CLINICO-PATHOLOGIC AND IMMUNOHISTOCHEMICAL PROFILES OF MALIGNANT AND POTENTIALLY MALIGNANT VERRUCOPAPILLARY LESIONS OF THE ORAL CAVITY

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FACULTY OF DENTISTRY
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ABSTRACT

Introduction: Verruco-papillary lesion (VPL) and non-VPL may be clinically and histologically similar. Problems separating these lesions are compounded by poorly oriented tissue sections and biopsies failing to demonstrate lesional margins. ***Objectives:*** To determine the clinico-pathologic and immunohistochemical profiles of VPL and compare it with non VPL by utilizing a set of 4 immunohistochemical panel (p53, Ki67, matrix metalloproteinase-1 (MMP-1) and E-cadherin). It is a further objective to evaluate these selected immunohistochemical panel as potential markers for differentiating between VPL and non-VPL. ***Methods:*** Twenty-four cases of VPL and twenty-nine cases of non VPL were studied. Diagnoses were confirmed by two oral pathologists. Formalin-fixed, paraffin-embedded archival tissues of these cases were used for immunohistochemistry (avidin–biotin immunoperoxidase technique) of p53, ki-67, e-cadherin and MMP-1. ***Results:*** We found that most of the VPL (70.8%) patients were 60 years and above while most of non-VPL (69%) patients were less than 60 years old (p=0.004). The male to female ratio were 1:3.8 and 1:1.4 for VPL and non VPL patients, respectively. Indian ethnic group were the highest in both VPL and non-VPL cases with betel quid chewing the most associated habit. Location wise, VPL were predominantly located on buccal mucosa (45.8%) while most of non-VPL (75.9%) cases were located on the tongue & floor of the mouth. This findings was statistically significant (p=0.001). There was a significant higher lymph node positivity in non-VPL (85.7%) compared to VPL (36%) cases (p=0.001). The nuclear staining of p53 and Ki-67 was seen in a majority of VPL compared to non-VPL cases. There was no statistically significant difference between the scores for percentage staining of these 2 markers in VPL and non-VPL. There was a diffuse membranous staining exhibiting E-cadherin expression in both VPL and non-VPL cases. A slightly lower combined percentage and intensity scores in non-VPL (96.6%) compared to VPL (100%) cases

was observed. These findings are however, not statistically significant. MMP-1 expressions are seen in the cytoplasm of both VPL and non-VPL cases. There were significantly higher combined percentage and intensity score of MMP-1 in VPL (91.7%) cases compared to non-VPL (62.1%) cases (p=0.013). ***Conclusion:*** Among the four IHC markers investigated in this study, MMP-1 demonstrated a significantly high expression for VPL compared to non-VPL. Although a properly oriented hematoxylin–eosin-stained section including normal marginal tissue is considered to be the gold standard for differentiation of VPL and non-VPL potentially malignant and malignant disorders, the outcome of this study suggests that for selected markers, immunohistochemistry may serve as a useful diagnostic adjunct in interpreting the histopathology of difficult cases.

ABSTRAK

Pengenalan: Lesi papilari-verukus (LPV) dan bukan LPV mungkin mempunyai ciri-ciri histologi dan klinikal yang sama. Masalah untuk membezakan lesi-lesi ini adalah di sebabkan biopsi tidak dapat menunjukkan margin lesi dengan baik dan orientasi keratan bahagian tisu yang tidak baik. ***Objektif:*** Untuk menentukan profil patologi-klinikal dan immunohistokimia bagi LPV dan membezakannya dengan bukan LPV, dengan menggunakan panel immunohistokimia (p53, Ki67, matriks metalloproteinase (MMP-1) dan E-Cadherin). Kemudian menilai panel immunokimia ini sebagai petanda untuk membezakan LPV dengan bukan LPV. ***Kaedah:*** Dua puluh empat kes LPV dan dua puluh sembilan kes bukan LPV dikaji. Dignosis disahkan terlebih dahulu oleh ahli patologi. Material dari arkib yang terikat-formalin, terbenam-parafin digunakan untuk immunohistokimia (teknik avidin-biotin immunoperoksida). ***Keputusan:*** Kami mendapati lebih banyak kes LPV (70.8%) berumur 60 tahun dan keatas berbanding kes bukan LPV (69%) yang berumur di bawah 60 tahun (p=0.004). Nisbah lelaki kepada perempuan adalah 1: 3.8 dan 1: 1.4 masing-masing bagi kes LPV dan bukan LPV. Etnik India adalah paling tinggi di dalam kes LPV dan bukan LPV dengan tabiat mengunyah sireh adalah tertinggi. Berkaitan lokasi, LPV adalah paling bayak di mukosa bukal (45.8%) sementara kes bukan LPV (75.9%) paling banyak di lidah dan lantai mulut. Keputusan ini adalah signifikan secara statistik (p=0.001). Nodus limfa yang positif di dalam kes bukan LPV (85.7%) adalah lebih tinggi daripada kes LPV (36%) dan ini adalah signifikan secara statistik (p=0.001). Secara majoriti, lebih banyak penstainan nuklear oleh p53 dan Ki-67 dilihat dalam LPV berbanding kes bukan LPV. Peratusan skor bagi kedua-dua penanda ini di dalam kes LPV dan bukan LPV adalah tidak signifikan secara statistik. E-cadherin menunjukkan banyak penstainan membran dalam LPV dan kes bukan LPV. Gabungan skor peratusan dan intensiti dalam kes bukan LPV (96.6%) adalah sedikit rendah berbanding kes LPV (100%). Penemuan ini walau

bagaimanapun tidak signifikan secara statistik. Ekspresi MMP-1 dilihat di dalam sitoplasma kedua-dua kes LPV dan bukan LPV. Gabungan skor peratusan dan intensiti oleh MMP-1 didalam LPV (91.7%) adalah signifikan lebih tinggi berbanding kes bukan LPV (62.1%) (p=0.013). ***Kesimpulan:*** Di antara empat penanda yang dikaji di dalam penyelidikan ini, MMP-1 menunjukkan ekspresi tinggi yang signifikan bagi LPV berbanding bukan LPV. Walaupun orientasi keratan stain hematoksilin-eosin termasuk margin normal tisu yang betul adalah piawai emas untuk membezakan LPV dan bukan LPV, lesi berpotensi malignan dan lesi malignan. Penilaian penyelidikan ini mencadangkan bagi penanda tertentu, panel immunohistokimia ini mungkin berguna sebagai tambahan kepada diagnostik bagi menterjemah kes histopatologi yang sukar.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

FFPE	:	Formalin-fixed paraffin-embedded
IHC	:	Immunohistochemistry
LOH	:	Loss of heterozygosity
MMP-1	:	Matrix metalloproteinase-1
MOCDTBS	:	Malaysia oral cancer database & tissue bank system
OCRCC	:	Oral Cancer Research & Coordinating Center
OPMD	:	Oral potentially malignant disorder
OSCC	:	Oral squamous cell carcinoma
OVC	:	Oral verrucous carcinoma
OVH	:	Oral verrucous hyperplasia
PSCC	:	Papillary squamous cell carcinoma
RBZ	:	Rosnah Binti Mohd Zain
SCC	:	Squamous cell carcinoma
TGK	:	Thomas George Kallarakkal
VPL	:	Verrucopapillary lesion
VP	:	Verrucopapillary

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Squamous cell carcinoma (SCC) is the most common malignancy of upper aerodigestive tract. Most of them are conventional-type which composed of infiltrating nest of atypical squamous cells. Morphologically, oral SCC (OSCC) may present with and without verruco-papillary (VP) features. Those with VP features may present with different histologies and may have different growth patterns. Histologically, papillary SCC, verrucous carcinoma and conventional OSCC with VP features are the verrucopapillary variants. The histologies of the non-VP variants are mainly the conventional oral SCC without VP features, with other variants being basaloid SCC, spindle cell carcinoma, adenosquamous carcinoma and adenoid SCC.

Similarly, oral potentially malignant disorders (OPMD) may present with the planar variety as in the homogenous and speckled leukoplakia and the verruco-papillary variants such as the verrucous leukoplakia and the exophytic verucous hyperplasia (Zain et al., 2013).

Both of the non-VP variants and VP variants of OSCC and OPMD will be referred to in this write up as verrucopapillary lesions (VPL) and non-verrucopapillary lesions (non-VPL). Of note, these 2 types may appear identical clinically (Shear & Pindborg, 1980). Moreover the histologic differentiation and interpretation may also be made difficult due to poor orientation, failure to sample the lesional margin and small specimen size post primary biopsy. To overcome this, suggestions to use immunohistochemical staining as a diagnostic adjunct has been suggested (Klieb & Raphael, 2007; Wu, Putti, & Bhuiya, 2002).

Being able to distinguish these lesions at incisional biopsy would be of great significance in determining the final treatment of these lesions. In addition, the

classification of these variants would require knowing the clinico-pathologic features of each type, their risk factors and their respective growth patterns.

CHAPTER 2: REVIEW OF THE LITERATURE

2.1 Epidemiology of oral cancer

Over 400,000 new cases of cancer of oral cavity and pharynx are diagnosed annually which make them sixth most common malignancy worldwide. South and South-East Asia countries showed higher number of cases compared other developing countries. These serious health issues were less common in developed countries.

In Malaysia, oral cancer is not listed in the top ten most common cancers but it is the fourth commonest cancer in Indian female and eighth commonest in Indian male (Azizah Ab M, Nor Saleha I.T, Noor Hashimah A, Asmah Z.A, & Mastulu W, 2016). This is related to betel quid chewing habits in Indian female, and alcohol and smoking habits in Indian male.

Oral cancer affects more men than women, about 1.5:1 ratio in most countries worldwide. This trend can be seen especially in high incidence countries such Sri Lanka, India, Pakistan and Bangladesh. It is most probably due to men are more related to risk habits such as tobacco and alcohol.

In term of age, majority of cases occur in people at the age 50 and above (Warnakulasuriya, 2010). Studies have shown that the risk of developing cancer increased with age but cases have been reported to occur at the age below 40 in high incidence country as mentioned above.

Death rates among oral cancer patients are higher compared to other cancer types although it is fairly treatable. This is because patients usually presented to the hospital at late stage. Rogers et al. (2009) reported that 50-60% of five year survival rates for cancer of oral cavity, tongue and oropharynx.

While patients with cancer of the lip showed the best outcome with 90% of 5 years survival. The outcome of patients presented at late stage of the cancer and keep on delaying professional consultation will usually ended having poorer prognosis.

2.2 Risk factors

The etiologies of oral cancer are multifactorial. The most established etiology are tobacco, betel quid usage and excess consumption of alcohol (IARC, 2004) whether acting separately and synergistically. Blot et al. (1988) reported that oral cancer risk to be more than 80% when tobacco and alcohol habits are combined. It is shown that smokeless tobacco also caused pharyngeal and oral cancer which supports the carcinogenicity of all form of tobacco. Oropharynx and tonsil cancer in young patients (under 45 years of age) has been associated with HPV infection (D'souza et al., 2007). About 25% of young patients, who probably had no history of high risk factors developed oral cancers and the cause remain unknown (Llewellyn, Linklater, Bell, Johnson, & Warnakulasuriya, 2004).

Figure 2.1: Established risk factors of oral cancer- Areca nut chewing, smoking and alcohol drinking

[Picture adapted from (IARC, 2004) and www.google.com]

2.3 Clinico-pathologic features of oral squamous cell carcinoma (OSCC)

More than 90% of the malignancy in the oral cavity is squamous cell carcinoma. These includes floor of the mouth, tongue, buccal mucosa, hard and soft palate and alveolar rim (Barnes, 2005).

White or red mucosal change known as leukoplakia and erythroplakia can transformed into OSCC (Figure 2.2). While other lesions may appear with combination of red and white clinically termed as speckled erythroplakia or speckled leukoplakia and erythroleukoplakia. These white or red mucosal lesion has been classified as "potentially malignant disorders" due to increased risk of transforming or already harboring invasive carcinoma (Chi, Day, & Neville, 2015).

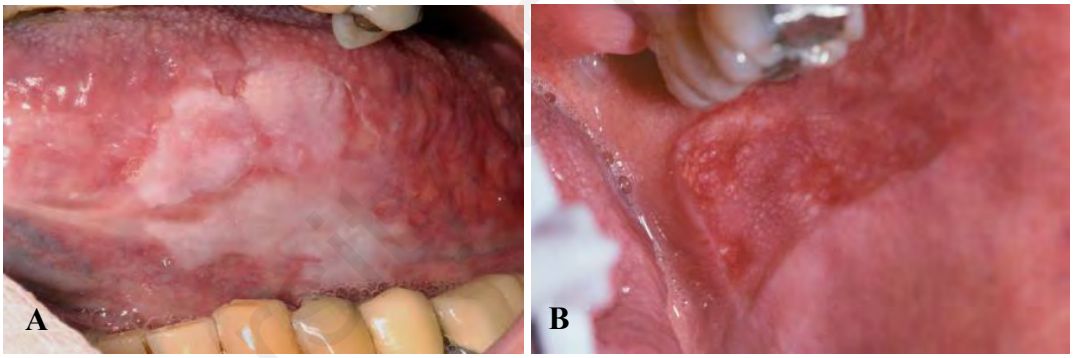


Figure 2.2: Leukoplakia at left lateral border of tongue (A) and erythroplakia at left posterior buccal mucosa (B)

[Picture adapted from (Chi et al., 2015)]

OSCC can also be presented with non-healing ulcers without red or white surrounding mucosal change (Figure 2.3). Mucosal changes to deep ulcerated lesion and increasing irregular surface indicates invasion process has been taken place. Further growth will result in raised, rolled edge lesion with endophytic and exophytic mass. Later progression of the disease, will results in pain and tenderness (Chi et al., 2015).

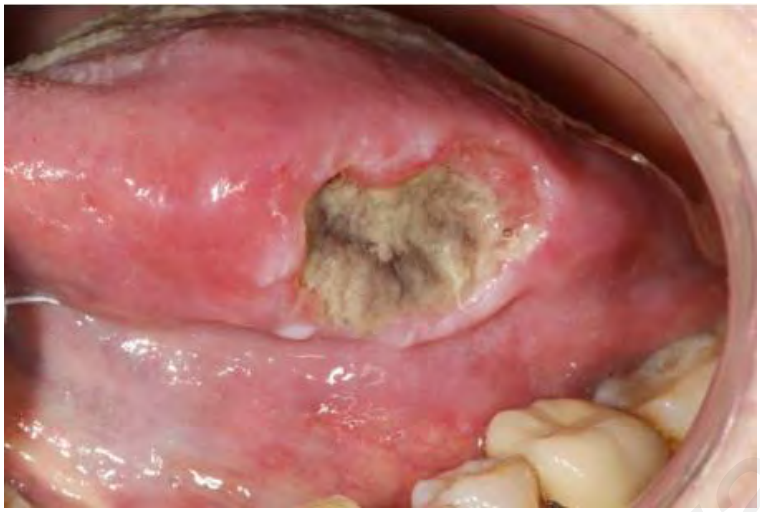


Figure 2.3: OSCC at left lateral border of tongue. A deep, necrotic ulceration.

[Picture adapted from (Chi et al., 2015)]

SCC of the tongue is the commonest site in the western countries, which accounts about 40% to 50% of all cases. Dorsum of the tongue rarely affected while ventero-lateral and lateral aspect of the tongue are the most common site for carcinomas. Floor of the mouth is the second most common oral cancer site while hard palate, gingiva and buccal mucosa are less common site. It is seen that most buccal mucosa and tongue SCC are associated with betel quid usage especially in countries with high prevalence of this habit (Chi et al., 2015).

The American Joint Committee on Cancer staging system (TNM-staging) is the most widely used classification-system for describing the anatomical extent of the disease (Edge & Compton, 2010). By using this classification, treatment can be stratified and prognosis of the patient can be determined. It consists of tumour size (T), regional lymph node involvement (N) and distant metastasis (M). TNM-staging also directly related to the survival of patient with higher TNM-stage related to poor survival.

Table 2.1 Staging and TNM Classification for Oral and Oropharyngeal Carcinoma

STAGE	TNM CLASSIFICATION
0	Tis N0 M0
I	T1 N0 M0
II	T2 N0 M0
III	T3 N0 M0
	T1 N1 M0
	T2 N1 M0
	T3 N1 M0
IV	
IVA	T4a N0 M0
	T4a N1 M0
	T1 N2 M0
	T2 N2 M0
	T3 N2 M0
	T4a N2 M0
IVB	T4b Any N M0
	Any T N3 M0
IVC	Any T Any N M1

[Table adapted from (Edge & Compton, 2010)]

The essential histologic feature of OSCC is the invasion of the surrounding tissues by the malignant epithelial cells. These invading cells penetrate the basement membrane into the connective tissue and form separate tumour island. The individual cells show cellular pleomorphism, large irregular shape nuclei, nuclear hyperchromatism, increase in nuclear cytoplasmic ratio, increased mitosis and abnormal mitosis, loss of cellular adhesion and cohesion, intraepithelial keratinization and loss of well-ordered architecture of epithelium (Cawson & Odell, 2002).

The degree of malignant cells to differentiate to form keratin and prickle cells dictates the grades of the OSCC. Three grades of differentiation are well, moderate and poor. Well differentiated carcinoma characterized by cells with pale eosin stain of cytoplasm or may form concentrate layer of keratin pearls. While in poorly differentiated carcinoma, cells are more darkly stained, irregular shape and show little evidence of a normal squamous pattern. Poorly differentiated tumour carry poorer prognosis due to its metastatic behavior and tend to infiltrate widely (Cawson & Odell, 2002).

2.4 Verrucopapillary Lesions (VPL) of oral cavity

A variety of VPLs affect the oral mucosa which include benign, potentially malignant and frankly malignant disorders. These lesions typically presented as slowly enlarging, pink or white, warty and exophytic overgrowths. When the clinical appearance is of multiple projections or stalks, much like a sea anemone, they are usually said to be papillary; conversely, when the lesions are white and keratotic with a roughened surface, they are said to be verrucous (Costa LJ, Da Silveira FR, Batista JM, & Birman EG, 1994). Frequently it is difficult for clinician to differentiate each one of them as they may also mimic an invasive pattern of squamous cell carcinoma (SCC) (Kallarakkal, Ramanathan, & Zain, 2013). Benign VPL such as verruciform xanthoma or squamous papilloma, are usually more difficult to diagnose because it is smaller in size (less than 10mm). Most of benign VPL are Human Papilloma Virus (HPV) related (Thomas & Barrett, 2009). Whereas those which are potentially malignant such as oral verrucous hyperplasia (OVH) and proliferative verrucous leukoplakia (PVL) together with malignant VPL such as oral verrucous carcinoma (OVC) and papillary squamous cell carcinoma (PSCC) poses greater diagnostic challenge, with addition of confusing terminology.

2.5 Classification of Verrucopapillary Lesions (VPL)

Most VPL shares gross morphological features and the definitive diagnosis relies upon identification of microscopically evident architectural and cytological characteristics of exophytic papillary features. Previous literatures have proposed various types of VPL classification as shown in figure 2.4.:-

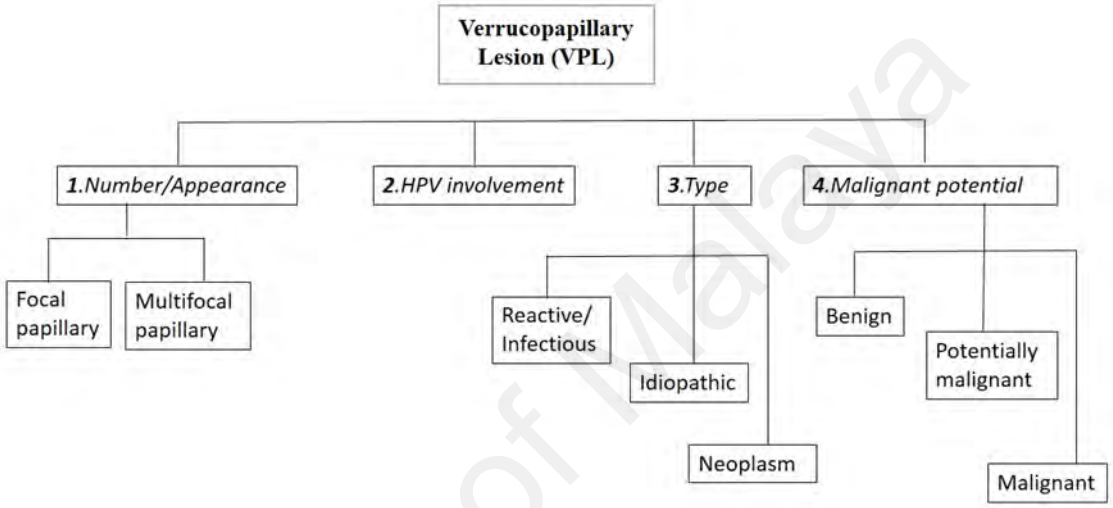


Figure 2.4: Classification of VPL from the literature

1. Based on the number and appearance of lesion

Eversole LR and Papanicolaou SJ (1983) have classified VPL based on the number and appearance which was focal and multifocal papillary lesion. Focal papillary lesions are single while multifocal characterized by multiple lesion with intervening zones of normal appearing mucosa. (Table 2.2)

Table 2.2: Focal and multifocal papillary lesions

Focal Papillary and Verrucous Lesions	Multifocal Papillary and Verrucous Lesions
Squamous Papilloma	Papillary Hyperplasia
Verruca Vulgaris	Florid Papillomatosis
Molluscum Contagiosum	Nevis Unis Lateris
Verruciform Xanthoma	Verrucous Carcinoma
Sialadenoma Papilliferum	Papillary Exophytic Squamous Cell Carcinoma
Keratoacanthoma	Multiple Condylomata
Condyloma Acuminatum	Focal Epithelial Hyperplasia
Squamous Cell Carcinoma	Focal Dermal Hypoplasia Syndrome
Warty Dsykeratoma	Multiple Hamartoma Syndrome
	Pyostomatitis Vegetans
	Acanthosis Nigricans
	Verruciform Leukopiakia
	Keratosis Follicularis

2. Based on the involvement of human papilloma virus (HPV) as an etiological factor.

Eversole LR (2000) has classified VPL based on the involvement of HPV as an etiological factor . Generally most of the papillary lesions are associated with HPV, but there are still some which are not associated with it. These HPV's which cause papillary and verrucous lesions are transmissible with direct mucosal contact, which

normally happen when there is breached of the mucosa. The type of HPV's and related lesions are listed in Table 2.3.

Table 2.3: VPL associated with HPV and without known viral association

Human papilloma viruses and Head and Neck Lesions	Papillary Oral Lesions without Known Viral Association
HPV 2, 4 -Verruca vulgaris	Papillary hyperplasia (Papillomatosis)
HPV 6,11 -Condyloma acuminatum, squamous papilloma	Verruciform Xanthoma
HPV 13, 32 -Focal epithelial hyperplasia	Cowden syndrome
HPV 16- Proliferative verrucoid leukoplakia subtypes	Nevus unius lateris
HPV 6, 11, 16 -Verrucous carcinoma	
HPV 16, 18 - Squamous cell carcinoma	

3. Based on the type of the lesion.

Regezi, Scuibba, and Rogers (2003) have classified VPL based on the type of lesions which are reactive/infectious, neoplasm and idiopathic. (Table 2.4)

Table 2.4: VPL related to reactive/infectious, neoplasm and idiopathic

Reactive/Infectious Lesion	Neoplasms	Idiopathic Lesions
Squamous Papilloma	Keratoacanthoma	Pyostomatitis Vegetans
Papillary hyperplasia	Verrucous Carcinoma	Verruciform Xanthoma
Condyloma Latum		
Condyloma Acuminatum		
Focal Papillary Hyperplasia		

4. Based on their malignant potential

Thomas and Barrett (2009) have proposed classification of VPL based on their malignant potential and the lesions are listed in Table 2.5.

Table 2.5: Benign, potentially malignant and malignant VPL

Benign	Potentially Malignant	Malignant
Viral papillomas	Verrucous hyperplasia	Verrucous carcinoma
Squamous papilloma; Verruca vulgaris; Condyloma acuminatum	Papillary dysplasia	
Focal epithelial hyperplasia (Heck's disease)	Proliferative (verrucous) leukoplakia	
Fibro-epithelial polyps		
Verruciform xanthoma		
Papillary hyperplasia		
Pyostomatitis vegetans		
Sialadenoma papilliferum		
Acanthosis nigricans		
Darier's disease		

Accurate diagnosis of these verrucopapillary lesions remains an important problem. There are many ways to classify them based on the literature and variable terms are used to describe probably the same lesion. Yet the most important thing is proper clinical and microscopic examination in order to achieve correct definitive diagnosis so that proper treatment can be commenced.

2.6 Clinico-pathologic characteristic of VPL

The clinico-pathologic characteristic of benign VPL with known etiological factors will not be described in this review. The focus will be more on malignant VPL and potentially malignant VPL which are more complicated due to its confusing and unsatisfactory terminology.

2.6.1 Oral Verrucous Hyperplasia (OVH)

Shear and Pindborg in 1998 were the first to describe oral verrucous hyperplasia as whitish or pink elevated oral mucosal plaque or mass with either a verrucous or papillary surface. It was also known as a potentially malignant disorder presenting with keratosis and/or varying grades of dysplasia (Thomas & Barrett, 2009). Oral verrucous carcinoma (OVC) and OVH closely resembled each other clinically and pathologically. OVH and OVC were believed to have same biological potential because OVH has been considered as an early form of OVC (Shear & Pindborg, 1998).

Wang et al. (2009) found a high association of OVH lesions with areca quid chewing and cigarette smoking habits. These lesions occur most common on the buccal mucosa and affecting patients in the fifth to seventh decade of life. Their case series reported a mean malignant transformation duration of 54.6 months malignant transformation rate of 3.1% (Wang et al., 2009).

Shear & Pindborg (1998) has classified OVH into sharp and blunt clinical variants. Sharp variant characterized by narrow, long and heavily keratinized verrucous processes which appear white due to heavy keratinization. Many of the authors refer this entity as verrucous leukoplakia. While the blunt variant consist of verrucous processes that are flatter, broader and not heavily keratinized. In other literature, Wang et al. (2009) has classified OVH into mass or plaque-typed. They found that mass-typed OVH lesion

requires more immediate treatment because of higher malignant transformation compared to plaque-typed.

Verrucous hyperplasias histologically has more prominent epithelial dysplasia in a significant percentage of patients compared with verrucous carcinoma and conventional squamous cell carcinoma (Sciubba, 1995). The similarities between OVC and OVH as described by Batsakis and P.Suarez (2000) has make it more difficult to differentiate between them. Some authors have described OVH as morphological variant of OVC while others consider it as an irreversible precursor of OVC. They also recommended that both lesions should be managed in the same manner.

Traditional treatment of OVH is total excision but Y.-C. Chang and Yu (2013) has reported that OVH can be treated successfully with photodynamic therapy combined with cryotherapy.

From the literature it was evident that the terminologies used to describe these lesions were confusing. Some author termed verrucous leukoplakia for sharp variant OVH and was also reflected in the report by Wang et al., (2009) who termed the plaque-type OVH as oral verruciform leukoplakia and preferred to reserve the diagnosis of OVH only for the mass type lesions.

2.6.2 Oral Verrucous Carcinoma (OVC)

Lauren V. Ackermann is the first man to described OVC which was also known as "Ackermann's tumour" or "Verrucous Carcinoma of Ackermann" (Ackerman, 1948). Although it is rare, it commonly appears in the oral cavity. In addition, it is also known to occur in the pyriform sinus, larynx, esophagus, paranasal sinuses and nasal cavity, external auditory meatus, skin, lacrimal duct, vulva, vagina, cervix, uterine, scrotum, penis, perineum, and the leg (Spiro, 1998). OVC is predominantly seen in the sixth

decade of life more in males compared to females. In terms of tumor biology, if not treated appropriately, OVC has the ability to become locally aggressive. Oliveira et al., (2006) reported that regional and distant metastasis in OVC is rare even with local tissue progression. So far, ethio-pathogenesis of OVC is still unclear but several studies have reported relationship of alcohol, tobacco usage, including both smokeless and inhaled tobacco and human papilloma virus (HPV) with OVC. HPV types 6,11,16 and 18 has been detected using polymerase reaction (PCR), restriction fragment analysis and DNA slot-blot hybridization in OVC which confirmed HPV involvement (Shroyer, Greer, Fankhouser, McGuirt, & Marshall, 1993).

In term of sites, cheek and gingival mucosa (mandible) are most commonly affected. Macroscopically OVC presented with broad-based warty & fungating mass (Figure 2.4). Histologically, it presented with exophytic and endophytic growth pattern with verrucous hyperkeratosis. The rete ridges are usually broad, widened and elongated with 'elephant foot' appearance and full depth extension of plugs of keratin may be present. Dense lymphoplasmacytic host response, well demarcated, 'pushing' invasion front are also commonly seen. The transition zone between abnormal and normal epithelium, which is sudden with deep extension of the abnormal rete ridges relative to normal epithelium marked the critical features of OVC (Slootweg & Muller, 1983).

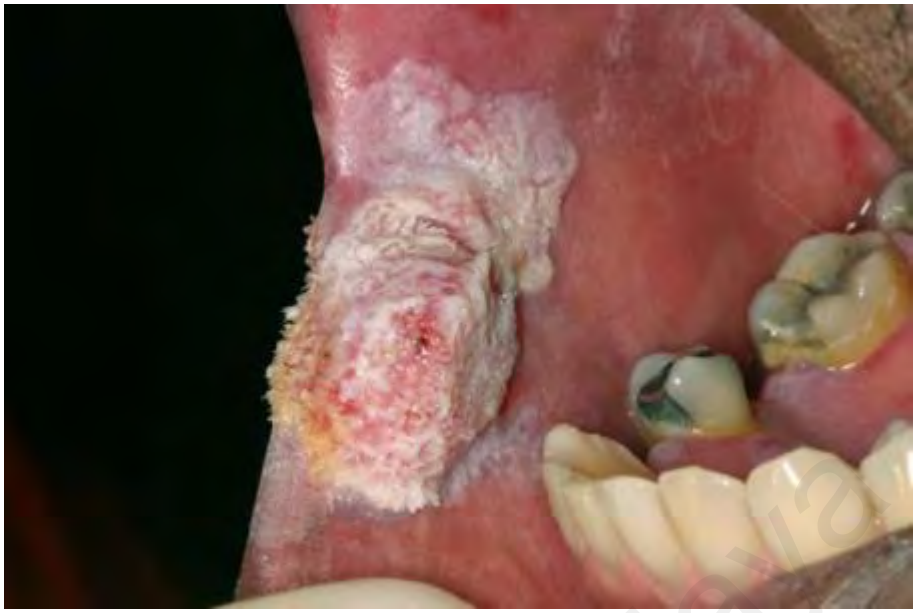


Figure 2.5: Verrucous carcinoma at right anterior labial/buccal mucosa

[Picture adapted from (Chi et al., 2015)]

The most preferable treatment for OVC is surgical excision with adequate margin. While some investigators believed in the role of radiotherapy alone or as an adjunct to surgery as treatment options. Contrarily, B.B Koch et al. (2001) believed that irradiation is less effective in the treatment of OVC and more likely to cause anaplastic differentiation and more aggressive cancer which lead to recurrence. To date, the literature focusing on the clinico-pathological features of OVC is still not robust. Hence further evaluation need to be done.

2.6.3 Oral Verrucous Hyperplasia versus Oral Verrucous Carcinoma

OVH and OVC may clinically appear similar but few histological features can help to distinguish them. Main histological features are rete morphology, growth pattern and degree of cytological atypia. OVH main characteristics are exophytic growth or projection, hyperplastic rete ridges which usually ragged, pointed anastomosing and slender. In comparison, OVC has both exophytic and endophytic growth pattern, elongated and broad rete ridges that resemble elephant's feet. Another important features is marked cytological atypia in OVH compared to OVC (Slootweg & Muller, 1983).

Table 2.6: Summary of OVH versus OVC

Histological features	Verrucous hyperplasia	Verrucous carcinoma
Growth pattern	Exophytic	Exo- & Endo-phytic
Rete morphology	Hyperplastic rete: pointed,ragged,slender & anastomozing	Broad elongated, resemble 'elephant feet'
Degree of cytological atypia	Marked cytological atypia	Little cytological atypia

2.6.4 Papillary squamous cell carcinoma (PSCC)

Initially, Crissman et al. (1988) were the first to propose the term papillary carcinoma which later was renamed as Papillary SCC in the current World Health Organization (WHO) classification. Papillary SCC is an uncommon variant of SCC occurring most frequently in the oropharynx, sinonasal track and oral cavity (Bao et al., 2012). It is noted to be more common in older men (Batsakis & P.Suarez, 2000) and has been described also in other parts of the body including thymus, skin, cervix, uterine, and conjunctiva of the eye (Li, Petit, & Zakka, 1980).

Until now, the pathogenesis and risk factors for Papillary SCC remains unclear, though much has been known about conventional SCC and other variants. For most conventional oral SCC, betel quid chewing, alcohol usage and tobacco are considered as the main etiologic agents for the development of disease (K. W. Chang, Chang, & Lai, 1989; Jacob, Straif, Thomas, & et, 2004). Other well-established etiologic factor is human papilloma virus (HPV) which associated with non-keratinizing (basaloid) SCC in younger patients and most common in oropharynx.Interestingly, there has also been speculation regarding the possible role of HPV in the initiation of Papillary SCC (Begum & Westra, 2008; Cobo, Talavera, & Concha, 2008).

Grossly, PSCC is largely exophytic and appears papillary, warty or even similar to verrucous carcinomas and verrucous hyperplasia (Crissman et al., 1988; Suarez, Adler-Storthz, & Luna, 2000). Histologically, PSCC are characterized by extremely exophytic and friable surface. They appear to have filiform papillary projections covered by an immature cytologically atypical squamous epithelium and surface keratinisation is either absent or rare (Batsakis & P.Suarez, 2000; Begum & Westra, 2008; Ferlito, Devaney, & Rinaldo, 1999). Throughout the entire thickness of the PSCC epithelium, filled with stratified squamous cells that has numerous mitotic figures, lack of

maturation, has overt features of malignancy, increase nuclear/cytoplasmic ratios, and nuclear irregularities (Ferlito et al., 1999). In addition, the papillae consist of thin fibrovascular core surround by immature, neoplastic basaloid cells or pleomorphic cells. Typically, minimal keratosis is seen. Lesions are known as non-invasive PSCC or papillary dysplasia if proven no invasion of the atypical epithelium (McClatchey & Zarbo, 1994). Whereas, lesions are called invasive type PSCC if both non-keratinizing ribbon-like and keratinizing cord-like pattern of the invasive SCC is seen. It is difficult to define invasion in case with superficial biopsies (Thompson, Wenig, & Heffner, 1999). Frank invasion will typically presented with single or multiple nests of tumour cells with dense lymphoplasmacytic inflammation at the tumour-stroma interface and foci of necrosis and hemorrhage are common (McClatchey & Zarbo, 1994).

The common treatment for PSCC involved wide resection (including neck lymph node dissections) or radiation with and without chemotherapy.

Patient with PSCC are generally believed to have better prognosis compared to conventional SCC (Slootweg & Muller, 1983), despite the presence of many contradicting findings in the literature. In a series of 12 patients with PSCC, including 6 with advanced stage, Ding et al. (2013) reported that 3-year survival rate was 91.7% while the 5-year survivor rate was 76.4%. However, both Jo VY, Mills SE, Stoler MH, and EB (2009) and Suarez et al. (2000) reported high recurrence rate with an increase incidence of secondary tumours seen in PSCC compared to the conventional SCC (CSCC). Hence, those with PSCC were also considered having poorer prognosis but these may also represent a CSCC where the application of PSCC criteria may have differed, thus the importance of criteria definition in order to discriminate between PSCC and CSCC.

2.7 Challenges in diagnosing VPL

Achieving diagnosis for all these VPL (PSCC, OVC and OVH) can be challenging as many conventional SCC itself exhibits exophytic growths that histologically may resemble the VPL. In addition, numerous complex papillary and filiform structures extend in all planes, often making the assessment of true tissue invasion somewhat difficult. Lacking good normal margins in incisional biopsies, which only represent small portions of the tumours, worsens this, hence an underlying conventional carcinoma cannot be excluded. Making definitive diagnosis of VPL is importance as it will influence the treatment plan and prediction of prognosis of the disease.

2.8 Hallmark of cancer and carcinogenesis

To conduct study related to cancer markers, first we have to understand the complex process of carcinogenesis. This involved where it starts and how actually it is triggered.

Looking into cellular level, the growth of cells is stimulated by growth factors. These cells have limitation in their growth capability. There are always molecular signal to stop them from dividing when they are damaged. In other hand, if they cannot be repaired, the programmed cell death (apoptosis) will be activated. While for the survival of the cells, they need the oxygen and nutrient from a good blood supply. Each of the said process needs several protein regulations. If the critical protein become abnormal or malfunction because of DNA damage, cells can developed into cancer. The DNA damage usually causes by somatic mutation or acquired. These occur in a series of steps, which Hanahan and Weinberg (2011) refer to as hallmarks. These hallmarks of cancer comprise of six biological capabilities which include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011) which was described below:-

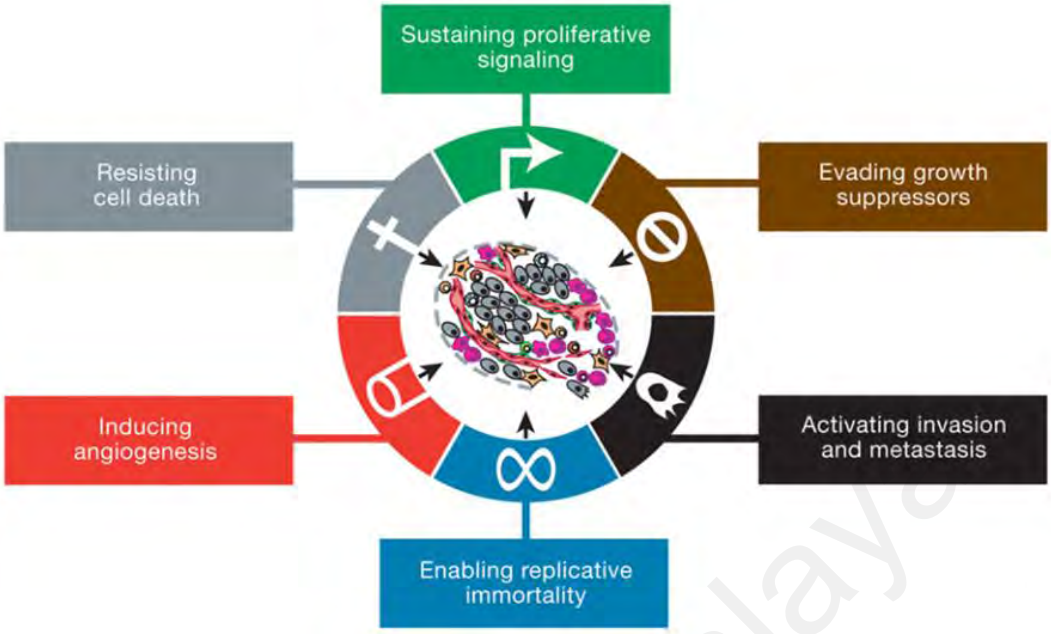


Figure 2.6: Summary of hallmarks of cancer

[Picture adapted from (Hanahan & Weinberg, 2011)]

2.8.1 Sustaining Proliferative Signaling

Normal cells usually requires hormones, growth factors and other molecules that send signals for them to multiply, while cancers cells do not require all this signals to grow. The cancer cells are unique in such that they produced their own signal (autocrine signaling) to multiply continuously and removing the "switch" that stop the excessive growth. Hence the normal cell regulation is totally altered in cancer cells causing uncontrolled cell division and growth within the tumour. These are due to malfunction or altered proteins that control them.

2.8.2 Evading Growth Suppressors

Normal cells are tightly controlled by processes within them which prevent them from dividing and growing. In the processes, there were proteins also known as tumour suppressor genes which received the information from the cell and send signal to initiate or stop the growing process. Whereas in cancer cells, these tumour suppressor proteins

are abnormal which generally cause the cells resistant to growth-preventing signals. These cancer cells will continue to divide even the cells were badly damaged. Another mechanism to prevent over division of normal cells is by 'contact inhibition'. Normal cells will stop dividing when the cells are occupying the whole space and in touch with each other. Cancer cells by passed this mechanism and will continue to multiply even the cells are in contact and space is full.

2.8.3 Resisting Cell Death

Damaged cells which cannot be repaired will undergo programmed cell death known as 'apoptosis'. This special characteristic is required for living organism to maintain the proper tissue turn over and useful when cell is infected or damaged. Cancer cells do not apoptose and will continue to grow and multiply even they were damaged. This is due to loss of ability to detect abnormal cells thus proper signaling to activate the apoptosis is inhibited. Protein involved in apoptosis may be altered in cancer cells which also contribute to the inhibition of signaling to start proper apoptosis.

2.8.4 Enabling Replicative Immortality

Cancer cells will not die after certain number of divisions (immortal) compared to non-cancer cells. This indefinite growth of cancer cells consist of cells with damaged chromosomes.

Non-cancer cells will not able divide after sometime and will go into senescence or die. DNA at the end of a chromosome, known as 'Telomeres", is responsible in this process where it will shorten with every cell division. This Telomeric DNA will continue to shorten until it is short enough to activate senescence and stop dividing. Cancer cells have the ability to alter enzymes that increases the length of telomerase. Therefore they will continue to divide continuously and will not go into senescence stage.

In addition, cancer cells can become immortal by shutting down their pRB and p53 tumour suppressor proteins. These allow them to duplicate without limit and become immortal.

2.8.5 Sustained angiogenesis

Any cells should receive enough oxygen and nutrient from the blood supply to maintain survival. To ensure this, cells are able to initiate a process known as angiogenesis which new formation of blood vessels. Angiogenesis occur in the normal tissue especially during development of embryos, female reproductive cycle and during wound repair. In growing tumour, cancer cells are able to exploit this physiological process to make sure that they have enough oxygen supply from the new blood vessels. This is by increased production of factor that promotes blood vessels formation and tries to reduce production of factors that inhibit blood vessels formation.

2.8.6 Activating Invasion and Metastasis

Cancer cells have the ability to invade neighboring tissue and metastasize to distant tissue or other body parts. This well-known characteristic determine the behavior of the tumour whether it is benign or malignant. In order to metastasize, cancer cells have to undergo several changes typically alteration of shape, their attachment to other cells and to the extracellular matrix. For example, loss of cell to cell adhesion due to under expression of E-cadherin potentiates invasion and metastasis. Beside local invasion, cancer cells can invade the lymphatic, neural, spread into the circulatory and start to invade distant tissue.

2.9 Potential diagnostic and predictive markers for VPL

An important help could be offered by molecular approach when there is difficulty in differentiating these VPL. Many studies have been conducted in order to search for potential predictive markers for VPL.

2.9.1 Loss of heterozygocity (LOH)

Utilizing microsatellite analysis, Poh et al. (2001) found that the LOH pattern of OVH and OVC was sharply different from reactive hyperplasia. Thus, it was further suggested that microsatellite analysis might be useful in differentiating OVH/OVC from reactive epithelial hyperplasias to avoid repeated biopsies in difficult diagnostic situations.

Studies have shown a similar incidence of genetic abnormalities, which is loss of heterozygocity (LOH) in PSCC and VC as well as well-differentiated conventional type SCC (Suarez et al., 2000). Compared to other variants of SCC, these tumours have an increase LOH specifically on the long arm of 11^{th} chromosome. This reported finding however was not statistically significant (Choi, Roberts, & Johnigan, 2004).

2.9.2 p53

p53 is a gene that encodes protein that function as a tumour suppressor and regulates the cell cycle. It is located at chromosome 17p13.1 and plays an important role in cell cycle control and apoptosis. Hence, it suppresses cancer cells in multicellular organisms. p53 also known as "the guardian of the genome' due to its role in maintaining stability and preventing genome mutation (Strachan and Read, 1999).

When p53 become defective, abnormal cells are able to proliferate and turned into cancer. It is believed that all human tumours contain almost 50% of mutated p53. Normal cells will have low p53 protein. Other stress signals or DNA damage may cause

increase in p53 proteins which initiate the major function of DNA repair, apoptosis and growth arrest. Normal functions of this p53 protein will stops further cells duplication and prevent replication of damaged DNA. Thus DNA repair can be initiated by transcription of proteins involved. As for the damaged cells, which cannot be repaired, they will undergo apoptosis as the last resort. Nevertheless, high level of p53 may cause accelerated aging process by excessive apoptosis besides suppressing tumour.

In the studies related to oral lesions, Klieb & Raphael (2007) found that, more diffuse nuclear staining of p53 in the OVC cases compared to the OVH cases ($P<0.001$). More diffuse staining in OVC was seen above the basal layer while only exceptional staining in OVH. Whereas Wu et al. (2002) found stronger expression of p53 in SCC compared with both OVH and OVC.

2.9.3 E-cadherin

Human epithelial E-cadherin gene also known as CDH1 gene encodes a classical cadherin protein of the cadherin superfamily and is located at chromosome 16q22.1. This protein is responsible in calcium-dependant cell-cell adhesion and consists of a transmembrane region, five extracellular cadherin repeats and a highly conserved cytoplasmic tail. It is important for E-cadherin molecules to maintain its relationship, so adherent junctions of epithelial cells contact can be formed and maintained. By losing this E-cadherin-mediated-adhesion, invasion can occur and benign lesions can transform into malignancy. Therefore cancer progression, rapid proliferation and invasion/metastasis were thought cause by mutation of this gene.

In addition, others evidence showed that E-cadherin may involve in the group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors known as "*wnt* signaling pathway". This pathway also involved other key molecules, such as beta-catenins and adenomatous poliposis coli gene products.

Major histological features that related to carcinoma cells are loss of intercellular adhesion, loss of normal architecture and increase of cellular motility. Under expression or defect of E-cadherin gene was found in most carcinoma and known to cause loss of integrity of this cellular adhesion. In addition most human tumour showed direct correlation between loss of cellular morphology and loss of cellular adhesion. Thus, alteration of this gene has played a role in tumour invasion.

Studies in head and neck SCC have found that E-cadherin expression was inversely associated with invasion, metastasis and poor prognosis. Expression in OVC (83.3%) has been reported to be significantly greater than that in poorly differentiated SCC (37.5%) (Tang ZG, Zou P, & XL, 2003). This finding also supported by Klieb and Raphael (2007) who found diffuse membranous staining throughout the epithelium in all cases of both OVC and OVH.

2.9.4 Ki-67

The Ki67 antigen was firstly identified by Scholer and Gerdes back in early 1980s. It is also known as MK167 which is responsible in encoding two protein isoforms with molecular weights of 345 and 395 kDa. Cell proliferation is highly associated with the expression of human Ki-67 protein. This is shown by the present of Ki-67 protein in all active phases of the cell cycle (G1, S, G2 and M) but not in resting phase (G0). While a sudden decrease of Ki-67 level is seen in the late phase of mitosis (anaphase and telophase). On the other hand, Ki-67 protein can be used as a marker of tumour aggressiveness because of overexpression of it is related to the proliferative activity of intrinsic cell population in carcinoma. Several studies have been conducted which found Ki-67 as a reliable prognostic markers for cancers of the soft tissue, breast, prostate, lung, central nervous system and cervix (Lian Tao Li, Guan Jiang, Qian Chen, & Jun Nian Zheng, 2014). When used as a marker, most of the Ki-67 antigen will be

specifically detected within the nucleus and its protein will be shifted to the surface of the chromosomes when cell undergo mitosis.

Focusing on the oral lesions, Takeda et al. (2001) reported that PSCC had a high Ki-67 labeling index (53.2- 59.0%), almost the same as that of conventional SCC (56.7-70.4%). Whereas mean percentage of Ki-67 expression was significantly high in verrucous carcinoma but there was no significant difference among CSSC, PSCC and microinvasive squamous cell carcinoma. Based on these results, author has suggested that biological behavior of CSSC is analogous to PSCC.

2.9.5 MMP-1

Human matrix metalloproteinase (MMP-1) gene is responsible in encoding MMP-1 penzyme which also known as fibroblast collagenase and interstitial collagenase. This gene is localized on chromosome 11q22.3 and part of a cluster of MMP genes. Initially, most MMP's released an inactive proproteins which later activated by the binding of extracellular proteinases. But intracellularly, within the constitutive secretory pathway, MMP-1 enzyme is activated by furin. In addition, these enzyme weakly degrades structural proteins of the extracellular matrix by cleaving alpha 1-proteinase inhibitor.

The functions of MMP family in normal physiological processes are breakdown of extracellular matrix such as in reproduction, embryonic development and tissue remodeling. It also involved in disease processes such as arthritis and metastasis. Specifically in carcinoma, lymphatic and blood vessels are channels for invasion and metastatic spread. MMPs are suggested to play a major role in tumour invasion and metastasis because it involved in the breakdown of the extracellular matrix.

During tumour invasion, cancer cells break through the basement membrane and enter connective tissue, which is a crucial basic characteristic, and varies with changes

in the interactions of cells, matrix, and matrix-degrading enzymes, among which MMPs are the most important. Collagenases are MMPs that mainly degrade types I, II, and III collagen in connective tissues. At present, collagenase-type MMPs include MMP-1 (collagenase-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3). Many cells including fibroblast, macrophages, keratinocytes, and epithelial cells, express MMP-1. It is also expressed by basal keratinocytes at wound margins (Chang-Ta Chiu et al., 2008).

In the studies of oral lesions, MMPs have been reported to be up-regulated during invasion in SCC, and in oral SCC, MMP-1 mRNA has been detected in fibroblastic cells of tumoral stroma (Impola U, Uitto VJ, & Saarialho-Kere U, 2004). To support this, Klieb & Raphael (2007) reported that there was statistically significant increased staining within adjacent stromal cells for matrix metalloproteinase-1 ($P<0.05$) in the OVC cases compared to OVH. Thus, there is a need for further validation of this immunohistochemistry (IHC) panel.

CHAPTER 3: AIM & OBJECTIVES

3.1 Aim

This study aims to investigate the clinico-pathologic and immunohistochemical profiles of malignant and potentially malignant verrucopapillary lesions (VPL) of the oral cavity treated in the Department of Oral and Maxillofacial Surgery, Dental Faculty, University of Malaya, and other government hospitals listed under the Malaysian Oral Cancer Database & Tissue Bank System (MOCDTBS) between 2004 and December 2016.

3.2 Objectives

i. To determine the clinico-pathologic profiles of VPLs and compare with clinico-pathologic profiles of non-VPLs

ii. To determine the immunohistologic profiles of VPLs and compare with immunohistologic profiles of non-VPL

iii. To evaluate selected immunohistochemical panel as potential markers for differentiating between VPLs and non-VPLs.

CHAPTER 4: MATERIALS AND METHOD

4.1 Materials

4.1.1 Study population

A retrospective search of the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS) at Oral Cancer Research & Coordinating Center (OCRCC), Faculty of Dentistry, University of Malaya on verrucopapillary lesions (VPL) and conventional Squamous Cell Carcinoma (SCC) cases diagnosed from 2004 to 2016 was conducted to identify suitable cases for this study. Patients included from MOCDTBS were those that have been treated in Oral and Maxillofacial Surgery Department, Faculty of Dentistry, University of Malaya and Department of Oral and Maxillofacial Surgery, Hospital Tengku Ampuan Rahimah, Klang.

Inclusion criteria:

1. **VPL group**: Conventional OSCC with papillary projection, Papillary OSCC, Verrucous Carcinoma and Verrucous Hyperplasia (clinically Exophytic Verrucous Hyperplasia).
2. **Non-VPL group**: non-Verrucopapillary conventional OSCC, Epithelial hyperplasia/keratosis with/without dysplasia (clinically planar leukoplakia).

Exclusion criteria:

1. VPL or non-VPLs which were recurrent lesions
2. VPL or non-VPLs with incomplete data

4.1.2 Tumour tissues and diagnosis

Formalin-fixed Paraffin embedded (FFPE) samples from incisional and surgical specimen of VPL were retrieved from the archives of the Oral Pathology Diagnostic laboratory and the MOCDTBS at OCRCC, Faculty of Dentistry, University of Malaya. All specimens had been routinely fixed in formalin and embedded in paraffin. This project was approved by the local institutional ethics committee [DF OS1518/0076(P)].

All cases were initially reviewed by an oral pathologist (RBZ) to subdivide into VPL and non-VPL group. Placements into VPL and non-VPL groups were based on the clinical description in the clinical charts and histological slides of the case. Criteria of the VPL cases were as in Rosnah et al. (2016). Another oral pathologist (Thomas George Kallarakkal-TGK) also reviewed the VPL cases and the diagnoses of VC, PSCC and CSCC with papillary features were achieved by consensus of both Oral Pathologists (RBZ & TGK).

4.2 Methods

4.2.1 Sociodemographic and clinico-pathologic data

Sociodemographic, clinical and pathologic data were extracted from the MOCDTBS at OCRCC. Patient follow-up data were a combination of available data from MOCDTBS and records of previous cases treated in Oral Cancer Clinic at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Malaya.

4.2.2 Tissue Processing

4.2.2.1 Sectioning

Serial sections (4μm) were cut from paraffin blocks on a microtome. Crease was removed on the water bath at 40°C and mounted on silanized glass slides. Slides were dried and stored until require during immunostaining.

4.2.2.2 Immunohistochemistry (IHC)

(a) ***Avidin-biotin-peroxidase Complex (ABC) immunostaining***

All the experiments in this study were conducted using ABC immunostaining method based on Hsu et al. (1981). First step in this method, 4μm formalin-fixed paraffin-embedded tissue sections samples were deparaffinized in the oven at 30^0C overnight and at 60^0C one hour before the immunostaining. Later, sections are incubated with a primary antibody to the antigen of interest. In this study, selected study samples were immunostained with primary antibody as shown in the table 4.1 with final diluent factor using appropriate positive control. All immunostaining was conducted by the author by using Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+,Rb/Mo Kit.

Table 4.1: Antibodies used for immunohistochemistry experiments

Monoclonal antibody/ source	Diluent factor	Positive control
Ki-67 (Dako)	1: 100	Adenocarcinoma
p53 (Dako)	1: 100	Breast carcinoma
E-Cadherin (Dako)	1: 100	Normal oral mucosa
MMP-1 (MERCK)	1: 10 000	Breast carcinoma

The procedure continued with adding the secondary antibody to bind with primary antibody so that biotin molecules can be localized at the site of the antigen. Finally, Diaminobenzidine (DAB) was added to the sample to form brown deposits which help visualization in the microscopic examination. Dark brown precipitates within the nucleus were seen for p53 and Ki-67, membranous for E-Cadherin, and in the cytoplasm for MMP-1.

4.2.3 Scoring

The stained slides were examined and scored by the author and one oral pathologist (RBZ) by consensus. Protein expression scoring was graded as follows for p53, Ki-67, E-Cadherin and MMP-1: “negative” (0) indicates absence of brown precipitate in cells; the positives were labeled as (1) if there were a few (<10%) scattered cells with precipitate; (2) for small foci of clustered positive cells, but still less than 10% of the total number; (3) constituted large areas (10–50%) of positivity; and (4) designated 50% to 100% positivity. For E-Cadherin and MMP-1, scoring based on staining intensity was further conducted and graded as follows: 0 = if there is no staining; 1 = if there is little staining; 2 = for moderate staining; and 3 = for high staining intensity. Final score for E-Cadherin and MMP-1 was achieved from the percentage expression score, times intensity score.

4.3 Statistical Analysis

Data entry and data analysis was done using Statistical Program for Social Science (SPSS) Version 23.0 (SPSS Inc., 2015). Analysis was done according to the specific objectives of the study. Data were first checked and cleaned. The distribution and frequencies were examined. Descriptive statistics were used to describe all the dependent and independent variables. Means and standard deviations (SD) were calculated for all continuous variables. Categorical variables were calculated as frequency and percentages.

For the first objective, sociodemographic, and clinico-pathological profile parameters were analysed using frequency and % (for categorical variables). Chi Square test was used for appropriate P values.

For second and third objectives, immunohistochemical profile parameters were group using Receiver Operating Characteristic (ROC) curve analysis based on sensitivity and specificity. After grouping done, all descriptive statistics were analyzed using frequency and % (for categorical variables). Finally Chi Square test was used for appropriate *p* values.

A *p*-value <0.05 is considered as statistically significant.

CHAPTER 5: RESULTS

5.1 Socio-demographic characteristics

Twenty-four cases for VPL and twenty-nine cases for non VPL were selected for this study. The distribution of age, sex, ethnic and habits are depicted in Table 5.1 below. VPL group of patients ranges from the age of 38 to 79 years old (mean age, 64.3 ±11.23 years). The age range of patients in non VPL group was 20 to 81 years old (mean age, 54.1 ±12.3 years). Most of the VPL patients (70.8%) were 60 years old and above while most of non-VPL patients (69%) were less than 60 years old. This findings was statistically significant (p=0.004). The male to female ratio were 1:3.8 and 1:1.4 for VPL and non VPL patients, respectively. In this study, Indian (70.8%) was the most number of patients followed by Chinese (20.8%) and Malays (8.3%) in the VPL group. Similar to VPL, the non VPL group also showed the highest number of Indian (44.8%) patients followed by Malays (37.9%) and Chinese (17.2%). These findings with regards to ethnic distribution were found to be statistically significant (p=0.042). Many VPL patients had indulged in betel quid chewing habits (60.9%) with less betel-quid chewers in the non-VPL group of patients (31.8%).

Table 5.1: Statistics of socio-demographic data in VPL & non VPL groups.

	VPL n (%) **N**=24	**Non VPL** n (%) **N**=29	**Total** n (%) **N**= 53	***p*-value**
Age (mean ±SD)	64.3 ±11.23 years	54.1 ±12.3 years		
<60 years old	7 (29.2)	20 (69)	27 (50.9)	0.004
≥ 60 years old	17 (70.8)	9 (31)	26 (49.1)	
Total	24(100)	29(100)	53 (100)	
Gender				
Male	5 (20.8)	12 (41.4)	17 (32.1)	0.111
Female	19 (79.2)	17 (58.6)	36 (67.9)	
Total	24 (100)	29 (100)	53 (100)	
Race				
Malay	2 (8.3)	11 (37.9)	13 (24.5)	
Chinese	5 (20.8)	5 (17.2)	10 (18.9)	0.042
Indian (*3 Singh*)	17 (70.8)	13 (44.8)	30 (56.6)	
Total	24 (100)	39 (100)	53(100)	
Habits				
Smoking only	2 (8.7)	4 (18.2)	6 (13.3)	
Alcohol drinking only	0 (0)	0 (0)	0 (0)	
Betel quid chewing only	14 (60.9)	7 (31.8)	21 (46.7)	
Smoking + alcohol	3(13)	3 (13.6)	6 (13.3)	
Alcohol + Betel quid	1 (4.3)	1 (4.5)	2 (4.4)	0.418
Smoking + alcohol + betel quid	0 (0)	1 (4.5)	1 (2.2)	
No habits	3 (13)	6 (27.3)	9 (20)	
Total	23 (100)	22(100)	45* (100)	

*Missing data on habits for 8 patients

5.2 Clinico-pathologic characteristics

Clinico-pathologic findings are summarized in Table 5.2. Most cases of VPL were located on the buccal mucosa (45.8%) while most of non-VPL cases were located on the tongue & floor of the mouth (75.9%). This findings was statistically significant (p=0.001).

In term of size of lesion, 54.2% of VPL presented with more than 4cm followed by 45.8% with size 4cm or less. In contrast, 58.6% of non-VPL presented with size 4cm or less followed by 47.2% with size more than 4cm. There are no statistical differences in these findings.

All cases were treated with surgical excision alone or together with neck dissection. In VPL group, 29.2% were treated by surgical excision alone and 70.8% were done together with neck dissection. While in non-VPL group, 7.1% were treated by surgical excision alone and most of the cases were treated by surgical excision with combination of neck dissection (92.9%).

In the neck dissection cases, most of non-VPL (85.7%) presented with significant positive neck lymph node when compared with the VPL group (36%). This finding was found to be statistically significant (p=0.001)

Table 5.2: Statistics of clinico-pathologic of VPL & non VPL groups

	VPL n (%)	**Non VPL** n (%)	**Total** n (%)	***p*-value**
Location				
Tongue + Floor of the mouth	4 (16.7)	22 (75.9)	26 (49.1)	0.001
Gingiva + Palate	9 (37.5)	2 (6.9)	11 (20.8)	
Buccal mucosa	11 (45.8)	5 (17.2)	16 (30.2)	
Total	24 (100)	29 (100)	53 (100)	
Lesion size				
≤ 4cm	11 (45.8)	17 (58.6)	28 (52.8)	0.353
> 4 cm	13 (54.2)	12 (41.4)	25 (47.2)	
Total	24 (100)	29 (100)	53 (100)	
Treatment				
-Surgical excision	7 (29.2)	2 (6.9)	9 (17)	0.062
-Surgical excision and neck dissection	17 (70.8)	27 (93.1)	44 (83)	
Total	24 (100)	29 (100)	53 (100)	
Neck node				
Positive	9 (37.5)	24 (82.8)	33 (62.3)	0.001
Negative / No neck dissection	15 (62.5)	5 (17.2)	20 (37.7)	
Total	24 (100)	29 (100)	53 (100)	

5.3 Immunohistochemical profile

Immunohistochemical findings for VPL and non-VPL groups are summarized in Table 5.3. The result presented here was based on the expressions of the markers (% positive cells) in the lesions. For p53, positive expression was seen 91.7% in VPL and 79.3% in non-VPL. For Ki-67, 100% positive expression was seen in both VPL and non-VPL group. E-Cadherin showed 100% positive expression in VPL and 96.6% in non-VPL. Similar to Ki-67, MMP-1 showed 100% positive expression in both VPL and non-VPL groups. The overall expressions of all markers were higher in VPL compared with non-VPL group as presented in Figure 5.1.

Table 5.3: Immunohistochemical expression (% positive cells) of p53, Ki-67, MMP-1and E-Cadherin in VPL & non VPL groups

Score	p53 (%)		Ki-67 (%)		E-Cadherin (%)		MMP-1 (%)	
	VPL	Non VPL	VPL	Non VPL	VPL	Non VPL	VPL	Non VPL
0	8.3	20.7	0	0	0	3.4	0	0
1	12.5	10.3	12.5	27.6	8.3	6.9	0	0
2	20.8	6.9	50	34.5	25	17.2	4.2	6.9
3	33.3	13.8	33.3	34.5	25	27.6	4.2	13.8
4	25	48.3	4.2	3.4	41.7	44.8	91.7	79.3

0 = absent, **1** = <10% few scattered cells with precipitate, **2** = < 10% for small foci of clustered positive cells, **3** = 10–50% of positivity, **4** = 50% to 100% positivity

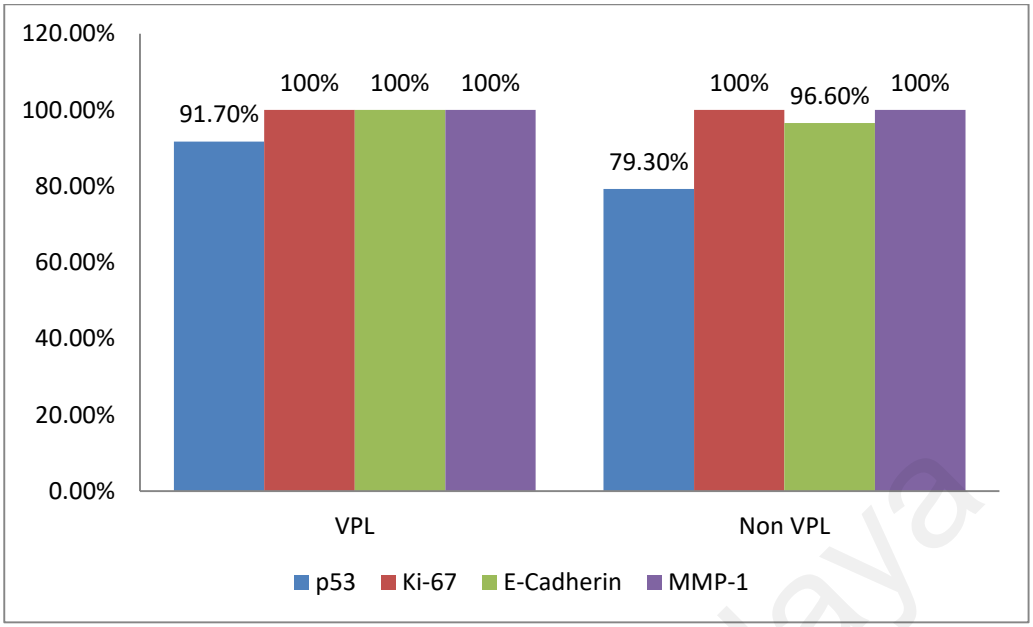


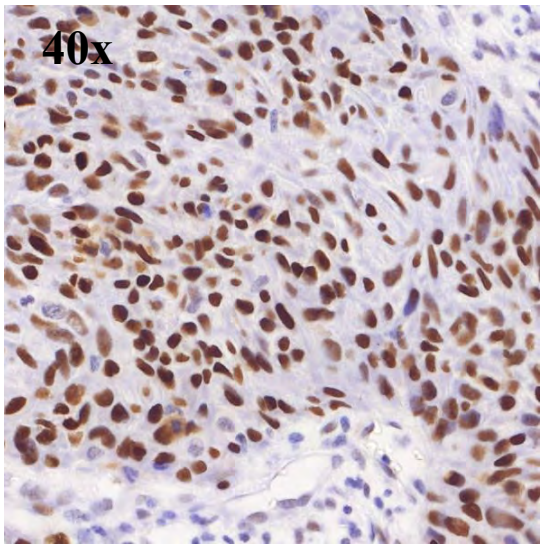
Figure 5.1: Overall percentage of all markers expressed positivity in VPL and non-VPL groups

For E-Cadherin and MMP-1, further scoring based on staining intensity was conducted and Table 5.4 summarized our findings. Overall, both E-Cadherin and MMP-1 presented with 100% positive staining. For E-Cadherin, most strong staining was found in non-VPL (69%) compared to VPL (62.5%). In contrast, in MMP-1, most strong staining was found in VPL (45.8%) compared to non-VPL (24.1%).

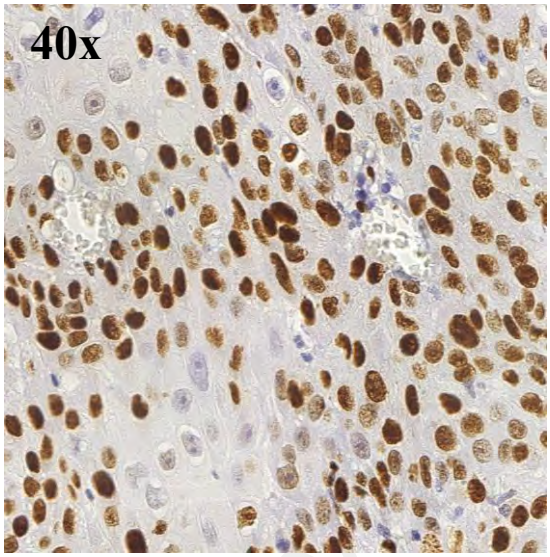
Table 5.4: Immunohistochemical expression (staining intensity) of MMP-1 and E-Cadherin in VPL & non VPL groups

Score	E-Cadherin (%)		MMP-1 (%)	
	VPL	Non VPL	VPL	Non VPL
0	0	0	0	0
1	16.7	6.9	8.3	24.1
2	20.8	24.1	45.8	51.7
3	62.5	69	45.8	24.1

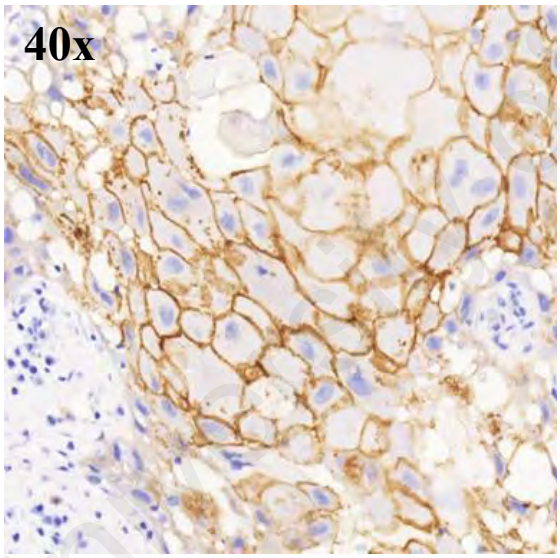
0 = absent, **1** = weak staining, **2** = moderate staining, **3** = strong staining



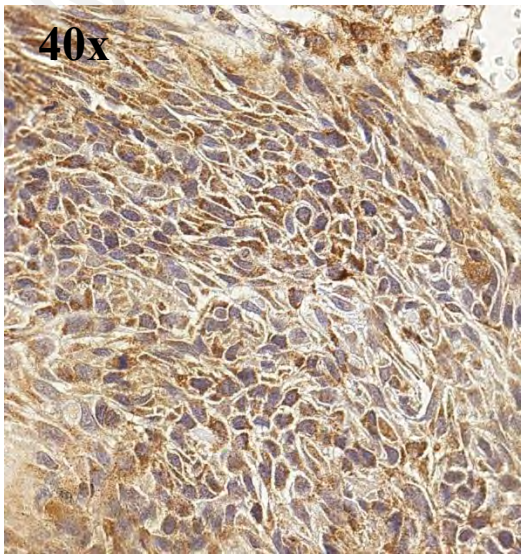
P53: nucleus staining



Ki-67: nucleus staining

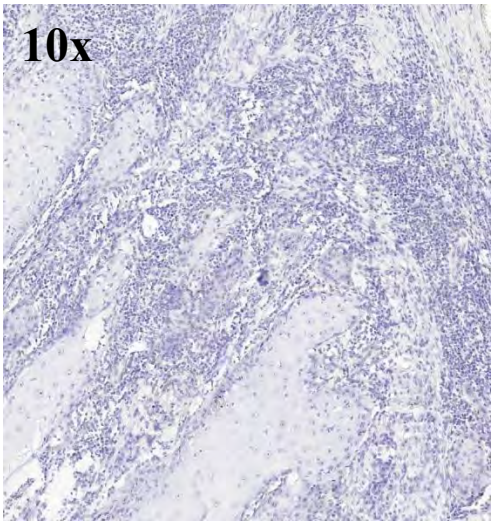


E-Cadherin: membranous staining

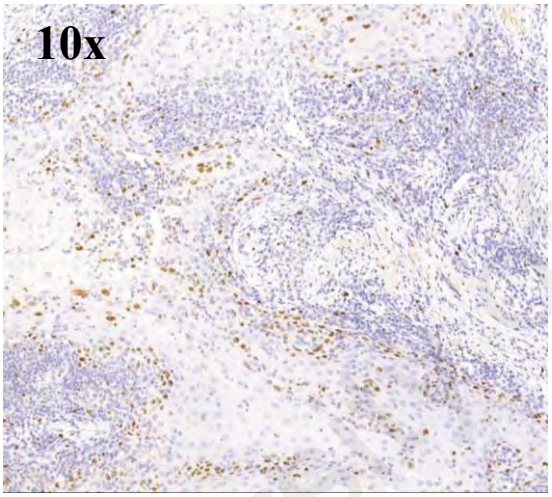


MMP-1: cytoplasm staining

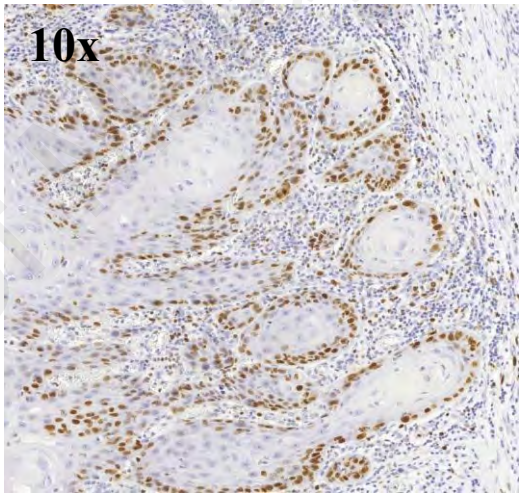
Figure 5.2: Expression of p53, Ki-67, E-cadherin & MMP-1 markers



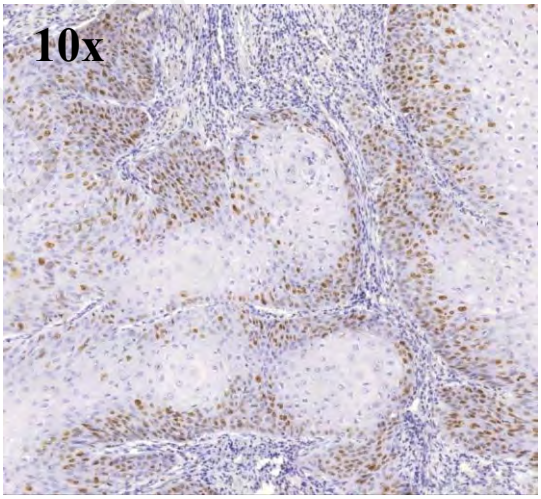
(Score 0: Absent**)**



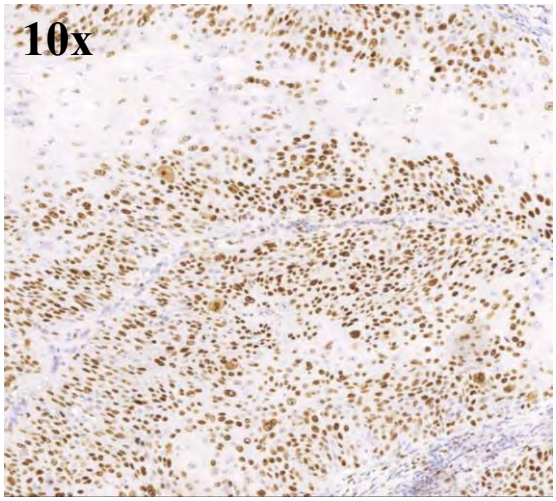
(**Score 1**) : <10% few scattered cells



(**Score 2**) : < 10% for small foci of clustered positive cells

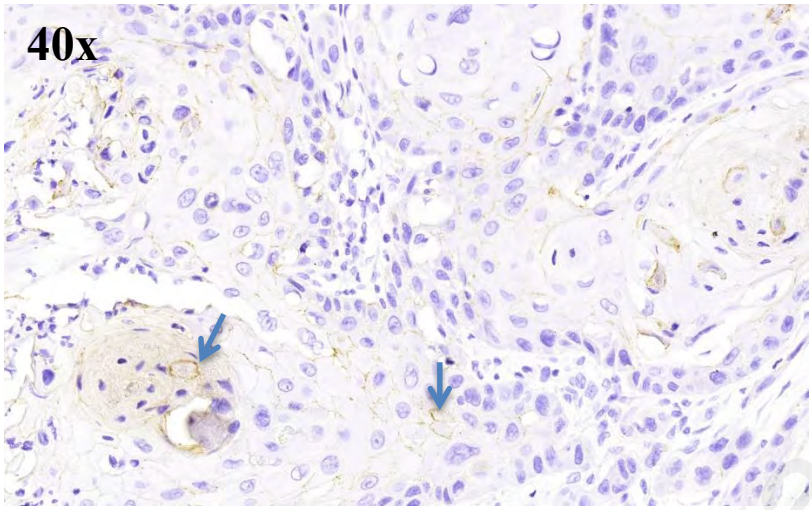


(**Score 3**): 10–50% of positivity

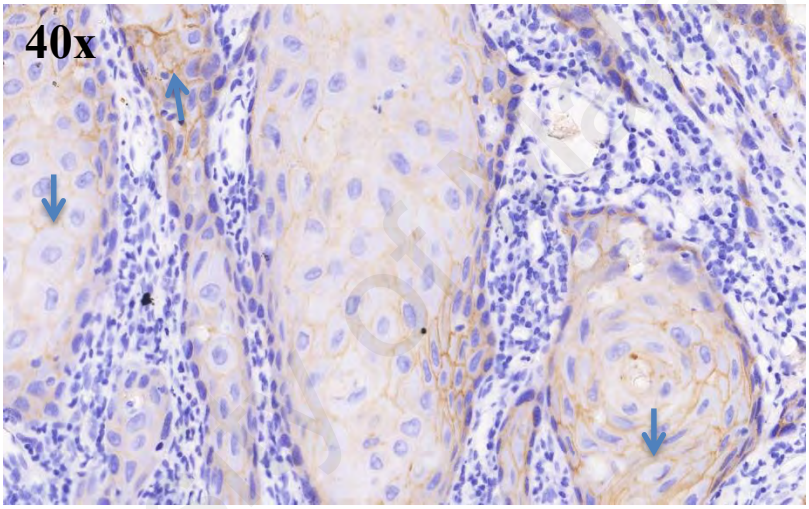


(**Score 4**): 50% to 100% positivity

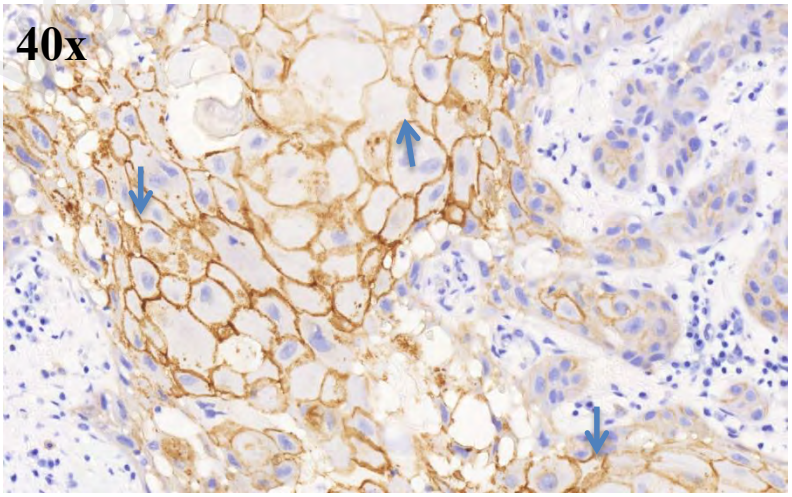
Figure 5.3: % of positive cell scoring for p53, Ki-67, E-Cadherin & MMP-1



(**Score 1**): Weak staining (arrows showing membrane staining)



(**Score 2**): Moderate staining (arrows showing membrane staining)



(**Score 3**): Strong staining (arrows showing membrane staining)

Figure 5.4: Staining intensity scoring for E-Cadherin & MMP-1

Both E-Cadherin and MMP-1 scores from percentage positive cells and staining intensity were combined to get a final score for each marker.

The final score of each marker expression then were further grouped into low and high expressions. This is done by using ROC curve analysis to determine the cutoff point of low and high expression score based on sensitivity and specificity. The summary of the findings are summarized in the Table 5.5.

The findings were high expression of p53 (91.7%) in VPL compared to non VPL (79.3%) although result was not statistically significant. Ki-67 and E-Cadherin showed similar results with high expression of Ki-67 in VPL (87.5%) compared with non VPL (72.4%) and high expression of E-Cadherin in VPL (100%) compared with non-VPL (96.6%). Both these findings were not statistically significant. However, MMP-1 result showed statistically significant high expression in VPL (91.7%) compared to non-VPL (62.1%) with p=0.013.

Table 5.5: Final expression score of all markers in VPL & non VPL group

Semi-quantitative scoring	**VPL** n (%)	**Non VPL** n (%)	**P-value**
p53 (Nuclear staining)			0.269
Low	2 (8.3)	6 (20.7)	
High	22 (91.7)	23 (79.3)	
Total	24 (100)	29 (100)	
Ki-67 (Nuclear staining)			0.308
Low	3 (12.5)	8 (27.6)	
High	21 (87.5)	21 (72.4)	
Total	24 (100)	29 (100)	
E-Cadherin (Membranous staining)			1.0
Low	0	1 (3.4)	
High	24 (100)	28 (96.6)	
Total	24 (100)	29 (100)	
MMP-1 (Cytoplasmic staining)			0.013
Low	2 (8.3)	11 (37.9)	
High	22 (91.7)	18 (62.1)	
Total	24 (100)	29 (100)	

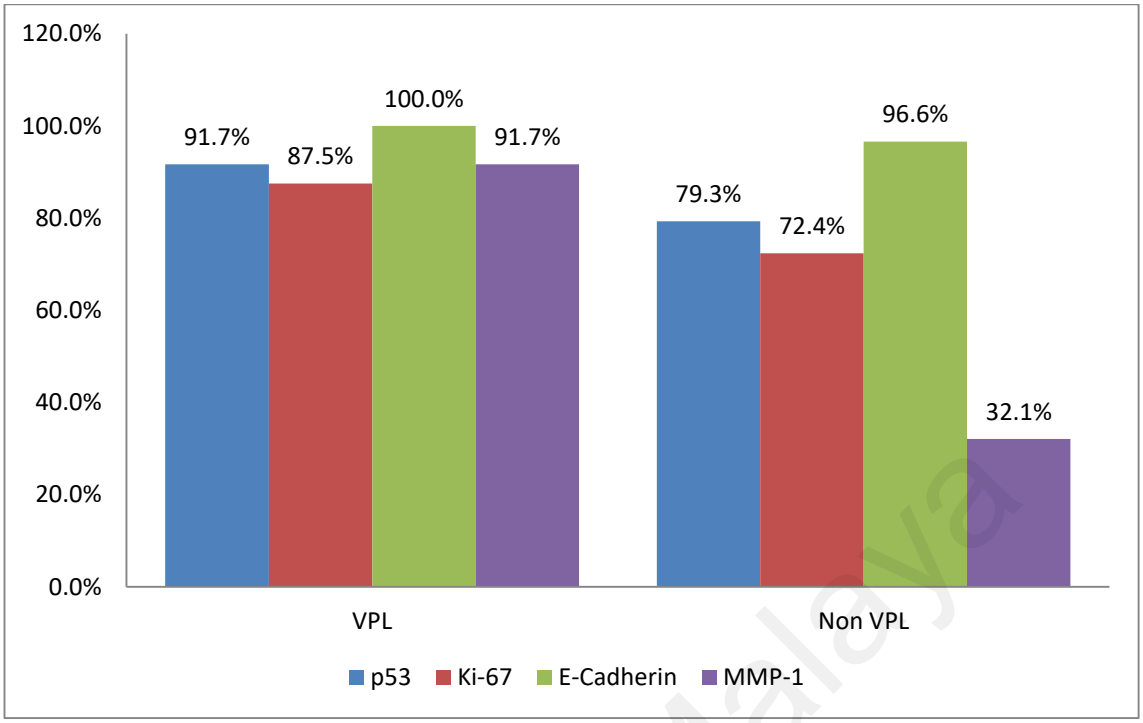


Figure 5.5: Summary of high expression of markers in VPL compared with non VPL groups.

To further evaluate the behavior of all VPL and non-VPL lesions, we subdivided the lesions into three groups and compared it with lymph node metastasis. First group consist of VH, VC and PSCC, second group CSCC with papillary features only and third group CSCC only. The findings for this part of the study were summarized in Table 5.6. It was observed that, (VH+VC+PSCC) group (66.7%) and CSCC with papillary features group (61.1%) showed the highest percentage of negative lymph node compared to CSCC group. While CSCC group presented with highest percentage of positive lymph node (82.8%) compared to other groups. Both of the findings were statistically significant with p=0.03.

Table 5.6: Group comparison of (VH+VC+PSCC), CSCC with papillary features and CSCC with lymph node metastasis

	Group 1 VH+VC+PSCC	Group 2 CSCC with papillary features	Group 3 CSCC without papillary features		
	n (%)	**n (%)**	**n (%)**	**Total n (%)**	***p*-value**
Lymph node					
Positive (+)	2 (33.3)	7 (38.9)	24 (82.8)	33 (62.3)	0.003
Negative (-) / No ND	4 (66.7)	11 (61.1)	5 (17.2)	20 (37.7)	
Total	6 (100)	18 (100)	29 (100)	53 (100)	

*VH: verrucous hyperplasia, VC: verrucous carcinoma, CSCC: conventional squamous cell carcinoma. ND: neck dissection (assumed to be negative nodes through clinical confirmation)

CHAPTER 6: DISCUSSION

Recent studies have addressed the role of p53, Ki-67, E-cadherin and MMP-1 markers in development of cancers. Reports pertaining to the head and neck region were confined mostly to CSCC. Only few studies have look into the comparison between VPL & non-VPL group. This study provides a comparison of these markers, in VPL and non-VPL groups. Together, the clinico-pathologic and immunohistological profile between VPL & non VPL groups were compared.

6.1 Sociodemographic profile

Looking into age results, generally patients with VPL were significantly older (> 60 years old) than patients with non-VPL (p=0.004). This finding were similar with D.T. Oliveira et al. (2006) who found most of oral verrucous carcinoma (VPL) seen in sixth decades of life and papillary SCC (VPL) which were commonly seen in older patient (J.G. Batsakis & P.Suarez, 2000).

Many parts of Asia including migrant Asian community chewed betel quid (paan) as common habit, which estimated 600 to 1200 million users globally (IARC, 2004). The common practice was to wrap the betel leaf with slake lime and areca nut which may be combined with sweeteners, tobacco or spices. This 'cocktail' produces pleasing psychostimulatory effect. It is also commercialized with the availability of freeze-dried betel quid substitutes (eg, gutka, pan masala) which prepackaged. In Malaysia, male are the most related to smoking habit while betel quid chewing habit are more related to female. This explained why most number of our VPL and non-VPL patients were female with most associated habit of betel quid chewing in the Indian ethnic group significantly were the commonest (p=0.001).

6.2 Clinico-pathologic profile

The study's findings presented that the common site of involvement related to VPL was buccal mucosa which was also in agreement with other studies (Slootweg & Muller, 1983; Wang et al., 2009). For non-VPL, most of cases were located on the tongue & floor of the mouth. Tumour arising at the buccal mucosa mostly due to betel quid being kept at the buccal mucosa. While patients with cigarette smoking and alcohol are more pre-disposed to tumour at the floor of mouth (Dhar et al., 1999).

Due to its exophytic nature, VPL group were larger in size compared to non-VPL which is part of the finding in the current study, although it is not statistically significant. Concern should be placed on benign VPL which is equal or larger than 10 mm because it may exhibit a risk for malignant transformation and present with difficulty in diagnosis (Hwang et al., 2012).

Most of VPL can be successfully treated by surgical excision and has good prognosis. Oral verrucous carcinoma for example was reported having rare regional or distance metastasis (Oliveira et al., 2006). While papillary SCC was shown by Yewei Ding et al. (2013) to have better prognosis probably due to limited invasion of the tumour. In our study, surgical excision in combination with neck dissection was the choice of treatment in non-VPL compared to VPL group. This reflects that non-VPL need to be treated more aggressively than VPL group. Furthermore, non-VPL was seen having statistically significant positive neck lymph node compared to VPL group (p=0.001), which support non-VPL invasiveness.

Study conducted by Shah (1990) recommended supra-omohyoid neck dissection (clearing levels I, II, and III cervical lymph node) for negative node patients with primary squamous cell carcinomas (non-VPL) of oral cavity. This was due to levels I, II, and III were at highest risk for metastasis. In other hand, we suggested that neck

dissection may be avoided in VPL patients with negative node because of current study showed that VPL cases presented with more negative lymph node compared to non-VPL cases which statistically significant (p=0.001).

6.3 Immunohistochemical profile

To our knowledge only few studies have been published on analyzing the immunohistochemical profile of VPL and non-VPL. Overall this current study found that, p53, Ki-67, E-cadherin and MMP-1 was highly expressed in VPL compared to non VPL group. However, only MMP-1 was statistically significant (p=0.013).

p53 mutation has been related to abnormal cell proliferation lead to cancer. Study by Wu et al. (2002) found stronger expression of p53 in SCC compared with both OVH and OVC (both VPL). Contrarily, the current study found higher expression of p53 in VPL compared to non VPL. However the result was not statistically significant ($p > 0.05$). Wu et al. did not subdivided SCC cases into CSCC and CSCC with papillary features. Furthermore, his SCC group had probably included CSCC with papillary features too. Our result differs from Wu et al. due to the fact that we have included both PSCC and CSCC with papillary features in the same group with OVH and OVC. Hence, this IHC panel is warranted for further evaluation.

This result was contradicted with our results which found higher expression of p53 in VPL compared to non VPL, but our result was not statistically significant ($p > 0.05$). Thus further evaluations of this IHC panel need to be done.

Ki-67 can be used as a marker due to its overexpression has been associated with proliferative activity of intrinsic cell population in malignant tumour. Takeda et al. (2001) reported that there was high significant difference of Ki-67 expression among PSCC (VPL) in comparison with verrucous carcinoma, while PSCC had a high Ki-67 labeling index (53.2- 59.0%), almost the same as that of conventional SCC (56.7-70.4%). Our study showed that, Ki-67 high expression percentage was slightly higher in VPL (87.5%) compared to non-VPL (72.4%) although statistically not significant. In addition, Zargaran, Eshghyar, Baghaei, and Moghimbeigi (2012) concluded that Ki67

expression showed Ki67 (Mib-1) is not a good immunohistochemical marker to assess invasion status and to differentiate OVC (VPL) from well-differentiated OSCC (non - VPL).

Down regulation or low expression of E-Cadherin is related to loss of integrity of the adherent's junctions, loss of the epithelial morphology and with the acquisition of metastatic potential by the carcinoma cells. In other words, E-cadherin expression was inversely associated with invasion, metastasis and poor prognosis. From our study we found that, E-Cadherin high expression was 100% in VPL compared to 96.6% in non-VPL although it is not statistically significant. Study by Tang ZG et al. (2003), reported that E-Cadherin expression in OVC (83.3%) were significantly greater than that in poorly differentiated SCC (37.5%). In other hand, Klieb and Raphael (2007) also found diffuse membranous staining of E-cadherin throughout the epithelium in all cases of both OVC and OVH. From all these findings, we can suggest that VPL behavior is less invasive and disturbances of adhesion molecules appear to be a property of more severe and advanced neoplasia.

MMP-1 over expression has been known to be related to tumour invasion. Impola U et al. (2004) have reported the presence of MMP-1 mRNA in fibroblastic cells of tumoral stroma. MMP-1 is expressed by both epithelial cells and connective tissue cells of VPL and non-VPL lesions. In our study, we found that MMP-1 in cytoplasm of epithelial tumour cells were significantly highly expressed in VPL (91.7%) compared to non VPL (62.1%) with a *p* value of 0.013. Another study by Klieb and Raphael (2007) only compared OVC with OVH (both VPL) and found that significant increase in staining within adjacent stromal cells in OVC more than OVH. But up till recently no study do comparison of MMP-1 in VPL with non VPL except us. Thus, we suggest there is need for further validation of this MMP-1 panel.

The presence of the regional lymph node metastasis in patients with oral cancer is one of the contributing factors to increase of staging and mortality (Rikardsen, Bjerkli, Uhlin-Hansen, Hadler-Olsen, & Steigen, 2014). In order to evaluate the difference in behavior of CSCC with papillary features with CSCC without papillary features, all VPL & non VPL were subdivided into three different groups namely: Group 1 consists of VH+VC+PSCC; Group 2 consists of CSCC with papillary features only and Group 3 consists of CSCC with no papillary features (as in Table 5.6). Both Group 1 (CSCC with papillary features) (66.7%) and Group 2 (VH+VC+PSCC) (61.1%) showed significantly higher percentage of negative lymph node (66.7% and 61.1%) respectively compared to Group 3 (CSCC without papillary features). Significantly high percentage of positive lymph node ($p< 0.05$) was observed for Group 3 as compared to Groups 1 and 2. These significant correlations was at p=0.003. These findings indicated that CSCC with papillary features may be similar in behavior as compared to other VPLs while CSCC with papillary features seemed to show different behaviors from the CSCC without papillary features. Hence this study may suggest that CSCC with papillary features can be treated less aggressively than CSCC without papillary features and may not require prophylactic neck dissection in case of negative lymph node.

6.4 Limitation

This study was carried out under various limitations. The most significant of all was time limitation. If the study was carried out in a longer time, more samples could have been recruited. This is important as the higher the cases number the more accuracy in profiling the sociodemographic, clinicopathologic and immunohistochemistry difference between VPL and non VPL. The limited sample size also limited the analysis that could be carried out.

Besides that, challenges raised because of insufficient data as some data are collected direct from the patient folders as they are not part of the MOCDTBS. In an effort to complete the data, difficulty arises when patient refused to be interviewed and we have to respect patient's decision. This is probably due to psychosocially effect after patients was diagnosed having malignancy.

In addition, there are limited numbers of studies investigating markers which compare VPL with non VPL. Choices of markers to be used also limited due to insufficient references. Hence, this study is to validate other studies.

CHAPTER 7: CONCLUSION

To conclude, firstly, this study showed that most of the VPL (70.8%) patients were 60 years and above while most of non-VPL (69%) patients were less than 60 years old ($p < 0.05$). The male to female ratio were 1:3.8 and 1:1.4 for VPL and non VPL patients, respectively. Indian ethnic group were highest in both VPL and non-VPL cases with betel quid chewing the most associated habits. Location wise, VPL were predominantly located on buccal mucosa (45.8%) while most of non-VPL (75.9%) cases were located on the tongue & floor of the mouth. This findings was statistically significant ($p<0.05$). There was statistically significant higher lymph node positivity in non-VPL (85.7%) compared to VPL (36%) cases ($p <0.05$).

Secondly, the applied four markers (p53, Ki67, E-Cadherin and MMP-1) to cases of VPL and non-VPL by immunohistochemical methods showed that the nuclear staining of p53 and Ki-67 was seen in a majority of VPL compared to non-VPL cases. There was no statistical difference between the scores for percentage staining of these 2 markers in VPL and non-VPL ($p >0.05$). There was a diffuse membranous staining exhibiting E-cadherin expression both VPL and non-VPL cases. A slightly lower combined percentage and intensity score in non-VPL (96.6%) compared to VPL (100%) cases. These findings are however, not statistically significant ($p>0.05$). MMP-1 expressions are seen in the cytoplasm of both VPL and non-VPL cases. There was a significantly higher combined percentage and intensity score of MMP-1 in VPL (91.7%) cases compared to non-VPL (62.1%) cases ($p<0.05$).

Thirdly, although the goal standard for differentiating VPL and non-VPL is adequate and well orientated hematoxylin-eosin stained section, MMP-1 immunohistochemistry panel may be helpful as an adjunct in difficult cases. This difficult case includes

inability to have good specimen orientation or not enough samples from an incisional biopsy.

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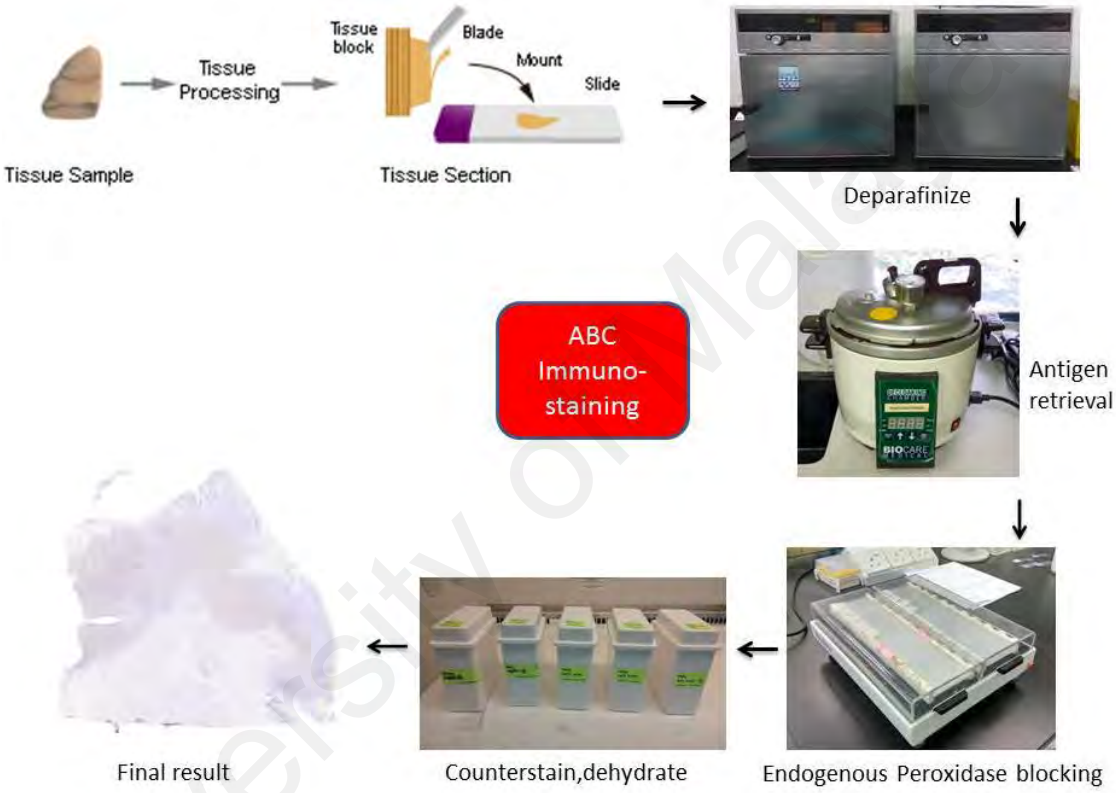
APPENDICES

Appendix A

Picture of IHC workflow:-



Appendix B

Immunostaining sequence:-

Deparaffinise	**Time**	**Check**
30^{0}C overnight and 60^{0}C one hour before		
Xylene 1	5mins	
Xylene 2	4mins	
100% etoH	3mins	
95% etoH	3mins	
70% etoH	3mins	
Immerse in running water	3mins	
Antigen retrieval		
Citrate buffer pH6 – Pressure cooker 99^{0}C at 20mins or microwave 121^{0}C 30sec, 90^{0}C 10sec		
Cool down -10mins whole plastic container,5mins outside	15mins	
Endogenous Peroxidase blocking		
Blocking H2O2	10mins	
Wash PBST 2x		
Add **primary Ab** cover all slides	1 hour	
Wash PBST 2x		
Add **secondary Ab** cover all slides (light off)	1 hour	
Wash PBST 2x		
DAB	5mins	
Running water	5mins	
Counterstain,dehydrate		
Hematoxylin	1mins	
Running water	3mins	
Acid alcohol	10sec	
Running water	10sec	
2% sodium acetate	5sec	
Running water	10sec	
95% alcohol 2x	2mims	
100% alcohol 2x	2mins	
Xylene 1	3mins	
Xylene 2	3mins	
Coverslip		