DEVELOPMENT OF REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION AND PCR ASSAYS FOR DETECTION OF HUMAN PAPILLOMAVIRUS 16 IN ORAL SQUAMOUS CELL CARCINOMA

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

Symbol	Meaning
ΔG	Gibb free energy
µg	Microgram
µl	Microliter
µM	Micro molar
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
AT	Adenine-thymine
ATCC	American Type Culture Collection
ASCO	American Society of Clinical Oncology
B3	Backward outer primer
BIP	Backward inner primer
BLAST	Basic local alignment search tool
bp	Base pair
Bst	*Bacillus stearothermophilus*
CDC	Centres of Disease Control and Prevention
cDNA	Complement deoxyribonucleic acid
CDK	Cyclin-dependent kinases
CI	Coefficient Interval
CO_2	Carbon dioxide
CV	Coefficient variation
DAB	3,3' Diaminobenzidine
Df	Turbidity measurement
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
E2F	Transcription factor
EBV	Epstein Barr virus
ECR	Early control region
EDTA	Ethylenediaminetetraacetic acid
F3	Forward outer primer
FDA	Food and Drug Agency
FFPE	Formalin-fixed paraffin embedded
fg	Fentogram
FIP	Forward inner primer
g	Gram
GC	Guanine-cytosine
Gy	Gray
HDA	Helicase-dependent amplification
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HNB	Hydroxynaphthol blue
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HPV-OSCC	HPV associated OSCC
HPV+OSCC	HPV positive OSCC

Symbol	Meaning
HPLC	High performance liquid chromatography
H&E	Hematoxylin and Eosin
IARC	International Agency for Research on Cancer
ICD-10	International Classification of Disease (ICD-10)
ICTV	International committee on taxonomy of viruses
IDT	Integrated DNA technologies
IgG	Immunoglobulin type G
IHC	Immunohistochemistry
ISH	*In-situ* hybridisation
ITF	Invasive Tumour Front
L	Litre
LAMP	Loop-mediated isothermal amplification
LB	Loop backward primer
LCR	Long control region
LF	Loop forward primer
LED	Light emitting diodes
Ltd.	Limited
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute
ml	Milliliter
mM	Milimolar
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NBF	Neutral buffer formalin
NASBA	Nucleic acid sequence-based amplification
NCBI	National Center for Biotechnology Information
NCI	National Cancer Institute
ng	Nanogram
$(NH_4)_2SO_4$	Ammonium sulfate
nm	Nanometer
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
NPV	Negative predictive value
OCRCC	Oral Cancer Research & Coordinating Centre
OD	Optical density
OPMDs	Oral potentially malignant disorders
OPSCC	Oropharyngeal squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
PBS	Phosphate buffer saline
PC	Personal computer
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pH	Potential hydrogen
PPV	Positive predictive value
pRB	Retinoblastoma protein

Symbol	Meaning
QC	Quality control
qLAMP	Real-time loop mediated isothermal amplification
qPCR	Quantitative polymerase chain reaction
RCA	Rolling circle amplification
RE	Restriction enzyme
RFLP	Restriction-fragment length polymorphism
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCC	Squamous cell carcinoma
SD	Standard deviation
SDA	Strand displacement amplification
SEA	South East Asia
SEER	Surveillance, Epidemiology, and End Results
SNR	Signal-to-noise ratio
SPSS	Statistical product and service solutions
ssDNA	Single-stranded deoxyribonucleic acid
Ta	Annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
Tm	Melting temperature
TNM	Classification of Malignant Tumours
TORS	Transoral robotic surgery
Tt	Threshold/amplification time
U	Unit
UK	United Kingdom
UM	Universiti Malaya
URR	Upstream regulatory region
USA	United States of America
USB	Universal Serial Bus
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Volts
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
X	Times or multiplication
x g	Relative centrifugal force

PEMBANGUNAN ASAI AMPLIFIKASI ISOTERMA BERPENGANTARA GELUNG MASA-NYATA DAN PCR UNTUK PENGESANAN PAPILLOMAVIRUS MANUSIA 16 DALAM KARSINOMA MULUT SEL SKUAMUS

ABSTRAK

Penglibatan papillomavirus manusia genotip 16 (HPV-16) dalam pembangunan karsinoma mulut sel skuamus (OSCC) telah didokumentasikan dengan baik. Pengesanan virus ini adalah penting untuk pengkelasan OSCC positif dan negatif kerana ia mempengaruhi prognosis pesakit. Kaedah pengesanan konvensional untuk HPV berisiko tinggi bergantung pada penanda surogat pewarnaan p16 imunohistokimia (p16 IHC) yang mempunyai kelemahan kerana proses yang panjang, memerlukan 20-24 jam untuk disiapkan, kadar spesifikasi yang rendah (46% hingga 78%), interpretasi pewarnaan yang subjektif (berjulat antara 5% hingga 75%), memerlukan pakar patologi untuk interpretasi keputusan dan kit yang mahal. Sehubungan dengan itu, dalam usaha untuk menambahbaik ujian diagnostik bagi pengesanan HPV-berkaitan kanser mulut, kami telah membangunkan asai amplifikasi isoterma berpengantara gelung masa-nyata (qLAMP) untuk pengesanan pantas, sensitif, spesifik dan kuantitatif untuk pengesanan HPV-16 dalam OSCC. Fasa pertama kajian difokuskan pada pembangunan asai qLAMP dan PCR, manakala dalam fasa kedua, asai yang telah dibangunkan dinilai dengan menggunakan sampel klinikal OSCC (tisu, n = 63; air liur, n = 13; dan darah, n = 59) dan sampel dari subjek yang sihat (air liur, n = 50). Pewarnaan rutin hematoxylin dan eosin (H&E) dan p16 IHC pada tisu parafin tertanam (FFPE) dijalankan untuk menilai tahap histologi tumor dan

menentukan positiviti HPV berisiko tinggi, masing-masing. Nilai Kappa ditentukan di antara dua penilai untuk pewarnaan p16. Nilai kepekaan, kekhususan, nilai ramalan positif (PPV), nilai ramalan negatif (NPV) dan ketepatan asai qLAMP dinilai dan dibandingkan dengan asai PCR dan pewarnaan p16. Dalam kajian ini, asai qLAMP yang dibangunkan berjaya mengenalpasti HPV-16 secara spesifik tanpa reaksi silang dengan strain HPV yang berlainan, virus respiratori dan bakteria mulut. Amplifikasi positif dapat dikesan seawal minit ke 21:18 dan keseluruhan proses boleh disiapkan dalam masa satu jam. Nilai LOD bagi asai qLAMP dan PCR adalah 4.68 X 10^1 dan 4.68 X 10^3 salinan dalam setiap tindak balas, masing-masing. Ujian qLAMP yang dibangunkan dapat mengesan HPV-16 positif dalam tiga sampel tiga tisu (4.7%) dan air liur (23%) pesakit OSCC, manakala PCR dapat mengesan dua (3.17%) HPV-16 positif dalam sampel tisu dan satu (7.69%) dalam air liur dengan nilai beban virus di antara 4.68 X 10^1 sehingga 4.68 X 10^4. Pewarnaan p16 menunjukkan tiga positif dengan nilai skor H adalah berjulat dari 40 hingga 225% dan tumor ini adalah gred terbeza yang baik. Nilai persetujuan Kappa adalah sangat baik (κ = 1.0) bagi dua penilai pewarnaan p16. Nilai kepekaan, kekhususan, PPV, NPV dan ketepatan pengujian qLAMP terhadap p16 adalah 100%. Nilai kepekaan, kekhususan, PPV, NPV dan ketepatan pengujian PCR terhadap p16 adalah 67%%, 100%, 100%, 98% and 98%, masing-masing. Kesimpulannya, asai qLAMP yang dibangunkan adalah sangat sensitif dan spesifik, dan cepat untuk pengesanan HPV-16 dalam OSCC. Kajian ini adalah novel kerana ia adalah yang pertama melaporkan penggunaan kedua-dua tisu dan air liur sebagai matriks pengantara untuk mengesan HPV-16 dalam OSCC, dan platform pengesanan menggunakan masa-nyata untuk pengesanan nilai beban virus jangkitan berbanding dengan kit pengesanan HPV-16 sedia ada.

DEVELOPMENT OF REAL-TIME LOOP-MEDIATED ISOTHERMAL AMPLIFICATION AND PCR ASSAYS FOR DETECTION OF HUMAN PAPILLOMAVIRUS 16 IN ORAL SQUAMOUS CELL CARCINOMA

ABSTRACT

Human papillomavirus genotype 16 (HPV-16) involvement in the development of oral squamous cell carcinoma (OSCC) has been well-documented. Its detection is crucial to classify OSCC into positive and negative cases; as this affects prognosis. The conventional method of detection for high-risk HPV relies upon p16 immunohistochemistry (IHC) as a surrogate marker which has drawbacks on its lengthiness, low specificity level (46 to 78%), broad range of staining intensity cut-off value (range 5 to 75%), requirement of expertise and costly. Thus, in an effort to improve the diagnostic test for HPV-related oral cancer, we have developed a real-time LAMP (qLAMP) assay for rapid, sensitive, specific and quantitative detection of HPV-16 in OSCC. The first phase was focused on the development of qLAMP and PCR assays, while in the second phase the developed assays were evaluated using OSCC clinical samples (tissue, n=63; saliva, n=13; and blood, n=59) and healthy (saliva, n=50). The hematoxylin and eosin (H&E) and p16 IHC staining using formalin-fixed paraffin embedded (FFPE) tissues was done to evaluate the tumour histological grading and determine high-risk HPV positivity, respectively. The Kappa value was determined between two raters for p16 staining. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of qLAMP were evaluated, in comparison with PCR and p16 IHC. It was found that the developed qLAMP assay successfully amplified HPV-16, and no cross-reaction with other HPV strains, respiratory viruses and oral bacterial. The positive amplification

starts as early as 21:18 minute and the whole process can be completed within one hour. The LOD for qLAMP and PCR assays were 4.68 X 10^1 and 4.68 X 10^3 copies per microliters, respectively. The developed qLAMP assay detected HPV-16 positivity in three tissue (4.7%) and saliva (23%) samples from OSCC patients, while the PCR assay detected two (3.17%) HPV-16 positives in tissue and one (7.69%) in saliva samples, with the HPV-16 viral load ranging from 4.68 X 10^1 to 4.68 X 10^4. The sensitivity, specificity, PPV, NPV and accuracy of qLAMP assay towards p16 IHC were 100%. The sensitivity, specificity, PPV, NPV and accuracy of in-house PCR assay were 67%%, 100%, 100%, 98% and 98%, respectively. p16 IHC staining showed three positivity of tissues with the H score ranged from 40 to 225% and well-differentiated grade. Very good agreement ($\kappa = 1.0$) was found between two raters for evaluation of p16 IHC staining. In conclusion, the developed qLAMP was highly sensitive and specific, and rapid for the detection of HPV-16 in OSCC. This study is novel as it is the first report describing the use of both tissue and saliva as the sample matrix for detection of HPV-16 in OSCC and the detection platform using real-time to quantify the viral load of the infection in comparison to the current available HPV-16 detection kit.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Oral cancer is ranked as the 17th among the most common cancer worldwide (Globocan, 2018). More than 95% of oral cancers are oral squamous cell carcinomas (OSCC). OSCC is defined as a malignant epithelial neoplasm arising from the oral cavity and exhibits squamous differentiation characterised by the formation of keratin/or the presence of intercellular bridges (Dhanuthai *et al.*, 2018). Worldwide, OSCC affects 350,000 people each year (National Cancer Institute, 2018). It occurs most often in people over the age of 40 years old and affects more than twice as many men as women (Fox *et al.*, 2018). Established risk factors for OSCC include smoking, alcohol consumption, betel quid chewing, sunlight, dietary habits, chronic candidal infection, genetic predisposition and compromised immune system secondary to organ transplant (Lin *et al.*, 2011; Talhout *et al.*, 2011). Recent findings have demonstrated the presence of high-risk human papillomavirus in OSCC and their ability to immortalise oral keratinocytes by bringing transformation of epithelial cells (Ajila *et al.*, 2015; Chen *et al.*, 2016). Majority of HPV associated OSCC (HPV-OSCC) cases are caused by HPV-16 and 18 with the most infected sites are tonsil and base of the tongue (Kabeya *et al.*, 2012).To date, there have been more than 200 types of HPV subtypes discovered (Pinidis *et al.*, 2016). The low-risk HPV subtypes (HPV-6, 11, 2, 43, 44, 54, 61, 70 and 72) are associated with a variety of benign oral papillomatous lesions including oral squamous papilloma, oral condyloma accuminatum, oral verruca vulgaris, and focal epithelial hyperplasia (Méndez-Martínez *et al.*, 2020).

HPV-OSCC cases are rapidly growing and have been reported worldwide (Zhou *et al.*, 2019). These increasing trends have been observed in many countries, despite the decreased in tobacco consumption (Metgud *et al.*, 2012). The reported prevalence of HPV-OSCC varies considerably because of the differences in the anatomical site, ethnicity, detection methods and geographical setting (Ajila *et al.*, 2015). Worldwide prevalence of HPV-OSCC is 3.9% (World Health Organization, 2018) but, a higher prevalence rate was observed in many countries, ranged from 60% - 41% (Plummer *et al.*, 2016). In Malaysia, there are few studies reported the prevalence of HPV-OSCC ranged from 21-51.4% (Heah *et al.*, 2012; Kerishnan *et al.*, 2016; Saini *et al.*, 2011).

Persistent infection with high-risk HPV is a prerequisite for the development of OSCC. Twelve HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) are classified by the International Agency for Research on Cancer (IARC) as being carcinogenic to humans. Among these, HPV-16 is the predominant type, which accounts for more than 70% of all cancer diagnosed worldwide (IARC, 2018). Moreover, HPV-16 is the most common type found in OSCC, representing >95% of all HPV-positive SCC and also the predominant type found in OSCC affecting Malaysian population (Kerishnan *et al.*, 2016; Saini *et al.*, 2011).

Detection of HPV in OSCC have therapeutic and prognostication implications, which allows a better estimate for prognosis assessment and potential use in targeted therapy. Review of the literature concluded that the presence of HPV in OSCC constitutes a positive prognostic marker for the disease. In essence, HPV-positive patients have better disease-free survival rate compared to HPV negative group as it has demonstrated a higher degree of sensitivity towards radiotherapy and chemotherapy

(Ang *et al.*, 2010; Götz *et al.*, 2016). Thus, it is recommended to distinguish HPV-OSCC from non-HPV-OSCC. Additionally, dose reduction has been suggested for the treatment in HPV-OSCC cases. The standard chemo-radiotherapy regimen for the treatment of OSCC is considerably an overtreatment for HPV-OSCC and may result in toxicity and reduced quality of life (Mirghani and Blanchard, 2017). Thus, the discrimination of HPV-OSCC from non-HPV-OSCC is important towards delivering accurate treatment for these two groups of patients (Bhargava *et al.*, 2012; Gildener *et al.*, 2014).

Several methods have been described for high-risk HPV detection and genotyping. Currently, p16 immunohistochemistry (IHC) is the routine diagnostic test used in the clinical setting (Mirghani *et al.*, 2015). Other commonly used methods are in-situ hybridization (ISH) and molecular detection assays (Bishop *et al.*, 2012). Detection of HPV by PCR can be done using consensus primers, such as PGMY09/PGMY11 and GP5+/GP6+ or specific primers targeting on the specific gene (Tawe *et al.*, 2018). Various types of isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), helicase-dependent amplification (HDA), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA) have also been reported for HPV detection (Yan *et al.*, 2014).

1.2 Rationale of the study

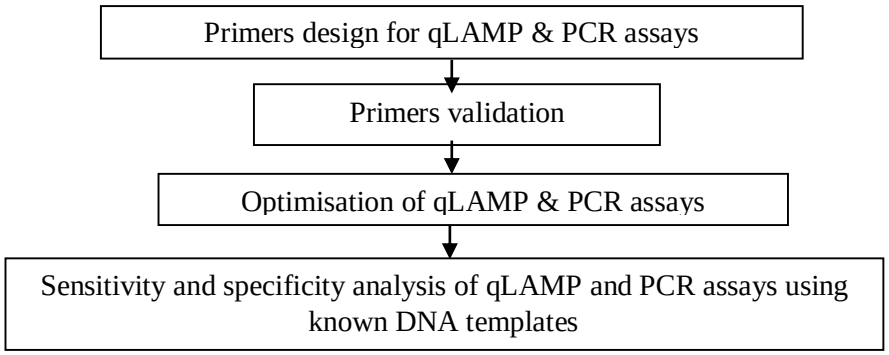
Identification of HPV-OSCC is routinely done using p16 IHC, which identify the presence of p16 protein, a cell cycle-regulating protein, often overexpressed in tumours infected with high-risk HPV. Nevertheless, the protocol is time-consuming; requiring 20 – 24 hours to complete all the procedures and a pathologist to validate and confirming the test results. p16 IHC has lack of specificity which were reported ranging from 46% to 78%. The result interpretation of staining intensity is considerably subjective, where the cut-off value for overexpression of p16 staining varies from ⩾5% to ⩾75 (Gronhoj *et al.*, 2014). The p16 staining kit is also expensive since it requires an antibody for detection (Klussmann *et al.*, 2003; Westra, 2014). For molecular technique method, PCR is favourable due to sensitivity and specificity of the results provided. Nevertheless, PCR also demonstrated to have various disadvantages. It is time-consuming due to required post-amplification and highly sensitive to inhibitor such as haemoglobin, urea, phenol, ethanol, collagen, myoglobin, IgG and proteinases (Schrader *et al.*, 2012; Ting-Yin, 2011). PCR also might not be suitable in primary clinical settings in developing countries or for field use, because of the sophisticated instrumentation required, elaborate and complicated assay procedures (Schache *et al.*, 2013).

Due to all the above-mentioned pitfalls, there is a growing demand for the simpler and economical molecular test for HPV status determination in OSCC patients. LAMP is a versatile isothermal amplification tool that can be applied in various fields. It provides a specific, sensitive, rapid and simple method for DNA synthesis. One of the most attractive features of this LAMP assay is that the results can be observed and determined by dye-mediated visualisation. After a short amplification time, a white

precipitate of magnesium pyrophosphate as a by-product is produced and it can be observed by the naked eye without any special processing or electrophoresis step (Notomi *et al.*, 2000). Moreover, LAMP assay is not relatively affected by inhibitors remaining in the DNA extraction step. Relatively simple DNA extraction assays (*i.e.*, boiling) can be used for DNA extraction instead of commercial DNA extraction kits (Abbasi *et al.*, 2016; Hayashida *et al.*, 2015) can be cost-saving.

Thus, to improve high-risk HPV detection in oral cancer, the present study aimed to develop a real-time LAMP (qLAMP) assay for rapid, sensitive, specific and quantitative detection of HPV-16 in OSCC. The first phase was focused on the development of qLAMP and PCR assays. The second phase was the evaluation of the developed assay using OSCC clinical and healthy samples in comparison with developed PCR and p16 IHC staining. The experiment design of the study is shown in Figure 1.1.

Phase I: Development of qLAMP and PCR assays



Phase II: Evaluation of qLAMP assay on clinical samples

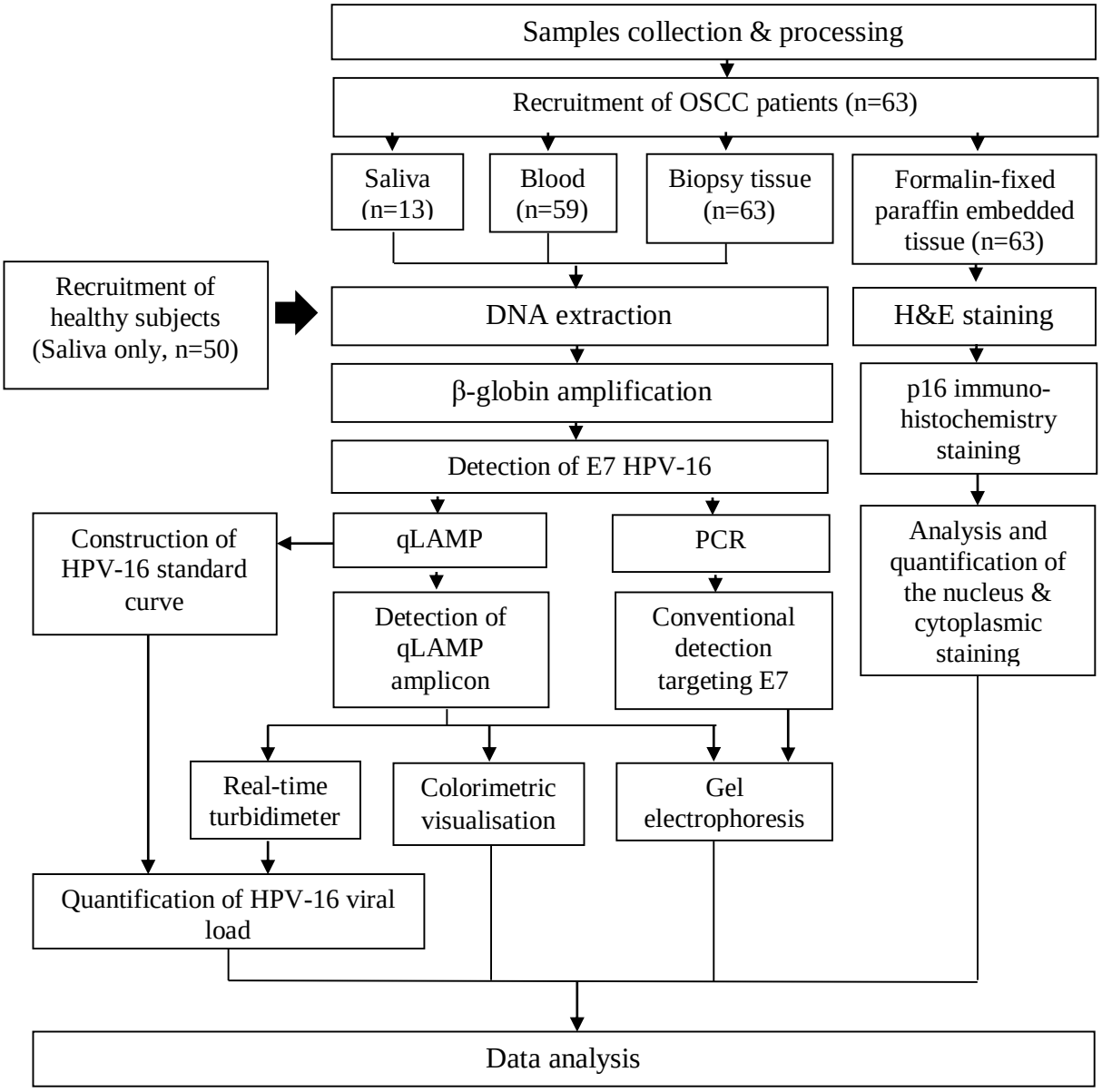


Figure 1.1 Flowchart of the study

1.3 Objectives of the study

General objective:

To develop a real-time loop-mediated isothermal amplification (qLAMP) and polymerase chain reaction (PCR) assays for detection of HPV-16 in OSCC.

Specific objectives:

i. To design and validate specific primers targeting E7 gene of HPV-16 for qLAMP and PCR assays.

ii. To optimize and develop qLAMP and PCR assays for HPV-16 detection in clinical samples of OSCC.

iii. To determine HPV-16 viral load in clinical samples using a constructed HPV-16 standard curve

iv. To evaluate and compare the diagnostic performance of developed HPV-16 qLAMP assay with p16 IHC using clinical samples of OSCC.

v. To evaluate and compare the diagnostic performance of the developed HPV-16 qLAMP assay with PCR using clinical samples of OSCC.

CHAPTER 2

LITERATURE REVIEW

2.1 Oral squamous cell carcinoma (OSCC)

Oral squamous cell carcinoma (OSCC) is defined as carcinoma with squamous differentiation arising from the mucosal epithelium. Approximately, 90% of the OSCCs are in the oral cavity region (World Health Organization, 2018). The term "OSCC" is often used interchangeably with oral cancer (Markopoulos, 2012). Based on the International Classification of Disease Revision 10, (ICD-10), classification of oral cancer is given by codes of C00-C06 for lip and oral cavity, while C09-C10 for oropharynx. Oral cavity consists of the front two-thirds of the tongue, the upper and lower gums, the lining of the cheeks (buccal mucosa) and lips (labial mucosa), the floor of the mouth, ventral surface of the tongue, the 'bony' top of the mouth (hard palate) and the small area behind the last (wisdom) teeth (retromolar trigone). Oropharynx, which located behind the oral cavity includes the back third of the tongue, the soft palate, the side and back walls of the throat, and the tonsils (Chai *et al.*, 2015; Feller *et al.*, 2013).

2.1.1 Epidemiology of OSCC

Cancer incidence and mortality are rapidly growing worldwide. The reasons are complex but reflect both ageing and growth of the population, as well as changes in the prevalence and distribution of the main risk factors for cancer which are associated with socioeconomic development (Bray *et al.*, 2018). Based on the epidemiology data extracted from GLOBOCAN database 2018, it was estimated that 18.1 million new

cases and 9.6 million cancer deaths occurred worldwide in 2018. Among these, cases involving the lip and oral cavity (C00-C06) accounted for 354 864 (2.20%) of new cases and 177 384 (2.01%) of deaths. Meanwhile, cases for oropharynx (C09-C10) encountered for 92 887 (0.51%) of new cases and 51 005 (0.53%) of death. To date, oral cancer and oropharynx cancer have been ranked as the 17th and 25th worldwide, respectively (Globocan, 2018). OSCC is predominantly found among males as compared to females where 320 892 new cases per year found in men and 126 859 in women. The highest incidence rates were seen in Papua New Guinea (25.0 per 100,000), followed by Melanesia as a whole (19.0 per 100,000), Maldives (11.0 per 100,000), Sri Lanka (10.3 per 100,000), Bangladesh (9.4 per 100,000) and India (7.2 per 100,000) (Bray *et al.*, 2018; Globocan, 2018).

In Malaysia, 667 new oral cancer cases were reported on 2018 and 327 deaths were recorded. The 5-year prevalence is 6%, ranking oral cancer as the 19th among 36th listed cancers by GLOBOCAN. Although OSCC is predominant among males, in Malaysia, the prevalence of OSCC is found higher in females compared to males. A study among Malaysian population demonstrated the male-to-female ratio of 0.92:1 and a similar finding was reported among Thailand population (Khan *et al.*, 2008). As Malaysia and Thailand are neighbouring countries, residents are exposed to a similar type of environment and share some cultural practices, so both countries elicit a female predilection (Dhanuthai *et al.*, 2017). In Malaysia, oral cancer was reported due to habits of betel quid chewing in Indian ethnicity (Razak *et al.*, 2010).

2.1.2 Risk factors of OSCC

There are certain stimuli known to induce OSCC either genetically or systematically, which can be divided into three broad groups, namely chemical, physical and viral infections. Among these three stimuli, chemical carcinogens are the main culprit contributed to the problem in which tobacco and alcohol are the most commonly abused (Hanahan and Weinberg, 2011).

2.1.2 (a) Tobacco/betel quid

Tobacco is a leafy plant grown around the world. There are many chemicals found in tobacco leaves created by burning (as in cigarettes). Chemical produces by smoking such as nitrosamines, polycyclic aromatic hydrocarbons, aldehydes, aromatic amines, phenols, nitro compounds, volatile hydrocarbons, including other organic and inorganic compounds can cause serious harm to the body (Talhout *et al.*, 2011). Smokers have nine times greater risk to develop OSCC compared to non-smoker which 80% of the reported OSCC patients are smokers (Gupta and Johnson, 2014). Although several tobacco agents play a role in the development of the tumour, the potent effects of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) are unique. Metabolically activated NNK and NNN induce deleterious mutations in oncogenes and tumour suppression genes by forming DNA adducts, which could be considered as tumour initiation. Meanwhile, the binding of NNK and NNN to the nicotinic acetylcholine receptor promotes tumour growth by enhancing and deregulating cell proliferation, survival, migration, and invasion, thereby creating a microenvironment for tumour growth. These two unique aspects of NNK and NNN synergistically induce cancers in tobacco-exposed individuals (Xue *et al.*, 2014). Continuous exposure to tobacco may interrupt genetic metabolism where

the cells begin to divide at a higher rate. In unusual patterns, this could change the maturation of epithelium cells which either be disturbed or become dysplastic. Eventually, the damaged mucosa remains deteriorated (Markopoulos, 2012). Each cigarette contains about 10 milligrams of nicotine. Nicotine increases dopamine level, a neurotransmitter that enhances pleasurable feeling which can lead to addiction (Talhout *et al.*, 2011). However, most of the harmful components are not from the nicotine, but from chemicals in burning tobacco (Lin *et al.*, 2011).

Smokeless tobacco is defined as unburned tobacco, also known as chewing tobacco, oral tobacco, spit or spitting tobacco, dip, and snuff (Gupta and Johnson, 2014). The products contain nicotine and 28 known carcinogens which also contribute to OSCC (Khan *et al.*, 2014). The most widely used smokeless tobacco is betel quid named "khaini" or "gutka". It is a combination of powdered tobacco and slaked lime paste, sometimes with added areca nut (Guha *et al.*, 2014). Betel quid is one of the four most commonly used psychoactive substances worldwide. People usually wrapped in betel leaf (derived from the *Piper betel* vine) with at least one of two basic ingredients: chewing tobacco and sliced fresh or dried areca nut (Hernandez *et al.*, 2017). The leaves then are smeared with aqueous lime and various additives. Users who chew or suck tobacco in their mouth will absorb chemical compounds and nicotine through the lining of the mouth (Gupta and Johnson, 2014; Ogbureke and Bingham, 2013). These smokeless tobacco has been considered a substitute to smoke tobacco, however, previous studies demonstrated that there was similar exposure to the tobacco-specific carcinogen nicotine-derived nitrosamine ketone in smokers and smokeless tobacco users (Hecht *et al.*, 2007; Xue *et al.*, 2014).

In one meta-analysis study, a significant relationship was found between oral cancer and betel quid chewing alone (Lee *et al.*, 2012). Betel quid chewing added with tobacco was three to five times more likely to develop oral cancer compared to betel quid without tobacco (Guha *et al.*, 2014; Gupta and Johnson, 2014). Exposure-response analyses showed that the risk of oral cancer increased with increasing daily amount and duration (years) of chewing betel quid in India, Taiwan and China. In the Indian subcontinent, betel quid associated with oral cavity was 5.38–11.13 (95% CI) and in Taiwan was 4.86–24.84 (95% CI) (Bray *et al.*, 2018; Guha *et al.*, 2014).

2.1.2 (b) Alcohol

According to the International Agency for Research on Cancer (IARC), alcohol has been shown consistently associated with increased risk of oral cancer, both independently and synergistically with tobacco. Numerous studies have been reviewed extensively suggested that alcohol play an important role in the development of OSCC. People consuming alcohol 170 g per day was said to have 10 times risk in OSCC compared to lighter drinker (IARC, 2014).

One possible mechanism through which alcohol consumption may influence the risk of OSCC is via conversion of ethanol to acetaldehyde. Alcohol dehydrogenase 3 (ADH3) converts ethanol to acetaldehyde, which is a suspected oral carcinogen. The ADH3*1 allele is associated with increased conversion of ethanol to acetaldehyde, but whether the risk of OSCC is increased among ADH3*1 carrier, or whether the risk of OSCC attributable to alcohol consumption is unclear (Ram *et al.*, 2011). One meta-analysis study found a strong direct trend of increasing risk with increased alcohol consumption for oral and pharyngeal cancer where alcohol contributed to the entry of

carcinogens into an exposed cell, hence altering the oral mucosa cells (Hashibe *et al.*, 2007). Carcinogenesis may also relate to nutritional deficiencies associated with alcoholism because alcohol leads to impaired absorption of nutrient and vitamin (Bagnardi *et al.*, 2014).

Heavy drinking, smoking and their synergistic effects are the major risk factors for SCC in Western settings (Lin *et al.*, 2011). However, in lower-income countries, such as in parts of Asia and Sub-Saharan Africa, the major risk factors for OSCC have yet to be elucidated. Meanwhile, a suspected additional risk factor for OSCC in Southern America (*i.e.*, in Uruguay, Brazil, and Argentina) is due to drinking very hot mate (Bray *et al.*, 2018).

2.1.2 (c) Nutritional intake

According to World Health Organization (WHO) reports (2016), 35 – 55% of human cancer and approximately 15% of OSCC can be attributed to dietary deficiencies or imbalances (Stewart and Kleihues, 2016). The relationship between nutrition and OSCC can be approached from two different points of views: (1) the direct effect of carcinogens presence in food and food additives (*i.e.*, direct carcinogenesis) and (2) *in-vivo* synthesis of carcinogens caused by changes in metabolism due to altered dietary habits (*i.e.*, indirect carcinogenesis) (Taghavi and Yazdi, 2007).

With regards to OSCC, among the dietary factors inhibit cancer growth are fibre, antioxidants (β-carotene, tea, fresh fruits, and vegetables) and micronutrients (vitamin C, E, and K, zinc, folate). The anti-cancer effects of fresh vegetables and fruits on OSCC have been confirmed by some previous studies, which were associated to their

contents of carotenoids, vitamin A, vitamin C, folic acid, flavonoids, fibre and other antioxidants(Chen *et al.*, 2017; Ogbureke and Bingham, 2013; Taghavi and Yazdi, 2007). Additionally, there were also several studies reported a relationship between seafood intake, particularly fish and inhibition of oral cancer risk (Chen *et al.*, 2017; Toledo *et al.*, 2010; Zheng *et al.*, 2013). Fish and seafood contain n-3 polyunsaturated fatty acids, mineral salts and proteins which could inhibit tumour progression through their anti-inflammatory effects and inhibition of oxygen free radicals (MacLean *et al.*, 2006).

Monounsaturated fatty acids like n-9 oleic acid, as the main source of fat, behave as a tumour promoter in oral cancer (Zheng *et al.*, 2013). Foods rich in fat content such as pork, bacon, pasta, cheese, red meat, fried foods, meat fried, or cooked at high temperature or in the microwave were significantly associated with the risk of oral cancer (Bovell-Benjamin *et al.*, 2010; Toporcov *et al.*, 2004). Fried foods, meat fried or cooked at high temperature or in a microwave can be carcinogen because of the production of heterocyclic amines (Taghavi and Yazdi, 2007; Toledo *et al.*, 2010). Possible mechanisms through which fatty acids may influence carcinogenesis include effects on membrane integrity, increase in lipid peroxidase and impairment of nutrient metabolism (Chen *et al.*, 2017).

2.1.2 (d) Viral infections

Recent findings showed that viral infections are significantly found associated with OSCC. Majority of oral viral infection manifesting as oral cancer is caused by Human papillomavirus (HPV) and Epstein-Barr virus (EBV; HHV-4) (Gillison *et al.*, 2015; Metgud *et al.*, 2012; Speicher *et al.*, 2016). The presence of EBV in squamous cell

lesions in the oral cavity was proven by Metgud *et al.* where 60% of SCC was EBV positive by PCR and *in-situ* hybridisation (ISH) (Metgud *et al.*, 2012). Another study reported that EBV infection among oral cancer patients in Taiwan population was 82.5% by microarray analysis (Yen *et al.*, 2009). HPV, particularly HPV-16 has also been established for the development of OSCC especially at the base of the tongue and the tonsillar area in the younger individuals (D'Souza *et al.*, 2007; Dhanuthai *et al.*, 2017). The proportions of HPV-positive OSCC were reported in many countries, including South Korea (60%), North America (51%), East Europe (50%), Japan (46%), Northwest Europe (42%), Australia (41%), South Europe (24%), China (23%), India (22%) and Malaysia (20.4%) (Kerishnan *et al.*, 2016; Plummer *et al.*, 2016).

2.1.2 (e) Other risk factors

Other minor risk factors could contribute to OSCC. People with poor oral hygiene or dental care may have an increased risk of oral cavity cancer (Oji and Chukwuneke, 2012). Poor dental health or ongoing irritation from poorly fitting dentures and chronic hyperplastic candida infection, especially in people who use alcohol and tobacco products may contribute to an increased risk of oral and oropharyngeal cancer (Singhvi *et al.*, 2017). Some studies indicated a correlation between oral cancer and bacterial load which was associated with poor oral hygiene, chronic periodontitis and poor dental status (Ahrens *et al.*, 2014; Fitzpatrick and Katz, 2010).

Excessive and unprotected exposure to the sun is linked with cancer on the lip area. Sunlight, through actinic radiation, promotes cancer development along the vermilion border of the lip (Wood *et al.*, 2011). Sunlight-induced cancers are much more common in fair-skinned individuals exposed to the outdoor life than in individuals

with darker pigmentation, where it appears that darker pigment protects against actinic radiation damage (Amaro-Ortiz *et al.*, 2014).

People with a weakened immune system may have a higher risk of developing oral and oropharyngeal cancer (Coghill *et al.*, 2016). Human immunodeficiency virus (HIV) infection and organ transplant patients have a high incidence of developing oral cancer as compared to the general population (Junaid, 2015). Among HIV-positive immunocompromised individuals, HIV-associated oral malignancies have been reported where the disease manifests itself first in the oral cavity region (Bajpai and Pazare, 2010).

2.1.3 Pathogenesis of OSCC

During the tumorigenic process, tumor suppressor genes and oncogenes are involved (Jurel *et al.*, 2014). Tumor suppressor genes such as p53, PTEN and BRCA1 are responsible to promote tumour development when inactivated, while oncogenes such as HER2, EGRF and BRAF inhibitor are responsible to promote tumour development when activated (Khor *et al.*, 2013). Tumor suppressor genes can be inactivated through either mutation, loss of heterozygosity or deletion, or by epigenetic modifications (Schache *et al.*, 2011). On the other hand, oncogenes can be activated through gene overexpression caused by gene amplification, increased transcription or changes in structure due to mutations that lead to increased transforming activity (Knopf *et al.*, 2015).

Angiogenesis is an important factor for the cancer cell. It is a growth of new blood vessels around the cells, functioning to supply blood to the tumour cells. Through

blood, the cells will have enough oxygen and other essential nutrients to grow (Wadhwan *et al.*, 2015). Among all types of angiogenic factors, vascular endothelial growth factor (VEGF) expression, particularly VEGF-A and VEGF-C have been reported in OSCC and are dominantly associated with metastasis (Friedrich *et al.*, 2010; Kapoor and Deshmukh, 2012).

2.1.4 Clinical features of OSCC

The aetiology of OSCC is a multifactorial, sequential process that starts from normal epithelium to invasive and metastatic cancer (Bagan *et al.*, 2010). Cancer starts from potentially malignant lesions, such as oral leukoplakia and erythroplakia, followed by progression through mild, moderate, and severe degrees of epithelial dysplasia to carcinoma *in-situ* and finally to invasive SCC (Farah *et al.*, 2014). Erythroplakia is identifiable red patches while leukoplakia is white patches, a subtle, painless and asymptomatic lesion (Isaäc, 2015). In comparison between these two patches, erythroplakia have a higher risk of malignant transformation than leukoplakia (Shukla, 2014).

Other clinical presentations of oral cancer include non-healing ulcer with induration-fixed to underlying tissue, exophytic or endophytic growth, persistent, non-healing extraction, numbness of socket tongue or other areas of the mouth, swelling at jaw causing difficulty in wearing denture and hoarseness of voice (Bagan *et al.*, 2010; Prasad *et al.*, 2018; van Zyl and Bunn, 2012). At the late stage of cancer, bleeding, loosening of teeth, difficulties in swallowing (dysphagia), painful swallowing (odynophagia), speech impairment (dysarthria), loss of weight and development of a neck mass can be observed (Heijnen *et al.*, 2016; Son *et al.*, 2015). Neck mass is a sign

of lymph node metastasis where usually develops in the ipsilateral (on the same side) cervical lymph nodes, and distant metastasis to the lung, though any part of the body may be affected (Patil *et al.*, 2013).

2.1.5 Histological grading system of OSCC

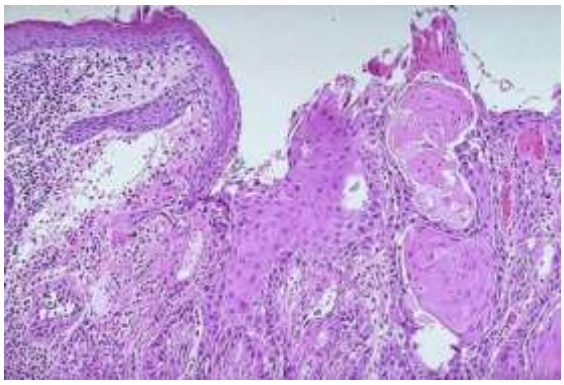
The first grading system was proposed by Broders which classified tumour into four different grades according to the degree of differentiation and keratinisation of tumour cells (Broaders, 1920). However, OSCC is typically a heterogeneous tumour that presents cell populations in different stages of differentiation. Therefore, Jakobsson *et al.* developed a multifactorial grading system that was later modified by Anneroth *et al.*. This system considers keratinisation, nuclear pleomorphism, number of mitoses, pattern and stage of invasion and lymphoplasmacytic infiltration within the entire thickness of the tumour (Anneroth *et al.*, 1987; Jakobsson *et al.*, 1973).

The grading systems have been continuously revised by experts to determine the most efficient method to predict patients' outcomes. Several years later, Bryne *et al.* introduced the concept where the more invasive area of the tumour, known as the invasive tumour front (ITF) would be the most relevant to prognosis. These authors proposed a method in which only part of the tumour was analysed using the same multifactorial criteria proposed previously (Bryne *et al.*, 1989). In 1992, Bryne *et al.*, demonstrated that the reproducibility of the system could be improved by excluding the mitotic count from the system while the prognostic value remained highly significant (Bryne *et al.*, 1992). The most recent WHO guideline suggested a grading system based on the degree of cell differentiation by grouping OSCC into three categories: well, moderately and poorly differentiated tumour. This system is typically

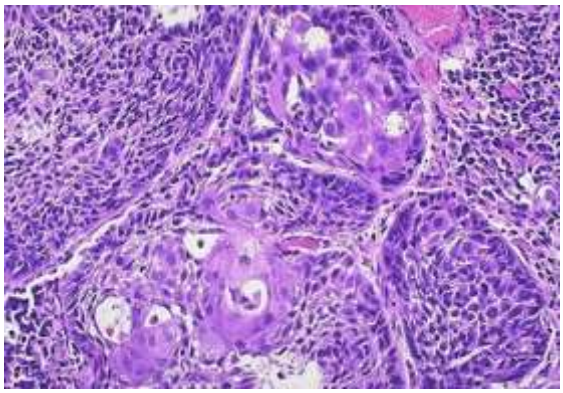
used in routine diagnosis and research; nevertheless, its prognostic factor is controversial in the literature (Wagner *et al.*, 2017).

Figure 2.1 showed the histopathological characterisation of SCC according to the degree of differentiation; well-differentiated tumour (grade 1), moderately-differentiated tumour (grade 2) or poorly-differentiated tumour (grade 3) (Krafts, 2009). In a well-differentiated tumour, the tumour cells resemble normal epithelial cells, arranged in an orderly stratification and heavy keratinisation can be found in pearl formations. In a moderately-differentiated tumour, the cells are less stratified, less keratinized and the tumour contains prickle cells. In grade 3, the cells are poorly differentiated and need further additional IHC cytokeratin staining (Alamgir *et al.*, 2016; Padma *et al.*, 2017).

A



B



C

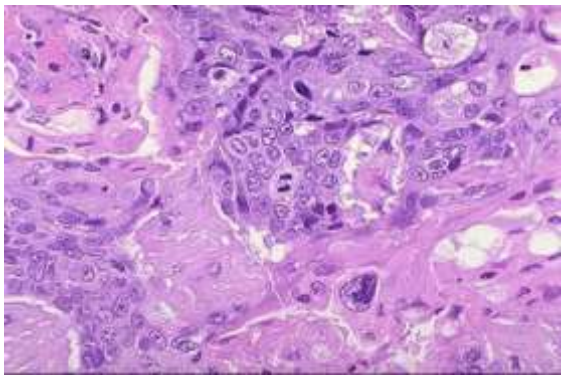


Figure 2.1 Histopathological characterisation of squamous cell carcinoma according to the degree of differentiation. A) Well-differentiated, B) Moderately-differentiated, C) Poorly-differentiated (adopted from Krafts, 2009).

2.1.6 Classification and staging of OSCC

To facilitate treatment planning and predictive outcome, tumour, node, and metastases (TNM) staging system is recommended. The TNM system is the most widely used system for cancer reporting in most hospitals and medical centres (Paul, 2012). Based on the TNM system, T (T; T0-T4) refers to the size and extent of the main tumour, N (N; N0-N3) refers to the regional lymph node metastasis and M (M; M0-M1) refers to distant metastasis from oral cancer to secondary sites such as lung, vertebra, femur, pelvis and liver (de Bree *et al.*, 2000). The stage is strongly correlated to the prognosis of the patient and is the main contributor to establishing the proper treatment strategy. The individual T, N and M values classify the tumour to either of four stages (stage I-IV), one where the higher stage is strongly associated with worse prognosis (National Cancer Institute, 2015). The staging of the tumour is associated with the timing of the diagnosis. The earlier the diagnosis, the lower the tumour stage, and hence, a better survival rate (83.7%) is noted compared with a lower survival rate with a late diagnosis, leading to a higher stage III-IV (38.5%) (Naseer *et al.*, 2016).

2.1.7 Treatment and Prevention of OSCC

2.1.7 (a) Treatment

Generally, treatments for early to moderate stage of OSCC (cancer stage I to III) are done by surgical and radiotherapy or in a combination of radiotherapy with chemotherapy. However, in Stage IV cancer, multidisciplinary non-surgical approaches are used to prolong survival and maintain the quality of life (Choi and Myers, 2008). The prognosis of HPV-OSCC patients is better compared to non-HPV-OSCC since they are more susceptible and respond better to the treatment. When both groups exempted for chemotherapy, the rate of successful treated HPV-OSCC was

82% compared to only 55% among the non-HPV-OSCC (Gillison *et al.*, 2000; Selcuk, 2016). *In-vitro* study using HPV positive cell line with the exposure of radiation showed increased in apoptosis and decreased in cell survival (Gupta *et al.*, 2009; Liu *et al.*, 2018). Recent studies are focusing on the reduction of toxicity-related treatment for OSCC patient that include dose reduction of radiotherapy, usage of cetuximab instead of cisplatin for chemoradiation, transoral robotic surgery (TORS) and immunotherapeutic strategies (Deschuymer *et al.*, 2018; Kofler *et al.*, 2014; Mirghani and Blanchard, 2017).

There is a growing amount of data supporting the hypothesis that HPV-OSCC has a better survival rate due to a higher sensitivity to chemotherapy and radiotherapy as compared to non-HPV-OSCC. Studies showed that the gene expression levels are significantly different (347 differentially expressed genes) in HPV-positive versus HPV-negative cancers. Differences are particularly among the genes involved in DNA regulation and repair, cell cycle regulation, and chemotherapy/radiotherapy-sensitivity. These differences allow for increased sensitivity to radiation in HPV-positive cells, hence lead to an improved response to radiation therapy (Liu *et al.*, 2018; Zhang *et al.*, 2014).

In radiotherapy, the standard dosage ranges from 60-70 gray (Gy) of ionising radiation and the reduction in radiation dosage results in less toxicity. In HPV-OSCC, the use of 54 Gy has successfully treated the patient. Apart from that, the reduction also decreases the cost of the treatment (Buckwalter *et al.*, 2007). For chemotherapy, the best agent for this strategy is by using cisplatin. It is the gold standard agent for head and neck squamous cell carcinoma (HNSCC) although severe complications are noted

on renal, hematologic, neurologic and gastrointestinal toxicities. Hence, for HNSCC, a less toxic agent such as cetuximab is used to replace cisplatin for toxicity reduction. This showed a promising result where cetuximab plus radiation was used in treating HNSCC patients (Kabolizadeh *et al.*, 2012). Moreover, analysis of the 5-year study suggested that cetuximab may be useful in HPV-positive patients (Bonner *et al.*, 2006). Besides, Phase I clinical trial at the University of Pittsburgh (UPCI 12-084) used the combination of ipilimumab (the targeted drug, monoclonal antibody specific to CTLA-4) plus cetuximab with radiation. Immunologic strategies are among the ongoing studies for HPV-OSCC and HPV+ cervical cancer, which involve immunomodulators (toll-like receptors), cytokines (IL-2, IL-12, GM-CSF and IFN-alpha), non-viral HPV peptide and protein vaccines, DNA vaccines, tumour cellular vaccine, dendritic cell vaccines, bacterial vectors, T cell therapy and targeted immunotherapy by a live viral vector (Gildener *et al.*, 2014).

2.1.7 (b) Prevention

Among the prevention strategies is the development of vaccines against HPV. Since 2006, three vaccines have been approved by the Food and Drug Association (FDA, USA) namely Gardasil, Gardasil9 and Cervarix. Gardasil is a quadrivalent vaccine used to immunise against HPV-16, 18, 6,11, while Cervarix is a bivalent vaccine for HPV-16, 18. Gardasil9 is the latest vaccine approved in 2014 for immunisation against HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58 (Satterwhite *et al.*, 2013). All these three vaccines have one thing in common, they protect against HPV-16 and 18. These type of HPV cause about 70% of cervical cancers and the majority of other HPV-attributable cancers (Ferlay *et al.*, 2018). These prophylactic HPV vaccines are 90% to 100% effective in preventing HPV infections and associated anogenital

precancerous lesions (Barr *et al.*, 2008, FDA, 2014). However, these vaccines efficacies against oral HPV infection and related diseases is unknown.

WHO recommends the young teenagers (at ages 12) to receive HPV vaccination before any sexual activity for preventing cervical cancers (Markowitz *et al.*, 2009). Three intramuscular doses in 6 months for men and women are recommended. The USA was the first country started the vaccination programme in 2006 for their 12-17 school girls, followed by Australia in 2007, United Kingdom in 2008 and Sweden in 2010 (Chandra *et al.*, 2011). Malaysia started the vaccination program in 2010. These HPV vaccines are meant for prevention of cervical cancer with the prevention rates up to 70% (Ezat *et al.*, 2013).

A recent report (June 2017) from the National Cancer Institute (NCI) showed that HPV vaccination may decrease the oral HPV infection. When the USA started the first vaccination program in 2006, 2600 people were selected to form a study on oral cancer with HPV association. The results showed that the prevalence of oral infections with HPV was 1.61% in unvaccinated young adults versus 0.11% in vaccinated young adults; an 88% reduction in HPV prevalence with vaccination (Chaturvedi *et al.*, 2018).

Besides vaccination programme, prevention by education and social awareness also play an important role. As HPV-OSCC is a sexually transmitted disease, the protection methods like condoms and barrier protections during the sexual activity must be used. Social awareness and education about the role of the HPV in the development of OSCC and the protection methods must be achieved (CDC, 2016).