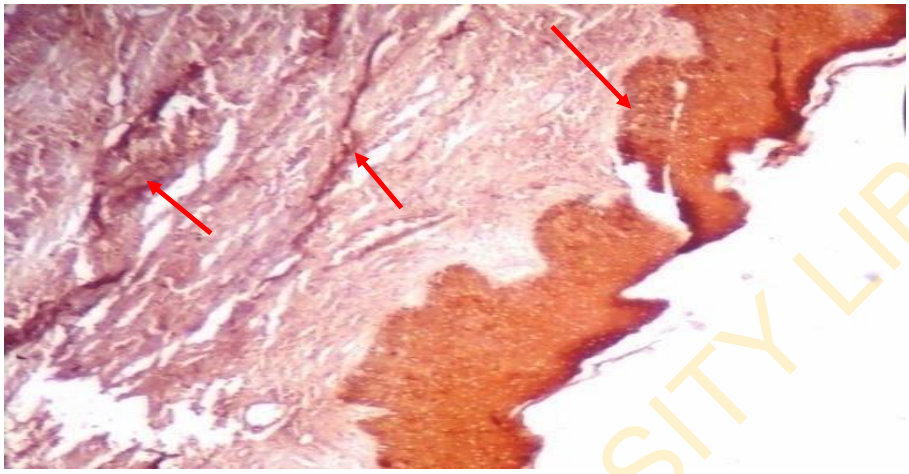

Figure 4.2c: Moderately differentiated squamous cell carcinoma showing brownish +ve CK AE1/AE3 protein expression in arrow (100x Mag)

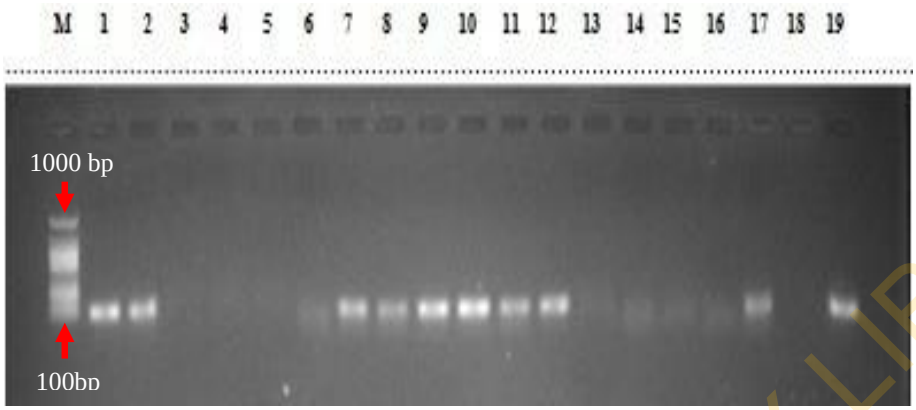

Figure 4.3a: Methylation specific-PCR products amplified by methylated primer showing C_pG island hypermethylation in *RUNX3*.

M is 100bp ladder; Lanes 1, 2, 7, 8, 9, 10, 11, 12, 17 and 19 are hypermethylated OSCC tumor samples; Lanes 3, 4, 5, 6, 13, 14, 15, 16 and 18 are unmethylated OSCC tumor samples.

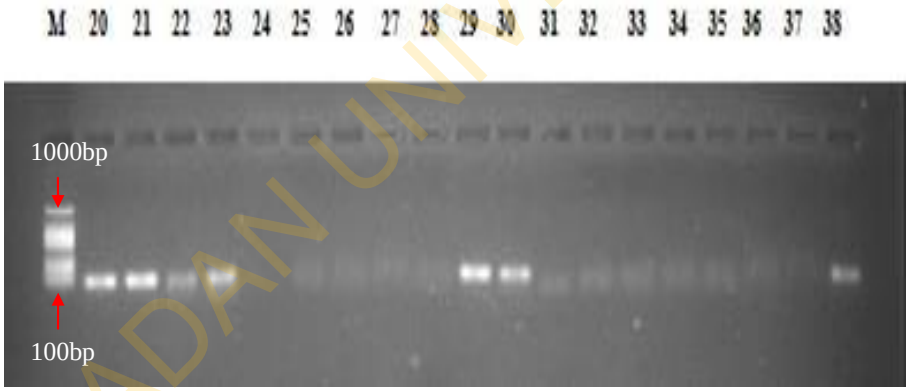

Figure 4.3b: Methylation specific-PCR products amplified by methylated primer showing C_pG island hypermethylation in *RUNX3*.

M is 100bp ladder; Lanes 20, 21, 22, 23, 29, 30 and 38 are hypermethylated OSCC tumor samples; Lanes 24, 25, 26, 27, 28, 31, 32, 33, 34, 35, 36 and 37 are unmethylated OSCC tumor samples.

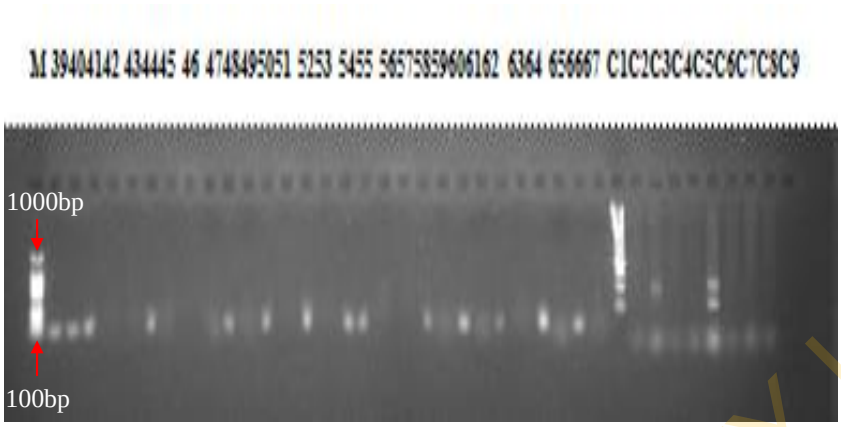

Figure 4.3c: Methylation specific-PCR products amplified by methylated primer showing C_pG island hypermethylation in *RUNX*3.

M is 100bp ladder; Lanes 39, 40, 41, 44, 48, 50, 52, 54, 55, 58, 60, 64 and 66 are hypermethylated OSCC tumor samples; Lanes 42, 43, 45, 46, 47, 49, 51, 53, 56, 57, 59, 61, 62, 63, 65 and 67 are unmethylated OSCC tumor samples; Lanes C1 - C9 are unmethylated cancer-free samples.

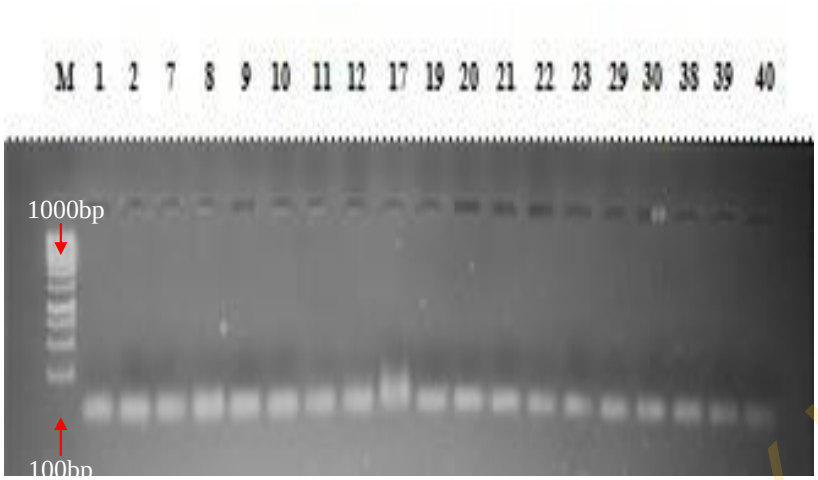

Figure 4.3d: Methylation specific-PCR products amplified by unmethylated primer.

M is 100bp ladder; Lanes 1, 2, 7, 8, 9, 10, 11, 12, 17, 19, 20, 21, 22, 23, 29, 30, 38, 39 and 40 are unmethylated randomly selected OSCC tumor samples.

Table 4.7: Distribution of CpG island promoter hypermethylation of *RUNX3* in OSCC by gender, age and histology class

Variables	Number of cases	Hypermethylated (%)	p-value
Gender			0.157
Male	43	21 (48.8)	
Female	24	9 (37.5)	
Age			0.223
0.223			
≥ 60	32	13 (40.6)	
< 60	32	16 (50.0)	
Undisclosed	3	1 (33.3)	
Histology class			0.199
Well differentiated	17	10 (58.8)	
Moderately differentiated	46	19 (41.3)	
Poorly differentiated	4	1 (25.0)	

Table 4.8: Distribution of CpG island promoter hypermethylation of *RUNX3* in OSCC by tumor site

Variables	Number of cases	Hypermethylated(%)	p–value
Tumor site			0.010*
Tongue	13	6 (46.2)	
Antrum	2	1 (50.0)	
Facial mass	6	3 (50.0)	
Maxilla	10	5 (50.0)	
Lip	4	2 (50.0)	
Mandible	14	9 (64.3)	
Floor of mouth	2	2 (100.0)	
Commissure	1	1 (100.0)	
Buccal mass	2	1 (50.0)	
Palate	8	0 (0.0)	
Oropharynx	1	0 (0.0)	
Undisclosed	2	0 (0.0)	
Parotid	1	0 (0.0)	
Medial canter	1	0 (0.0)	

*significant at $p \leq 0.05$ using Pearson's χ^2 test

4.4 Pattern of Single-nucleotide Polymorphism of *RUNX3* in OSCC

4.4.1 Frequency of genotypes of *RUNX3* and its association with OSCC

Two single-nucleotide polymorphisms, rs7528484 and rs760805 of *RUNX3* were genotypically tested in 74 OSCC samples. While rs7528484 polymorphism was successfully genotyped (Figures 4.4a, 4.4b and 4.4c), rs760805 polymorphism on the other hand failed to genotype (Figures 4.5a, 4.5b and 4.5c). Consequently three genotypes, 52.7% (39) homozygote normal (CC), 28.4% (21) heterozygote mutant (CT) and 18.9% (14) homozygote mutant (TT) were observed for rs7528484 polymorphism of *RUNX3* in the OSCC samples. Statistically relevant association was seen between gender and genotype (p = 0.04) and between histology class and genotype (p = 0.05) (Table 4.9). No connection was observed between age and genotype (p = 0.31) (Table 4.9) and between tumour site and genotype (p = 0.54) (Table 4.10).

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4.4.2 Frequency of alleles of *RUNX3* and its association with OSCC

From the successfully genotyped rs7528484 polymorphism of *RUNX3* in 74 OSCC samples, two alleles (C and T) variously distributed by gender, age, histology class and tumour site were observed. As with genotypes, statistically relevant association was also seen between gender and alleles (p = 0.04) (Table 4.11) and between histology class and alleles (p = 0.05) (Table 4.11). No connection was observed between age and alleles (p = 0.31) (Table 4.11) and between tumour site and alleles (p = 0.54) (Table 4.12).

4.4.3 Odds of predicting OSCC by genotypes and alleles of *RUNX3*

Odds ratios were statistically computed for the identified genotypes and alleles. The homozygote normal genotype (CC) had an odds ratio of (OR 0.52, 95% CI: 0.3436 - 0.6964), heterozygote mutant (CT) genotype had odds ratio of (OR 0.28, 95% CI: 0.1889 - 0.3711) and homozygote mutant (TT) genotype had an odds ration of (OR 0.18, 95% CI: 0.1118 - 0.2482). The normal allele (C) had an odds ratio of (OR 0.33, 95% CI: 0.26 - 0.40), while the mutant allele (T) had an odds ratio of (OR 0.66, 95% CI: 0.52 - 0.80) (Table 4.13).

Figure 4.4a: RFLP-PCR products showing genotypes of rs7528484 polymorphism in *RUNX3*.

M is 100bp marker; Lanes 1, 2, 8, 11, 12, 17, 18, 21, 22, 23, 24, 25, 26, 29, 30, 31 and 32 are homozygote normal genotype CC; Lanes 4, 5, 6, 7, 9, 10, 13, 14, 15, 16, 27 and 28 are heterozygote mutant genotype CT; Lanes 3, 19 and 20 are homozygote mutant genotype TT.

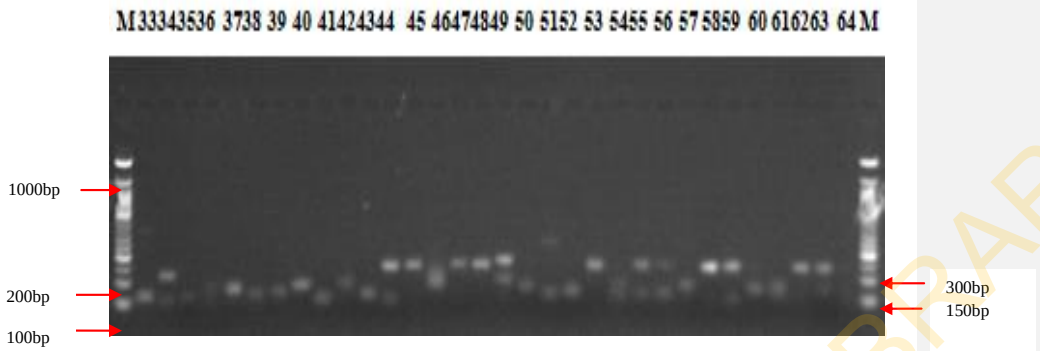

Figure 4.4b: RFLP-PCR products showing genotypes of rs7528484 polymorphism in *RUNX3*.

M is 100bp marker; Lanes 33, 35, 36, 37, 38, 39, 40, 41, 42, 43, 46, 50, 52, 54, 57, 60, 61 are homozygote normal genotype CC; Lanes 34, 44, 49, 51, 55, 56, 59, 63, 64 are heterozygote mutant genotype CT; Lanes 45, 47, 48, 53, 58, 62 are homozygote mutant genotype TT.

Figure 4.4c: RFLP-PCR products showing genotypes of rs7528484 polymorphism in *RUNX3*.

M is 100bp marker; Lanes 66, 68, 70, 72, 74 is homozygote normal genotype CC; Lanes 65, 67, 69, 71, 73 is homozygote mutant genotype TT.

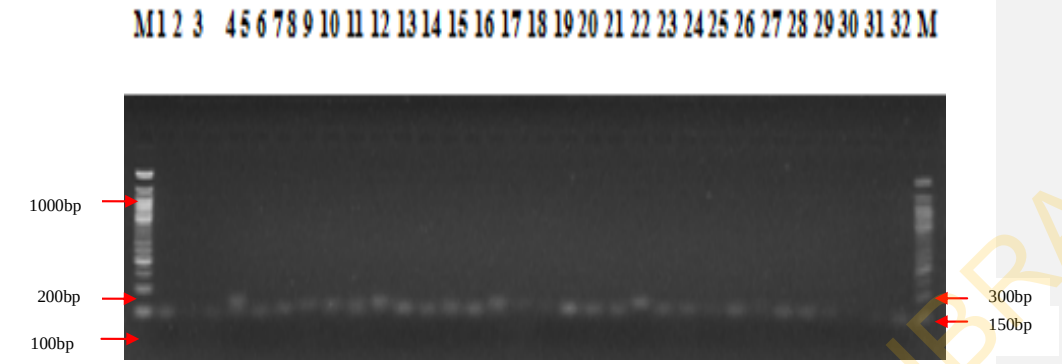

Figure 4.5a: RFLP-PCR failed to genotype rs760805 polymorphism in *RUNX3*. M is 100bp marker; Lanes 1 – 32 are OSCC tumor samples.

Figure 4.5b: RFLP-PCR failed to genotype rs760805 polymorphism in *RUNX3*. M is 100bp marker; Lanes 33 – 64 are OSCC tumor samples.

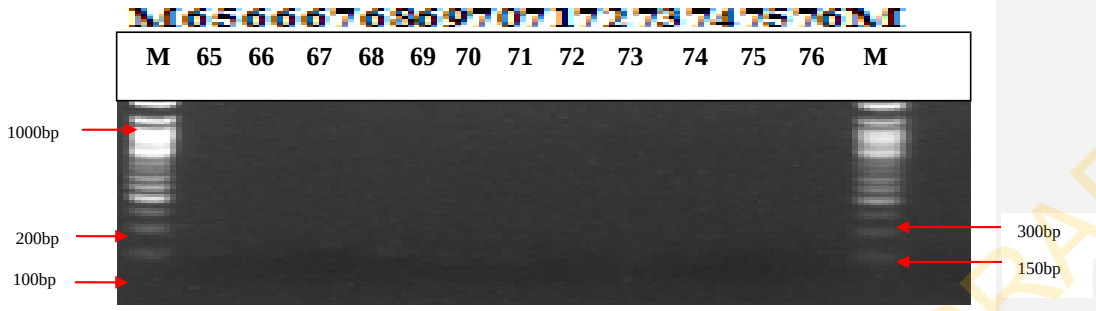

Figure 4.5c: RFLP-PCR failed to genotype rs760805 polymorphism in *RUNX3*.

M is 100bp marker; Lanes 65 – 74 are OSCC tumor samples; Lanes 75 – 76 are cancer-free samples.

Table 4.9: Distribution of rs7528484 polymorphism genotypes of *RUNX3* in OSCC by gender, age and histology class

Variables	Number of cases	Number of genotype CC (%)	Number of genotype CT (%)	Number of genotype TT (%)	p-value
Total	74	39 (52.7%)	21 (28.4%)	14 (18.9%)	
Gender					0.04*
Male	44	26 (59.1%)	12 (27.3%)	6 (13.6%)	
Female	26	12 (46.2%)	9 (34.6%)	5 (19.2%)	
Undisclosed	4	1 (25.0%)	0 (0.0%)	3 (75.0%)	
Age					0.31
≥60	26	13 (50.0%)	9 (34.6%)	4 (15.4%)	
<60	41	22 (53.7%)	12 (29.3%)	7 (17.1%)	
Undisclosed	7	4 (57.1%)	0 (0.0%)	3 (42.9%)	
Histology class					0.05*
Well differentiated	19	10 (52.6%)	6 (31.6%)	3 (15.8%)	
Moderately differentiated	46	25 (54.3%)	15 (32.6%)	6 (13.0%)	
Poorly differentiated	5	3 (60.0%)	0 (0.0%)	2 (40.0%)	
Undisclosed	4	1 (25.0%)	0 (0.0%)	3 (75.0%)	

Table 4.10: Distribution of rs7528484 polymorphism genotypes of *RUNX3* in OSCC by tumor site

Variables	Number of cases	Number of genotype CC (%)	Number of genotype CT (%)	Number of genotype TT (%)	p-value
Total	74	39 (52.7%)	21 (28.4%)	14 (18.9%)	
Tumor site					0.54
Tongue	11	8 (72.7%)	2 (18.2%)	1 (9.1%)	
Facial mass	5	2 (40.0%)	2 (40.0%)	1 (20.0%)	
Mandible	17	8 (47.1%)	7 (41.2%)	2 (11.7%)	
Palate	5	3 (60.0%)	2 (40.0%)	0 (0.0%)	
Buccal mucosa	3	1 (33.3%)	1 (33.3%)	1 (33.4%)	
Medial canter	1	1 (100.0%)	0 (0.0%)	0 (0.0%)	
Maxilla	6	3 (50.0%)	3 (50.0%)	0 (0.0%)	
Antrum	3	2 (66.7%)	0 (0.0%)	1 (33.3%)	
Lip	6	3 (50.0%)	2 (33.3%)	1 (16.7%)	
Cheek mass	1	0 (0.0%)	1 (100.0%)	0 (0.0%)	
Parotid	4	2 (50.0%)	1 (25.0%)	1 (25.0%)	
Vocal cord	1	0 (0.0%)	0 (0.0%)	1 (100.0%)	
Oropharynx	1	1 (100.0%)	0 (0.0%)	0 (0.0%)	
Nasopharynx	3	2 (66.7%)	0 (0.0%)	1 (33.3%)	
Undisclosed	7	3 (42.9%)	0 (0.0%)	4 (57.1%)	

Table 4.11: Distribution of alleles of rs7528484 polymorphism of *RUNX3* by gender, age and histology class

Variables	Number of C allele (%)	Number of T allele (%)	p-value
Total	99(66.9%)	49(33.1%)	
Gender			0.04*
Male	64(65.0%)	24(49.0%)	
Female	33(33.0%)	19(39.0%)	
Undisclosed	2(2.0%)	6(12.0%)	
Age			0.31
≥60	35(35.0%)	17(35.0%)	
<60	56(57.0%)	26(53.0%)	
Undisclosed	8(8.0%)	6(12.0%)	
Histological class			0.05*
Well differentiated	26(26.0%)	12(24.5%)	
Moderately differentiated	65(66.0%)	27(55.1%)	
Poorly differentiated	6(6.0%)	4(8.2%)	
Undisclosed	2(2.0%)	6(12.2%)	

Table 4.12: Distribution of alleles of rs7528484 polymorphism in *RUNX3* by tumor site

Variables	Number of C allele (%)	Number of T allele (%)	p-value
Total	99(66.9%)	49(33.1%)	
Tumor site			0.54
Tongue	18(18.2%)	4(8.2%)	
Facial mass	6(6.1%)	4(8.2%)	
Mandible	23(23.2%)	11(22.4%)	
Palate	8(8.1%)	2(4.1%)	
Buccal mucosa	3(3.0%)	3(6.1%)	
Medial canter	2(2.0%)	0(0.0%)	
Maxilla	9(9.1%)	3(6.1%)	
Antrum	4(4.0%)	2(4.1%)	
Lip	8(8.1%)	4(8.2%)	
Cheek mass	1(1.0%)	1(2.0%)	
Parotid	5(5.1%)	3(6.1%)	
Vocal cord	0(0.0%)	2(4.1%)	
Oropharynx	2(2.0%)	0(0.0%)	
Nasopharynx	4(4.0%)	2(4.1%)	
Undisclosed	6(6.1%)	8(16.3%)	

Table 4.13: Odds of predicting OSCC by genotypes and alleles of rs7528484 polymorphism in *RUNX3* gene

Variables	OR (95% CI)
Genotypes	
Homozygote normal (CC)	0.52 (0.3436 – 0.6964)
Heterozygote mutant (CT)	0.28 (0.1889 – 0.3711)
Homozygote mutant (TT)	0.18 (0.1118 – 0.2482)
Alleles	
Normal allele (C)	0.33 (0.26 – 0.40)
Mutant allele (T)	0.66 (0.52 – 0.80)

OR (Odds Ratio); CI (Confidence Interval)

CHAPTER FIVE

DISCUSSION

5.1 Demographic Distribution of OSCC in Ibadan

Current literature suggests that OSCC is becoming a frightful community health issue not only in Nigeria but worldwide. The reasons for this include its rising incidence especially in the younger population, high mortality rate; indeed the failure to improve survival rate despite advances in treatment and accumulating proof that suspected possible causes alone probably may not account for a sizeable sum of the malignancy (Lawal *et al*., 2017; Khan *et al*., 2018). Undoubtedly, early detection is essential but remains a challenge. Investigation into the molecular processes behind OSCC tumorigenesis is therefore essential for the discovery of new biomarkers which could result in improved treatment, drastically reduced mortality rate and increased survival rate. Thus, in this study, we have looked at the demographic pattern of OSCC in terms of gender prevalence, age group distribution, histological class preponderance and tumour site predilection with the aim of using these findings for predictive diagnosis of OSCC. We have also tried to elucidate molecular predictive diagnostic biomarkers for OSCC.

Although, slightly more females (798) presented with various types of benign and malignant neoplasia than males (729) resulting in a 1.1: 1 ratio between January 2004 and December, 2015 which is the period covered by this study. However the figure for males diagnosed as OSCC exceeded that for females and this amounted to a 1.4:1 ratio. The preponderance of the masculine gender as sufferers of OSCC was consistent with reports over time from various geographical areas of the world (Neville and Day, 2002; Al-Rawi and Talabani, 2008; Menach *et al*., 2014; Khan and Khan, 2015). To further buttress this assertion, a male frequency of 76.6 % was reported by Alves *et al*., (2017); 74 % by Siriwardena *et al*., (2015); 73 % by Gajurel *et al*., (2019); 76.53 % by Tandon *et al*.,

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(2018); 67 % by Pires *et al*., (2013); 80.1 % by Anwar *et al*., (2020); 50.5 % by Kitamura *et al*., (2012); 71% by Santoro *et al*., (2015); 56 % by Al Ashalah and Alagraa, (2020). The prevalence of OSCC among males may not be unconnected with the fact that indulgence in socio-cultural habits such as alcohol consumption and cigarette smoking which are obvious risk factors is supposedly common in males than in females (Tandon *et al*., 2017). However, the gap in prevalence between males and females was narrower in our study than what was reported in previous studies from other parts of the world. This may be linked to more women getting actively involved in social habits that expose them to cancer causing agents (Tandon *et al*., 2018). These habits include; more women indulging in cigarette smoking, alcohol consumption, and unconventional sexual practices such as coition with multiple sex partners and oro-anal sex which predisposes them to HPV infection. This infection has been positively correlated with oropharyngeal cancer in young people (Siriwardena *et al*., 2015). Thus, increased spate of Human Papilloma Virus infection in women has been identified as a factor responsible for this reduced gap in prevalence between males and females (Siriwardena *et al*., 2015). This study was however limited by the lack of information on the lifestyle habits such as cigarette smoking and alcohol consumption of our OSCC patients'.

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Records retrieved from the Dental Center of the University College Hospital, Ibadan showed that there was a rise in annual occurrence of OSCC from 6 cases in 2004 to 18 and 13 cases in 2014 and 2015 respectively. Similar trend from studies in other climes had been noted by Chidzonga and Mahomva (2006); Rekha *et al*., (2013) and Menach *et al*., (2014). It was claimed by Adisa *et al*., (2011) that access to facilities for multiple approaches to handling of these subjects was accountable for the higher number of subjects seeking dental care at the University College Hospital, Ibadan, Nigeria than in other centers in Nigeria. This together with increased level of awareness of dental health could have contributed to this increased occurrence.

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The moderately differentiated histology class was the commonest type of OSCC in the population covered by this study. Meanwhile, in other studies the reports were inconsistent. For instance, Al Ashalah and Alagraa, (2020) reported a preponderance of

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the well differentiated OSCC with a 40 % frequency; Santoro *et al*., (2015) reported a preponderance of the moderately differentiated OSCC with a frequency of 47.4 %; Kitamura *et al*., (2012) reported a preponderance of the well differentiated OSCC with a frequency of 68.6 %; Pires *et al*., (2013) reported a preponderance of the moderately differentiated OSCC with a frequency of 45.5 %; Tandon *et al*., (2018) reported a preponderance of the well differentiated OSCC with a frequency of 66.73 %; Siriwardena *et al*., (2015) reported a preponderance of the well differentiated OSCC with a frequency of 55.5 %. The reasons for this inconsistent histological pattern may be difficult to substantiate, it is however our opinion that early or delayed presentation at the health facility may contribute to the degree of OSCC differentiation where the well differentiated histological type coincides with early presentation; and the poorly differentiated histological type coincides with late presentation. Again, since different geographical areas have their peculiar dietary and nutritional preferences, this could also be a factor that may have contributed to OSCC differentiation.

Furthermore, it was noted that in each case, there was a male preponderance, which also agrees with reports from Zimbabwe (Chidzonga and Mahomva, 2006) and Kenya (Menach *et al*., 2014) where it was documented that the well differentiated class was the commonest, and with a similar higher male preponderance. The poorly differentiated type in both reports was also the least common with a higher male preponderance.

In this study, it was observed that OSCC is particularly associated with the middle-aged and the elderly especially those in their 5^{th} - 8^{th} decade of life regardless of histology class. This observation is consistent with reports from similar studies emanating from other centers (Chidzonga and Mahomva, 2006; Rekha *et al*., 2013; Khan and Khan, 2015; Okoh and Okoh, 2017). Specifically, Alves *et al*., (2017) reported that 53.9 % of OSCC sufferers in their study fell within the 51 - 70 years age group; 30.9 % fell within the 51 - 60 years age group (Siriwardena *et al*., 2015); 31.9 % fell within the 51 - 60 years age group (Gajurel *et al*., 2019); 62.2 % fell within the 40 - 60 years age group (Tandon *et al*., 2018); 46 % fell within the 41 - 60 years age group (Pires *et al*., 2013); 65 % were older than 65 years (Kitamura *et al*., 2012); 52.6 % were older than 63 years (Santoro *et al*.,

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2015); 44 % fell within the 60 - 69 years age group (Al Ashalah and Alagraa, 2020). A active and chronic exposure to acclaimed risk factors such as tobacco use in different forms; cigarette smoking; HPV infection; diet and nutrition (Pires *et al*., 2013), may be some of the explanations that account for this pattern. Fairly high frequencies for young adults in their 3rd and 4th decades of life (21 - 40 year age group) were also observed, which agrees with the reports from South Eastern Nigeria (Okoh and Okoh, 2017) and the Western world (Feller and Lemmer, 2012). This was not unconnected with exposure to socio-cultural risk elements at a very young age or other ambiguous risk parameters which may play a decisive function in the onset of tumourigenesis in this cohort (Siriwardena *et al*., 2015). Poor dental hygiene at a young age may also account for this development. Another important factor could be infection with Human Papilloma Virus as a result of oral sex and having unprotected coition with single or multiple partners (Siriwardena *et al*., 2015).

Regardless of the histology class, OSCC affects all parts of the oral cavity with the highest predilection observed for the palate, followed by the mandible, maxilla and the tongue. Indeed, highest mandibular predilection was reported in a Zimbabwean population (Chidzonga *et al*., 2006), laryngeal predilection in an Indian population (Rekha *et al*., 2013) and predilection for the tongue was reported in different populations and in the Western world (Feller and Lemmer, 2012) respectively. To further show that predilection of OSCC for anatomic site is variable, Ashalah and Alagraa, (2020) reported that both the oropharynx and cheek had the highest predilection of 24 % in their study; 76 % for floor of the mouth by Frohwitter *et al*., (2016); 60 % for the tongue by Kitamura *et al*., (2012); 68.8 % for the buccal mucosa by Anwar *et al*., (2020); 37 % for the tongue by Pires *et al*., (2013); 31.4 % for the buccal mucosa by Tando *et al*., (2017); 42 8 % for the tongue by Gajurel *et al*., (2019); 41.9 % for the buccal mucosa by Siriwardena *et al*., (2015). This seems to imply an influence of geographic distribution and nutritional/dietary differences as suggested by Feller and Lemmer (2012). There may also be need to rule out Human Papilloma Virus infection as a possible predisposing factor. However, this study was unable to do this for lack of such information.

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The moderately differentiated histology class was prevalent in the mouth, parotid, buccal mucosa as well as commissure, while the poorly differentiated histology class was prevalent in the oropharynx. No statistically significant association between gender, age, tumor site and OSCC was noted, thereby ruling out patient demographic variables as predictive diagnostic biomarkers for OSCC.

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5.2 Pattern of Expression of CK AE1/AE3 and EMA in OSCC

Research is continuing on the mechanism of OSCC pathogenesis. All the differentiated classes of OSCC from our study samples expressed cytokeratin (CK AE1/AE3). A similar pattern of expression for cytokeratin (CK AE1/AE3) in OSCC was observed by Khan *et al*., (2018). Similarly, Gupta and Ramani (2016) while conducting immunohistochemical evaluation on replica biopsies of OSCC observed strong positive staining for CK AE1/AE3 in the epithelium of primary tumours. Immunohistochemical staining for CK AE1/AE3 also showed the existence of minute metastasis and remote tumour cells in oral tissue samples previously diagnosed as OSCC negative (Dhawan *et al*., 2016). In oral spindle cell carcinoma, CK AE1/AE3 was also shown to positively stain both the carcinoma and spindle cell components (Romanach *et al*., 2010).

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Aside from this, other subsets of cytokeratin have been positively expressed in OSCC. Among them are; CK8/18, CK19, CK5/6, CK10 and CK14 as reported by Fillies *et al*., (2007) and Frohwitter *et al*., (2016). The expression of these subsets was confined to the cytoplasm of the positive cells. The degree of expression of CK8/18 in OSCC was reported to be as high as 66.7% (Fillies *et al*., 2007). Kitamura *et al*., (2012) had also reported a 96.2 % and 2.9 % positive expression of the CK17 and CK13 subsets of cytokeratin in OSCC cases. They also reported that the well differentiated OSCC strongly expressed CK17 more than the moderately- and porly- differentiated OSCC cases. While reporting on the CK19 subset of cytokeratin, Santoro *et al*., (2015) not only showed that OSCC was positively immunoreactive but that its expression was heterogeneous. Based on these findings, it was speculated that the probable reason for the expression of CK AE1/AE3 proteins in all three histological classes may be because of the heterogeneous nature of the population of cells that make up OSCC (Frohwitter *et al*., 2016). We also postulate that another likely reason why CK AE1/AE3 proteins was expressed in all three

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histological classes of OSCC could be because of the ubiquitous nature of these proteins in tumour cells from the oral cavity.

Even though EMA is considered to be abroad spectrum protein capable of being expressed in a wide range of carcinomas (Al Ashalah and Alagraa, 2020),; it was observed from our study samples that while the well differentiated and moderately differentiated classes expressed EMA, the poorly differentiated class failed to express EMA. In their study, Al Ashalah and Alagraa (2020) reported a 0 % non-expression of EMA in the well- and moderately- differentiated OSCC cases respectively and a 12 % non-expression in the poorly differentiated histologic class; a 24 % and 12% weak expression of EMA in the well- and moderately- differentiated OSCC cases respectively was also reported in their study. They also reported a 4 % and 12 % moderate expression of EMA in the well-; and moderately- differentiated OSCC cases respectively. They further reported a 12 % and 8 % strong expression of EMA in the well- and moderately- differentiated OSCC cases respectively. A positive expression in the well differentiated squamous cell carcinoma was reported by Aldelaimi (2014). Expression of EMA has also been noted in other types of tumours. A positive expression was observed in carcinoma of squamous epithelial cells of the skin by Ramezani *et al*., (2016). In addition, a general positive expression was also acknowledged in clear cell squamous cell carcinoma by Ricci *et al*., (2019) and oral spindle cell carcinoma by Romanach *et al*., (2010). These data could support the use of EMA stains for preliminary diagnostic predictions as they allow a clear cut distinction between the poorly differentiated histology class of OSCC and the other classes. For the non-expression of EMA in the poorly differentiated histological class, we hypothesize that this could be linked to the loss of this protein as the cancer becomes more invasiveness and progresses in malignancy.

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5.3 Pattern of CpG Island Methylation of *RUNX3* Gene in OSCC

Aberrant C_pG island methylation of the DNA sequences responsible for transcription is known to silence some genes as efficiently as in other pathways such as histone modification, chromatin remodeling; and entire human DNA search for such methylations have revealed that as much as ten percent of these nucleic acid sequences become methylated in human cancer (Costello *et al*., 2000). The involvement of transcriptional

regulation to prevent expression of quite a lot of genes such as *p16, hMLH1* (Kane *et al*., 1997) as well as *E-cadherin* (Yoshiura *et al*., 1995) among others, by DNA methylation in cancer development have been established. Furthermore, methylation of *SERPINE1* gene (Gao *et al*., 2010); *14-3-3σ* gene (Bhawal *et al*., 2007); *SFRP-2/SFRP-4/SFRP-5/wif-1/DKK-3* genes (Pannone *et al*., 2010) to mention a few correlated with OSCC occurrence.

In this study, methylation of *RUNX3* in OSCC and its association with gender, age, tumor site and histology class were examined. Contrary to the low (1.38%) frequency of hypermethylation of *RUNX3* in OSCC reported by Silva dos Reis *et al*., (2020); we detected a great amount (45%) of hypermethylation of this gene in our OSCC tissue samples. A 20% frequency of hypermethylation of *RUNX3* had also been reported in eosophageal carcinoma being anatomically close to the oral cavity by Saikia *et al*., (2017). Observations similar to this had been made in malignancies of the colorectum (Ku *et al*., 2004, Shin *et al*., 2018); lung (Li *et al*., 2004); intestine (Hu *et al*., 2010); liver (Mori *et al*., 2005) and with oral squamous cell carcinoma in a Brazillian cohort (Cordeiro-Silva *et al*., 2012). This therefore suggests that epigenetic aberration through promoter hypermethylation of R*UNX3* may likely be a pathway for the inducement of this cancer through gene inactivation. The high amount of *RUNX3* hypermethylation detected in our study could have been due to the strict adherence to the DNA extraction protocol as directed by the manufacturer of the kits used. Proper preservation of tissues and tissue blocks used in this study with the resultant conservation of tumour cells may have also played a role in our high detection rate. Tissue blocks with very few tumour cells could also give rise to low detection rate. The method of assessing methylation activity could also potentially impact on the variability of results. Some of the limitations of these methods as suggested by Lim *et al*., (2014) include; their tendency to give false positive results due to incomplete bisulfite conversion, formation of primer dimer, non-specific binding of primers and the subjective assessment of adequate binding intensity of amplicons that are methylated. Our keeping to standard operating procedures may have contributed to the prevention of these events.

We also found that hypermethylation of *RUNX3* in OSCC had no significant correlation with gender, age and histology class; this agrees with observations made in gastric

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malignancy (Hu *et al*., 2010); colorectal cancer (Shin *et al*., 2018); and OSCC in a Brazillian study (Cordeiro-Silva *et al*., 2012). Promoter hypermethylation of *RUNX3* in our OSCC samples was considerably correlated with tumor location which is in contrast with what was reported in the Brazillian study (Cordeiro-Silva *et al*., 2012). Furthermore, it was observed in a previous study that even though promoter methylation of *RUNX3* is significantly associated with gastric cancer, yet no correlation exists between age and gender with promoter methylation of this gene (Fan *et al*., 2011). This compares to what was observed for small solitary pulmonary nodules (Zhao *et al*., 2019). In another study involving promoter methylation of *RUNX3* gene in non-small cell lung cancer, meta-analysis showed that age correlates with *RUNX3* promoter methylation whereas, gender showed no correlation (Liang *et al*., 2014). As a matter of speculation, these inconsistencies may be in large part due to the impact of risk factors such as cigarette smoking, alcohol consumption, low socio-economic status, Human Papilloma Virus infection, diet/nutrition among others on the gene. Although highly sensitive, MSP being a non-quantitative method has the disadvantage of not being efficient in making clinical correlations as against semi-quantitative methods such as methylation specific high resolution melting (MS-HRM) (Lim *et al*., 2014).

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The data from the present study have shown that aberrant promoter hypermethylation of *RUNX3* occurs frequently in OSCC and that this is significantly correlated with tumor location. This suggests therefore a possible mechanism for *RUNX3* inactivation, thus giving rise to OSCC development. Consequently promoter hypermethylation of *RUNX3* may be an important diagnostic biomarker for OSCC. In spite of this, the non-availability of data detailing the lifestyle habits of OSCC patients whose samples were used in this study was observed to be a limiting factor. Potentially, prolonged exposure to these risk factors could have further explained the significant correlation between aberrant promoter hypermethylation of *RUNX3* with tumor location.

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5.4 Pattern of Single-nucleotide Polymorphisms of *RUNX3* Gene in OSCC

Several studies have evaluated the relationship between SNPs occurring in several genes and the development of malignancy. Among these studies, Wen *et al*., (2018) established that a major association exists between the single-nucleotide polymorphisms of the

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following genes; *MTHFR*, *ADIPOQ*, *CCND1*, *CASC8 CCAT2*, *miR - 27a*, *miR - 196a*^{2}, *LOC105376400*, *BMP2*, *BMP4*, *GREM1*, *ADPOR1*, *SMAD7*, *TGF-β1*, *KRAS* and sporadic colorectal cancer susceptibility.

Further revelations include the discovery that polymorphisms in the ATM gene are linked with development of damage in the major blood vessels of the heart in the Han Chinese people (Ding *et al*., 2017), and polymorphisms in *PIK3CA*, *AKT1* and *mTOR* genes may be related to bladder cancer development in an Iranian population (Bizhani *et al*., 2018). In relation to oral malignancy Tsai *et al*., (2011) showed significant connection between SNPs in *Cyclin D1* gene and oral cancer development in Taiwan. Ge *et al*., (2018) also showed that the mutant allele (T) and homozygote mutant genotype (TT) of *MTHFRC677T* polymorphism were highly correlated with an increased tendency to develop OSCC in China. Similarly, Li *et al*., (2015) found that functional polymorphisms in *COX - 2* are correlated with a tendency to have this cancer. An *et al*., (2007), also showed that SNPs in the central nucleotide excision repair genes (*NER*) consisting of several subtypes may collectively contribute to susceptibility to OSCC. In addition, Yang *et al*., (2018) showed that polymorphism in the ressistin gene (*RETN*) was significantly correlated with onset and further growth of OSCC, while Al-Hadyan *et al*., (2012) found that polymorphisms in the *p21*, *C31A*, *Ku80 A2790G* and *MDM2 T309G* genes were associated with predisposition to this cancer in Saudi Arabia.

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Out of the two polymorphisms (rs7528484 and rs760805) investigated in the *RUNX3* gene in our OSCC study population, only rs7528484 polymorphism was successfully genotyped with the homozygote normal (CC), heterozygote mutant (CT) and homozygote mutant (TT) genotypes occurring in the following proportions respectively; 52.7%, 28.4% and 18.9%. The normal (C) and mutant (T) alleles occurred in the following proportions respectively; 66.9% and 33.1%. This is akin to the account of Gao *et al*., (2016), who successfully genotyped rs7528484 polymorphism in *RUNX3* in cervical cancer and cervical intraepithelial neoplasia. We speculate that failure to genotype polymorphism rs760805 in the *RUNX3* gene in our OSCC samples could possibly be linked to standardization issues which include inadequacies in the amplification running time, gel and buffer concentrations, enzyme concentration; inappropriate temperature and target

DNA and insufficient digestion time. If enzyme concentration is inadequate, this has the tendency of triggering failure in recognizing the polymorphic sites. In such a situation extensive optimization may be needed to prevent such eventualities.

Statistically note worthy association was also established between gender and genotype (p=0.04); histology class and genotype (p=0.05); gender and allele (p=0.04); histological class and allele (p=0.05). In our study population, individuals carrying the mutant genotypes (CT and TT) with genotypic odds ratios less than one were more susceptible to developing OSCC than the normal genotype (CC) with genotypic odds ratio less than one. Similarly, individuals carrying the mutant allele (T) with allelic odds ratio less than one were more susceptible to developing OSCC than individuals carrying the normal allele (C) with allelic odds ratio less than one. This is in consonance with an observation in studies involving *MTHFR* polymorphisms and OSCC where it was established that persons with the CT and TT genotypes exhibited more vulnerability to this cancer than individuals with the CC genotype; and that persons carrying the T allele had an increased possibility of having OSCC than persons with the C allele (Ge *et al*., 2018). The results here may have been more profound but for the lack of information on the lifestyle habits of patients' whose samples were used in this study. It is suggested that future investigations should be mindful of these limitations and endeavour to incorporate the missing information.

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CHAPTER SIX

SUMMARY AND CONCLUSIONS

6.1 Conclusions

This study was conducted to identify molecular predictive markers for OSCC from patients in Ibadan. The data obtained indicate that in this environment, OSCC is more preponderant in the male than in the female, with peak prevalence in people aged between 61 and 70 years. The most frequently vulnerable anatomic location is the palate with the moderately differentiated squamous cell carcinoma being the most frequent histology class. The data aslo demonstrated that there is no association between gender, age, tumor anatomic site and OSCC development and thus none of these parameters can be used to predict OSCC.

The failure of the poorly differentiated histology class to express EMA protein lends credence to the diagnostic usefulness of EMA and CK (AE1/AE3) antibodies in distinguishing between the histology classes. We have obtained evidence in this study of aberrant promoter hypermethylation at the C_pG islands in *RUNX3* gene; however, no association was established between aberrant hypermethylation and patients' demographic variables of gender, age, histology class.

There is significant association between aberrant promoter hypermethylation at the C_pG islands in *RUNX3* gene and tumor anatomic site and thus proving useful as a diagnostic predictor of OSCC. In addition, three genotypes; homozygote normal (CC), heterozygote mutant (CT) and homozygote mutant (TT) and two alleles (C and T) in rs7528484 polymorphism of *RUNX3* gene were identified in this study. The heterozygote (CT) and homozygote (TT) mutant genotypes together with the mutant (T) allele in conjunction with gender and histology class were identified to be associated with susceptibility in

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OSCC. These results indicate that the presence of aberrant promoter hypermethylation at the C_pG islands in *RUNX3* and rs7528484 polymorphisms in *RUNX3* plays a significant role in susceptibility to OSCC.

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6.2 Recommendations

Samples in this study were obtained from a single institution thereby limiting the number of OSCC samples obtained. It is therefore suggested that a collaborative cross-regional multi-centre study should be undertaken. More SNPs and methylation sites could be genotyped. Indeed, a GWAS or microarray format may yield more information on gene expression.

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6.3 Contributions to Knowledge

1. Gender, age and tumour location lack predictive diagnostic value in OSCC patients from Ibadan, Nigeria
2. CpG island promoter hypermethylation in *RUNX3* gene was prevalent in OSCC patients from Ibadan, Nigeria
3. CpG island promoter hypermethylation in *RUNX3* gene in combination with gender, age and histology class lacks predictive diagnostic value in OSCC patients from Ibadan, Nigeria
4. CpG island promoter hypermethylation in *RUNX3* gene in combination with tumour location was predictive in OSCC patients from Ibadan, Nigeria
5. Homozygote normal, heterozygote mutant and homozygote mutant genotypes of *RUNX3* gene were identified in OSCC patients from Ibadan, Nigeria
6. rs7528484 polymorphism in *RUNX3* gene in combination with gender and histology class was predictive of OSCC from Ibadan, Nigeria
7. Epithelial membrane antigen (EMA) protein selectively predicts the poorly differentiated squamous cell carcinoma in OSCC patients from Ibadan, Nigeria

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