EXPRESSION OF KI-67, CORNULIN AND ISG15 IN NON-INVOLVED MUCOSAL SURGICAL MARGINS AS PREDICTIVE MARKERS FOR RELAPSE IN ORAL SQUAMOUS CELL CARCINOMA (OSCC)

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ABSTRACT

Introduction: Relapse in OSCC is often observed in histologically non-involved surgical margins. This indicates possible presence of field alteration in the non-involved surgical margins, which in turn leads to local recurrence or second primary tumour. The identification of specific biomarkers that could predict relapse of OSCC would help the clinicians in treatment planning for patients. **Objectives:** The objectives of this study were to evaluate the expression of Ki-67, Cornulin and ISG15 in the non-involved surgical margins and its association with clinicopathological prognosticators and relapse of OSCC. **Methods:** Immunohistochemistry was used in staining of non-involved mucosal surgical margins from study (relapse) group (n = 23), control (non-relapse) group (n = 32) and normal oral mucosa (n = 5) with Ki-67, Cornulin and ISG15. Association between expression of markers with clinicopathological prognosticators and relapse in OSCC was analysed statistically using Chi-square tests. Binary logistic regression analysis was used to determine predictors of relapse in OSCC. **Results:** In the study group, significant low expression of Cornulin (p = 0.032) and ISG15 (p = 0.047) was observed. Low expression of Cornulin was also significantly associated with relapse (p = 0.004) of OSCC and primary tumours involving non-tongue sites (p = 0.013). Surgical margins exhibiting high expression of Ki-67 was significantly reduced in female patients (p = 0.041). Clinicopathological prognosticators such as age above 57.5 years ($p < 0.001$), Chinese ethnicity (p = 0.009), Indian ethnicity (p = 0.007), alcohol use (p = 0.025), epithelial dysplasia in surgical margins (p = 0.045) and type III and IV pattern of invasion of tumour (p = 0.007) were significantly associated with relapse of OSCC. Binary logistic regression analysis showed decreased expression of Cornulin (p = 0.018) and increased patient's age (p = 0.008) were predictors of relapse in OSCC, with 34-fold risk and 18-

fold risk, respectively. **Conclusions:** Relapse of OSCC could be predicted by decreased expression of Cornulin in the non-involved surgical margins. Validation of role of Ki-67 and ISG15 in predicting relapse of OSCC would require larger cohorts. Taken together, Cornulin as a predictor in relapse of OSCC was suggested.

Keywords: OSCC, Relapse, Ki-67, Cornulin, ISG15

TAJUK: PENGEKSPRESAN KI-67, CORNULIN DAN ISG15 DI MARGIN MUKOSA PEMBEDAHAN BEBAS TUMOR SEBAGAI PENANDA RAMALAN KEBERULANGAN KARSINOMA MULUT SEL SKUAMUS

ABSTRAK

Tujuan kajian: Keberulangan karsinoma mulut sel skuamus (OSCC) sering berlaku di margin pembedahan bebas tumor. Ini menandakan perubahan medan berkemungkinan berlaku di margin pembedahan yang bebas tumor, dan seterusnya mengakibatkan keberulangan setempat atau tumor primer kedua. Pengenalan biomarker tertentu yang dapat meramalkan keberulangan OSCC akan membantu pegawai klinikal membuat perancangan untuk rawatan pesakit. **Objektif:** Objektif-objektif kajian ini adalah untuk menilai pengekspresan Ki-67, Cornulin dan ISG15 dalam margin pembedahan yang bebas tumor serta hubungannya dengan penunjuk-penunjuk prognostik klinikopatologi serta keberulangan OSCC. **Kaedah:** Margin pembedahan mukosa untuk kumpulan dikaji (dengan keberulangan) (n = 23), kumpulan kawalan (tanpa keberulangan) (n = 32) serta mukosa mulut normal (n = 5) diwarnakan dengan cara imunohistokimia untuk Ki-67, Cornulin dan ISG15. Hubungan antara pengekspresan Ki-67, Cornulin dan ISG15 dengan penunjuk-penunjuk prognostik klinikopatologi dan keberulangan OSCC dianalisis dengan ujian Chi-square. Analisis regresi logistik binari juga dijalankan untuk menentukan peramal keberulangan OSCC. **Keputusan:** Dengan signifikannya kumpulan dikaji menunjukkan pengekspresan Cornulin (p = 0.032) dan ISG15 (p = 0.047) yang rendah. Pengekspresan Cornulin yang rendah dan signifikan adalah juga berkaitan dengan keberulangan OSCC (p = 0.004). Di kalangan pesakit wanita, margin pembedahan yang menunjukkan pengekspresan Ki-67 yang tinggi adalah kurang tetapi signifikan (p = 0.041), manakala pengekspresan Cornulin yang rendah adalah dikaitkan dengan signifikannya bersama lokasi tumor utama OSCC selain daripada lidah (p = 0.013). Keberulangan OSCC juga berkaitan secara signifikan dengan penunjuk-penunjuk prognostik klinikopatologi seperti umur pesakit melebihi 57.5 tahun (p < 0.001), etnik Cina (p = 0.009), etnik India (p = 0.007), penggunaan alkohol (p = 0.025), displasia

epitelium di margin pembedahan (p = 0.025) dan corak serangan tumor jenis III dan IV (p = 0.007). Peramal keberulangan OSCC seperti pengekspresan Cornulin yang berkurangan (p = 0.018) menunjukkan 34 kali ganda risiko keberulangan OSCC, manakala peningkatan umur pesakit (p = 0.008) menunjukkan 18 kali ganda risiko keberulangan OSCC, seperti yang dihasilkan daripada analisis regresi logistik binari. **Kesimpulan:** Keberulangan OSCC dapat diramalkan oleh pengekspresan Cornulin yang rendah dalam margin pembedahan. Pengesahan peranan Ki-67 dan ISG15 sebagai peramal keberulangan OSCC memerlukan kumpulan kajian yang lebih besar. Secara keseluruhannya, Cornulin dicadangkan sebagai peramal keberulangan OSCC.

Kata-kata kunci: OSCC, Keberulangan, Ki-67, Cornulin, ISG15

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ASR	:	Age standardised rate
C1Orf10	:	Chromosome 1 Open Reading Frame 10
CCND1	:	Cyclin D1
CHEK2	:	Checkpoint kinase 2
CI	:	Confidence Interval
CNA	:	Copy number alteration
DAB	:	3,3'-diaminobenzidine
DNA	:	Deoxyribonucleic acid
ECS	:	Extracapsular spread
ED	:	Epithelial dysplasia
FFPE	:	Formalin-fixed paraffin embedded
FOM	:	Floor of mouth
GLOBOAN	:	Global Burden of Cancer
H&E	:	Haematoxylin and eosin
HNSCC	:	Head and neck squamous cell carcinoma
HPE	:	Histopathological examination
HPV	:	Human papillomavirus
IARC	:	International Agency for Research on Cancer
ICC	:	Intraclass correlation coefficient
ICD	:	International Classification of Diseases
IFN	:	Interferon
IHC	:	Immunohistochemistry
IL	:	Interleukin
IRS	:	Immunoreactive scores
ISG15	:	Interferon-stimulated gene 15
IT	:	Index tumour
LAMA5	:	Laminin Subunit Alpha 5
LI	:	Labelling index
LOH	:	Loss of heterozygosity
LR	:	Local recurrence
MEC	:	Medical Ethics Committee
MOCDTBS	:	Malaysian Oral Cancer Database and Tissue Bank System
MSI	:	Macrosatellite instability
MRC	:	Minimal residual cancer
NNN	:	Nitroso-nor-nicotine
NNK	:	4-(methylnitrosoamino)-1-(3-pyridyl)-1 butanone
NOM	:	Normal oral mucosa
OCRCC	:	Oral Cancer Research and Coordinating Centre
OH	:	Oral health
OPMD	:	Oral potentially malignant disorders
OSCC	:	Oral squamous cell carcinoma
PAH	:	Polycyclic aromatic hydrocarbons
PNI	:	Perineural invasion
POI	:	Pattern of invasion
qRT-PCR	:	Quantitative real time-polymerase chain reaction
ROC	:	Receiver operating characteristic
ROS	:	Reactive oxygen species

SCC	:	Squamous cell carcinoma
sd	:	Standard deviation
SEA	:	South East Asian
SFT	:	Second field tumour
SPT	:	Second primary tumour
TNF	:	Tumour necrosis factor
UADT	:	Upper aerodigestive tract
UK	:	United Kingdom
UPP	:	Ubiquitin/26S proteasome pathway
USA	:	United States of America
UVR	:	Ultraviolet radiation
VGFR	:	Vascular endothelial growth factor
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Recent epidemiological data shows oral cancer accounts for 300373 new cases and 145353 cancer deaths in 2012 globally. More than half of these oral cancers (estimated 168850 cases) were diagnosed in Asia (Ferlay et al., 2015). Throughout the world, oral cancer, grouped together with oropharyngeal cancer, is the sixth most common cancer (Warnakulasuriya S., 2014).

Incidence of oral cancer varies markedly within specific geographical regions around the world. The areas with high incidence rates for oral cancer are found in Southern Asia (India, Pakistan, Sri Lanka, Taiwan and China), eastern and western Europe (e.g. Hungary, Slovakia, Slovenia and France), Latin America, and the Caribbean (e.g. Brazil, Uruguay and Puerto Rico). Oral cancer incidence is usually higher among males (5.5 cases per 100000 populations ~~-~~ per year) than females (2.5 cases per 100000 populations~~)~~; however, the distribution is reversed in India and Thailand (El-Naggar et al., 2017).

Most oral cancers cases occur in the fifth and sixth decades of life (El-Naggar et al., 2017). In Malaysia, the incidence of oral cancer is higher among the Indian ethnic population and is highest in Indian females where the age standardised rate (ASR) is 10.2/100,000 among Indian females. More recently, 667 new cases of oral and lip cancer and 327 deaths have been reported in Malaysia (IARC, 2019).

According to the National Cancer Registry (2007), oral cancer may involve any intraoral sites. It commonly occurs on the buccal mucosa, tongue, floor of the mouth and lips (Omar & Tamin, 2011). The site of oral cancer incidence is determined by the high-risk habits of the populations (El-Naggar et al., 2017). In Asian countries such as India and Thailand, tongue, buccal mucosa and gingiva are the most common sites of oral cancer (Gupta et al, 2016). Oral squamous cell carcinoma (OSCC) is a carcinoma with squamous differentiation arising from the oral mucosal epithelium and is the most

common form of oral cancer. More than 90 percent of cancers in the oral cavity are OSCCs (El-Naggar et al., 2017). OSCC accounts for 24% of all malignancies in the upper aerodigestive tract (UADT) (Carvalho et al., 2003). Among Asian populations, OSCC most commonly affects the buccal mucosa due to betel-quid and tobacco chewing habits (El-Naggar et al., 2017).

Common risk factors in the development of OSCC include smoking, betel-quid and tobacco chewing as well as excessive alcohol consumption. A combination of smoking and alcohol consumption induces synergistic effects in promoting OSCC development (Salaspuro & Salaspuro, 2004). Use of all forms of tobacco (smoking, chewing and dipping) has been associated with OSCC, contributing to over 85% of oral cancer deaths among men in the industrialised countries (Johnson, 2001). As smoking rates decline, the incidence of intraoral cancer is found to be decreasing in some countries (El-Naggar et al., 2017). In certain populations, tobacco chewing and betel-quid chewing are important risk factors for oral cancer, especially in Southeast Asian countries. A study conducted by Zain et al. (1999) has shown that betel quid chewing is a major factor for developing oral premalignant lesions in the Malaysian population. In 2007, the International Agency for Research on Cancer (IARC) concluded that Human Papillomavirus particularly type 16, is a causative factor in the development of oropharyngeal cancer but the association is only seen in a small minority (3%) of OSCC cases (Gillison et al., 2015).

Risk factors especially alcohol and tobacco use have been reported to lead to genetic variation in tumour suppressor genes such as p53, oncogenes such as Ras and many other genes which control cellular processes. These alterations are associated with genetic instability due to defective chromosomal segregation, copy number alteration (CNA), loss of heterozygosity (LOH), telomerase stabilities, regulation of cell cycle checkpoints and DNA damage repairs (Ali et al., 2017). Genetic markers such as LOH, chromosomal instability and mutation in TP53 gene can be detected by immunohistochemistry, in situ

hybridisation and DNA amplification techniques. (Braakhuis et al., 2003)

Conventional treatment for OSCC includes surgery, radiotherapy, and chemotherapy. Surgical management, which is the most common treatment for OSCC, often leads to severe morbidity due to disfiguring and functional side effects (Furness et al., 2011). Surgery combined with chemotherapy and radiotherapy can improve overall survival, particularly in patients with advanced oral cancers. Induction chemotherapy may prolong survival by up to 20% and adjuvant concomitant chemoradiotherapy can improve survival by up to 16% (Furness et al., 2011). However, approximately one-third of patients treated with surgery and adjuvant therapy will experience local or regional recurrence and/ or distant metastasis (Greenberg et al., 2003). In general, a five-year survival rate of 50% is seen among patients with OSCC. Over the years, there has been an improvement in OSCC survival rate; however, this increase is not prominent. For example, the 5-year survival rate recorded for OSCC in Netherlands moderately improved from 56% to 62% from 1989 - 1994 to 2007 – 2011 (Braakhuis et al., 2014). Survival rate depends on tumour size, nodal involvement, and success of initial treatment where an incompletely resected primary tumour might lead to recurrence or development of new tumour (Meyyappan et al., 2015). Other reported factors that would affect the recurrence of OSCC include staging of tumour, tumour site, histological grade, pattern of invasion and perineural invasion (Camisasca et al., 2011).

Relapse is defined by Gleber-Netto et al. (2015) as "the return of a disease after treatment". Among OSCC patients, frequency of relapse is as high as 63.6%. It is also shown that relapse in the oral cavity is associated with higher mortality rate than in other head and neck sites (Agra et al., 2008). Relapse in OSCC can be categorised into local recurrence (LR) where the tumour cells are not completely removed during surgery and regrow, and second primary tumour (SPT) where there is an independent carcinogenesis leading to development of new tumour (Braakhuis et al., 2002). For LR in OSCC, it is

defined by Braakhuis et al. as a tumour that develops within three years and at less than two centimetres distance of the primary tumour. LRs not fulfilling these criteria are known as SPT.

Surgical margins which are clear or not involved by tumour cells are related to a lower recurrence rate. Hence, the main goal in an OSCC resection is to achieve clear margins while sparing as much normal tissue as possible to preserve the functions. On the other hand, presence of epithelial dysplasia at the mucosal resection margins although tumour cells are not evident, is also associated with less satisfactory prognosis. Most authors have considered that moderate and severe epithelial dysplasia at inked resection margins have biological significance similar to that of early invasive carcinoma (Shah A. K., 2018). However, the significance of mild epithelial dysplasia is still questionable (Shah A. K., 2018), although it has been suggested that presence of mild epithelial dysplasia in surgical margins of OSCC resection is risky for a local recurrence (Weijers et al., 2002).

Field cancerisation, which is also known as field effect or field defect, is characterised by molecular alteration in the surgical margins. The concept of field cancerisation was introduced by Slaughter et al. in 1953. It is postulated that a field refers to the presence of one or more areas consisting of epithelial cells that have genetic alterations although it appears normal histologically. This phenomenon has been reported in many cases where recurrence of tumour as well as development of second primary tumour have taken place (Shah A. K., 2018). Field cancerisation is not seen in a conventional histopathological assessment using a standard light microscope; hence this necessitates a more reliable and predictive method for examination of resection margins. Therefore, research targeting towards detection of molecular and genetic alterations in histologically non-involved margins has been carried out (Shah A. K., 2018).

Molecular studies have also been done on tumour-adjacent histologically normal tissue to evaluate field cancerisation. Studies have shown that in one third of oral and

oropharyngeal cancer cases, tumour-associated genetic alterations are found in histologically normal surgical margins, which indicate that the genetically altered field remained in patients although the lesional tissues have been sufficiently removed (de Carvalho et al., 2012). More recent studies have shown different types of molecular alterations in tumour-free OSCC surgical margins and association with recurrence, these include LOH at 9p21 and 17p13 (Wang et al., 2016), LOH at 1q21.3 (Salahshourifar et al., 2015), amplification of proto-oncogene c-myc (Wang et al., 2017) and amplification of ISG15 gene due to CNA of DNA (Vincent-Chong et al., 2012).

Tumour markers have a role in the diagnosis, prognosis, formulating treatments and in the detection of recurrence of cancer (Prasad et al., 2013). Several studies have been carried out to investigate useful tools or markers to predict relapse in OSCC. For example, Oliveira et al. (2010) has shown that there is significant association between p53 expression and tumour recurrence in mucosa and invasive front of head and neck squamous cell carcinoma (HNSCC). Increased expression of p53 and elF4E markers in surgical margins is associated with recurrence in OSCC (Singh et al., 2016). Markers such as S100A2, Claudin-7 and E-cadherin have also been studied for local recurrence of OSCC (Melchers et al., 2015; and Gonzalez-Moles et al., 2000). Decreased expression of Cornulin (Salahshourifar et al., 2015) and increased expression of ISG15 (Vincent-Chong et al., 2012) markers in surgical margins are also related with recurrence of OSCC.

Given these facts, the purpose of this study was to determine the presence and effect of field cancerisation in OSCC based on the surgical margins.

1.2 Aims

The aim of this study was to investigate the presence of genetically altered epithelial cells that are at risk for malignant transformation in histologically non-involved surgical margins of OSCC.

1.3 Specific objectives

The specific objectives of this study were:

1. To evaluate the expression of Ki-67, Cornulin and ISG15 in histologically non-involved surgical margins of OSCC.
2. To evaluate the association between clinicopathological prognosticators that promote the relapse of OSCC and the expression of Ki-67, Cornulin and ISG15 in histologically non-involved surgical margins of OSCC.
3. To evaluate the association between expression of Ki-67, Cornulin and ISG15 in histologically non-involved surgical margins of OSCC and relapse in these patients.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of oral cancer

Oral cancer is a type of head and neck cancer and refers to any cancerous tissue growth located in the oral cavity (Werning JW, 2007). More than 90% of cancers in the oral cavity are oral squamous cell carcinomas (OSCCs). OSCC is a carcinoma with squamous differentiation arising from the oral mucosal epithelium and is the most common form of oral cancer (El-Naggar et al., 2017).

2.2 Epidemiology of oral cancer

Oral cancer is a significant public health threat with emergence of 350000 new cases annually worldwide. More than half of all oral cancers in the world occur in Asia where an estimated 168,850 new cases were diagnosed in this geographical region alone. Of these, approximately 11% were from South East Asia (SEA) region where the incidence of oral cancer has been regarded as disturbingly high for many years (Cheong et al., 2017). Globally, oral cancers (ICD, 10th edition C00-08) accounted for 300,373 new cancer cases and 145,353 cancer deaths in 2012 (Ferlay et al, 2015).

Most oral cancer cases occur in individuals aged 50 to 70 years. However, slight increase in oral cancer incidence among younger populations has been reported in countries such as United States of America and United Kingdom (El-Naggar et al., 2017). According to a study by the British Dental Association earlier in year 2000, approximately six percent of oral cancers were diagnosed in younger patients aged less than 45 years old. In India, oral cancer usually occurs prior to the age of 35 years which is mainly due to early practice of tobacco chewing (Johnson, 2001). In Malaysia, a gradual increase in oral cancer prevalence was observed in individuals aged 40 years old and above among males and females (Omar & Tamin, 2011).

Globally, while oral cancer is predominantly diagnosed among males, it can be as common or even more common in females than in males, in many SEA populations. This is attributed to increased usage of smokeless tobacco and betel quid chewing among

women in countries such as Thailand, Laos and Malaysia (Cheong et al., 2017). A mortality rate of 1.9 deaths per 100000 population per year was estimated by the Global Burden of Cancer (GLOBOCAN) in 2012 (El-Naggar et al., 2017). The mortality to incidence ratio in SEA is among the highest in Asia, and in 2012, the mortality due to oral cancer in SEA was estimated as 8508 cases, where 5014 and 3494 were men and women, respectively. However, as cause-specific mortality is poorly documented, in most countries in SEA, the numbers are likely under-reported (Cheong et al., 2017).

2.3 Aetiology of oral cancer

2.3.1 Smoking/ tobacco usage

By far, smoking is the most important aetiology of oral cancer. Use of all forms of tobacco (smoking, chewing and dipping) have been associated with OSCC, contributing to over 85% of oral cancer deaths among men in the industrialised countries (Johnson, 2001). Globally, it was shown that oral cancer risk for smokers is ten times higher than for non-smokers (Warnakulasuriya et al., 2005), while a case-control study showed that the odds for heavy smokers getting oral cancers stood at a ratio of 20.7 (Rodriguez et al., 2004). Risk of smokeless tobacco in causing oral cancer is somehow controversial, especially in those consuming Swedish snuff (El-Naggar et al., 2017). In Asian countries such as India, Thailand and Malaysia, tobacco is also mixed with areca nut and/ or other substances, wrapped in a betel leaf and betel quid is left in contact with the oral mucosa apart from chewing (Cheong et al., 2017). In Malaysia, smoking habit is most prevalent in the Malay ethnic group. However, an almost similar distribution of tumours of the tongue and buccal mucosa was observed amongst the Malays, notably due to their inclination towards the habits of both smoking betel quid chewing (Ghani et al., 2018). Among the reasons why tobacco smoking could lead to oral cancer is the presence of more than 300 different carcinogens in tobacco smoke, the major contributory ones being polycyclic aromatic hydrocarbons (PAH), benzo-α-pyrene, tobacco specific nitrosamines

including nitroso-nor-nicotine (NNN) and 4-(methylnitrosoamino)-1-(3-pyridyl)-1 butanone (NNK). These carcinogens act as DNA adducts that stimulate oral mucosal epithelium which harms the chromosomes and led to DNA mutations (IARC, 2004). Smoking and duration of smoking cessation with risk of developing OSCC is dose dependent (El-Naggar et al., 2017).

2.3.2 Alcohol consumption

Based on the findings by the International Agency for Research on Cancer (IARC), excessive and regular alcohol intake increased the risk of oral cancer (IARC, 2004). A combination of smoking and alcohol consumption would be able to induce synergistic effects in promoting OSCC development. This accounted for more than 75% of oral cancer cases reported in developed countries (Rodriguez et al., 2004). Acetaldehyde, which was a primary metabolic product of ethanol, was shown to be carcinogenic by IARC. In the oral cavity, extrahepatic metabolism of alcohol to acetaldehyde is evident. There are enzymes in the oral cavity that allows accumulation of acetaldehyde in oral tissues. Subsequently, Kurkivuori et al. (2007) proposed that common oral bacteria such as *Streptococcus salivarius*, *S. intermedius* and *S. mitis* is able to produce high amounts of acetaldehyde, which is detected in human saliva, hence predisposing to oral cancer. This in turn explained the mechanism of carcinogenesis among individuals with poor oral hygiene. Warnakulasuriya et al. (2008) had carried out a pilot immunohistochemistry (IHC) study that assessed alcohol- induced changes to the oral epithelium in OSCC and dysplasia patients. Generation and subcellular distribution of ethanol- induced DNA-protein alteration was studied. These proteins were shown to present in oral epithelial cells in patients with OSCC and history of alcohol abuse, hence ethanol- induced carcinogenesis was proven.

2.3.3 Betel quid chewing

Betel quid, which was also known as "pan" or "paan", was composed of betel leaf, areca nut and slaked lime, and may contain tobacco. The preference on content of betel quid varies among geographical regions of the world, for example in India and neighbouring countries, dry areca nut pieces or tobacco may be chewed alone, as a mixture of areca nut, tobacco and slaked lime, or tobacco and slaked lime. In the south-eastern part of China, unprocessed fresh areca nut is treated with maltose and lime, cut into pieces and chewed with a few drops of cassia oil (IARC, 2004). Some individuals would place betel quid in the mouth to let it remain in contact with the oral mucosa or slowly sucked it rather than chewing it. Regardless of this, it is still considered as betel quid chewing habit (IARC, 2004).

Betel quid chewing habit is an important risk factor for oral cancer, especially in Asian countries for example India, Thailand, Taiwan and Malaysia. In areas like north-east of Thailand, betel quid chewing is the strongest risk factor for oral cancer and the habit is popular among the females and youngsters. Hence the incidence of OSCC among women is increasing. This finding was in contrast with studies based in other regions of the world, where there were more oral cancer cases seen among the males (Cheong et al., 2017). In Malaysia, betel quid chewing is a major factor for developing oral premalignant lesions in the Malaysian population. The habit is commonly practised by the Indian community, elderly Malays, and the indigenous people from peninsular and east Malaysia (Ghani et al., 2018). Anyhow, the mechanism of oral carcinogenesis due to betel quid chewing has not been clearly elucidated yet.

Regular chewing could induce chronic irritation and inflammation, which would damage the mucosal epithelium. Arecoline, the primary alkaloid of areca nut, had shown to induce pro-carcinogenic alterations including nitrosamines and reactive oxygen species (ROS) production, modulation of matrix metalloproteinases, inhibition of collagenase,

upregulation of heat shock proteins and increased release of inflammatory cytokines such as IL-1β, IL-6 and TNF-α. These would lead to genetic instability and initiate carcinogenesis through structural changes in the oral mucosa which further allows other betel quid compounds to diffuse through (Hernandez et al., 2017).

2.3.4 Human Papillomavirus

Approximately 20% of oral cancers are thought to be attributable to Human Papillomavirus (HPV) infection. In 2007, the International Agency for Research on Cancer (IARC) concluded that HPV particularly type 16, is a causative factor in the development of oropharyngeal cancer but was only seen in small minority (3%) of OSCC cases (Gillison et al., 2015). In 2010, according to Marur et al. (2010), HPV type 16 and 18 were identified as high-risk oncogenic HPV types which were considered as a major risk factor in oropharyngeal cancers. Prevalence of high-risk HPV in oral cancer varies in between continents, with majority of cases observed in Asia (33.77%) followed by USA (19.65%) and Europe (16.19%). Highest number of HPV-positive oral cancer cases is involving the tongue (50%) followed by palate (42%) (Yete et al., 2017). HPV positivity in oral cancers has been associated with prognosis, younger age of onset, and reduction in tobacco habits in several developed countries (O'Rorke et al., 2012). Mechanism of HPV in oral carcinogenesis is unclear. It was observed that HPV infected cells lacked mutations attributed to traditional risk factors such as smoking and alcohol consumption. E6 and E7 oncoproteins from high-risk HPV inactivates tumour suppressor genes such as p53 and pRb, and upregulated cellular proteins (Ras, Myc, p16, NF-kB and AP-1) Loss of p53-mediated apoptosis increases cell proliferation, immortalisation and malignant transformation (Yete et al., 2017).

2.3.5 Sunlight

Sunlight exposure is a well-known risk factor for lip cancer, especially in Western Australia. Lip cancer accounts for as many cases as all intraoral sites together (El-Naggar

et al., 2017). Ultraviolet radiation (UVR) is a risk factor for cancer especially the skin. Mechanisms of carcinogenesis by UVR include DNA damage, mutagenesis, immunosuppression, and interaction with viruses such as HPV. It is observed that HPV E6 and E7 proteins may impede the repair of UVR-induced DNA damage in HPV-infected cells (Schwarz, T., 2005). A recent study found that UVR exposure was significantly correlated with incidence of oral, pharyngeal, and cervical cancer in 16 states in USA (Godar et al., 2014). In contrast to this, Adams et al. (2016) observed an inverse association between UVR exposure and incidence rates of oral, pharyngeal and cervical cancer and melanoma in 18 registered regions for instance Hawaii, New Mexico, Los Angeles etc., under the National Cancer Institute's Surveillance, Epidemiology, and End Results programme (SEER) of USA.

2.3.6 Poor oral health

World Health Organisation (2003) defined oral health as "a state of being free from chronic mouth and facial pain, oral and throat cancer, oral infection and sores, periodontal (gum) disease, tooth decay, tooth loss, and other diseases and disorders that limit an individual's capacity in biting, chewing, smiling, speaking, and psychosocial wellbeing". Poor oral health (OH) has been associated with oral cancer; however, it has not been proven to be an independent risk factor (El-Naggar et al., 2017). Lately, in a systematic review, Mathur et al. (2018) had identified factors contributing to poor OH which included irregular teeth brushing habits, lack of dental visits, poor socioeconomic status, lower level of education, tobacco, and alcohol consumption. Poor OH might not directly cause OSCC but it could catalyse carcinogenesis. For example, one of the main pathogens causing acute periodontitis, *Porphyromonas gingivalis*, has been reported to promote the invasion and metastasis of oral cancers. The bacteria played a role in oral carcinogenesis and metastasis by activation of promatrix metalloproteinase, and by anergy and apoptosis of activated T cells (Galvao-Moreira & da Cruz, 2016).

2.3.7 Diet

Daily diet rich in fruits and vegetables is thought to have some protective effect against oral carcinogenesis (El-Naggar et al., 2017). It is well established that high consumption of fresh vegetables, fruits, fish and seafood could protect against oral cancer (Chen et al., 2017). Vitamins A, E, C and beta carotene found in fruits and vegetables have important antioxidant properties which include neutralisation of metabolic products, inhibition of chromosomal aberration as well as interference in activation of procarcinogens (Giovannelli et al., 2002). In a case-control study by Edefonti et al. (2010) in Italy, it was shown that among oral cancer patients (n = 804), diets rich in animal origin and animal fats are positively, and those rich in fruit and vegetables and vegetable fats are inversely related to oral and pharyngeal cancer risk. High intake of red and processed meat is also associated with increased risk of oral cancer (Toporcov et al., 2004).

2.4 Treatment and survival of oral cancer

Conventional treatment for OSCC includes surgery, radiotherapy, and chemotherapy. Surgical management, which is the most common treatment for OSCC, often lead to severe morbidity due to disfigurement and functional side effects. Surgery combined with chemotherapy and radiotherapy could improve overall survival, particularly in patients with advanced OSCC (Furness et al., 2011). Yanamoto et al. (2012) also showed in their study that neoadjuvant chemotherapy was a predictor of recurrence in OSCC. However, approximately one-third of patients treated with surgery and adjuvant therapy would still experience local or regional recurrence and/or distant metastasis (Greenberg et al., 2003). Success of treatment is multifactorial; apart from patients' existing medical conditions such as heart problem and diabetes as reported by Vazquez-Mahia et al. (2012), it also depended on multiple clinicopathological prognosticators.

2.4.1 Gender

OSCC is known to affect more males than females with an approximate ratio of 1.5:1, respectively. Nearly a quarter of the newly diagnosed cancers in males from Sri Lanka, India, Pakistan and Bangladesh are located in the head and neck region (Jerjes et al., 2017). However, number of female OSCC patient have increased in certain regions, likely due to practice of risky habits, for instance increased prevalence of OSCC among females in northern Thailand was due to prominent betel quid chewing habit (Cheong et al., 2017).

2.4.2 Age at first OSCC

About 6% of oral cancers occur in young people under the age of 45 years (Jerjes et al., 2010). A study comparing the relative survival of young people (under 45 years of age) with oral cancer compared with the survival of older people (45 years and older) showed a higher five-year relative survival among young people compared with the older group. Younger patients responded better to treatment of OSCC (Warnakulasuriya et al., 2007). Worse treatment outcome and survival was related to older patients due to comorbidities (Moye et al., 2015).

2.4.3 Primary tumour site

The most commonly reported oral cancer sites include the floor of the mouth (FOM) and lateral borders of the tongue. The tongue was the most common (40-50%) site for OSCC in European and American population. Asian population usually suffered from cancer of the buccal mucosa due to betel quid/tobacco chewing habits; Buccal mucosa squamous cell carcinoma (SCC) constitute 40% of OSCC in Sri Lankan population (Jerjes et al., 2010).

2.4.4 Tumour size

The tumour size usually affects choice and outcome of treatment where increased tumour size was associated with cervical involvement, high recurrence rate and poor prognosis (Woolgar et al., 2006). It also affected the surgeon's ability to achieve complete

resection, especially in deep invading tumours and clearance of surgical margins. However, Larsen et al. (2009) did not find association between tumour size and nodal involvement.

2.4.5 Depth of invasion

Depth of invasion was defined as distance between normal mucosal surface and the deepest point of invasion. In a meta-analysis, sixteen relevant studies were examined for the cut-off tumour thickness points (3, 4, 5 and 6 mm); there was a statistically significant difference between the 4 mm and 5 mm tumour thickness cut-off points and cervical lymph node involvement in OSCC (Woolgar J.A., 2006). It is now widely accepted that tumour thickness is more accurate predictor of sub-clinical nodal metastasis, local recurrence and survival than tumour size (Woolgar J.A., 2006). The average depth of lesion among those who had recurrence was 8.3 mm, in comparison to only 6 mm in those without recurrence.

2.4.6 Lymph node involvement

Size and multiplicity of lymph nodes are taken into account when assessing prognosis from cancer. The incidence of occult metastases to the neck could range from 15% to 60% depending on the different diagnostic procedures adapted. Clinically, lymph nodes are assessed for location, number, size, shape, consistency and fixation. Lymph nodes are considered to be malignant if their size is greater than 1 cm, and particularly if they are firm and fixed. Postoperative evaluation of the tumour specimen allows pathological staging (Warnakulasuriya S., 2014). The presence of nodal metastasis is the most important prognostic factor for oral cancers Approximately 50% reduction in five-year survival rate was seen with the development of lymph node metastasis in patients with OSCC. Contralateral neck metastasis may be associated with higher distant metastasis as spread of tumour across the midline confirms aggressive behaviour of OSCC (Tankere et al., 2000). Extracapsular spread (ECS) (microscopic or macroscopic) was related mostly

to prognosis. It was recommended that microscopic ECS should be incorporated into pathological staging system as the capsular rupture showed the most significant prognostic influence (Woolgar et al., 2003).

2.4.7 Tumour staging

The 'TNM' classification of the International Union Against cancer (UICC) relates well to the prognosis and overall survival, earlier the tumour stage, better the prognosis and less complicated was the treatment (Sharma et al., 2016). A logistic regression analysis revealed that higher the pathological TNM stage, worse the prognosis (Jerjes et al., 2010). There was a growing concern that TNM staging is insufficient to accurately map or classify OSCC, whose biological impact may be related to volume and pathological aggressiveness of disease (Woolgar J.A., 2006).

2.4.8 Differentiation of tumour

There was consistent evidence of the value of tumour grade in determining prognosis: Higher grades of OSCC gave poorer prognosis. Grading was based on the degree of resemblance of the invading carcinoma to the normal epithelium and its ability to form keratinising islands (Warnakulasuriya S., 2014). The most aggressive area (at × 100 magnification field) was graded as well, moderately or poorly differentiated. Most OSCCs are moderately differentiated. However, the system suffers from inter-examiner variability and sampling errors.

2.4.9 Invasive front and pattern of invasion

An infiltrative margin, as opposed to a smooth pushing margin, has been shown to be an adverse prognostic feature in the tongue, the supraglottis and the FOM. More cells at the invasive front were proliferating compared to the centre, confirming that this part of the tumour was likely to be more informative in determining the prognosis (Dissanayaake et al., 2003). However, a study on 68 OSCC patients confirmed that the pattern of invasion (POI) was not significantly related to local recurrences (Weijers et al., 2004).

2.4.10 Lymphovascular and nerve invasion

Lymphovascular and peri-/intraneural invasion showed a significant association with tumour size, histological grading, pattern of invasion, nodal involvement, status of the surgical margins, overall prognosis and survival (Scully & Bagan, 2009). It has been proposed that tumour emboli were more difficult to form in the small channels of superficial areas than in the wider lymphatics of deep tissue, hence tumour thickness may play a vital role in lymphovascular invasion (Fagan et al., 1998).

2.4.11 Bone and cartilage invasion

Bone and cartilage invasion affected prognosis of OSCC. It was previously suggested that a T4N0 OSCC of gingiva or alveolar ridge showed better prognosis than the other stage IV categories, as risk of nodal metastases in these sites was low (Woolgar J.A., 1999). However, it was later observed that an infiltrative, but not an erosive POI predicted local recurrence and survival, hence it was suggested by the authors that an infiltrative bone invasion should be a prerequisite for pT4 status (Woolgar et al., 2006). Study by Jerjes et al. (2010) had shown an association between mandibular cortical plate invasion and locoregional metastasis in OSCC patients.

2.5 Surgical margins

The goal in a surgery for OSCC is to obtain an optimal clearance of the tumour while sparing as much normal tissue as possible to preserve function and limit morbidity. Several studies have revealed that obtaining a clear margin in surgical resection was related to a lower recurrence rate (de Carvalho et al., 2012; Meyyappan et al., 2015). The UK Royal College of Pathologists have issued the latest version of standard and datasets for histopathology reporting of mucosal malignancies of the oral cavity, by Helliwell & Woolgar (2013). According to the standards, surgical margins were designated as clear when it was > 5 mm away from tumour, close margin when 1 – 5 mm away from tumour and < 1 mm from tumour would be involved. Additionally, the authors also reported that

incomplete resection or the presence of epithelial dysplasia at the margin was associated with a significantly increased risk of local recurrence. Margin status is a predictor of recurrence and may require consideration of adjuvant therapy (Helliway & Woolgar, 2013; Warnakulasuriya S., 2014). Failure to achieve a clear surgical margin would result in an increased risk of local recurrence (LR) and subsequently reduced chance for survival. Therefore, the importance of obtaining histologically clear surgical margins has been a foundation for surgical treatment of all OSCC. However, surgical margins should not be considered as clear when there is microscopic evidence of tumour or moderate or severe ED at the surgical margins; these margins should be considered involved (Shah, A.K., 2018).

2.6 Oral epithelial dysplasia

Conventional histopathological examination for the presence of oral epithelial dysplasia (ED) is considered as gold standard in predicting malignant transformation. In oral ED, cells of normal oral epithelium are replaced by cells showing immature morphology with a resemblance to cells usually seen in malignancy. Presence of ED in oral mucosa indicates a risk of malignant transformation especially in oral potentially malignant disorders (OPMD), positive correlation was also observed between increased severity of ED and malignant transformation. However, non-dysplastic lesion may also transform (Speight et al., 2017).

Traditionally oral ED has been graded into mild, moderate and severe ED by taking into account a combination of architectural and cytological changes in the involved epithelium (El-Naggar et al., 2017). One major limitation of using existing histological criteria for dysplasia to predict neoplastic transformation potential would be the inherent subjectivity of the grading system itself. Multiple studies have demonstrated low-to-moderate inter-observer consensus for dysplasia grade among experienced oral pathologists. Hence, a binary classification (low/ high risk) system for grading oral ED

was proposed by Kujan et al. (2006) to improve inter-observer agreement between pathologists. However, the biological significance of this system needs to be investigated in longitudinal studies to ascertain its value in the prediction of malignant transformation risk of OPMD.

The clinical impact of ED at the surgical margin is controversial. Sopka et al. (2013) reported that the presence of moderate or severe ED at the margins is strongly correlated to worse disease-free survival. In a report by Gokavarapu et al. (2014), it was stated that ED involving the surgical margin is an important clinical finding indicating field cancerisation and influencing survival in association with a history of tobacco use. However, the prognostic importance of mild and moderate ED at the margins is relatively unknown.

A premalignant field may need a longer time to progress into a new tumour than a tumour that develops from remaining tumour cells. An oral premalignant lesion might need up to 67 or 96 months, respectively, to progress to invasive cancer (Tabor et al., 2001).

2.7 Relapse in oral cancer

According to Gleber-Netto et al. (2015), relapse is defined as the return of disease after treatment. Relapse can be categorised into local recurrence (LR) and second primary tumour (SPT). Among OSCC patients, frequency of relapse is as high as 63.6% while the frequency among head and neck SCC patients ranges from 16% to 52%. It was also shown that relapse in the oral cavity was associated with higher mortality rate than in other head and neck sites (Agra et al., 2008). Relapse in OSCC is multifactorial, it is associated with patient's sociodemographic characteristics and clinicopathological prognosticators, for example age and comorbidities (Moye et al., 2015), practising of high-risk habits (Ghani et al., 2018) and pattern of invasion of tumour (Camisasca et al., 2011).

2.7.1 Field cancerisation

Relapse in OSCC is related to the concept of field cancerisation introduced by Slaughter et al. (1953), where it was described as presence of grossly normal but histologically abnormal tissue surrounding a cancerous lesion, attributed to exposure of mucosa to carcinogenic agents. the carcinogenic agents had preconditioned the oral mucosa and produced irreversible changes. As exposure to carcinogenic agents did not happen at the same time, therefore the affected field may break down at different time intervals to produce cancer at multiple locations. This also explained the situation where cancer suddenly developed on normal oral mucosa. Lateral expansion of the field was observed and that was why local recurrence still happened postoperatively. Due to unclear definition of field cancerisation, Braakhuis et al. (2005) proposed a new definition for field cancerisation as presence of one or more fields consisting of epithelial cells that have genetic alteration that are irrefutably linked to the process of carcinogenesis. A field does not show invasive growth, the hallmark criterion for oral cancer. Size of a field could be > 7 cm away from tumour (Braakhuis et al., 2005).

2.7.2 Local recurrence

A clinical classification of local recurrence (LR) and second primary tumour (SPT) was proposed by Braakhuis et al. (2002). For LR in OSCC, it was defined as a tumour that developed within three years and at a distance < 2 cm from the index tumour (IT). After surgical removal of an HNSCC, patients had a considerable risk for developing a relapse at histologically tumour-free margins due to relatively small number of cancer cells that remained at the margins. These tiny cells were not able to be detected using light microscope and have been designated as minimal residual cancer (MRC). Molecular study in this context was to determine the clonality of the cells as MRC was expected to show similar genetical aberration with the IT. Clinically MRC aided in treatment planning if patient developed a relapse. Presence of MRC mandated postoperative radiotherapy or

re-excision in view of high risk of local recurrence (Braakhuis et al., 2005). Another concern regarding MRC was presence of dysplasia at the surgical margins. A severe dysplasia or carcinoma-in-situ was often considered an indication for further treatment.

2.7.3 Second primary tumour

Patients with OSCC are at high risk of developing a second primary tumour (SPT). In a retrospective study on 727 patients with OSCC, the prevalence of at least one SPT in the respiratory and upper digestive tract was 10%. Patients were found to be at risk for an SPT at a rate of 2.8% per year during at least 10 years. Apart from that, patients with an IT in the inferior part of the oral cavity (FOM, lower alveolus) and habits such as smoking or alcohol consumption, were at higher risk of developing an SPT (Jovanovic et al., 1994). SPT was defined earlier by Warren & Gates (1932) as follows: i) each of the tumours must present a definite picture of malignancy; ii) each of the tumours must be distinct; and iii) the probability of one being a metastasis of the other must be excluded. Histological examination often showed the tumour was malignant, but with this method, it was difficult to prove that the lesions were distinct. To exclude the possibility of a LR, an SPT was defined as having a distance of at least 2 cm between the IT and the SPT, and should have occurred at least three years after the diagnosis of the IT. SPTs could be divided into synchronous SPTs, which developed simultaneously within 6 months after the IT, and metachronous SPTs, which developed six months after the IT. However, most SPTs were metachronous and developed during follow-up of HNSCC patients after curative treatment of the first tumour.

Based on the new classification proposed by Braakhuis et al. in 2005, when a SPT showed unrelated genetic pattern, it was considered a true SPT; it was known to be a secondary field tumour, when the genetic pattern of the lesion and the underlying field was similar. SPT often have a negative impact on the prognosis and survival in OSCC patients. Marcos et al. (2007) reported a significantly lower five-year survival in patients

treated for an SPT in the head and neck region. As an SPT was able to arise in distant sites such as oesophagus and the lungs, specialised radiography techniques such as computed tomography, magnetic resonance imaging and positron emission tomography had been used extensively to detect a SPT distant from IT (Ashwini et al., 2015).

2.7.4 Secondary field tumour

This concept was proposed by Braakhuis et al. (2005). A secondary field tumour (SFT) was defined as a tumour that had developed from the same field as the IT. This could arise from LR or SPT based on the clinical classification, and it showed some similarity in genetic alteration with the IT. Fields with genetically altered cells could be up to 7 cm in diameter and are not visible to the surgeons (Tabor et al., 2002). These facts explained how a field was often left behind when an HNSCC was resected. The presence of a field with genetically altered cells was likely to be a continuous risk factor for another carcinoma. Indeed, evidence was available to show that cancer has developed from fields that remain in patients after surgery of the initial carcinoma. Various mechanisms have been proposed to explain the common clonal origin of these tumours, such as shedding of preneoplastic cells into the saliva and implantation at other sites and lateral migration of isolated preneoplastic cells (Bedi et al., 1996). However, due to the contiguous nature of the field(s), this would be the most possible explanation for development of an SFT. In an event of SFT, frequent surveillance with diagnostic biopsy, molecular assessment and possible chemoprevention is suggested (Braakhuis et al., 2005).

There are a few potential chemotherapeutic agents, a commonly used one is 13 cis-retinoic acid. It has been shown to upregulate the retinoic acid receptor β, leading to a good clinical response in head and neck premalignant lesions. High doses of 13 cis-retinoic acid has led to a regression in leukoplakia and prevention of SPT. However, despite clinical regression of premalignant lesions, genetic alterations in mucosal fields

remain unchanged. This indicated that targeted therapy with ability of repairing genetic damage in altered cells is needed (Alok et al., 2014).

2.8 Tumour markers

Tumour markers have demonstrated a role in the diagnosis, prognosis, formulating treatments and in the detection of recurrence of cancer (Prasad et al., 2013). Most of the studies have used formalin-fixed paraffin-embedded (FFPE) margins, but also extra biopsies and brushed cells have also been taken from grossly normal mucosa adjacent to tumour. Various techniques, all with their pro's and con's, have been used. Protein markers were detected by immunohistochemistry (IHC), as was done on various genes which included p53, CCDN1, p16, CHEK2 and LAMA5. This technique was relatively easy to perform, but problems related to objectivity of the scoring, setting cut-off points and reproducibility occurred. DNA-based techniques are based on DNA copy number alteration (CNA), promoter- methylation (such as in MGMT, p16, DAPK1), allelic imbalance with microsatellite markers or mutation analysis. Compared to IHC, measurement of DNA-based markers has a higher level of objectivity due to a better reproducibility and standardized cut-off levels (e.g. presence or absence of a certain alteration). Moreover, DNA-markers often have a more direct link to carcinogenesis. Nevertheless, the application of DNA-based techniques requires a relatively high level of technical expertise (Prasad et al., 2013).

Multiple IHC studies have been carried out to investigate useful tools or markers to predict relapse in OSCC, these include immunostaining for p53 and Ki-67. P53 has been widely used to study relapse of OSCC, and it was shown to be important in the regulation of apoptosis (Alok et al., 2014). Oliveira et al. (2011) showed that there was a significant association between p53 expression and tumour recurrence in mucosa and invasive front of head and neck SCC. Increased expression of p53 and elF4E markers in surgical margins was also associated with recurrence in OSCC (Singh et al., 2016). Other markers

such as Cornulin, ISG15, Survivin, CD44v6, cytokeratin 4, S100A2, Claudin-7, E-cadherin, Cyclin D1 and EGFR have also been used in previous IHC studies to predict recurrence in OSCC (Braakhuis et al., 2010; Gonzalez-Moles et al., 2010; Vincent-Chong et al., 2012; Melchers et al., 2015; Salahshourifar et al., 2015; Gupta et al., 2016). Decreased expression of Cornulin (Salahshourifar et al., 2015) and increased expression of ISG15 (Vincent-Chong et al., 2012) markers in OSCC tissue were also associated with recurrence of OSCC.

2.8.1 Ki-67

Ki-67 phosphoprotein is encoded by MKI67 gene in human body. It is present in the cell nucleus during all the cellular division phases in different tissues of different species, by organising and maintaining structures of deoxyribonucleic acid (DNA) and ribosome synthesis during the cell division. Expression of Ki-67 is seen in all active phases of the cell cycle (G1, S, G2, and mitosis), but was absent from resting cells (G0)., hence it was believed to be a reliable marker for cell proliferation (Reddy & Saxena, 2010). Proliferative capacity of Ki-67 is known as labelling index (LI) (Liu et al., 2000). Immunostaining with antibodies to Ki-67 antigen is well established as a quick and efficient method for evaluating growth fractions of various tumour types (Birajdar et al., 2014). Other immunohistochemical markers which were previously used to study cell proliferation included poliferating cell nuclear antigen (PCNA), cyclin D and Centromere Protein F (CENP-F). However, due to its intensity of staining and pattern of expression, Ki-67 is more reliable compared to other markers (Liu et al., 2000).

In OSCC tumour tissue, Ki- 67 is shown as a marker which is significantly related to proliferation of OSCC epithelial cells (Mineta et al., 1999). Therefore, it has been widely used as a biomarker of OSCC carcinogenesis to determine proliferation index of normal, premalignant and malignant tissues (Iamaroon et al., 2004). Regarding oral epithelial dysplasia (ED), immunohistochemical analyses have shown that Ki-67 is involved in

early stage of malignant transformation (Birajdar et al., 2014). Oral ED often display degrees of alteration of cellular maturation in the epithelium and increased proliferative activity in the suprabasal layers, and these features were positively correlated with Ki-67 positivity within the epithelium. Increased Ki-67 LI in oral dysplastic lesions was shown to be correlated with disease progression and poor clinical outcome (Thomson et al., 2008). Apart from its role in tumour or lesional tissue, prognostic value of Ki-67 has been studied in histologically normal mucosa which is slightly distant away from the primary tumour in patients who are surgically treated for OSCC. Due to effect of field cancerisation, genetically altered cells in a field often possessed high proliferative capacity, and this was associated with increased Ki-67 positivity (Braakhuis et al., 2003). In an evaluation of surgical margins in patients with early tongue SCC, Okazaki et al. (2007) noticed that patients with increased Ki-67 expression at surgical margins exhibiting ED eventually developed recurrent tumours after couple of years of follow up. This study suggested that ED associated with increased Ki-67 expression was likely to undergo malignant transformation. In addition, a prospective study by Montebugnoli et al. (2009) had shown that Ki-67 proliferative status in distant mucosa in OSCC patients was significantly higher than that in the controls. Among 11 OSCC patients with Ki-67 overexpression, four of them presented with local recurrence and three suffered from lymph node metastasis. Therefore, the authors suggested that Ki-67 was a potential predictor of survival in OSCC.

2.8.2 Cornulin

Cornulin, also known as Chromosome 1 Open Reading Frame 10 (C1Orf10) or squamous epithelial heat shock protein 53, belongs to the family of S100 fused-type protein. It is located in chromosome 1q21 locus (Xu et al., 2000). Cornulin plays an important role in the differentiation of epidermis by encoding for proteins involved in calcium signalling. It was called “Cornulin” due to its localisation in the cornified layers

of epithelium. In a study by Li et al. (2018), Cornulin was upregulated in psoriatic skin lesions due to proliferation of epidermal cells. In contrast to this, Cornulin was reported to be downregulated in skin eczema due to its role in late epidermal differentiation (Lieden et al., 2008). Studies on human cancers have shown that Cornulin was downregulated in oesophageal and cervical SCC as well as OSCC (Xu et al., 2000). Chen et al. (2013) reported that downregulation of Cornulin was associated with lymph node metastases, advanced clinical stage and decreased survival rate in oesophageal SCC.

In OSCC, downregulation of Cornulin was often observed, as upregulation of Cornulin was expected in damaged oral epithelial cells due to habits such as betel quid chewing. This could be related to genetic alteration in OSCC. Imai et al. (2005) reported that overexpression of Cornulin in oral cancer cell lines had caused a significant decline in cell proliferation by arresting cell cycle progression at the G1/S phase, with downregulation of cyclin D1 expression. Their results suggested that Cornulin played a role in controlling cell cycle progression, its downregulation might be linked to tumour progression. However, the mechanism of Cornulin downregulation remained unknown. In 2009, Schaaij-Visser et al. discovered an allelic loss in chromosome arms 3p, 9p, 11q and 17p, this was associated with low expression of Cornulin in severely dysplastic oral mucosal tissue and loss of expression in OSCC tumour tissue. This was followed by Salahshourifar et al. (2015) where the authors discovered loss of heterozygosity (LOH) and microsatellite instability (MSI) at 1q21.3 in OSCC. These findings have successfully improved our understandings in the downregulation of Cornulin associated with OSCC. The authors had also discovered that expression of Cornulin in surgical margins was inversely correlated with risk of relapse in head and neck SCC, therefore they suggested that Cornulin staining of the surgical margins could be used in postsurgical treatment planning for patients with OSCC. Patients who had unresected preneoplastic fields which were indicated by low expression of Cornulin immunohistochemically, were required to

be followed up for a long period of time. Suggestion of Cornulin to be a predictor of relapse in head and neck SCC was proposed by the authors.

Among all the mutations found in OSCC, the p53 tumour suppressor gene is most frequently mutated. Different kinds of stresses including oncogenic stimuli, ionising radiation, ultraviolet radiation, hypoxia, cytokines and growth factors can activate the p53 through different pathways. P53 protein can initiate DNA repair proteins and stop the cell cycle at the G1/S regulation point when the DNA is damaged or activate apoptotic processes if the DNA damage is beyond repair. When mutated, it allows cells to progress through DNA damage checkpoints and these cells would replicate along with mutations (Ali et al., 2017). It was reported that p53 could be activated by DNA damage in oesophageal squamous cell carcinoma. In a cell line study of oesophageal squamous cell carcinoma, along with Cornulin expression, p53 was also upregulated. Subsequently G1/S transition was inhibited (Chen et al., 2013).

2.8.3 Interferon- stimulated Gene 15 (ISG15)

Interferon-stimulated gene 15 (ISG15) is a member of the ubiquitin-like protein superfamily. The expression of ISG15 is stimulated by type I Interferon (IFN) and induced by bacterial or viral infection through the Janus kinase/ signal transducer and activator of transcription signalling pathway (Bektas et al., 2008). ISG15 is involved in extra- and intracellular regulatory activities. Extracellular ISG15 stimulated the production of IFN-gamma from lymphocytes and increased proliferation of natural killer cells. Intracellularly, ISG15 formed covalent conjugation with proteins through an enzymatic activity similar to ubiquitin protein conjugation system. This process is known as “ISGylation”. It promoted stabilisation of the conjugation complex of ISG15 and the involved protein by interfering with the ubiquitin/26S proteasome pathway (UPP). The ISGylation process targeted large number of proteins regulating diverse cellular pathways such as RNA splicing, cytoskeleton organisation and regulation, chromatin remodelling,

stress responses, and translation (Saitoh et al., 2006). Hence a deregulated expression of ISG15 could be related to carcinogenesis as ISGylated proteins would usually remain stable for a sustained period (Laljee et al., 2013).

Overexpression of ISG15 in breast cancer cells at RNA and protein level was reported by Bektas et al. (2008). There was a significant correlation between ISG15 overexpression and unfavourable prognosis. Chen et al. (2016) has reported marked overexpression of ISG15 in nasopharyngeal carcinoma. Tumour recurrence and poor prognosis were correlated with overexpression of ISG15. According to Laljee et al. (2015), there was overexpression of ISG15 in up to 80% of OSCC tissues collected from Indian patients while Sumino et al. (2016) reported that overexpression of ISG15 in OSCC was associated with poorer five-year survival rate in a study in Japan. It was also suggested by the authors that ISG15 could be further validated for its role as a biomarker in OSCC.

As for its association with p53, Huo et al. (2017) noticed that ISG15 silencing induced cell cycle arrest through stabilisation of p53, which allowed the prolonged time for cells to repair damaged DNA in OSCC. Hence it was proven that ISG15 downregulation enhanced tumour suppressive effect of p53.

CHAPTER 3: METHODOLOGY

3.1 Study design

This was a retrospective case control study conducted to predict relapse in surgically treated OSCC using immunohistochemistry (IHC). A total of 34 OSCC cases which fulfilled all the inclusion and exclusion criteria were included in this study. These cases were surgically treated OSCC cases from year 2000 onwards. Non-involved surgical margins were selected from each case. Expression of selected markers in these surgical margins was determined using immunostaining of formalin fixed paraffin embedded (FFPE) sections of OSCC surgical margins. Expression of markers in the selected surgical margins of each case was scored and analysed using statistical methods.

3.2 Sample types

Samples involved in this study were surgical margins of OSCC cases treated in Klang Valley. According to the Malaysian Department of Statistics, Klang Valley area refers to the capital city, Kuala Lumpur and its suburbs as well as adjoining cities and towns in the state of Selangor, with population of 7.2 million. Included were 19 cases treated in the Oral the Maxillofacial Surgery Clinic, Hospital Tengku Ampuan Rahimah, Klang; while 15 cases were from the Oral Surgery Clinic in the Department of Oral and Maxillofacial Clinical Sciences, Faculty of Dentistry, University of Malaya. Sociodemographic, clinicopathological and follow-up data of all the OSCC cases were extracted from patients' histopathological reports as well as from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS). MOCDTBS is coordinated by the Oral Cancer Research and Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya. FFPE tissues of the surgical margins for all the study and control cases, as well as normal oral mucosal tissues which served as a standard for comparison, were retrieved from MOCDTBS.

This study was approved by the Medical Ethics Committee (MEC), Faculty of Dentistry, University of Malaya with Ethics Committee / IRB reference number of DF OS1712/0035(P).

3.3 Sample selection

3.3.1 Sample size calculation

Sample size calculation was performed using G*Power software, version 3.1.9.4. Based on the effect size of 0.695 and power of 0.80 as shown in the reference article by Zargoun I. (2017), the estimated sample size was 33 for each group. Due to limited cases that fulfilled the inclusion and exclusion criteria, the estimated sample size was not achievable.

3.3.2 Sample selection for study group

Sample selection for study group was based on the following inclusion and exclusion criteria. The inclusion criteria were:

1. Cases with a primary tumour located in the oral cavity.
2. Cases that had been treated by surgery with or without postoperative chemotherapy or radiotherapy.
3. Cases with histologically non-involved surgical margins. Only cases with surgical margins that were ≥ 1 mm 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.

Exclusion criteria were:

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

Study group (with relapse) consisted of 15 OSCC cases which were diagnosed with OSCC from year 2000 onwards and developed a relapse within the following five years of follow up. Initially 17 cases which fulfilled all the inclusion criteria were selected; however, due to missing FFPE tissue blocks, two cases were excluded.

3.3.3 Sample selection for control group

Control group consisted 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 also strictly based on the same inclusion and exclusion criteria as listed above. There were 22 cases included in the control group which were selected based on the criteria; however, only 19 cases were included in the study. One of the cases was excluded due to involved surgical margins as observed in one of the new haematoxylin and eosin (H&E) stained reference sections examined while the remaining two cases were excluded due to missing FFPE tissue blocks.

3.3.4 Selection of surgical margins

Multiple FFPE tissue blocks of surgical margins from the selected 34 cases of OSCC were available. This could be explained by an example of a right lateral border of tongue OSCC, where the excised main tumour would have four mucosal margins i.e. anterior, posterior, superior, and inferior margins and a deep connective tissue margin. Hence, a two-staged randomisation of sampling was performed. First stage of random sampling was carried out to select two out of all available and different margins for Ki-67 and Cornulin staining. This process was carried out using the RANDBETWEEN function of Microsoft Excel (Office 365 version). When selected margins had multiple representative FFPE tissue blocks, this was followed by second stage of random sampling with same method to select one FFPE tissue block for that margin. It would have been ideal to study all the representative sections from all the mucosal margins of all the cases in both the study and control groups. However, this was not possible as it would necessitate IHC

staining of a lot more sections which was beyond the scope of the study and partly due to the non - availability of the FFPE tissue blocks. As for IHC staining with ISG15, when there were two available surgical margins in one case, one margin was randomly selected for immunostaining using the same method as mentioned above, due to insufficient anti-ISG15 antibody. Hence, we had only 34 slides stained with ISG15 instead of 55 slides. Illustration of sample selection protocol is shown in Appendix A. A table of all the selected cases with respective 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 purpose. All tissues were screened to ensure that these 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 five samples of NOM were suitable to be utilised in this study as control samples.

Four 4 µm sections were obtained from each FFPE tissue block of selected surgical margins. The first section from all the surgical margins were stained with H&E stain to reassess the distance of surgical margins from the tumour, as involved margins needed to be excluded from the study. The subsequent sections were stained with Ki-67, Cornulin and ISG15. For the control samples, additional three 4 µm sections were obtained, and these were used for immunohistochemical staining with Ki-67, Cornulin and ISG15.

3.4 Epithelial dysplasia grading

All H&E stained sections in both study and control groups were graded for epithelial dysplasia (ED) by two independent observers. Grading of ED was performed on all the selected surgical margins according to the criteria listed in "World Health Organization (WHO) Classification of Head and Neck Tumours 4th edition" as well as the criteria proposed by Kujan et al. (2006), respectively. An overall dysplasia grade for the patient

was defined as the highest grade of dysplasia graded in any of the surgical margins of that patient (Warnakulasuriya et al., 2008). The observers were blinded to clinical and treatment outcome details of each case. Consensus was achieved for all the graded cases. All cases in both study and control groups were then dichotomised into two groups as illustrated below in Figure 3.1, based on the grades of ED observed within the surgical margins.

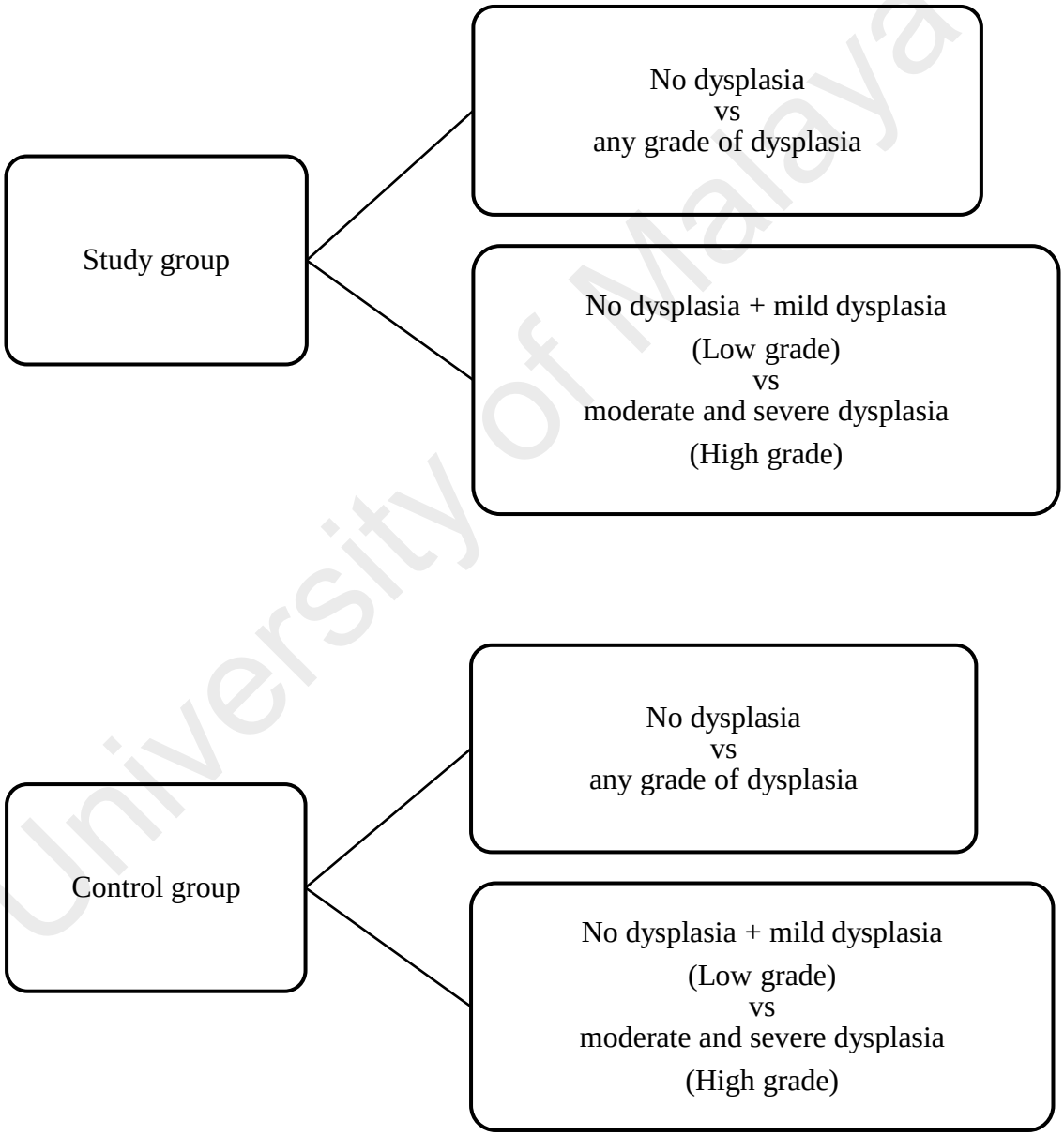

Figure 3.1: Dichotomisation of samples based on epithelial dysplasia

3.5 Laboratory procedures

3.5.1 Haematoxylin and Eosin staining

One of the sections obtained from each FFPE tissue blocks for all cases were stained with H&E based on the protocol recommended by manufacturers. Firstly, sections were deparaffinised and rehydrated, followed by haematoxylin and eosin staining after washing. The stained sections were dehydrated and cleared with xylene before mounting. Detailed description for each step in the procedure were as listed in Appendix C.

3.5.2 Immunohistochemical staining

Subsequently, IHC procedures were performed on the remaining sections. Immunohistochemical staining procedures using monoclonal mouse anti-human Ki-67 antigen, clone MIB-1 (M724029, Agilent DAKO, USA), polyclonal rabbit Cornulin antibody (11799-1-AP, Proteintech, USA) and polyclonal rabbit anti-ISG15 antibody (HPA004627, Sigma-Aldrich, USA) were performed on the unstained sections from FFPE tissue blocks by applying the Dako Real EnVision Detection System and Peroxidase/DAB+ (Dako, USA) according to the manufacturers' protocols. A prior optimisation for each antibody was carried out to determine optimum concentration of antibody, antigen retrieval method and condition of primary antibody incubation, which are as tabulated in Table 3.1.

Table 3.1: Types of primary antibody used with associated materials and procedures

Primary antibody	**Ki-67**	**Cornulin**	**ISG-15**
Clone	Monoclonal mouse anti-human Ki-67 antigen, clone MIB-1	Polyclonal rabbit Cornulin antibody	Polyclonal rabbit anti-ISG15 antibody
Tissue for optimisation and positive control	OSCC tissue	Normal oral mucosa	Hepatocellular carcinoma tissue

Table 3.1 (continued)

	Ki-67	**Cornulin**	**ISG-15**
Dilution	1:300	1:300	1:100
Antigen retrieval	Pressure cooker 121°C for 30 seconds followed by 90°C for 10 seconds.	Microwave oven 99°C for 20 minutes.	Pressure cooker 125°C for 4 minutes.
Buffer solution for antigen retrieval	Tris-Ethylenediaminetetraa-cetic acid (EDTA) buffer, pH9	Citrate buffer, pH6	Citrate buffer, pH6
Primary antibody incubation	1 hour at room temperature	1 hour at room temperature	Overnight (16 hours) at 4°C
Wash buffer	Tris-buffered saline	Phosphate-buffered saline	Phosphate-buffered saline

Briefly, unstained sections obtained from FFPE tissue blocks for all samples were deparaffinised and rehydrated in a stepwise manner. Antigen retrieval was carried out using selected devices and buffer solutions as recommended by the manufacturers. The sections were then immersed in blocking solution (Dako Corporation, CA, USA) to block the endogenous peroxidase activity for 10 minutes at room temperature followed by washing with buffer solutions. Incubation of primary antibodies was carried out based on the recommended protocols, followed by secondary antibody incubation. Lastly, sections were stained with 3'3 diaminobenzidine (DAB) substrate chromogen, (Dako Corporation, CA, USA) and then counterstained with Mayer's haematoxylin, dehydrated and mounted with a xylene-based mounting medium. Tissues used as positive control for each marker were similar as those used for optimisation. Negative control was performed using the same condition and tissues; except that the primary antibody was replaced with wash

buffer solutions. Detailed IHC procedures for each antibody were described as in Appendix D.

3.6 Quantification of immunohistochemistry

Scoring of immunostainings with Ki-67, Cornulin and ISG15 was performed independently by two independent observers. The observers were blinded to details of the samples. Cells with brown staining under the microscope were considered as positive. For expression of Ki-67 in each margin, a total number of 500 cells were counted and included for scoring from a maximum of five randomly selected fields in the sections at highest magnification of 400x. Number of positively stained cells within these 500 cells was determined. Selection of the cells was done by using ImageJ software (LOCI, University of Wisconsin). Percentage of Ki-67 positivity was then calculated and was indicated as the labelling index (LI).

Semiquantitative scoring technique was applied in this study to quantify perception of IHC marker expression on an ordinal scale, and to combine scores of different parameters (for example, intensity of staining and staining coverage area) into a total score. The total score would be used for statistical analysis. Immunoreactive score (IRS) was one of the commonly used semiquantitative scoring techniques, produced by multiplication between proportion and intensity of positive cells (Fedchenko & Reifenrath, 2014). This has been widely used in studies involving various IHC markers. IRS was determined in Cornulin and ISG15 staining to determine score for all sections, in which intensity of stained cells was multiplied with proportion of stained cells For Cornulin immunostaining, intensity of staining was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining) while the proportion of stained cells was graded as 0 (no stained cells), 1 ($\leq$ 5%), 2 (6 – 50%) and 3 ($\geq$ 51%). (Salahshourifar et al., 2015) The total score ranged from 0 to 9. For ISG15 staining, a similar grading for intensity of staining was applied,

while the proportion of stained cells was graded as 0 (≤ 10%), 1 (11 – 50%), 2 (51 – 80%) and 3 (≥ 81%). Hence, total score ranged from 0 to 12 (Bektas et al., 2008).

A calibration exercise was carried out prior to scoring by two independent observers. As there were two independent observers, two sets of scores were submitted for each antibody. Average scores were obtained from these two sets of scores for statistical analysis later. 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 25, IBM). Intraclass correlation coefficient (ICC) tests were performed to observe interobserver agreement in scoring of expression of Ki-67, Cornulin and ISG15. Based on the analysis the interobserver agreements for scoring of Ki-67, Cornulin and ISG15 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 average measures of ICC test for each marker.

Table 3.2: Average measures of ICC test for each marker

	Ki-67 (*p* value)	Cornulin (*p* value)	ISG15 (*p* value)
Study group	0.916 (0.000)	0.974 (0.000)	0.982 (0.000)
Control group	0.708 (0.000)	0.916 (0.000)	0.873 (0.000)
Normal oral mucosa	0.921 (0.015)	0.887 (0.029)	0.878 (0.033)

Significance level: $p = 0.05$

Kappa statistics was performed to assess interobserver agreement in grading of epithelial dysplasia. The results from Kappa statistical test showed interobserver agreement of 0.683 for epithelial dysplasia (ED) grading, which was considered as good according to Dawson & Trapp (2004). Interobserver agreement was also measured for dysplasia grading with binary grading system using the same test and the results was

0.713. This was also considered as good. The results were all statistically significant (p = 0.000) as shown in Table 3.3.

Table 3.3: Measure of interobserver agreement in ED grading

Epithelial dysplasia grading system	Value	Approximate significance (p value)
WHO (2017) grading system	0.683	0.000
Binary grading system (Kujan et al, 2006)	0.713	0.000

Significance level: p = 0.05

Cut-off value for high and low expression of each marker was determined by carrying out Receiver Operating Characteristic (ROC) curve analysis. Scores of each sample were plotted to generate ROC curves. The closest score to the point with maximum sensitivity and specificity was selected as the cut-off value. IRS for each marker was divided into high and low expressions; low expression consisted of scores below or equal to the cut-off value, while high expression was defined as scores above the cut-off value. The cut-off values are listed in Table 3.4.

Table 3.4: Cut-off value for Ki-67, Cornulin and ISG15 expressions

Markers	Area under the curve	Cut-off value
Ki-67	0.518	27.75
Cornulin	0.666	4.95
ISG15	0.298	7.05

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

1. Low and high expression of all markers in surgical margins.
2. Association between expression of Ki-67, Cornulin and ISG15 in surgical margins with clinicopathological prognosticators of OSCC.

3. Association between expression of Ki-67, Cornulin and ISG15 in surgical margins with relapse in OSCC.
4. Association between clinicopathological prognosticators and relapse in OSCC.

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

CHAPTER 4: RESULTS

4.1 Sociodemographic findings

A total of 34 OSCC cases comprising of 15 study cases and 19 control cases were included. Cases included in the study group showed relapse within five years of follow up postoperatively, while the cases included in the control group did not show any relapse. Of the 34 patients, there were 25 females and 9 males with a mean age at presentation of 58.38 years and an age range of 36 to 78 years. Patients were categorised within study and control groups based on the cut-off value of 57.5 years obtained from ROC curve analysis. Majority (60%) of the patients were of Indian ethnic origin. Table 4.1 demonstrates the distribution of patients according to age, gender, ethnicity and habits respectively.

Table 4.1: 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 ± standard deviation, range : 58.38 ± 11.37, 36 – 78		
≤ 57.5 years	3 (20.0)	5 (21.7)
> 57.5 years	12 (80.0)	14 (78.0)
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.

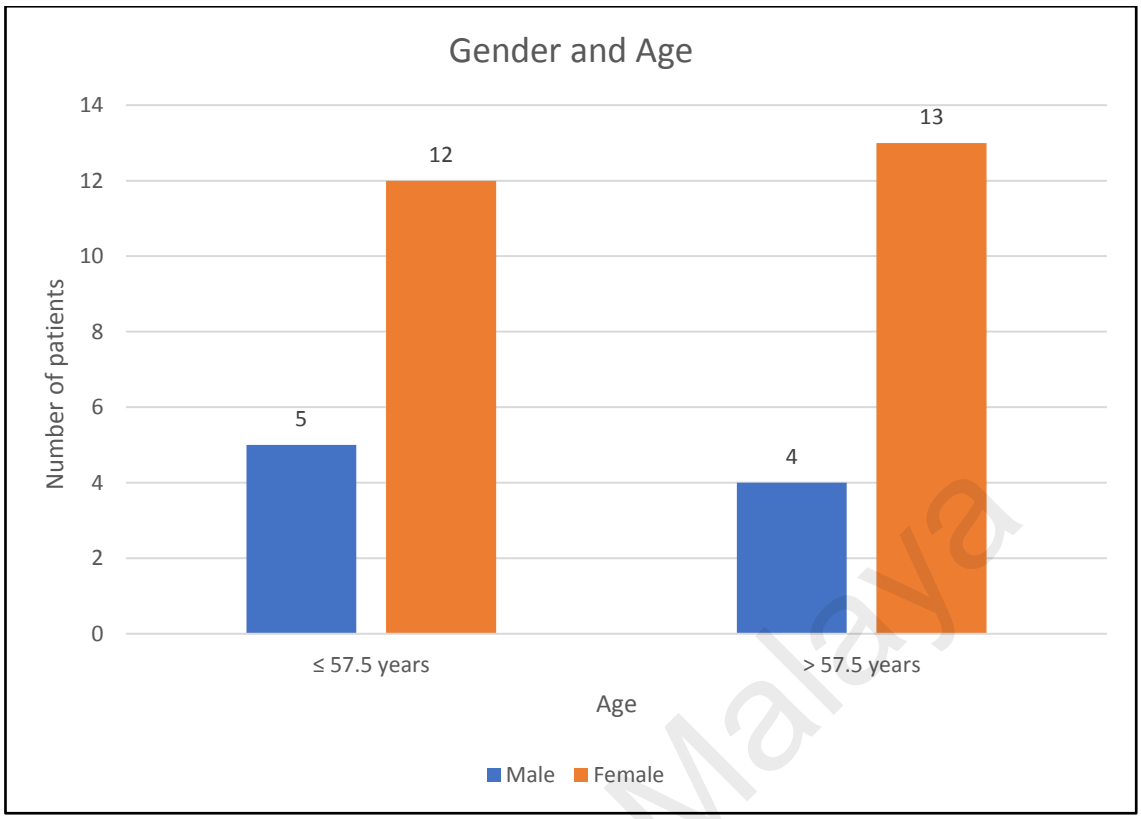

Figure 4.1: Distribution of patients according to gender and age.

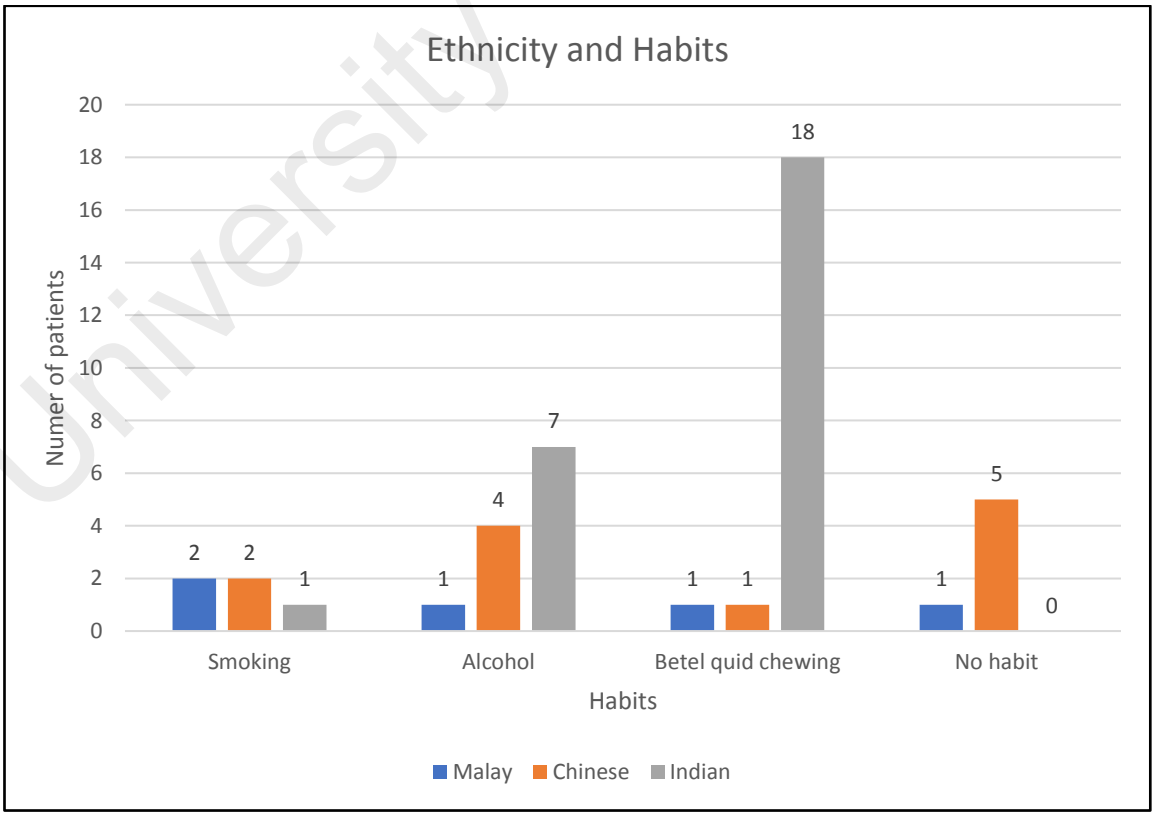

Figure 4.2: Distribution of patients according to ethnicity and habits.

4.2 Clinicopathological findings

Patients in the study group had relapse (n = 15) while the patients within the control group (n = 19) did not show relapse. Among the study group patients, seven patients had SPT while the remaining eight patients showed LR. Approximately one third of the patients in both study and control groups had tongue as primary site of OSCC. Other reported primary tumour sites included lip, buccal mucosa, alveolar mucosa, retromolar and gingivobuccal complex. Observed relapse sites included retromolar region, tongue, buccal mucosa, mandibular gingiva, floor of mouth and flap margin.

Most of the patients in both groups showed a moderately differentiated primary OSCC (n = 20), followed by well-differentiated (n = 12) and poorly differentiated OSCC (n = 2). Pattern of invasion observed was predominantly Type III (n = 19) followed by Type IV (n = 9). Perineural invasion (n = 2) and vascular invasion (n = 4) were seen in both groups. Nine patients had metastasis of OSCC to the neck. Clinicopathological prognosticators of study and control groups are as described in Table 4.2 below.

Table 4.2: Clinicopathological prognosticators of study and control groups

Variables	Study group n (%)	Control group n (%)
Number of patients	15 (100)	19 (100)
Primary tumour site		
Tongue	5 (33.3)	7 (36.8)
Non-tongue*	10 (66.7)	12 (63.2)
Second primary tumour	8 (53.3)	-
Local recurrence	7 (46.7)	
Tumour differentiation		
Well differentiated	3 (20.0)	9 (47.4)
Moderately differentiated	11 (73.3)	9 (47.4)
Poorly differentiated	1(6.7)	1 (5.2)

Table 4.2, continued

Variables	Study group n (%)	Control group n (%)
Pattern of invasion		
Type I	1 (6.67)	3 (15.8)
Type II	0 (0)	4 (21.1)
Type III	11 (73.3)	8 (42.1)
Type IV	3 (20.0)	3 (15.8)
Unknown	0 (0)	1 (5.2)
Perineural invasion		
Yes	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 neck		
Yes	4 (21.1)	5 (26.3)
No	7 (57.8)	14 (73.7)
Unknown**	4 (21.1)	-

*Lip, buccal mucosa, alveolar mucosa, retromolar and gingivobuccal complex.
**Information was unable to be retrieved.

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

4.3 Epithelial dysplasia grading

Among all non-involved surgical margins (n = 55) in which ED grading was performed, 24 surgical margins did not show dysplasia (43.64%), followed by 22 cases of mild ED (40%). Out of 55 surgical margins, there was only one (1.82%) severe ED, which was from one of the cases in the control group. Moderate ED was equally seen in both study and control groups (n = 4). More cases without relapse showed no ED (n = 13) than cases with relapse (n = 10). Total number of surgical margins with any grade of ED in the control group was 19 while in the study group, the total number of surgical margins

with any grade of ED was 13. Distribution of different grades of ED in both study and control groups are shown in the bar chart below (Figure 4.3).

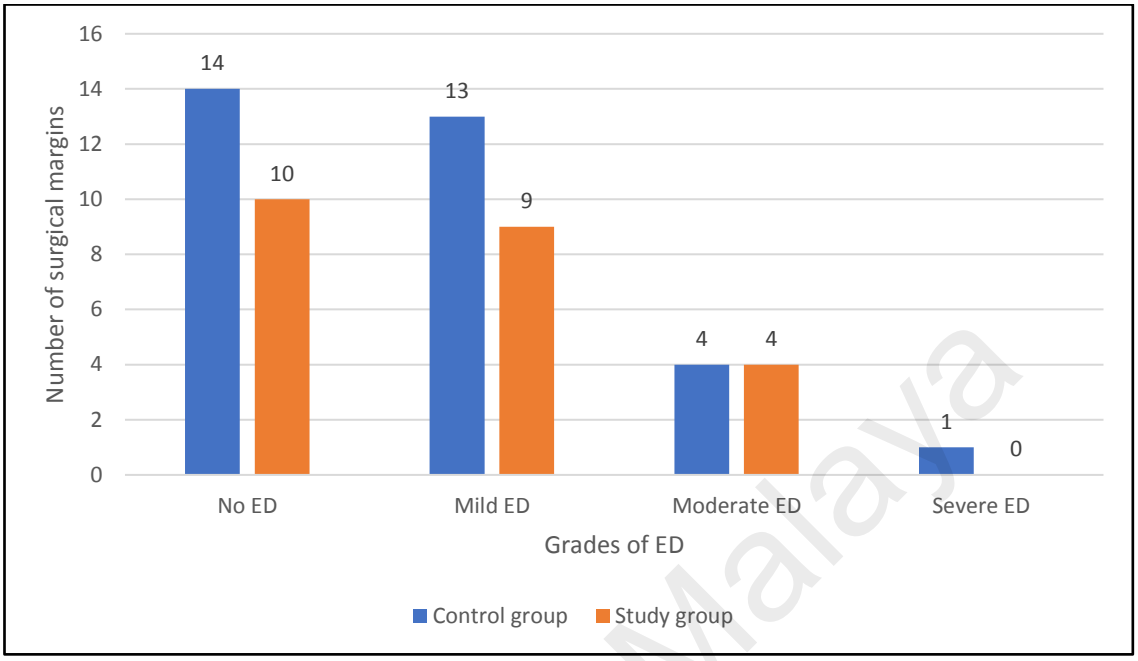

Figure 4.3: Distribution of ED in study and control group

4.4 Expression of Ki-67, Cornulin and ISG15 in surgical margins

Mean immunoreactive scores (IRS) were obtained from all non-involved surgical margins for both study and control groups for all markers. Expression of Ki67 was higher in the study group compared to the control group. Inversely, expression of Cornulin and ISG15 was lower in the study group when compared to the control group. Shapiro-Wilk test was used to evaluate normality of data distribution for all markers due to small sample size in each group ($n < 50$).

Comparison was made for expression of all markers between study and control groups. Independent t-test was used to analyse expression of Cornulin as the data was normally distributed and it showed expression of Cornulin in the control group was significantly greater than that in the study group ($p = 0.032$). As the data was not normally distributed, Mann-Whitney U test was applied in the analyses of Ki-67 and ISG15 expressions. The increase in expression of ISG15 in the control group was statistically significant ($p = 0.047$); however, no significant association was observed in the expression of Ki-67

between the study and control groups. The results of statistical analyses are as tabulated in Table 4.3. Significant *p* values are bolded.

Table 4.3: Expression of Ki-67, Cornulin and ISG15 in surgical margins

Mean score	Ki-67	Cornulin	ISG15
	Mean ± sd [95% CI]	Mean ± sd [95% CI]	Mean ± sd [95% CI]
Study group	14.82 ± 11.20 [9.98, 19.67]	5.32 ± 2.51 [4.23, 6.40]	1.85 ± 2.37 [0.54, 3.17]
Control group	13.26 ± 8.10 [10.34, 16.19]	6.66 ± 1.65 [6.06, 7.25]	2.83 ± 2.22 [1.75, 3.90]
Study * control	0.824[m]	**0.032[t]**	**0.047[m]**

Significance level: $p = 0.05$. sd: Standard deviation. CI: Confidence interval. m: Mann Whitney U test. t: Independent t-test.

The variability of the pattern and intensity of immunostaining for Ki-67, Cornulin and ISG15 in study group and control group is shown in Figure 4.4, 4.5 and 4.6, respectively.

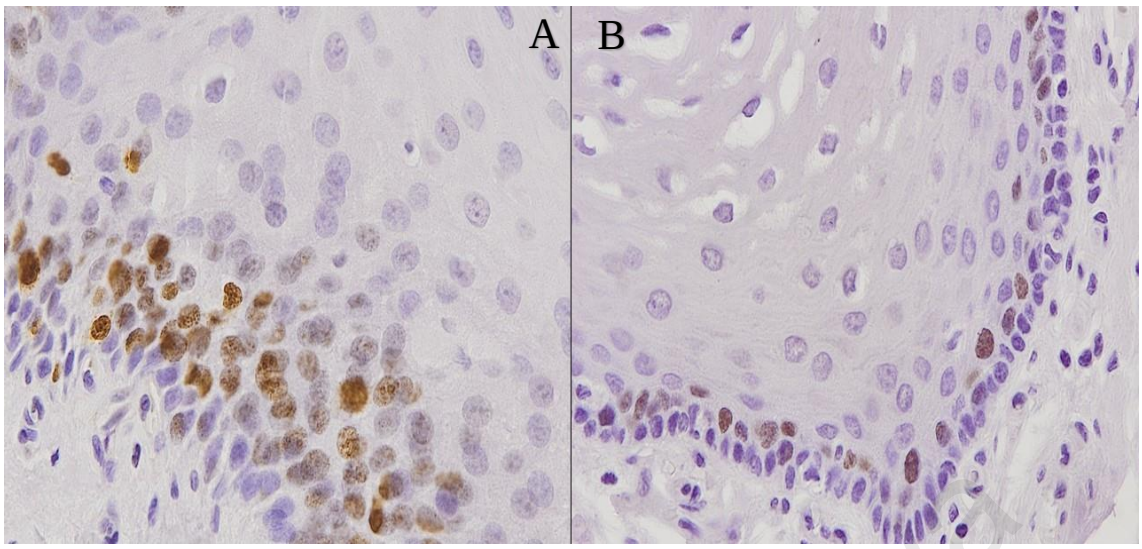

Figure 4.4: Staining with Ki-67 in study group and control group.

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

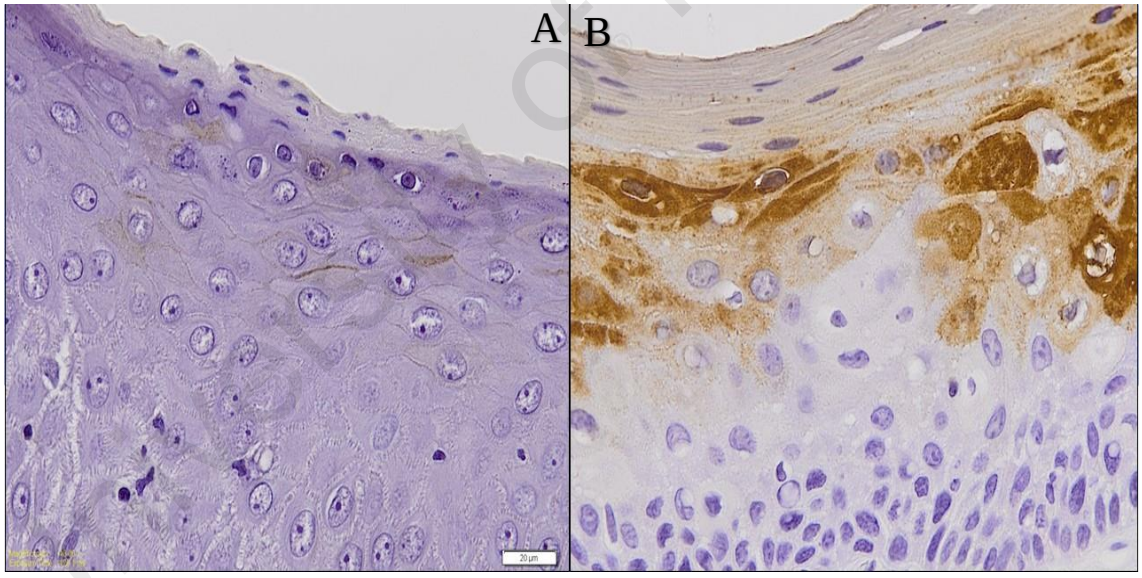

Figure 4.5: Staining with Cornulin in study group and control group.

(A) Lack of Cornulin staining in the study group. (400x) (B) Moderate to strong cytoplasmic staining was observed within the granular and upper spinous layer in the control group. (400x)

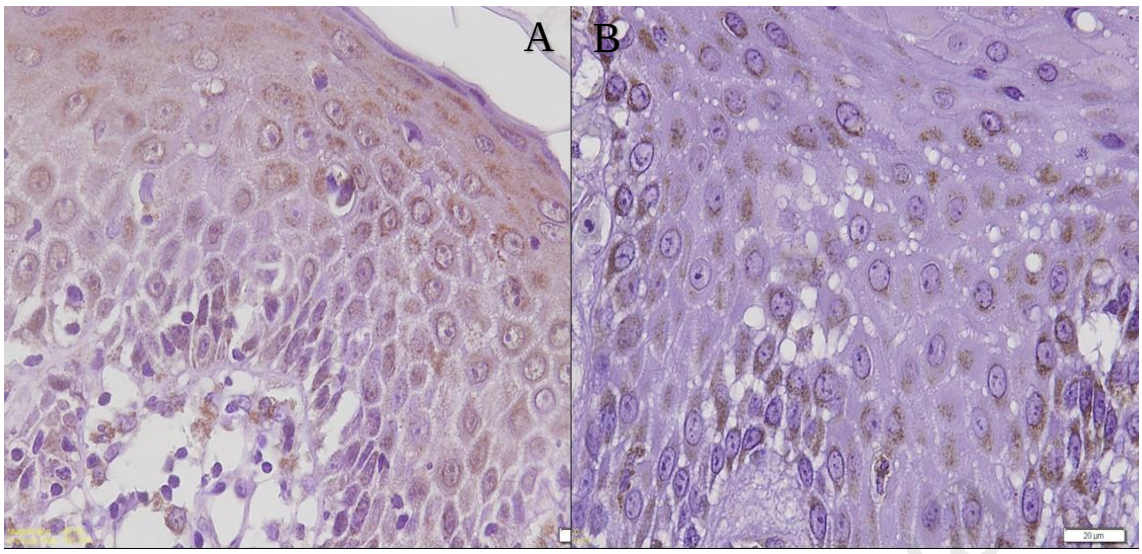

Figure 4.6: Staining with ISG15 in study group and control group.

In general, moderate cytoplasmic staining was observed in the epithelial cells in both study (A) and control (B) groups. (400x)

4.5 Expression of Ki-67, Cornulin, ISG15 and relapse of OSCC

Statistical analyses with Chi-square tests (Pearson Chi-square test or Fisher's Exact test, whichever applicable) was performed. In patients with relapse of OSCC, there was a slight increase in the number of surgical margins exhibiting high Ki-67 expression (n = 2) and high ISG15 expression (n = 1); however, the difference between the control group was not statistically significant.

Low Cornulin expression was observed in the study group and there was a statistically significant difference between the control group which showed a high expression of Cornulin (p = 0.004). In Table 4.4, number and percentage of surgical margins showing low and high expression of Ki-67, Cornulin and ISG15 are shown. Significant p value is bolded.

Table 4.4: **Expression of Ki-67, Cornulin and ISG15 and relapse in OSCC**

	Ki-67		Cornulin		ISG15	
	Low	High	Low	High	Low	High
Relapse n (%)						
No	30 (54.5)	2 (3.6)	4 (7.3)	28 (50.9)	18 (52.9)	1 (2.9)
Yes	19 (34.5)	4 (7.3)	11 (20)	12 (21.8)	13 (38.2)	2 (5.9)
P value	0.223[f]		**0.004**[c]		0.571[f]	

Significance level: $p = 0.05$. c: Pearson Chi-Square test. f: Fisher's Exact test

Based on the level of expression of each marker, number of patients in both study and control groups are also shown in bar chart below (Figure 4.7). For high expression of Ki-67 and ISG15, only one and two patients respectively developed relapse of OSCC. Seven patients who had OSCC relapse showed low expression of Cornulin.

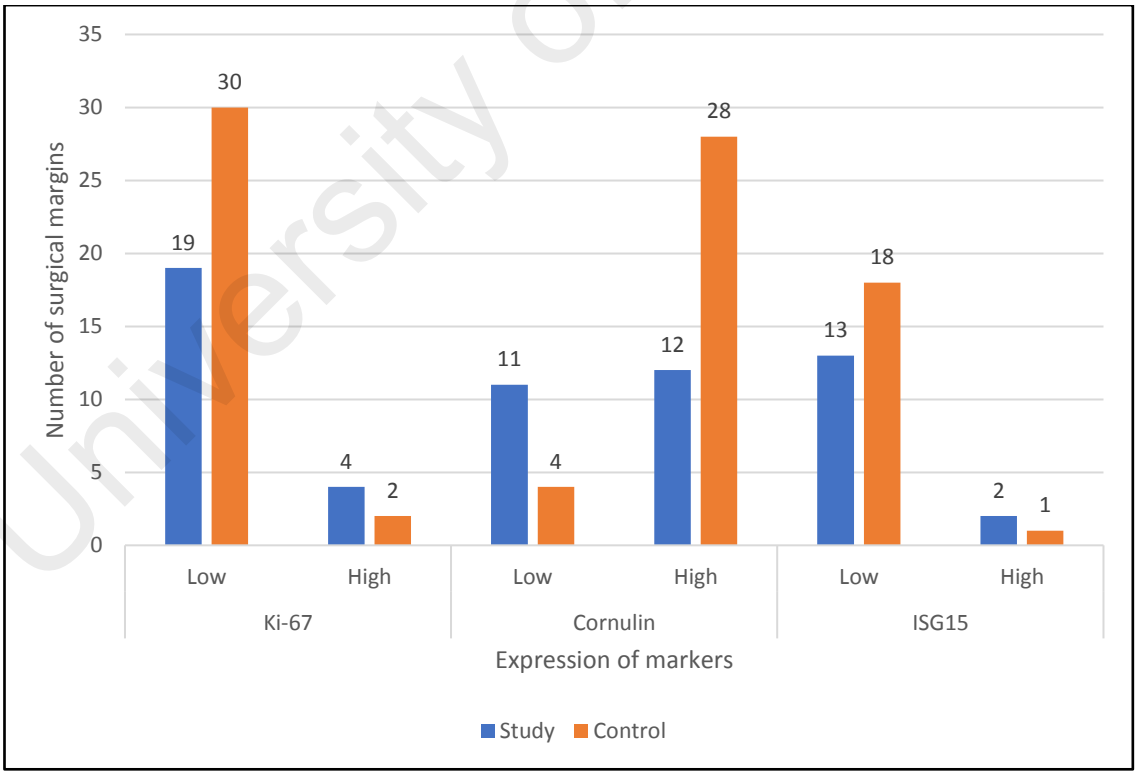

Figure 4.7: Expression of Ki-67, Cornulin, ISG15 and its association with relapse in OSCC

4.6 Expression of Ki-67, Cornulin, ISG15 and its association with clinicopathological prognosticators

Statistical analyses with Chi-square tests (Pearson Chi-square test or Fisher's Exact test, whichever applicable) showed significant difference between expression of Ki-67 in histologically non-involved surgical margins and gender of the patient. Expression of Ki-67 was significantly low among female patients. Expression of Cornulin in histologically non-involved surgical margins was also significantly associated with primary tumour site, where the expression of Cornulin was significantly low in tongue SCC. These are shown in Table 4.5. Significant *p* values are bolded.

Apart from that, there was no other significant association observed between expression of markers and other clinicopathological prognosticators.

Table 4.5: Expression of Ki-67 and Cornulin and its association with gender and tumour site

		Ki-67		Cornulin	
Clinicopathological prognosticators	n = 55	Low n (%)	High n (%)	Low n (%)	High n (%)
Gender	Male	11 (20)	4 (7.3)		
	Female	38 (69.1)	2 (3.6)		
	p value	**0.041[f]**			
Tumour site	Non-tongue			13 (23.6)	20 (36.4)
	Tongue			2 (3.6)	20 (36.4)
	p value			**0.013[c]**	

Significance level: $p = 0.05$. c = Pearson Chi-square test. f = Fisher's Exact test

4.7 Association between expression of Ki-67, Cornulin, ISG15, clinicopathological prognosticators and relapse in OSCC

Binary logistic regression analysis was carried out to study the association between expression of all markers, clinicopathological prognosticators and relapse in OSCC. Prior to that, the association between clinicopathological prognosticators and relapse in OSCC was studied to determine additional clinicopathological prognosticators which might be influential in relapse of OSCC. Statistical analysis was performed using Chi-square tests (Pearson Chi-square test or Fisher's Exact test, whichever applicable). Significant association was observed between relapse of OSCC and age of patients ($p = 0.000$ or $p < 0.001$), where patients aged 57.5 years and above were significantly increased in the study group. There was significant increase in Chinese patients in the study group ($p = 0.009$); however, significant reduction in number of Indian patients ($p= 0.007$) and non-alcoholics ($p = 0.025$) was also observed in the study group. In patients with relapse of OSCC, a significant reduction in Type I and II POI and increase in Type III and IV POI was seen ($p = 0.007$).

Surgical margins with any grade of ED were significantly lesser in OSCC relapse cases ($p = 0.045$); however, when comparison was done on surgical margins without ED and mild ED versus moderate ED and severe ED, there was no significant association noted ($p = 1.000$) by using Fisher's Exact test, although there was slight decrease in the number of surgical margins without ED and mild ED, within the study group. Other clinicopathological prognosticators which included gender of patients, Malay ethnicity, habits of smoking and betel quid chewing, tumour site, tumour differentiation, perineural invasion, vascular invasion and metastasis to neck did not show significant association with relapse in OSCC. The results are shown in Table 4.6, significant *p* values are bolded.

Table 4.6: Clinicopathological prognosticators and relapse in OSCC

Clinicopathological prognosticators	Categories	Study	Control	*P* value
		n (%)		
Age (years)	≤ 57.5	5 (9.1)	24 (43.6)	**0.00**[c]
	> 57.5	18 (32.7)	8 (14.5)	
Ethnicity	Chinese	10 (18.2)	4 (7.3)	**0.009**[c]
	Non-Chinese	13 (23.6)	28 (50.9)	
	Indian	9 (16.4)	24 (43.6)	**0.007**[c]
	Non-Indian	14 (25.5)	8 (14.5)	
Alcohol	No	11 (20.8)	26 (49.1)	**0.025**[c]
	Yes	10 (18.9)	6 (11.3)	
Pattern of invasion	Type I and II	2 (37.5)	13 (24.1)	**0.007**[c]
	Type III and IV	21 (38.9)	18 (33.3)	
ED at surgical margins	No ED	10 (18.2)	13 (23.6)	**0.045**[c]
	any grade of ED	13 (23.6)	19 (34.5)	
	No ED + Mild ED	19 (34.5)	27 (49.1)	1.000[f]
	Moderate + Severe ED	4 (7.3)	5 (9.1)	

Significance level: $p = 0.05$. c: Pearson Chi-Square test.

In binary logistic regression analysis, the probability of relapse was calculated using expression of selected markers and clinicopathological prognosticators which were significant in relapse of OSCC. The model was 86.5% accurate in its predictions of OSCC relapse. Hosmer and Lemeshow test results confirmed that the model was a good fit for the data ($p = 0.955$). Coefficients for the model's predictors are presented in Table 4.7. Significant *p* values are bolded.

Table 4.7: Predictor Coefficients for the Model Predicting OSCC relapse

Clinicopathological prognosticators	*P* value	OR [95% CI]
Decreased expression of Cornulin	**0.018**	34.70 [1.86, 647.3]
ED	0.184	0.22 [0.022, 2.074]
Age	**0.008**	18.52 [2.174, 157.8]
Chinese	0.984	0. 97 [0.037, 25.33]
Indian	0.226	0.12 [0.004, 3.76]
Alcohol	0.058	8.41 [0.933, 75.85]
Pattern of invasion	0.244	4.28 [0.370, 49.54]

CI = Confidence interval; OR = Odds ratio

As demonstrated in Table 4.7, expression of Cornulin and age were the predictors which significantly improved the model's predictive capability. The odds ratio for decreased Cornulin expression indicated that there was a predicted 34-fold risk of OSCC relapse. There was also an 18-fold risk of OSCC relapse noted in relation to patients who were aged above 57.5 years. Other predictors such as ethnicity (Chinese and Indian), alcohol consumption, epithelial dysplasia and pattern of invasion of tumour did not appear to significantly influence the probability of OSCC relapse.

CHAPTER 5: DISCUSSION

5.1 Sociodemographic and clinicopathological prognosticators with OSCC

The global incidence of OSCC is increasing especially in Asian countries, partly attributed to practise of high-risk habits which includes smoking, alcohol consumption and betel quid chewing. Surgery is the preferred treatment in multidisciplinary setting along with radiotherapy and chemotherapy. However, OSCC remains challenging due to its aggressive characteristics which leads to relapse of the disease. Majority of the locally advanced tumours relapse within the first two years post-operatively (Irawati & Hao, 2015).

Hence, we studied the sociodemographic and clinicopathological features in OSCC patients including those who had developed a relapse within five years of follow up. Sociodemographic parameters included in this study were age, gender, ethnicity and habits. In the present study, our OSCC patients were 58 years old on an average when diagnosed with OSCC, which was in concordance with the findings from a multicentre-study involving a few Asian countries and Canada, where the mean age of OSCC patients was 50 – 59 years (Danuthai et al., 2017). Strikingly, there were two patients in our study who were below 40 years old. According to Kapila et al. (2017), there seemed to be a change in the demographic trend where OSCC was increasingly seen in younger individuals, leading to increasing prevalence of "early-onset SCC" which may be defined as SCC occurring in individuals younger than 40 years.

We noticed that total number of female patients was greater than male patients in our study. This finding was in concordance with OSCC epidemiological studies by Cheong et al. (2017) and Dhanuthai et al. (2017) where number of females exceeded males in Brunei, Laos and a few centres in Thailand; however, males generally outnumbered females overall.

In the present study, we identified a few clinicopathological prognosticators that were significantly associated with relapse of OSCC, which included age of patient, ethnicity, alcohol consumption, POI and ED at surgical margins. The other clinicopathological prognosticators included in this study were primary tumour site, tumour differentiation, perineural invasion (PNI), vascular invasion and metastasis to the neck.

Mean age was 63.9 ± 13.1 years in the study group while in the control group, mean age was 53.2 ± 8.2 years. There were more patients who were above 57.5 years in the study group, indicating that older patients were at a higher risk of developing a relapse of OSCC. The binary logistic regression analysis in our study showed increased age of patient was a predictor of relapse in OSCC. In one of the Malaysian studies that consisted of 19 OSCC patients who were 40 years and below, 21.1% of patients had developed recurrence (Ahmad et al., 2009). Earlier, recurrence in OSCC was reported after 10 years following treatment; this was thought to be related to prevalent drinking and smoking habits in the older age group, hence increased risk of recurrence and smoking-related cancers (Siegelmann-Danieli et al., 1998). Vazquez-Mahia et al. (2012) had reported a recurrence rate of 44.9% in 118 patients with OSCC. Comorbidities were significantly associated with recurrence. The study showed that presence of additional disorders (hepatic, diabetes and heart disorders) played an important role because patients with coexisting pathologies showed shorter recurrence-free intervals. This was later supported by Moye et al. (2015) that worse outcome of HNSCC treatment among elderly patients was partly associated with higher comorbidity status related to unspecified medical conditions. In contrast to our finding, there were less patients who were 60 years and above (33.6%) having OSCC recurrence than non-recurrence group (66.4%), in a study based in Tianjin, China (Wang et al., 2013).

In our study, Chinese ethnicity (p = 0.009) and Indian ethnicity (p = 0.007) significantly correlated with relapse of OSCC. We noticed that there were more Indian

patients compared to other ethnicities. Indians were also the highest number of patients having betel quid chewing and alcohol consumption habit. As discussed by Ghani et al. (2018), betel quid chewing habit was exclusively common in Malaysians of Indian origin. It is also the biggest attributable factor for OSCC among Malaysians, with estimation of reduction in oral cancer burden in this country by 23% if the habit is eliminated among the general population. We also noticed that more Chinese patients had combined habits of smoking and alcohol consumption, which was the most common practice among the populations. The significant association between ethnicity (Indian and Chinese) and relapse of OSCC could be explained due to the prevalence of these habits in the populations.

Statistical comparison of alcohol consumption habit with OSCC relapse showed significantly increased number of non-alcoholics in the control group than the study group. This indicated risk of relapse in OSCC was associated with alcohol consumption. In a multi-ethnic prevalence study recently in Malaysia, a 1.36-fold risk (adjusted for age, gender, ethnicity and socioeconomic status) of developing oral cancer with drinking habit alone was observed. A 5.49-fold and 20.33-fold risk of oral cancer was also observed with habit of drinking with smoking and drinking with chewing, respectively. However, association between drinking habit and relapse in OSCC could not be established (Ghani et al., 2018). In a meta-analysis by Miller et al. (2006), SPT associated with upper aerodigestive tract (UADT) cancer was related to the duration and severity of the patient's drinking history prior to diagnosis. In addition, risk of SPTs was 50% higher in patients who continued to drink more than 14 drinks per week after treatment, after adjusting for smoking status.

In the present study, we noticed POI of most OSCC cases was predominantly Type III, while Type I and II POI was significantly reduced in OSCC cases with relapse, indicating presence of more Type III and IV POI in relapse cases. This result agreed with findings

in a study by Camisasca et al. (2011) in Brazil, where only Type IV and type V POI were present in OSCC recurrence group, the result was statistically significant (p = 0.02). In our study we did not observe any Type V POI. Batool et al. (2015) had demonstrated that among 50 retrospective cases of OSCC in Lahore, Pakistan, 70% of patients having recurrence showed a Type III, IV and V POI where greatest number of patients showed Type III POI, which was similar to our study. The worst pattern of invasion in OSCC was significantly associated with overall survival as well as local recurrence. (Nason et al., 2009) proposed that POI would influence adequacy of margin resection. It was implicated that in tumours that invade deeply as nests and cords of cells usually required a wider margin compared to tumours with broad and flat-pushing invasive front.

It is well known that the residual tumour cells in the surgical margins due to incomplete resection may lead to LR of OSCC (Yang et al., 2019). However, despite having histologically clear (> 5 mm away from tumour) surgical margins, LR would still occur (Helliwell & Woolgar, 2013). Hence, we postulated that there might be presence of field cancerisation in histologically non-involved/ tumour-free surgical mucosal margins, which were not able to be detected under a light microscope. Due to retrospective nature of the study, it was not possible to obtain tissue samples directly from the surgical resection margins in patients. Therefore, the existing FFPE tissue blocks of surgical margins were used in the study, in view that these surgical margins were located adjacent to the remaining non-involved tissue in the patients.

To exclude the possibility of OSCC relapse due to residual tumour, we included only non-involved surgical margins as research samples, in which the distance of the margin was at least > 1 mm away from the tumour. Definition of an involved margin according to Helliwell & Woolgar (2013) is that the margin is < 1 mm away from an invasive carcinoma. In this context, ED was not a consideration in the definition of a histologically non-involved margin. However, presence and any grade of ED were considered as a

prognosticator for OSCC relapse in our study. We noticed that most of the non-involved mucosal surgical margins were not dysplastic (n = 24), followed by mild ED (n = 22), moderate ED (n = 8) and severe ED (n = 1). Severe ED was seen in the control group with no relapse of OSCC.

Our study showed significant association between ED in surgical margins and relapse of OSCC. However, there was no significant difference when four different groups of ED (no ED/ mild ED/ moderate ED/ severe ED) or dichotomisation of ED (no/ mild ED vs moderate/ severe ED) were compared with relapse of OSCC. Similarly, this was also observed by Holmstrup et al. (2006) in their study of long-term outcome of oral premalignant lesions. They concluded that dysplasia grading did not have any influence on the risk of malignant transformation. Schaaij-Visser et al. (2009) found that the grades of dysplasia in the surgical margins did not show a significant association with local disease-free survival. Histopathologic grading of dysplasia was subjective and showed limited value to predict relapse in individual cases, only severe ED might be considered an indication for adjuvant postoperative radiotherapy. Alternatively, genetic markers such as LOH and mutated TP53 could be determined in DNA isolated from the surgical margins, and these would be able to predict relapse (Schaaij-Visser et al., 2009). In the past few years, surgical margins with ED have been repeatedly reported to carry a risk of malignant transformation and subsequently local recurrence of OSCC (Jerjes et al., 2010; Warnakulasuriya S., 2014). Most authors had considered that moderate and severe ED at the resection margins demonstrated biological significance akin to that of early invasive carcinoma. However, the presence of mild ED at the margin was of questionable significance (Shah et al., 2019). Although a signification association was noted between ED and relapse; this was beyond our scope of investigation. Our aim was to investigate the field alteration in the surgical margin instead of the pre-existing morphological alterations. Presence of ED in our non-involved surgical margin samples was inevitable.

Due to limited samples, we were not able to include only surgical margins without ED. Absence of ED in other surgical margins from the same OSCC was not confirmed as well.

In our study, there were also many cases of OSCC relapse without ED in surgical margins. Out of 32 margins in the control group, we noticed ED in 18 margins (56.2%), in which 13 margins were mildly dysplastic, four margins were moderately dysplastic, and one was severely dysplastic. While in the study group, 43.5% of margins were not dysplastic. This could be partly explained due to our sampling technique. Only one or two of the surgical margins were randomly selected for this study, there were other surgical margins which we had not included due to random sampling or missing FFPE tissue blocks. Presence of ED was possible in the remaining surgical margins of the OSCC cases, and those could have contributed to OSCC relapse.

OSCC relapse in absence of ED could be explained by possible presence of small clusters of residual tumour cells that are undetectable on routine histopathological examination, this is known as minimal residual cancer (MRC). Another cause of relapse in cases without ED in the surgical margins was a remaining field of preneoplastic cells which is known as field cancerisation (Braakhuis et al., 2010). In the process of carcinogenesis, a field of preneoplastic epithelium precedes the development of cancer and expands laterally. These fields are usually not visible and does not cause any symptoms despite their large dimension. An important clinical implication is that the field often remains after surgery. Hence, in a course of time, a cell within the field develops cancer due to series of genetic hits that leads to SPT or LR, depending on the timing of development and its location from primary or IT. Secondary tumour which develops from the field is also known as second field tumour (SFT). A preneoplastic field might or might not show histological characteristics of ED.

Therefore, histologically normal appearing mucosa may be the forerunner of molecularly altered premalignant lesion which was yet to develop

morphological/cytological changes consistent with dysplasia (Speight et al., 2007). Oral premalignancy or dysplasia could also accumulate genetic and epigenetic changes within a clonal population of cells. The resulting genetic alterations could affect numerous genes and lead to phenotypic changes such as cell death, increased proliferation, increased angiogenesis and ability to metastasise (Lingen et al., 2011). LOH analyses were carried out by several researchers, and it was conclusive that ED was strongly associated with LOH. It was suggested that around five genetic mutations were required to transform a normal epithelial cell to a malignant one. LOH in 3p, 9q and 17p was frequently seen in dysplastic lesions (Braakhuis et al., 2003). Mao et al (1992) noticed LOH at 9p21 and 3p14 were associated with progression of premalignant lesions. LOH at 4q and 17p showed approximately 50-fold increase in progression to OSCC (Zhang et al., 2013). However, if patients were related to risky habits such as smoking, they might be having similar genetic alterations with non-smokers although some researchers thought unique genetic mutation might be seen in oral ED of non-smokers (Rao et al., 2018). Furthermore, LOH profile of the primary lesion should be consistently present in the recurrent lesions and should be validated in the context of field cancerisation (Lingen et al., 2011).

In our study, we noticed seven SPT cases and eight cases with LR among OSCC patients with relapse. Out of seven SPTs, there were two SPTs which occurred on the contralateral side of IT. The IT in both cases involved the buccal mucosa, while the contralateral SPT involved the buccal mucosa and gingiva, respectively. Both patients were Indian females practising betel quid chewing habit. One of the patients practised alcohol drinking as well. Both OSCC IT showed moderate differentiation. Lymph node metastasis was absent in one of the patients, while status of lymph node metastasis of another patient was unknown. As diagnostic criteria of an SPT was relapse after three years and the location being more than two centimetres away from index tumour

(Braakhuis et al., 2010), these patients were diagnosed as having SPT. As proposed by Ha & Califano (2003), prolonged exposure to carcinogens could alter the state of epithelium, making it susceptible to development of multifocal carcinoma. This could explain the presence of SPT in the contralateral mucosa in our study due to exposure of oral mucosa to carcinogens from the generalised effect of betel quid chewing.

A comparison of ED and clinicopathological prognosticators showed significant increase in number of smokers among patients having any ED, in the control group. Smoking was associated with an increased risk of oral cancer and other oral potentially malignant disorders, with risks increasing in a dose-dependent pattern and declining with the duration of smoking cessation (Morse et al., 2007). A 3.8-fold risk of developing high-risk oral ED was observed in smokers taking any form of tobacco. Interestingly tobacco use with areca nut and tobacco chewing showed a 0.8-fold risk and 0.7-fold risk of developing high risk ED, respectively (Aroquidasse et al., 2016). The authors also found a steady increase in development of high-risk ED with increased duration of tobacco use. In our study, duration of smoking was barely specified in the patient's histopathological examination (HPE) reports, and it was not available in the database. A dose-dependent relationship between smoking and development of oral cancer could be established if the information was available.

Among all OSCC cases, we noticed 35.3% of tongue SCC whereas OSCC in other subsites was higher in number. This was in contrast to many other studies where tongue was a highly prevalent site for OSCC (Pires et al., 2013; Gupta et al., 2016; Rao et al., 2013). Certain regions in Asian countries observed that buccal mucosa was the commonest site of OSCC as reported in Mangalore (Salian et al., 2016), Maharashtra, India (Giri et al., 2013), and Khon Kaen, Thailand (Vatanasapt et al., 2011). Apart from the small sample size in our study, the discordance observed could be due to the prevalence of habits practised. For instance, highly prevalent betel quid chewing habit

among OSCC patients was associated with a high frequency of buccal mucosa SCC. (Salian et al., 2016). Most of the patients in our study showed moderately differentiated OSCC, but lack of PNI and vascular invasion. About 26% of patients had metastasis of tumour to the neck. Several parameters have been implicated in prognosis and recurrence of OSCC, including tumour differentiation, PNI, lymphatic invasion, bone invasion, location of tumour, depth of invasion, and status of surgical margins, as seen in most of the clinicopathological studies for OSCC. These factors have been correlated with OSCC recurrence (Camisasca et al., 2011). Further analyses by the authors showed additional clinicopathological factors such as location of OSCC in the tongue, tobacco use, tumour grading and POI should be considered for OSCC treatment and follow-up. Due to insufficient data, we were not able to include other prognosticators such as tumour size and staging, depth of invasion of tumour and extracapsular spread in lymph nodes; these factors were also considered to be important in recurrence and survival of OSCC (Wang et al., 2013).

5.2 Expression of Ki-67, Cornulin and ISG15 with OSCC

Ki-67 has been known as a useful proliferative marker to accurately measure tumour growth and aggressiveness (Birajdar et al., 2014). It is also a good prognosticator of OSCC even in normal oral mucosa distant from primary tumour (Kumar et al., 2019). Based on the available evidence, this marker was used to compare against immunostaining of Cornulin and ISG15 in our study. We observed that the labelling index (LI) of Ki-67 in non-involved surgical margins was higher in patients with OSCC relapse than those without relapse, although this was not significantly different (p = 0.824). Our finding was similar to that by Kumar et al. (2019) where increased Ki-67 LI was seen in negative margins of OSCC recurrence cases, although the result was not statistically significant. It was also observed by the authors that expression of Minichromosome Maintenance Protein 2 (MCM 2) in the surgical margins of OSCC

recurrence cases compared to normal oral mucosa was significant, suggesting MCM 2 to be a more sensitive and novel marker in predicting the locoregional recurrence. In a study by Wangsa et al. (2008), increased Ki-67 expression was seen in the OSCC tissue of cases with local recurrence, and the result was statistically significant. Similar result was also observed by Mohri et al. (2016) in a multivariate analysis where the authors discovered Ki-67 as an independent predictor of local recurrence in T1 and T2 tongue SCC. Wang et al. (2013) noticed OSCC cases showing high expression of Ki-67 were increased in OSCC recurrence group compared to non-recurrence group. RACK1 gene was also investigated in the study and it was found to be as useful as Ki-67 in predicting recurrence. Epithelial cells within the genetically altered field in surgical margins would exhibit high proliferative capacity; hence increased Ki-67 staining was observed thereby accounting to relapse of OSCC in cases with non-involved surgical margins (Lingen et al., 2011). This would be further discussed in the text later.

In the present study, Ki-67 positive cells in normal oral mucosa (NOM) control samples were occasionally seen in the basal and suprabasal layers of the epithelium, which was not in agreement with most of the studies, where the distribution of Ki-67 positive cells were confined to basal or occasionally parabasal layers only (Preethi et al., 2014; Nagappa et al., 2016). The NOM tissue included in our study was obtained from sites adjacent to impacted third molars during minor oral surgical removal procedures. Our study also showed statistically significant increase in labelling index of Ki-67 (28.05 ± 11.7) in NOM control tissue compared to non-involved surgical margins of patients with or without relapse of OSCC. This opposed the findings in other studies where the labelling index of Ki-67 in normal gingival tissues was significantly lower than that in chronic gingivitis and chronic periodontitis (Nagappa et al., 2016), or in pericoronal tissue (Rahman et al., 2013). It was suggested that the inflammation in the pericoronal tissue upregulated the cell turnover and led to proliferation, which might result in cystic

formation. However, the mechanisms underlying these changes have not yet been explained but could reflect an imbalance between cell proliferation and apoptosis.

We noticed expression of Ki-67 was significantly associated with gender in our study. Number of female patients showing low expression of Ki-67 in the surgical margins was much greater than that with high Ki-67 expression. Number of male patients exhibiting low Ki-67 expression was also lower than females. This phenomenon was also observed by Jalayer et al. (2012) when expression of Ki-67 in relation to age and gender in OSCC was studied. A positive correlation between Ki-67 expression in female patients was noted; however, this could not be explained by the authors. Another possible explanation for this finding was, we had more female patients (n = 25) than male patients (n = 9) in our study. Greater difference (n = 27) was seen between number of males and females with low Ki-67 expression while small difference was seen between number of males and females with high Ki-67 expression (n = 2). In most of the available studies on OSCC, number of male patients usually exceeded female patients, due to habits such as smoking and alcohol consumption. However, due to widely practised betel quid chewing habit among females, increasing number of females diagnosed with OSCC have been reported especially in South East Asian (SEA) countries. This was proven when betel quid chewing habit was commonly observed in rubber estates among the Indian population in Malaysia as well as Northern Thailand. Highest percentage of women smokers was seen in Laos and Brunei across all SEA countries involved in the study (Cheong et al. (2017). A study in Brazil showed increased number of females having OSCC with changes in male to female ratio from 6:1 to 2:1 over the time, this could probably due to changes in social and daily activities associated with modern women social profile and way of living, leading to higher exposure to carcinogenic agents, such as tobacco and alcohol consumption and exposure to biological agents, such as high-risk HPV subtypes (Pires et al., 2013).

The Cornulin (CRNN) gene is referred to as squamous epithelial heat shock protein-53 and functions as a stress-responsive factor. It is also involved in terminal epidermal differentiation. Molecular studies of squamous epithelial heat shock protein-53 have shown that its expression helps to maintain the barrier function in the squamous epithelium in response to injury and functions as a survival factor in humans, for example upregulation of Cornulin was observed in response to squamous epithelial cell injury in the buccal mucosa of smokers (Kupfer et al., 2010). Overexpression of Cornulin was also seen in psoriasis lesions of the skin, induced by M5 pro-inflammatory cytokines namely IL-17A, IL-22, IL-1α, oncostatin M and TNF- α. Expression of Cornulin increases cell proliferation by inducing cyclin D1. Thus, it seems that upregulation of Cornulin plays a critical role to control environmental pressures and to prevent formation of lesions on the epithelial tissues. Cornulin expression was usually downregulated in cancerous lesions as seen in OSCC (Salahshourifar et al., 2015) and oesophageal SCC (Hsu et al., 2014).

In our study, NOM control samples showed intense staining of Cornulin in the superficial epithelium which included the keratin, granular and spinous cell layers. Gradual decrease in intensity and proportion of staining was observed in the samples of control group followed by the study group. The finding was similar to that by Santosh et al. (2019) in which the pattern of staining was described as stepwise reduction in expression. Expression of Cornulin was confined to squamous cells. As the outer layers of the epithelium were responsible in confronting with stresses and stimuli, increased expression and intensity of staining in response to squamous epithelial cell injury was observed (Salahshourifar et al., 2015).

Our findings also showed significant downregulation of Cornulin (p = 0.004) in the surgical margins of patients with OSCC relapse compared to those without relapse. This finding agreed with study by Schaaij-Visser et al. (2009) where downregulation of Cornulin in the surgical margins of previously resected HNSCC (oral cavity and

oropharynx) was associated with an increased risk for relapse. When upregulation of Cornulin was expected in damaged oral epithelial cells due to habitual risk factors, downregulation of Cornulin was noted in OSCC. This reflected that it might not be able to respond to DNA damages that might be induced by habitual risk factors, possibly attributed to mutation of Cornulin or LOH in relevant locus in the chromosome (Salahshourifar et al., 2015).

Santosh et al. also mentioned that downregulation of Cornulin was associated with lymph node metastasis in OSCC. In our study, expression of Cornulin and lymph node metastasis was not significantly related, although there were three patients with lymph node metastasis exhibiting low Cornulin expression. In contrast to most of the studies, Weinberger et al. (2010) discovered an overexpression of Cornulin in the normal tissue adjacent to HNSCC in patients with occult cervical lymph node metastasis.

In our study we also noticed a significant association between expression of Cornulin and primary tumour sites. Among 15 surgical margins exhibiting low expression of Cornulin, 11 surgical margins were from the patients with relapse which showed only one OSCC case involving the tongue. OSCC involving tongue in our study was associated with high Cornulin expression. However, Salahshourifar et al. (2015) noticed one non-tongue OSCC case among three OSCC cases which showed low Cornulin expression. In a study by Schaaij-Visser et al. (2009), significant association was observed between low expression of Cornulin and relapse in HNSCC which included OSCC and oropharyngeal SCC; however, specific tumour site was not mentioned. Due to small number of tongue SCC cases in our study, we were not able to conclude that low expression of Cornulin was seen in primary OSCC of the tongue.

By performing a binary logistic regression analysis, we found that Cornulin expression in surgical margins was one of the predictors for relapse of OSCC. We agreed with the suggestion by Schaaij-Visser et al. that Cornulin immunostaining of the surgical margins

could be used in the decision-making process to determine the surveillance policy for treated HNSCC patients during follow-up, due to its potential prognostic value.

ISG15 is an interferon regulated gene induced as a primary response with regards to various microbial and cell stress stimuli and encodes a 15kDA interferon inducible ubiquitin-like protein. An appropriate undisturbed regulation of ISG15 gives rise to a tumour suppressive effect (Andersen & Hassel, 2006). In the present study, both study and control groups demonstrated higher expression of ISG15 than the NOM. However, expression of ISG15 in surgical margins of control group was significantly higher than that in the study group. These results were not in concordance with previous studies where overexpression of ISG15 was usually seen in OSCC tissue (Laljee et al., 2013; Sumino et al., 2016). A study by Laljee et al. (2013) suggested ISG15 to be a clinically relevant OSCC diagnostic biomarker as 80% of OSCC tissue samples showed an overexpression of ISG15. Sumino et al. (2016) had shown moderate to strong staining of ISG15 in all OSCC tissue samples and 33 out of 34 samples of oral dysplastic lesions. However, the grade of ED was not mentioned in dysplastic lesions. Overexpression of ISG15 in their study was associated with micro-copy number alteration (CNA), this was observed in all (n = 27) OSCC samples using quantitative real time-polymerase chain reaction (qRT-PCR). It was suggested by the authors that this gene could be considered as a potential biomarker in oral cancer.

To the best of our knowledge, this was the first study to investigate expression of ISG15 in non-involved surgical margins to predict relapse in OSCC. Most of the published literature on ISG15 and oral cancer included studies on OSCC tissue rather than the surgical margins. Sumino et al (2016) commented on the staining of tumour and its adjacent epithelium. The authors noticed the epithelium barely picked up the stains, but the tumour tissue did. The distance of adjacent normal epithelium from the tumour was not specified.

In a study by Zhang et al. (2017) it was observed that the expression of ISG15 was higher in OSCC tissue than in adjacent grossly normal tissue. The authors discovered that miR-138 which was involved in regulation of protein translation and mRNA stability, was able to bind to ISG15 3'UTR (three prime untranslated region). After binding to ISG15, miR-138 expression became lower in cancer tissue than in the adjacent tissue. In contrast to that, ISG15 became overexpressed in cancer tissue compared to adjacent tissue. Overexpression of ISG15 negatively regulated the ubiquitin proteasome pathway (UPP) and poly-ubiquitin protein level. The level of poly-ubiquitination was a key factor to degrade target protein by proteasome. Hence, ISG15 was able to promote the invasion and migration of tumour through ubiquitin induced protein degradation. Elevated expression of miR-138 in the adjacent normal tissue could induce tumour suppression effect and inhibit invasion, migration and proliferation of OSCC cells (Zhang et al., 2017). This could explain the finding in our study that expression of ISG15 was lower within surgical margins in the study group. We agreed with suggestions by the authors that MiR-138 may play a role in regulating the upstream factors in the UPP, and it may potentially serve as a novel biomarker for the early diagnosis and prognosis of OSCC.

Although amplification and overexpression of ISG15 in OSCC tumour tissue (Vincent-Chong et al., 2012; Sumino et al., 2013) and its downregulation in the surgical margins of OSCC (Zhang et al., 2016) had been reported, the mechanism of this gene in OSCC remains controversial. This necessitates further analysis to clarify its role in OSCC. In addition, the finding in relation to ISG15 in our study may also partly be attributed to small sample size compared to Ki-67 and Cornulin as only one representative surgical margin was included for each OSCC case, due to insufficient anti-ISG15 antibody. We recommend further validation to be carried out using surgical margins of OSCC.

We noticed that in the binary logistic regression analysis, decreased expression of Cornulin and age of patients showed odds ratio of 34.7 and 18.5, respectively. The results implied that expression of Cornulin and age of patients were predictors for relapse of OSCC, after having other clinicopathological prognosticators such as ethnicity, alcohol consumption, ED and POI of tumour adjusted. Our finding on association between age and relapse of OSCC was in contrast to that by Xu et al. (2018). The authors had concluded that age was not an independent risk factor for OSCC, although advanced age (above 75 years) was significantly associated with recurrence of OSCC.

In a study by Hsu et al. (2014), a multivariate analysis for overall survival in oesophageal SCC showed negative CRNN expression along with nodal involvement and distant metastasis remained an independent predictor for poor overall survival ($p = 0.047$). Hazard ratio of positive Cornulin staining versus negative Cornulin staining was 0.683.

In a retrospective analysis of 517 OSCC cases with negative surgical margins by Safi et al. (2017), the authors noticed only histological grading of OSCC had been demonstrated as an independent risk factor for locoregional recurrence in the multivariate analysis. Poor histological grading showed a 2.08 to 2.10-fold higher risk of recurrence than well or moderate grading in OSCC. Histological grading was neither a predictor nor associated with relapse of OSCC in our study. In addition, Sridharan et al. (2019) had analysed 49 early tongue SCC cases who developed LR. A proportional hazards regression model has established that distance between tumour to the closest margin and PNI were associated with LR. Observed hazard ratio was 0.36 and 1.92 respectively. However, in our study, duration of disease and onset of recurrence were not included.

5.3 Limitations of study

Due to the retrospective nature and limited number of cases that could be included and non-availability of FFPE tissue blocks required, a strong conclusion could not be drawn from this study even though significant results were observed in relation to expression of

Cornulin. Studies with larger cohort of cases are required to validate our findings. Secondly, correlation with tumour characteristics, tumour staging, and survival analysis was not done in this study due to insufficient available data. Part of the sociodemographic data was missing, especially patients' habits. Discrepancies were also observed in the HPE reports, as some of the important information was not reported. As we were having insufficient anti-ISG15 antibody due to unexpected events, homogenous sample size for all markers could not be created and we believed this could have affected our results.

CHAPTER 6: CONCLUSION

The present study was able to show presence of genetically altered epithelial cells in histologically non-involved mucosal surgical margins that were associated with relapse in OSCC. Downregulation of Cornulin was able to predict relapse in OSCC.

In this study, expression of Ki-67, Cornulin and ISG15 was demonstrated in histologically non-involved mucosal surgical margins. Although not statistically significant, expression of Ki-67 in surgical margins of OSCC relapse was higher than that in non-relapse cases. Significant decreased expression of Cornulin in the margins was associated with relapse in OSCC. Unfortunately, we were not able to draw a conclusion based on the decreased expression of ISG15 in non-involved surgical margins of OSCC relapse cases. We strongly recommend future investigations to validate this finding.

In our study, expression of Cornulin and age of patients were 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, Cornulin and ISG15 in predicting a relapse in OSCC as well as their association with clinicopathological prognosticators of OSCC and analysis of survival with larger cohorts. Taken together, we suggest Cornulin as a predictor in relapse of OSCC.

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