EXPRESSION OF KI-67, FSCN1 AND TNFRSF12A IN HISTOLOGICALLY NON-INVOLVED MUCOSAL SURGICAL MARGINS AS PREDICTIVE MARKERS FOR LOCAL RELAPSE IN ORAL SQUAMOUS CELL CARCINOMA

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Field of Study: Oral pathology (Oral Squamous Cell Carcinoma)

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ABSTRACT

Introduction: The presence tumour-related genetic alterations in histologically non-involved mucosal surgical margins was linked to development of local relapse in OSCC. **Aims:** The present study aimed to determine the association between expression of Ki-67, FSCN1 and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC patients and local relapse. **Materials and methods:** The study involved a total of 34 OSCC cases including 15 cases of patients who had relapse within the 5 years follow-up period and 19 cases of patients who did not had relapse. Two-stage sampling was carried out to select the margins' site, followed by the representative sections from the selected site. All the selected sections were subjected to FSCN1, TNFRSF12A and Ki-67 immunostaining. The immunostaining for FSCN1 and TNFRSF12A were evaluated by a semi-quantitative approach (HSCORE), while Ki-67 immuno-positivity was scored by the percentage of positive staining cells in randomly selected fields (Labelling index). The correlation of FSCN1, TNFRSF12A and Ki-67 expressions and clinicopathological parameters with OSCC relapse were analysed by Chi-square test. Binary logistic regression was performed to predict the relationship of OSCC relapse and the significantly associated parameters. **Results:** Our results showed that expression of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal surgical margins was weak to moderate in both study and control groups. OSCC relapse in this study was significantly associated with patients' age ($p < 0.001$), pattern of invasion ($p = 0.007$), Chinese ethnicity ($p = 0.013$), presence of epithelial dysplasia ($p < 0.001$), and alcohol consumption habits ($p = 0.025$). Age was the only predictor of relapse from the binary logistic regression analysis with an 11-fold risk of OSCC relapse noted in relation to patients who were aged above 57.5 years. **Conclusion:** The expression of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal surgical margins in the

present study was not associated with relapse in OSCC. However, patients' age may be a reliable predictor for OSCC relapse.

ABSTRAK

Pendahuluan: Kehadiran kelompok sel yang kelihatan normal dari segi histologi tetapi mengandungi perubahan dan ketidaksetabilan genetik dalam margin pembedahan yang bebas dari tumor dikaitkan dengan kejadian kambuh karsinoma sel skuamus mulut (OSCC). **Tujuan**: Kajian ini bertujuan untuk mengenal pasti hubungan antara ekspresi Ki-67, FSCN1 dan TNFRSF12A dalam margin pembedahan pesakit OSCC yang bebas dari sel kanser dan kejadian kambuh OSCC. **Bahan dan kaedah:** Kajian ini melibatkan sejumlah 34 kes OSCC termasuk 15 kes pesakit yang mengalami kambuh dalam tempoh susulan 5 tahun dan 19 kes pesakit yang tidak mengalami kambuh. Pensampelan dua peringkat dilakukan untuk memilih dua laman margin, diikuti dengan pemilihan keratan dari setiap laman margin yang dipilih. Penstainan imunohistokimia FSCN1, TNFRSF12A dan Ki-67 dilakukan ke atas sampel tissue yang telah dipilih. Ekspresi FSCN1 dan TNFRSF12A diskor secara semi-kuantitatif (HSCORE). Bagi Ki-67 pula, peratusan sel positif dalam kawasan yang dipilih secara rawak (Indeks Pelabelan) dikira. Korelasi ekspresi FSCN1, TNFRSF12A dan Ki-67 dan parameter klinikopatologi dengan kambuh OSCC dianalisis menggunakan ujian Chi-square. Regresi logistik binari dilakukan untuk meramalkan risiko kekambuhan OSCC berdasarkan parameter yang berkaitan secara signifikan. **Hasil:** Hasil kajian ini menunjukkan bahawa ekspresi FSCN1, TNFRSF12A dan Ki-67 dalam margin pembedahan yang bebas dari sel kanser secara histologi adalah lemah dan sederhana pada kedua-dua kumpulan kajian dan kawalan. Kambuh OSCC dalam kajian ini dikaitkan dengan usia pesakit ($p < 0{,}001$), corak invasi sel kanser ($p = 0{,}007$), etnik Cina ($p = 0{,}013$), adanya displasia epitelium ($p < 0{,}001$), dan pengambilan alkohol ($p = 0.025$). Risiko kambuh OSCC adalah 11 kali ganda bagi pesakit yang berusia melebihi 57.5 tahun berdasarkan analisis regresi logistik binari. **Kesimpulan:** Ekspresi FSCN1, TNFRSF12A dan Ki-67 dalam margin pembedahan yang bebas sel kanser dalam

kajian ini tidak dikaitkan dengan kambuh di OSCC. Walau bagaimanapun, usia pesakit mungkin menjadi ramalan yang boleh dipercayai untuk kambuh OSCC.

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Table of contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADH	:	Alcohol dehydrogenase
AJCC	:	American Joint Committee for Cancer
ALDH	:	Aldehyde dehydrogenase
ANT	:	Adjacent non-tumour
ASR	:	Age standardized rate
BT	:	Brachytherapy
CAP	:	Current College of American Pathologists
CCRN	:	Counulin
CCRT	:	Concurrent chemotherapy
CDKN2A	:	Cyclin-dependent kinase inhibitor 2A
DAB	:	3,3'-diaminobenzidine
DFS	:	Disease free survival
DNA	:	Deoxyribonucleic acid
DOI	:	Depth of invasion
EBRT	:	External beam radiotherapy
EBV	:	Epstein–Barr virus
ECS	:	Extracapsular spread
ED	:	Epithelial dysplasia
END	:	Elective neck dissection
FFPE	:	Formalin-fixed paraffin embedded
FIJI	:	Fiji for Image J
FOM	:	Floor of mouth
FSCN1	:	Fascin
GLOBOCON	:	Global Burden of Cancer
H&E	:	Haematoxylin and eosin
HNC	:	Head and neck cancer
HNSCC	:	Head and neck squamous cell carcinoma
HPE	:	Histopathological examination
HPV	:	Human papillomavirus
HSV	:	Herpes Simplex Virus
IARC	:	International Agency for Research on Cancer
ICC	:	Interclass correlation coefficient
IFN	:	Interferons
Ig	:	Immunoglobulin
IHC	:	Immunohistochemistry
IL	:	Interleukin
LHR	:	Lymphocytic host response
LI	:	Labelling index
LNR	:	Lymph node ratio
LOH	:	Loss of heterozygosity
LR	:	Local recurrence
LRFS	:	Local recurrence-free survival

LVI	:	Lympho-vascular invasion
MMP	:	Matrix metallopeptidases
MOCDTBS	:	Malaysian Oral Cancer Database and Tissue Bank System
NOM	:	Normal Oral mucosa
OCRCC	:	Oral Cancer Research and Coordinating Centre
OS	:	Overall survival
OSCC	:	Oral squamous cell carcinoma
PDGF	:	Platelet Derived Growth Factor
PNI	:	Perineural invasion
POH	:	Poor oral health
PORT	:	Post-operative radiotherapy
RNA	:	Ribonucleic acid
ROC	:	Receiver operating characteristic
ROS	:	Reactive oxygen species
RT	:	Radiotherapy
SFT	:	Second field tumour
SM	:	Surgical margins
SPT	:	Second primary tumour
TNF	:	Tumour necrosis factor
TNFRSF12A	:	Tumour necrosis factor receptor superfamily member 12A
WHO	:	World health organization
WPOI	:	Worst pattern of invasion

CHAPTER 1 : INTRODUCTION

1.1 Introduction

The aim of cancer treatment has always been to cure patients by eradicating the malignancy and preventing spread of the disease, leaving patients in a tumour free state. Surgery is accepted as the principal treatment for cancers in general, including oral squamous cell carcinoma (OSCC), with the goal to completely excise the tumour, leaving behind healthy, normal tissue. OSCC is the commonest head and neck cancer referring to malignancy of squamous cell origin occurring on the lip, palate, tongue, buccal mucosa, gingiva, retromolar trigone and floor of the mouth. Surgical removal of OSCC should adequately incorporate all of the macroscopic as well as the presumed microscopic extent of the disease. Postoperative radiotherapy (PORT) with or without concurrent chemotherapy is reserved for large tumours (pT3-T4) as well as small tumours (pT1-T2) with unfavourable histopathological findings such as cervical lymph node involvement, extra-nodal extension and microscopically involved surgical margins (PDQ Adult Treatment Editorial Board, 2019). Other than tumour stage and treatment modalities, tumour relapse is also a major factor affecting prognosis of OSCC. The 3-years survival rates for both early (stage I and II) and late stage (stage III and IV) OSCC has improved over the past 4 decades (1973-2014); in tandem with the increasing use of adjuvant chemoradiotherapy and elective neck dissection in node-negative OSCC patients (Cheraghlou et al., 2018). The 5-years overall survival rates for OSCC is about 70%-80% in early-stage disease and decreases to half in late-stage disease (Geum et al., 2013; González-García, 2016; Pericot et al., 2000). The 2 and 5-years survival rates were significantly lower in the recurrence group than in the non-recurrence group (67.6% vs.

88.0%, 31.8% vs. 79.9%, P < 0.001) (Wang et al., 2013). Tumour recurrence is return of the disease after completion of treatment within or at a distance from the primary site. The recurrence rate for OSCC varies between 30% to 45% with most of the recurrence occurring locally, within 24 to 36 months after initial treatment (Fan et al., 2017; Geum et al., 2013; Kernohan et al., 2010; Vázquez-Mahía et al., 2012). Local recurrence (LR) presents a challenge for clinicians and is devastating to patients as it is highly associated with morbidity following salvage surgery and reduction in overall prognosis (Brockstein et al., 2004; Pignon et al., 2007). To complicate further, about 50% of OSCC patients with recurrences are unsalvageable due to the advanced tumour stage at presentation and involvement of local vital structures (Kademani & Dierks 2006). Thus, a tool that could accurately stratify the risk of tumour recurrence is essential as the identified patients would benefit from a stringent surveillance protocol.

At present, TNM staging system (American Joint Committee for Cancer [AJCC], 2018) is a standard tool for classifying risk of recurrence and survival based on anatomical extent of the tumour. Published prognosticators for LR include tumour size (pT), nodal involvement (pN), surgical margin (SM) status, and pattern of invasion (Toratani et al., 2019), of which only pTN is included in the TNM staging system. SMs status is an important factor for local disease control as the presence of tumour in or close to the resected margins increases the likelihood of tumour recurrence (Sutton et al., 2003). Nevertheless, locoregional recurrences were also reported to develop from cases with histologically clear SMs (Gokavarapu et al., 2017). An explanation for this is that malignant transformation of epithelial cells in OSCC is a result of accumulated gene dysregulation that precedes phenotypic change of the epithelium (Feller et al., 2013). Correspondingly, molecular studies have shown that there are common tumour-related genes shared between the primary tumour and the contiguous non-neoplastic epithelium (Braakhuis et al., 2002). The size of this genetically altered field, termed field

cancerization was reported to be up to 7cm (Tabor et al., 2002). Thus, histologic findings in uninvolved SMs may not be accurate to predict the risk of local recurrence. Additionally, the recurrence may be the result of a second malignancy characterized clinically as a second tumour that may arise at least 2cm away from the primary tumour and/or within 3 years after the completion of treatment. The distinction between an LR and an SPT is further refined based on pattern of genetic alteration (Gleber-Netto et al., 2015). Recurring tumours that exhibit similar genetic alterations as the primary tumour is inferred as clonally related and classified as tumour recurrence while tumours that are genetically dissimilar from the primary tumour reflects a true SPT, a tumour that possibly originates from a separate pre-cancerized field. In addition, there are a group of second tumours emanating from the same precursor cells as the initial tumour but diverge during the later stages of carcinogenesis process. These tumours share only a part of the cancer-related gene alterations with the primary tumour and are termed as second field tumours (SFTs). Thus, detection of pre-cancerous fields within SMs through molecular assessment is of utmost importance in order to improve detection of patients at risk of relapse (Braakhuis et al., 2010;de Carvalho et al., 2012; Feller et al., 2013).

Various molecular markers have been studied to explore the possible clinical utility of the concept of field cancerization in predicting relapse. Immunohistochemistry (IHC) and DNA-based techniques were employed to investigate the altered protein expression and DNA copy number change, DNA ploidy, promoter methylation, and mutation analysis. Among them, TP53 mutation, DNA ploidy, loss of heterozygosity at various chromosomal arms, promoter-methylation of CDKN2A (p16) and expression level of Counulin (CCRN) and cytokeratin 4 (KRT4) were shown to have significant correlation with development of recurrences in a number of case-control studies (Braakhuis et al., 2010). However, most studies relied on established biomarkers, lacking extensive genome-wide analysis. Such selected biomarkers might represent late genetic alterations

associated with early carcinogenesis, hence, potentially be missed in surgical margin samples taken during treatment of primary tumour (Dakubo et al., 2007). Good biomarkers should be those that are expressed early in the development of a primary lesion, maintained throughout the progression of the lesion and expressed distinctively from normal epithelium (Article et al., 2018). Conway et al. (2015) managed to separate the genes in tumour cells that were differently expressed from normal epithelium in matched samples. The genes were ranked following average weighting, and the top 5% (1066) genes differently expressed in tumour were shown to be enriched in processes involving actin-filaments and cytoskeletons, the cellular tools for orientation, migration and invasion. Among them, TNFRSF12A and FSCN1 verified as cancer-related genes attest to unfavourable prognostic markers in head and neck cancer as well as have evidence of expression at protein level and reliable IHC staining pattern (The Human Protein Atlas, n.d). Hence, it would be plausible to investigate whether dysregulation of these genes in histologically non-involved surgical margins is related to relapse in OSCC.

Apart from invasive phenotype, the malignant transformation of the altered field may also result from change in cellular proliferative activity. Nuclear protein Ki-67 is a reliable cell proliferation marker as its expression correlates with tumour proliferation and invasion. It is expressed in proliferating cells from the G1 to the M phase of the cell cycle and not detected in resting cells. In OSCC, high Ki-67 expression was associated with poor tumour differentiation, worst pattern of invasion type, lymph node metastasis, tumour size and poor overall survival and disease free survival (Jing et al., 2018; Lopes et al., 2017). Since not all gene mutations would result in alteration in protein expression, IHC studies are necessary to assess the protein expression levels of these particular genes in SMs to prove their role in the development of local relapse.

The molecular profile of SMs is of equal importance as the histologic findings and carries a significant clinical implication. This is because pre-cancerous field may

remain in patients after surgical resection of the primary tumour, and may extend for a greater width than the accepted 5mm clear margin. This altered field is a forerunner of malignant change and identifying this field early at primary treatment stage may spare patients from mortality and morbidity associated with local relapse. The knowledge of genomic alteration in OSCC should be translated through exploration of molecular markers, in practical setting to identify the reliable predictors for local recurrence.

1.2 Aim

This study aimed to determine the association between expression of Ki-67, FSCN1 and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC patients and relapse.

1.3 Objectives

1. To evaluate the association of clinicopathological prognosticators of OSCC and local relapse in these patients.
2. To evaluate the expression of Ki-67, FSCN1, and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC.
3. To evaluate the association between clinicopathological prognosticators of OSCC and the expression Ki-67, FSCN1 and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC.
4. To evaluate the association between expression of Ki-67, FSCN1, and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC and local relapse in these patients.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of oral cancer

Oral cancer is a malignancy that arises from areas that extend from the skin-vermilion junctions of the lips to the junction of the hard and soft palates above and to the line of circumvallate papillae below (PDQ Adult Treatment Editorial Board, 2019). The anatomic subsites of the oral cavity include the labial mucosa, buccal mucosa, floor of mouth, alveolar ridge and gingiva, anterior two-thirds of the tongue (anterior to the circumvallate papillae), hard palate, and retromolar trigone. More than 90% of cancers in the oral cavity are Oral Squamous Cell Carcinomas (OSCCs). OSCC is a carcinoma with squamous differentiation that arises from the mucosal epithelium (Sloan et al., 2017, p.109).

2.2 Epidemiology of Oral Cancer

According to the GLOBOCAN (2018) database produced by the International Agency for Research on Cancer (IARC), cancer of the lip and oral cavity (ICD-10: C00-C06) is the 16th most common cancer in the world with 354,864 new cases reported in 2018 from which 246,420 cases were males and 108,444 were females. This is an increase of more than 50,000 cases reported in GLOBOCAN 2012 and the high incidence of oral cancer is consistently found in Asian countries (64.2%) (Bray et al., 2018) In 2018, five countries with the highest age standardized incidence rate (ASR) per 100 000 population of lip and oral cavity cancers were Papua New Guinea (20.4), Pakistan (12.2), Bangladesh (9.5), India (9.1) and Sri Lanka (7.6). The incidence of oral and lip cancer was higher among males (5.8 cases per 100 000) than females (2.5 cases per 100 000) with a global

mortality rate of 2.0 per 100 000 population per year. Lip and oral cavity cancer is ranked 15th in South eastern Asian region with 16, 818 new cases in 2018 and incidence and mortality rates (per 100 000 population per year) of 1.7 and 1.4 respectively (The Global Cancer Observatory [GCO], 2019a). In Malaysia, cancers of the lip and oral cavity ranked 19th with 667 new cases (2.2 cases per 100 000) and 327 deaths (1.1 per 100 000) (GCO, 2019b).

The most common sites for oral cancer are the tongue, floor of the mouth and gingiva, accounting more than half of all oral cancers (Sloan et al., 2017, p.109). However, buccal mucosa is the most common affected site among Asian population due to the prevailing risk factors such as tobacco and betel quid chewing.

2.3 Aetiology of Oral Cancer

2.3.1 Smoking tobacco

Smoking tobacco is defined as habitual use of tobacco in the form of smoke inhalation of cigarettes, cigars, bidi, and reverse smoking habit (Tilakaratne et al., 2019). Globally, tobacco use causes more than 7 million deaths per year (World Health Organization, 2020) and the use of tobacco in both smoke and smokeless form has been identified as a risk factor for development of oral cancer (Cheong et al., 2017). Cigarette smoke consists of more than 60 carcinogens specifically 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) that are accountable for mutations in oncogenes and tumour suppressor genes that initiate tumorigenesis (Hecht 1999; Xue et al., 2014). A meta-analysis conducted by Sadri and Mahjub (2007) involving 15 case-control studies showed that the odds of developing oral cancer are about five times higher among those who smoke compared with those who do not. A similar association was observed in United States

2.3.2 Alcohol consumption

Epidemiological studies adopting different designs and involving various populations have consistently reported that alcohol consumption is strongly associated with an increase in risk of oral and pharyngeal cancer. Women and men who drinks more than 8 and 15 drinks per week respectively have 5-fold higher risk of oral cavity and pharynx cancer (National Cancer Institute [NCI], 2018). Goldstein et al, (2010) in their extensive review on alcohol consumption and oral cancer concluded that simultaneous exposure to alcohol and tobacco demonstrate a synergistic effect and duration of alcohol consumption is not related to the risk of oral and pharyngeal cancer. The authors also found that those who stopped alcohol drinking habit had reduced risk of developing oral cancer compared to current drinker. Alcohol induces carcinogenesis through several mechanisms. Alcohol is oxidized to acetaldehyde, a group 1 carcinogen by alcohol dehydrogenase (ADH). Acetaldehyde is then metabolized to acetate by aldehyde dehydrogenase (ALDH). Acetaldehyde causes DNA damage, gene mutation and interferes with DNA synthesis and repair. Additionally, genetic polymorphism resulting in defect of ADH and ALDH enzymes could lead to increased ADH activity or impaired ALDH function that in turn cause acetaldehyde accumulation and increase the risk for alcohol-related cancers. (Kumar, M et al., 2016)

2.3.3 Betel quid chewing

There are 600 million betel quid chewers worldwide, mainly in South East and South Asia, the Indo-Pakistan subcontinent, mainland China, Taiwan, and on the South Pacific region (such as Papua New Guinea) (Gupta and Warnakulasuriya, 2002). Betel quid chewing is a habit that is practiced using a mixture of substances such as areca nut and slaked lime wrapped in betel leaf. This habit is related to the occurrence and development of oral/pharyngeal cancers as well as oral potentially malignant disorders

such as leukoplakia and oral submucosa fibrosis (Kumar et al., 2016). GLOBOCAN 2018 data demonstrates a regional pattern for lip and oral cavity cancer. Populations with high betel quid chewing have a higher incidence rate of lip and oral cancers than other countries. In vitro studies on oral mucosal fibroblasts showed that some of the betel quid ingredients are genotoxic, cytotoxic, and also stimulate cell proliferation (Chen et al., 2017). Investigators also showed that various kind of DNA damage are induced by reactive oxygen species (ROS), methylating agents, and reactive metabolic intermediates from betel quid.

2.3.4 Viral infection

Latent and chronic viral infections could induce neoplastic change through interference to the host's cell cycle machinery by the viral genes and gene products. The viruses associated with oral cancer are Human Papillomavirus (HPV) and Human Herpes Virus, mainly Epstein–Barr virus (EBV) and Herpes Simplex Virus (HSV). A meta-analysis by Warnakulasuriya (2003) revealed that the incidence of HPV-positive OSCC varies in the literature (0-100%). The variability can possibly be due to several factors such as demographic differences; sample types (biopsy, smear); different preparation methods (fixed or frozen specimens); HPV detection methods with different levels of sensitivity (in situ hybridization vs polymerase chain reaction) and especially the erroneous classification of tongue lesions in respect to their oropharyngeal location (Vargas-Ferreira et al., 2012). Sexual transmission of HPV is a main aetiological factor for OSCC in young male adults. Malignant transformation of the keratinocytes is induced by oncogenic proteins encoded by E6 and E7 genes from high-risk HPV genotypes (HPV-16 and -18), that are able to disrupt cell cycle regulatory proteins such and p53 and pRB by stimulating their degradation (Markopoulos, 2012). EBV has also been implicated with OSCC in few studies. A case-control study by Acharya et al. (2015) showed that

Epstein-Barr virus prevalence is correlated with OSCC and seems to be enhanced by betel quid chewing. A meta-analysis by Lima et al. (2019) supports the hypothesis of EBV association with OSCC and suggested that EBV latent transcripts (latent membrane protein 1, EBV nuclear antigen 1 and 2, and EBV-encoded small RNAs) have an important role in the tumorigenesis. A meta-analysis by She et al. (2017) identified an association between EBV infection and increased risk of OSCC. Additionally, the herpes simplex viruses (HSVs), types 1 and 2, have also been investigated in the past for possible associations with human cancers. High levels of anti-HSV1 (IgA and IgM) have been detected in patients with oral cancer than in control subjects. It has been reported that combination of cigarette smoking and HSV seropositivity is associated with a higher risk of oral cancer (Gupta & Metgud, 2013). The molecular and histopathological characteristics of these viral-related tumours have yet to be clearly defined to improve diagnosis and treatment. HPV and herpes viruses have been extensively studied thus far and are now considered to be the most likely "synergistic viruses" in OSCC.

2.3.5 Diet and nutrition

The working group of International Agency for Research on Cancer (IARC) has affirmed that diet does influence the development of certain cancers. Certain foods and food groups such as red and processed meat as well as saturated fat have been associated with increased risk for stomach, colorectal and breast cancer. Diet comprising high vegetables, fruits, fibers and cereals on the other hand were reported to have protective effects. The association between diet and oral cancer is nonetheless scarce and inconclusive. A study on Brazilian population by Willya et al. (2017) found that OSCC is significantly associated with diet rich in red and processed meat, oil and fat food, dairy products and alcohol while cereals and tubers reduced the risk of developing OSCC. A study on Italian population showed that a daily meal consisting at least six Mediterranean-

type dietary items (plant foods, fruits, cereals, olive oil, wine and low intake of meat and dairy products) favourably affects the risk of cancers of the upper aerodigestive tract including OSCC when compared with those whose daily intake has less of these items (Bosetti et al., 2003). However, the impact of individual food components and micro-nutrients on carcinogenesis remains unclear at present. Although remission or regression of oral leukoplakia using β-carotene with/without vitamin A has been shown in many studies, its impact on OSCC is not proven (Kumar et al., 2016). Since the dietary effect on OSCC can only be depicted through retrospective survey or descriptive studies, data reported should be interpreted with caution.

2.3.6 Poor oral health

Poor oral health (POH) is one of the extrinsic factors associated with development of OSCC. Causative factors for POH include irregular teeth brushing habits, less number of dental visits, poor socioeconomic status, lower level of education, tobacco, and alcohol consumption. While most studies have reported a strong correlation between POH and OSCC, some researchers agreed that POH is rather an additive factor in causation of oral cancer. Oji and Chukwuneke (2012) proposed that POH may be the only causative factor of OSCC according to their case-control questionnaire-based study involving 60 OSCC patients who had no smoking tobacco and alcohol habits. The study however did not include histopathological parameters and the cases selected were biased towards those with 'chewing stick' habit rather than the standard toothbrushing regime. A meta-analysis by Zeng et al. (2015) revealed that tooth brushing using proper tools i.e toothbrush at least twice daily has been reported to reduce the risk of head and neck cancer to half. Nonetheless, there was no level 1 evidence but only 18 case-control studies included in the analysis. POH may indirectly promote carcinogenesis by enhancing the effect of other known carcinogens instead. For example, the accumulation of plaque and

certain microbial colonies in the oral cavity following poor oral hygiene habit may cause retention or interfere with metabolism of carcinogenic substances found in tobacco (nitric oxide), alcohol (aldehyde) and areca nut (Mathur et al., 2019).

2.3.7 Chronic irritation/inflammation

Chronic mucosal irritation is characterized by low-intensity, persistence and repeated irritation caused by teeth, dentures, and functional alterations such as swallowing. It has been proposed to be an etiological factor for oral cancer but available evidence is limited and controversial. Publications exploring the association between chronic mucosal irritation and OSCC mainly comprised of retrospective case–control and descriptive studies and case series. Singhvi et al (2017) in their review did not find any level 1 evidence to support the notion. The review showed that ill-fitting denture is considered a risk factor for development of OSCC and in such cases, OSCC often occur over the lateral border of tongue. Similarly, a meta-analysis by Manoharan et al. (2014) found that ill-fitting dentures significantly increase the risk of OSCC but there was no correlation between duration of denture use and OSCC development. Sun et al. (2016) studied key inflammatory mediators namely IFN-γ IL-10 and tumour associated macrophages, involved in the progression of oral lichenoid keratosis and oral lichen planus to OSCC and found that immunosuppression could be induced by chronic inflammation and consequently fosters tumorigenesis of OSCC rather than initiating it. The biological pathways of chronic irritation in the development of OSCC are nevertheless not completely understood. There are many confounding factors that need to be addressed, further studies adopting standardized methodology are essential to prove the relationship.

2.4 Relapse in Oral Cancer

2.4.1 Field cancerization

The concept of field cancerization was proposed by Slaughter et al. in 1953 following their observation of multifocal OSCC in 783 patients. The authors hypothesized that OSCC developed from multifocal patches of abnormal tissue bordering the tumour and the persistence of this abnormal tissue after surgery is accountable for local recurrence and development of a new distinct malignancy. Recent studies have addressed the genomic and molecular basis of this concept and it is now established that field cancerization refers to clonal expansion of cells which share a collection of early genetic alterations, that could progress to malignancy following multiple intricate molecular events (Braakhuis et al., 2002). This concept is used to stratify the recurring malignancies as a true recurrence, a second field or second primary tumour depending on the genetic aberration harboured by the second tumour; whether they are clonally related (LR or SFT) or independent of the index tumour (SPT). Early genetic change is shared by cells in a local anatomical area in response to carcinogen exposure. As the cells develop into an expanding clone, the lesion laterally displaces the normal epithelium creating a field of pre-cancerized epithelium (Feller et al., 2013). The subsequent genetic events in various part of the altered field may differ from one another resulting in formation of subclones that harbour different phenotypic alterations (Califano et al., 1996). Malignancy eventually arises from a subclone that has acquired a particular selective growth advantage.

Microscopically, the field lesion may appear normal or display an aberrant morphology characteristic of dysplasia. The study by Tabor et al. (2003) discovered genetically altered cells not only in all of the moderate and severely dysplastic lesions but also within normal epithelium (no dysplasia) and in two-thirds of the epithelium reported

as mild dysplasia. The genetically altered cells also have higher proliferation activity demonstrated by Ki-67 immunohistochemistry (IHC) staining. Recent studies revealed that the field lesions bear a wide range of genetic aberrations including deletion of key chromosomal regions at 3p, 4q, 8p, 9p, 13q and 18q, amplification of cyclin genes, mutations and LOH affecting P53 gene (Article et al., 2018). Identification of the genetic alterations related to malignant transformation is essential to understand the biological initiation, behaviour, and progression of OSCC. Knowledge on the genetic status of a primary tumour or the tissues surrounding the tumour may have prognostic significance as it could be used to detect residual clonal populations and predict LR.

2.4.2 Local recurrence

Local recurrence (LR) is described as cancer that develops within less than 2 cm away from the index tumour, within a 3-year time period (Braakhuis et al., 2002). Mucke et al. (2009) on the other hand characterized LR as return of OSCC near the primary site in the absence of cervical metastasis, having similar or higher histologic grade than the initially diagnosed tumour and developed at least six months after completion of treatment of the primary tumour. Braakhuis et al. (2002) recommended a new definition for LR based on the genetic profile of the primary and secondary tumours. In this classification, LR is reserved for second tumours that arise in the same or adjacent site of the primary tumour and harbours similar genetic alterations. LR may arise from the remaining histologically undetected cancer cells at the margin, known as minimal residual cancer (MRC) (Tabor et al., 2004) or from monoclonal cell populations disseminated throughout the field of pre-cancerized epithelium adjacent to the tumour (Bedi et al., 1996). Additional genetic hits may lead to clonal divergence in which the genes that dysregulate later in the carcinogenesis process differ from the primary tumour. Thus, Braakhuis et al. (2003) favoured the term “second field tumour” (SFT) to be used

in cases where a second tumour arises from the same field in which a first tumour has developed. Taken together, an LR can be designated as SFT and can only be proven otherwise by molecular studies.

However, unless molecular analysis is carried out, the distinction between locally recurring tumour and second primary tumour is often based on subjective clinical decision making. Rohde et al. (2020) carried out a systematic review to search for a standardized definition of LRs in HNSCC and found no uniform definition for locally recurrent HNSCC currently exists. The authors proposed a reproducible criteria to define LR in oropharyngeal carcinomas that includes the most used criteria for LRs and based on standard clinical examination. They suggested that LRs are tumours that arise within 3 cm of the primary lesion, no more than 3 years from the completion of treatment of the primary lesion, and have same p16-status. Although suboptimal, a standardized clinical criteria is crucial for further molecular and epidemiologic studies that compare the prognostic features, treatment effects, incidence rates, risk factors, detection methods and genomic characteristics between LRs and SPT.

In study by Yanamoto et al. (2012), patients with LR were defined as those who developed a second tumour adjacent to the primary tumour after 6 weeks and within 5 years after the initial definitive treatment, had significantly lower overall survival rate compared to patients without LR (33.3% and 94.3% respectively). The prognosis of patients with recurrence is also inferior than those with SPT (Spencer et al., 2001; Spencer et al., 2008). Significant prognostic factors for overall survival of patients with recurrent OSCC include age, clinical stage at first diagnosis, and recurrence-free interval (Chang et al., 2017). Previous retrospective studies have identified several clinico-pathologic factors predictive for development of LRs in OSCC. These include postoperative radiotherapy, bone involvement, surgical margin status, extracapsular spread, lymph node ratio, age, coexisting diseases, tumour staging, histological grading and pattern of

invasion (Naval-gi, 2009; Safi et al., 2018; Shaw et al., 2004; Vázquez-Mahía et al., 2012, Yanmoto et al., 2012).

2.4.3 Second primary tumour

The occurrence of more than one squamous cell carcinomas in different sites of the head and neck is not unusual. Second primary tumours (SPT) are those that develop independently from the initially diagnosed head and neck squamous cell carcinoma (HNSCC). It is identified based on the criteria described by Warren and Gates (1932) that are: (a) Each of the tumours must present a definite picture of malignancy, (b) each must be distinct, and (c) the probability of one being a metastasis of the other must be excluded. Most researchers use an arbitrary distance of 2cm and/or a minimum of 3 years from the diagnosis of first tumour to distinguish SPT from local recurrence (Braakhuis et al., 2005). SPT in OSCC patients commonly arise in the oral cavity and oropharynx but non-head and neck region such as lung and oesophagus may also be affected (Hsu et al., 2008; Rogers et al., 2019; Sassi et al., 2010). Few studies revealed a gap of more than 5 years between the diagnosis of first OSCC and development of SPT, implying the need of a longer follow-up with vigilant inspection for new lesions (Hsu et al., 2008; Rogers et al., 2019; Sassi et al., 2010). The 5 years disease specific survival rates for patients with SPT is lower compared to those who are disease free (Naval-gi, 2009; Xi et al., 2013). Hsu et al., (2008) also found that in patients who had tongue and larynx SCC, the overall survival rate was lower if the SPT developed in head and neck region compared to SPT that occurred in other areas such as lung and oesophagus. Data on the risk factors for SPTs in OSCC patients varies between studies. While Ko et al., (2016) reported a significant association between areca quid chewing, tongue tumours, and nodal metastasis with the development SPTs, Naval-gi (2009) was unable to associate SPTs with any clinico-pathologic factors. Rogers et al., 2019 reported early stage of primary cancer as the only

significant factor for development of a second primary within 10 years, reflecting the superior long-term survival amongst low risk OSCC patients as a risk factor for SPTs.

The criteria by Warren and Gates (1932) however did not describe any objective term as what could make the second tumour distinct from the first one. Thus, a new classification based on molecular genetics has been proposed. Second tumours that arise from precursor cells that are genetically different from the primary tumour are true SPTs (Braakhuis et al., 2002). SPTs genetically distinct from primary tumours are believed to develop from an autonomous event. Discrimination of these lesions through molecular analysis may improve cancer treatment as the distinct genetic mutations of SPT might enable use of molecular targeted therapy that was rendered ineffective during the occurrence of the first tumour and prevent radical treatment.

2.4.4 Second field tumour

Recent findings on molecular genetics allow for a more refined classification of the tumours that can arise once an HNSCC has been surgically removed. A cancer that develops from a field that is genetically related to the primary tumour, is termed as "Second Field Tumour", SFT. SFT develops from genetically altered fields that remain in patients after surgery while SPTs harbour a completely different genetic profile from the primary tumour (Braakhuis et al., 2005). Tabor et al. (2001) analysed the tumour-associated genetic alterations in non-continuous mucosa adjacent to the tumour (HNSCC) as well as in the surgical margins. The authors discovered that field of aberrant cells found in the normal mucosa can also be detected in the surgical margins in a quarter of the cases studied, and 1 of the cases developed recurrence. This led to the understanding that HNSCC could arise from a contiguous genetically altered field (monoclonal origin). However, clonal divergence can occur with subsequent genetic mutations, causing the second tumour to acquire additional tumour-related markers (Braakhuis et al., 2002).

Thus, a patient can develop a true local recurrence, an SFT, or SPT based on the genetic correlation between the first and subsequent tumours. A new tumour that develops in the anatomic region near the initial carcinoma and displays a similar genetic profile is regarded as SFT. SFT reflects the intricate molecular events of field cancerization in which the impact of carcinogen exposure varies along the length resulting in independent transformation of the clonal field and multiple malignancies may arise once the invasive potential is gained.

2.5 Clinicopathological factors for local recurrence

2.5.1 Primary tumour site

Many researchers considered anatomical location of OSCC as one of the predictors for LR in their studies. Jerjes et al. (2010) and Gontarz et al., (2013) observed a strong correlation between recurrence and primary tumours of the tongue and floor of mouth (FOM). The disease specific survival for patients with loco-regional recurrence was poorer when the primary tumour was on the tongue and FOM (Jerjes et al., 2010). The association between tumour site and poor local regional control is mainly explained by the lymphatic drainage of these locations and inability of complete surgical removal. Tumour site is also correlated with unfavourable histopathological features that consequently affect the local regional control. Rai and Ahmed (2016) revealed that OSCC on the palate, tongue and floor of the mouth tend to be poorly differentiated at diagnosis compared to tumours on the buccal mucosa that were often well differentiated at presentation. Intra-oral tumour site is also associated with advanced clinical stage (III and IV) compared to lip SCC (Santos et al., 2016). On the contrary, there were studies that found no statically significant correlation between tumour site and recurrence (Rosenquist, 2007; Buchakjian, 2018).

2.5.2 Tumour stage

Despite the emerging significance of a molecular phenotype in prognostication of OSCC, anatomic extend described by size of the primary tumour (T), nodal involvement (N), and distant metastasis (M) has long and is still be considered as an important predictor for survival and loco-regional relapse in OSCC. In a large retrospective study involving 500 OSCC patients González-García et al. (2009) found that large tumours (pathological T3-T4) and advanced pathological stage (stage III and IV) were more likely to develop local recurrence. T staging also showed to be significantly associated with local relapse of OSCC occurring within 1 to 2 years after resection, with histological evidence that the tumour arises from cancer cells remaining deep within resected site (Toratani et al., 2019). Nevertheless, Ghantous (2018) in their analysis of 159 OSCC cases showed that only pT staging and not pN status or the overall pathological staging was significantly correlated with local recurrence. The 8th edition of TNM classification system by American Joint Committee of Cancer (AJCC, 2018) has incorporated depth of invasion (DOI) into the T category of OSCC staging, thus reframing the staging system making it a better predictor for overall survival and risk of local recurrence. DOI is the distance from the deepest level of invasion to the reconstructed mucosal surface and accordingly the previously defined T1 tumour is upstaged to T2 if DOI is more than 5mm and a cut-off value of 10mm for upstaging T2 to T3 (AJCC, 2018). Following the update, many studies have showed significant association of the newly defined pT with local recurrence (Faisal et al., 2018).

2.5.3 Nodal involvement (pN) and extracapsular spread

Cervical lymph node metastasis (pN status) is a major factor in treatment planning for patients as it significantly affects the disease specific survival outcome. Many publications have demonstrated the significant impact of number and size of positive

nodes, occult node metastasis as well as extracapsular spread on the survival rate (Majumdar et al., 2017). Studies on the association of nodal status and LRs however provide conflicting evidence. Buchakjian et al. (2018) and Jerjes et al. (2010) reported nodal involvement as an independent predictor for LR. The latter also demonstrated nearly half (44.2%) of the LR cases were in pN2 stage. Ghantous (2018) on the contrary found no correlation between LR and pN stage. The latest edition (8^{th}) of American Joint Committee on Cancer TNM staging (AJCC, 2018) has included extracapsular extension in the pN category with an aim to better prognosticate patients. Extracapsular spread (ECS) is related to loco-regional recurrence, distant metastasis and overall survival (Jerjes et al., 2010). Subramaniam, et al. (2019) restaged 643 OSCC cases following the eight edition of AJCC TNM staging system (AJCC, 2018) and found an increased hazard ratio for recurrence as the pN staging increases. The study also showed an inverse relationship between disease free survival and pN staging (pN0 =74%, pN1,=53%, pN2a=50%, pN2b=47%, pN2c=24% and pN3b=0%). Likewise, meta-analysis by Mermod et al. (2016) revealed that ECS not only has a negative impact on LR but is also associated with distant metastasis in HNSCC patients. In addition, several investigators suggested lymph node ratio (LNR) to be considered as another nodal staging system. LNR is the ratio of positive lymph nodes to the total number of lymph nodes removed. LNR has been shown to predict locoregional recurrence better than the conventional nodal staging system (Safi et al., 2018; Subramaniam et al., 2019). This is supported by systematic review and meta-analysis by Huang et al., (2019) that revealed LNR as an independent prognostic factor for LR free survival. On the contrary, de Ridder et al., (2016) showed that the number of tumour-positive lymph nodes but not LNR appears to be a more reliable factor than LNR, provided a minimum number of lymph nodes were assessed. Number of isolated lymph nodes was also reported to have significant influence in prognosis of cN0 OSCC patients who had elective neck dissection (END). Ebrahimi et al. (2014) revealed nodal yield less

than 18 in END increased the risk for locoregional recurrence (HR 1.53; 95 % CI 1.04-2.26; p = 0.032).

2.5.4 Tumour grade/histopathological grade

Histopathologic grading of OSCC is based on the assessment of cancer cell differentiation reflected by the degree of resemblance of the tumour to the normal epithelium and the extent of keratin formation. WHO scoring system comprising of degree of keratinization, cellular and nuclear pleomorphism and frequency of mitosis is used to grade the tumour into four categories: Grade I (well differentiated), Grade II (moderately differentiated), Grade III (poorly differentiated) and Grade IV (undifferentiated). Poorly differentiated carcinomas are often associated with aggressive biological behaviour and henceforth lead to inferior disease outcome. Adverse clinicopathological characteristics such as lympho-vascular invasion, perineural invasion, tumour stage, tumour depth, lymph node status and metastasis are significantly associated with poorly differentiated OSCC of buccal mucosa (Padma et al., 2017). The study also reported tumour grade as an independent risk factor for LR. Similarly, Buchakjianin et al. (2018) found that apart from surgical margin status, tumour grade is an independent predictor of LR. Safi et al. (2018) demonstrated increased risk of local recurrence as the tumour grade increased. Patients with poorly differentiated tumour had 2 fold higher risk of developing recurrence than well and moderate grade tumours. In terms of individual morphological parameters in tumour grading, moderate to high degree of keratinization is significantly associated with LR in early stage (T1-T2) tongue SCC (Migueláñez-Medrán et al., 2019). However, there are studies that failed to prove any significant correlation between histopathological grade and LR (Nagal-gi, 2009; Yanamoto et al., 2012).

2.5.5 Worst pattern of invasion

Worst pattern of invasion (WPOI) is examined at the tumour/host interface and often linked with tumour biologic behaviour. Bryne et al. (1998) classified WPOI into 4 types to denote the manner in which the tumour invades the underlying connective tissue. WPOI type 1 represent tumours that have broad pushing border. Tumours having finger-like pushing fronts is defined as WPOI Type 2. WPOI type 3 and type 4 are tumours with large invasive islands of more than 15 cells and small invasive tumour islands consisting less than 15 cells respectively. WPOI type 5 is described by Brandwein-Gensler et al. (2005) as presence of discrete tumour satellites separated by at least 1mm of normal intervening stroma from the main tumour front and regarded by the authors to be accountable for the development of LR. Brandwein-Gensler et al. (2010) in the subsequent study showed that WPOI is a significant and independent predictor for LR and regarded it as one of the high risk histopathological features. The findings were further validated by Li et al. (2013) in which WPOI alone was significantly predictive for loco-regional recurrence and the probability of developing loco-regional recurrence was 42% with WPOI type 5. Sinha et al. (2018) also showed that higher WPOI (type 4 and 5 grouped together) is significantly associated with loco-regional recurrence. Similarly, Almangush et al. (2015) reported WPOI as a significant pathological predictor for loco-regional recurrence in cT1N0-cT2N0 OSCC. However, other studies have reported conflicting results. Yanamoto et al. (2012) reported WPOI type 4 as a significant predictive factor for LR by univariate analysis but not an independent factor in multivariate analysis. Spiro et al. (1999) on the other hand revealed that WPOI has a significant impact on survival but not local recurrence.

2.5.6 Perineural invasion

Perineural invasion (PNI) was initially described by Batsakis in 1985 as tumour cell invasion in, around and through the nerves (Batsakis, 1985). It is now accepted that PNI is the finding of tumour cells in close proximity of at least one third of the nerve circumference (Fagan et al., 1998) or within any of the three layers (epineurium, perineurium, endoneurium) of the nerve sheath (Liebig et al., 2009). PNI is recognised as a negative disease prognosticator and is associated with other adverse histopathologic features. It is one of the histopathologic parameters in Brandwein–Gensler's (2010) histologic risk model for OSCC; the other parameters are WPOI and lymphocytic host response. Nonetheless, reports on its value as predictor for LR are equivocal. In a study involving 292 OSCC patients, PNI (of nerves >1 mm in diameter) was significantly associated with increased local recurrence and reported to be an independent risk factor for LR (Brandwein-Gensler et al., 2005). Fagan et al., (1998) reported that PNI increased risk of LR, decreased disease-specific survival and was an independent predictor of ECS in 142 HNSCC patients treated with resection with or without adjuvant therapy. Other studies demonstrated correlation between PNI and other prognosticators such as disease free survival, lymph node metastasis and ECS (Miller et al., 2012; Varsha et al., 2015). Faisal et al. (2018) studied the role of DOI in LR and identified PNI as another independent risk factor for local recurrence in their cohort. On the contrary, Safi et al., (2018) reported no significant association between locoregional recurrence and perineural invasion. In another study, PNI was found to be a significant factor for loco-regional recurrence only in univariate analysis (Gokavarapu et al., 2017). Despite the conflicting data, PNI is regarded as an adverse feature and is considered as an indication for adjuvant radiotherapy.

2.5.7 Lymphocytic host response

Enhanced immune surveillance and adaptive immunity have protective effects on cancer patients (Yu & Fu, 2006). The prognostic value of cancer inflammation was revealed by Brandwein-Gensler et al. in 2005 and later on in 2010 whereby limited lymphocytic host response (LHR) in the tumour stroma was significantly correlated with LR. The authors developed a risk model and classified LHR as strong, intermediate and limited. Strong LHR shows continuous dense rim of lymphoid infiltrate, intermediate LHR displays patches of dense lymphoid infiltrate and little or no host response denote the limited LHR. A study on a series of 94 tongue SCC showed that strong LHR significantly correlated with complete response to radio therapy and fewer recurrences (37%) (Lundqvist et al., 2012). Using the same risk model on 64 early stage (stage I and II) tongue SCC, Sinha et al. (2016) conversely found no significant correlation between LHR and LR. LHR was also not a predictive factor for LR in a study of 479 early (T1N0-T2N0) tongue SCC cases as reported by Almangush et al. (2015). Recent studies suggest that the type, not the quantity, of immune cells may be a more critical determinant for the prognosis. Kindt et al. (2016) showed that a high quantity of Langerhans cells in both intra-tumoural and stromal tissue of HNSCC cases is associated with longer recurrence-free survival. Other than density of LHR, tumour-infiltrating lymphocytes, CD8/CD4 ratio, presence of T- regulatory cells and tumour- associated macrophages were also reported to have prognostic implications (Majumdar et al., 2017).

2.5.8 Bone invasion

Bone invasion in OSCC reflects tumour aggressiveness and denote an advanced disease. It remained as one of the criteria by which T4 tumours are defined since the 1st Manual for Staging of Cancer (1977). It is noteworthy that superficial erosion of the bone/tooth socket is insufficient to classify gingival primaries as T4. Bone invasion by

the malignant epithelial cells may progress by either an infiltrative or an erosive histological pattern. The erosive pattern exhibits a broad, pushing tumour front while the infiltrative pattern shows nests and cords of tumour cells along an irregular tumour front. Wong et al., (2000) revealed that the infiltrative pattern presents a more aggressive behaviour compared to erosive pattern, with an increased risk of LR, regional recurrence, distant metastasis, and shorter disease-free survival. Shaw et al (2004) similarly reported infiltrative pattern as predictor for LR. Nonetheless, there are studies that identified bone invasion as an independent factor for LR regardless of the invasion pattern (Naval-gi, 2009; Shaw et al., 2004). However, studies examining the prognostic impact of bone invasion in OSCC have shown conflicting results. Buchakjian et al. (2018) in their study on 426 OSCC patients did not find any correlation between bone invasion and LR. Likewise, out of 115 early OSCC patients (T1-T2) reviewed by Jerjes et al. (2010), only 5 had bone invasion and out of that only 1 had locoregional recurrence. The inconsistence outcomes may be attributed by the extent of bone invasion: cortical or medullary invasion. Ebrahimi et al. (2014) observed a weak association between medullary invasion and LR (p= o.o6) but unlike cortical invasion, medullary invasion has a significant impact on overall survival. A systematic review by Li et al. (2017) demonstrated mandibular medullary invasion but not cortical invasion as an independent prognostic factor. Nonetheless, bone invasion has been correlated with other adverse histopathologic features such as tumour size and PNI. Bone invasion alone may not be a strong predictor for LR but its presence is notably an indicator for intervention and closer surveillance.

2.5.9 Adjuvant therapy

Surgery is the mainstay of treatment for resectable OSCC. However, post-operative radiotherapy therapy (PORT) [external beam radiotherapy (EBRT) and/or brachytherapy (BT)] with or without adjuvant systemic therapy (chemotherapy and/or

target agents) is carried out depending on the disease presentation and pathological findings, aiming to improve loco-regional control. Generally, PORT is indicated for T3 or T4 tumours; close and involved surgical margins, presence of lympho-vascular invasion (LVI) and/or perineural invasion (PNI), and positive lymph nodes with or without extracapsular extension (Huang et al., 2013). Nisi et al. (1998) revealed that while LR was not significantly affected by adjuvant RT, the rates for local control after 5 years was higher in adjuvant RT group (92%) than the group with surgery alone (74%). Higher incidence of tumour recurrence and inferior disease-free survival were seen in patients with locally advanced head and neck cancer (HNC) treated with PORT alone compared to patients who received postoperative concurrent chemotherapy and radiotherapy (CCRT) (Bernier et al., 2004; Cooper et al., 2004). Similarly, a study on the effect of PORT with concurrent chemotherapy on 68 OSCC patients found that CCRT group had a significantly higher 5-year recurrence free survival rate than the PORT group (75.4% vs. 42.6%) (Fan et al., 2017). Shrime et al., (2010) revealed that the impact of PORT on pT1N1 OSCC is in fact insignificant compared to that in patients with pT2N1, especially in patients with tongue and the floor of the mouth SCC. However, PORT or CCRT used to enhance disease outcome is associated with radiation-induced toxicity that include osteoradionecrosis, dysphagia and xerostomia. Cheng et al. (2018) reported prolonged PORT time and extreme radiation dose >70Gy or $\leq$ 50 Gy had an adverse impact on locoregional free survival. Cooper et al. (2012) also reported no added long term advantage on patients treated with CCRT compared to PORT alone in terms of local regional control (28.8% vs. 22.3%, P = 0.1) except in a subgroup of patients with ECS and involved surgical margins.

2.5.10 Surgical margin status

The surgical margins or resection margins are boundaries of the surgical specimen excised by the surgeon. According to approximation of tumour cells, mucosal and deep surgical margins (deep margin includes submucosa, skeletal muscle, and bone) are classified into clear, close, and involved: a margin of greater than 5 mm is clear, a margin of 1 to 5 mm is close, and a margin less than 1 mm is involved (Durham, 2019). Achieving cancer clearance at the primary site is paramount as the presence of close and involved surgical margins carry a higher risk of LR and is associated with decreased disease-free (DFS) and overall survival (OS) (Dillon et al., 2015; Ghantous, 2018; Kurita et al., 2010; Naval-gı, 2009; Toratani et al., 2019; Yanamoto et al., 2012; Mitchel et al 2018). Recent studies on surgical margin status and disease outcome have roused debates as to how much distance from invasive tumour denotes a clear margin. A meta-analysis by Anderson et al. (2015) showed a pooled estimate absolute risk reduction of 21% for margins $\geq$ 5mm compared to margins that were less than 5mm. Surgical margin status also remained as a statistically significant predictor of LR-free survival (LRFS), DFS and OS after adjustment for other clinic-pathologic factors in a retrospective study involving 612 OSCC patients conducted by Jain et al. (2020). Additionally, the authors revealed that the conventional <1mm used to define positive margin was sufficient to discriminate patients outcome in term of LRFS and OS. Wong et al., (2012) on the contrary found no minimal distance of surgical margin that could predict LR but observed a significant association between surgical margin $\leq$ 1.6mm and disease specific survival. Interestingly, the investigators in both groups suggested the cut-off of 2 mm as determinant for post-operative adjuvant therapy. Stathopoulos and Smith (2017) also believed that the approach of using close resection margins as a predictor for local recurrence and adverse prognosis need to be reviewed as their study showed no significant difference in local recurrence between patients having close and clear surgical margins.

There are many factors that confound the actual correlation. These include molecular and histopathologic status of the surgical margin and clinicopathological characteristics of the tumour, such as anatomical location that complicate complete tumour removal (Thomas et al., 2019). Surgical margins are considered negative based on histologic assessment of the pathological specimen; however, this method lacks sensitivity in identifying histologically normal cells that harbour cancer-related gene mutations (de Carvalho et al., 2012). Neither clear margins nor normal mucosa can be considered as such until the genetic aberration is assessed. However the validation of molecular markers for routine examination of surgical margins of OSCC has yet to be established. To date, surgical margin of 5mm is considered optimal and changes in the current standards should be deferred until further investigations are done.

2.5.11 Epithelial dysplasia at surgical margins

Oral epithelial dysplasia is a spectrum of architectural and cytological epithelial changes caused by accumulation of genetic changes and is associated with an increased risk of progression to SCC (Reibel, 2017, p.112). The malignant transformation rate of dysplastic epithelium range from 1% to 25% (Reibel, 2017, p.112). It is a microscopic diagnosis with immense clinical importance as the risk of malignant transformation increases with higher grades of dysplasia (Warnakulasuriya et al., 2011). However, the implication of epithelial dysplasia in surgical margin remains controversial. Gokavarapu et al., (2017) studied 425 OSCC cases with uninvolved margins and found that dysplasia of any grade has no significant correlation with local recurrence. However, margins with severe epithelial dysplasia were excluded in the study to eliminate impact of re-resection with recurrence. In contrast, Kurita et al., (2010) found that severe epithelial dysplasia was a significant factor for local recurrence in OSCC cases with clear margin. DFS and LR for early (T1-2,N0) tongue SCC patients with moderate or severe dysplasia at the

margin was shown to be inferior than those with mild or no dysplasia at the margin (Sopka et al., 2013). Additionally, the authors revealed that moderate epithelial dysplasia was an independent prognosticator for LR. While most studies reported insignificant role of mild dysplasia, Pu et al (2016) observed a significant improvement in recurrence free survival in a group of patients having surgical margins with mild dysplasia that underwent re-excision as compared to the group without re-excision.

The histologic grading of oral epithelial dysplasia used at present however is subject to inter-and intra-examiner variability and carries no biologic significance. Smith et al. (2009) reviewed 13 publications on biomarkers in dysplasia and suggested that LOH, survivin, matrix metalloproteinase-9, and DNA ploidy may be associated with risk of progression. Out of that, DNA aneuploidy was consistently reported as an important predictive factor for cancer progression (Alaizari et al., 2018; Speight & Eng, 2018). DNA aneuploidy may be a more reliable tool to predict risk of malignant change in surgical margins than dysplasia grading.

2.6 Tumour markers

Tumour markers are substances that are produced either by the tumour itself or by the tumour microenvironment or host in response to the presence of malignancies. These markers could be proteins, genes or biochemical indicators that are altered quantitatively or qualitatively in precancerous or cancerous conditions. The clinical utility of tumour markers can be generally categorized into four groups: screening and early detection, as diagnostic aids, prognosis and prediction of therapeutic response, recurrence and disease surveillance (Sharma, 2009). For OSCC, histopathology remains the gold standard for diagnosis and therapeutic decision, but prediction of clinical outcome based on the conventional clinical and pathological parameters remain difficult, owing to the heterogenous genetic events involved (Vinício et al., 2010). A wide range of molecular

markers involved in cell cycle regulation, apoptosis, cell migration, cell adhesion and tumour microenvironment have been identified as prognostic biomarkers for OSCC. The established markers include cyclin dependent kinase, survivin, CD44, BUBR1, and heat shock proteins (Vinício et al., 2010).

Copy number alterations of genes related to pathogenesis and prognosis of OSCC are also considered as important biomarkers. Among that, overexpression of FSCN1 and TNFRSF12A genes have been associated with malignant transformation and invasion in OSCC. Conway et al. (2015) revealed that FSCN1 and TNFRSF12A were among the genes most informative in separating normal, dysplasia and tumour tissue. Zhang et al. (2018) also identified FSCN1 and TNFRSF12A as part of the top 5 genes that positively correlated with disease state (normal vs Dysplasia vs OSCC) which were altered in 44% of 510 OSCC patients in the study. Since genetic alterations have also been observed in peri-tumoural tissue, it would be worthy to investigate if the overexpression of FSCN1 and TNFRSF12A could be identified in histologically non-involved SMs. Given that second malignancy could develop from the genetically altered field, it is hypothesized that overexpression of these markers in histologically non-involved SMs might correlate with relapse in OSCC. Cellular proliferation is another fundamental mechanism in carcinogenesis (van Diest et al., 1998). Assessment of the proliferative activity is possible by IHC staining with Ki-67 antigen and regarded as means of predicting malignant transformation of dysplastic epithelium (Girod et al., 1998). Thus, proliferative activity reflected by high Ki-67 labelling index is also predicted to be increased in pre-cancerized field in the histologically non-involved SMs.

2.6.1 FSCN1

FSCN1 (Fascin Actin-Bundling Protein 1) is a protein coding gene that encodes for Fascin, a highly conserved 55-kDa actin bundling protein that plays an important role

in the assembly and stability of cell protrusions and other actin- based structures that aid in cell motility, migration and invasion (UniProt, n.d). Fascin is involved in the formation and stability of filopodia or microspikes used for cell migration or growth extension in skeletal myoblasts, fibroblasts and neurons (Hashimoto et al., 2011). In vascular smooth muscle cells, Fascin is part of the dynamic podosomes that facilitate cell migration in response to Platelet Derived Growth Factor (PDGF) receptor following vascular injury. The expression of Fascin is low or absent in normal adult epithelial cells but expressed in most carcinomas. Actin bundling by Fascin is upregulated by cell-surface adhesion receptors, growth factors, cytokines, and signalling proteins. Functional studies using cell lines and animal models discovered that Fascin is responsible for tumour growth, metastasis and cell motility and its depletion is related to reduction in filopodia, resulting in cell migration inhibition (Hashimoto Y et al 2011). Given that cellular migration and invasion are hallmarks for malignant transformation (Siriwardena et al., 2018), we hypothesized that FSCN1 would be overexpressed and could be investigated in the determination of field cancerization in peri-tumoural tissue.

Immunohistochemical (IHC) studies have shown that overexpression of Fascin is associated with carcinomas of aggressive and metastatic phenotype (Hashimoto et al., 2005; Hashimoto et al., 2006; Zhang et al., 2006; Zigeuner, 2006). A systematic review and meta-analysis by Tan et al. (2013) found that FSCN1 is significantly associated with increased risk of mortality in breast, colorectal and oesophageal carcinomas as well as lymph node metastasis in colorectal and gastric carcinomas. Expression of Fascin in OSCC is also implicated with adverse prognosis. Overexpression of FSCN1 in OSCC samples was strongly correlated with lymph node metastasis ($p=0.027$), tumour recurrence ($p<0.001$) and poor overall survival ($p=0.013$) (Lee et al., 2007). OSCC cells with FSCN1 overexpression result in significant increase in cell migration, cell invasion and MMP-2 activity (Alam et al., 2012). A study on a series of 129 oral and

oropharyngeal carcinomas revealed that intensity and distribution of Fascin immunostaining correlated with tumour size, lymph node metastasis, distant metastasis, clinical staging and histological grading (Chen et al., 2007). The authors however found no correlation between Fascin expression and tumour location. The role of Fascin in OSCC was demonstrated by Rodrigues et al. (2017) through IHC and real-time quantitative PCR analysis in ex vivo OSCC samples and cell lines. Knockdown of Fascin in OSCC cells promoted cell adhesion and hampered migration, invasion and epithelial-mesenchymal transformation (EMT). Authors also reported an inverse relationship between Fascin overexpression and disease-specific survival. Likewise, Chen et al. (2019) demonstrated reduction in cell viability and transmigration in vitro and impaired tumour growth in vivo following Fascin knockdown in tongue SCC. The study also observed a significant correlation between high Fascin expression and pN, clinical stage and relapse. Interestingly, the study also reported higher Fascin expression in tongue SCC than in corresponding adjacent non-tumour (ANT) tissue and normal tongue tissue. However, no comparison was made between the expression of Fascin in ANT and normal tissue. This is of interest as FSCN1 dysregulation may be more pronounced in the ANT than in healthy normal mucosa and its correlation with other clinico-pathologic parameters particularly local recurrence is yet to be explored.

2.6.2 TNFRSF12A

Tumour necrotic factor receptor super family 12A (TNFRSF12A) a type-I transmembrane protein with a single extracellular cysteine-rich region comprising six cysteine residues in its extracellular domain (Brown et al., 2006). It is a member of the TNF receptor family which binds to Tumour necrosis factor (TNF)-related weak inducer of apoptosis (TWEAK) with high affinity (Winkles et al., 2007). Thus TNFRSF12A is also known as TWEAKR, the specific receptor for TWEAK ligand. In the literature,

TWEAKR is also used interchangeably with fibroblast growth factor-inducible Fn14, a small transmembrane protein discovered one year earlier than TWEAKR by Feng et al., (2000). The nucleotide sequence of these two proteins is identical but due to the degree of amino acid sequence divergence from other TNF receptor family members, Fn14 was not classified as a member of the TNF receptor family (Wiley et al., 2001). In most publications, Fn14, TNFRSF12A and TWEAKR are used synonymously denoting the cognate receptor for TWEAK. TNFSF12A is expressed on many tissue types including mesenchymal, epithelial and endothelial cells but not on B and T lymphocytes regardless of their activation state. It is also expressed on tissue progenitor cells such as liver and neuronal progenitors, embryonic stem cells, and immature erythrocytes (Wiley et al., 2001).

The expression of TNFRSF12A is relatively low in normal tissue but is upregulated following tissue injury. Immunohistochemical studies have shown that TNFRSF12A is overexpressed in a wide variety of human tumour cell types including breast, pancreatic, glioma and oesophageal, relative to the normal tissue (Burkly et al., 2007). TWEAK/ TNFRSF12A signalling pathway induced pivotal pro-tumorigenic effects such as cell proliferation, migration and invasion, angiogenesis and apoptosis (Winkles et al., 2007). TWEAK/TNFRSF12A has been reported to induce the proliferation of hepatocellular carcinoma cells in dose dependent manner (Kawakita et al., 2004). However, there were studies that demonstrated a cytokine dependent TWEAK/TNFRSF12A induced tumour cell death (apoptosis) in certain cell lines. This included HT-29 (colon adenocarcinoma), HSC3 (OSCC) cells and Kym-1 cells and PC-3 prostate cancer cells (Hu et al., 2017). TWEAK/TNFRSF12A signalling pathway also promotes cell invasion through activation of specific molecules. For instance, invasion potential of glioma cells following TWEAK/TNFRSF12A binding is due to subsequent activation of RhoA, Rac1, and Cdc42, the key regulators of cell migration that is involved

in the induction of lamellipodia and filopodia protrusion as well as the formation of stress fibers (Hu et al., 2017). Collectively, this shows that TNFRSF12A-associated signalling complex differs between tumour cell lines as these complexes may linked to different activation pathways that trigger the corresponding cellular responses specific to the cell lines. The definite role of TNFRSF12A in OSCC has not been confirmed but its overexpression in OSCC was reported in few studies (Acharya et al., 2019; Samman et al., 2015). The immunoreactivity of TNFRSF12A is higher in OSCC tissue samples compared to healthy oral mucosa and dysplastic lesions, and is significantly associated with WPOI, surgical margins status, high tumour budding and poor tumour differentiation at invasive front (Acharya et al., 2019). Based on the published evidence, we postulate that role of TNFRSF12A in OSCC is related to cellular migration and invasion and its overexpression in peri-tumoural tissue predisposes the genetically altered field to malignant change.

2.6.3 Ki-67

Ki-67 antigen is a nuclear protein in humans that is associated with cellular proliferation. It is encoded by the MKI67 gene (Schonk et al., 1989) and is present during all active phases of the cell cycle (G1, S, G2 and M), but is absent in quiescent cells (G0) (Scholzen and Gerdes, 2000). Ki-67 expression varies throughout the different cell cycle phases. Its expression is low in G1 and S phase and rises to its peak level during mitosis. The exclusive expression of this protein made it an excellent operational marker to determine the growth fraction of a given cell population. The growth fraction is the proportion of cells born into the proliferative category (Alison 1995). In malignancy, expression of Ki67 protein is related to the proliferative activity of native cell populations, allowing it to be used as a marker of tumour aggressiveness (Brown and Gatter, 2002). The fraction of Ki-67 positive cells, indicated by Ki-67 labelling index (LI)

is the percentage of immune-positive cells within the counted 100 tumour cells (Li et al., 2015). The usefulness of the Ki-67 LI has been well established for various types of malignancies. Ki-67 expression correlates with the course of the disease, disease specific survival and tumour recurrence in multiple myeloma, prostate cancer, colorectal cancer and breast carcinoma (Luo et al., 2019; Scholzen and Gerdes, 2000). Meta-analysis by Xie et al., (2016) found that high Ki-67 expression was a negative prognostic factor in OSCC but the prognosis was better in group who received surgical treatment with CCRT. Birajdar et al., (2014) observed an increase in supra-basilar Ki-67 immunostaining with increasing grade of dysplasia and proposed that Ki-67 could be a reliable marker to predict malignant transformation of oral epithelial dysplasia (OED). Additionally, Ki-67 was shown to be a reliable surrogate marker to demonstrate loss of heterozygosity (LOH) in clinically and histologically normal mucosa surrounding as well as distant from the primary OSCC. Tabor et al. (2003) found a good correlation between LOH analysis and Ki-67 LI in the normal mucosa adjacent to the tumour while Montebugnoli et al. (2009) demonstrated high Ki-67 LI in mucosa contralateral to the index tumour. These findings confirmed the presence of genetically altered field as proposed by Slaughter in 1953. Gissi et al., (2016) later found that high Ki-67 expression in non-neoplastic mucosa contralateral to the primary OSCC was associated with appearance of LR, SPT and lymph node metastasis. Thus, the evaluation of Ki67 expression in mucosa contiguous to the primary OSCC, may become a potential predictor of disease free survival with further investigations and confirmation by long-term follow-up data.

CHAPTER 3 : METHODOLOGY

3.1 Study design

This was a retrospective case control study investigating the expression of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal surgical margins to predict local relapse amongst surgically treated OSCC patients. This study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya [DF OS1825/0087(P)].This research was financed by Research Management Centre, Faculty of Dentistry, MAHSA University (RMC/CA06/2018).

3.2 Sample types

This study involved OSCC cases treated in Oral and Maxillofacial Surgery Clinic, Hospital Tengku Ampuan Rahimah, Klang and Oral Surgery Clinic, Department of Oral and Maxillofacial Clinical Sciences, Faculty of Dentistry, University of Malaya. Surgically treated OSCC cases from year 2000 onwards from the respective centres were screened and data on status of surgical margins were extracted from the patient's histopathological reports. Socio-demographic, clinicopathological and follow-up data of all the cases were obtained from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS). MOCDTBS is coordinated by Oral Cancer Research and Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya. Formalin-fixed paraffin embedded (FFPE) tissues of cases included in the study, as well as five normal oral mucosal samples which served as a standard for comparison were also retrieved from MOCDTBS.

3.3 Sample selection

3.3.1 Sample size calculation

G*Power software, version 3.1.9.4. was used to calculate the sample size for this study. Based on the effect size of 0.695 and power of 0.80 (Zargoun 2017), the estimated sample size was 33 cases for each group. However, due to limited number of cases that fulfilled the inclusion and exclusion criteria, the estimated sample size was not achievable.

3.3.2 Sample selection for study group

A total of 15 primary OSCC cases diagnosed from the year 2000 onwards were selected based on the inclusion criteria. The inclusion and exclusion criteria were as follows:

1. Cases with a primary tumour located in the oral cavity
2. Cases that had been treated by surgery with or without post-operative chemotherapy or radiotherapy.
3. Cases with histologically non-involved mucosal surgical margins. Only cases with mucosal surgical margins that were $\geq$ 1mm away from the tumour were designated as histologically non-involved margins.
4. Cases in which a relapse was diagnosed during the 5-year follow-up period.

The exclusion criteria were:

1. Cases with mucosal surgical margins involved by tumour. The margin was <1mm away from the tumour.
2. Cases where patients received pre-operative chemotherapy or radiotherapy.
3. Cases where relapse was diagnosed within six months post-operatively.

3.3.3 Sample selection for control group

The control group comprised of OSCC cases diagnosed during the same period but did not develop any relapse in the subsequent five years of follow up. Selection of these cases were strictly based on the same inclusion and exclusion criteria as listed above. 19 OSCC cases with available FFPE tissue blocks that fulfilled the listed criteria were selected as the control group for this study.

3.3.4 Stratification of the relapse cases into LR/SFT and SPT

Within the relapse group, the cases were further stratified into LR/SFT or SPT based on the clinical criteria (Braakhuis, et al., 2002). Cases in which the second tumour developed not more than three years from the diagnosis of the primary tumour and was located within 2cm from the previously excised primary tumour were designated as LRs. As Braakhuis et al. (2003) proposed that these LRs which arise from the same field as the primary tumour may develop additional genetic hits in which the genes that dysregulate later in carcinogenesis differed from the primary tumour these tumours that fulfilled the above mentioned criteria for LR could also be an SFT and hence were designated as LR/SFT. Second tumours that developed at a site more than 2cm from the primary tumour and after 3 years from the completion of the initial treatment were designated as SPTs. There were 16 and 7 non-involved mucosal surgical margins in the LR/SFT and SPT category respectively. The expression of each markers in the non-involved mucosal surgical margins for both the clinical categories of relapse was compared and statistical analysis using Chi-square test (Fisher's Exact test) was performed.

3.3.5 Selection of mucosal surgical margins

Each of the 34 OSCC cases selected for the study had at least more than one surgical margin. For example, a tumour excision for SCC on the right lateral border of

tongue would have four mucosal surgical margins i.e anterior, posterior, medial, inferior and a deep connective tissue margin. In addition, each of the mucosal surgical margins would have multiple sections resulting in multiple FFPE tissue blocks of mucosal surgical margins available per case. In this study, a two-staged random sampling was carried out to select two representative mucosal surgical margins for each case using the RANDBETWEEN function of Microsoft Excel (Office 365 version). The first stage of sampling was performed to select two non-involved mucosal surgical margins for example posterior and medial, followed by a second stage of random sampling utilizing the same method to select a representative section from the selected mucosal surgical margins. Ideally there should have been 68 slides (two from each of mucosal surgical margins) studied, however due to missing FFPE tissue blocks, a total of 55 FFPE tissue blocks were selected from the 34 OSCC cases. Illustration of sample selection protocol is shown in Appendix A. A table of all the selected cases with representative margins is also appended in Appendix B.

FFPE tissue blocks of normal oral mucosa (NOM) which were available in the archives of MOCDTBS were retrieved. Sectioning followed by H&E staining was done for screening purposes to ensure that these tissues could be included in the study. These tissues were previously collected during crown lengthening procedures or surgical removal of impacted third molars and they were devoid of epithelial dysplasia. After screening, only four samples of NOM were suitable to be utilised as control samples.

One section each of 5µm thickness was taken from the FFPE tissue block of the selected mucosal surgical margins. The sections were stained with H&E stain to reassess the distance of mucosal surgical margins from the tumour to confirm each of the mucosal surgical margins randomly selected fulfilled the inclusion criteria of $\geq$1mm away from the tumour. An additional of three FFPE tissue sections were obtained from each block and were stained with FSCN1, TNFSF12A and Ki-67.

3.4 Epithelial dysplasia grading

All H&E stained sections in both the study and control groups were graded for epithelial dysplasia (ED) by two independent observers. The observers were blinded to the clinical and treatment outcomes details of each case. ED grading was performed according to the criteria listed in the "World Health Organization (WHO) Classification of Head and Neck Tumours 4th edition" as well as the criteria proposed by Kujan et al (2006). Consensus was achieved for all the graded cases before being dichotomised into two groups as illustrated below in Figure 3.1.

3.4.1 WHO criteria for epithelial dysplasia

The WHO 2017 classification recognizes eight cytological and architectural disturbance as diagnostic criteria for oral epithelial dysplasia. The criteria used are as the following:

Architectural changes:

1. Irregular epithelial stratification
2. Loss of polarity of basal cell
3. Drop-shaped rete-ridges
4. Increased number of mitotic figures
5. Abnormally superficial mitotic figures
6. Premature keratinization in single cells
7. Keratin pearls within rete ridges
8. Loss of epithelial cell cohesion

Cytological changes:

1. Abnormal variation in nuclear size
2. Abnormal variation in nuclear shape
3. Abnormal variation in cell size

4. Abnormal variation in cell shape
5. Increased N:C ratio
6. Atypical mitotic figures
7. Increased number and size
8. Hyperchromasia

According to WHO grading system, dysplasia is divided into grades of mild, moderate and severe. Epithelium that exhibits architectural change limited to the lower third of the epithelium and accompanied by minimal cytological atypia is designated as mild grade dysplasia. Moderate epithelial dysplasia on the other hand is when the architectural disturbance is observed up to middle third of the epithelium but the degree of cytological atypia is taken into consideration in upgrading it to severe dysplasia. Thus, severe epithelial dysplasia is recognized when the architectural disturbance with associated cytological atypia involves greater than two third of the epithelium or up to middle third but with marked cytological atypia. An overall dysplasia grade for the patient would be the highest grade of dysplasia graded in any of the mucosal surgical margins of those patients (Warnakulasuriya et al., 2008).

3.4.2 Binary grading system

Kujan et al (2006) proposed and evaluated a binary grading scheme following the diagnostic criteria used in WHO classification 2005 that stratify the lesions into either "low-risk" or "high-risk" based on the association between progression to malignancy and histological features. The cut-off point for a "high-risk" lesion was based on observing at least four architectural changes and five cytological changes. The cut-off point for "low-risk" lesion is associated with observation of less than four architectural changes or less than five cytological changes. In the present study, the histological

assessment was based on the diagnostic criteria for epithelial dysplasia listed in the WHO classification 2017.

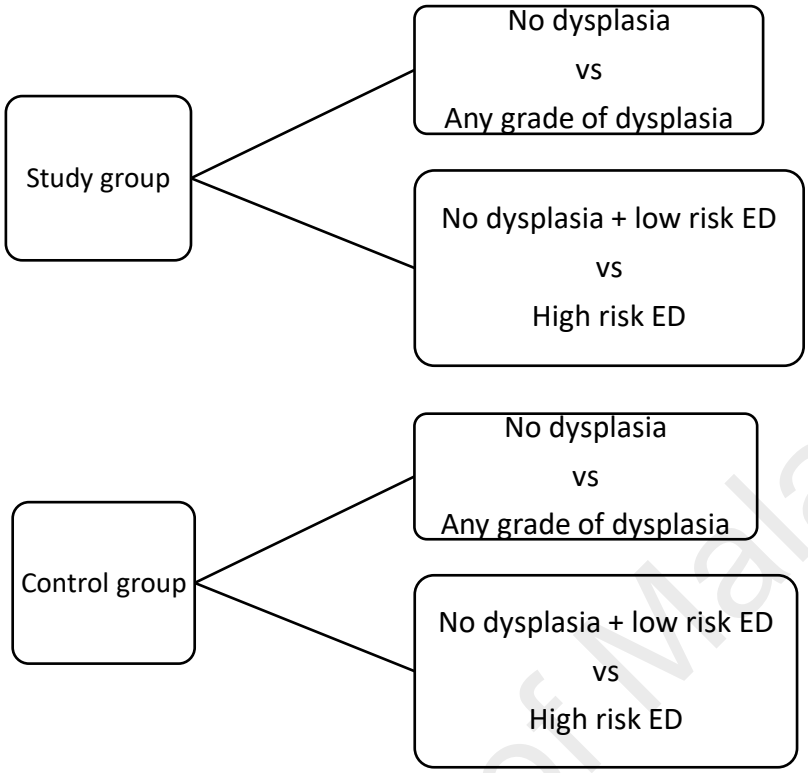

Figure 0-1: Dichotomization of samples based on epithelial dysplasia grading

3.5 Laboratory procedures

3.5.1 Haematoxylin and Eosin staining

One section each from the selected FFPE tissue blocks was stained with H&E following the protocol recommended by the manufacturers. The sections were firstly deparaffined and rehydrated before staining with haematoxylin and eosin. The stained sections were dehydrated and cleared with xylene prior to mounting. Detailed description for each step in the procedure were as listed in Appendix C.

3.5.2 Immunohistochemistry

Immunohistochemistry staining procedures using monoclonal mouse anti-human Ki-67 antigen, clone MIB-1 (M724029, Agilent DAKO, USA), monoclonal rabbit anti-Fascin antibody [EP5902] (ab126772, Abcam, Cambridge MA, USA) and monoclonal rabbit anti-TWEAKR/Fn14 antibody [EPR3179] (ab109365, Abcam, Cambridge MA, USA) were performed on the unstained sections from FFPE tissue blocks by applying Dako Real EnVision Detection System and Peroxidase/DAB+ (Dako, USA) according to manufacturer's protocols. Optimisation for each antibody was carried out on the control tissues to determine the optimum concentration of antibody, antigen retrieval method and incubation period for the primary antibody. Control tissues for TNFSF12A (human placenta) antibodies were obtained from Pathology's Unit for Research Support and Education (PURSuE), Department of Pathology, Faculty of Medicine, University of Malaya, while the control tissue for Ki-67 (OSCC) and FSCN1 (skin and lymph node) were obtained from Oral Pathology Diagnostic and Research Laboratory (OPRDL), Faculty of Dentistry, University of Malaya. The FFPE tissue sections were mounted on silanized glass slides and incubated at 37 ^{o}C overnight and at 60 ^{o}C 1 hour prior to procedure for deparaffinization. The antibody dilution, antigen retrieval buffer, wash buffer, pH and temperature used are as tabulated in Table 3.1. Details of the immunohistochemistry procedure performed is described in Appendix D.

Table 0.1 : Types of primary antibodies and reagents used with associated procedures

Primary antibody	**FSCN1**	**TNFSF12A**	**Ki-67**
Type	Rabbit monoclonal to Fascin (ab126772)	Rabbit monoclonal to TWEAKR/Fn14 (ab109365)	Monoclonal mouse anti-human Ki-67 antigen, clone MIB-1
Control tissue	Lymph node/skin	Human placenta	OSCC

Table 3.1 (continued)

Dilution	1:1000	1:100	1:300
Antigen retrieval	Microwave oven for 20 minutes at 99°C	Microwave oven 20 minutes at 99°C	Pressure cooker 121 °C for 30 seconds followed by 90 °C for 10 seconds
Buffer solution for antigen retrieval	Citrate buffer, pH 6	Citrate buffer, pH 6	Tris-Ethylenediaminetetra-acetic acid (EDTA) buffer, pH9
Incubation period	1 hour at room temperature	1 hour at room temperature	1 hour at room temperature
Wash buffer & pH	Phosphate buffer pH 7.4	Phosphate buffer pH 7.4	Tris-buffered saline

3.6 Quantification of Immunohistochemistry

The immunostaining for FSCN1 and TNFRSF12A were evaluated using a semi-quantitative index known as HSCORE by two independent observers. The observers were blinded from the outcome of the patients. Five high power fields (x400 magnification) were randomly chosen for all immuno-stained slides. Epithelial cells showing brown cytoplasmic staining was considered as FSCN1 and TNFRSF12A positive while Ki-67 immuno-positivity was denoted by brown nuclear staining. In each field, one hundred cells were counted sequentially and graded 0 to 3 depending on the intensity of staining (0 = negative; 1 = weak staining; 2 = moderate intensity; 3 = strong staining) using "Cell Counter" plugin of morphometric software Fiji is Just Image-J (Fiji) (Vijayashree, Aruthra, & Ramesh Rao, 2015). This plugin allows simultaneous categorisation and quantification. The final score was obtained by multiplying percentage of positive cells with the intensity score (1 = weak; 2 = moderate; 3 = strong). The maximum H SCORE achievable is 300, calculated using the following formula:

H SCORE = ΣPi x i,

where i is the intensity of staining and Pi is the percentage of stained cells for each given i.

The expression of Ki-67 in each SM was scored by counting the number of positive cells from a total of 500 cells from a maximum of five randomly selected fields at highest magnification of 400x. The percentage of positive cells was calculated and indicated as the labelling index (LI).

A calibration exercise to set a standard for staining intensity, *i* , was conducted prior to scoring. The final HSCORE for FSCN1 and TNFRSF12A and LI for Ki-67 were the average of the two sets of scores/percentage obtained from the 2 observers. The scores were categorised into high and low expression groups based on cut-off points determined statistically as discussed in the following sections.

3.7 Statistical analysis

Statistical analysis was performed by using SPSS software (version 26, IBM). Interclass correlation coefficient (ICC) test was performed to observe the interobserver agreement in scoring the expression of FSCN1, TNFRSF12A and Ki-67. ICC estimates and their 95% confidence intervals were calculated based on a mean-rating (k = 2), absolute-agreement, 2-way mixed-effects model. Based on the analysis, the interobserver agreements for scoring FSCN1, TNFRSF12A and Ki-67 in both study and control groups were all statistically significant (p <0.05) and ranked as good to excellent (Koo & Li., 2016). Table 3.2 shows the average measure of ICC test for each marker.

Table 0.2: Average measure of ICC for each marker

	FSCN1(*p* value)	TNFRSF12A (*p*-value)	Ki-67(*p* value)
Study group	0.946(.000)	0.881(.000)	0.839(.000)
Control group	0.889(.000)	0.852(.000)	0.841(.000)

Normal oral mucosa	0.999 (0.000)	0.898 (.000)	0.888 (0.032)

Significance level: p=0.05

The interobserver agreement on both three-tier WHO (2017) and binary (Kujan et al., 2006) dysplasia grading system was assessed by performing the Kappa statistics. The result from Kappa statistical test demonstrate interobserver agreement of 0.717 and 0.716 for three-tier WHO (2017) and binary grading respectively. The result was statistically significant and considered as substantial agreement (Landis & Koch, 1977).

Table 0.3: Measure of interobserver agreement in ED grading

Epithelial dysplasia (ED) grading system	Value	Approximate significant (p value)
WHO (2017)	0.717	0.000
Binary grading system (Kujan et al., 2006)	0.716	0.000

Significance level: p=0.05

Receiver operating characteristic (ROC) curve analysis was carried out to determine the cut-off for high and low expression of each marker in predicting local relapse. The HSCORE and LI for each samples were plotted to generate the ROC curve and the closest score to the point with maximum sensitivity and specificity was selected as the cut-off value. The HSCORE and LI were dichotomized into low and high expression based on the acquired cut-off value; low expression consisted of scores below and equal to the cut-off value, while high expression denoted the scores that were above the cut-off value.

Table 0.4: Cut-off value for FSCN1, TNFRSF12A and Ki-67 expressions

Markers	Area under the curve	Cut-off value
FSCN**1**	0.501	16.80
TNFRSF12A	0.403	90.65
Ki-67	0.520	18.01

Normality of data distribution was confirmed using SPSS software. Independent t-test and Mann-Whitney U test were applied to evaluate the expression of all markers in mucosal surgical margins. Pearson Chi-square test and Fisher's Exact test were applied to evaluate:

1. Association between clinic-pathological prognosticators and relapse in OSCC.
2. Low and high expression of all markers in the mucosal surgical margins.
3. Association between expression of FSCN1, TNFRSF12A and Ki-67 in mucosal surgical margins with clinicopathological prognosticators of OSCC.
4. Association between expression of FSCN1, TNFRSF12A and Ki-67 in mucosal surgical margins with relapse in OSCC.

Binary logistic regression analysis was performed to assess the correlation between the expression of markers in non-involved mucosal surgical margins, clinic-pathological prognosticators and relapse in OSCC. *P* value was regarded as statistically significant only when it was less than 0.05.

CHAPTER 4 : RESULT

4.1 Sociodemographic findings

A total of 34 OSCC patients comprising of 15 study cases and 19 control cases were included. Cases in the study group were those who had relapse within the 5 years follow up period postoperatively, while the cases in the control group experienced no relapse. There were 25 female and 9 male patients with a mean age at presentation of 58.38 years and an age range from 36 to 78 years. ROC curve analysis was carried out to identify the cut-off age of patients that could accurately predict OSCC relapse in this study. The analysis showed that age above 57.5 years was 78.3% specific and 75% sensitive in predicting the occurrence of relapse in this study (p = 0.001). Majority (61.7 %) of the patients were of Indian ethnicity. Table 4.1 demonstrates the distribution of patients according to age, gender, ethnicity and habits respectively.

Table 0.1: Sociodemographic data

Variables	Study group n (%)	Control group n (%)
Number of patients Total number of patients (n=34)	15 (100)	19 (100)
Age (years) Mean $\pm$ standard deviation, range:	58.38 $\pm$ 11.37, 36 - 78	
$\leq$ 57.5 years	3 (20.0)	14 (73.7)
> 57.5 years	12 (80.0)	5 (26.3)
Gender		
Male	5 (33.0)	14 (73.7)
Female	10 (67.0)	5 (26.3)
Ethnicity		
Malay	1(6.7)	4(21.1)
Chinese	6(40.0)	2(10.5)
Indian	8(53.3)	13(68.4)
Habits*		
Smoking	3(13.6)	2(9.1)
Alcohol	8(36.4)	4(18.2)
Betel quid chewing	7(31.8)	13(59.1)

No habit	3(13.6)	3(13.6)
Unknown**	1(4.6)	-

* Seven patients from study group and three patients from control group were having two habits.
** Information was unable to be retrieved.

4.2 Clinicopathological findings

There were 15 cases of relapse while 19 cases had no relapse in this study. Of that 15 patients, 5 were identified as having SPT while 10 of them were classified as having LR/SFT. Tongue was a frequent primary site for OSCC for patients in both the study and control groups with one third and almost half of the patients in the study and control group respectively having primary tongue SCC. Other reported primary tumour sites included retromolar region, alveolar mucosa, buccal mucosa, lip and gingivobuccal complex. Observed relapse sites included retromolar region, tongue, buccal mucosa, mandibular gingiva and flap margins.

The pathological features of OSCC included for statistical analysis in the present study were retrieved from the histopathological examination (HPE) report and the histopathology of the primary tumour was not re-assessed. Patients in both groups mostly had OSCC exhibiting moderate differentiation (n=20) followed by well-differentiated (n=12) and poorly differentiated OSCC (n=2). The pattern of invasion (POI) reported in all of the cases in this study was based on the classification by Bryne et al. (1998). The POI was predominantly type III, observed in 19 patients followed by type IV POI (n =9). Two patients in the control group had perineural invasion while 4 patients, two from control and study groups respectively had vascular invasion. Four and five patients in the study and control group respectively had cervical metastasis. One patient each from the study and control group received adjuvant radiotherapy. Clinicopathological prognosticators of study and control groups are described in Table 4.2 below.

Table 0.2: Clinicopathological prognosticators of study and control groups

Variables	Study group (n = 15)		Control group (n = 19)	
	n	(%)	n	(%)
Primary tumour site				
Tongue	5	(33.3)	7	(36.8)
*Non-tongue	10	(66.7)	12	(63.2)
Second primary tumour	7	(53.3)	-	
Local recurrence/ Second field tumour	8	(46.7)	-	
Tumour differentiation				
Well differentiated	3	(20)	9	(47.4)
Moderately differentiated	11	(73.3)	9	(47.4)
Poorly differentiated	1	(6.7)	1	(5.2)
**Pattern of invasion				
Type I	1	(6.67)	3	(15.8)
Type II	0	(0.0)	4	(21.1)
Type III	11	(73.3)	8	(42.1)
Type IV	3	(20.0)	3	(15.8)
***Unknown	0	(0.0)	1	(5.2)
Perineural invasion				
Yes	0	(0.0)	2	(10.5)
No	15	(100.0)	17	(89.5)
Vascular invasion				
Yes	2	(13.3)	2	(10.5)
No	13	(86.7)	17	(89.5)
Metastasis to the neck				
Yes	4	(21.1)	5	(26.7)
No	7	(57.8)	14	(73.7)
***Unknown	4	(21.1)	-	
Adjuvant radiotherapy				
Yes	1	(6.67)	1	(5.26)
No	13	(86.67)	18	(94.74)
***Unknown	1	(6.67)	-	

* Lip, buccal mucosa, alveolar mucosa, retromolar and gingivobuccal complex
** Bryne et al (1998) classification
*** Information was unable to be retrieved

Detailed sociodemographic data and clinicopathological prognosticators for each patient is appended (Appendix E).

4.3 Epithelial dysplasia grading

Among the 55 non-involved mucosal SMs, 29 (52.7 %) SMs did not have dysplasia while the remaining 26 (47.3%) in which 11 SMs in the control group and 15 SMs in the study group, were dysplastic. In order to reduce the statistical impact of outlier data, only the binary ED grading was used in further statistical analysis as there were only 3 SMs showing moderate and severe dysplasia respectively. There were 24 (43.6%) SMs that showed low risk ED and 2 (3.6%) SMs, one from each group, demonstrated high risk dysplastic features. Distribution of different grades of ED in control and study groups is shown in the Table 4.3 and bar chart below.

Table 0.3: Distribution of epithelial dysplasia in mucosal surgical margins of study and control groups

*Binary epithelial dysplasia grading	Study (n =23)	Control (n = 32)
	n (%)	n (%)
No dysplasia	8 (14.5)	21 (38.2)
Dysplasia of any risk	15 (27.3)	11 (20.0)

* Based on Kujan et al (2006) criteria for binary grading

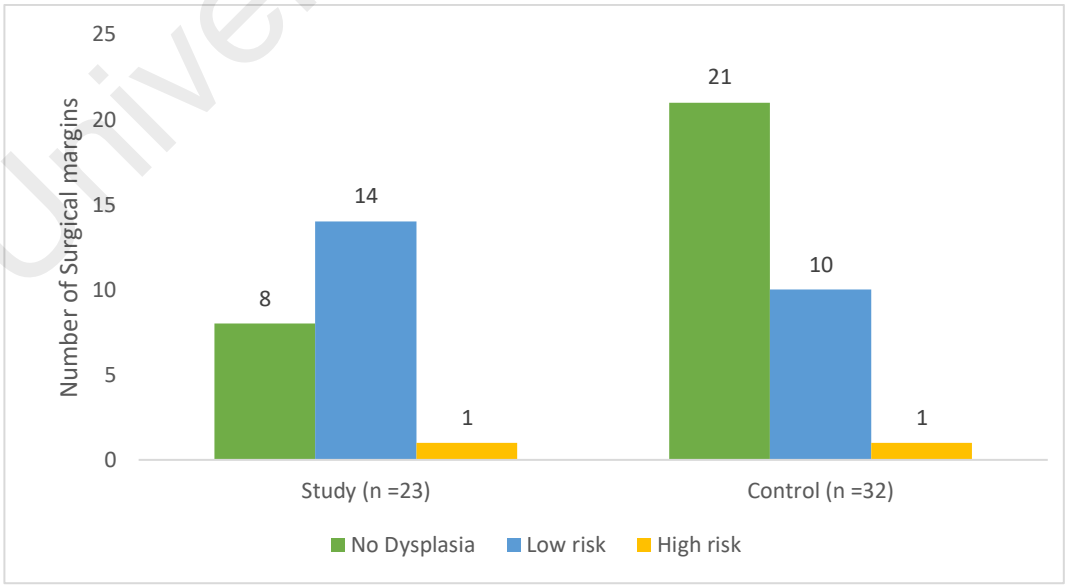

Figure 0-1: Distribution of epithelial dysplasia in mucosal surgical margins

4.4 Association between sociodemographic and pathological prognosticators of OSCC and local relapse

The association between clinicopathological prognosticators and relapse in OSCC was analysed to determine whether any clinicopathological prognosticators might be associated with the occurrence of relapse. Statistical analysis was performed using Chi-square test (Pearson Chi-square or Fisher's Exact test). Significant association was observed between relapse in OSCC and presence of ED, patients' age, ethnicity, alcohol consumption habit and pattern of invasion. Relapse was significantly more among Chinese patients ($p = 0.013$), patients aged above 57.5 years ($p < 0.001$) as well as those with type III and IV pattern of invasion ($p = 0.007$). Significant reduction in relapse was noted among non-alcoholics ($p = 0.025$). The result with are shown in Table 4.4.

Mucosal surgical margins with any grade of ED were significantly higher in the relapse group ($p = 0.024$). However, when the mucosal surgical margins with no dysplasia and low risk ED were grouped and compared with those with high risk ED, there was no significant difference noted between relapse and non-relapse group. Other clinicopathological prognosticators which included gender of patients, Malay and Indian ethnicity, habits of smoking and betel quid chewing, tumour site, tumour differentiation, perineural invasion, vascular invasion, adjuvant therapy and cervical metastasis did not show association with relapse in OSCC. The result for the statistical insignificant data is appended (Appendix F).

Table 0.4: Association between sociodemographic and clinicopathological prognosticators with relapse in OSCC

Sociodemographic/ clinicopathological prognosticators	Relapse		*p* - value
	Yes (n =23) n (%)	No (n= 32) n (%)	
Age			
≤ 57.5	5 (9.1)	24 (43.6)	<0.001
> 57.5	18 (32.7)	8 (14.5)	

Ethnicity			
Chinese	10 (18.2)	4 (7.3)	0.013[p]
Non-Chinese	13 (23.6)	28 (50.9)	
Alcohol			
Yes	10 (18.9)	6 (11.3)	0.025[f]
No	11 (20.8)	26 (49.1)	
Pattern of invasion			
Type I and II	2 (3.7)	13 (24.1)	0.007[p]
Type III and IV	21 (38.9)	18 (33.3)	
*ED at surgical margins			
No ED	8 (14.5)	21 (38.2)	0.024[p]
Any grade of ED	15 (27.3)	11 (20.0)	
No ED + Low risk ED	22 (40.0)	31 (56.4)	1.000[f]
High risk ED	1 (1.8)	1 (1.8)	

Significant level: *p*= 0.05. p = Pearson Chi-square. f = Fisher's Exact Test
*Based on Kujan et al (2006) criteria for binary grading

4.5 Expression of Ki-67, FSCN1 and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC

The total number of histologically non-involved mucosal surgical margins in the relapse and non-relapse group were 23 and 32 respectively. Mean HSCORE was obtained from all non-involved mucosal surgical margins for both the study and control groups for all markers. The expression of FSCN1 and Ki67 was higher in the study group than in the control group. However, the expression of TNFRSF12A was lower in the study group compared to the control group. Shapiro-Wilk test was used to evaluate the normality of the data distribution for all markers due to the small sample size in the study and control groups (n <50).

The expressions of FSCN1, TNFRSF12A and Ki-67 between the study and control groups were compared. As the data for all markers were not normally distributed, Mann-Whitney U test was used to evaluate if the difference in immuno-expression of the markers between the two groups was significant. However, there was no significant

difference observed between the expression of FSCN1, TNFRSF12A and Ki-67 between the control and study groups. The results of the statistical analyses are as tabulated in Table 4.5.

Table 0.5: Expression of FSCN1, TNFRSF12A and Ki-67 in mucosal surgical margins

Markers	Study group Mean ± sd	Control group Mean ± sd	*p* – value
FSCN1	12.71 ± 25.32	10.50 ± 21.01	0.993
TNFRSF12A	13.83 ± 22.77	21.09 ± 26.61	0.219
Ki-67	16.81± 11.92	15.11 ± 8.94	0.798

Significance level: $p = 0.05$. sd: Standard deviation. Test: Mann Whitney U test.

The variability of the pattern and intensity of immunostaining of FSCN1, TNFRSF12A and Ki-67 in the study and control groups is shown in Figure 4.2, 4.3 and 4.4 respectively.

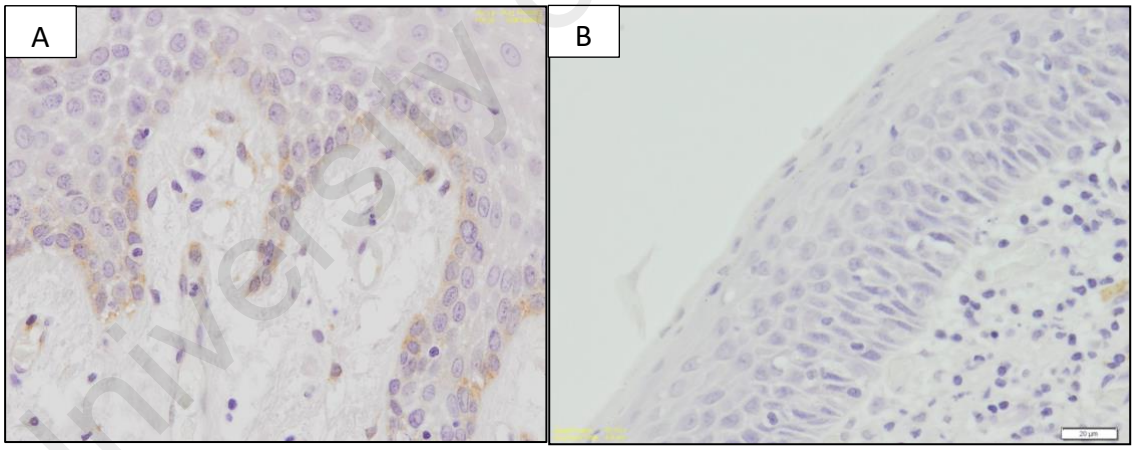

Figure 0-2: Staining with FSCN1 in study and control group

(A) Weak to moderate cytoplasmic staining was observed in the basal and parabasal layers in the study group. (400x) (B) Lack of FSCN1 staining in the control group. (400x)

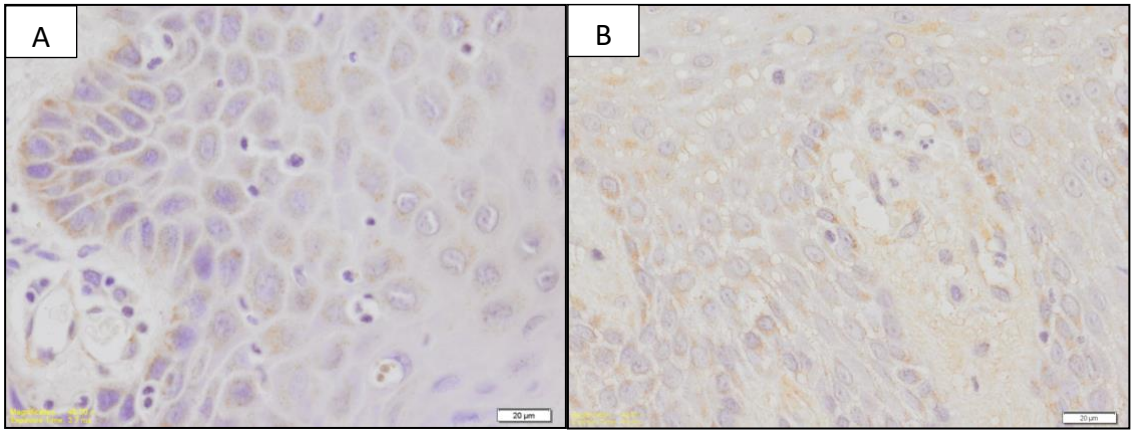

Figure 0-3: Staining with TNFRSF12A in study and control group

Weak to moderate cytoplasmic staining was observed in the basal and parabasal layer in the study group. (400x) (B) Weak to moderate cytoplasmic staining was observed within the basal, parabasal and spinous layers in the control group. (400x)

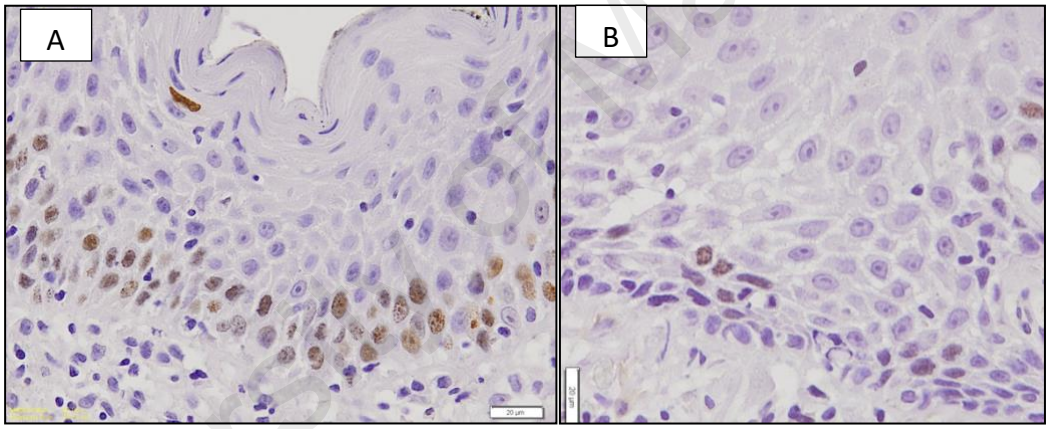

Figure 0-4: Staining with Ki-67 in study and control group

(A) Moderate to strong staining was seen in the basal and suprabasal layers in the study group. (400x) (B) Weak to strong nuclear staining was seen within parabasal layer in the control group

4.6 Association between clinicopathological prognosticators and expression of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal surgical margins

Statistical analysis with Chi-square test (Pearson chi-square or Fisher's Exact test) showed that expression of FSCN1 was significantly associated with patients' ethnicity

and tumour site where high FSCN1 expression was observed among Indian patients and non-tongue tumours. There was a significant association between expression of Ki-67 with patients' gender in which low expression of Ki-67 was observed among female patients. These are shown in Table 4.6.

There was no other significant association between the expression of markers and other clinicopathological prognosticators.

Table 0.6: Expression of FSCN1 and Ki-67 in histologically non-involved mucosal surgical margins and its association with ethnicity, gender and tumour site

Markers	Ki-67 n (%)		FSCN1 n (%)		*p* value
	High	Low	High	Low	
Gender					
Female	9 (16.4)	31 (56.4)	-		0.021
Male	9 (16.4)	6 (10.9)			
Ethnicity					
Indian	-		10 (18.2)	21 (38.2)	0.015
Non-Indian			1 (1.82)	23 (41.8)	
Tumour site					
Tongue	-		0 (0.0)	22 (40.0)	0.02
Non-tongue			11 (20.0)	22 (40.0)	

Significant level: p= 0.05. Statistical test: Pearson chi-square

4.7 Association between expression of Ki-67, FSCN1, and TNFRSF12A in histologically non-involved mucosal surgical margins of OSCC and local relapse in these patients.

Statistical analysis with Chi-square test (Pearson chi-square or Fisher's Exact test) was performed. In both relapse and non-relapse groups, the number of mucosal surgical margins exhibiting low expression of FSCN1, TNFRSF12A and Ki-67 was higher than those exhibiting high expression. The number of mucosal surgical margins exhibiting high expression of TNFRSF12A (n = 9) and Ki-67 (n = 1) were equal in both relapse and

non-relapse groups. The number of mucosal surgical margins showing high expression of FSCN1 was greater in relapse (n = 6) cases compared to non-relapse (n =5) cases even though the results were not significant. The number of mucosal surgical margins showing high and low expression of FSCN1, TNFRSF12A and Ki-67 are shown in Table 4.7 and figure 4.5.

Table 0.7: Expression of FSCN1, TNFRSF12A and Ki-67 and relapse in OSCC

Markers	Expression of marker	Relapse (n = 23)		No relapse (n =32)		*p* - value
		n	(%)	n	(%)	
FSCN1	High	6	(26.09)	5	(15.63)	0.496
	Low	17	(73.91)	27	(84.37)	
TNFRSF12A	High	1	(4.35)	1	(3.13)	1.00
	Low	22	(95.65)	31	(96.87)	
Ki-67	High	9	(39.13)	9	(28.13)	0.561
	Low	14	(60.87)	23	(71.87)	

Significant level: *p*= 0.05. Statistical test: Pearson chi-square

The number of mucosal surgical margins exhibiting high expression of FSCN1 was equal in both SPT and LR/SFT categories. More than half of the mucosal surgical margins in the SPT category exhibited high Ki-67 expression but none showed high expression of TNFRS12A. The differences in the expression of markers between LR/SFT and SPT groups observed were not statistically significant. The number and percentage of mucosal surgical margins showing high and low expression of FSCN1, TNFRSF12A and Ki-67 within the LR/SFT and SPT categories are shown in Table 4.8 and Figure 4.6.

Table 0.8: Expression of FSCN1, TNFRSF12A and Ki-67 in SPT and LR/SFT group

Markers	Expression of marker	SPT (n=7)		LR/SFT (n=16)		*p* - value
		n	(%)	n	(%)	
FSCN1	High	3	(42.86)	3	(18.75)	0.318
	Low	4	(57.14)	13	(81.25)	

TNFRSF12A	High	0	(0.00)	1	(6.25)	1.00
	Low	7	(100.0)	15	(93.75)	
Ki-67	High	4	(57.14)	5	(31.25)	0.363
	Low	3	(42.86)	11	(68.75)	

Significant level: p= 0.05. Statistical test: Pearson chi-square

4.8 Association between expression of FSCN1, TNFRSF12A, Ki-67, clinic-pathological prognosticators and relapse in OSCC

Binary logistic regression analysis was carried out to investigate the relationship between expression of all markers, clinicopathological prognosticators and relapse in OSCC. In the analysis, the probability of relapse was calculated using the clinicopathological prognosticators that were shown to be significantly correlated with OSCC relapse in the Chi-square test. Since no significant association was observed between the expression of FSCN1, TNFRSF12A and Ki-67 in the mucosal surgical margins and relapse, the expression of these markers was excluded in the binary logistic regression analysis.

This study model was 80.8% accurate in its prediction of OSCC relapse. Hosmer and Lemeshow test results confirmed that the model was a good fit for the data (p = 0.256). Coefficients for the model's predictors are presented in Table 4.9. Significant p values are bolded.

Table 0.9: Predictor coefficients for the Model Predicting OSCC relapse

Clinicopathological prognosticators	p values	OR [95%CI]
Age	**0.008**	11.47 [1.89, 69.70]
Chinese	0.118	4.77 [0.67, 33.80]
Alcohol consumption	0.170	3.59 [0.578, 22.232]
ED	0.063	0.196 [0.035, 1.094]
POI	0.090	9.72 [0.704, 134.316]

CI = Confidence interval; OR = Odd ratio.

As demonstrated in Table 4.8, age was the predictor of relapse which significantly improved the model's predictive capability. There was an 11-fold risk of OSCC relapse noted in relation to patients who were aged above 57.5 years. Other predictors such as Chinese ethnicity, alcohol consumption, epithelial dysplasia in mucosal surgical margins and pattern of invasion of tumour did not appear to significantly influence the probability of OSCC relapse.

CHAPTER 5 : DISCUSSION

This retrospective study investigated the significance of sociodemographic, clinical and histopathological characteristics in OSCC patients as well as expression of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal surgical margins as predictors of relapse in OSCC. Based on the concept of field cancerization, we postulated that dysregulation of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal SMs partake in the development of tumour relapse. The clonal relationship between the primary and second tumour was not established in this study, thus the stratification of relapse into LR, SFT and SPT was exclusively based on clinical criteria. Estimation of protein expression of FSCN1 and TNFRSF12A in the primary tumour tissue was trivial as the prediction of relapse in this study was grounded on the role of the selected markers in malignant transformation of the genetically altered field.

5.1 Sociodemographic and clinicopathological prognosticators and relapse in OSCC

The 5 years survival rate for OSCC have improved since 1960 but the survival rate of OSCC patients with recurrence remain poor as only about 30% of them would survive for more than 5 years (Jadhav & Gupta 2013). Several sociodemographic and clinicopathological characteristics of OSCC have been implicated with the risk of recurrence which include age, tobacco use, site, tumour differentiation and lymphocytic host response (Camisasca et al., 2011). In the present study, we analysed the sociodemographic, clinical and pathological features in OSCC patients including those who had developed relapse within the 5 years of follow up.

Sociodemographic parameters included in this study were age, gender, ethnicity and habits. The age of the patients in our study ranged from 38 to 78 years with a mean

of 58 years. This is in concordance with the findings from a multicentre study by Dhanuthai et al. (2018) involving few Asian countries and Canada and a study on OSCC among Malaysians by Ghani et al. (2011), where the mean age of OSCC patients was 58 years. There were 2 patients aged below 40 years in this study which reflects the current trend of increasing incidence of 'early-onset SCC', arbitrarily defined as SCC occurring in individuals younger than 40 years old (Müller et al., 2008; Gupta et al 2016; Kapila, et al., 2017). Majority of the patients in this study were females and of Indian ethnicity. This again reflected the sociodemographic characteristics of OSCC in Malaysia where there the majority of OSCC patients were females and of Indian ethnicity (Ghani et al., 2011).

In the present study, patients' age, ethnicity, alcohol consumption, POI and ED at the mucosal surgical margins were identified as clinicopathological prognosticators significantly associated with relapse in OSCC. Tumour differentiation, PNI, VI, bone invasion, cervical metastasis, adjuvant therapy, primary tumour site, gender, tobacco use and betel quid chewing showed no association with OSCC relapse in our study cohort.

Most of the patients who had relapse in this study aged more than 57.5 years at first diagnosis with a mean age of 63.9 $\pm$ 13.1 years. The binary logistic regression analysis showed that age above 57.5 years was a predictor of relapse in OSCC. Patients who had no relapse were younger with a mean age of 53.2 $\pm$ 8.2 years, indicating older patients were at higher risk of developing OSCC relapse. This finding was in contrary to studies by Wang et al. (2013) and Vázquez-Mahía et al. (2012) who not only reported that age was not significantly related with OSCC relapse but patients who had recurrence in both studies were younger than those with no recurrence. Nevertheless, development of relapse in older patients may be due to several factors that include presence of comorbidities such as diabetes, hepatic and heart disorders that are common among elderly. Vázquez-Mahía, I et al. (2012) found that presence of co-existing morbidities was the most relevant

prognostic factor for relapse. Similarly, higher age and comorbidity score were found to be independent risk factors for worst overall survival in a study on OSCC patients based in Thuringia, Germany (Göllnitz et al., 2016). The prognostic significance of comorbidites in head and neck cancer was reported to correlate with age below 65 years, tongue and laryngeal tumour and early stage (stage I and II) in a population-based study by Alho et al., (2007). However data on associated comorbidities of OSCC patients was not analysed in this study, thus further inference could not be drawn.

In our study, Chinese ethnicity ($p = 0.013$) significantly correlated with relapse in OSCC. While the published evidence concerning inter-ethnicity survival and prognosis in OSCC is scarce, we cannot exclude the possibility of age, smoking habits and alcohol consumption as contributing factors to the finding in this study cohort. A study on multi-ethnic variation in practice of oral cancer risk habits in Malaysia showed that concurrent smoking and alcohol drinking were the most common habits amongst the Chinese ethnicity in Malaysia (Ghani et al., 2018). Five out of six of Chinese patients who had relapse in our study were aged above 60 years and 2 of them had combined smoking habit and alcohol consumption.

Additionally, the number of non-alcoholic patients were significantly higher in the control group compared to the study group in the present study, implying a positive correlation between alcohol consumption habit and development of OSCC relapse. Data from epidemiological studies involving different populations have consistently supported the strong association between alcohol consumption and increased risk of oral cancer. Evidence for the impact of alcohol drinking on recurrence is nonetheless conflicting. Fortin et al. (2009) showed that survival rates and local control of OSCC among alcohol drinkers were poorer to that in patients who were not exposed to the habit. A multicentric study based in Italy by Leoncini et al., (2015) found a significant correlation between excessive alcohol use and increased hazard of death from any cause but no significant

association was reported for alcohol drinking and recurrence and second primary tumour in head and neck cancer. An epidemiological study based in Brazil by Souza Cruz et al. (2014) reported alcohol consumption and the presence of metastasis as significant factors for increased recurrence and mortality of OSCC patients. The poor survival among drinkers compared to non-drinkers in OSCC has been linked to the overexpression of hypoxia-inducible factor-1-alpha (HIF-1α), a biomarker related to tumour invasion, metastasis, and progression of a variety of human cancers (Lin et al., 2008).

In the present study, the predominant worst POI was type III (n = 18). POI type I and II were frequently seen in the control group and the difference in the distribution of POI type III and IV and POI type I and II between the control and study groups were significant. This finding is expected as several studies have reported tumour infiltrating in small islands and in a non-cohesive manner as poor prognosticators in OSCC (Almangush et al., 2015; Sheahan et al., 2003). Dissanayaka et al., (2012) found that POI was the best prognosticator for both local recurrence and overall survival in their study that involved a cohort of 193 Sri Lankan patients. Spiro et al., (1999) on the contrary did not observe significant correlation between POI and LR but reported that the likelihood for nodal and distant metastasis was significantly higher with POI type III and IV. The authors also noted a significant reduction in overall survival with increasing grade of POI. POI is essentially a significant histopathological feature in OSCC as it correlates with tumour cell cohesiveness, motility, loss of contact inhibition, excretion of enzymes, and other factors associated with aggressiveness in an experimental model Crissman (1986). The impact of POI on the prognosis of OSCC other than LR was nonetheless beyond the scope of this study.

In regards to the prognostic significance of epithelial dysplasia in malignant transformation, we examined the effect of dysplasia in SMs on OSCC relapse. Our study showed that 12 out of 15 OSCC patients in the relapse group had dysplastic SMs as

opposed to the control group that mostly had microscopically normal SMs. There was a significant difference in the distribution of SMs showing no, low and high-risk dysplasia in relapse and non-relapse group. Only 34.5% of SMs in the control group had either low or high-risk ED compared to 65.2% of SMs in the study group. Our study showed a statistically significant association between OSCC relapse and presence of ED in histologically non-involved mucosal SMs. Strikingly, the severity of ED grading (low risk and high risk) did not influence the development of relapse in the present study. We noted that high risk ED in SMs did not provide greater risk for relapse. However, it should be noted that group of patients in our study with high risk ED in the SMs was rather small (n = 2). Other endogenous and environmental factors could account for the risk of malignant transformation of the dysplastic epithelium in SMs. Nonetheless, ED was not a predictor for OSCC relapse in this study based on binary logistic regression analysis. Consistent with our finding, significant risk for relapse imposed by dysplasia in SMs was reported by Weijers et al., (2001). Kurita (2010) found that presence of ED in SMs was a significant predictor for LR and LR was observed only in cases with severe dysplasia and not mild or moderate dysplasia in their study involving 146 OSCC patients. On the contrary, Buchakjian et al., (2018) in their study of a large cohort of OSCC patients (n = 426) found that the presence of tumour but not ED of any grade in SMs was an independent predictor of LR. The conflicting findings in the published literature on the effect of ED in SMs could be due to a relatively large interobserver variability in histopathological ED grading and disparity in study design and cancer reporting. Current College of American Pathologists (CAP) guidelines (2017) have recommended that both severe and moderate dysplasia at SMs of oral cavity cancers be reported as positive margins. Researchers would also omit cases with severely dysplastic SMs in their study as to reduce the effect of re-resection in their analysis.

Oral ED is conventionally graded into mild, moderate and severe based on the extent of architectural changes and cytological atypia listed in the WHO diagnostic criteria. However, there is great variability in interpretation of the presence and degree of significance of the individual diagnostic criteria among pathologists. Several studies have demonstrated great variability in inter-observer diagnosis and grading of OED with results showing only poor to moderate agreement as well as difficulties in achieving accurate reproducible agreement between the pathologists (Karabulut et al., 1995; Abbey et al., 1995). A new binary dysplasia grading has been proposed by Kujan et al., (2006) to reduce the subjectivity in ED reporting. This grading system which uses four architectural and five cytological features to distinguish low and high risk lesions demonstrated an increased inter-observer agreement ($\kappa = 0.5$) as compared to the WHO grading ($\kappa = 0.22$). Although the customary three-tier grading system is widely used, the binary system complements the WHO classification system, and it has merit as it helps clinicians to make critical clinical decisions particularly in cases with moderate dysplasia (Ranganathan & Kavitha, 2019). Thus, binary grading system by Kujan et al., was used to grade ED in SMs in this study not only to improve inter-observer agreement but also to investigate any correlation between SMs with high risk ED and OSCC relapse. However, significant statistical correlation could not be observed as there was only one relapse case that was from the SPT group that showed high risk ED in the SMs.

The presence of ED is accepted as one of the most important predictors of malignant transformation in oral potentially malignant disorders (OPMDs). However, only a minority of ED progress to carcinoma, whereas other dysplastic lesions remain unchanged for years or resolve over time (Napier and Speight, 2008). The malignant transformation rate amongst patients histologically diagnosed as oral epithelial dysplasia undergoing long-term follow-up was only 10.5% (Shariff and Zavras, 2015). Given that oncogenesis of OSCC is a complex multistep process and not all dysplastic epithelium

embark in malignant transformation, several molecular aberrations have been identified as predictive markers for progression of ED to carcinoma. These include DNA aneuploidy, expression of p53 and Ki-67, LOH at specific chromosomal loci and alteration in microRNA expression (Bradley et al., 2010). Conway et al., (2015) demonstrated that malignant transformation of dysplasia presumably depends on genes that dysregulate later in the oncogenic events. These genes, as revealed by the authors are those associated with cellular motility and invasion which include FSCN1 and TNFSF12A that were analysed in the present study. However, there was no association between expression of FSCN1, TNFRSF12A and Ki-67 and presence of dysplasia in SMs in this study. High expression of these markers also did not correlate with severity in ED grading. We noted that both dysplastic and non-dysplastic SMs mostly showed low FSCN1, TNFRSF12A and Ki-67 expression. Investigating the potential use of the selected markers as prognostic markers for ED is nonetheless beyond the scope of this study.

OSCC relapse in this study was nevertheless not influenced by post-operative radiotherapy, tumour differentiation, vascular invasion, cervical metastasis and PNI. Due to incomplete data, the effect of pTNM stage, extra-capsular spread in lymph nodes and depth of invasion on relapse could not be assessed in this study. Lack of correlation between the assessed clinicopathologic parameters in this study was believed to be due to small and unequal sample size. There was only one OSCC patient in the study and control group respectively that was reported to receive adjuvant radiotherapy, 2 patients showed PNI and both were in the control group, 2 patients in each group had vascular invasion and 4 and 5 patients had cervical lymph node metastasis in the study and control group respectively. The modest representative for each clinicopathological parameter certainly did not reflect the actual effect on OSCC relapse. As most of the cases did not exhibit high risk histopathological features such as PNI, VI and all were cases with clear

or close SMs, the number of patients who received adjuvant radiotherapy was low. The negative correlation observed in this study is insufficient to allow us to disagree with the published evidence.

5.2 Expression of FSCN1, TNFRSF12A and Ki-67 and relapse in OSCC

Ki-67 is a tool for quick estimation of the proportion of proliferating cells in a neoplasm. As its expression increases according to the severity of dysplasia and tumour differentiation, it was postulated that Ki-67 might be a reliable predictor for malignant transformation (Birajdar et al., 2014; Dwivedi et al., 2013; Takkem et al., 2018). In the present study, we investigate the potential use of Ki-67 as a predictor for development of OSCC relapse. We noted that the mean LI of Ki-67 in non-involved mucosal SMs was higher in patients with OSCC relapse than those without relapse, although the difference was not statistically significant ($p = 0.798$). This observation was similar to that by Kumar et al., (2019) in which LI for Ki-67 was higher in the recurrence group compared to non-recurrence and likewise, the difference was not statistically significant. Nonetheless, the prognostic significance of Ki-67 in OSCC has been testified in several studies. Jing, Y et al., (2019) found that high Ki-67 LI was significantly associated with tumour differentiation, worst POI, lymph node metastasis and serve as an independent predictor for overall survival, disease-free survival and recurrence-free survival for OSCC patients. Wangsa D et al., (2008) revealed that high-proliferative activity denoted by high Ki-67 LI in stage I tongue SCC was associated with an increased risk of recurrence. Although high Ki-67 LI was not associated with relapse in the present study, we noted that the percentage of mucosal surgical margins with high Ki-67 LI was more in the relapse group than in the control group (39.13% vs 28.13%). Given that non-involved mucosal SMs may harbour a field of genetically altered cells and dysregulation in cellular proliferation is part of oncogenic change, the observed difference was anticipated.

Additionally, the number of female patients showing low Ki-67 LI in the mucosal surgical margins was greater than male patients and the difference was statistically significant. This finding inferred an association between Ki-67 expression and gender among OSCC patients. While the relationship between Ki-67 expression in OSCC and clinical features were assessed in several studies, significant correlation between Ki-67 expression and gender was not observed. A study on influence of clinicopathological parameters on Ki-67 staining in OSCC by Jalayer et al. (2014) showed that Ki-67 positive cells counts was not associated with gender nor OSCC histopathological differentiation. Jalayer et al., (2012) noted no significant difference in the expression of Ki-67 between males and females, but when compared between different age groups, a positive correlation between Ki-67 expression and gender and patients aged below 60 years was observed. The authors were not able to justify their observation. Pertaining to expression of Ki-67 in non-neoplastic samples, higher Ki-67 LI in non-dysplastic oral leukoplakia tissues was significantly associated with older age, tobacco habits and non-homogenous leukoplakia but not gender (Mondal, Mnadal & Sarkar 2016). The wide difference in number of female (n = 25) and male (n = 9) patients in the present study might explain the significant variation in distribution of Ki-67 LI between the gender. Ki-67 expression might also be influenced by habits and age of the respective gender but such evaluation was beyond the scope of this study.

The invasive ability of cancer cells results from the formation of a protrusive cellular structure called invadopodia (Eddy et al., 2017). Cancer cells use invadopodia to degrade extracellular matrix and infiltrate the basement membrane and surrounding tissue (Lee et al., 2017). FSCN1 is an actin-bundling protein that serves as an integral component of invadopodia in which it provides stability to actin and releases proteases important for invasive migration (Li et al., 2010). Overexpression of FSCN1 in OSCC was reported as an adverse prognostic indicator of disease-specific survival and correlated

with disease progression (Rodrigues et al. 2017). In our study, the mean HSCORE for FSCN1 in histologically non-involved mucosal SMs of OSCC patients with relapse was higher than in the control group; 12.71 + 25.32 and 10.50 + 21.01 respectively. Likewise, the number and percentage of SMs in the relapse group showing high FSCN1 expression were greater compared to the non-relapse group in which high FSCN1 expression was observed in 26.1% SMs with relapse and in 15.6% of those without relapse. The observed differences were not significant. Similar to the present study, Rodrigues et al. (2017) observed a statistically non-significant higher percentage of OSCC patients with recurrence showing strong FSCN1 positivity compared to those with low expression; 48.8% and 28.4% respectively. Alam et al. (2012) on the other hand reported a significant correlation between FSCN1 overexpression and increased recurrence and lymph node metastasis in OSCC. The authors linked the finding to their in vitro observation that showed FSCN1 overexpression in OSCC derived cells led to an increase in cell membrane protrusions, disorganization of cell-cell contacts and alteration in actin organization.

FSCN1 expression was also reported to differ between NOM, dysplastic oral mucosa and OSCC tissue. Expression of FSCN1 in NOM in this study (n = 4) showed weak to moderate staining restricted to the basilar layer except for one hyperplastic NOM that exhibited moderate to strong FSCN1 expression extending to the supra-basal epithelial layer. The FSCN1staining pattern in NOM depicted in this study was similar to that by Natesan et al. (2019) in which FSCN1 immuno-expression of low intensity was observed in basal layer of NOM. The authors also revealed that the distribution of FSCN1 immuno-staining within dysplastic epithelium increased with increasing grade of dysplasia and its expression significantly correlated with tumour differentiation. Based on the published evidence, FSCN1 expression is informative in separating normal, pre-neoplastic and neoplastic tissue. Since cancer-related genetic aberration preceded

phenotypic change, FSCN1 upregulation in histologically non-involved SMs may be useful to predict malignant transformation. However, genes involved in matrix degradation and invasive capacity was shown to dysregulate later in the pathological process (Conway et al., 2015) and FSCN1 may be one of the genes involved.

FSCN1 expression in mucosal SMs was however not associated with ED nor tumour differentiation in our study. We noticed that high FSCN1 expression significantly correlated with tumour site and Indian ethnicity. Most of the SMs that showed high FSCN1 expression were from Indian OSCC patients and non-tongue OSCC.

It has been shown that TNFRSF12A expression is elevated in several solid tumours such as brain, breast and liver (Winkles et al., 2006). Despite the various oncogenic processes allied to TNFRSF12A, there is currently insufficient information regarding its expression and role in OSCC. In the present study, expression of TNFRSF12A in non-involved mucosal SMs was higher in the non-relapse group than in the relapse group. We could not justify if the result is contradictory with the existing literature as to the best of our knowledge, this is the first study to investigate IHC expression of TNFRSF12A in non-involved SMs to predict OSCC relapse. TNFRSF12A expression in the normal and dysplastic epithelium was shown to be significantly lower than in OSCC (Acharya et al. 2019). Likewise, the staining pattern observed in this study was consistent with that in non-neoplastic mucosa reported by Acharya et al. (2019) in which TNFRSF12A was weak to moderately expressed in basal and suprabasal keratinocytes. TNFRSF12A expression was also reported to be strongly enhanced in tumour tissue compared to non-neoplastic tissue of non-small cell lung carcinoma, breast cancer, hepatocellular cancer, pancreatic cancer, malignant melanoma as well as glioblastoma multiforme (Wajant 2013). TNFRSF12A is also expressed in normal epidermis and skin appendices and elevated in inflammatory benign and malignant skin conditions, such as psoriasis and skin SCC (Sabour et al., 2012).

Binding of TNFRSF12A with its cognate ligand, tumour necrosis factor (TNF) superfamily member named TWEAK (TNF-like weak inducer of apoptosis) results in activation of multiple downstream signalling pathways pivotal in tumour development and progression such as angiogenesis, motility and invasion (Hu et al., 2017). The subsequent cellular responses, however, depends on the cell type, physiologic circumstances and interaction with other cytokines (Blanco-Colio, 2014). In breast carcinoma cell invasion model, TNFRSF12A/TWEAK binding led to inhibition of cell invasion but when TNFRSF12A was silenced, TWEAK could still potentiate the increase in cellular invasion possibly via another unidentified receptor (Gaudineau et al., 2012). Although dysregulation of TNFRSF12A has been observed in OSCC (Conway et al., 2015; Zhang et al., 2018), the definite role of TWEAK/TNFRSF12A cytokine receptor axis in the oncogenic process of OSCC has yet to be established. OSCC of aggressive phenotype was believed to have higher TNFRSF12A expression as Acharya et al. (2019) observed significant correlation observed between high TNFRSF12A expression and several clinicopathological parameters of prognostic significance such as POI, tumour budding and poor invasive front grading. Given that LR was also linked to high risk histopathologic parameters, examining the correlation between the expression of TNFRSF12A in histologically non-involved SMs and development of tumour relapse could improve our understanding on the potential role of this recently recognized prognosticators. We believe that the role of TNFRSF12A in invasion and motility of subclones in pre-cancerized SMs might be more meaningful if the expression of TWEAK is also studied.

5.3 Limitation

Due to relative small sample size (n = 34) in this study, in part caused by the inclusion criteria that only included OSCC relapse cases with histologically non-involved

mucosal surgical margins and non-availability of FFPE tissue blocks required, significant association could not be observed between expression of FSCN1, TNFRSF12A and Ki-67 and OSCC relapse in this study. Studies with larger cohort and equal size study and control group are required to validate our findings. Correlation with tumour staging, and survival analysis was not done in this study due to insufficient available data. Part of the sociodemographic data such as patients' habits was also missing. Discrepancy was also observed in the HPE reports, as some of the important information such as depth of invasion was not reported.

CHAPTER 6 : CONCLUSION

The expression of FSCN1, TNFRSF12A and Ki-67 in histologically non-involved mucosal surgical margins in the present study was not associated with relapse in OSCC. Although not statistically significant, expression of Ki-67 and FSCN1 in the study group was higher than in the control group. Unfortunately we were not able to draw a definite conclusion based on the decreased expression of TNFRSF12A in non-involved mucosal surgical margins of OSCC relapse cases. We strongly recommend future investigations to identify the definite role of TNFRSF12A in OSCC and the cascade of events related to its activation.

In our study, age of patients was able to predict OSCC relapse after having other clinicopathological prognosticators such as ethnicity, alcohol consumption and POI of tumour adjusted.

We recommend validation of role of KI-67, FSCN1 and TNFRSF12A in predicting relapse in OSCC as well as their association with clinicopathological prognosticators of OSCC and survival analysis in a larger cohort.

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