MAGEB2 ANTIBODY AS POTENTIAL DIAGNOSTIC AND PREDICTIVE TOOL IN THE PROGRESSION OF ORAL CANCER

NORATIKAH BT AWANG HASYIM

FACULTY OF DENTISTRY

UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

MAGEB2 ANTIBODY AS POTENTIAL DIAGNOSTIC AND PREDICTIVE TOOL IN THE PROGRESSION OF ORAL CANCER

NORATIKAH BT AWANG HASYIM

RESEARCH PROJECT SUBMITTED TO THE FACULTY OF DENTISTRY UNIVERSITY OF MALAYA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF CLINICAL DENTISTRY ORAL MEDICINE AND ORAL PATHOLOGY

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2020

UNIVERSITI MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Noratikah bt Awang Hasyim

Registration/Matric No: DGH170002

Name of Degree: Master of Clinical Dentistry Oral Medicine and Oral Pathology.

Thesis: MAGEB2 antibody as a potential diagnostic and predictive tool in the progression of oral cancer.

Field of Study: Oral cancer.

I do solemnly and sincerely declare that:

(1) I am the sole author / write of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exist was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of all the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's signature Date
Name:
Designation:

MAGEB2 ANTIBODY AS POTENTIAL DIAGNOSTIC AND PREDICTIVE TOOL IN THE PROGRESSION OF ORAL CANCER

ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) represents more than 90% of all oral cancer. Despite advances in diagnostic and therapeutic modalities, mortality and morbidity rates have not improved in the last decade. Hence, alternative diagnostic and therapeutic approaches are urgently needed. Cancer testes antigens (CTA) are proteins that are expressed in various malignant tumours but its expression in normal tissue is restricted to testes and occasionally placental trophoblast. Due to its exclusive presence in different types of malignant tumors, and their ability to trigger immune responses, CTA are considered promising biomarkers for cancer vaccines. A previous protein array study reported that MAGEB2, a subset of CTA was significantly associated with better prognosis in OSCC patients. **Objectives:** The objective of this study were to compare the expressions of MAGEB2 antibody in the tissues of normal oral mucosa (NOM), oral potentially malignant disorder (OPMD), and OSCC patient as well as to evaluate the association of MAGEB2 antibody with socio-demographics and clinico-pathological characteristics in OSCC patients, and lastly to determine the association of MAGEB2 expressions with overall survival in OSCC patients. **Methods:** Immunohistochemical (IHC) staining with MAGEB2 antibody was performed on 10 NOM, 20 OPMD, and 57 OSCC tissues. Kruskal-Wallis test was used to compare MAGEB2 expression between NOM, OPMD, and OSCC tissue. Diagnostic accuracy of MAGEB2 in distinguishing NOM, OPMD, and OSCC tissue and prognostic accuracy of MAGEB2 with socio-demographic and clinico-pathologic characteristics were determined using receiver operating characteristic (ROC) curve. Kaplan-Meier survival analysis was used to determine the association between MAGEB2 expressions with overall survival (OS).

Results: MAGEB2 expression was seen in 81% of OSCC tissue. The least MAGEB2 expression was observed in OPMD tissue. MAGEB2 expression was significantly higher in OSCC compared to OPMD tissue ($p = 0.014$). However, there is no significant difference between MAGEB2 expression in NOM Vs OSCC and NOM Vs OPMD tissue. MAGEB2 was able to distinguish OSCC from OPMD tissue with diagnostic accuracy of 61% sensitivity and 80% specificity. There is no significant correlation between MAGEB2 protein expression with socio-demographic, clinico-pathologic characteristics, and OS in OSCC patients. However, a trend of better overall survival in tissues with high MAGEB2 expression was observed in this study.

Conclusions: MAGEB2 is a potential diagnostic biomarker in distinguishing OPMD from OSCC tissues. However, there is no significant association between MAGEB2 expression with socio-demographic, clinico-pathological, and OS in OSCC patients. A larger and equal number of sample size along with inclusion of various OPMD cases are recommended for future study.

Keywords: Cancer testes antigens, MAGEB2, immunohistochemistry, oral squamous cell carcinoma.

MAGEB2 ANTIBODI SEBAGAI ALAT DIAGNOSTIK POTENSI DAN PREDIKTIF DALAM PROGRES KANSER ORAL

ABSTRAK

Pengenalan: Karsinoma sel skuamosa oral (OSCC) mewakili lebih daripada 90% daripada semua kanser mulut. Walaupun terdapat kemajuan dalam kaedah diagnostik dan terapi, kadar kematian dan morbiditi tidak menunjukkan perubahan dalam dekad yang terakhir. Oleh itu, pendekatan diagnostik dan terapi alternatif sangat diperlukan. Antigen kanser testis (CTA) adalah protein yang dinyatakan dalam pelbagai ketumbuhan malignan tetapi ekspresinya dalam tisu normal hanya terhad kepada testis dan kadang-kadang dalam trofoblas plasenta. Oleh sebab kehadirannya yang eksklusif dalam pelbagai jenis ketumbuhan malignan, dan kemampuannya untuk meggerakkan tindak balas imun, CTA dianggap sebagai biomarker yang berpotensi untuk vaksin barah. Kajian larutan protein sebelumnya melaporkan bahawa MAGEB2, subset CTA dikaitkan dengan prognosis yang lebih baik dalam pesakit OSCC. **Objektif**: Objektif kajian ini adalah untuk membandingkan ekspresi antibodi MAGEB2 dalam tisu mukosa oral normal (NOM), gangguan berpotensi malignan oral (OPMD), dan pesakit OSCC serta menilai kaitan antibodi MAGEB2 dengan sosio- demografi dan ciri klinikal-patologi pada pesakit OSCC, dan terakhir untuk menentukan hubungan ekspresi MAGEB2 dengan keseluruhan kelangsungan hidup pada pesakit OSCC. **Kaedah**: Pewarnaan imunohistokimia (IHC) dengan antibodi MAGEB2 dilakukan pada 10 tisu NOM, 20 OPMD, dan 57 OSCC. Ujian Kruskal-Wallis digunakan untuk membandingkan ekspresi MAGEB2 antara tisu NOM, OPMD, dan OSCC. Ketepatan diagnostik MAGEB2 dalam membezakan tisu NOM, OPMD, dan OSCC dan ketepatan prognostik MAGEB2 dengan ciri sosio-demografi dan

klinikal-patologi ditentukan menggunakan keluk ciri operasi penerima (ROC). Analisis survival Kapplan-Meier digunakan untuk menentukan hubungan antara ekspresi MAGEB2 dengan keseluruhan survival (OS). **Hasil**: Ekspresi MAGEB2 dilihat pada 81% tisu OSCC. Ungkapan MAGEB2 paling sedikit diperhatikan dalam tisu OPMD. Ekspresi MAGEB2 jauh lebih tinggi dalam OSCC berbanding dengan tisu OPMD (p = 0.014). Walau bagaimanapun, tidak ada perbezaan yang signifikan antara ekspresi MAGEB2 dalam tisu NOM Vs OSCC dan NOM Vs OPMD. MAGEB2 dapat membezakan OSCC dari tisu OPMD dengan ketepatan diagnostik iaitu 61% kepekaan dan 80% kekhususan. Tidak ada hubungan yang signifikan antara ekspresi protein MAGEB2 dengan sosio-demografi, ciri klinikal-patologi, dan OS pada pesakit OSCC. Walau bagaimanapun, trend survival keseluruhan yang lebih baik dalam tisu yang menunjukkan ekspresi MAGEB2 yang tinggi diperhatikan dalam kajian ini. **Kesimpulan**: MAGEB2 adalah biomarker diagnostik yang berpotensi dalam membezakan OPMD dari tisu OSCC. Namun, tidak ada hubungan yang signifikan antara ekspresi MAGEB2 dengan sosio-demografi, klinik-patologi, dan OS dalam pesakit OSCC. Saiz sampel yang lebih besar dan seragam dan penyertaan pelbagai kes OPMD disyorkan untuk kajian masa depan.

Kata kunci: Antigen testis barah, MAGEB2, imunohistokimia, karsinoma sel skuamosa oral.

ACKNOWLEDGEMENTS

All praises and thanks to Allah, the Most Beneficient, the Most Merciful for His showers of blessings that allows successful completion of this research project. I would like to express my deep and sincere gratitude to my research supervisor, Dr. Anand Ramanathan for his determination, invaluable guidance, and constructive critism throughout this research. I am extending my heartfelt appreciation to my co-supervisor, Associate Professor Dr. Zuraiza Mohamad Zaini for her advice, encouragement, and feedback in this study. I would like to thank Prof Rosnah Mohd Zain from MAHSA University for her financial assistance for this research.

A research would not complete without an appropriate statistical analysis that is fundamental in every research. Hence, I want to express my thanks in abundance to my statistician, Dr. Mahmood Danaee from Research Office, Faculty of Medicine, University of Malaya for his guidance in selection of appropriate statistical test, data analysis, and interpretation of the results. In addition, I also want to give credit to Dr. Wan Maria Nabillah Ghani from Oral Cancer Research and Coordinating Centre (OCRCC) for her assistance in providing relevant data throughout this research. I also thank all the staff of Oral Pathology Diagnostic and Research Laboratory (OPDRL) for helping during the process of data collection and assisting me in laboratory works all this while.

I would like to dedicate this research to my son, Harith Ukail for being the ultimate source of motivation for me. To my loving colleagues, for their constant support and understanding, Finally, my thanks to all the people who have directly or indirectly contribute to this research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC	Area under the curve
CEA	Carcinoembryonic antigen
CI	Confidence Interval
COE	Clinical oral examination
CTA	Cancer testes antigens
cTNM	Clinical tumour staging
DAB	3,3'-diaminobenzidine
DNA	Deoxyribonucleic acid
DOI	Depth of invasion
DPX	Dibutylphthalate Polystyrene Xylene
ECS	Extracapsular spread
ENE	Extranodal extension
FFPE	Formalin-fixed paraffin embedded
GLOBOCAN	Global Burden of Cancer
H&E	Hematoxylin and Eosin
HNSCC	Head and neck squamous cell carcinoma
HPE	Histopathological examination
HPV	Human papilloma virus
IARC	International Agency for Research in Cancer
ICC	Intraclass correlation coefficient
ICD	International Classification of Diseases
IHC	Immunohistochemistry
IL-8	Interleukin-8
IRS	Immunoreactive scores
LHR	Lymphocyte host response
LOH	Loss of heterozygosity
LR	local recurrence
LVI	Lymphovascular invasion
MAGEB2	Melanoma antigen family B2
MAHSA	Malaysian Allied Health Sciences Academy
MOCDTBS	Malaysian Oral Cancer Database and Tissue Bank system
MTR	Malignant transformation rate
NOM	Normal oral mucosa
NSCLC	Non-small cell lung cancer
NSGCT	Non-seminomatous germ cell tumour
OC	Oral cancer
OCRCC	Oral Cancer Research and Coordinating Centre
OPSCC	Oropharngeal squamous cell carcinoma
OPDRL	Oral Pathology Diagnostic and Research Laboratory
OR	Odds ratio
OS	Overall survival
OSCC	Oral squamous cell carcinoma
OPMD	Oral potentially malignant disorder
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
pN	Pathological lymph node
PNI	Perineural invasion
POI	Pattern of invasion

PSA	Prostate specific antigen
pT	Pathological tumour
pTNM	Pathological tumour staging
Rb	Retinoblastoma
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerise chain reaction
SCC	Squamous cell carcinoma
TAAs	Tumour associated antibodies
TP53	Tumour protein 53
TSNA	Tobacco specific nitrosamine
UM	Universiti of Malaya
USA	United States of America
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WPOI	Worst pattern of invasion

CHAPTER 1 : INTRODUCTION

1.1 Background

Oral cancer, a subset of head and neck cancer is a heterogeneous disease with known multifactorial aetiology. In Malaysia, although oral cancer (ICD-10 codes C00-C06) is not listed among the top ten most common cancers, oral cancer is ranked 4th and 8th most common cancer in female and male Indian according to the Malaysia National Cancer Registry report (Azizah, Nor Saleha, Noor Hashimah, Asmah, & Mastulu, 2016). The alarming high incidence among Indian populations is mainly due to betel-quid chewing habit. Oral squamous cell carcinoma (OSCC) represents more than 90% of cancers diagnosed in the oral cavity (El-Naggar, 2017; El-Naggar, Chan, Grandis, Takata, & Slootweg, 2017). In spite of advancement in diagnostic and therapeutic approaches in oral cancer, the overall survival of OSCC patients in Malaysia is still low compared to other developed countries. A study in England reported the 5-year overall survival (OS) was 56% in OSCC patients (Rogers et al., 2009). Meanwhile in Malaysia, the 5-year OS was 13.4% that was three times worse than OS for OSCC patients in England (W. M. N. Ghani et al., 2019). Accordingly, development of alternatives for oral cancer detection and treatment is urgently needed. A major drawback in OSCC is the failure for early detection as a majority of the patients were diagnosed at advanced stages (Zainal Ariffin & Nor Saleha, 2011).

Biomarker is defined as a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process and disease, such as cancer (Henry & Hayes, 2012). Several previous studies demonstrated specific targets for immunotherapeutic approaches that could be useful in the control of cancer progression (Rosenberg, Dudley, & Restifo, 2008). However, limited studies were done in head and

neck cancer. Cancer testes antigens (CTA) are proteins that are expressed in various malignant tumours but expression in normal tissue is restricted to testes and occasionally placental trophoblast (Caballero & Chen, 2012). Thierry Boon and his colleagues first discovered CTA in 1991 when they successfully cloned the first antigen that made significant advancement in tumour immunology (van der Bruggen et al., 1991). More than 200 CTAs have been identified (Simpson, Caballero, Jungbluth, Chen, & Old, 2005). Many CTAs have shown to evoke cellular and serological immune responses in human (Scanlan, Simpson, & Old, 2004). CTAs are considered as potential target for cancer vaccines due to its presence in various malignant tumours and capability to initiate immune response. Several clinical trials have been and are being conducted using CTAs as vaccine targets to stimulate autologous immune responses in patients with various tumors (Davis et al., 2004). As a consequence, global research that looks into various aspects of CTA in cancer is rapidly growing. However, very little is known about the presence of CTAs in head and neck cancer especially in the oral cancers.

1.2 Rationale

A previous unpublished protein array study conducted by oral cancer research coordinating centre (OCRCC) team showed a series of top ten upregulated tumour associated antibodies (TAAs) were identified by protein array analysis study involving sera of OSCC patients. The top ten upregulated TAAs were TP53, MAGEA4, MAGEB2, NRIP3, MAGEB4, SH2B1, GSTT1, PTP1B, GTP2H2D and MAPK3. Of the ten TAAs, five TAAs (TP53, SH2B1, PTP1B, MAGEB2 and NRIP3) were significantly associated with better prognosis after accounting for the socio-demographic and clinic-pathological parameters in OSCC patients. Potential use of these markers in cancer was explored from the extensive literature review.

Surprisingly, no previous research has been done to evaluate protein expression of MAGEB2 in OSCC tissue. Along this line, it was decided that it is worthwhile to assess the expression of this biomarker in normal oral mucosa (NOM) and oral potential malignant disorder (OPMD) tissue as well. Unfortunately, due to financial constraint, only one biomarker, which is MAGEB2, was selected in this study. Thus, the intention of this study was to investigate and compare protein expression of MAGEB2 in NOM, OPMD, and OSCC tissue, analyzing its potential association with socio-demographic and clinico-pathological characteristics, as well as the overall survival.

1.3 Aim

To analyze the expression of MAGEB2 antibody in the tissues of NOM, OPMD, OSCC patients.

1.4 Specific objectives

1. To compare the expression of MAGEB2 antibody in the tissues of NOM, OPMD, OSCC patients.
2. To evaluate the association between MAGEB2 expressions with socio-demographics and clinico-pathological characteristics in OSCC patients.
3. To evaluate the association between MAGEB2 expressions with overall survival in OSCC patients.

1.5 Study hypothesis

There is a difference in the expression of MAGEB2 antibody in the tissue of NOM, OPMD, and OSCC patients. There is an association between MAGEB2 expression with socio-demographics, clinico-pathological characteristics, and overall survival in OSCC patients.

1.5.1 Null hypothesis

1. There is no difference in the expression of MAGEB2 antibody in the tissue of NOM, OPMD, and OSCC patients.
2. There is no association between MAGEB2 expressions with socio-demographics and clinico-pathological characteristics in OSCC patients.
3. There is no association between MAGEB2 expressions with overall survival in OSCC patients.

CHAPTER 2 : LITERATURE REVIEW

2.1 Introduction

Oral cancers (OC) are malignant neoplasm of the oral cavity comprised of squamous cell carcinoma, salivary gland, and odontogenic malignancy. Squamous cell carcinoma (SCC) constitutes about 84-97% of all oral cancers (Ariyoshi et al., 2008; Bhurgri et al., 2003; Kruaysawat, Aekplakorn, & Chapman, 2010; Sawair et al., 2007). OSCC may arise from the pre-existing oral potentially malignant disorder (OPMD) or from normal oral mucosa (NOM). Ironically, not all mucosa with OPMD will transform into OSCC. Patients with risk habits like alcohol, smoking, and betel-quid chewing are at higher risk of acquiring OPMD mucosal changes and OSCC. OC is a chronic disease and burden to developing and developed countries, and associated with high cost of treatment and high mortality. Loss of function in terms of chewing efficiency, speech difficulties, and aesthetic concerns leads to reduction in the quality of life in OC patients.

2.2 Oral potentially malignant disorder (OPMD)

2.2.1 Definition

Oral potentially malignant disorders (OPMDs) are clinical presentations of oral mucosal disorders that carry an increased risk of malignancy in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal oral mucosa (El-Naggar et al., 2017).

2.2.2 Classification

The latest WHO classification of head and neck tumours in 2017 described the spectrum of OPMDs comprised of erythroplakia, erythroleukoplakia, leukoplakia,

oral submucous fibrosis, dyskeratosis congenita, smokeless tobacco keratosis, palatal lesions associated with reversed smoking, chronic candidiasis, lichen planus, discoid lupus erythematosus, syphilitic glossitis, and actinic keratosis (lip only) (El-Naggar et al., 2017). Biopsy is a gold standard in diagnosing OPMDs for several reasons. First, to exclude other pathologies (OSCC and other benign reactive lesions) that might have similar presentation with OPMDs. Second, to assess the degree of epithelial dysplasia. Lastly, to identify presence of candidal hyphae within the oral epithelium as OPMDs is commonly superimposed with candida infections. In current practice, degree of epithelial dysplasia is used as prognostic factor to assess the risk of malignant transformation although not all lesions with high-grade epithelial dysplasia will turn into OSCC.

2.2.3 Biomarkers in OPMD

Substantial numbers of studies regarding potential biomarkers to predict MTR in OPMDs in histologic or chair side setting have been conducted over the years. However, none of them has been proven to be used in clinical practice. Out of more than 2500 publications that address biomarkers in dysplasia, about 113 biomarkers have been analysed. The most common biomarkers studied are p53, proliferation markers Ki 67 and PCNA, cell cycles proteins, loss of heterozygosity (LOH), cell surface and stromal proteins. Previous studies in OPMDs suggested that LOH, survivin, matrix metalloproteinase-9, and DNA ploidy may be associated with risk of progression to OSCC Analysis of DNA content (ploidy) provides insight into genetic aberrations that are highly associated with malignancy (Williams & Amon, 2009). Study by Alaizari showed that aneuploidy with dysplastic lesions was associated with higher grade of epithelial dysplasia in which 3.12- fold increased risk of malignant transformation was seen in aneuploidy lesions (Alaizari,

Sperandio, Odell, Peruzzo, & Al‐Maweri, 2018). Overall, these findings suggest that aneuploid dysplastic lesions have a higher risk of malignant progression. Aneuploidy analysis in OPMDs lesion is a promising prognostic test in future as it can be measured by using image cytometry resulting in rapid and reliable noninvasive tests for risk assessment of oral lesions (Yang et al., 2017).

2.3 Oral squamous cell carcinoma (OSCC)

2.3.1 Definition

Oral squamous cell carcinoma (OSCC) is a carcinoma with squamous differentiation that arises from the oral mucosal epithelium. The incidence of OSCC is seen highest in the fifth to sixth decades of life and associated with risk factors, such as smoking, drinking alcohol, and betel-quid chewing. OSCC may arise *de novo* or from clinically evident OPMD (El-Naggar et al., 2017).

2.3.2 Epidemiology

2.3.2.1 Incidence

Lip and oral cavity cancer represents 354,864 of new cancer cases with 177,384 death worldwide (Bray et al., 2018). The GLOBOCAN project in 2018 estimated 447,751 new cases for oral and oropharynx cancer at eighth position and accounts for 2.5% of all cancer cases (Bray et al., 2018; Ferlay et al., 2018). The incidence of oral cancer varies according to geographical regions. The prevalence of oral cancers is high in Pacific Islands (Papua New Guinea) and Southern Asia (India and Sri Lanka) (Bray et al., 2018). OC epidemiological data in 11 South East Asian (SEA) countries showed severe lack of methodical OC burden reporting with only five of the eleven countries have national cancer registries. Majority of the SEA countries

were listed within top 20 countries with high incidence OC yielding ASRs of 9.0/100,000 for both oral and oropharyngeal cancers (Cheong et al., 2017). A national epidemiological survey of oral mucosal lesions of Malaysian population during 1993 to 1994 period revealed 0.04% prevalence of OSCC (Zain et al., 1997). Another epidemiological survey among Malaysian geriatric population in Klang district demonstrates prevalence 0.4% for OSCC (Ali, Razak, Latifah, & Zain, 1995). The fact that huge discrepancy (10 times fold) exists between the two surveys could be contributed by small sampling involving only one district in the latter study. Based on the GLOBOCAN in 2018, the numbers of new oral cancer cases in Malaysia were 43837 with 26397 deaths recorded (Ferlay et al., 2018). About 60% of oral cancer patients in Malaysia died due to the disease and this percentage is 10% higher than the worldwide percentage.

2.3.2.2 Trends

Since 1970s, global incidence of OC was steadily increasing over the years as reported in European countries like Scotland and Denmark (G. Macfarlane, Boyle, & Scully, 1987; G. J. Macfarlane, Boyle, & Scully, 1992; Møller, 1989). In the UK, a sudden 35% rise of OC was observed between 1995 and 2005 and significant rise was observed in younger age group (Warnakulasuriya & Greenspan, 2020). Portugal also showed the trend of rising OC between 1998 and 2007 in the age-standardized incidence of OC with 1.96% increment per year in both gender (Monteiro, Antunes, Bento, & Warnakulasuriya, 2013). Meanwhile in United States of America (USA), the incidence of HPV-related OSCC increased significantly from 1973- 2004. In contrast, the incidence of HPV-unrelated OSCC showed stable and decreasing trends for the same periods of time (Chaturvedi, Engels, Anderson, & Gillison, 2008). In Asian region, countries like Pakistan, Taiwan, Khonkaen

province of Thailand had rise in the incidence of OC. In contrast, USA reported declining age-adjusted rates for OC among both white and black Americans (Chaturvedi et al., 2013). However, there was a marked increased in the incidence of tongue cancer in both sexes (Tota et al., 2017). The rise of tongue cancer incidence was also reported in Nordic countries (Annertz, Anderson, Palmér, & Wennerberg, 2012). The reason for increasing trend of tongue cancer remains ambiguous. Meanwhile in Japan, earlier low incidence of OC showed significant increase since 1970. OC data in Japan demonstrates 3.5-fold increase in age-standardize-rates for male and 2.0-fold increase for women from 1975 to 2013 ("Cancer Statistics in Japan. Center for Cancer Control and Information Services, National Cancer Center, Japan. https://ganjoho.jp/en/ professional/statistics/table_download.html.,"). On the other hand, the incidence of lip cancer seems declining in Australia that previously had high incidence (Anura Ariyawardana & Johnson, 2013). In France, there was a drop in OC incidence amongst males but not in females. Data showed that France women had increasing trend of palatal and oropharyngeal cancer (Ligier et al., 2011). India and Sri Lanka showed declined incidence of OC but certain cities like Mumbai and Ahmedabad had increasing incidence of OC and the numbers estimated will rise in the future (A Ariyawardana & Warnakulasuriya, 2011; Shridhar et al., 2016). The decreasing trends in OC also seen in countries like Philippine and Chiangmai province of Thailand (Laudico et al., 2010; Reichart, Dietrich, Khongkhunthian, & Srisuwan, 2003). In summary, the incidence of OC varies between countries and even between states of the same country. The drop in the incidence may reflect the successfulness of the oral cancer awareness programmes. However, the cause of increase in OC incidence in certain populations should be investigated in depth to identify the root cause of the problem.

2.3.3 Aetiology

Despite of the high incidence, oral cancer is preventable by avoiding known risk factors. The main risk factors for oral cancer are smoking, alcohol consumption and betel-quid chewing.

2.3.3.1 Tobacco

Tobacco consumptions exist in many forms. Tobacco is the main risk factors for OSCC. However, how individuals use tobacco will determine the sites of OC involvement.

(a) Smoking cigarettes

Smoking is associated with increased risk of upper respiratory and upper digestive tract cancers due to carcinogens present in cigarettes (IARC, 1986). The risk of OC related to dose-response relationships for frequency (number of cigarettes/day) and duration (years) of tobacco smoking. Smoking at early age is also associated with increased risk of OC (IARC, 2004). Smoking cessation is associated with reduction in OC risk. Former smoker has OC risk of 1.4 compared to current smoker with a 3.43-fold increase in OC risk (Bagnardi et al., 2015). 35% reduction in risk of OC was seen less than 5 years of quitting smoking and the odds ratio is similar to never smoking after more than 20 years of quitting smoking (Marron et al., 2010).

(b) Smoking cigar and pipes

Smoking cigars and pipe had similar magnitudes of risks developing OC in comparison to cigarette smoking (IARC, 2004).

(c) Smoking bidis

Bidis are mainly practiced in South Asia and composed of coarse and uncured tobacco hand-rolled in *tendu* or *temburini* leaf and usually smoked without filters (IARC, 2004). Bidi smoking is associated with dose-response relations for frequency and duration, similar to cigarette smoking (IARC, 2012). A meta-analysis of 10 studies revealed the odds ratio (OR) of bidi smoking is 4.0, the highest among types of tobacco habits practiced. However, this may be due to the variables for sex, age, ethnicity, duration of smoking and alcohol was not adjusted prior to study, unlike other studies conducted for other types of tobacco consumption (Rahman, Sakamoto, & Fukui, 2003).

(d) Chewing tobacco

In certain parts of the continent, individuals practice smokeless tobacco in the forms of chewing or dipping (snuff). Smokeless tobacco may cause oral cancer but in a few studies the risk is negligible (El-Naggar et al., 2017).

(e) Involuntary smoking

The association of involuntary smoking or second hand smoke with increased risk of lung cancer was well established but not with OC (IARC, 2012). Numerous studies conducted to find the association between involuntary smoking with risk of OC showed mixed results. Study by Lee et al. revealed no association of involuntary smoking with increased risk of OC whilst a multicenter case control study in Western Europe showed positive association between never tobacco smokers when exposed to involuntary smoking at work for more than 15 years with increased risk oral and oropharyngeal cancers (Lee et al., 2008; Lee et al., 2009). More research is necessary to conclude if there is any possible association between

involuntary smoking and risk of OC in the future. Quantification on the amount of second hand smoke exposed is subjective and needs to be clarified.

(f) Electronic cigarettes

Electronic cigarettes or widely known as e-cigarretes was first invented in China in 2003 and was introduced in European countries in 2006 (Bareham, Ahmadi, Elie, & Jones, 2016). Since then, e-cigarettes have gained popularity especially among the young adults as major smoking companies advertise it as an alternatives to quit smoking and lack of carcinogens compared to conventional cigarettes. This battery operated device contains glycerine and/or propylene glycol solvent, various artificials flavours, and with or without nicotine. The e-liquid solution was heated to form inhalable aerosol (Dinakar & O'Connor, 2016). A recent systematic review showed poor association between e-cigarettes in the pathogenesis of head and neck cancers (Flach, Maniam, & Manickavasagam, 2019). Several studies in that review reported cytotoxic effects of e-cigarettes, increased production of reactive oxygen species (ROS), decrease in total antioxidant capacity, and reduced expression of DNA repair proteins leading to DNA double strand breaks in cell lines. Harmful effects of e-cigarettes is due to presence of various chemical compounds such as tobacco specific nitrosamines (TSNA), aldehydes, trace metals, volatile organic compounds, phenolic compunds, polycyclic aromatic hydrocarbons, flavoured e-liquids especially menthol, and tobacco alkaloids (Flach et al., 2019). TSNA and nickel have been implicated in the oral carcinogenesis. The amount of toxic substances varies with e-cigarette brands, solvents, device voltage, and e-liquid flavours. Hence, whether the levels of toxic substances in conventional and electronic cigarretes are equivalent is questionable. Up to date, there is no good evidence that supports e-cigarettes are less harmful than conventional cigarettes.

Hence, a longitudinal study is recommended to investigate the role of e-cigarettes in the pathogenesis of OC in the future.

2.3.3.2 Alcohol

Alcohol beverages exist in the forms of beer, liquor, wine, and local alcohol products. Since 1988, alcohol was identified as an established carcinogen for OC (IARC, 1988). The dose-response trend of alcohol consumption is similar to smoking habits, which depends on frequency and durations of habits (Hashibe et al., 2007). The dose-response relationship was seen consistent among study designs, gender, and geographical locations. Cessation of alcohol habits reduces the risk of OC with risks almost similar to those who never drink after 10 years of alcohol cessation (Marron et al., 2010). Alcohol is more of a synergistic agent than a primary carcinogen for OC.

2.3.3.3 Betel-quid chewing

Betel-quid is a combination of areca nut with or without tobacco that often mixed with slaked lime, betel inflorescence, condiments, sweetening agents, and spices (El-Naggar et al., 2017). Areca nut was recognized by IARC as a carcinogen for OC (IARC, 2012). The carcinogenic component in areca nut is the presence of arecoline specific nitrosamines (Warnakulasuriya, Trivedy, & Peters, 2002). Lime in betel quid plays a role as tumour promoter by hydrolysing alkaloids present in the areca nut into cytotoxic derivatives (Shah, Chaturvedi, & Vaishampayan, 2012). In Taiwan, the adolescent chew betel-quid habitually and this practice was reflected by high incidence of OC with an OR of 10.97 (Yen, Lin, Wang, Wang, & Liu, 2008). In India, a meta-analysis on betel-quid with tobacco reported summary estimate of 8.47 (95% CI: 6.49, 11.05) where as for betel quid without tobacco the summary estimate was much lower, 2.41 (95% CI: 1.82, 3.19) for the risk of OC. A dose

response relationship was observed for the frequency and amount of quid chewed daily. However, the risk of OC still persists even after quitting the habit. Habits of starting to chew quid before the age of 20 years and chewing 10 betel-quid daily increases the risk of OC (Balaram et al., 2002).

(a) Interactions between tobacco, alcohol, and betel-quid

Alcohol acts synergistically with smoking resulting in more additive risk of developing OSCC. Individuals that smoked more than 20 cigarettes per day and consumed 3 or more alcoholic beverages per day were considered having the highest risk for OSCC (OR = 15.49; 95% CI: 7.24, 33.14) (Hashibe et al., 2009). Three-way interactions of tobacco smoking, alcohol drinking, and betel-quid chewing have been evaluated. A meta-analysis from Taiwan and India reported that the summary risk for individuals who practiced all three habits was 40.09 (95% CI = 35.06, 45.83). Meanwhile, the summary risk estimates for individual who had two habits were 6.29 (95% CI-5.41, 7.32) for smoking and drinking, 10.44 (95% CI = 8.02, 13.60) for drinking and betel quid chewing, and 16.01 (95% CI = 13.67, 18.75) for smoking and betel quid chewing. The data showed combination of smoking and betel-quid chewing habit pose the highest risk, followed by drinking and betel quid chewing, and the least risk for smoking and drinking (Petti, Masood, & Scully, 2013).

2.3.3.4 Human papilloma virus (HPV)

HPV is a circular double stranded DNA and having a small diameter of 50 micrometer (Campisi et al., 2007). HPV is categorized under five evolutionary phylogenetic genera known as alpha, beta, gamma, mu, and nu. (Egawa, Egawa, Griffin, & Doorbar, 2015). The alpha HPV can be further divided into high risk and

low risk HPV. High risk HPV is associated with potentially malignant lesions whilst low risk HPV is related with benign lesions such a squamous cell papilloma. At least 10 high risk HPVs (Type 16, 18, 31, 33, 45, 51, 52, 56, 58, 59) and six low-risk HPVs (Type 11, 32, 44, 53, 57, 58, 61) were isolated in head and neck squamous cell carcinoma (HNSCC). HPV subtypes of 16 and 18 were recognized as virus associated with progression in head and neck cancers. HPV 16 was detected in approximately 90% of all HPV positive oropharnyngeal squamous cell carcinoma (OPSCC) (Ndiaye et al., 2014). HPV is accepted as the main risk factor for oropharyngeal cancer. About 3% of OSCC was associated with HPV (El-Naggar et al., 2017). HPV has the predilection to infect the skin and ano-genital mucosa and mostly transmitted by sexual intercourse, followed by vertical spread (prenatal trans placental route) and self-inoculation. HPV is less common in individuals who smoke or drink alcohol but frequently found in individuals who had multiple sexual partners (P. C. H. Chen, Kuo, Pan, & Chou, 2002). The prevalence of HPV varies among different populations. HPV was found in 25% of the cases in India and up to 85.7% in Taiwan (P. C. H. Chen et al., 2002; Nagpal, Patnaik, & Das, 2002). The odds ratio (OR) for OC in Taiwan due to HPV-16 (11.21) was significantly higher than HPV-18 (P. C. H. Chen et al., 2002). In Malaysian population, the OR was lower (4.3) (Saini et al., 2011).

The ability of HPV to generate persistent infection and evade host immune response is the most important factor of progression into cancer. Transcriptional activity of HPV oncogenes E6 and E7 plays an essential role in disease causation and progression by HPV. E6 and E7 oncogene are associated with activation of p53 and pRb pathway. However, research on the mechanism by which HPV promotes oral mucosal epithelial transformation is scarce (Warnakulasuriya & Greenspan, 2020).

2.3.3.5 Dietary habits

In Malaysia and Indonesia, high consumption of fermented, milk derivatives, poultry, meat and salted food combination was related to increase risk of OC whilst a protective effect was seen with a food rich in antioxidant (fruits and vegetables) (Amtha et al., 2009; Helen-Ng et al., 2012). Meanwhile, in Turkey, margarine and egg intake was related with higher risk for OC whereas consumption of fish and raw vegetables reduces the risk for OC (Güneri et al., 2005).

2.3.3.6 Sunlight

Sunlight, particularly the ultraviolet light is the main risk factors for lip cancer, which is predominantly seen in Australia (El-Naggar et al., 2017).

2.3.3.7 Poor oral health

Poor oral health is associated with OSCC but is not proven as independent risk factor (El-Naggar et al., 2017). Poor oral hygiene was evaluated by status of dentition (missing teeth) and periodontal disease. In India, poor oral hygiene is associated for 32% increased risk for OC in men and 64% in women (Balaram et al., 2002). Risk of OC also increased if patient wears denture for more than 15 years and without regular dentist follow-up (Güneri et al., 2005). This is related with long term wearing of ill-fitting denture that is subjected chronic irritation to oral mucosa.

2.3.4 Socio-demographic

2.3.4.1 Age

Most of oral cancer affects elderly group aged 50 to 70 years old (El-Naggar et al., 2017). This showed the incidence of OC increases with advanced age. Rarely, OC was reported in children as young as 10 years old without any known risk habits (Solanki, Gandhi, Koshy, & Mathew, 2012). Recently, there is higher incidence of OC in younger age at diagnosis. Studies from Western countries reported about 4-6% of OC occur in a younger age group of less than 45 years old (Llewellyn, Johnson, & Warnakulasuriya, 2001). The incidence of OC in India affecting younger patients of less than 40 years is 7.5%, slightly higher than the Western population (Sherin, Simi, Shameena, & Sudha, 2008).

2.3.4.2 Gender

Globally, oral cancer has slight male predilection (Rao, Mejia, Roberts-Thomson, & Logan, 2013). Other studies from various countries showed conflicting results in relation to gender bias. A report from Karachi, Pakistan shows no sex difference in OC (Bhurgri et al., 2003). Meanwhile, Asian population particularly India and Thailand showed female predilection with male to female ratio of 1:2 and 1:1.56 respectively (El-Naggar et al., 2017). Data from previous studies revealed predilection of tongue cancer among young male (J. Chen, Eisenberg, Krutchkoff, & Katz, 1991). The gender variation closely associated with the risk habits practiced by certain groups. For instance, in Karachi about 70% of both female and male had betel quid chewing habit and this correspond to the no gender predilection in OC incidence (Bhurgri et al., 2003). The male predilection for tongue carcinoma was previously regarded closely associated with habits of smoking and drinking alcohol. However, the differences in habits become

less as smoking and drinking alcohol is socially acceptable amongst women in this era.

2.3.4.3 Ethnicity

In Malaysia, the highest risk for oral cancer is in Indians, followed by the Indigenous people of East Malaysia (Zain et al., 1997).

2.3.5 Clinical features

Clinical oral examination (COE) of oral cancer can be done by visual inspection and palpation of the suspicious lesion followed by examination of the neck nodes. The symptoms often depend on the size of the cancer. Small lesion is usually asymptomatic and difficult to be recognized by the patient and even the clinician resulting in diagnostic delay. Large cancer may cause pain, discomfort, reduced mobility of tongue, and paraesthesia (El-Naggar et al., 2017). White, red, mixed lesions, nodular growth, and ulcer are examples of the clinical presentations of oral cancer. Persistent ulceration, swelling, red, white, and mixed lesions of more than 2 weeks with induration at margins are the red flags of early transformation into oral cancer. Any source of trauma should be eliminated and lesion should be reviewed again after 2 weeks. The understanding of other oral mucosal lesions mimmickers that can have similar presentations as oral cancer is essential to arrive at definitive diagnosis (El-Naggar et al., 2017). Hence, biopsy is a gold standard to diagnose oral malignancy.

2.3.6 Histopathology subtypes

Basaloid, adenosquamous, lymphoepithelial variant of OSCC are considered as high-grade oral malignancy. Basaloid OSCC is characterized as having frequent metastases but prognosis is almost similar as the conventional OSCC.

Adenosquamous carcinoma is an aggressive, highly infiltrative, frequently metastasizes, and having the worse prognosis than conventional OSCC. A rare subtype of OSCC, lymphoepithelial carcinoma is associated with 70% of risk lymph node metastases. However, not all lymphoepithelial carcinomas were related to EBV infection (El-Naggar et al., 2017). On the other hand, spindle cell OSCC typically occur as a result of post radiation therapy and in case of second primary tumour. Of all variants of OSCC, verrucous carcinoma has the most favourable prognosis characterized by exophytic verrucous lesion with endophytic pushing invasive front, minimal atypia and no metastatic potential. However, this entity may co-exist with OSCC or may progress to invasive conventional OSCC. The other OSCC subtypes with favourable prognosis are papillary OSCC and carcinoma cuniculatum. Papillary OSCC has two types, which are keratinizing and non-keratinising types. Carcinoma cuniculatum is a well differentiated and locally destructive OSCC commonly occur on mucoperiosteum, forming deep burrowing pattern but rarely metastasizes. Lastly, acantholytic OSCC may mimics adenoid appearance. The cutaneous variant of acantholytic OSCC may affects the lip (El-Naggar et al., 2017).

2.3.7 Adjunctive tools

2.3.7.1 Immunohistochemistry in OSCC

AE1/AE3, CK5/6, p63, p40 are useful OSCC markers, especially in a case of poorly differentiated OSCC in which squamous differentiation is minimal or may absent (El-Naggar et al., 2017).

2.3.8 Clinico-pathologic characteristics of OSCC

2.3.8.1 Site

The most common sites for OSCC are tongue, floor of the mouth, and gingiva (Rao et al., 2013). The site of OSCC also depends on the practiced habits. For example, in Asian populations the most common site for OSCC is buccal mucosa due to tobacco and betel quid chewing (El-Naggar et al., 2017).

2.3.8.2 Grading

OSCC can be histologically graded as described by Broders, that takes into account a subjective assessment of the degree of keratinisation, mitoses, cellular and nuclear pleomorphism (Broders, 1920). This grading system was later adopted by WHO that recommends categorization of OSCC into three categories that includes grade 1 (well differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated) based on WHO grading system (Pindborg, Reichart, Smith, & Van der Waal, 2012). In tumours with heterogenous tumour grading, evaluation of the worst tumour grading determines the final grading. However, the grading system shows poor correlation with patients' outcome and response to treatment (Pindborg et al., 2012; Po Wing Yuen et al., 2002). The assessment of the grading is subjective in nature that relies on morphology of the tumour cells without consideration of stromal changes and host immune response (Pindborg et al., 2012). More than 90% of oral and oropharyngeal SCC are moderately differentiated (Julia A Woolgar, 2006).

2.3.8.3 Pathological tumour size (pT)

Tumour size is related to size or extent of the primary tumour. Generally, the prognosis of OC worsens when tumor is larger. Tumour thickness and depth of invasion (DOI) have been used interchangeably although these two parameters were measured differently. DOI is calculated from the level of basement membrane of the closest adjacent mucosa to the deepest point of tumour invasion. On the other hand, tumour thickness is the distance from the outermost surface epithelium to the deepest point of tumour invasion. Tumour thickness changes when the tumour is ulcerated (reduced thickness) or exophytic (increase thickness). DOI is a better prognostic parameter than tumour thickness. The incorporation of DOI parameters into T category will affect the pathological tumour (pT) and tumour staging (pTNM). For every 5 mm increase in DOI, T category will increase by one level (Lydiatt et al., 2017). Classification of T category based on American Joint Committee on Cancer (AJCC), 8th edition is as follows (Lydiatt et al., 2017):

TX : Primary tumor cannot be assessed.

Tis : Carcinoma in situ.

T1 :Tumor size ≤2 cm; Depth of invasion (DOI) ≤5 mm.

T2 : Tumor ≤2 cm, DOI >5mm and ≤10mm / tumor >2cm but ≤4 cm and DOI ≤10 mm.

T3 : Tumor >4 cm / any tumor DOI >10 mm.

T4 : Moderately advanced or very advanced local disease.

T4a : Moderately advanced local disease:

Lip: tumor invades through cortical bone or involves the inferior alveolar nerve, floor of mouth, or skin of face.

Oral cavity: tumor invades adjacent structures only (eg, through cortical bone of the mandible or maxilla, or involves the maxillary sinus or skin of the face).

*Superficial erosion of bone/tooth socket alone by a gingival primary is not sufficient to classify a tumor as T4.

T4b : Very advanced local disease. Tumor invades masticator space, pterygoid plates, or skull base and/or encases the internal carotid artery.

2.3.8.4 Pathological lymph node (pN)

Metastases to cervical lymph nodes related to poor prognosis in HNSCC. Hence, every OC patients should be examined thoroughly to determine metastatic deposits to lymph nodes. Extranodal extension (ENE) was added in AJCC 8th edition as predictor parameters for lymph node metastases apart from number and size of metastatic deposits. ENE is defined as metastatic tumour cells from a lymph node that extends outside the lymph node capsule into the surrounding connective tissue. ENE can be further classified as major and minor ENE based on the diameter of tumour spread. Minor ENE is defined as extension ≤2 mm from the capsule. Major ENE is defined as either tumor extension in a lymph node upon examination and palpation of gross specimen or >2 mm beyond the capsule which includes extension of carcinoma in soft tissues. Pathological lymph node staging based on AJCC 8th edition was as follows (Lydiatt et al., 2017):

NX : Regional lymph nodes cannot be assessed.

N0 : No regional lymph node metastasis.

N1: Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension, and ENE-negative.

N2 : Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension, and ENE-positive/ >3 cm but ≤6 cm in greatest dimension, and ENE-negative/ metastases in multiple ipsilateral lymph nodes, ≤6cm in greatest dimension and ENE-negative/ metastasis in bilateral or contralateral lymph nodes, ≤6cm in greatest dimension, ENE-negative.

N2a : Metastasis in a single ipsilateral or contralateral lymph node ≤ 3 cm in greatest dimension and ENE-positive/ metastasis in a single ipsilateral lymph node > 3 cm but ≤ 6 cm in greatest dimension and ENE-negative.

N2b : Metastasis in multiple ipsilateral lymph nodes, ≤6cm in greatest dimension and ENE-negative.

N2c : Metastasis in bilateral or contralateral lymph nodes, ≤6cm in greatest dimension and ENE-negative.

N3 : Metastasis in a lymph node > 6 cm in greatest dimension and ENE-negative/ metastasis in a single ipsilateral lymph node > 3 cm in greatest dimension and ENE-positive/ metastasis in multiple ipsilateral, contralateral, or bilateral lymph nodes, with any ENE-positive.

N3a : Metastasis in a lymph node > 6 cm in greatest dimension and ENE-negative.

N3b : Metastasis in a single ipsilateral node > 3 cm in greatest dimension and ENE-positive/ metastasis in multiple ipsilateral, contralateral, or bilateral lymph nodes, with any ENE-positive.

2.3.8.5 Pathological tumour staging (pTNM)

The TNM classification describes the extent of disease by involvement of anatomical sites of primary, regional, or distant sites. This classification can be further combine into groups for further analysis and comparison with survival. For instance, a worse prognosis was observed in patients with stage IV than stage II cancer (Lydiatt et al., 2017). A study by Kreppel et al., showed that pTNM had a better prognostic characteristics than clinical TNM (cTNM) (Kreppel et al., 2016). In general, carcinoma in situ is considered as stage 0, meanwhile tumors present at the site of origin are classified as stages I and II. Stage III or IV indicates local extension or spread of the primary tumor to adjacent lymph nodes whilst stage IV implies presence of distant metastases (Lydiatt et al., 2017).

2.3.8.6 Pattern of invasion (POI)

Pattern of tumor invasion (POI) is defined as the manner of neoplastic cells invading the underlying connective tissue at the tumor-host interface. Bryne described four types of POI. Type 1 is characterized by tumour invasion in a broad pushing manner. Type 2 represents tumor invasion with broad pushing fingers or separate large tumor islands with a stellate appearance. Type 3 represents invasive tumour islands with number of cells greater than 15 cells per island. Type 1-3 POI was regarded as cohesive type. Meanwhile, type 4 represents tumor islands lesser than 15 cells per island, single cell invasion, and single-cell filing pattern regardless of size of the tumour islands (Bryne, Koppang, Lilleng, & Kjærheim, 1992; Bryne et al., 1989). Type 5 POI was introduced later and was defined as tumor satellites measuring 1 mm or farther from the closest intervening tumor island. The surrounding connective tissue must appear normal without evidence of fibrosis. Brandwein-Gensler coined the term worst pattern of invasion (WPOI) that represents the highest POI score present. WPOI 4 and WPOI 5

were significantly associated with poor OS when compared with WPOI 1-3. WPOI has been significantly related with lymph nodes metastases whereby WPOI 4 and WPOI 5 were associated with higher risk of nodal involvement (Brandwein-Gensler et al., 2005).

2.3.8.7 Perineural invasion (PNI)

Historically, PNI is defined as invasion in, around, and through peripheral nerves. Batsakis proposed that tumour invasion targets along planes of least resistance in the connective tissue coverings of the nerve (Batsakis, 1985). This definition is well accepted by many pathologists and researchers, but pose a broad definition of PNI and lacks objectivity. Later, Dunn et al (2009), suggests that in the presence of a malignancy, PNI may be diagnosed when cytologically malignant cells are present in the perineural space. Total or almost total circumferential of nerve surrounded by tumour cells and intraneural invasion are key features in the assessment of neural invasion when questionable cases are encountered (Dunn, Morgan, & Beer, 2009). Liebig et al defines PNI as the presence of tumour cells in any of three layers of nerve sheath and in cases whereby tumor is adjacent to a nerve, involvement of at least 33% of nerve circumference is needed to diagnose PNI (Liebig, Ayala, Wilks, Berger, & Albo, 2009). Diagnosis of PNI is mainly by histopathological examination by pathologists. PNI of small and large nerves was associated with poor overall survival (OS) and LR. There is an association between PNI with tumour site, size, POI, lymph node metastasis, close/involved resection margins, and survival (Julia A Woolgar, 2006).

2.3.8.8 Lymphovascular invasion (LVI)

Lymphovascular invasion (LVI) was referred as the presence of neoplastic cells within endothelial-lined blood vessels or invasion of the media of a blood vessel

with ulceration of the tunica intima. Several studies have shown a significant association of LVI with tumour site, tumour size, perineural invasion (PNI), pattern of invasion (POI), lymph node metastasis, status of resection margins, local recurrence (LR), and survival (Julia A Woolgar, 2006). Although certain researchers omitted LVI assessment in OSCC tissue as they regard it as difficult to define and identify with certainty, the assessment of LVI is still worthwhile since the presence of tumour cells in the vascular network means higher likelihood of tumour metastases.

2.3.8.9 Bone invasion

Bone invasion can be classified as bone infiltration and erosion. Bone involvement with penetration of tumour cells into the cortical bones of maxillary or mandibular jawbones indicates stage T IVa status, which implied poor prognosis. An infiltrative bone invasion, but not an erosive, was predictive for LR and survival (Julia A Woolgar, 2006).

2.3.8.10 Lymphocyte host response (LHR)

Three-tiered scoring (continuous band, large patches, little/ none) improves interobserver reproducibility compared to four-tiered scoring of lymphocytic response. A study by Brandwein-Gensler demonstrated a strong, inverse relationship between lymphocytic infiltrate and LR. In that study, weak lymphocyte infiltrate at the tumour to host interface is strongly associated with LR and death (Brandwein-Gensler et al., 2005).

2.3.8.11 Sarcolemmal spread

Similar to PNI and LVI, the tumour cells might spread along the sarcolemmal sheaths of muscle fibres. Sarcolemmal spread usually occurs in advanced stage of cancer, probably due to deep location of the muscle bundles (Julia Anne Woolgar & Triantafyllou, 2009).

2.3.8.12 Surgical margin status

The surgical resection margins include mucosal and deep connective tissue margins. The surgical margin clearance is assumed when the distance of the surgical margins to the closest tumour is more than 5mm. Positive margin is when the distance of the surgical margins to the closest tumour is 5mm and less indicating close margin and 1mm or less indicating involved margin. Woolgar suggested that even 5 mm surgical margin clearance may not be adequate if the tumour cells invades in dyscohesive manner into widely separated islands and individual cells (Julia A Woolgar, 2006).

Positive margin was associated with poor prognosis, in which local recurrence rate (LRR) for patients with positive margins ranges from 22% to 80% (Brandwein-Gensler et al., 2005). Tissue shrinkage after tissue fixation and processing also need to be taken into consideration as this results in a reduction of 30–47% in the margin length seen under the microscope (Johnson, Sigman, Funk, Robinson, & Hoffman, 1997). Surgical margin status is a robust prognosticator for OSCC. 5-year survival rate showed only 11% of patients with an involved margin were alive or dead free of disease, compared to 47% of patients with close margins and 78% with clear margins. Adjuvant radiotherapy for patients with involved or close surgical margins does reduce the risk of local

recurrence similar with clear margins (Julia A Woolgar, 2006). Hence, surgical intervention is preferred in close or involved surgical margin.

2.3.8.13 Survival

Prognosis of early and late-stage oral cancer has significantly improved between 1973 and 2014. In early-stage oral cancer, 3-year survival increased from 78.0% (1973 to 1980) to 92.2% (2011 to 2014). This increasing trend of improved survival rate is also seen among patients with late-stage disease, 3-year survival ranged from 51.9% (1973 to 1980) to 70.3% (2011 to 2014). For patients with late-stage disease, this improved prognosis maybe associated with increasing usage of chemoradiotherapy as adjuvant therapy and increasing utilization of neck dissection (Cheraghlou, Schettino, Zogg, & Judson, 2018).

2.3.9 Diagnostic tools for oral cancer detection

Early detection of cancer is crucial in determining the prognosis of cancer patients. Clinical oral examination is the main early diagnostic screening method for oral cancer. Developing new diagnostic tools and discovering new reliable markers is critical for early assessment and detection of oral cancer. New diagnostic tools are being increasingly investigated, for example the detection of proteomic signatures in saliva and blood as a diagnostic and prognostic tool in oral cancer (Gallo et al., 2016). Diagnostic blood test using tumour-associated antigens in body fluid samples has been widely used to detect various cancers, such as prostate-specific antigen (PSA) for prostate cancer (Gretzer & Partin, 2003), carcinoembryonic antigen (CEA) for colon cancer (Crawford, Colliver, & Galandiuk, 2003), CA15-3 for breast cancer (Cheung & Robertson, 2003), CA125 for ovarian cancer (Anderiesz & Quinn, 2003), and CA19-9 for the diagnosis of the gastrointestinal

cancer (Trompetas, Panagopoulos, Priovolou-Papaevangelou, & Ramantanis, 2002). However, these tumour-associated antigens are not an ideal cancer biomarker due to lack of sensitivity and specificity. On the other hand, autoantibodies are more stable and persistent and therefore have the potential to be used as diagnostic markers in detection of cancer (Desmetz, Mangé, Maudelonde, & Solassol, 2011).

2.3.10 Biomarkers in OSCC

In cancer studies, tumor markers are known as substances that can be found in the blood, tissues, or other body fluid, and their level may increases in certain types of neoplasm. An exceptionally high level of tumour markers may present in specific types of neoplasms while certain markers may present in various tumours. Tumor markers can either be produced directly from tumor cells or from normal cells. Tumour markers are useful tools to monitor recurrence after surgical intervention of a tumour (Cervino et al., 2019).

p53, also known as tumor suppressor protein 53 (TP53 gene), is a tumor suppressor that controls cell cycle progression, differentiation, DNA repair, and apoptosis. P53 involves in antitumor mechanisms in which p53 activates the repair of damaged DNA, ability to initiate apoptosis, inducing the transcription of Noxa, in case DNA damage is irreparable. However, if the DNA is repairable, p53 is degraded and the cell cycle will be recovered. Human papilloma virus (HPV) indirectly involves in alteration of p53 functions. HPV encodes a protein, which binds p53 and thus inactivating it. p53 is one of the widely studied biomarkers in OSCC and the introduction of p53 into cells with protein deficiency has shown rapid death of cancer cells that represents potential use in cancer treatment. 25-69% of gene encodes p53 was mutated in OSCC. An increased expression of p53 was observed in 40-67% of HNSCC. The expression of p53 above the basal cell layer is considered as an early change in the progression of OSCC. It is an

indicator of the development of carcinoma and hence a potential diagnostic biomarker (Tsuji et al., 1995).

The retinoblastoma (Rb) pathway also plays an important role in regulating cell cycle progression. 66% of OSCC and 64% of OPMD cases showed loss of Rb expressions (Edwards, 2013). Many proteins are known to regulate programmed cell death (apoptosis). Some proteins were members of the Bcl-2 family. At least 15 proteins belong of Bzl-2 group having anti-apoptotic (Bcl-2, Bcl-X) and pro-apoptotic (Bax, Bak) functions that regulates cell cycle. Increase expressions of Bcl-2 and Bcl-X were seen in dysplastic lesions and OSCC (Cervino et al., 2019). Survivin, is an inhibitor of apoptosis was expressed in 80% of OSCC and related with an aggressive phenotype. Expression of miRNA was altered in tumor tissue compared to normal tissue, indicating involvement of these molecules in carcinogenesis (Takamizawa et al., 2004). Angiogenesis in cancer is the result of proangiogenic signals, for example vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and interleukin 8 (IL-8) and antiangiogenic signals, like interferons and proteolytic fragments such as angiostatin and endostatin (Cervino et al., 2019).

Alterations of several biomarkers were reported in OSCC. For instance, an elevated EGFR gene copy number increases the risk of OSCC whereas high miR-21 expressions are important prognosticators in OSCC. Meanwhile, osteopontin plays an important role in tumor invasion and metastasis. In comparison to normal adjacent tissues, DEPDC1B is highly expressed in OSCC that related to cell migration and tumour invasion. EZH2 expression is an independent risk predictor for OSCC. Better survival was observed in upregulated gene deltaNp63, EIC, and podoplanin (Cervino et al., 2019).

2.3.11 MAGEB2

2.3.11.1 Background

Melanoma antigen family B2 (MAGEB2) is a member of the cancer testis antigens (Scanlan et al., 2004). The location of MAGEB family is in the last exon of chromosome 6 (Lurquin et al., 1997). Type I MAGE (MAGE-I) proteins contain 200-amino acid-long conserved domain (MAGE homology domain, MHD) that identifies the family (Askew, Bai, Hnat, Minges, & Wilson, 2009). MAGE-I genes are grouped in 3 clusters of human chromosome X, which are MAGE-A, MAGE-B, and MAGE-C. Expression of MAGEB2 gene is generally absent in normal tissue except for testis and placenta (Scanlan et al., 2004). In normal cells, suppression of MAGE-I gene transcription is mainly maintained by promoter methylation (De Smet, Lurquin, Lethé, Martelange, & Boon, 1999).

MAGEB2 has been implicated in carcinogenesis and identified as one of the potential cancer biomarkers. MAGEB2 expression is found in various cancers, for example head and neck squamous cell carcinoma (HNSCC) (Pattani et al., 2012), lung carcinoma (Jang et al., 2001), renal cell carcinoma (Krämer et al., 2005) and multiple myeloma (van Duin et al., 2011). However, the function of MAGEB2 in tumor cells is mostly unknown. MAGE family of genes has potential cancer vaccine targets and has shown to induce humoral and cell mediated immune responses. Coordinated antibody and T cell responses have been observed for MAGE-A and SS-X antigens (Caballero & Chen, 2012). Multiple vaccines (peptide based vaccine and recombinant protein vaccines) have been carried out in clinical trials targeting MAGEA3 and NY-ESO-1. In melanoma patients vaccinated with peptide vaccine for NY-ESO-1 and MAGEA3 have shown partial to complete tumour regression. On the other hand, recombinant protein vaccine was beneficial for a larger patient population as its ability to induce broader

CD4+ and CD8+ immune responses and unrestricted by the HLA types of patients. A phase II clinical trial using recombinant protein MAGEA3 vaccine in non-small cell lung cancer (NSCLC) has shown improved disease-free survival. Additional phase II clinical trial for melanoma and phase III trial for NSCLC on MAGEA3 proteins have been conducted to evaluate immune response and results are being interpreted (Caballero & Chen, 2012). In mouse model, a vaccine raised against murine MAGEB1/2 has been shown to prevent metastasis formation in breast cancer. In mice vaccinated with MAGEB2, an elevated level of IFNγ was observed that indicate the immunogenic effects of MAGEB2 vaccine (Sypniewska et al., 2005).

Human show 50% similarity in terms of amino acid identity if compared with mouse MAGEB2 proteins. Pattani et al., (2012) reported a correlation between human MAGEB2 promoter demethylation and MAGEB2 gene expression in HNSCC. In that study, MAGEB2 overexpression showed growth promoting effect in a minimally transformed oral keratinocyte cell line but not in HNSCC cell lines. MAGEB2 overexpression in tumors is an attractive target for cancer therapy since it can generate antigenic peptides leading to immunogenicity that makes it a potential candidate for vaccination therapy (Pattani et al., 2012). However, further studies are required to corroborate this finding and clarify the exact role of MAGEB2 in HNSCC.

2.3.11.2 Expression of cancer testes antigens (CTA) in head and neck cancer

In head and neck cancer, only three studies have been done previously to analyze the protein expressions of CTA. Two of the studies only investigated one primary site with limited number of samples. The first research that studied the expressions of various CTA in 63 cases of laryngeal SCC found that at least one CTA was observed in 67% of cases. The highest CTA expression was seen in MAGE-A family, which present up to 60%. A significant correlation (p=0.01) between at

least 1 CTA (MAGE-A4, MAGE-C1, MAGE-A1, MAGE-A3, MAGE-C2, NY-ESO-1, and GAGE) with advanced tumour stage (stage III &IV) was observed. However, no association between the expressions of the CTA with survival was reported (Figueiredo et al., 2011). Next, Montoro and colleagues revealed protein expressions of MAGE-A4 and MAGE-C1 were 56.5% and 47.8% respectively in 23 patients with OSCC. There was no association between the expressions of CTA with studied clinico-pathological variables and survival (Montoro et al., 2012). Lastly, a large-scale study involving 453 HNSCC cases from 3 subsites (oral cavity, oropharynx, larynx) reported that the protein expression of MAGE-A family, MAGE-C family, and NY-ESO-1 was seen in approximately 30, 7, and 4% of tumours, respectively. They also found that MAGE-A family and NY-ESO-1 was associated with poor survival (Laban et al., 2014).

2.3.11.3 Biological mechanisms of MAGEB2 in cancer

Earlier in 2012, MAGEB2 expression was associated with growth enhancing effects in normal oral keratinocytes (Pattani et al., 2012). Later, a study by Peche et al., (2015) in human cancer cell lines (osteosarcoma and colorectal carcinoma) and in vivo melanoma cells among mouse revealed that MAGEB2 involves in promoting cell proliferation independently of cell type and p53 status. In their study, only human MAGEB2 expression was associated with up regulated E2F1 transcriptional activity whilst no significant effect was seen in mouse cells. In human, MAGEB2 triggered cell growth and proliferations, which was independent of p53 status whereas in mouse, MAGEB2 cause massive cell death. These finding showed that the exact molecular mechanism of MAGEB2 was different in mouse and human cells. MAGEB2 expression indirectly affects the E2F pathway by forming a protein complex with HDAC1 that will suppress E2F activity. In the absence of MAGEB2 expression, HDAC1 and E2F1 form a part of

protein complex. This HDAC1-E2F1 interaction was reduced in the presence of MAGEB2 by formation of MAGEB2-HDAC1 complex that enhances E2F activity. This is possible by direct interaction of MAGEB2 with HDAC1 that prevents mobilization of HDAC1 to the E2F1 protein complex, therefore increasing the fraction of free and transcriptionally active E2F resulting enhanced E2F1 activity. Similar interactions with HDAC1 were also observed in various oncoproteins such as LEF1 and HMGA2 (Peche et al., 2015). E2F activity is highly sensitive to HDAC activity because a number of proteins inhibit its activity through HDAC recruitment, such as pRb, Kap1, Sin3B, Ebp1 and ELL. Previous studies showed that elevated E2F activity is closely associated with invasion, metastasis, and, with poor survival. In certain conditions, E2F1 has the ability to initiate cell death by activation transcription of pro-apoptotic genes. It has been reported that MAGEB2 function is independent from p53 expression. Instead, MAGEB2 associates with HDAC1 to exert specific regulatory activity on different transcription factors (Peche et al., 2015).

Ribotoxic stress activates cell cycle arrest through the regulation of E2F, p53, and c-myc proteins signaling pathways. Drugs triggering ribotoxic stress relocalize specific ribosomal proteins from the nucleoli to the nucleoplasm as a response mechanism to restrict high translational demand and cell proliferation. Ribosomal proteins have been found to reduce c-myc activity, target MDM2, increase p53 activity, and decrease E2F activity. Peche et al., reported that endogenous MAGEB2 expression plays a critical role to enhance cell cycle progression under ribotoxic stress. MDM2 plays a crucial role in nucleolar checkpoint because it is targeted by ribosomal proteins and as a consequences down-regulating E2F activity and activating p53 pathway. However, MAGEB2 was able to regulate E2F activity independently without presence of MDM2. Then, they concluded that MAGEB2 behaves as a cell proliferation-promoting protein as its expressions enhance the cell proliferations in human cancer cell

lines (osteosarcoma and colorectal cancer) and *in vivo* melanoma mouse model (Peche et al., 2015).

CHAPTER 3 : METHODOLOGY

3.1 Study design

This is a cross-sectional study to investigate the expression of MAGEB2 antibody in the tissue of 20 normal oral mucosa (NOM), 20 oral potentially malignant disorder (OPMD), 60 oral squamous cell carcinoma (OSCC). The formalin fixed paraffin embedded (FFPE) tissue used were retrieved from the archival records of Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS), coordinated by Oral cancer Research & Coordinating Center (OCRCC-UM), Faculty of Dentistry, University of Malaya. This study was conducted in a collaborative effort between OCRCC-UM, Faculty of Dentistry, University of Malaya and Faculty of Dentistry, MAHSA University. This research was approved by the Medical Ethics Committee of Faculty of Dentistry, University of Malaya (Ethics Committee/IRB reference number: DF OS1910/0043 (P)).

3.2 Materials

3.2.1 Samples

3.2.1.1 Sample size calculation

Sample size estimation was achieved by comparing sample size from the other comparable study (Zamunér et al., 2015). However due to budget constraint, the total sample number was limited to 100, comprised of 20 normal oral mucosa (NOM), 20 oral potentially malignant disorder (OPMD), and 60 oral squamous cell carcinoma (OSCC).

3.2.1.2 Tissue samples

(a) Normal oral mucosa (NOM)

20 NOM cases were selected randomly from year 2018. FFPE together with patients' data and information were retrieved from Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS) and Oral Pathology Diagnostic and Research Laboratory (OPDRL) archival records and database. Data extracted includes:

1. Socio-demographic characteristics: age, gender, ethnicity, risk habits (alcohol, smoking, betel quid chewing).
2. Histopathological diagnosis.

(b) Oral potentially malignant disorder (OPMD)

20 OPMD cases were selected randomly from year 2008 to year 2019. FFPE together with patients' data and information were retrieved from Oral Pathology Diagnostic and Research Laboratory (OPDRL) archival records and database. Data extracted includes:

1. Socio-demographic characteristics: age, gender, ethnicity, risk habits (alcohol, smoking, betel-quid chewing).
2. Histopathological diagnosis.

(c) Oral squamous cell carcinoma (OSCC)

60 OSCC cases were selected randomly from year 2012 to 2017. FFPE together with patients' data and information were retrieved from MOCDTBS, coordinated by OCRCC, Faculty of Dentistry, University of Malaya. Data extracted includes:

1. Socio-demographic characteristics: age, gender, ethnicity, risk habits (alcohol, smoking, betel-quid chewing).

2. Clinico-pathologic characteristics of OSCC: tumour site, tumour grading, pathological tumour size (pT), pathological lymph node metastases (pN), pathological tumour staging (pTNM), pattern of invasion (POI), perineural invasion (PNI), lymphovascular invasion (LVI), bone invasion, lymphocytic host response (LHR), sarcolemmal spread, surgical margin status, treatment, and survival status.

3.2.1.3 Inclusion and exclusion criteria

A detailed inclusion and exclusion criteria for NOM, OPMD, OSCC is shown in Table 3.1, 3.2, 3.3:

Table 3.1 : Inclusion and exclusion criteria of NOM.

Inclusion criteria	Exclusion criteria
FFPE of patients diagnosed histologically as normal tissue.	Patients with co-existing other types of malignancy.
Patients with complete socio-demographic data.	Patients with incomplete socio-demographic data.

Table 3.2 : Inclusion and exclusion criteria of OPMD.

Inclusion criteria	Exclusion criteria
FFPE of patients diagnosed histologically as OPMD with or without dysplasia.	Patients with co-existing other types of malignancy.
Patients with complete socio-demographic data.	Patients with incomplete socio-demographic data.

Table 3.3 : Inclusion and exclusion criteria of OSCC.

Inclusion criteria	Exclusion criteria
FFPE of patients diagnosed histologically as OSCC (primary or recurrent cancer).	Patients with co-existing other types of malignancy.
Patients treated by surgery and/ or adjuvant chemoradiotherapy.	Patients with incomplete socio-demographic, clinico-pathological, and survival status data.
Patients with complete socio-demographic, clinico-pathological, and survival status data.	

3.2.1.4 Selection of biomarkers

A previous unpublished protein array study conducted by a group of researchers in OCRCC has identified five TAAs in the serum of OSCC patients. High expression of the five TAAs, which includes TP53, SH2B1, NRIP3, MAGEB2 and PTP1B, were significantly associated with better prognosis after accounting for the socio-demographic and clinico-pathological parameters. In this study, one of the TAAs, which is MAGEB2 was selected to validate and elucidate the role of MAGEB2 in OSCC.

3.3 Methods

3.3.1 Specimen processing

100 FFPE tissue blocks comprising of 20 NOM, 20 OPMD, 60 OSCC were retrieved from the MOCDTBS and OPDRL archival records. Two sections with 4-micrometer thickness were sectioned from FFPE blocks and mounted on the poly-L-lysine coated slides. One tissue section was stained with Hematoxylin & Eosin and another tissue section was used to stain the MAGEB2 antibody in for immunohistochemistry (IHC).

3.3.2 Tissue staining

3.3.2.1 Hematoxylin and Eosin (H&E) stain

Hematoxylin and Eosin (H&E) staining was performed to one of the sections with 4 micrometre thickness from the selected FFPE blocks. The H&E slides were examined to assess the availability of the tissue prior to commencement of IHC procedure. The H&E staining method was described in Appendix A.

3.3.2.2 Immunohistochemistry (IHC)

IHC staining was performed in 10 NOM, 20 OPMD, 60 OSCC tissues. The remaining ten normal tissue slides were not stained with MAGEB2 due to insufficient antibody. Three OSCC cases were excluded due to insufficient tissue present. Table 3.4 summarized the primary antibody, control tissue, optimum dilution, incubation period, antigen retrieval method, and washing buffer utilized in our study. Secondary antibody used was from DAKO REAL™ EnVision™/HRP, Rabbit/Mouse. A detailed IHC protocol was described in Appendix B.

Table 3.4 : Primary antibody, control tissue, optimum dilution, incubation period, antigen retrieval method, and washing buffer used in the study.

Primary Antibody	Rabbit polyclonal MAGEB2
Manufacturer	Novus Biologicals (product code NBP2-62688)
Control Tissue	Human (Testis tissue)
Dilution	1:100
Antigen retrieval buffer & pH	Citrate buffer (pH6.0)
Incubation period	1 hour
Wash buffer & pH	PBS (pH7.2)

3.4 Analysis

3.4.1 Calibration

One oral pathologist (AR) and one oral pathologist trainee (NAAH) were involved in assessing the IHC scoring. The assessors were blinded to the diagnosis and clinico-pathological data. Calibration exercise was conducted between the two assessors before scoring the IHC slides. Intraclass coefficient (ICC) was used to evaluate inter-observer agreement between the two assessors.

3.4.2 Scoring of IHC staining

A semi-quantitative scoring method using immunoreactive score (IRS) was selected in this study. Final IRS is a product of multiplication between percentage of positive cells and intensity of cells. The value ranges from 0 to 12 (Remmele, 1987). The proportion of positive cells and intensity of scoring was displayed in Table 3.5.

Table 3.5 : IRS scoring method.

I (Intensity of staining)	P (Percentage of positive cells)	IRS (Multiplication of I and P)
0 = no	0 = no positive cells	0 – 1 = negative
1 = mild	1 = <10% of positive cells	2 – 3 = mild positivity
2 = moderate	2 = 10-50% positive cells	4 – 8 = moderate positivity
3 = strong	3 = 51-80% positive cells	9 – 12 =strong positivity
	4 = >80% positive cells	

3.4.3 Statistical analysis

Statistical analysis was performed by SPSS software (version 26, IBM). Intraclass coefficient (ICC) was performed to determine the interobserver agreement in scoring the expression of MAGEB2. Based on the interobserver agreement in ICC, the average measures of agreement for each study group was above 0.8 and statistically significant (p<0.05), indicating good agreement as shown in Table 3.6.

Table 3.6 : Intraclass coefficient (ICC) of interobserver agreement for each study group.

Disease status	NOM (p-value)	OPMD (p-value)	OSCC (p-value)
Average measures	0.806 (0.011)	0.887 (0.000)	0.835 (0.000)

Significance level: $p < 0.05$

3.4.3.1 First objective

Test of normality was used to determine the normality of data distribution. Next, Kruskal-Wallis test was used to test if there is a difference in the mean score of MAGEB2 expression between NOM, OPMD, and OSCC tissue. Diagnostic accuracy of MAGEB2 in distinguishing OSCC from OPMD tissue was determined using receiver operating characteristic (ROC) curve.

3.4.3.2 Second objective

Test of normality was used to determine the normality of data distribution. Next, independent sample t-test (parametric) and Mann-Whitney test (non-parametric) was used to compare the mean score of MAGEB2 expression with socio-demographic and clinco-pathologic characteristics. Prognostic accuracy of MAGEB2 expression in distinguishing socio-demographic and clinico-pathological characteristics was determined using receiver operating characteristic (ROC) curve.

3.4.3.3 Third objective

Kaplan-Meier survival analysis was used to establish the associations of MAGEB2 expressions in OSCC patients with overall survival (OS). All statistical analyses was conducted using the SPSS statistical package (SPSS version 26). *P*- value was regarded as statistically significant when the value was less than 0.05.

CHAPTER 4 : RESULTS

4.1 Socio-demographic characteristics of total samples

A total of 87 samples were included in this study comprising of 10 NOM, 20 OPMD, and 57 OSCC patients. The age ranges from 20 to 94 years with a mean age of 56.92. Median age was 60 years and was used as cut-off value for age group in this study. In this study 60% of the total sample were females. Almost half of the sample was Indians followed by Chinese, Malay, and others (Sikh). About two-third of the sample did not practice any risky habits like smoking, drink alcohol, and chew betel-quid. Table 4.1 depicts the distribution of total sample based on the socio-demographic parameters.

Table 4.1: Socio-demographics characteristics of total samples.

Characteristics	Overall n=87 (%)	NOM n=10 (%)	OPMD n=20 (%)	OSCC n=57 (%)
Age group (years)				
≤ 59	42 (48.3)	10 (100.0)	11 (55.0)	21 (36.8)
> 59	45 (51.7)	-	9 (45.0)	36 (63.2)
Gender				
Male	35 (40.2)	2 (20.0)	9 (45.0)	24 (42.1)
Female	52 (59.8)	8 (80.0)	11 (55.0)	33 (57.9)
Ethnicity				
Malay	18 (20.7)	2 (20.0)	4 (20.0)	12 (21.1)
Chinese	27 (31.0)	2 (20.0)	8 (40.0)	13 (22.8)
Indian	41 (47.1)	6 (60.0)	7 (35.0)	32 (56.1)
Others	1 (1.1)	-	1 (5.0)	-
Smoking				
Yes	22 (25.3)	2 (20.0)	1 (5.0)	19 (33.3)
No	65 (74.7)	8 (80.0)	19 (95.0)	38 (66.7)
Alcohol				
Yes	19 (21.9)	1 (10.0)	3 (15.0)	15 (26.4)
No	68 (78.2)	9 (90.0)	17 (85.0)	42 (73.7)
Betel-quid				
Yes	23 (26.4)	-	4 (20.0)	19 (33.4)
No	64 (73.6)	10 (100.0)	16 (80.0)	38 (66.7)

4.2 Site distribution and histopathological diagnosis

4.2.1 NOM

A total of 10 NOM tissues were recruited in this study. Gingiva (8/10) was the most common site and all of them were taken from MOCDTBS, which were reserved as normal control tissues for research purposes. Meanwhile the other two NOM tissues from lip (1/10) and gingiva (1/10) were taken from archival records of OPDRL.

4.2.2 OPMD

From 20 OPMD cases, 80% of them were taken from buccal mucosa (16/20), followed by tongue (2/20), lip (1/20), and soft palate (1/20). Majority of the cases were diagnosed as oral lichen planus (12/20) followed by mild epithelial dysplasia (6/20) and moderate epithelial dysplasia (2/20). All of the OPMD cases were retrieved from archival records of OPDRL.

4.3 Clinico-pathological characteristics of OSCC samples

Table 4.2 summarizes the clinico-pathological characteristics of 57 OSCC patients recruited in this study. From the gathered data, the most common sites for OSCC were buccal mucosa, tongue, and gingiva. Parameters like tumour size of T3-T4 with absence of lymph node metastases, and advanced tumour staging (stage III and IV) were observed in more than half of OSCC patients.

Table 4.2 : Clinico-pathological characteristics of OSCC sample.

Characteristics	OSCC N=57 (%)
Tumour site	
Buccal mucosa	33 (37.9)
Tongue & floor of the mouth	24 (27.5)
Lip	2 (2.3)
Others	28 (32 .0)
Tumour grading	
Well	6 (10.5)
Moderate	49 (86.0)
Poor	2 (3.5)
Tumour size (pT)	
T1-T2	26 (45.6)
T3-T4	31 (54.4)
Lymph node metastasis (pN)	
Present	24 (42.1)
Absent	33 (57.9)
Tumour staging (pTNM)	
Stage I & II (early)	19 (33.3)
Stage III & IV (advanced)	38 (66.7)
Pattern of invasion (POI)	
Cohesive	2 (3.5)
Non-cohesive	55 (96.5)
Perineural invasion (PNI)	
Present	18 (31.6)
Absent	39 (68.4)
Lymphovascular invasion (LVI)	
Present	7 (12.3)
Absent	50 (87.7)
Bone invasion	
Present	15 (26.4)
Absent	42 (73.7)
Lymphocytic host response (LHR)	
Strong	15 (26.3)
Intermediate	35 (61.4)
Weak	7 (12.3)
Sarcolemmal spread	
Present	42 (73.7)
Absent	15 (26.3)
Surgical margin status	
Clear	13 (22.8)
Not clear (close and involved)	44 (77.2)
Treatment	
Surgery	55 (96.2)
Surgery and radiotherapy	2 (3.5)
Survival status	
Alive	31 (54.4)
Deceased	21 (36.8)
Lost to follow-up	5 (8.8)

4.4 Immunohistochemical staining in NOM, OPMD, and OSCC tissue

Immunohistochemistry (IHC) staining for MAGEB2 antibody was localized in the cytoplasm and nucleus of the epithelial cells. Nucleus or cytoplasm of cells that stained with brown colour was considered as positive. IHC scoring was conducted using IRS technique. Intensity and proportion of immunoreactive cells scored from two observers were recorded. The average score for each sample was obtained. Figure 4.1, 4.2, 4.3, and 4.4 illustrates the photomicrograph of anti-MAGEB2 in control tissue of human testes, NOM, OPMD, and OSCC tissue.

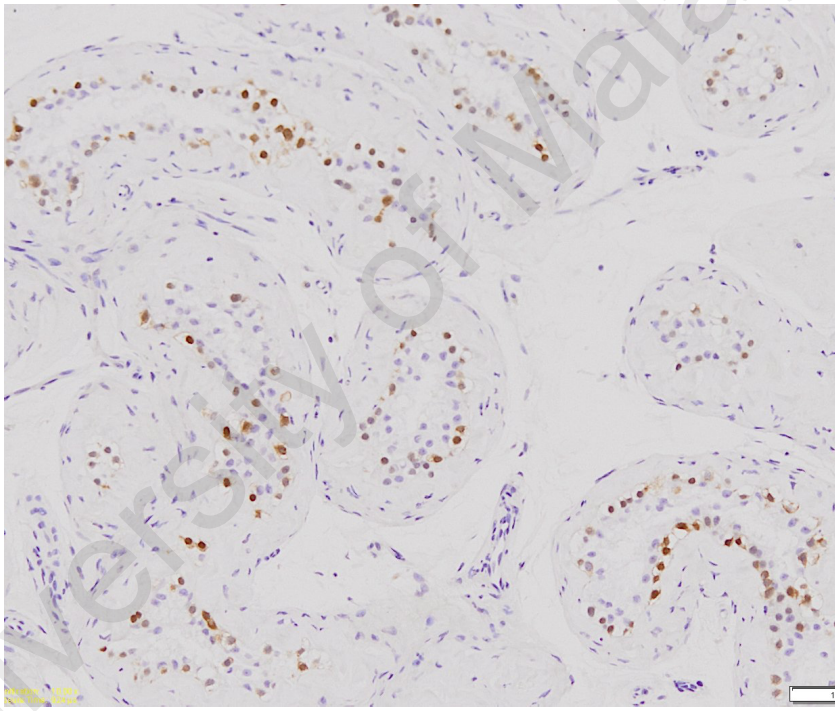

Figure 4.1 : Photomicrograph in positive control human testes tissue (seminiferous tubules) showing localization of strong anti-MAGEB2 immunostaining in the nucleus and cytoplasm of spermatogonia cells (Original magnification: 200x).

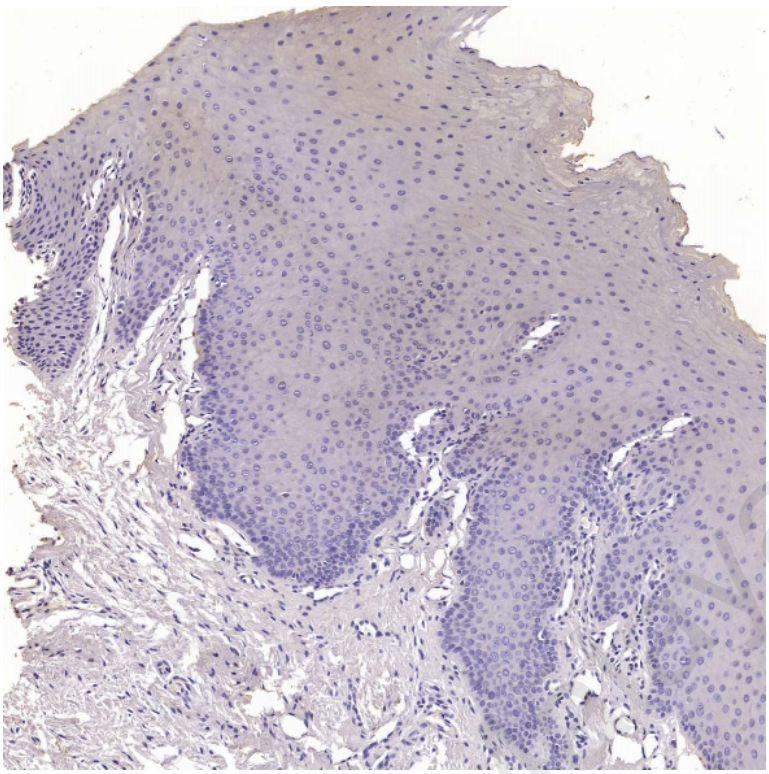

Figure 4.2 : Photomicrograph of the normal oral mucosa tissue exhibiting focal mild cytoplasmic anti-MAGEB2 immunostaining within the epithelium (Original magnification: 100x).

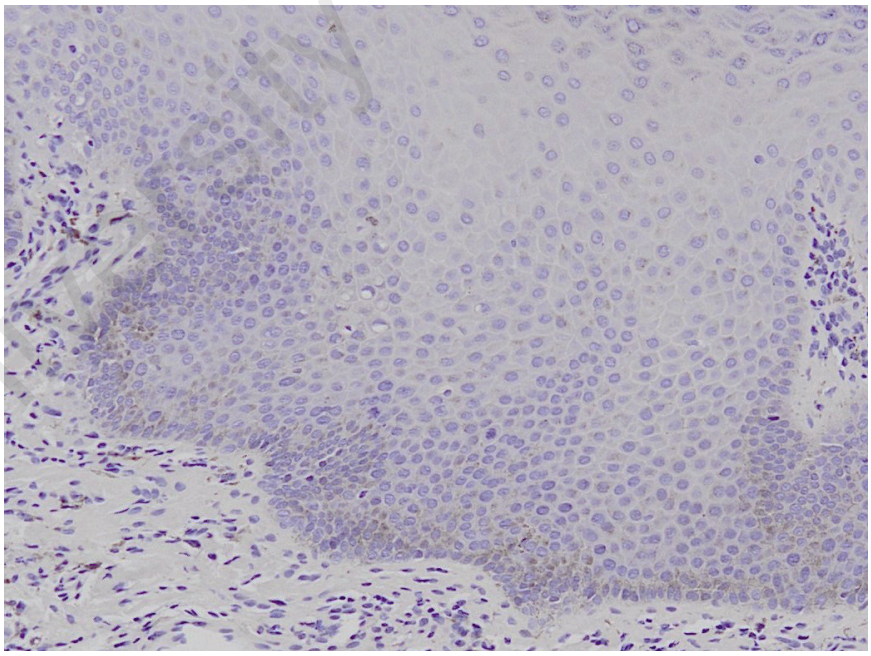

Figure 4.3 : Photomicrograph of the mucosa exhibiting mild epithelial dysplasia showed focal mild cytoplasmic anti-MAGEB2 immunostaining within the lower third of the epithelium (Original magnification: 100x).

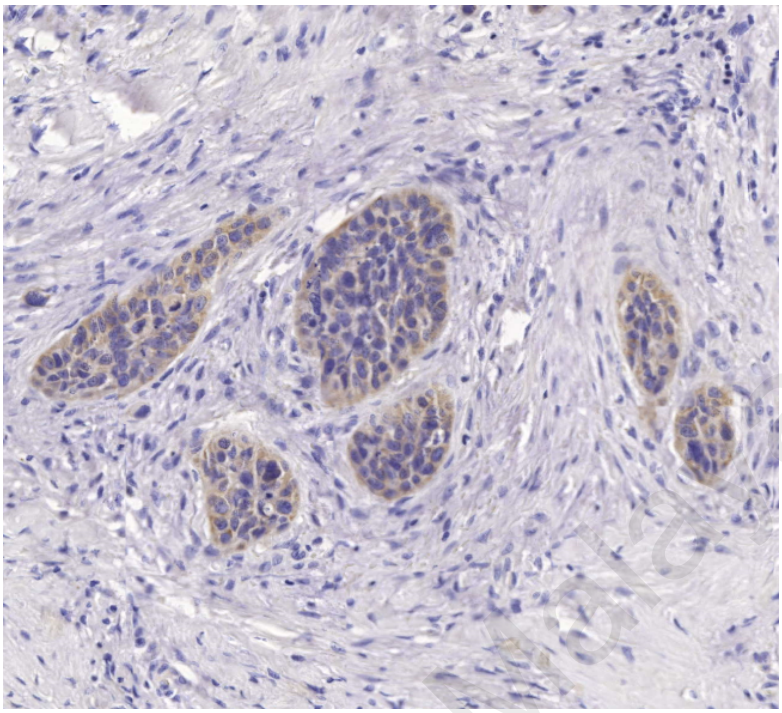

Figure 4.4 : Photomicrograph of the malignant tumour cells exhibiting moderate cytoplasmic and focal nuclear anti-MAGEB2 immunostaining (Original magnification: 200x).

4.4.1 Distribution of MAGEB2 expression in NOM, OPMD, and OSCC tissue

The value of mean score of MAGEB2 expressions ranges from 0 to 8. The most frequent mean score is 2 followed by 4. The overall mean score of MAGEB2 expressions was depicted in Figure 4.5. Regarding distribution of MAGEB2 expression in NOM, OPMD, and OSCC tissue about three-quarter of all tissue samples showed immunopositivity towards MAGEB2. The percentage of tissue with positive MAGEB2 expression in NOM and OSCC tissue was similar, about 80%. About 60% of MAGEB2 was expressed in OPMD tissue. In terms of intensity of staining, more than half of all immunopositive tissue exhibits mild positivity (56%). This pattern of expression was observed in NOM and OPMD tissue. On the other hand, OSCC tissue demonstrates similar percentage of mild and moderate positivity, which is 40%. However, none of the tissue samples had strong positivity.

The data for the distribution of MAGEB2 expression in NOM, OPMD, and OSCC tissue was displayed in Table 4.3. The highest MAGEB2 expression was observed in OSCC (Mean= 3.1; 95% CI 2.6 - 3.6) followed by normal tissue (Mean= 2.2; 95% CI 1.7 - 3.2). In contrary to our prediction, the least MAGEB2 expression was observed OPMD tissue (Mean= 1.7; 95% CI 1.1 – 2.4). In this study, the highest mean immunoreactive score for MAGEB2 expression was 8, which represents the upper end spectrum of moderate positivity and was observed exclusively in OSCC tissue. Meanwhile, the highest MAGEB2 expression in both OPMD and NOM was 4, represents the lower end of the spectrum of moderate positivity. Table 4.4 summarized the mean score of MAGEB2 antibody in NOM, OPMD, and OSCC tissue.

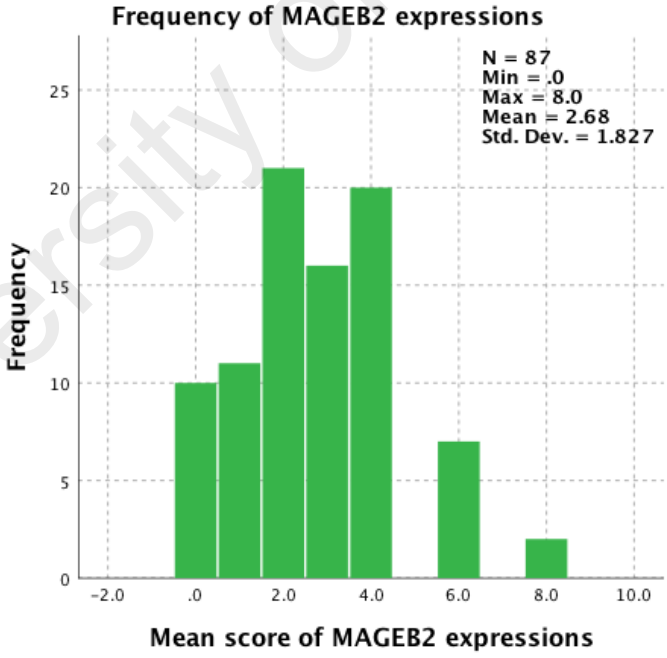

Figure 4.5 : Frequency of MAGEB2 expressions.

Table 4.3 : Distribution of MAGEB2 expression in NOM, OPMD, and OSCC tissue.

Variables	Number of samples, N	Negative N(%)	Positive			
			Mild positive N(%)	Moderate positive N(%)	Strong positive N(%)	Total
NOM	10	2 (20%)	5 (50%)	3 (30%)	0 (0%)	8 (80%)
OPMD	20	8 (40%)	9 (45%)	3 (15%)	0 (0%)	12 (60%)
OSCC	57	11 (19%)	23 (40%)	23 (40%)	0 (0%)	46 (81%)
Total	87	21 (24%)	37 (56%)	29 (44%)	0 (0%)	66 (76%)

Table 4.4 : Mean score of MAGEB2 expression in NOM, OPMD, and OSCC tissue.

Mean score	N	Mean	Standard Deviation	95% Confidence Interval
NOM	10	2.200	1.4376	1.172 - 3.228
OPMD	20	1.725	1.3521	1.092 - 2.358
OSCC	57	3.096	1.9050	2.591 - 3.602
Total	87	2.678	1.8268	2.289 - 3.067

4.5 First objective

4.5.1 Comparison of MAGEB2 expressions in NOM, OPMD, and OSCC tissue.

Test of normality was performed using Shapiro-Wilk test (N<50) in the normal and OPMD tissue resulting in *p*- value of 0.254 and 0.025 respectively. Kolmogorov-Smirnov test (N>50) was used to test normality of data distribution in OSCC tissue showed *p*- value of 0.001. Since the *p*- value was statistically significant ($p < 0.05$), in OPMD and OSCC group, therefore the data was not normally distributed. Kruskal-Wallis non-parametric test was used to test if the mean score for MAGEB2 expression differs between the three study groups since the data was not normally distributed. Bonferroni procedure was used to adjust significant value for disease pair that was significantly different and the data was summarized in Table 4.5. Figure 4.6 illustrates

independent Kruskal-Wallis test to compare mean score of MAGEB2 expressions in NOM, OPMD, and OSCC tissue. The *p*- value of OPMD and OSCC was 0.014, which is less than 0.05 indicating significant difference. Hence, MAGEB2 expressions in OSCC were significantly higher compared to OPMD group. Inversely, the *p*-value of NOM Vs OPMD and NOM Vs OSCC was 1.0 and 0.614 respectively. Therefore, there was no significant difference between MAGEB2 expressions in NOM Vs OPMD and NOM Vs OSCC group.

Table 4.5 : Pairwise comparisons of MAGEB2 expression with disease status using Kruskal-Wallis test.

Disease group pair	Standard. Error	*p*-value
NOM Vs OPMD	9.677	1.000
NOM Vs OSCC	8.566	0.614
OPMD Vs OSCC	6.494	**0.014**

Significance level: $p < 0.05$
Significance values have been adjusted by the Bonferroni correction for multiple tests.

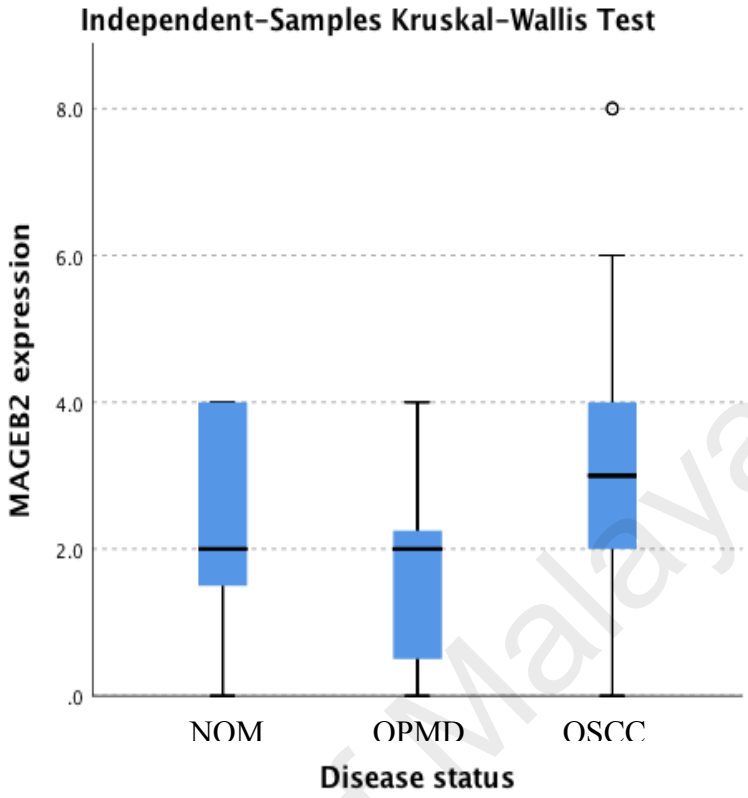

Figure 4.6 : Box plot for MAGEB2 expressions in NOM, OPMD, and OSCC tissue.

4.5.2 Association between NOM, OPMD, and OSCC with socio-demographic and tumour site.

From chi square analysis, there is a significant association between disease status (NOM, OPMD, and OSCC) with clinical parameters like age, ethnicity, smoking, and site groups as seen in Table 4.6. Multinomial logistic regression was conducted to reanalyze the association between the mean score of MAGEB2 expression with NOM-OPMD and NOM-OSCC pair groups after adjustment of confounding factors (age, ethnicity, smoking, and site groups). Table 4.7 summarizes output from the multinomial logistic regression.

Table 4.6 : Association between disease status with socio-demographic and clinical site.

Variables	Category	Disease status, N (%)			*p*-value
		Normal	OPMD	SCC	
Age group	≤ 59	10 (23.8%)	11 (26.2%)	21 (50%)	**0.001**
	>59	0 (0%)	9 (20%)	36 (80%)	
Ethnicity group	Indian	2 (4.9%)	7 (17.1%)	32 (78%)	**0.049**
	Non-Indian	8 (17.4%)	13 (28.3%)	25 (54.3%)	
Gender	Female	8 (15.4%)	11 (21.2%)	13 (63.5%)	0.412
	Male	2 (5.7%)	9 (25.7%)	24 (68.6%)	
Smoking	No	8 (12.3%)	19(29.2%)	38 (58.5%)	**0.036**
	Yes	2 (9.1%)	1 (4.5%)	19 (86.4%)	
Drinking alcohol	No	9 (13.2%)	17 (25%)	42 (61.8%)	0.419
	Yes	1 (5.3%)	3 (15.8%)	15 (78.9%)	
Betel-quid chewing	No	10 (15.6%)	16 (25%)	38 (59.4%)	0.064
	Yes	0 (0%)	4 (17.4%)	19 (82.6%)	
Site group	Others	9 (16.7%)	4 (7.4%)	41 (75.9%)	**0.000**
	Buccal mucosa	1 (3.0%)	16 (48.5%)	16 (48.5%)	

Significance level: $p < 0.05$
Chi square analysis.

Table 4.7 : Multinomial logistic regressions after adjustment of confounding factors.

Variables	Standard error	*p*-value	95% Confidence interval
NOM Vs OPMD	0.388	0.645	0.391 - 1.790
NOM Vs OSCC	0.402	0.288	0.697 - 3.371

Significance level: $p < 0.05$

From Table 4.7, the *p*-value of both NOM Vs OPMD and NOM Vs OSCC pair groups were not statistically significant. Therefore, even after adjustment of the confounding factors, there is no significant association between NOM Vs OPMD and NOM Vs OSCC.

4.5.3 Diagnostic accuracy of MAGEB2 expression in distinguishing OPMD from OSCC tissue

Diagnostic accuracy of MAGEB2 expressions in distinguishing OPMD from OSCC was determined from ROC curve. Figure 4.7 show ROC curve plot was above than the 45-degree diagonal line indicates that MAGEB2 was able to discriminate OPMD from OSCC tissue. ROC curves are useful for comparing diagnostic ability

of screening tests. From Table 4.8, only OPMD Vs OSCC pair showed significance difference of *p*- value of 0.005 and AUC value of 0.711. AUC is an effective method to summarize diagnostic accuracy of a test. AUC values range from 0 to 1, whereby a value of 0 represents perfectly inaccurate test whereas value of 1 reflects a perfectly accurate test. In general, an AUC of 0.5 suggests the test has no discriminatory ability, 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and value of more than 0.9 is considered outstanding. A value of 0.5 for AUC indicates that the ROC curve will fall on the diagonal 45-degree plot and therefore suggests that the diagnostic test has no discriminatory ability (Hosmer & Lemeshow, 2000). Our study showed that AUC value of 0.711 indicates that MAGEB2 was an acceptable diagnostic marker to discriminate OPMD from OSCC tissue. Table 4.9 showed that value 2.75 was chosen as cut-off point value resulting in 61.4% sensitivity and 80% specificity. Therefore, MAGEB2 was able to distinguish OPMD from OSCC tissue with 61.4% sensitivity and 80% specificity.

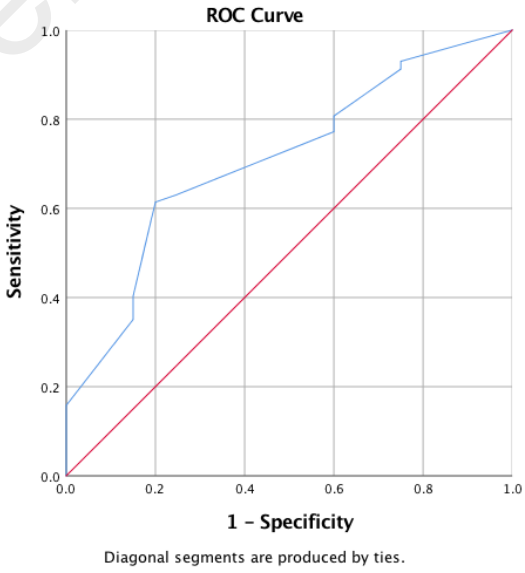

Figure 4.7 : ROC curve of MAGEB2 expression in distinguishing OPMD from OSCC tissue.

Table 4.8 : Area Under the Curve (AUC) of MAGEB2 expressions between NOM, OPMD, and OSCC tissue.

Variables	Area Under the Curve (AUC)	Standard error	*p*-value	95% Confidence interval
NOM Vs OPMD	0.413	0.112	0.441	0.193 - 0.632
NOM Vs OSCC	0.625	0.093	0.208	0.443 - 0.807
OPMD Vs OSCC	**0.711**	0.065	**0.005**	0.584 - 0.837

Significance level: $p < 0.05$
Under non-parametric assumption
Null hypothesis: True area = 0.5

Table 4.9 : Sensitivity and specificity value of MAGEB2 expression in distinguishing OPMD and OSCC.

Cut-off value	Sensitivity	1 - Specificity	1-[1-Specificity]	Sensitivity + Specificity
-1	1	1	0	1
0.25	0.93	0.75	0.25	1.18
0.75	0.912	0.75	0.25	1.162
1.25	0.807	0.6	0.4	1.207
1.75	0.772	0.6	0.4	1.172
2.25	0.632	0.25	0.75	1.382
2.75	0.614	0.2	0.8	1.414
3.25	0.404	0.15	0.85	1.254
3.75	0.351	0.15	0.85	1.201
5	0.158	0	1	1.158
7	0.035	0	1	1.035
9	0	0	1	1

4.6 Second objective

4.6.1 Prognostic accuracy of MAGEB2 in distinguishing socio-demographic and clinico-pathological characteristics.

The diagnostic accuracy for MAGEB2 in distinguishing the two outcomes from socio-demographic (age, gender, ethnicity, smoking, drink alcohol, betel-quid chewing) and clinico-pathological (site, pT, pN, pTNM, POI, PNI, LVI, bone invasion, LHR, sarcolemmal spread, surgical margin status, treatment, survival) parameters was determined using receiver operating characteristic (ROC) curve. Area under the curve (AUC) for these parameters (sociodemo-graphic and clinico-pathological) was determined from the ROC curve plot and displayed in Table 4.10.

Table 4.10 : Area Under the Curve (AUC) of MAGEB2 in relation to socio-demographics and clinic-pathological parameters.

Variables	AUC	Standard error	*p*-value	95% Confidence interval
Age group	0.619	0.082	0.137	0.459 -0.779
Ethnicity group	0.493	0.079	0.930	0.338 -0.648
Gender	0.530	0.081	0.704	0.372 -0.688
Smoking	0.470	0.086	0.710	0.300 -0.639
Drink alcohol	0.486	0.092	0.870	0.305 -0.667
Betel-quid chewing	0.366	0.075	0.101	0.220 -0.512
Site	0.484	0.081	0.852	0.326 -0.642
Grading	0.277	0.155	0.288	0.000 -0.581
pT	0.507	0.078	0.923	0.355 -0.659
pN	0.561	0.076	0.438	0.411 -0.710
pTNM	0.539	0.084	0.629	0.376 -0.703
POI	0.691	0.136	0.362	0.423 -0.958
PNI	0.446	0.087	0.514	0.275 -0.610
LVI	0.507	0.100	0.952	0.311 -0.703
Bone invasion	0.401	0.080	0.257	0.245 -0.557
LHR	0.374	0.136	0.285	0.107 -0.642
Sarcolemmal spread	0.510	0.086	0.913	0.341 -0.678
Surgical margin	0.457	0.099	0.641	0.264- 0.650
Treatment	0.623	0.112	0.558	0.404-0.841
Survival status	0.467	0.078	0.679	0.313- 0.621

Significance level: $p < 0.05$
Under non parametric assumption
Null hypothesis: true area = 0.5

Based on table 4.10, from all the parameters, only AUC value for POI and age group was above 0.5. Other parameters showed that AUC value equals to 0.5, whilst the AUC value for betel-quid chewing and recurrence is less than 0.5. However, the *p*- value for all the parameters is more than 0.05, hence the AUC results were not statistically significant. Therefore, the initial null hypothesis of true area under the ROC curve equals to 0.5 was accepted since p-value is more than 0.05. This indicates that MAGEB2 has no discriminatory ability to distinguish outcomes from socio-demographic and clinico-pathological characteristics.

4.7 Third objective

4.7.1 Overall survival (OS) in OSCC patients

Based on Kaplan-Meier survival analysis (Figure 4.8), the 5-year overall survival for OSCC patient was 43%. The mean and median survival estimates was 39 months (95%CI: 30.3- 48.0) and 30 months (95%CI: 18.5- 41.5) respectively.

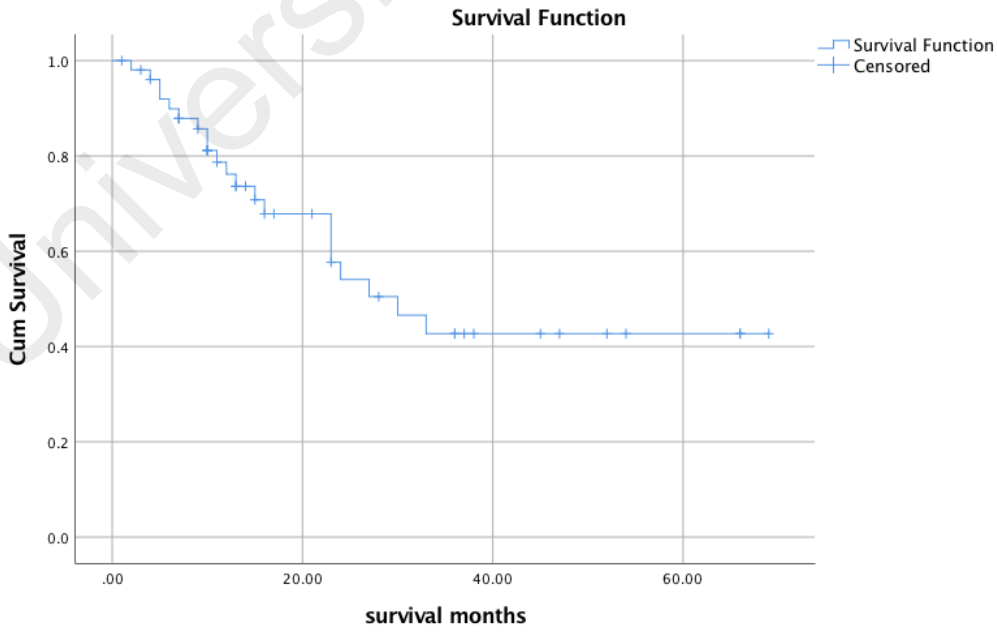

Figure 4.8: Kaplan-Meier overall survival plot in OSCC patients.

4.7.2 Association between MAGEB2 expressions with overall survival in OSCC patients

From 52 OSCC patients, 21 of them died. The remaining 5 patients were lost to follow-up. The number of death events is seen more in tissue that exhibits high (12/52) MAGEB2 expression compared to tissue with low (9/52) MAGEB2 expression. This may be due to unequal representatives of tissue with high and low MAGEB2 expression. In the present study, the number of patients having high (33/52) MAGEB2 expression is more than the patients with low (19/52) MAGEB2 expression. The overall mean survival estimates was 39 months. The mean survival estimates for low MAGEB2 expression was 37 months whilst for high MAGEB2 expression, the mean survival estimates was 47 months. This finding demonstrates that better overall survival was observed in tissue with high MAGEB2 expressions.

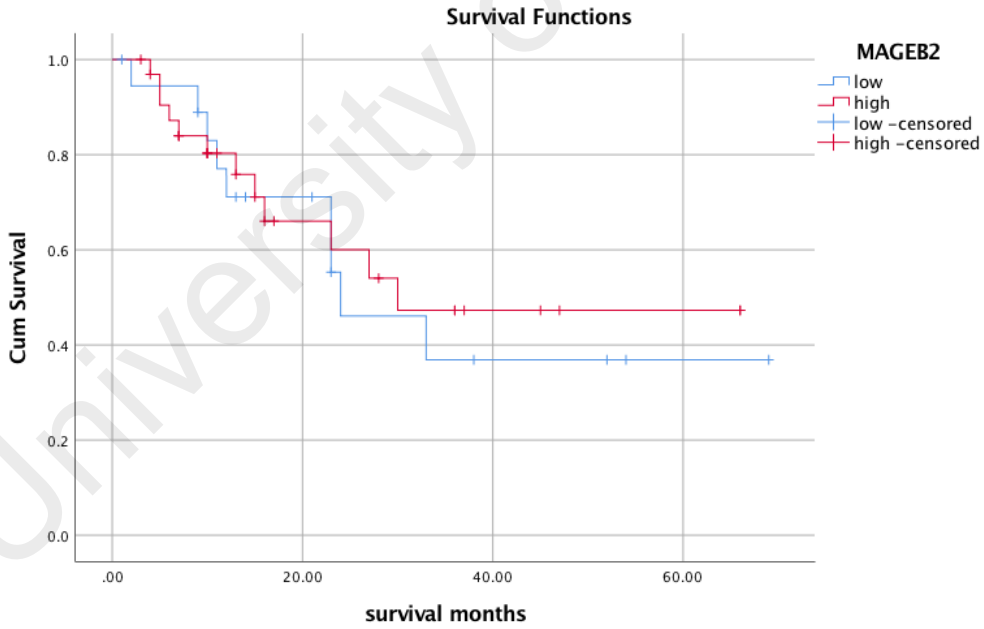

Figure 4.9 : Kaplan-Meier overall survival plot for MAGEB2

From the Kaplan-Meier plot (Figure 4.9), at time zero, the cumulative survival is 1.0 that implies 100% of participants were still alive. At 20 months, the cumulative survival is 0.71 (71%) for low MAGEB2 expression and 0.66 (66%) for high MAGEB2 expression. This shows that at 20 months, low MAGEB2 expression is associated with

better overall survival compared to patient with high MAGEB2 expression. Inversely, at 60 months the cumulative survival is 0.47 (47%) for patients with high MAGEB2 expression and 0.37 (37%) for the low MAGEB2 expression group. This latter finding contradicts with initial observation when the tissues with high and low MAGEB2 expression were compared at 20 months. Therefore, better survival overall survival is observed in patients with high MAGEB2 expression compared to low MAGEB2 expression at 60 months but not at 20 months. Log Rank (Mantel Cox) analysis revealed no significant difference (p = 0.75) between the low and high MAGEB2 expression group with survival estimates. Although there is no significant associations between patients with high and low MAGEB2 expression with overall survival in OSCC patients, an improved overall survival was noticed in patients with high MAGEB2 expression.

CHAPTER 5 : DISCUSSION

5.1 Introduction

World Health Organization (WHO) in 2015 reported that cancer is one of the leading causes of death before age 70 years in more than half of global populations (Bray et al., 2018). Despite significance advances in cancer therapy, the survival rates have not improved tremendously. Late detection of cancer and frequent locoregional metastases are the main factors associated with treatment failure in cancer patients. Although globally lip and oral cavity cancer was not as common as other cancers, the incidence was higher in South East Asia due to different cultural practices such as chewing betel quid and low socioeconomic background. The conventional treatment of OSCC is surgery with adjuvant chemoradiotherapy.

The recent discovery of tumour-targeted treatments using tumour antigens opened a new door for novel forms of treatment of cancer. Immunotherapy is a growing cancer treatment due to antitumoral effect caused by immunologic memory (Zamunér et al., 2015). Hence, immunologic intervention may assists in the control of cancer progression and recurrence. Recent FDA approval for Sipuleucel-T in prostate cancer and Ipilimumab in metastatic melanoma has triggers substantial interest in applying immunotherapy approaches to different types of cancer (Cheever & Higano, 2011; Traynor, 2011).

Cancer testes antigens (CTA) were recognized as promising tumour antigens due to its high immunogenicity and specific expression pattern. CTA have attracted attention as potential targets for tumor immunotherapy, but few reports are available in the literature about their expression, especially regarding the MAGE family in head and neck cancers (Figueiredo et al., 2011).

5.2 MAGEB2 expressions in NOM, OPMD, and OSCC

Our study showed that MAGEB2 expression was seen highest in OSCC tissue, followed by NOM and OPMD tissue. MAGEB2 expression was significantly higher in OSCC compared to OPMD tissue (p = 0.014). However, there is no significant difference between MAGEB2 expression in NOM and OSCC. Similarly, no significant difference of MAGEB2 expression was also observed in NOM and OPMD tissue. ROC curve analysis demonstrates that MAGEB2 was able to differentiate between OSCC and OPMD tissue at 2.75 cut off-point value with 61% sensitivity and 80% specificity. Unfortunately, this marker was unable to discriminate between OSCC from normal tissue and OPMD from normal tissue. Although, from statistical analysis there is no significant difference in NOM Vs OSCC and NOM Vs OPMD, in clinical setting these entities was completely different and can be easily distinguished from each other. The reasons behind the inability of this marker to distinguish OSCC from normal tissue and OPMD from normal tissue may be due to unequal sample size in normal (n=10) compared to OPMD (n=20) group. At the beginning of the study, 20 NOM tissues were selected however due to insufficient antibody, only 10 normal tissues were able to proceed with MAGEB2 IHC staining. The recommended MAGEB2 antibody (Novus) dilution from the manufacturer is 1:500- 1:1000, but the antibody dilution used in this study to achieve optimal staining was 1:100, which is five to ten times more concentrated than the recommended dilution. Therefore, the insufficient antibody was contributed by unexpected antibody dilution ratio utilized in this study than the initial antibody dilution as recommended by the manufacturer.

To the best of our knowledge, this is the first study comparing protein expression of MAGEB2 in NOM, OPMD, and OSCC tissue. About 81% of OSCC tissue in our study expressed MAGEB2 antibody. A comprehensive study conducted to

determine the expression of various CTA in HNSCC using RT-PCR reveals that 11 CTA genes were expressed in HNSCC, and five of them, namely MAGEB2, MAGEA1, MAGEB6, SPANX-CD, and CXORF48 were CTA testes restricted gene. The mRNA expression of MAGEB2 in HNSCC was 44.9% whereas none was expressed in normal tissue (Zamunér et al., 2015). The percentage of OSCC tissue in our study that expressed MAGEB2 antibody was almost two-fold than study conducted by Zamunér (2015). The discrepancy in the MAGEB2 expression could be for few reasons. First, expressions of MAGEB2 at mRNA and protein level were not equal. A study of MAGE genes in lung carcinoma reported that mRNA expressions might not necessarily indicate protein expressions (Jang et al., 2001). In agreement to that, Gordeeva and colleagues reported that human protein expression of MAGEB2 was slightly less (63%) than at mRNA level (89%). Hence, the levels of mRNA expression of MAGEB2 may not correspond to MAGEB2 expression at protein level (Gordeeva, 2018). Second, Zamuner and colleagues used RT-PCR method and fresh frozen tissue in their study whilst in our study IHC method using FFPE was utilized to quantify MAGEB2 expression at protein level. Next, all oral cancer in this study was diagnosed as OSCC meanwhile in Zamuner et al., study, their HNSCC samples were from 3 main sites: oral cavity, oropharynx/ hypopharynx, and larynx. Hence the MAGEB2 expressions may differ according to tumour location.

Scanlan reported that the expression of MAGEB2 was seen in various tumours but none was expressed in normal tissue except testes and placenta (Scanlan et al., 2004). Zamuner also described similar finding in his study involving comprehensive analysis of CTA in HNSCC showed no expression of MAGEB2 in normal control samples at mRNA level (Zamunér et al., 2015). MAGEB2 was known as “testes restricted” CTA as it was absent in normal tissues but can be detected in several tumors. The findings from previous studies contradict with the present study whereby expression of MAGEB2

antibody was observed in 80% and 60% of NOM and OPMD respectively. Although in this study MAGEB2 was expressed in NOM and OPMD tissue, their expression was much lower compared to OSCC tissue. Moderate positive MAGEB2 expression was observed in 40% (23/57) of OSCC, 30% (3/10) in normal and 15% (3/20) in OPMD tissue. Strong positive MAGEB2 expression was not seen in all three groups. Therefore, this study showed that MAGEB2 protein expression was not specific to OSCC tissue, but was also observed in normal and OPMD tissue as well. Zamuner also reported up to 10% of other CTA expressed in normal samples, these include PRAME, SPANX-CD, MAGEA1, MAGEC2, and CRISP2 (Zamunér et al., 2015).

5.3 MAGEB2 expressions in other types of cancer

The expression of MAGEB2 varies in different types of cancer. In testicular cancer, MAGEB family was expressed in 89% of the cases, particularly seminomas. Low or negative expressions of MAGEB was observed in non-seminomatous germ cell tumour (NSGCT) (Gordeeva, 2018). A study in other disease, for example non-small cell lung carcinoma (NSCLC) documented that MAGEB2 was highly expressed in squamous cell carcinoma (SCC) variant and may represents potential targets in the treatment. However, no prognostic association was observed in protein expression of MAGEB2 with socio-demographic and clinico-pathological characteristic, except for smoking habits (Jin et al., 2018).

5.4 Diagnostic accuracy of MAGEB2

We found out that MAGEB2 was able to distinguish OSCC from OPMD tissue with diagnostic accuracy of 61.4 % sensitivity and 80% specificity. Zamuner (2015) elucidated slightly lower sensitivity (44.9%) of but perfect specificity (100%) of MAGEB2 expressions in HNSCC samples compared to normal tissue. In their study,

combination two to five CTA panels (MAGEB2, MAGEA1, MAGEB6, SPANX-CD, and CXORF48) results in enhanced sensitivity ranging from 57.3% to 85.4%, whilst the specificity of certain combinations can be as high as 100% making this panels as potential targets for immunotherapy. Hence, combinations of more than one CTA panels were able to increase the sensitivity value. The sensitivity of an instrument/ tool refers to the ability of the test to correctly identified patients with disease whilst specificity refers the ability of the test to correctly identify those patients without the disease (Lalkhen & McCluskey, 2008). Although MAGEB2 was able to distinguish OSCC from OPMD tissue but not in normal tissue, we postulate that the possibility for MAGEB2 to discriminate between NOM Vs OSCC and NOM Vs OPMD will be higher if the sample size for the normal tissue increases. Moreover, the sensitivity and specificity of a test to discriminate OMPD from OSCC will be higher with a combination of several biomarkers.

5.5 Prognostic accuracy of MAGEB2

This study also demonstrates MAGEB2 was unable to discriminate prognostic outcomes from socio-demographic (age, gender, ethnicity, smoking, drink alcohol, betel quid chewing) and clinico-pathological (tumour site, pT, pN, pTNM, POI, PNI, LVI, bone invasion, LHR, sarcolemmal spread, surgical margin status, treatment, and survival status) parameters. Along these lines, other CTA (for instance MAGE-A1, SSX-1, CTp11 and HCA587) showed no significant association with clinico-pathological parameters in hepatocellular carcinoma (Zhao et al., 2004). Moreover, other various CTA for example; NY-ESO-1, MAGE-A3, and KK- LC-1 in non-small cell lung cancer; and SPAG9 in epithelial ovarian cancer showed no significant association with clinico-pathological parameters (Garg et al., 2007; Shigematsu et al., 2010; Zhao et al., 2004). On the other hand,

most of the previous studies reported positive expression of CTA was related to poor outcome in multiple myeloma, head and neck, ovarian, gastric, and lung cancers (Andrade et al., 2008; Cuffel et al., 2011; Gure et al., 2005; Jung et al., 2005; Yakirevich et al., 2003). Zamuner et al., reported that other CTA for example MAGEB6, CRISP2, and CXORF48 expressions were significantly correlated with poorer clinical disease outcome and MAGEA3/6 was an independent predictor for tumor recurrence. Zamuner et al., also proposes that the expression of these CTA in HNSCC may have initiated a spontaneous immune response that could impact the overall disease prognosis.

5.6 Association between MAGEB2 with overall survival

The 5-year overall survival rate for OSCC patients in this study was 43%. This finding is in concordance as reported in USA, whereby they reported 5-year OS of patients with OSCC was about 50% (Feller & Lemmer, 2012). In contrast, a survival analysis of OSCC patients in Malaysian population demonstrates worse survival with 1-year and 5-year OS was 67.2% and 13.4% respectively (W. Ghani et al., 2011). In terms of survival analysis, there was no significant association observed between high and low expression of MAGEB2 with overall survival (OS). Instead of that, a trend of better overall survival in tissues with high MAGEB2 expression was observed in our study. Along the same line, Zamuner et al., (2015) documented no significance difference between MAGEB2 expressions with OS. However, their results portrayed a trend of high MAGEB2 expression with poor overall survival. In addition, no correlation between MAGE expression and survival rate observed in testicular cancer (Gordeeva, 2018). Other CTA for example; NY-ESO-1, MAGE-A3, and KK- LC-1 in non-small cell lung cancer; MAGE-A1, CTp11, SSX-1, HCA587 in hepatocellular carcinoma; and SPAG9 in epithelial ovarian cancer showed no significant association with OS (Garg et al.,

2007; Shigematsu et al., 2010; Zhao et al., 2004). However, several previous studies in other diseases have reported a positive association of CTA with better survival outcome. Sharma and colleagues presented CT10 positive expression was associated with better survival in urothelial carcinoma (Sharma et al., 2006). In lung adenocarcinomas, improved survival rates were seen in the patients with highly expression of *MAGE-A3/4* (Grah, Katalinic, Juretic, Santek, & Samarzija, 2014). Similarly, in glioblastoma, expression of ACTL8, OIP5, XAGE3 and CTCFL was significantly associated with better OS. Meanwhile, a study in epithelial ovarian cancer patients revealed that *MAGE-C1* expression was correlated with improved progression-free survival (Daudi et al., 2014; Freitas et al., 2013). In addition, high CTA expression may be correlated with poor survival outcomes. In multiple myeloma and myxoid liposarcoma, NY-ESO-1 expression was associated with poor survival prognosis (Iura et al., 2015; Van Rhee et al., 2005)

Conflicting results on the MAGEB2 along with other CTA expression profiles in various cancers with its associations with clinico-pathological parameters and overall survival was contributed by diversity of the analyzed tumor samples (different ratio of undifferentiated and differentiated cells) and different histological origin of various tumours (Gordeeva, 2018).

5.7 Potential use of MAGEB2

Previous clinical trial conducted in ovarian cancer has evaluated several CTAs as targets for anticancer vaccines immunotherapy (Odunsi et al., 2003). Meanwhile in HNSCC, a clinical trial investigating a vaccine that targets MAGEA3 and HPV-16 antigens was conducted by a group of researchers in United States. Zamuner and colleagues suggested that CTAs might be used as a potential immunotherapy treatment that targets multiple antigens in HNSCC. However, further studies are required to further elucidate

the ability of MAGEB2 to provoke humoral and cell-mediated immune responses to demonstrate immunogenicity in the human tissue before it can be considered as a potential cancer vaccine target in OSCC (Zamunér et al., 2015).

MAGE proteins are considered as potential antigens to produce anti-tumor vaccine. A vaccine developed against mouse MAGEB2 in breast cancer has been shown to prevent metastasis (Peche et al., 2015; Sypniewska et al., 2005). In mouse, the mRNA and protein expression of MAGEB2 was 99% and 100% respectively (Gordeeva, 2018). Peche et al., (2015) compared to MAGEB2 expression on E2F1 transcriptional activity in human and mouse model. They found out that E2F1 transcriptional activity was up regulated only in human, whereas no significant effect was observed in mouse model. In contrary to significant cell death in mouse model, human MAGEB2 results in limited rate of cell proliferations and independently of p53 status. Hence Peche and colleagues suggested different molecular mechanisms of tumour cell proliferations were observed in mouse and human MAGE-B gene expression (Peche et al., 2015). In our study, we were unable to explain why higher protein expression of MAGEB2 was observed in OSCC compared to OPMD tissues. Hence, further study in the future may explore the mechanisms of MAGEB2 in NOM, OPMD, and OSCC tissue at gene level.

5.8 Limitations

There are some limitations to the present study, for example limited numbers of samples in NOM (n=10) in comparison to OPMD tissue (n=20). We speculate a better significant result between expressions of MAGEB2 in NOM Vs OSCC tissues if the number of samples in NOM increases to create a more equal number of sample size. The limited number of OSCC tissues is because some selected cases were excluded in the present study due to several factors, for example missing FFPE blocks in the

archival records, unavailability of viable tissue in FFPE blocks, and FFPE blocks were eaten by rats. Furthermore, another challenge that we faced in this study was to obtain a complete and updated data for certain clinical parameters such as recurrence status of OSCC patients as the data was absent in MOCDTBS database. Another limitation in this study was that most of the OPMD cases were histologically diagnosed as oral lichen planus, which does not represent the heterogenous spectrum of OPMD.

5.9 Recommendations

We recommend that for future study, a more equal number of sample size for NOM, OPMD, and OSCC tissue samples to be selected. Moreover, it will be interesting to evaluate the expression of MAGEB2 in OPMD tissue with high-grade dysplasia and superficially invasive OSCC. In clinical practice, discrimination between these two entities is indeed a diagnostic challenge for the oral pathologist. In future, development of a novel biomarker to distinguish difficult histopathological cases is beneficial and may serves as adjunct tool in clinical practice.

CHAPTER 6 : CONCLUSION

In conclusion, our study showed that protein expression of MAGEB2 was found in approximately 81% of OSCC tissues. The least MAGEB2 expression was observed in OPMD tissue. Protein expression of MAGEB2 was significantly higher in OSCC compared to OPMD tissue ($p = 0.014$). MAGEB2 was able to differentiate between OSCC and OPMD tissue with diagnostic accuracy of 61% sensitivity and 80% specificity. On the other hand, there is no significant correlation between MAGEB2 protein expression with socio-demographic and clinico-pathologic characteristics in OSCC patients. Although no significant association observed between high and low expression of MAGEB2 with overall survival, a trend of better overall survival in tissues with high MAGEB2 expression was observed in our study. We suggest for subsequent study, a larger and homogenous sample size having uniform representatives from each categories of NOM, OPMD, and OSCC tissue. Moreover, inclusion of various OPMD cases is recommended for future study.

REFERENCES

Alaizari, N. A., Sperandio, M., Odell, E. W., Peruzzo, D., & Al‐Maweri, S. A. (2018). Meta‐analysis of the predictive value of DNA aneuploidy in malignant transformation of oral potentially malignant disorders. *Journal of Oral Pathology & Medicine, 47*(2), 97-103.

Ali, T. T., Razak, L., Latifah, R. R., & Zain, R. B. (1995). An epidemiological survey of oral mucosal lesions among elderly Malaysians. *Gerodontology, 12*(1), 37-40.

Amtha, R., Zain, R., Razak, I. A., Basuki, B., Roeslan, B. O., Gautama, W., & Purwanto, D. J. (2009). Dietary patterns and risk of oral cancer: a factor analysis study of a population in Jakarta, Indonesia. *Oral Oncology, 45*(8), e49-e53.

Anderiesz, C., & Quinn, M. A. (2003). Screening for ovarian cancer. *The Medical Journal of Australia, 178*(12), 655-656.

Andrade, V. C., Vettore, A. L., Felix, R. S., Almeida, M. S., de Carvalho, F., de Oliveira, J. S. R., . . . Zago, M. A. (2008). Prognostic impact of cancer/testis antigen expression in advanced stage multiple myeloma patients. *Cancer immunity: a journal of the Academy of Cancer Immunology, 8*.

Annertz, K., Anderson, H., Palmér, K., & Wennerberg, J. (2012). The increase in incidence of cancer of the tongue in the Nordic countries continues into the twenty-first century. *Acta oto-laryngologica, 132*(5), 552-557.

Ariyawardana, A., & Johnson, N. W. (2013). Trends of lip, oral cavity and oropharyngeal cancers in Australia 1982–2008: overall good news but with rising rates in the oropharynx. *BMC cancer, 13*(1), 333.

Ariyawardana, A., & Warnakulasuriya, S. (2011). Declining oral cancer rates in Sri Lanka: are we winning the war after being at the top of the cancer league table? *Oral diseases, 17*(7), 636-641.

Ariyoshi, Y., Shimahara, M., Omura, K., Yamamoto, E., Mizuki, H., Chiba, H., . . . Seto, K. (2008). Epidemiological study of malignant tumors in the oral and maxillofacial region: survey of member institutions of the Japanese Society of Oral and Maxillofacial Surgeons, 2002. *International Journal of Clinical Oncology, 13*(3), 220.

Askew, E. B., Bai, S., Hnat, A. T., Minges, J. T., & Wilson, E. M. (2009). Melanoma antigen gene protein-A11 (MAGE-11) F-box links the androgen receptor NH2-terminal transactivation domain to p160 coactivators. *Journal of Biological Chemistry, 284*(50), 34793-34808.

Azizah, A. M., Nor Saleha, I. T., Noor Hashimah, A., Asmah, Z. A., & Mastulu, W. (2016). Ministry of Health Malaysia National cancer registry report: Malaysia cancer statistics-data and figure 2006-2011.

Bagnardi, V., Rota, M., Botteri, E., Tramacere, I., Islami, F., Fedirko, V., . . . Pasquali, E. (2015). Alcohol consumption and site-specific cancer risk: a comprehensive dose–response meta-analysis. *British journal of cancer, 112*(3), 580-593.

Balaram, P., Sridhar, H., Rajkumar, T., Vaccarella, S., Herrero, R., Nandakumar, A., . . . Gajalakshmi, V. (2002). Oral cancer in southern India: The influence of smoking, drinking, paan‐chewing and oral hygiene. *International journal of cancer, 98*(3), 440-445.

Bareham, D., Ahmadi, K., Elie, M., & Jones, A. W. (2016). E-cigarettes: controversies within the controversy. *The Lancet Respiratory Medicine, 4*(11), 868-869.

Batsakis, J. (1985). Nerves and neurotropic carcinomas. *The Annals of otology, rhinology, and laryngology, 94*(4 Pt 1), 426.

Bhurgri, Y., Bhurgri, A., Hussainy, A. S., Usman, A., Faridi, N., Malik, J., . . . Pervez, S. (2003). Cancer of the oral cavity and pharynx in Karachi-identification of potential risk factors. *Asian Pacific journal of cancer prevention, 4*(2), 125-130.

Brandwein-Gensler, M., Teixeira, M. S., Lewis, C. M., Lee, B., Rolnitzky, L., Hille, J. J., . . . Wang, B. Y. (2005). Oral squamous cell carcinoma: histologic risk assessment, but not margin status, is strongly predictive of local disease-free and overall survival. *The American journal of surgical pathology, 29*(2), 167-178.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians, 68*(6), 394-424.

Broders, A. C. (1920). Squamous-cell epithelioma of the lip: a study of five hundred and thirty-seven cases. *Journal of the American Medical Association, 74*(10), 656-664.

Bryne, M., Koppang, H. S., Lilleng, R., & Kjærheim, Å. (1992). Malignancy grading of the deep invasive margins of oral squamous cell carcinomas has high prognostic value. *The Journal of pathology, 166*(4), 375-381.

Bryne, M., Koppang, H. S., Lilleng, R., Stene, T., Bang, G., & Dabelsteen, E. (1989). New malignancy grading is a better prognostic indicator than Broders' grading in oral squamous cell carcinomas. *Journal of Oral Pathology & Medicine, 18*(8), 432-437.

Caballero, O. L., & Chen, Y.-T. (2012). Cancer/testis antigens: potential targets for immunotherapy *Innate Immune Regulation and Cancer Immunotherapy* (pp. 347-369): Springer.

Campisi, G., Panzarella, V., Giuliani, M., Lajolo, C., Di Fede, O., Falaschini, S., . . . Lo Muzio, L. (2007). Human papillomavirus: Its identikit and controversial role in oral oncogenesis, premalignant and malignant lesions. *International journal of oncology, 30*(4), 813-823.

Cancer Statistics in Japan. Center for Cancer Control and Information Services, National Cancer Center, Japan. https://ganjoho.jp/en/ professional/statistics/table_download.html.

Cervino, G., Fiorillo, L., Herford, A. S., Romeo, U., Bianchi, A., Crimi, S., . . . Santoro, R. (2019). Molecular biomarkers related to oral carcinoma: Clinical trial outcome evaluation in a literature review. *Disease markers, 2019*.

Chaturvedi, A. K., Anderson, W. F., Lortet-Tieulent, J., Curado, M. P., Ferlay, J., Franceschi, S., . . . Gillison, M. L. (2013). Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. *Journal of clinical oncology, 31*(36), 4550.

Chaturvedi, A. K., Engels, E. A., Anderson, W. F., & Gillison, M. L. (2008). Incidence trends for human papillomavirus-related and-unrelated oral squamous cell carcinomas in the United States. *Journal of clinical oncology, 26*(4), 612-619.

Cheever, M. A., & Higano, C. S. (2011). PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. *Clinical Cancer Research, 17*(11), 3520-3526.

Chen, J., Eisenberg, E., Krutchkoff, D. J., & Katz, R. V. (1991). Changing trends in oral cancer in the United States, 1935 to 1985: a Connecticut study. *Journal of oral and maxillofacial surgery, 49*(11), 1152-1158.

Chen, P. C. H., Kuo, C., Pan, C. C., & Chou, M. Y. (2002). Risk of oral cancer associated with human papillomavirus infection, betel quid chewing, and cigarette smoking in Taiwan—an integrated molecular and epidemiological study of 58 cases. *Journal of Oral Pathology & Medicine, 31*(6), 317-322.

Cheong, S. C., Vatanasapt, P., Yi-Hsin, Y., Zain, R. B., Kerr, A. R., & Johnson, N. W. (2017). Oral cancer in South East Asia: Current status and future directions. *Translational Research in Oral Oncology, 2*, 2057178X17702921.

Cheraghlou, S., Schettino, A., Zogg, C. K., & Judson, B. L. (2018). Changing prognosis of oral cancer: An analysis of survival and treatment between 1973 and 2014. *The Laryngoscope, 128*(12), 2762-2769.

Cheung, K., & Robertson, F. (2003). Objective measurement of remission and progression in metastatic breast cancer by the use of serum tumour markers. *Minerva chirurgica, 58*(3), 297.

Crawford, N. P., Colliver, D. W., & Galandiuk, S. (2003). Tumor markers and colorectal cancer: utility in management. *Journal of Surgical Oncology, 84*(4), 239-248.

Cuffel, C., Rivals, J. P., Zaugg, Y., Salvi, S., Seelentag, W., Speiser, D. E., . . . Bron, L. (2011). Pattern and clinical significance of cancer‐testis gene expression in head and neck squamous cell carcinoma. *International journal of cancer, 128*(11), 2625-2634.

Daudi, S., Eng, K. H., Mhawech-Fauceglia, P., Morrison, C., Miliotto, A., Beck, A., . . . Gnjatic, S. (2014). Expression and immune responses to MAGE antigens predict survival in epithelial ovarian cancer. *PLoS ONE, 9*(8), e104099.

Davis, I. D., Chen, W., Jackson, H., Parente, P., Shackleton, M., Hopkins, W., . . . Murphy, R. (2004). Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4+ and CD8+ T cell responses in humans. *Proceedings of the National Academy of Sciences, 101*(29), 10697-10702.

De Smet, C., Lurquin, C., Lethé, B., Martelange, V., & Boon, T. (1999). DNA methylation is the primary silencing mechanism for a set of germ line-and tumor-specific genes with a CpG-rich promoter. *Molecular and cellular biology, 19*(11), 7327-7335.

Desmetz, C., Mangé, A., Maudelonde, T., & Solassol, J. (2011). Autoantibody signatures: progress and perspectives for early cancer detection. *Journal of cellular and molecular medicine, 15*(10), 2013-2024.

Dinakar, C., & O'Connor, G. T. (2016). The health effects of electronic cigarettes. *New England Journal of Medicine, 375*(14), 1372-1381.

Dunn, M., Morgan, M. B., & Beer, T. W. (2009). Perineural invasion: identification, significance, and a standardized definition. *Dermatologic surgery, 35*(2), 214-221.

Edwards, P. C. (2013). Oral cancer screening for asymptomatic adults: do the United States Preventive Services Task Force draft guidelines miss the proverbial forest for the trees? *Oral surgery, oral medicine, oral pathology and oral radiology, 116*(2), 131-134.

Egawa, N., Egawa, K., Griffin, H., & Doorbar, J. (2015). Human papillomaviruses; epithelial tropisms, and the development of neoplasia. *Viruses, 7*(7), 3863-3890.

El-Naggar, A. K., Chan, J. K., Grandis, J. R., Takata, T., & Slootweg, P. J. (2017). *WHO classification of head and neck tumours*: International Agency for Research on Cancer.

Feller, L., & Lemmer, J. (2012). Oral squamous cell carcinoma: epidemiology, clinical presentation and treatment.

Ferlay, J., Colombet, M., Soerjomataram, I., Siegel, R., Torre, L., & Jemal, A. (2018). Global and regional estimates of the incidence and mortality for 38 cancers: GLOBOCAN 2018. Lyon: International Agency for Research on Cancer. *World Health Organization*, 394-424.

Figueiredo, D. L., Mamede, R. C., Spagnoli, G. C., Silva Jr, W. A., Zago, M., Neder, L., . . . Saggioro, F. P. (2011). High expression of cancer testis antigens MAGE‐A, MAGE‐C1/CT7, MAGE‐C2/CT10, NY‐ESO‐1, and gage in advanced squamous cell carcinoma of the larynx. *Head & neck, 33*(5), 702-707.

Flach, S., Maniam, P., & Manickavasagam, J. (2019). E‐cigarettes and head and neck cancers: A systematic review of the current literature. *Clinical Otolaryngology, 44*(5), 749-756.

Freitas, M. R. P., Malheiros, S. M. F., Stavale, J. N., Biassi, T. P., Zamuner, F. T., de Souza Begnami, M. D. F., . . . Vettore, A. L. (2013). Expression of cancer/testis antigens is correlated with improved survival in glioblastoma. *Oncotarget, 4*(4), 636.

Gallo, C., Ciavarella, D., Santarelli, A., Ranieri, E., Colella, G., MUZIO, L. L., & RUSSO, L. L. (2016). Potential salivary proteomic markers of oral squamous cell carcinoma. *Cancer Genomics-Proteomics, 13*(1), 55-61.

Garg, M., Chaurasiya, D., Rana, R., Jagadish, N., Kanojia, D., Dudha, N., . . . Suri, S. (2007). Sperm-associated antigen 9, a novel cancer testis antigen, is a potential target for immunotherapy in epithelial ovarian cancer. *Clinical Cancer Research, 13*(5), 1421-1428.

Ghani, W., Zain, R. B., Jalil, N., Razak, I., Raja-Latifah, R. J., & Samsuddin, A. (2011). Sociodemographic characteristics, clinicopathological parameters and survival among Malaysians with oral squamous cell carcinoma (OSCC). *Oral Oncology, 47*(Spp. 1), S38.

Ghani, W. M. N., Ramanathan, A., Prime, S. S., Yang, Y.-H., Razak, I. A., Abdul Rahman, Z. A., . . . Kallarakkal, T. G. (2019). Survival of oral cancer patients in different ethnicities. *Cancer investigation, 37*(7), 275-287.

Gordeeva, O. (2018). *Cancer-testis antigens: Unique cancer stem cell biomarkers and targets for cancer therapy.* Paper presented at the Seminars in Cancer Biology.

Grah, J. J., Katalinic, D., Juretic, A., Santek, F., & Samarzija, M. (2014). Clinical significance of immunohistochemical expression of cancer/testis tumor-associated antigens (MAGE-A1, MAGE-A3/4, NY-ESO-1) in patients with non-small cell lung cancer. *Tumori Journal, 100*(1), 60-68.

Gretzer, M. B., & Partin, A. W. (2003). PSA markers in prostate cancer detection. *Urologic Clinics of North America, 30*(4), 677-686.

Güneri, P., Çankaya, H., Yavuzer, A., Güneri, E. A., Erişen, L., Özkul, D., . . . Boyacioğlu, H. (2005). Primary oral cancer in a Turkish population sample: association with sociodemographic features, smoking, alcohol, diet and dentition. *Oral Oncology, 41*(10), 1005-1012.

Gure, A. O., Chua, R., Williamson, B., Gonen, M., Ferrera, C. A., Gnjatic, S., . . . Old, L. J. (2005). Cancer-testis genes are coordinately expressed and are markers of poor outcome in non–small cell lung cancer. *Clinical Cancer Research, 11*(22), 8055-8062.

Hashibe, M., Brennan, P., Benhamou, S., Castellsague, X., Chen, C., Curado, M. P., . . . Wünsch-Filho, V. (2007). Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Journal of the National Cancer Institute, 99*(10), 777-789.

Hashibe, M., Brennan, P., Chuang, S.-c., Boccia, S., Castellsague, X., Chen, C., . . . Fabianova, E. (2009). Interaction between tobacco and alcohol use and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiology and Prevention Biomarkers, 18*(2), 541-550.

Helen‐Ng, L. C., Razak, I. A., Ghani, W. M. N., Marhazlinda, J., Norain, A. T., Raja Jallaludin, R. L., . . . Zain, R. B. (2012). Dietary pattern and oral cancer risk‐a factor analysis study. *Community dentistry and oral epidemiology, 40*(6), 560-566.

Henry, N. L., & Hayes, D. F. (2012). Cancer biomarkers. *Molecular oncology, 6*(2), 140-146.

Hosmer, D. W., & Lemeshow, S. (2000). *Applied logistic regression*: Wiley New York.

IARC. (1986). *Tobacco smoking. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans* (Vol. 38).

IARC. (1988). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 44: Alcohol Drinking, IARC Press Lyon.

IARC. (2004). *Tobacco smoke and involuntary smoking. IARC monographs on the evaluation of carcinogenic risks to humans* (Vol. 83): IARC.

IARC. (2012). *A review of human carcinogens: personal habits and indoor combustions. Monographs on the Evaluation of Carcinogenic Risks to Humans.* (Vol. 100): World Health Organization.

Iura, K., Kohashi, K., Hotokebuchi, Y., Ishii, T., Maekawa, A., Yamada, Y., . . . Oda, Y. (2015). Cancer‐testis antigens PRAME and NY‐ESO‐1 correlate with tumour grade and poor prognosis in myxoid liposarcoma. *The Journal of Pathology: Clinical Research, 1*(3), 144-159.

Jang, S. J., Soria, J.-C., Wang, L., Hassan, K. A., Morice, R. C., Walsh, G. L., . . . Mao, L. (2001). Activation of melanoma antigen tumor antigens occurs early in lung carcinogenesis. *Cancer research, 61*(21), 7959-7963.

Jin, S., Cao, S., Grigorev, A., Li, J., Meng, Q., Wang, C., . . . Yu, Y. (2018). Establishment of cancer/testis antigen profiling based on clinicopathological characteristics in resected pathological stage iii non-small cell lung cancer. *Cancer management and research, 10*, 2031.

Johnson, R. E., Sigman, J. D., Funk, G. F., Robinson, R. A., & Hoffman, H. T. (1997). Quantification of surgical margin shrinkage in the oral cavity. *Head & Neck: Journal for the Sciences and Specialties of the Head and Neck, 19*(4), 281-286.

Jung, E. J., KIM, M. A., Lee, H. S., YANG, H. K., Lee, Y. M., Lee, B. L., & Kim, W. H. (2005). Expression of family A melanoma antigen in human gastric carcinoma. *Anticancer research, 25*(3B), 2105-2111.

Krämer, B. F., Schoor, O., Krüger, T., Reichle, C., Müller, M., Weinschenk, T., . . . Stevanovic, S. (2005). MAGED4–expression in renal cell carcinoma and identification of an HLA-A* 25-restricted MHC class I ligand from solid tumor tissue. *Cancer biology & therapy, 4*(9), 943-948.

Kreppel, M., Nazarli, P., Grandoch, A., Safi, A.-F., Zirk, M., Nickenig, H.-J., . . . Zöller, J. E. (2016). Clinical and histopathological staging in oral squamous cell carcinoma–Comparison of the prognostic significance. *Oral Oncology, 60*, 68-73.

Kruaysawat, W., Aekplakorn, W., & Chapman, R. S. (2010). Survival time and prognostic factors of oral cancer in Ubon Ratchathani Cancer Center. *Journal of the Medical Association of Thailand= Chotmaihet thangphaet, 93*(3), 278.

Laban, S., Atanackovic, D., Luetkens, T., Knecht, R., Busch, C. J., Freytag, M., . . . Knuth, A. (2014). Simultaneous cytoplasmic and nuclear protein expression of melanoma antigen‐A family and NY‐ESO‐1 cancer‐testis antigens represents an independent marker for poor survival in head and neck cancer. *International journal of cancer, 135*(5), 1142-1152.

Lalkhen, A. G., & McCluskey, A. (2008). Clinical tests: sensitivity and specificity. *Continuing Education in Anaesthesia Critical Care & Pain, 8*(6), 221-223.

Laudico, A. V., Mirasol-Lumague, M. R., Mapua, C. A., Uy, G. B., Toral, J. A. B., Medina, V. M., & Pukkala, E. (2010). Cancer incidence and survival in Metro Manila and Rizal province, Philippines. *Japanese journal of clinical oncology, 40*(7), 603-612.

Lee, Y.-C. A., Boffetta, P., Sturgis, E. M., Wei, Q., Zhang, Z.-F., Muscat, J., . . . Winn, D. M. (2008). Involuntary smoking and head and neck cancer risk: pooled

analysis in the International Head and Neck Cancer Epidemiology Consortium. *Cancer Epidemiology and Prevention Biomarkers, 17*(8), 1974-1981.

Lee, Y.-C. A., Marron, M., Benhamou, S., Bouchardy, C., Ahrens, W., Pohlabeln, H., . . . Castellsague, X. (2009). Active and involuntary tobacco smoking and upper aerodigestive tract cancer risks in a multicenter case-control study. *Cancer Epidemiology and Prevention Biomarkers, 18*(12), 3353-3361.

Liebig, C., Ayala, G., Wilks, J. A., Berger, D. H., & Albo, D. (2009). Perineural invasion in cancer: a review of the literature. *Cancer: Interdisciplinary International Journal of the American Cancer Society, 115*(15), 3379-3391.

Ligier, K., Belot, A., Launoy, G., Velten, M., Bossard, N., Iwaz, J., . . . Guizard, A.-V. (2011). Descriptive epidemiology of upper aerodigestive tract cancers in France: incidence over 1980–2005 and projection to 2010. *Oral Oncology, 47*(4), 302-307.

Llewellyn, C., Johnson, N., & Warnakulasuriya, K. (2001). Risk factors for squamous cell carcinoma of the oral cavity in young people—a comprehensive literature review. *Oral Oncology, 37*(5), 401-418.

Lurquin, C., De Smet, C., Brasseur, F., Muscatelli, F., Martelange, V., De Plaen, E., . . . Boon, T. (1997). Two members of the HumanMAGEBGene family located in Xp21. 3 are expressed in tumors of various histological origins. *Genomics, 46*(3), 397-408.

Lydiatt, W. M., Patel, S. G., O'Sullivan, B., Brandwein, M. S., Ridge, J. A., Migliacci, J. C., . . . Shah, J. P. (2017). Head and neck cancers—major changes in the American Joint Committee on cancer eighth edition cancer staging manual. *CA: a cancer journal for clinicians, 67*(2), 122-137.

Macfarlane, G., Boyle, P., & Scully, C. (1987). Rising mortality from cancer of the tongue in young Scottish males. *The Lancet, 330*(8564), 912.

Macfarlane, G. J., Boyle, P., & Scully, C. (1992). Oral cancer in Scotland: changing incidence and mortality. *British Medical Journal, 305*(6862), 1121-1123.

Marron, M., Boffetta, P., Zhang, Z.-F., Zaridze, D., Wünsch-Filho, V., Winn, D. M., . . . Sturgis, E. M. (2010). Cessation of alcohol drinking, tobacco smoking and the reversal of head and neck cancer risk. *International journal of epidemiology, 39*(1), 182-196.

Møller, H. (1989). Changing incidence of cancer of the tongue, oral cavity, and pharynx in Denmark. *Journal of Oral Pathology & Medicine, 18*(4), 224-229.

Monteiro, L. S., Antunes, L., Bento, M. J., & Warnakulasuriya, S. (2013). Incidence rates and trends of lip, oral and oro‐pharyngeal cancers in P ortugal. *Journal of Oral Pathology & Medicine, 42*(4), 345-351.

Montoro, J. R. d. M. C., Mamede, R. C. M., Neder Serafini, L., Saggioro, F. P., Figueiredo, D. L. A., Silva Jr, W. A. d., . . . Zago, M. A. (2012). Expression of cancer‐testis antigens MAGE‐A4 and MAGE‐C1 in oral squamous cell carcinoma. *Head & neck, 34*(8), 1123-1128.

Nagpal, J. K., Patnaik, S., & Das, B. R. (2002). Prevalence of high‐risk human papilloma virus types and its association with P53 codon 72 polymorphism in tobacco addicted oral squamous cell carcinoma (oscc) patients of Eastern India. *International journal of cancer, 97*(5), 649-653.

Ndiaye, C., Mena, M., Alemany, L., Arbyn, M., Castellsagué, X., Laporte, L., . . . Trottier, H. (2014). HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. *The Lancet Oncology, 15*(12), 1319-1331.

Odunsi, K., Jungbluth, A. A., Stockert, E., Qian, F., Gnjatic, S., Tammela, J., . . . Santiago, D. (2003). NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. *Cancer research, 63*(18), 6076-6083.

Pattani, K. M., Soudry, E., Glazer, C. A., Ochs, M. F., Wang, H., Schussel, J., . . . Loyo, M. (2012). MAGEB2 is activated by promoter demethylation in head and neck squamous cell carcinoma. *PLoS ONE, 7*(9), e45534.

Peche, L. Y., Ladelfa, M. F., Toledo, M. F., Mano, M., Laiseca, J. E., Schneider, C., & Monte, M. (2015). Human MageB2 protein expression enhances E2F transcriptional activity, cell proliferation, and resistance to ribotoxic stress. *Journal of Biological Chemistry, 290*(49), 29652-29662.

Petti, S., Masood, M., & Scully, C. (2013). The magnitude of tobacco smoking-betel quid chewing-alcohol drinking interaction effect on oral cancer in South-East Asia. A meta-analysis of observational studies. *PLoS ONE, 8*(11), e78999.

Pindborg, J. J., Reichart, P., Smith, C., & Van der Waal, I. (2012). *Histological Typing of Cancer and Precancer of the Oral Mucosa: In Collaboration with LH Sobin and Pathologists in 9 Countries*: Springer Science & Business Media.

Po Wing Yuen, A., Lam, K. Y., Lam, L. K., Ho, C. M., Wong, A., Chow, T. L., . . . Wei, W. I. (2002). Prognostic factors of clinically stage I and II oral tongue carcinoma—a comparative study of stage, thickness, shape, growth pattern, invasive front malignancy grading, Martinez‐Gimeno score, and pathologic features. *Head & Neck: Journal for the Sciences and Specialties of the Head and Neck, 24*(6), 513-520.

Rahman, M., Sakamoto, J., & Fukui, T. (2003). Bidi smoking and oral cancer: a meta‐analysis. *International journal of cancer, 106*(4), 600-604.

Rao, S. V. K., Mejia, G., Roberts-Thomson, K., & Logan, R. (2013). Epidemiology of oral cancer in Asia in the past decade-an update (2000-2012). *Asian Pacific journal of cancer prevention, 14*(10), 5567-5577.

Reichart, P., Dietrich, T., Khongkhunthian, P., & Srisuwan, S. (2003). Decline of oropharyngeal cancer in Chiangmai province, Thailand, between 1988 and 1999. *Oral Oncology, 39*(6), 569-573.

Remmele, W. (1987). Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologe, 8*, 138-140.

Rogers, S. N., Brown, J. S., Woolgar, J. A., Lowe, D., Magennis, P., Shaw, R. J., . . . Vaughan, D. (2009). Survival following primary surgery for oral cancer. *Oral Oncology, 45*(3), 201-211.

Rosenberg, S. A., Dudley, M. E., & Restifo, N. P. (2008). Cancer immunotherapy. *N Engl J Med, 359*(10), 1072.

Saini, R., Tang, T.-H., Zain, R. B., Cheong, S. C., Musa, K. I., Saini, D., . . . Santhanam, J. (2011). Significant association of high-risk human papillomavirus (HPV) but not of p53 polymorphisms with oral squamous cell carcinomas in Malaysia. *Journal of cancer research and clinical oncology, 137*(2), 311-320.

Sawair, F. A., Al-Mutwakel, A., Al-Eryani, K., Al-Surhy, A., Maruyama, S., Cheng, J., . . . Saku, T. (2007). High relative frequency of oral squamous cell carcinoma in Yemen: qat and tobacco chewing as its aetiological background. *International journal of environmental health research, 17*(3), 185-195.

Scanlan, M. J., Simpson, A., & Old, L. J. (2004). The cancer/testis genes: review, standardization, and commentary. *Cancer Immun, 4*(1), 1.

Shah, G., Chaturvedi, P., & Vaishampayan, S. (2012). Arecanut as an emerging etiology of oral cancers in India. *Indian journal of medical and paediatric oncology: official journal of Indian Society of Medical & Paediatric Oncology, 33*(2), 71.

Sharma, P., Shen, Y., Wen, S., Bajorin, D. F., Reuter, V. E., Old, L. J., & Jungbluth, A. A. (2006). Cancer-testis antigens: expression and correlation with survival in human urothelial carcinoma. *Clinical Cancer Research, 12*(18), 5442-5447.

Sherin, N., Simi, T., Shameena, P., & Sudha, S. (2008). Changing trends in oral cancer. *Indian journal of cancer, 45*(3), 93.

Shigematsu, Y., Hanagiri, T., Shiota, H., Kuroda, K., Baba, T., Mizukami, M., . . . So, T. (2010). Clinical significance of cancer/testis antigens expression in patients with non-small cell lung cancer. *Lung cancer, 68*(1), 105-110.

Shridhar, K., Rajaraman, P., Koyande, S., Parikh, P. M., Chaturvedi, P., Dhillon, P. K., & Dikshit, R. P. (2016). Trends in mouth cancer incidence in Mumbai, India (1995- 2009): An age-period-cohort analysis. *Cancer epidemiology, 42*, 66-71.

Simpson, A. J., Caballero, O. L., Jungbluth, A., Chen, Y.-T., & Old, L. J. (2005). Cancer/testis antigens, gametogenesis and cancer. *Nature Reviews Cancer, 5*(8), 615-625.

Solanki, M. C., Gandhi, S., Koshy, G., & Mathew, G. C. (2012). Squamous cell carcinoma of maxilla in 10 year old boy: A rare case report. *International Journal of Pediatric Otorhinolaryngology Extra, 7*(1), 33-35.

Sypniewska, R. K., Hoflack, L., Tarango, M., Gauntt, S., Leal, B. Z., Reddick, R. L., & Gravekamp, C. (2005). Prevention of metastases with a Mage-b DNA vaccine in a mouse breast tumor model: potential for breast cancer therapy. *Breast cancer research and treatment, 91*(1), 19-28.

Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., . . . Nimura, Y. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer research, 64*(11), 3753-3756.

Tota, J. E., Anderson, W. F., Coffey, C., Califano, J., Cozen, W., Ferris, R. L., . . . Chaturvedi, A. K. (2017). Rising incidence of oral tongue cancer among white men and women in the United States, 1973–2012. *Oral Oncology, 67*, 146-152.

Traynor, K. (2011). Ipilimumab approved for metastatic melanoma. *American journal of health-system pharmacy: AJHP: official journal of the American Society of Health-System Pharmacists, 68*(9), 768.

Trompetas, V., Panagopoulos, E., Priovolou-Papaevangelou, M., & Ramantanis, G. (2002). Giant benign true cyst of the spleen with high serum level of CA 19-9. *European journal of gastroenterology & hepatology, 14*(1), 85-88.

Tsuji, T., Mimura, Y., Wen, S., Li, X., Kanekawa, A., Sasaki, K., & Shinozaki, F. (1995). The significance of PCNA and p53 protein in some oral tumors. *International journal of oral and maxillofacial surgery, 24*(3), 221-225.

van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., . . . Boon, T. (1991). A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science, 254*(5038), 1643-1647.

van Duin, M., Broyl, A., de Knegt, Y., Goldschmidt, H., Richardson, P. G., Hop, W. C., . . . Neuwirth, R. (2011). Cancer testis antigens in newly diagnosed and relapse multiple myeloma: prognostic markers and potential targets for immunotherapy. *Haematologica, 96*(11), 1662-1669.

Van Rhee, F., Szmania, S. M., Zhan, F., Gupta, S. K., Pomtree, M., Lin, P., . . . Shaughnessy, J. (2005). NY-ESO-1 is highly expressed in poor-prognosis multiple myeloma and induces spontaneous humoral and cellular immune responses. *Blood, 105*(10), 3939-3944.

Warnakulasuriya, S., & Greenspan, J. S. (2020) *Textbook of Oral Cancer: Prevention, diagnosis, and management*: Springer.

Warnakulasuriya, S., Trivedy, C., & Peters, T. J. (2002). Areca nut use: an independent risk factor for oral cancer: The health problem is under-recognised: British Medical Journal Publishing Group.

Williams, B. R., & Amon, A. (2009). Aneuploidy: cancer's fatal flaw? *Cancer research, 69*(13), 5289-5291.

Woolgar, J. A. (2006). Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncology, 42*(3), 229-239.

Woolgar, J. A., & Triantafyllou, A. (2009). Pitfalls and procedures in the histopathological diagnosis of oral and oropharyngeal squamous cell carcinoma and a review of the role of pathology in prognosis. *Oral Oncology, 45*(4-5), 361-385.

Yakirevich, E., Sabo, E., Lavie, O., Mazareb, S., Spagnoli, G. C., & Resnick, M. B. (2003). Expression of the MAGE-A4 and NY-ESO-1 cancer-testis antigens in serous ovarian neoplasms. *Clinical Cancer Research, 9*(17), 6453-6460.

Yang, X., Xiao, X., Wu, W., Shen, X., Zhou, Z., Liu, W., & Shi, L. (2017). Cytological study of DNA content and nuclear morphometric analysis for aid in the diagnosis of high-grade dysplasia within oral leukoplakia. *Oral surgery, oral medicine, oral pathology and oral radiology, 124*(3), 280-285.

Yen, T.-T., Lin, W.-D., Wang, C.-P., Wang, C.-C., & Liu, S.-A. (2008). The association of smoking, alcoholic consumption, betel quid chewing and oral cavity cancer: a cohort study. *European Archives of Oto-Rhino-Laryngology, 265*(11), 1403-1407.

Zain, R. B., Ikeda, N., Razak, I. A., Axéll, T., Majid, Z. A., Gupta, P. C., & Yaacob, M. (1997). A national epidemiological survey of oral mucosal lesions in Malaysia. *Community dentistry and oral epidemiology, 25*(5), 377-383.

Zainal Ariffin, O., & Nor Saleha, I. (2011). National cancer registry report: Malaysia cancer statistics-data and figure 2007. *Putrajaya, Ministry of Health Malaysia*.

Zamunér, F. T., Karia, B. T. R., De Oliveira, C. Z., Dos Santos, C. R., Carvalho, A. L., & Vettore, A. L. (2015). A comprehensive expression analysis of cancer testis antigens in head and neck squamous cell carcinoma revels MAGEA3/6 as a marker for recurrence. *Molecular cancer therapeutics, 14*(3), 828-834.

Zhao, L., Mou, D.-C., Leng, X.-S., Peng, J.-R., Wang, W.-X., Huang, L., . . . Zhu, J.-Y. (2004). Expression of cancer-testis antigens in hepatocellular carcinoma. *World journal of gastroenterology: WJG, 10*(14), 2034.