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DETECTION OF HUMAN PAPILLOMAVIRUS TYPES IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

ATANG BULANE

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DETECTION OF HUMAN PAPILLOMAVIRUS TYPES IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

by

ATANG BULANE

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in the

Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein

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February 2019

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DECLARATION

DECLARATION

I, Atang Bulane certify that the thesis hereby submitted by me for the degree PhD in Virology at the University of the Free State is my independent effort and had not previously been submitted for a degree at another university/faculty. I furthermore waive copyright of the thesis in favour of the University of the Free State.

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LIST OF ABBREVIATIONS

aa	Amino acid
ANRIL	Antisense noncoding RNA in the INK4 locus
ASR	Age standardised incidence rates
ATP	Adenosine triphosphate
Bcl	B-cell lymphoma
Bcl-2	B-cell lymphoma 2
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
CDK	Cyclin-dependent kinase
CDK2	Cyclin-dependent kinase Inhibitor 2
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
cDNA	Complemetary DNA
CIN	Cervical intraepithelial neoplasia
CK	Cytokeratin
CpG	Cytosine phosphate guanine
Ct	Cycle threshold
CyP	Cyclophilins
CXCR2	Chemokine receptor 2
CXCR4	Chemokine receptor 4
CXCR7	Chemokine receptor 7
C-terminus	Carboxyl-terminus
DNA	Deoxyribonucleic acid
DSG3	Desmoglein 3
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
E1-8	Early gene 1-8
E2F	E2 factor
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS5RNA	Growth arrest-specific 5RNA
GSK	GlaxoSmithKline
G0-2	Gap0-2
HE4	Human epididymis protein 4
HER2	Human epithelial growth factor 2
hDlg protein	Human disc large protein
HN	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HOTAIR	HOX transcript antisense intergenic RNA

HOTTIP	HOXA transcript at the distal tip
HOXC	Homeobox C
HOXD10	Homeobox D10
hScrib protein	Human Scribble protein
HPV	Human papillomavirus
ICTV	International Committee on Taxonomy of Viruses
ILs	Interleukins
ISH	In situ hybridization
LSCC	Laryngeal squamous cell carcinoma
LCR	Long Control Region
Linc RNA-21	Long intergenic non-coding RNA p21
LncRNA	Long non coding RNA
LSD1	lysine-specific histone demethylase 1
L1-2	Late gene 1-2
M	Mitosis phase
MAGE	Melanoma-associated gene
MCM7	Minichromosome Maintenance Complex Component 7
MEGA	Molecular Evolutionary Genetics Analysis
MiRNA	MicroRNA
MMPs	Metalloproteinases
mRNA	messager RNA
MUPP-1	Multi-PDZ domain protein 1
ncRNA	Non coding-RNA
NGS	Next generation sequencing
N-terminus	Amino-terminus
OPSCC	Oropharyngeal squamous cell carcinoma
ORF	Open reading frame
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
PHC	Personalised health care
PiRNAs	PIWI-interacting RNAs
PTEN gene	Phosphatase and tensin homolog
pRB	Retinoblastoma
PRC2	Polycomb Repressive Complex 2
ProGRP	Progastrin-releasing peptide
PV	Papillomavirus
$P16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
P670	Promotor 670
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus

RT-PCR	Reverse transcription PCR assay
RT-qPCR	Relative quantitative real-time
S	Synthesis phase
SA	South Africa
SCC	Squamous cell carcinoma
SiRNAs	Small interfering RNAs
SNPs	Single nucleotide polymorphism
TGN	Trans-Golgi network
TP53	Tumour protein p53
URR	Upstream Regulatory Region
WHO	World Health Organisation
Xist	X-inactive specific transcript
YYI	Ying-Yang
β	Beta
α	Alpha
γ	Gamma

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SUMMARY

Human papillomaviruses (HPVs) are the aetiologic agents for diverse clinical conditions ranging from benign lesions to cervical cancer. HPVs have also been associated with head and neck squamous cell carcinomas (HNSCC), in both men and women. HNSCC refers to carcinomas that occur in different sub-sites of the head and neck including the larynx, oral cavity, oropharynx and sinonasal cavity. Studies have shown that the prevalence of HPV infection varies in different population groups from geographically distinct regions, with much higher burden documented in developed countries compared to less developed countries. For instance, HPV-associated HNSCC age-standardized incidence rates (ASR) of over 1.25 per 100,000 were reported in countries in Europe and Northern America. However, the prevalence in less developed countries could be an underestimation due to limited studies. South Africa has a high incidence of oral squamous cell carcinoma, however not much is known about the prevalence of HNSCC cases due to HPV, or the HPV types associated with HNSCC.

HPV associated HNSCCs have a better prognosis compared with HNSCCs caused by other agents such as smoking and alcohol. Currently there are limited tests and biomarkers for early diagnosis and prognosis of the cancer. Therefore, a better understanding of the molecular pathways of carcinogenesis is essential in order to select accurate predictive biomarkers and for the development of effective treatment. Technological advancements in molecular science have improved the discovery of potential biomarkers in cancer, including non-coding RNAs (ncRNAs) which are divided into small non-coding (<200 bp) and long non-coding (>200 bp). Most studies on biomarkers for application in diagnosis of cancers have focused on small non-coding RNAs using tissue samples. While little is known about the association of long non-coding RNAs such as antisense noncoding RNA in the INK4 locus (ANRIL), HOXA transcript at the distal tip (HOTTIP) and HOX transcript antisense intergenic RNA (HOTAIR) with use of less invasive samples such as blood.

Therefore, the aims of the present study were firstly to determine the prevalence of HPV in head and neck cancer using archived specimens from patients with histologically confirmed HNSCCs submitted to the Department of Anatomical Pathology, Universitas Academic Laboratory in Bloemfontein, South Africa, between January 2004 and December 2014. HPV DNA was detected using conventional nested multiplex PCR targeting the L1 region and

confirmed using in house type specific E6 primer pairs and a heminested PCR. A total of 780/994 samples tested were found to have intact DNA based on amplification of a partial reference gene. The 780 samples were further tested for the presence of HPV DNA. In total 57/780 (7.3%) were positive for the HPV DNA. High risk (HR) HPV types were more frequently detected than low risk (LR) HPV types. HPV16 was the most frequently detected HR type, and was detected in 26/57 (45.6%) HPV DNA positive biospies.

Secondly, the study determined the phylogenetic relatedness and single nucleotide polymorphisms (SNPs) among the high risk HPV isolates using a Maximum Likelihood method for construction of phylogenetic trees. To determine the usefulness of partial L sequence data for identifying HPV lineages, a comparison was made using sequence data retrieved from GenBank for complete HPV genomes, complete L1 genes and partial L1 genes. Based on the outcome the ability to differentiate HPV lineages/sub-lineages was determined. HPV31 and 52 were classified with the L1 partial sequence data. With regards to HPV18 isolates, the partial L1 region was not diverse enough to identify sub-lineages. For HPV45, data for the partial L1 gene was able to discriminate sub-lineages within lineage B but not clearly within the A lineage. Similarly, for HPV16 and 33 clear discrimination of sublineages was not possible. However overall, although the partial L1 sequence data was too conserved for sub-lineage discrimination, it was possible to resolve lineages for some of the HPV types, particularly HPV31 and 52.

The identification of biomarkers for early detection of cancers, including HNSCC, would have benefit for any patient. Hence a previously described potential biomarker, HOTAIR was investigated. The expression levels of HOTAIR in laryngeal squamous cell carcinoma (LSCC) patients and healthy volunteers were determined using real time quantitative PCR (RT-qPCR) assay. Patient biopsies were also screened for HPV DNA. Total RNA was extracted from whole blood of patients presenting to Universitas Academic Hospital with histologically confirmed LSCC and healthy volunteers, while DNA was extracted from the LSCC biopsies. Most of the patients, 80% (4/5) had an advanced tumour (T3 and T4), with advanced stage cancer (stages III and IV). The majority of the patients 80% (4/5) did not show lymph node invasion nor metastasis. Histopathology revealed moderately differentiated squamous cell carcinoma in one patient while well differentiated histopathology was seen in the rest of the patients. The mean expression level of the biomarker was found to be significantly higher ($p=0.03$) in blood collected from patients with laryngeal cancer compared to controls (6.39± 2.13 versus 10.19±

2.24, respectively), with a 13.9-fold overexpression in laryngeal cancer patients' blood as compared to expression in blood from healthy participants. HPV DNA was detected in 1/5 biopsy samples. The results suggest that further investigation of expression levels of HOTAIR as a potential biomarker is warranted.

Keywords: HNSCC, HPV types, Prevalence, Variants, Biomarkers

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The first papillomavirus was discovered in 1933 and was called Shope papillomavirus (PV) or cottontail rabbit PV as it was identified from warty growths on cottontail rabbits (Shope and Hurst, 1933; Rous and Beard, 1935). It was shown that the virus exhibited neoplastic potential in domestic rabbits (Shope and Hurts, 1933; Rous and Beard, 1935). In 1949, the morphology of PV was described using electron microscopy (Strauss *et al*., 1949) and subsequently human papillomaviruses (HPV) were identified as causative agents of cervical cancer (zur Hausen, 2009). Later, there was evidence of women with cervical cancer having a 5-6 fold increased risk of developing oral cancer (Newell *et al*., 1975; Syrjänen *et al*., 1983). The first reports showing the association of HPVs with tongue and other oropharyngeal carcinomas were published in 1985 (de Villiers *et al*., 1985; Löning *et al*., 1985). Since then, numerous studies worldwide have confirmed the association.

1.2 Classification of Human Papillomavirus

PV, were initially grouped with polyomaviruses into one family called the *Papovaviridae* (Van Regenmortel *et al*., 2002). The classification was based on similar nonenveloped viruses with circular double-stranded DNA genomes, but later it was established that the two viral groups have different genome sizes and genome organisation, and that there were no major nucleotide and amino acid similarities (Bernard *et al*., 2010). Hence, they were separated into two families, *Papillomaviridae* and *Polyomaviridae*, by the International Committee on the Taxonomy of Viruses (ICTV) in their 7th report (Van Regenmortel *et al*., 2002). Papillomaviruses have a broad range of hosts including mammals and birds (Herbst *et al*., 2009; Lange *et al*., 2011).

In 2004, sixteen PV genera were identified and named using the Greek letters from alpha to pi (de Villiers *et al*., 2004; de Villiers, 2013). The sixteen genera include five human PVs (Alpha, Beta, Gamma, Mu and Nu), two bird PVs (Eta and Theta) and the nine PVs isolated from

different mammals (Bernard *et al*., 2010). There are more than 280 PV types and over 200 HPV types based on complete genome sequence data (de Villiers *et al*., 2004; Bernard, *et al*., 2010; Munday, 2014; Munday *et al*., 2015). Most of these HPV types group within three genera, α-papillomavirus, β-papillomavirus and γ-papillomavirus (de Villiers *et al*., 2004). Classification of the HPV into family, genus, species, types, subtypes and variants was largely based on the L1 open reading frame (ORF) variability since it is the most conserved gene within the genome of the virus (de Villiers *et al*., 2004; de Villiers, 2013). More recently complete genome analsyis has been used to confirm genetic relationships.

Classification of PVs is based on nucleotide sequence identity rather than serotype because the virus does not elicit robust antibody responses (Chan *et al*., 1995; de Villiers *et al*., 2004). HPVs are classified under different genera if they share less than 60% of the nucleotide sequence within the L1 ORF region (de Villiers *et al*., 2004). Within the genus, viral species share 60% to 70% nucleotide homology. A novel HPV type is considered if the whole genome has been cloned and the L1 ORF sequence differs by more than 10% from the closest known HPV type (de Villiers *et al*., 2004). HPV types are further clinically categorised as high and low risk types based on the ability of the virus to cause malignancy. HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73 and 82 are classified as high risk types according to World Health Organisation guidelines, while HPV6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 are classified as low risk. HPV26, 53 and 66 are listed as probable high risk (Muñoz *et al*., 2003). Differences in L1 gene identification of between 2% and 10% define molecular subtypes and less than 2% defines a variant (de Villiers *et al*., 2004; Bernard *et al*., 2006; Calleja-Macias *et al*., 2005; de Villiers, 2013). The viral variants are the result of nucleotide substitutions in a number of restricted positions within the whole genome.

HPVs are commonly known to cause a number of neoplasias in the anogenital tract, including cervical, anal and vulvar cancer (Backes *et al*., 2008; De Vuyst *et al*., 2009; de Martel *et al*., 2017; Hartwig *et al*., 2017). Recently there have been clinical and molecular studies that show the association of HPV with head and neck squamous cell carcinomas (HNSCCs). The molecular proof of infection with this virus, is based on the detection of HPV DNA and the transcription of the oncogenic genes (E6 and E7), the presence of integrated HPV DNA in the host cellular genome and the presence of considerable HPV DNA copy numbers in lesions (Chai *et al*., 2015). HNSCCs include tumours of different origins classified by the anatomical

sub-sites, with five times higher likelihood of HPV causing cancer in the oropharynx than the oral cavity, larynx or hypopharynx (Combes and Franceschi, 2014).

Based on HPV tropism, the virus is classified as cutaneous or mucosal, while based on oncogenic potential, the mucosal viruses are further sub-divided into low risk, high risk and probable high risk viral types as described above (de Villiers *et al*., 2004; Doorbar *et al*., 2015). Detection of HPV DNA in HNSCC samples may not always mean the virus is active. According to some studies done on E6 and E7 expression, E6 and E7 mRNA transcripts were detectable in only 11-50% of HPV DNA positive HNSCCs (Wiest *et al.*, 2002; Braakhuis *et al.*, 2004). The presence of HPV DNA in HNSCC samples without oncogenic E6 and E7 mRNA might suggest another mechanism responsible for the carcinoma. However, detection of HPV DNA is still used as an indication of prevalence of HPV and association with HNSCC particularly in instance where detection of mRNA transcripts may be limited by tissue type available such as archival tissues. The cellular tumour suppressor protein $pl6^{1NK4a}$ (p16) has been investigated as a potential biomarker for transforming HPV infections (Klussmann *et al*., 2003; Wang and Roden, 2013). Biomarkers according to the National Cancer Institute are a "biological molecule found in blood, other body fluids, or tissue that is a sign of a normal or abnormal process or of a condition or disease" (http://www.cancer.gov/dictionary/?CdrID=45618). Biomarkers are useful in terms of diagnosis and prognosis for differentiating affected patients from a person without the disease. This differentiation could be due to a number of factors such as germline or somatic mutations, transcriptional changes and post-translational modifications. Although considered a biomarker for cervical dysplasia, the role of p16 immunostaining as a biomarker of HPV infection in HNSCC has still to be clarified.

1.3 Epidemiology of HPV associated head and neck cancer

The prevalence of HPV in HNSCCs from geographically distinct regions has been shown to vary. According to recently published studies, about 38,000 to 45,000 cases of head and neck SCC annually are due to HPV (Castallsague *et al*., 2017; de Martel *et al*., 2017), with the majority of cases reported in developed regions such as United States (59.3%), Europe (31.1%) and Asia (17.5%) (Castallsague *et al*., 2016; Anantharaman *et al*., 2017). An Age Standardised Incidence Rate (ASR) of over 1.25 per 100 000 was reported in Northern America and Europe, while places such as China and Africa show a lower prevalence (Kreimer *et al*., 2005; Termine *et al*., 2008; de Martel *et al*., 2017). Studies on the prevalence of HPV in HNSCCs in Africa

are limited compared to North America and Europe, therefore the lower prevalence may not be a true reflection of the situation. Based on studies done in the western world, HNSCCs associated with HPV tend to affect younger male patients (less than 60yrs) who are also non-smokers (Gillespie *et al*., 2009; Garnaes *et al*., 2015).

In Sub-Saharan Africa countries, the burden of HNSCC has been reported as increasing (Ferlay *et al*., 2012; de Mortel *et al*., 2017), though the incidence of the HPV associated head and neck SCC in the region is still mostly unknown. In South Africa (SA), there is limited data on the prevalence of HPV in HNSCCs, with the majority of studies based on a small number of samples and focused on the oropharynx and oral cavity. According to these studies, HPV is implicated as a possible aetiological agent in HNSCCs (Hille *et al*., 1986; Matsha *et al*., 2002; Davidson *et al*., 2014; Müller *et al*., 2016). Therefore, it is apparent that a much larger analysis of the association of HPV with HNSCCs that focuses on all sites of the head and neck is needed to confirm the prevalence and the HPV types, lineages and sub-lineages that are circulating in our setting. To further improve the prognosis of patients with HNSCCs in our setting, studies that include investigating the molecular markers useful for routine use in managing patients with HNSCCs are also needed.

Geographic variations in incidence of head and neck SCC are mostly influenced by behaviour and various host and viral factors. These include immunological status, sexual activity in particular high rate of oral sex, multiple sex partners, gene polymorphisms, persistent infection of high risk HPVs, levels of oncogenic (E6 and E7) gene expression and levels of episomal compared to integrated viral genome in an infected host cells (Bernard *et al*., 2006; Gillison *et al*., 2008; D'Souza *et al*., 2009; Gao *et al*., 2014). The HPV virus is mostly associated with the three anatomic sub-sites of the head and neck, which are oropharynx, oral cavity and larynx (de Martel *et al*., 2017). According to de Martel and associates, it was reported that globally the highest incidence of the cancer is seen in oropharynx which includes, tonsils and the base of the tongue causing 29 000 cases per year, hence 30.8% rate of occurrence. In the oral cavity the virus has been detected in 4 400 cases, with 3 800 cases detected in the larynx annually (de Martel *et al*., 2017). Cancer patients with tumour at the oropharynx caused by HPV, have reported to have a considerably better overall survival, consequently good prognosis as compared to patients with HPV-negative oropharyngeal cancer (Ang *et al*., 2010; Marur *et al*., 2010; Olshan, 2010; Elrefaey *et al*., 2014). Among the high risk HPV types, there is high predominance of HPV16 and 18 in head and neck cancer, contributing towards an estimated

85% of the cancers (de Martel *et al*., 2017). In another review by Ragin and associates, the HPV prevalence in multiple races from different geographic regions was determined. The meta-analysis study was comprised of Asians from Asia, United States of America (USA) and Australia, whites from Europe, USA and Australia and black patients from USA, Europe and Australia. HPV16 or 18 was detected in 61% of the HPV infections in the oropharynx in whites, while 58% was detected in black oropharyngeal patients and lower rate of 25% was reported in Asians. The review further reported on the unexplained observation of nearly 15 times higher prevalence of HPV18 among black patients compared to white patients where prevalence of 1.1% was reported in oropharyngeal and 1.5% in non-oropharyngeal cancer (Ragin *et al*., 2017).

1.4 Human papillomavirus protein function

Human papillomaviruses encode for multiple viral proteins with different functions (Figure 1.1) (Jimenez *et al*., 2014). The virus relies on this small set of proteins to cause the infection and malignancy. Therefore, each protein performs multiple functions in order to regulate cellular gene expression, control cellular pathways, prompt DNA synthesis and prevent immune responses to viral presence. For example, the ability of HPV to cause cancer has been shown to be associated with the actions of the E6 and E7 proteins **(**Scheffner *et al*., 1990; Roman and Munger, 2013; Vande Pol and Klingelhutz, 2013**)** and the E5 in bovine papillomavirus is the primary transforming protein, while in HPV the E5 protein possesses weak transforming activity (DiMaio and Mattoon, 2001).

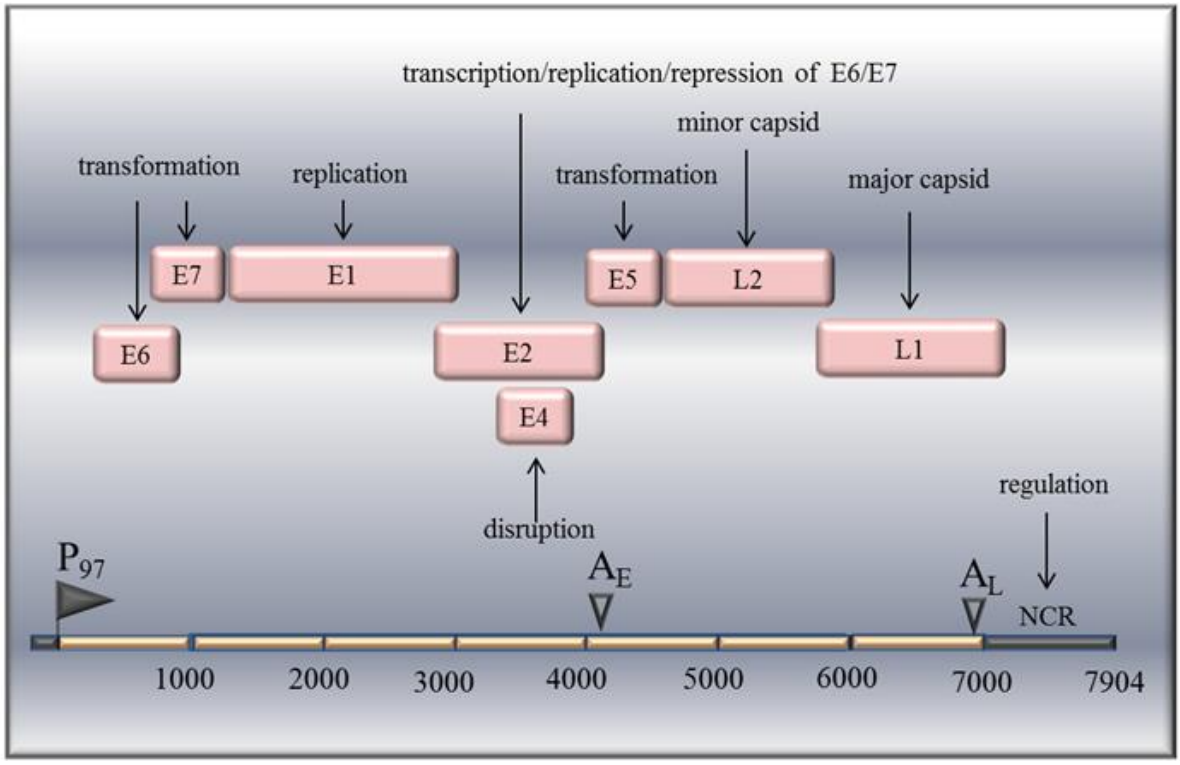

Figure 1.1: Schematic of HPV genome organization and viral proteins (Jimenez *et al.*, 2014)

During replication, E1 and E2 are the initial proteins to be expressed. The E1 protein is a 70 kDa polypeptide, the second most conserved sequence among PV genomes. The protein made up of three functional domains, which are: the N-terminus that induces cyclin-dependent kinase Inhibitor 2 (CDK2) phosphorylation, the C-terminus that acts as adenosine triphosphate (ATP) dependent helicase, and the central domain that is used to bind E1 to E2 protein to form a complex protein (E1-E2) (Garcia-Vallve *et al*., 2005; Wallace and Galloway, 2014). The formed complex then binds to the viral origin of replication (E1-E2-ori) and recruits cellular polymerase and accessory proteins to initiate replication (Figure 1.2) (Enemark *et al*., 2000; Schuck and Stenlund, 2015). The E1 protein is also known as the replication initiation helicase, which is responsible for viral DNA replication and exists as a monomer in solution (Ustav and Stenlund, 1991; Stenlund, 2003).

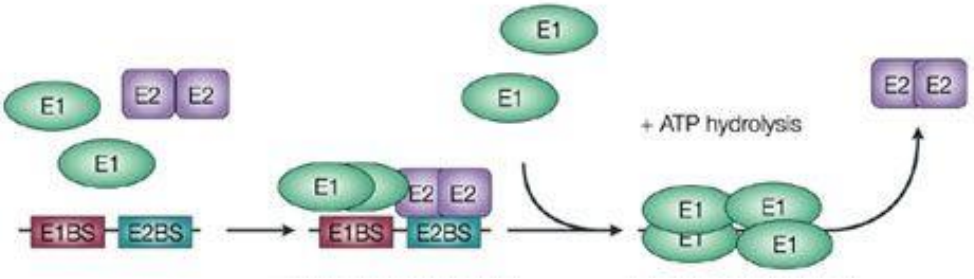

Figure 1.2: Steps in the initiation of HPV DNA replication (Stenlund, 2003)

The papillomavirus E2 protein is a multifunctional protein involved in viral replication, segregation and it is also a major viral regulator of transcription of the viral genome in infected cells (Pyeon *et al*., 2009). The E2 protein functions in a concentration dependent mode within the cell. At high concentration, it functions as a repressor while at low concentration it functions as an activator of homologous and heterologous promoters with E2 binding sites (Abroi *et al*., 1996; Schweiger *et al*., 2007; Doorbar *et al*., 2015). In transcription, the E2 protein functions as a transcription factor and ensures the HPV viral load is kept at a low concentration during the early stage of the viral life cycle (Steger and Corbach, 1997). During cell segregation, the E2 protein ensures that the newly replicated viral genomes survive basal cell division before mitosis.

The E4 ORF overlaps with the hinge-encoding region of E2. The gene does not have its own initiation codon and uses the E2 start codon (Longworth and Laimins, 2004). The protein is linked with multiple functions within the HPV infected cells, such as involvement in the alteration of the cytoskeleton network, which is responsible for structural integrity within the cell (Doorbar *et al*., 1991). The E4 protein also induces apoptosis in terminally differentiated cells using a leucine cluster whereby it interacts with the cellular mitochondria, which are thought to induce apoptosis, therefore ensures viral release from host cells (Raj *et al*. 2004; Yajid *et al*., 2017). The E4 protein also ensures maximum viral replication by inhibiting host genome replication during the viral-induced synthesis (S) phase of the cell cycle (Roberts *et al*. 2008).

The E6 and E7 proteins from high risk HPV types act as oncoproteins by disrupting genome stability to increase the chances of the infection progressing to malignancy (Duensing *et al*.,

2000), but no such function is seen with the same proteins from low risk HPV types. These proteins cause progression to malignancy by causing an impairment in cell cycle regulation and cell maturation (Gewin and Galloway, 2001; Barrow-Laing *et al*., 2010; Fu *et al*., 2010; Brimer *et al*., 2017). The two oncoproteins encode zinc finger proteins that interact with several cellular proteins to favour a replicative state within differentiated epithelial cells. The oncoproteins have structural similarities such as multiple domains of the Cys-x-x-Cys zinc-binding motif (Cole and Danos, 1987). These oncoproteins have different cellular functions with E7 assumed to be a 'major' transforming protein, while E6 has the complementary transforming effect (Chen *et al*., 1995; Flores *et al*., 2000).

HPV16 E5 proteins are small, transmembrane hydrophobic proteins, made up of about 83 amino acids (aa), and the ORF is transcribed from the episomal form of the viral DNA (zur Hausen and de Villiers, 1994; DiMaio and Mattoon, 2001; DiMaio, 2014). This protein is not expressed in all HPV genera, for example α-HPVs encode and express the E5 while the β-HPVs do not (Bernard *et al*., 2010). The E5 gene sequence is normally deleted during HPV integration into the host genome (DiMaio and Mattoon, 2001) therefore the protein is believed to exert its carcinogenic effects in early stages of the viral infection. The protein transforms the cell by interacting with the 16 KDal subunit of vacuolar-ATPase(V-ATPase), hence disrupting acidification of the endosome (Kim *et al*., 2010; Hemmat and Baghi, 2018)

The L1 and L2 HPV proteins are the two capsid proteins, where L1 is a major protein with the most conserved ORF and L2 is the minor capsid protein (Kirnbauer *et al*., 1993; Wang and Roden, 2013). Because of the conserved ORF, L1 is normally used in virus classification (de Villiers, 2013; Haga *et al*., 2013). The L1 protein can self-organise, resulting in the pentameric structure that forms the viral capsid (Ribeiro-Müller and Müller, 2014). These capsid proteins are expressed late in the viral life cycle which takes place in the highly differentiated suprabasal cells (Kirnbauer *et al*., 1993). After genome amplification, the HPV genome is packaged in chromatin into the viral capsid which is made up of both the L1 and L2 proteins (Kirnbauer *et al*., 1993; Johansson *et al*., 2012). It is also hypothesised that both the proteins accompany the viral genome to the host nucleus during infection entry and colocalise in the nucleus of infected cells after mitosis is completed (DiGiuseppe *et al.,* 2017).

1.5 Papillomavirus life cycle

All PVs follow similar life cycles, where the virus infects a specific type of epithelial cell called basal cells (Harwood *et al*., 2000; Hama *et al*., 2004). These cells serve as a protective barrier against the external environment, as they are part of cutaneous and mucosal tissue (skin, mouth, oesophagus and the anogenital tract) (Madison, 2003). The basal cells make up the bottom part of the epithelial layer and are capable of undergoing mitotic division in healthy epithelium **(**Hummel *et al*., 1992**)**. In a host, during mitosis, one daughter cell moves up through the epithelium to replace dead cells shed from the surface. As the cells move from the basal into the suprabasal layer, this marks the end of cell cycle and beginning of terminal differentiation and therefore the loss of the nucleus (Jones *et al*., 1997). The cell cycle occurs in four phases; the G1 phase where the cellular growth takes place, the S phase where host DNA replicates, the G2 phase where the cell prepares for division and the M phase where the chromosomes are separated and the new daughter cells are formed (Doorbar, 2005)

Apart from the E1 replication enzyme, PVs do not have their own replication machinery and therefore depend fully on the host replicative enzymes for propagation (Bedell *et al*., 1991; Pinidis *et al*., 2006). As a result, in order for the HPV to have successful infection, the virus must link their productive life cycle to that of the host, first by gaining entry into the basal keratinocyte cells (Doorbar, 2005; Lazarczyk *et al*., 2009). After entry into the cell, the virus takes over the host cell cycle and directs the host replicative system to achieve its own life cycle. The HPV life cycle is made up of three stages, namely the establishment, maintenance and productive stages (Figure 1.3).

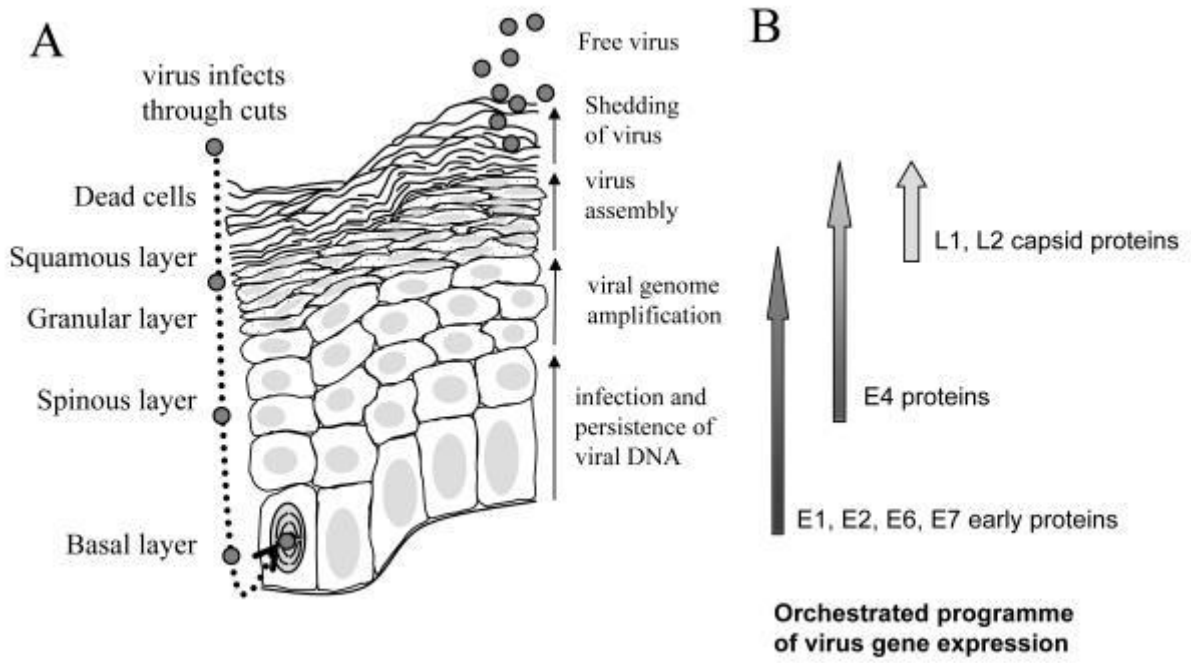

Figure 1.**3**: Replication of HPV in epithelial tissue (Graham, 2010)

The establishment stage is the first stage of the viral life cycle that occurs in the nucleus of the infected basal layer and the viral genome is amplified and remains as multicopies of circular extra-chromosomal (episomes) elements (Egawa, 2003). The second stage is the maintenance stage, where the viral genome replicates once in a cell cycle in proliferating basal layer keratinocytes (McBride *et al*., 2006) and in case of the high risk HPVs, the E6, E7 oncogenic transcripts are hardly detectable (Gadducci *et al*., 2013). In the last stage, which is the productive stage, the keratinocytes enter the stratum spinosum where the virus is exponentially amplified and packaged into progeny virions (Gadducci *et al*., 2013).

1.6 Pathogenesis of papillomavirus infection

The PVs target the undifferentiated multiplying basal cells of the epithelial mucosa. How the PVs gain entry into the basal cells of the host is still not clear and therefore remains an on-going area of research (Horvath *et al*., 2010). However, it is speculated that the virus entry takes place in a number of ways such as micro-wounds, hair follicles (Doorbar, 2005) and via the infection of the columnar cells in the case of the cervical infections (Boxman *et al*., 2001). In the case of de novo infection, the virion binds to the extracellular matrices which induces a conformational change and proteolysis occurs in order for the virion to bind to a coreceptor on the cell surface, resulting in particle uptake into the cell. The virus is internalised into the basal

cell by caveolae-mediated/clathrin-dependent endocytosis forming a compartment (endosome). The endosome causes the breakage of the disulfide cross-links of the L1 protein and the dissociation of the capsid by the acidic environment of the endosome and the detachment of L1 from L2-DNA complex by host cyclophilins (CyP) (Li *et al*., 1998; Day *et al*., 2004; Bienkowska-Haba *et al*; 2012; Doorbar *et al*., 2015; Senapati *et al*., 2016). The virus then moves to the trans-Golgi network (TGN) away from the lysosome/endosome (Day *et al*., 2013; Lipovsky *et al*., 2013). The movement is facilitated by minor capsid protein L2, by inserting itself into the cellular membrane then interacting with trafficking factors like retromer complex (DiGiuseppe *et al*., 2015; Popa *et al*., 2015). The viral DNA is delivered into the nucleus when the nuclear breakdown occurs during mitosis (Pyeon *et al*., 2009; Aydin *et al*., 2014; DiGiuseppe *et al*., 2016).

The virus then establishes productive infections in such a way that the viral life cycle is linked to the differentiating program of the infected host cell (Lambert, 1991; Ustav and Stenlund, 1991). Inside the nucleus of the basal cells, the virus stimulates a brief period of replication to increase the number of infected cells (Longworth and Laimins, 2004; Doorbar, 2006). This genome exists as extra-chromosomal episomes. At this stage, viral transcription is controlled by an early promoter (p97) within the long control region (LCR) via the E1 and E2 proteins (Lambert, 1991; Ustav and Stenlund, 1991).

The genomic regions of the virus responsible for the progression of the infection to tumour are the E6 and E7 transforming genes (Chen *et al.*, 1995; Flores *et al.*, 2000; Woodman *et al*., 2007; Howard and Chung, 2012). There are a number of possible mechanisms that the virus uses to transform cells, which include the infection of the host with large amounts of virus, ensuring that the virus will be capable of producing enough E6 and E7 oncoproteins (Smeets *et al*., 2007; Jung *et al*., 2010; Holzinger *et al*., 2012). In infections with a low viral load, as seen in some cervical cancers, mutations in repressive areas like the region Ying-Yang (YYI) are present. These mutations result in continued expression of E6 and E7 or production of more stable "chimeric" RNA, therefore resulting in greater synthesis of these oncoproteins (Schwarz *et al*., 1985; Wentzensen *et al*., 2004**).** In other tumour cells, mainly in the tonsil crypt epithelium, viral genomes are integrated into the host DNA therefore interrupting the E2 gene which is responsible for the inhibition of the E6 and E7 transcription by binding to the LCR (Begum *et al*., 2005). This leads to an elevated expression of the E6 and E7 oncogenes, resulting in immortalisation of the infected cells and the inhibition of the differentiation of

these cells (Jones *et al*., 1997; Park *et al*., 2011). Unlike low risk E6 and E7, high risk E6 and E7 proteins bind with high affinity to host cells proteins and therefore disturb the normal epithelial differentiation and apoptosis through the stimulation of cellular proliferation, DNA synthesis and inhibition of cell cycle control (Jones *et al*., 1997; Klingelhutz and Roman, 2012).

The E6 protein performs numerous functions leading to tumour formation, such as the degradation of the tumor protein p53 (TP53), which mediates cell cycle arrest by blocking the progression at the G1/S checkpoint and therefore abolishes this control (Thomas *et al*., 1999). The protein interacts with PDZ proteins such as multi-PDZ domain protein 1 (MUPP-1), human scribble protein (hSCRIB) and human disc large protein (hDlg) to stimulate cell proliferation. This increases the lifespan of infected cells by the activation of telomerase **(**Lee *et al*., 1997; Kiyono *et al*., 1998; Lee *et al*., 2000; Nakagawa and Huibregtse, 2000; Mischo *et al*., 2013; Rampias *et al*., 2014).

The E7 protein causes inactivation of the retinoblastoma (pRB) protein family. Therefore, disrupting its interaction with the E2 factor (E2F) at the G1 cell cycle stage, resulting in the release of the E2F transcription factor and more upregulation of the cell cycle genes, therefore disturbing the cell cycle control check points (Funk *et al.*, 1997; Jones *et al.*, 1997; Noya *et al.*, 2001; Liu *et al*., 2006). Inactivation of pRB also results in overproduction of p16 tumour suppressor protein, hence its use as a surrogate marker for detection of HPV infections (Bose *et al*., 2005; Marur *et al*., 2010; Schlecht *et al*., 2011; El-Naggar and Westra, 2012). The E7 protein also interacts with deacetylases, cyclins and cyclin-dependent kinase in order to alter cell control mechanism (Wang *et al*., 2013). Activation of DNA synthesis and cell replication mechanisms are then triggered, which are commonly inactive in mature epithelial cells, stimulating pathological cell growth which is called differentiation-dependent viral DNA amplification and takes place in the suprabasal cell (Bedell *et al.*, 1991).

After amplification of the genome, the virion is assembled. The L2 proteins are expressed first from the late promoter in the E7 ORF (p670) and help with the assembly of the L1 proteins (Zhou *et al*., 1991). The high specificity of DNA-binding of the E2 protein is used to aid in encapsidation of the viral genome (Day *et al*., 1998). The virion assembly takes place in the upper strata of the epithelial cells (Bedell *et al*., 1991; Hummel *et al*., 1992; Grassmann *et al*., 1996; Smith *et al*., 2007). The virions move to the squamous cells and are released and may

start a new infection cycle. Papillomaviruses are non-lytic and virions are not released until they reach the surface of the epithelial cells. Virion release is aided by the E4 protein by disturbing the integrity of keratin organisation (Wang *et al*., 2003; Doorbar, 2005). It is still not clear how E4 viral protein expression is controlled, it is speculated that the keratinocytes might be involved or the virus is influenced at the post-transcriptional level by cellular factors provided by differentiating cells (Zheng and Baker, 2006). The post-transcriptional regulation of the gene expression process mainly involves 5' capping, splicing and 3'polyadenylation or mRNA destabilisation (Collier *et al.*, 2002; Zhao *et al.*, 2007). Papillomavirus proteins are produced by alternative splicing and polyadenylation of the pre-mRNA.

1.7 Diagnosis of HPV positive HNSCC

HPV was the first virus identified to be associated with tumours in humans. HPV is associated with common warts, flat plane warts, plantar warts, epidermodysplasia verruciformis, anogenital cancer, laryngeal papillomas and oral papillomas (Bosch *et al*., 2008). The virus can also cause cancer in the head and neck preferably in the oral cavity and oropharynx (Kreimer *et al*., 2005; D'Souza *et al*., 2007). HPV types that infect a host and usually result in benign lesions are referred to as low risk types and those that infect a host and have a high risk of causing malignancy are called high risk types. The HPV virus also has the ability to co-exist with the host, and can therefore be detected in healthy humans or can be activated during immune suppression (Antonsson *et al*., 2000; Antonsson and Hansson, 2002).

HPVs cannot be cultured efficiently as they need cell differentiation to complete the replication cycle, and serological assays, while useful for epidemiological and vaccine studies, have limited utility as diagnostic assays. Therefore, molecular assays are frequently used for diagnosis (Coutlée *et al*., 2005). There are numerous molecular assays used for detection of HPV from clinical samples such as conventional PCR, real-time PCR and *in situ* hybridisation. There are few standardised protocols for sample processing and testing of HPV in HNSCC (Garland and Tabrizi, 2006: Lewis *et al*., 2018). The gold standard assay in the molecular diagnosis of oncologically relevant HPV is the detection of HPV mRNA in frozen specimens (D'Souza *et al*., 2010; Lewis, 2012; Blitzer *et al*., 2014). This assay is difficult to perform in a routine pathology laboratory since it requires good quality mRNA, extracted from a fresh specimen and stored at -80 °C. This gold standard is also not applicable in retrospective molecular studies since archived histological samples are used (Marur *et al*., 2010). In order to

compensate for this, numerous techniques have been developed that include use of different biomarkers for detection of active HPV infections in HNSCC (Marur *et al*., 2010; Hoffmann *et al*., 2012; Holzinger *et al*., 2012; Bussu *et al*., 2013). To date, the most frequently used biomarker is $p16^{INK4a}$ overproduction in HPV infected tissue detected using immunostaining techniques (Lewis *et al*., 2018). According to guidelines from the College of American Pathologists, in the routine setting, it is recommended that HR-HPV testing such as $p16^{INK4a}$ (surrogate marker) be performed on all newly diagnosed OPSCC patients, either on the primary tumour or regional lymph node metastases. The surrogate marker testing should also be performed on patients with metastatic SCC of unknown primary in a cervical upper or mid-jugular chain lymph node and head and neck fine needle aspirates SCC samples from OPSCC patients not previously tested (Lewis *et al*., 2018).

1.7.1 HPV DNA detection methods

In conventional PCR, consensus primers targeting a fragment of the HPV L1 gene are frequently used for amplification and product analysis is done either by sequencing, hybridisation or restriction fragment length polymorphism techniques (Dixit *et al*., 2011). Primers targeting the conserved L1 gene were designed to detect a broad range of HPV types. These primers include MY09/MY11, GP5+/GP6+, PGMY and SPF10 (Yi and Manos, 1990; De Roda Husman *et al*., 1995). The MY09/MY11 primers include several degenerate nucleotides resulting in a mixture of 25 primers capable of detecting a wide range of HPV types (Morris, 2005). The primer pair GP5+/GP6+ can detect multiple HPV types at a lower annealing temperature during amplification (Morris, 2005). To improve sensitivity with the L1 primers, a nested PCR is recommended such as use of PGMY primers and the GP5+/GP6+ primer pair (Evander *et al*., 1992; Husnjak *et al*., 2000). To achieve higher sensitivity, HPV detection is performed using primers that target the E6/E7 oncogenic gene sequences as the L1 gene can be lost during HPV genome integration in infection, while the E6/E7 genes are retained (Noffsinger *et al*., 1995). Unlike L1 primers, use of E6/E7 primers allow the detection of specific HPV genotypes as the genes harbour many sequence variations between HPV types (Bernard *et al*., 2006). The primers can be designed to distinguish between high risk and low risk HPV types. A major disadvantage of the method is that the detection of HPV DNA cannot distinguish between active and latent viruses.

An alternative to PCR for detection of HPV DNA in a tumour sample is *in situ* hybridisation assay (ISH). In this assay, HPV-type specific probes are used to allow direct visualisation of HPV genome in tissue samples, which may also help in differentiating between integrated and episomal DNA and oncologically and non-oncologically relevant infections (Samama *et al*., 2002; Marur *et al*., 2010; Lewis, 2012). The ISH results are evaluated using microscopy, with HPV positive epithelial cells showing precipitate within the nuclei (Venuti and Paolini, 2012). Additionally, intergrated HPV genomes result in a punctate signal while episomal genomes result in a diffuse signal (Venuti and Paolini, 2012).

1.7.1.1 HPV intratyping

Among the reported HPV types, HPV16 and HPV18 intratypes are the most studied (Burk *et al*., 2013). Identification of HPV variants among different HPV types, requires sequence analysis of complete or partial sequence ORFs such as E6, E7, E5, L2 and L1 or the upstream regulatory region (URR) (Berumen *et al*., 2001; De Boer *et al*., 2005; Chan *et al*., 2011; Chan *et al*., 2013; Chen *et al*., 2014; Xi *et al*., 2014). Currently, characterisation of HPV variants is mostly done based on PCR followed by Sanger sequencing (Chan *et al*., 2013; Chen *et al*., 2014; Xi *et al*., 2014). Based on high quality sequences from HPV types such as HPV16 and HPV18, it was established that intratype polymorphism is limited. The intratype polymorphism among HPV types is the result of single nucleotide polymorphisms (SNPs) (Cornet *et al*., 2012). The variant differences seen in HPV types is usually less than 2% within the L1 ORF and slightly more in the LCR (Ho *et al*., 1991; Yamada *et al*., 1997; de Villiers *et al*., 2004). There are numerous disadvatages of using PCR and Sanger sequencing techniques in variant determination, including the inability to detect co-infections, (Barzon *et al*., 2010; Barzon *et al*., 2012) and they also require prior knowledge of the HPV type before intratyping, as the methods use type specific primers for PCR (Chen *et al*., 2014; Xi *et al*., 2014; Combes and Franceschi, 2018). To overcome these issues, high throughput sequencing technologies such as next generation sequencing (NGS) and whole genome sequencing have been introduced. The technology allows the sequencing of multiple HPV types, variants and identification of small genetic variants of HPV in clinical samples (Conway *et al*., 2012; Meiring *et al*., 2012; Arroyo *et al*., 2013; Kukimoto *et al*., 2013; Yi *et al*., 2014; Garnaes *et al*., 2015). The whole genome sequencing allows identification of more SNPs and definition of HPV lineages and sub-lineages across the whole genome. The resulting genome diversity of the ORF varies between HPV types, with the L1 region being reported as conserved (Liu *et al*., 2017).

1.7.2 HPV mRNA detection methods

Real-time PCR is a technique that combines amplification and detection in one reaction through use of different fluorescent chemistries. These chemistries correlate amplicon concentration to fluorescence intensity in a reaction (Higuchi *et al*., 1993). The amplicon concentration is characterised by cycle threshold (Ct) which is the cycle number at which the target fluorescence signal appears above that of the background. The greater the starting complemetary DNA (cDNA) concentration, the faster the target signal will appear hence resulting in a lower Ct (Heid *et al*., 1996). The real-time PCR quantification can either be absolute or relative (Wong and Medrano, 2005). This technique allows quantification and detection of HPV genotypes using probe or syber green dye based assays (Roberts *et al*., 2008). Real time PCR can also assist in discrimination between HPV-related and HPV-unrelated cancers by targeting E6 and E7 mRNA (Yoshida *et al*., 2008; Blitzer *et al*., 2014). The assay can be performed as a one-step or two-step reaction. In a one-step reaction, cDNA synthesis and real-time PCR analysis is done in one reaction while in a two-step reaction, cDNA synthesis is performed as a separate reaction from the real-time PCR analysis. This technique has numerous advantages viz. it does not need post-amplification analysis, it can detect as little as a single copy of targeted transcript (Palmer *et al*., 2003), and has the ability to detect as low as 23% gene expression differences between samples (Gentle *et al*., 2001). A disadvantage of the assay is that it uses expensive reagents and equipment. Additionally, the assay is challenging to use as a routine test in detection of HPV in laboratories as it requires fresh-frozen samples because of the instability of RNA. Recently, HPV E6/E7 mRNA In situ hybridization (ISH) has been introduced as a potential detection assay in formalin-fixed, paraffin-embedded (FFPE) samples. The assay has shown success in a research setting, where in one study of 196 oropharyngeal squamous cell carcinoma (OPSCC) patients, 147/148 (99.3%) were found to be RNA ISH positive (Ukpo *et al*., 2011), while in another study, a sensitivity of 97% and specificity of 93% were obtained from FFPE OPSCC samples using RNA ISH (Schache *et al*., 2013).

While the use of mRNA detection methods is ideal in HPV detection, validation of the RNA extraction methods is essential to avoid false positive or negative results. For instance, it has been established in the literature that extraction methods that are based on magnetic beads result in higher RNA yield and purity compared to spin columns (Ovestad *et al*., 2011; Iscacciati *et al* 2014). This was confirmed in a study where three different RNA extraction methods (Nuclisens manual extraction kit from bioMérieux, HighPure viral nucleic acid kit

from Roche and RNeasy PlusMini kit from Qiagen) were compared. E6/E7 mRNA positivity rate was found to be higher in samples extracted using the bioMérieux method (62%, 31/50) (Fontecha *et al*., 2017).

1.7.3 $p16^{INK4a}$ immunohistochemistry

The $p16^{INK4a}$ protein functions as a CDK inhibitor and it is encoded by the CDKN2A gene (Rocco and Sidransky, 2001). Degradation of pRb proteins by the HPV E7 oncoprotein triggers the up-regulation of CDKN2A resulting in $p16^{INK4a}$ overexpression (Zhang *et al*., 1999; Shah *et al*., 2009; Lassen, 2010; Rischin *et al*., 2010). In comparison, in HPV-unrelated HNSCCs, degradation of the pRb pathway is not induced, therefore expression of CDKN2A is mostly downregulated (Wiest *et al*., 2002). As a result, $p16^{INK4a}$ immunostaining with HPV DNA detection has been used when establishing a diagnosis of HPV-related OPSCC cancer (Jordan *et al*., 2012) and several research groups have suggested use of $p16^{INK4a}$ as an initial screening marker for HPV infection in both non-oropharyngeal and oropharyngeal HNSCCs. A pooled meta-analysis study by Prigge and colleagues confirmed high sensitivity of $p16^{INK4a}$ in detecting cells transformed by HPV infection in oropharyngeal carcinoma, though the specifity obtained was moderate (Prigge *et al*., 2017). However, some studies have also shown that, $p16^{INK4a}$ overexpression has been encountered in HPV-unrelated HNSCCs, and while it is still unclear what causes the overexpression, some researchers relate it to mutations in the $p16^{INK4a}$/RB pathway (Lewis, 2012; Zhao *et al*., 2012; Blitzer *et al*., 2014). The molecular marker is also important for judging prognosis and is included in the Word Health Organization classification scheme (Kobayashi *et al*., 2018).

Therefore, the detection of either HPV DNA or overexpression of $p16^{INK4a}$ in HNSCC samples does not definitely associate the cancer with HPV, especially in patients with a history of smoking or alcohol consumption. Currently all the available molecular techniques for HPV diagnosis have technical limitations. Hence, establishment of more sensitive molecular assays are needed as biomarkers to be used in HNSCC associated with HPV, in combination with HPV DNA detection on fresh samples or FFPE samples.

1.7.4 Potential Biomarkers

Technology advancement has improved the discovery of potential biomarkers and therefore there has been an increase in identification of new biomarkers. Discovery of new biomarkers especially for early detection of HNSCC is of interest because it is estimated about 60% of patients with OPSCC are diagnosed in advanced clinical stages (Kessler *et al*., 2008). Therefore, to improve the survival and treatment outcome of these patients, an early diagnosis is required hence a better understanding of the cancer progression is mandatory so as to diagnose the cancer at an early stage and be able to offer target-specific therapy. HNSCCs are highly complex and involve multiple pathways (Mascolo *et al*., 2012; Towle and Garnis, 2012). These molecular changes induced in a patient vary by the origin of tumour cells, the degree of differentiation and invasive capacity of the cancer cell (Ge *et al*., 2013; Denaro *et al*., 2014) and therefore play a major role in tumour progression and an overall survival of cancer cells. Research studies have shown that these molecular changes can be of value in early and accurate diagnosis and in predicting prognosis in cancer (Gasche and Goel, 2012). Currently there are numerous potential prognostic or predictive biomarkers that have been discovered in HNSCCs (Lothaire *et al*., 2006; Chen *et al*., 2007; Poeta *et al*., 2007; Chang *et al*., 2008; Zhu *et al*., 2010; Langer, 2012; Chen *et al*., 2013; Rampias *et al*., 2013; Woods *et al*., 2014; Dahiya and Dhankhar, 2016).

1.7.4.1 Biomarkers in HNSCC

As mentioned in a previous section, there are a number of potential HNSCC biomarkers reported in literature, though most have never been clinically validated, therefore intensive research with large sample size, including different sample types such as, tissues and body fluids, and sensitivity and specificity testing are still needed. Among others, the HNSCC potential biomarkers include the chemokine receptors, methylation markers, metalloproteinases, interleukins, centrosome abnormalities, actin and mysin, cytokeratins, p53, melanoma-associated gene, HPV, eukaryotic translation factor 4E and non-coding RNAs, (Dahaiya and Dhankhar, 2016).

In laryngeal squamous cell carcinoma, the expression level of CXC chemokine receptor 2 (CXCR2) was reported to be significantly high in cancer tissues compared to paraneoplastic tissue. The increased levels of CXCR2 was associated with histological grade, lymph node

metastasis and five-year survival of the patients, hence a promising potential biological marker for the cancer (Bektas-Kayhan *et al*., 2012). Another chemokine receptor that showed a potential as a biomarker in the cancer of the head and neck is CXC chemokine receptor 7 (CXCR7), which is over expressed in tumour cells of the HNSCC compared to paraneoplastic tissue. The high levels of CXCR7 was found to correlate with tisssue biopsy histological differentiation stage and lymph node metastasis (Wang *et al*., 2004; Ueda *et al*., 2010). Furthermore, one study tested the importance of CXC chemokine receptor 4 (CXCR4) as a possible biomarker to predict prognosis and metastasis in the nasopharyngeal tumour tissue. The authors reported high levels of CXCR4 expression in nasopharyngeal tumour tissues, suggesting the increase to be directly proportional to the rate of metastasis detected in the carcinoma tissues hence a poor survival of the HNSCC patients (Wang *et al*., 2005). The finding was also in agreement with one other study where high levels of CXCR4 expression were significant in tissues from patients with cancer of head and neck compared to paraneoplastic tissues. The increase correlated with high risk of lymph node and distance metastasis (Ueda *et al*., 2010).

Another potential biomarker reported in the HNSCC early diagnosis, is methylation process, where the gene is activated because of hypermethylation of cytosine phosphate-guanine (CpG)-rich promoter site (Lin *et al*., 2005). This potential biomarker is still under investigation as the specificity and sensitivity of the methylation biomarker were found to differ in different studies on HNSCC. For instance, a 96% specificity for methylation was reported by one research group, while in another study 90% was reported where saliva was used as a sample of choice and 72% specificity from using serum samples (Sanchez-Cespedes *et al*., 2000; Carvalho *et al*., 2008). On the other hand, metalloproteinases (MMPs) such as MMP-1, the gelatinases (MMP-2 and MMP-9) and the stromelysins (MMP-3 and MMP-10) have been suspected of playing a role in head and neck SCC tumourigenesis and metastasis, therefore could be used as biomarkers for the cancer (Marcos *et al*., 2009; Cathcart *et al*., 2015). These enzymes are suspected to take part in degradation of the extracellular matrix hence promote the migration of tumour cells from the primary organ of infection to other tissues (Nelson *et al*., 2000; Ravanti, 2000; Rodrigo *et al*., 2005). Apart from that, metalloproteinases are also suspected of providing a favourable microenvironment for the tumourigenesis and growth (Nelson *et al*., 2000). High levels of MMP-2 and MMP-9 have been reported in numerous cancer types including HNSCC (Zucker *et al*., 1993; Garzetti *et al*., 1996; Linkov *et al*., 2007; Ali-

Labib *et al*., 2014). With MMP-9 reported useful in detecting 80% positivity of stage 1 HNSCC, though the marker showed poor specificity in differentiating between cancerous tumours and benign diseases (Ranuncolo *et al*., 2002; Somiari *et al*., 2006).

Interleukins (ILs), have also been associated with carcinogenesis, cancer progression and migration (Jia *et al*., 2018). In oral cavity or oropharyngeal SCC, IL-8 is a potential marker in early diagnosis of the cancer from salivary samples, while IL-6 can be detected from serum samples in early stages of the oral squamous cell carcinoma (OSCC) (Li *et al*., 2004; St John *et al*., 2004). These two ILs were further detected at high levels from other specimens such as malignant tissues from HNSCC patients (Chen *et al*., 1999; Cohen *et al*., 2009). Expression of melanoma-associated gene (MAGE) has also been proposed as a potential biomarker for the HNSCC (Jungbluth *et al*., 2000; Ries *et al*., 2008; Song *et al*., 2008). MAGE such as A3 and A4 have been detected at early invasive cancer using excisional biopsy (Metzler *et al*., 2009). Similarly, this expression was also seen in sputum specimens and 85.5%-90% of tissues from patients with HNSCC (Jungbluth *et al*., 2000; Lee *et al*., 2006; Ries *et al*., 2008; Song *et al*., 2008). According to reports from other researchers, MAGE-A1 to 6 expressions in sputum of patients dignosed with laryngeal and hypopharyngeal cancer predicts poor prognosis (Pastorcic-Grgic, 2010). Centrosome abnormalities, have also been detected in head and neck SCC. It has been reported that majority of tumour samples from HNSCC, have centrosome hyperamplification, hence it has been suggested that the centrosomal hyperamplification be included as a biomarker for the HNSCC (Gustafson *et al*., 2000). Centrosomal abnormalities were also detected in high frequency in OSCC in cells displaying high expression of spindle checkpoint protein (Thirthagiri *et al*., 2007).

Actin and myosin, have been reported to show high expression in exfoliated cells from saliva in patients with cancer in comparison with those with pre-cancerous lesions (de Jong EP *et al*., 2010). The cytoskeleton proteins help in cell invasion and motility during epithelial carcinogenesis (Friedl *et al*., 2009). High levels of actin isoforms have been detected in invasive basal cell carcinoma (Uzquiano *et al*., 2008), SCC of cervix (Li *et al*., 2009) and esophagus (Qi *et al*., 2005) and in invasive cancer at the oropharynx (Shi *et al*., 2006). While myosin abundance has been detected in tissues from OSCC sub-site (Lo *et al*., 2007). The cytokeratins (CKs) proteins, found in multiple tissues are the main component of intracytoplasmic cytoskeleton and are associated with overexpression in OSCC tissues than

normal mucosa (Barak *et al*., 2004), for instance, the overexpression of CK-17 mRNA in OSCC (Toyoshima *et al*., 2008; Toyoshima *et al*., 2009; Wei *et al*., 2009). The high expression of the CKs proteins has been linked to cancer progression and prognosis (Domachowske *et al*., 2000). This potential biomarker has also been detected in epithelial cells infected with respiratory syncytial virus (RSV) (Chu *et al*., 2000). Hence to validate it as a marker, CK-17, needs intensive analysis with larger samples. Eukaryotic translation factor 4E (eIF4E), is also a reported potential biomarker in HNSCC, useful in recurrence and survival prediction in patients with the cancer. The protein take part in protein synthesis intiation (Chen *et al*., 2004) and its overexpression has been linked to different stages of tumourigenesis. The high protein expression in cancers such as breast, lung, bladder and HNSCC has been associated with increase in cancer progression and unfavourable prognosis (De Benedetti and Graff ,2004; Crew *et al*., 2000; Mamane *et al*., 2004; Holm *et al*., 2008; Wang *et al*., 2009).

Though there are numerous HNSCC potential biomarkers reported, few of them have been intensively studied such as p53, $p16^{INKA4}$, HPV and non-coding RNA (Jalali *et al*., 2011; Marchese, *et al*., 2017; Vitzhum and Mell, 2018). The main function of the p53 protein pathway is to cause cell cycle arrest when there is a damaged DNA for repair or to cause apoptosis when cells are old or the DNA is irreparable (van Oijen and Slootweg, 2000) hence responsible for cellular integrity and maintenance (Bradford *et al*., 2014). Mutation in p53 genome is encountered in most carcinomas such as HNSCC (Jalali *et al*., 2011; Hardisson, 2003). In HNSCC, this mutation has been demonstrated in numerous published papers (Nathan *et al*., 2000; van Oijen and Slootweg, 2000; van Houten *et al*., 2002; Poeta *et al*., 2007; Bradford *et al*., 2014). Immunohistochemistry is used in the laboratory for the detection of the p53 expression in tissues suspected of cancer, though its pathogenesis in HNSCC still not understood (Gasco and Crook, 2003). Cancer patients showing no p53 mutation have been reported to have a better survival rate compared to those who are positive (Jalali *et al*., 2011), therefore this biological marker has the potential to be used as both a diagnostic and prognostic molecular marker in HNSCC patients. Human papillomavirus especially HPV16 and 18, the causative agents of cervical cancer (Kovachev and Slavov, 2016), have also been reported as promising diagnostic and prognostic biomarkers of HNSCC Viral (Perez-Ordoñez *et al*., 2006; Betiol *et al*., 2013). According to literature, the HNSCC associated with HPV has a better prognosis (Licitra *et al*., 2006). The virus has been documented to cause cancer through

its oncogenic proteins (E6 and E7). The E7 oncoprotein binds and causes degradation of pRb while E6 inactivates p53 through ubiquitin linked proteolysis (Vietía *et al*., 2014). In diagnosis, $p16^{INKA4}$ coupled with HPV DNA detection has proven useful in HPV associated HNSCC confirmation and prognosis (Zaravinos, 2014). Non-coding RNAs have been suggested as potential early biomarkers in HNSCC due to their stability in routine clinical specimens such as blood and tissues (Jin *et al*., 2013).

1.7.4.2 Non-coding RNAs

Cancer diagnosis and prognosis frequently focuses on the protein coding genes' effect due to alteration in their expression. About 65% of the human genome is transcribed and only 2% is translated into proteins (Carninci *et al*., 2005). The role of the non-coding genes in cancer initiation and progression is still unknown, as is their potential use as biomarkers (Chang *et al*., 2008; Zhu *et al*., 2010). At present, there are no assays available to monitor HNSCC patients for early detection of local recurrence or distant metastases, therefore there is a need to discover novel biomarkers that can be used to predict the clinical outcome (Denaro *et al* 2014). Non-coding RNAs have been studied as potential biomarkers. They are divided into two distinct groups based on their sizes: small ncRNAs (less than 200 bp) and long non-coding RNAs (200 bp and more) (Bolha *et al*., 2017). Small ncRNAs include small interfering RNAs (siRNA), PIWI-interacting RNAs (piRNAs) and microRNAs (miRNAs), while long non-coding RNAs are further divided into those that modulate the activity of transcription factors (GAS5RNA, B2 and Alu RNA, 7SK RNA, LincRNA-p21 and SRA) and those that regulate changes in the chromatin structure (Xist, ANRIL, HOTTIP, and HOTAIR) (Kugel and Goodrich, 2012; Ge *et al*., 2013). Among the small ncRNAs, the most studied as a potential biomarker for the HNSCC is miRNAs. Micro RNA are ribonucleic acid, classified as non-coding RNA taking part in regulation of gene expression hence affecting different physiological pathways (Ke *et al*., 2003). The miRNAs can act as oncogenes and tumour suppressor genes (Kent and Mendell, 2006). For instance, miR-106-25 cluster and miR-375 are proposed to take part in development and progression of the cancer at the head and neck while miR-451 could be a marker for recurrence (Hui *et al*., 2010). The miR-125a and miR-200a were also reported to be significantly lower in levels in patients with OSCC as to controls (Park *et al*., 2009).

The long non-coding RNAs are transcribed using RNA polymerase II, then undergo post transcription modifications such as splicing and polyadenylation processes. The gene is not further translated into protein, and is suspected of taking part in chromatin modification that leads to silencing and overexpression of target genes involved in tumour genesis pathways (Di Gesuald *et al*., 2014). The HOTAIR is among the most studied and promising potential biomarkers for head and neck SCC (Loewen *et al*., 2014; Zhou *et al*., 2014). This biomarker is 2.2 kb in size, located at the chromosome 2, transcribed from *HOXC* gene (antisense) and made up of six exons (Rinn *et al*., 2007; Tani *et al*., 2012). The HOTAIR interact with various chromatin modifying complexes such as Polycomb Repressive Complex 2 (PRC2) using the 5'-terminal binding domain (Bhan and Mandal, 2015) and lysine-specific histone demethylase 1 (LSD1). This results in gene regulation by the HOTAIR using chromatin dynamics (Liu *et al*., 2015; Li *et al*., 2016). In contrary some studies show that HOTAIR can also regulate genes without interacting with PRC2 (Portoso *et al*., 2017; Hajjari and Salavaty, 2015). HOTAIR can also regulate some miRNAs such as causing down regulation of the miR-331-3p resulting in increasing expression of the oncogene human epithelial growth factor 2 (HER2) (Liu *et al*., 2014). In HNSCC, HOTAIR promote tumourigenesis by several pathways, among others it regulates PTEN oncogene activity through promoter methylation (Li *et al*., 2013). Furthermore, is involved in epithelial mesenchymal transition (EMT) by inhibiting the expression of E-cadherin by means of interacting with EZH2 (Wu *et al*., 2015; Zheng *et al*., 2017). Overexpression of HOTAIR is also capable of inhibiting natural cell death by changing the mitochondrial membrane and mitochondrial calcium intake dependent cell death (Kong *et al*., 2015).

1.8 Treatment and prevention of HNSCC

Treatment for HNSCCs include surgery, chemotherapy and radiotherapy (Argiris *et al*., 2008; Leemans *et al*., 2011). Early stage localised cancer can be treated with either radiotherapy or surgery depending on the organ treated (Leemans *et al*., 2011; Martin *et al*., 2014; Suh *et al*., 2014). In the case of locally advanced HPV-positive HNSCCs, addition of chemotherapy in the treatment regimen has shown to result in five-year survival advantage of 6.5% and is frequently considered standard care (Blanchard *et al*., 2011). Furthermore, according to retrospective and prospective clinical trials, HPV-positive HNSCC patients have shown a five-year overall survival of around 80%, with greater disease free survival and locoregional control compared to patients who have tumours that are HPV-negative (with a five-year survival of

about 50%), independent of the treatment regimen (Lassen *et al*., 2009; Ang *et al*., 2010; Gillison *et al*., 2012; O'Sullivan *et al*., 2012; Ang *et al*., 2014; Blitzer *et al*., 2014). As it has been shown that patients with HPV-positive HNSCCs respond better to therapy, de-intensification of the therapy is being proposed and assessed in several ongoing clinical trials (Blitzer *et al*., 2014; Kang *et al*., 2015; Sepiashvili *et al*., 2015). There are two ways that de-intensification has been done. Firstly, the trials include de-intensification of chemotherapy by substitution with a chimeric monoclonal antibody against EGFR called cetuximab. The second means is de-escalating of radiotherapy dose in patients with HPV-positive tumours, either coupled with induction chemotherapy or minimally invasive surgery (Blitzer *et al*., 2014; Masterson *et al*., 2014).

To prevent HPV infection, three vaccines are available in most countries: Cervarix by GlaxoSmith Kline (GSK), Gardasil 4-valent and Gardasil 9-valent produced by Merck & Co (Ribeiro-Müller and Müller, 2014; Pitisuttithum *et al*., 2015). Cervarix protects against HPV16 and HPV18 while Gardasil-4 offers protection against oncogenic HPV16, HPV18 and genital condyloma causing HPV6 and HPV11, and Gardasil-9 elicits protective neutralising antibodies against oncogenic HPV16, 18, 52, 45, 33, 31, 58 and genital condyloma causing HPV6, 11 (Kumar *et al*., 2015; Takes *et al*., 2015). These vaccines have been shown to be protective against cervical intraepithelial neoplasia (CIN) and up to 90% of infiltrative cervical carcinoma protection through Gardasil-9, while their efficacy against HPV-related HNSCCs is still unknown. The vaccines are recommended for prophylactic use against HPV infections and their therapeutic application is uncertain (Kumar *et al*., 2015).

1.9 Problem identification

Previous studies have established that there is an association between HPV and HNSCCs. SA, amongst other countries, has a high incidence of HNSCCs. However, little is known of the association between HNSCCs and HPV in SA, as only limited studies have been conducted with conflicting results and small numbers of patients. Therefore, studies that could contribute to the understanding of HPV as a cause of HNSCCs and the prevalence in this country are required. There are few studies, and none conducted in the Free State province, that establish the prevalence of infection, or which HPV types are involved. Therefore, studies that could form a baseline by giving an indication of prevalence of the infection in the province are required. The data will provide useful information regarding HPV types circulating prior to

implementation of the vaccine aimed at reducing the incidence of cervical cancer. HNSCCs have a good prognosis if detected early, therefore detection of HPV nucleic acid, various biomarkers and their association with HPV have been investigated for diagnosis and prognostic application. HPV E7 oncoprotein inactivates the pRb protein resulting in increased expression of $p16^{INK4a}$ and some studies have shown that p16+ and novel biomarkers would play a useful role in prognosis and have public health implications. However, others have shown contrasting results, where overexpression of $p16^{INK4a}$ was also observed in HPV negative HNSCC patients. Hence identification of more accurate predictive biomarkers is needed and could be of great clinical value as they could be used in understanding the cell biology in HNSCC development and the knowledge could be used in novel therapeutic strategy development. Currently there are various studies that are being conducted in order to establish potential biomarkers, including studies investigating long non-coding RNA (lncRNA) as a potential biomarker. The lncRNA constitutes 60% of the non-coding RNA found in the human genome and several are linked to human disease. Previous studies have shown that HOTAIR, one of the lncRNAs, and the Homeobox10 (HOXD10) locus, a region regulated by HOTAIR, are potential biomarkers in HNSCCs, hence further studies are required to establish the possibility of using HOTAIR and HOXD10 as biomarkers for HNSCCs worldwide, especially in developing countries such as South Africa.

1.10 Aims and objectives

The aims of this study were to determine the prevalence of HPV in head and neck squamous cell carcinomas diagnosed at the Department of Anatomical Pathology, NHLS Universitas Academic Hospital in the Free State, South Africa using FFPE samples. To further investigate the phylogenetic relationship of the high risk HPV strains from the patients and finally identify potential biomarkers that can be used as an indicator of HNSCC (laryngeal cancer) in early diagnosis of the cancer.

The objectives of the study were to:

- Screen and genotype archived tissues from histologically confirmed HNSCC for HPV DNA using multiplex PCR, targeting L1 region.
- Investigate phylogenetic relatedness of high risk HPV types obtained.

- Investigate expression of HOX transcript antisense intergenic RNA as potential biomarker of disease using quantitative real-time PCR.

1.11 Structure of the Thesis

This thesis is structured as a sequence of research articles which will be submitted for publication in peer-reviewed scientific research journals. Chapter 2, which is on an investigation of the prevalence of HPV DNA in patients confirmed with HNSCC in our setting using a nested PCR represent the first article. The investigation was done on 780 FFPE tissue biopsies with intact DNA from different sub-sites of the head and neck. While Chapter 3, will represent the second article. This chapter, is focused on the intra-typing of the obtained six most prevailing high risk HPV types from the HNSCC in the previous study using heminested PCR and determination of the phylogenetic relatedness. To investigate phylogenetic relationship between the variants of different HPV types, phylogenetic trees were constructed using Maximum Likelihood method. The analysis was performed along with other available variant sequence data from the GenBank database. The reliability of the phylogenetic trees constructed using partial L1 was determined by comparing the topology of the trees to those constructed using complete HPV L1 and complete genome. The third article, presented in Chapter 4, is on the detection and comparison of the potential biomarker (HOTAIR) expression levels among the laryngeal carcinoma patients and healthy controls. This was done with the aim to study whether HOTAIR can be useful as prognostic/diagnostic marker in this cohort for early therapy initiation. The investigation was done on total RNA, isolated from blood of the participants using relative quantitative real time PCR. Lastly, in Chapter 5, an overall conclusion of the research study and future perspectives were discussed. In each chapter, referencing style used is in accordance with the journal considered for publication.

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CHAPTER 2

HUMAN PAPILLOMAVIRUS DNA IN HEAD AND NECK SQUAMOUS CELL CARCINOMA PATIENTS IN THE FREE STATE, SOUTH AFRICA

The editorial style of the Papillomavirus Research Journal was followed in this chapter

2.1 Abstract

Background: Human papillomaviruses (HPV) are the aetiologic agents for diverse clinical conditions. HPV has recently been associated with head and neck squamous cell carcinomas (HNSCC). At present, little is known about the prevalence of HPV in HNSCC in South Africa. Hence the aim of this chapter was to investigate the prevalence of HPV DNA in archived formalin fixed paraffin embedded (FFPE) tissue from patients with confirmed HNSCC using multiplex PCR.

Methods: A total of 994 FFPE tissue biopsies from different sub-sites of the head and neck were screened. DNA integrity was confirmed by amplification of the human beta globin gene. A nested multiplex PCR was used for detection of HPV DNA using primers PGMY09/11 and GP5+/6+ targeting the L1 gene. HPV types of positive samples were identified by sequence determination of amplicons and analysis of sequence data using BLAST and confirmed using heminested PCR targeting the E6 gene of the HPV.

Results: DNA integrity was confirmed in 780/994(78.5%) samples. Overall, HPV DNA was detected in 57/780 (7.3%) patients. Positive biopsies were from the larynx (18/259) 6.9%, oral cavity (20/329) 6.1%, sinonasal (4/25) 16.0%, oropharynx (13/120) 10.8%, nasopharynx (1/26) 3.8% and hypopharynx (1/17) 5.9%. HPV16 was the most commonly detected type, found in 26/57 (45.6%) of positive samples. Other types detected included HPV2, HPV6, HPV11, HPV13, HPV18, HPV31, HPV33, HPV35, HPV45, HPV52, and HPV59.

Conclusion: The overall rate of HPV infection of 7.3% in 780 samples was comparable to previous rates of detection using PCR on FFPE tissues in the country. The detection rate varied depending on the tumour location.

2.2 Introduction

Head and neck squamous cell carcinomas (HNSCCs) include squamous cell carcinomas of the oropharynx, oral cavity, larynx, pharynx, nose and paranasal sinuses [1,2]. They account for more than 90% of malignancies in the head and neck region [1]. Exogenous risk factors for HNSCC genesis include tobacco and alcohol abuse [3-5], however, some individuals develop HNSCCs without a history of exposure to these risk factors [1,5-6].

Human papillomaviruses (HPVs) have a circular, double-stranded, DNA genome of approximately 8000 base pairs (bp) [7]. Based on the risk for causing cervical carcinoma, HPVs are divided into low risk and high risk types [8]. Epidemiological and molecular studies have confirmed that HPVs are associated with a subset of HNSCCs, especially oropharyngeal carcinoma [9-12]. HPV as an aetiologic agent of HNSCC has been reported in numerous studies, with HPV16 being detected most frequently [4,13-15]. Countries located in North America and Europe have a relatively high (over 1.25 per 100,000) age-standardised incidence rate of HPV-attributable head and neck cancer [2]. The increase in prevalence of HNSCC has been linked to an increase in oral high-risk HPV infection, which is predominantly associated with changes in sexual behaviour and multiple oral sex partners [16]. The molecular evidence for associating HPV with HNSCCs originally based on the detection of HPV DNA, and subsequently substantiated by detecting transcription of the oncogenes (E6 and E7). In addition, there is some evidence that the HPV DNA may be integrated in the host cellular genome [14].

Studies to determine the prevalence of HPV associated HNSCC from different geographical areas have shown varied results. A systematic review of the literature suggests that taking into account the variability in study design, sample types analysed and methods used for detection between the studies, there is a higher HPV-related HNSCC burden in developed countries compared to the burden in developing countries [2]. Nevertheless, there is limited data in the literature regarding the role of HPV in HNSCC in developing countries, including countries in sub-Saharan Africa, where one third of the cancers are predicted to be due to infectious agents and HNSCCs are the sixth most common cancer [17,18].

In South Africa, limited studies on HPV associated HNSCC have been conducted, with the majority focusing on individual sites of the head and neck. The most extensively studied sites

are the oropharynx and oral cavity [18,19]. Early studies suggested that HPV was of limited importance as an agent of oropharyngeal squamous cell carcinoma (OPSCC). However with application of nucleic acid amplification tests, a higher incidence of HPV DNA was detected in patients with OPSCC, although most studies were performed on small cohorts of patients [20,21]. Hence, there is a need to determine the prevalence of HPV DNA in a larger cohort of samples from confirmed HNSCC cases from different sites. The availability of archival formalin fixed paraffin embedded (FFPE) tissues from patients with histologically confirmed HNSCC at the Universitas Academic Laboratories provided an opportunity to screen for HPV DNA.

2.3 Aim

The aim of this chapter was to determine the prevalence of HPV in head and neck squamous cell carcinomas diagnosed at the Department of Anatomical Pathology, NHLS Universitas Academic Hospital in the Free State, South Africa using FFPE samples

2.4 Objectives

- To Screen a total of 994 FFPE tissues from patients histologically confirmed with HNSCC for HPV DNA using multiplex PCR, targeting L1 region
- To confirm HPV positive samples with an in house heminested PCR targeting the E6 region
- To identify genotypes of HPV identified in the FFPE tissues

2.5 Materials and Methods

2.5.1 Sample collection

The sample group was comprised of specimens from patients with histologically confirmed HNSCC, submitted to the Department of Anatomical Pathology, Universitas Academic Laboratories in Bloemfontein, South Africa, between January 2004 and December 2014. A total 994 FFPE archived tissues were identified from the laboratory records, assigned a unique study number and were screened for HPV DNA. For each patient, the site of the carcinoma, gender and age of the patient were recorded. As testing was done retrospectively on residual tissue samples, individual patient consent was not required but permission was obtained from the National Health Laboratory Service. The study was approved by the Health Sciences

Research Ethics Committee of the Faculty of Health Sciences, University of the Free State (137/2013C).

2.5.2 Tissue sectioning and DNA extraction

Consecutive 10 µm sections were cut from each tissue block. Special precautions were taken to avoid possible cross-contamination. Paraffin was initially removed from the tissue by including a xylene-ethanol step prior to extraction of DNA from FFPE tissues. Briefly, 1 ml xylene was added to the FFPE tissue section in a microcentrifuge tube, vortexed for 15 seconds and centrifuged at 14000 x g for 5 minutes at room temperature. This step serves to remove paraffin from the tissue and was repeated three times. Residual xylene was removed with an absolute ethanol wash and the tissue was dried at 37 °C for 15 minutes. DNA was extracted using a commercially available DNA extraction kit (QIAmp®DNA Mini kit, Hilden, Germany), according to manufacturer's instructions. Integrity of the extracted DNA was confirmed by amplification of a region of a reference gene using the primer pair PCO_4 and GH_{20} which target a 268 base pair (bp) region of the human beta globin gene. Patient samples were screened for HPV DNA if human beta-globin was detected.

2.5.3 Positive controls

The efficacy of the DNA extraction was determined prior to screening samples. In a related study, HPV16, 18, 31, 45, 6, 11 were detected in fresh biopsy samples collected from patients with histologically confirmed SCCs. Based on sequencing of PCR amplicons and results from Linear Array HPV Genotyping assays (Roche Diagnostics, Indianapolis, USA) there were no coinfections in these controls. Paraffin embedded tissues, prepared at collection of the fresh biopsies, were available for each of these patient samples. DNA was extracted from the FFPE tissues using the extraction method described above, DNA integrity was confirmed by amplification of the reference gene and HPV DNA was detected in the samples using primers that target the L1 gene.

2.5.4 HPV amplification and genotyping

A nested PCR was performed on all human beta-globin positive samples using the consensus primers, PGMY09/11 and GP5+/6+ [22, 23] and a negative control was included in each PCR run. For the negative control, a 5µl volume of nuclease free water was added to the reaction instead of template. These primers amplify a region of the L1 gene of HPV. The amplification reaction was performed using GoTaq®Hot Start Polymerase (Promega, Madison, USA) following the

manufacturer's instructions. The following PCR conditions were used: initial denaturation, 95 °C for two minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and elongation at 72 °C for one minute and a final elongation at 72 °C for five minutes. Cycling conditions were similar for the nested reaction except the annealing temperature was decreased to 43 °C to accommodate the Tm of the primers, and the annealing time was extended to 1 minute.

The PCR products were separated by electrophoresis using a 2.5% agarose gel in 1xTAE buffer (pH 8.0) and visualized by staining with GelRed™ (Biotium Inc., Fremont, CA, USA). The sample products were run in parallel with the negative control, which was expected to show no amplification. The PCR products of the predicted size were excised from the gel and purified for nucleotide sequence determination using the Wizard®SV Gel and PCR clean-up system (Promega, Wisconsin, USA) according to manufacturer's instructions. DNA concentration was determined using a NANODROP 2000 spectrophotometer (Thermo Scientific, lllinois, USA). The purity was determined from the 260nm:280nm absorbance ratio. The nucleotide sequence of each purified amplicon was determined using Sanger sequencing. The reactions were performed using Big Dye Terminator sequencing ready reaction kit (Applied Biosystem, Foster City, CA, USA). Sample electrophoresis was performed and nucleotide sequence data was edited using Chromas Pro version 1.41 (Technelysium Pty Ltd, Australia). Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov, 2015) analysis was performed to compare the similarity between the nucleotide sequencing results obtained from the amplicons with the GenBank nucleotide sequence database. HPV positive samples were further confirmed with an in house heminested PCR and type specific primers that target the E6 gene (Tables 2.1 and 2.2). In the heminested reaction, one of the first round primers and an additional primer targeting an internal region of the amplicon were used.

2.5.5 E6 heminested type specific PCR

The reliability of HPV DNA detection and genotyping obtained using the nested PCR targeting the L1 region of the viral genome was confirmed using in-house type specific E6 primers (Table 2.1). Primers were designed by alignment of sequence data for 15 HPV isolates retrieved from GenBank using Clustal Omega version 1.2.1. A negative control was included in each run. To confirm the specificity of each primer, the oligonucleotide sequences were analysed using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The amplification reaction was performed using GoTaq®Hot Start Polymerase (Promega, Madison, USA) following the

manufacturer's instructions. The following cycling conditions were used: initial denaturation, 95 °C for two minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing temperatures (as shown in Table 2.1) for 30 seconds and elongation at 72 °C for one minute, and a final elongation at 72 °C for five minutes.

Table 2.1: In house PCR primer pairs for the E6 heminested type specific PCR

Primer name	Nucleotide sequence (5' to 3' direction)	Melting temperature (Tm)	Annealing temperatures (Ta)	Amplicon size (bp)
HPV6F	CCTCCACGTCGTCAACGACCA	68.5 °C	62 °C	174bp
HPV6R	AGGCTGCATATGGATAGCCGG	66.8 °C		
HPV11F	ATGGAAAGTAAAGATGCCTCCACGT	67.0 °C	62 °C	200bp
HPV11R	CAACAGGCACACGCTGCAAG	67.1 °C		
HPV16F	AGGACCCACAGGAGCGAC	66.3 °C	60 °C	147bp
HPV16R	TGCATAAATCCCGAAAAGCAAAGTC	65.3 °C		
HPV18F	ATGGCGCGCTTTGAGGATCC	68.0 °C	60 °C	191bp
HPV18R	GCAGCATGCGGTATACTGTCT	65.2 °C		
HPV31F	CGGCATTGGAAATACCCTACGA	65.1 °C	60 °C	141bp
HPV31R	GCACACACTCCGTGTGGTGTG	68.0 °C		
HPV33F	GAGAGGGAAATCCATTTGGAATATG	62.6 °C	58 °C	178bp
HPV33R	TCTTGAGGACACAAAGGTCTTTG	63.7 °C		
HPV52F	GAG GAT CCA GCA ACA CGA C	62.1 °C	53 °C	127bp
HPV52R	CTT GTA TAC CTC TCT TCG TTG	57.8 °C		
HPV13F	GCT TGT GCA TGC TGC TTA G	61.7 °C	56 °C	160bp
HPV13R	TCT CCA CTT CAC ACA ATG G	60.6 °C		
HPV59F	GAT CCT ACA CAA CGA CCA TAC-	59.9 °C	54 °C	167bp
HPV59R	GG TGT ACA GTC TCT ATA CAC	59.0 °C		
HPV2F	CA AGG GCA GGG ATG TCT GAG G	65.9 °C	61 °C	212bp
HPV2R	AAT CAG GCA TTT TCC GCA GGC	65.9 °C		

HPV45F	GGCGCGCTTTGACGATCCAAAG	68.7 °C	58 °C	136bp
HPV45R	TTGATATACCTCTGTGCGTTCC	62.9 °C		
HPV35F	CTG CAT GAT TTG TGC AAC G	61.3 °C	53 °C	142bp
HPV35R	GCT GGC CTT CTC TAT ATA C	57.5 °C		

*HPV6, 11, 16, 18, 31, 33, primer pairs were designed in a related study [41]

*HPV13, 59, 2, 45, 35, 52, were designed in the current study

A 1 µl aliquot of the first round reaction was used as template in the nested reaction while nuclease free water was used as negative control. The PCR reactions were performed using the following cycling conditions: initial denaturation, 95 °C for two minutes for one cycle, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing temperature as shown in Table 2.2 for 30 seconds and elongation at 72 °C for five minutes.

Table 2.2: Heminested in house PCR primers used for the second round PCR

Primer name	Nucleotide sequence (5'to 3' direction)	Melting temperature (Tm)	Annealing temperatures (Ta**)**	Amplicon size (bp)
HPV6F2	GCAAGAATGCACTGACCACTGCAG	68.4 °C	59 °C	90bp
HPV11F2	CTTTGCACACTCTGCAAATTCAG	63.8 °C	59 °C	133bp
HPV16F2	CCACAGTTATGCACAGAGCTGCAA	67.8 °C	63 °C	117bp
HPV18F2	GTGCACGGAACTGAACACTTCACT	67.6 °C	63 °C	141bp
HPV31F2	CTGCAAAGGTCAGTTAACAGAAAC	63.2 °C	61 °C	96bp
HPV33F2	CTGTGTTTGCGGTTTTTATCTAAAC	62.5 °C	62 °C	149bp
HPV52F2	GCA CGA ATT GTG TGA GGT G	61.3 °C	55 °C	102bp
HPV13F2	TAT ATA TAG TGT GGC GAG GAT C	59.6 °C	49 °C	195bp
HPV59F2	TCC TCT GCA TGA TAT TCG C	59.5 °C	50 °C	114bp
HPV2F2	AG AGG ATT TGC GAT TGC TCT G	62.8 °C	60 °C	136bp
HPV45F2	CCCTACAAGCTACCAGATTTG	61.2 °C	53 °C	107bp
HPV35F2	GAG GTA TAT GAC TTT GCA TG	56.2 °C	45 °C	55bp

*HPV6, 11, 16, 18, 31, 33, primer pairs were designed in a related study [41]

*HPV13, 59, 2, 45, 35, 52, were designed in the current study

2.5.6 Statistical analysis

Statistical analysis was performed with IBM SPSS version 25. Differences between ages were analysed using the independent samples t-test, while associations were analysed with Fisher's exact test.

2.6 Results

2.6.1 Patient data

DNA was extracted from 994 FFPE tissue biopsies from patients with histologically confirmed HNSCC from the Department of Anatomical Pathology, Universitas Academic Laboratories. DNA integrity was confirmed in 780/994 (78.5%) of the samples. The biopsies with intact DNA were from various sites (Table 2.3). Of the 780 biopsies, 631/780 (80.9%) were from males while 149/780 (19.1%) were from females.

Table 2.3: Number of HNSCC cases positive for human beta globin gene by site from a total of 994 samples analysed

Sites	Number/total (%)
Larynx	259/ 340(76.2%)
Oral cavity	329/341 (96.5%)
Oropharynx	120/224(53.6%)
Nasopharynx	26/32 (81.3%)
Hypopharynx	17/18 (94.4%)
Eye	2/2 (100%)
Sinonasal tract	25/35 (71.4%)
Trachea	1/1(100%)
Lymph node	1/1(100%)

2.6.2 Positive control

HPV DNA was detected in each of the positive controls after treating the sections with xylene prior to DNA extraction. Hence this method was used for all DNA extractions in the current study.

2.6.3 HPV genotyping

HPV isolates were genotyped based on sequence data obtained from a region of the L1 gene and alignment of data retrieved from GenBank. Confirmation of typing was achieved using sequence data obtained from partial E6 amplicons. A total of 57/780 (7.3%) of the patient samples were found to be positive for HPV DNA. The accuracy of the genotyping results was confirmed using an in house type specific heminested PCR targeting the E6 gene, with 100% concordance between results. The prevalence of HPV DNA was highest in the sinonasal tract and oropharynx (Table 2.4). Of the 120 oropharyngeal tumours, 43 were tonsillar carcinomas, of which eight (18.6%) were positive for HPV DNA.

Table 2.4: Location of the 57 HPV DNA positive samples obtained in the current study and the percent frequency per site

Site	**HPV DNA (+) /No tested (%)**
Larynx	18/259 (6.9%)
Oral cavity	20/329 (6.1%)
Oropharynx	13/120 (10.8%)
Nasopharynx	1/26 (3.8%)
Hypopharynx	1/17 (5.9%)
Sinonasal tract	4/25 (16.0%)

High risk (HR) HPV types were more frequently detected than low risk (LR) HPV types. HPV16 was the most frequently detected HR type, and was amplified from 26/57 (45.6%) of the positive samples (Figure 2.1). Other HR types detected included HPV18, 31, 33, 35, 45, 52 and 59. LR HPV6 was detected in 10/57 (17.5%) samples. HR types were detected in 41/57 (71.9%) positive samples from both males 29/41 (70.7%) and females 12/41(29.3%). In contrast, LR HPV types were only detected in males 16/57 (28.1%).

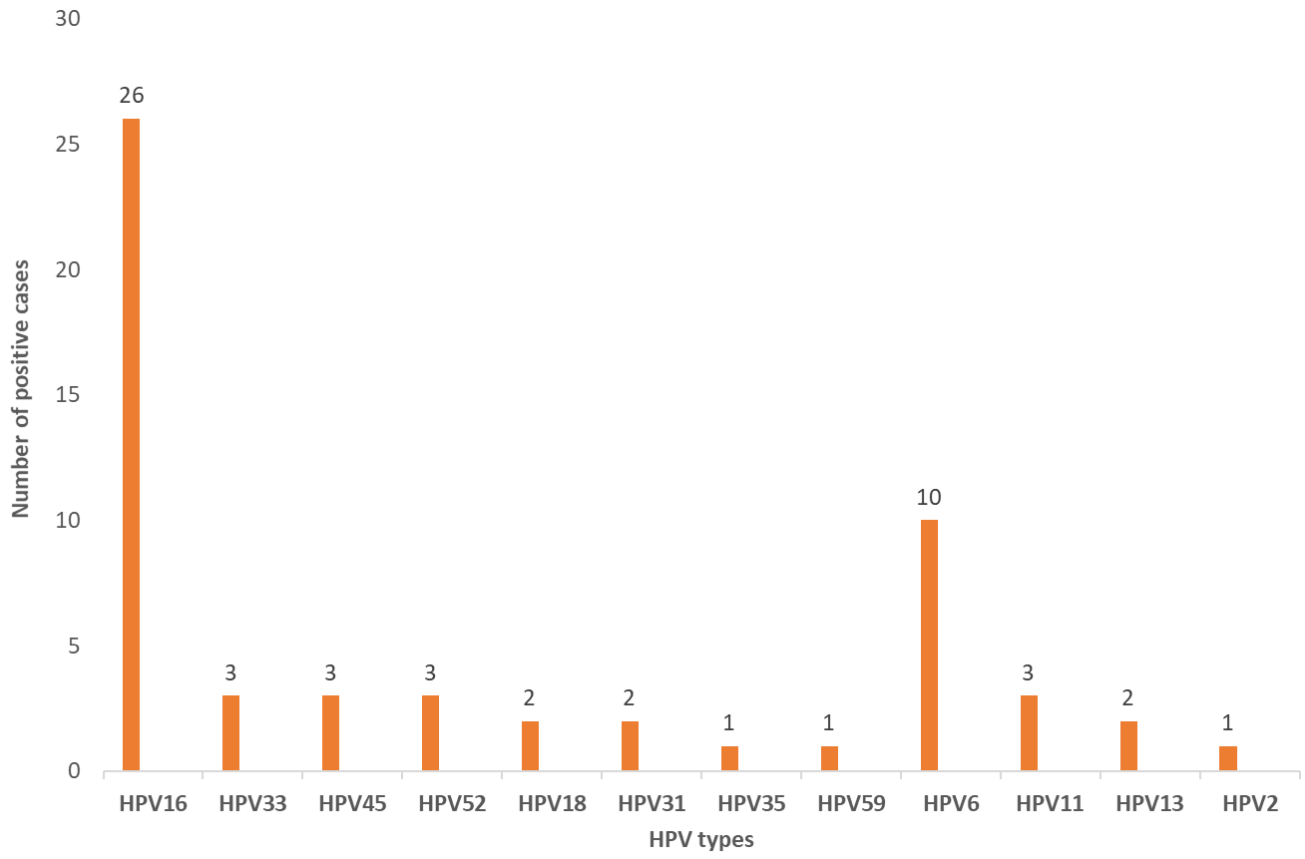

Figure 2.1: Frequency of HPV types detected in head and neck samples

Figure 2.2 shows the distribution of HPV type per site, with the oral cavity, oropharynx, sinonasal tract and larynx being the sites where HPV was most frequently detected. HPV16 was the most frequently detected HPV type in the oral cavity (50.0%), oropharynx (46.2%) and larynx (50.0%), while HPV45 was the most frequently detected type in the sinonasal tract (50%).

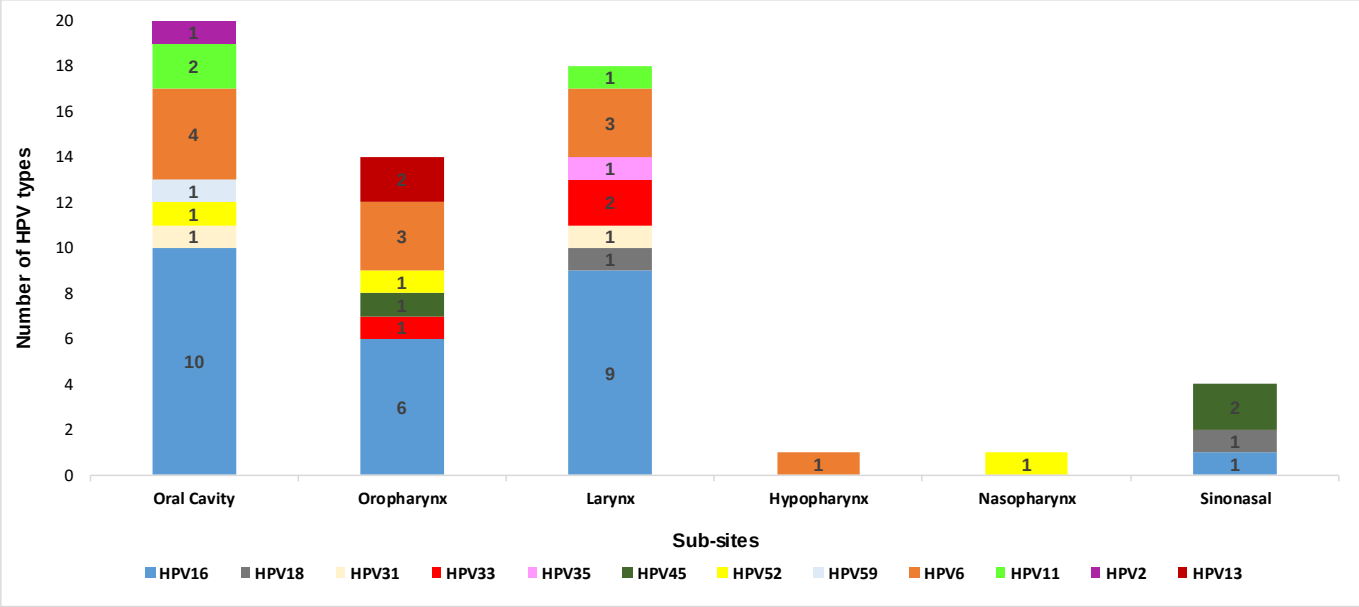

Figure 2.2: Frequency of HPV types identified from different regions of the head and neck

There was no significant difference in the age of the patients with HPV positive and HPV negative tumours for any of the tumour sites (Table 2.5).

Table 2.5: Age of the patients with HPV positive and HPV negative tumours

Site	HPV (+)		HPV (-)		
	Age (years)				
	Mean	Range	Mean	Range	p-value
Larynx	55.6	29-77	59.8	30-86	0.228
Oral cavity	57.2	43-73	58.5	24-91	0.585
Oropharynx	58.2	44-68	58.3	41-86	0.946
Sinonasal tract	46.3	36-63	57.2	35-82	0.140

HPV DNA was detected in 12/149 (8.1%) females and 45/631 (7.1%) males. The prevalence of HPV was significantly higher in males with oral cavity tumours and in females with oropharyngeal tumours (Table 2.6).

Table 2.6: Percentage of HPV DNA positive biopsies according to site and gender

Site	Male		Female		p-value
	HPV+/No tested (%)	HPV-/No tested (%)	HPV+/No tested (%)	HPV-/No tested (%)	
Larynx	15/228 (6.6%)	213/228 (93.4%)	3/31 (9.7%)	28/31 (90.3%)	0.460
Oral cavity	19/248 (7.7%)	229/248 (92.3%)	1/81 (1.2%)	80/81 (98.8%)	0.033
Oropharynx	8/102 (7.8%)	94/102 (92.1%)	5/19 (26.3%)	14/19 (73.7%)	0.026
Sinonasal	2/19 (10.5%)	17/19 (89.5%)	2/6 (33.3%)	4/6 (66.7%)	0.234

2.7 Discussion

While the incidence of HNSCC associated with smoking and alcohol consumption has been reported to have declined, especially in Western countries [24], there has been an increase in HNSCC associated with HPV infection, especially oropharyngeal carcinoma [25,26,27]. HNSCC due to HPV has been found to predominate in North America and Europe, with age standardized incidence rates (ASR) of over 1.25 per 100,000 [2]. Based on regional and country analysis, attributable fractions (AF) (proportion of HNSCC primarily caused by HPV) of over 40% have been reported in Northern America, Australia, New Zealand, Japan and the Republic of Korea, while much lower AF were observed in other countries including those from Sub-Saharan Africa [2]. This was further reiterated by a study conducted in Europe, where it was established that the 9-valent vaccine HPV types (HPV11, 6, 18, 16, 31, 33, 45, 52 and 58) are responsible for about 6,786 of 7,230 cases of HNSCC that occur annually due to HPV infection [28]. Currently, there is little African data on the role of HPV in HNSCC, with the majority of studies suggesting weaker association with HPV on the continent. For instance, in a PCR-based study using FFPE tissue from patients with confirmed HNSCC in Senegal, HPV DNA was detected in 3.4% of samples tested [29]. In a similar study performed in Ghana, 19.2% of FFPE tissues were positive [30], while in Nigeria no HPV DNA was detected in 149 FFPE head and neck biopsies tested using a Linear Array genotyping assay [31]. Similarly, no HPV DNA was detected in 51 FFPE samples from oral tongue and oropharynx squamous cell carcinoma patients tested using HPV genotype 16 specific PCR for the oncogenes E6 and E7 and p16 immunostaining [32].

To advance knowledge on HPV in HNSCC in Africa, we investigated the prevalence of HPV DNA in HNSCC patients in our South African setting for a period of 11 years (2004-2014). The rate of HPV DNA detection in our study was 7.8%, which was low compared to regions such as Europe and Asia. A prevalence of 40.0% was obtained in a meta-analysis conducted inEuropean populations, while 18% of salivary gland and head and neck tumour samples tested positive for HPV DNA in a Hungarian study and 23.5% HPV DNA prevalence was also obtained from 307 HNSCC cases from eight health care centers mainly from Northern Germany [33,34,35]. In Asia, where the HPV DNA analysis was performed mainly from the oropharynx, oral cavity, hypopharynx and larynx of the HNSCC patients, a prevalence of approximately 30% was obtained [38,39]. However, our results were in agreement with the low prevalence that has been reported in developing countries, including those in Africa [2, 29, 30, 31, 32]. While the few studies reported from SA have had small patient numbers and conflicting results, our observations were similar to some of the earlier reports describing HPV prevalence in the country [41,42,43,44,45]. In the recent study conducted in our setting on 112 HNSCC samples tested for HPV DNA following $p^{16INK4a}$, the authors reported HPV prevalence of 6.3% [41]. Moreover, in a retrospective study on 66 FFPE oral squamous cell carcinoma (OSCC) samples from patients in Ga-Rankuwa were screened for HPV DNA using radiolabelled probes for HPV6, 11, 16, 18 and further tested for HPV antigen by immunohistochemistry [42] and another study on 57 samples from black South African population with OSCCs were tested for HPV by PCR with primers targeting the E6 region of HPV6, 11, 16, 18 and confirmed by Southern blotting [43], both studies found HPV DNA in only one sample. The authors concluded that HPV did not play a major role in OSCC in the population studied. A further report suggesting that HPV is not significant in OSCC in the South African population tested 59 OSCC samples from lining mucosa anterior to the anterior tonsillar pillar, the masticatory mucosa of the gingiva and palate and specialized mucosa of the tongue using three nucleic acid hybridisation methods for detection of HPV, namely conventional in situ hybridization (ISH), a signal amplification ISH technique (Dako GenPoint™, Hamburg, Germany) and real-time PCR. HPV18 was detected in 7/59 samples by real-time PCR, while the other techniques were unable to detect any evidence of the virus. The authors felt that the positive results obtained might be due to contamination and concluded that HPV plays no significant role in OSCC patients in South Africa [44].

HPV DNA is mostly detected in the oropharynx especially the base of tongue and palatine tonsils and to a lesser extent in the oral cavity and larynx [2, 46, 47]. Our study was consistent

with global findings as a greater number of tumours from OPSCC (10.8%) tested positive for HPV DNA compared to other sites/subsites such as the oral cavity (6.1%) and larynx (6.9%). A higher prevalence of HPV was also found in the sinonasal tract. Out of 25 cases associated with malignant transformation in the site, HPV DNA was detected in four cases (16.0%) with amplification of HPV16, 18 and 45. The presence of HPV DNA in samples from the sinonasal tract has been reported in other studies conducted globally and it has been noted that high risk HPV, especially HPV16/18, were closely associated with tumour progression in the sinonasal tract [48, 49, 50, 51].

Demographic data from the worldwide cancer registry on the incidence of upper aero- digestive tract cancer caused by HPV versus tobacco smoking between 1983-2002, reported that patients who were HPV positive, especially with oropharyngeal carcinoma, were frequently found to be males, under the age of 60 years and non-smokers [52]. This was further confirmed by de Martel and colleagues in a review of HPV as a source of cancer world-wide, where they found that HPV positive tumours were more common in males, with the majority detected in the oropharyngeal region [2]. Our study found women to have a significantly higher HPV prevalence in oropharyngeal carcinomas and males a significantly higher HPV prevalence in oral cavity SCC (Table 2.6). Although there were fewer samples from females in the study (149 compared with 631) for which the reason could not be determined with any certainty. It was not possible to confirm whether the patients were tobacco smokers or not as clinical records were not available in this study. We did not find a significant difference in the age of the patients between those with HPV positive and HPV negative tumours for any of the sites. HPV16 was the most common HPV type identified, constituting 45.6% of HPV isolates, and was found to be distributed across most of the sites analysed (Figures 2.2 and 2.3). However, other high risk HPV types, viz. types 18, 31, 33, 35, 45, 52, and 59 were also detected. While HR HPV types have been reported as a possible aetiological agent in HNSCC, low risk HPV types (6, 11, 13 and 2) were also detected in some of the samples screened. These findings regarding the detection of high- and low-risk types from HNSCCs are similar to reports from other studies although the significance of LR HPV and association with HNSCC is unknown [2, 4, 28, 53, 54].

Information on the prevailing HPV types in HNSCC in South Africa is important for vaccine development. The prophylactic HPV vaccine (Cervarix®) which is currently in use in South African public schools against cervical cancer protects against HPV16 and 18 [55,56]. This

vaccine was shown to have close to 100% efficacy in preventing infection with HPV16 and HPV18, with an estimated reduction risk of cervical cancer by 50-70% [57]. Other available vaccines include Gardasil 4-valent and 9-valent (Merck & Co) [58,59]. With protection inference reported by Herrero et al. for the Cervarix vaccine on non-genital cancers, inclusion of these vaccines as part of national immunisation programme could provide additional benefit as they include multiple HPV types [60]. Gardasil 9-valent, with 90% potential protection against cervical cancers, could have significant impact in our setting since majority of the HPV types detected in our study (Figure 2.1) are part of the vaccine and known to be responsible for most HPV-associated HNSCC in European countries annually [28]. Introduction of this vaccine could prevent the spread of these HPV types hence reducing or maintaining the low prevalence of HPV associated with HNSCCs.

Despite the low HPV prevalence observed (7.8%), we were able to detect HPV DNA in FFPE samples from HNSCC patients analysed in the study. While the presence of HPV DNA alone cannot be used to confirm that the virus is responsible for the cancer [61,62], nonetheless, studies of this nature are informative in determining the prevalence of HPV types that are circulating in a particular setting. Oncogene expression is challenging to perform on FFPE tissues as most of the RNA is heavily degraded and fragmented during tissue fixation as compared to DNA [63]. Hence this finding calls for a detailed analysis on HPV in HNSCC in patients from South Africa that will include oncogenic gene expression analysis to confirm the involvement of the virus in the carcinogenesis.

2.8 Conclusion

The current study in which 994 cases of HNSCC were reviewed retrospectively, is the largest study on HPV prevalence to have been conducted so far in South Africa. According to the findings, the prevalence of HPV in HNSCC in our setting (7.8%) is in accordance with the majority of previously conducted studies in the country though lower relative to that reported globally. Although HPV16 and 18 have been implicated in squamous cell carcinogenesis and are known to be responsible for 85% of HNSCC globally, our findings show that HPV18 might be less important in this setting as only two samples tested positive for HPV18 DNA. In addition to the most common sites associated with head and neck cancer as described in previous studies, the sinonasal tract was also found to contribute the majority of cases in this population. Therefore, in order to fully understand the impact of HPV in cancer of the head

and neck in this population, future studies should continue to monitor changing trends in HPV infection, including genotypes and anatomical sites affected.

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CHAPTER 3

PHYLOGENETIC ANALYSIS OF HUMAN PAPILLOMAVIRUS VARIANTS FROM HEAD AND NECK SQUAMOUS CELL CARCINOMAS

The editorial style of the Scientific Reports Journal was followed in this chapter

3.1 Abstract

HPV variants show different oncogenic potential therefore large scale screening is required, which includes a large sample size to determine different variants for each HPV type circulating in a setting. Therefore, the current research explored phylogenetic relatedness and nucleotide variability among the six most prevalent high risk HPV types associated with HNSCC patients in our setting.

A total of 39 FFPE samples were previously identified to be positive for high risk HPV DNA and were further amplified by heminested PCR targeting the L1 region of the genome to obtain a longer L1 fragment suitable for intra-type analysis. Twenty samples were amplifiable and qualified for further analysis which included performance of nucleotide sequence analysis and phylogenetic investigation using the Maximum Likelihood method. Samples included HPV16 (n=12), HPV18 (n=2), HPV31 (n=1), HPV33 (n=2), HPV52 (n=1) and HPV45 (n=2). HPV31 and 52 partial L1 sequences resulted in phylogenetic trees similar in topology to those constructed with complete L1 and complete genome sequences, hence we were able to precisely classify the unknown isolates. The HPV31 isolate was classified as a B lineage while the HPV52 isolate clustered with the A lineage. In the other HPV types the amplified L1 fragment was too conserved to give an exact classification of the variants. In HPV18, the isolates could only be classified as an African (5228) and non-African (5267), while in HPV16, five samples were classified as non-European and seven as European variants. Use of partial L1 sequencing for HPV45 resulted in a phylogenetic tree clustering into A and B lineages, with lineage B further classified as B1 and B2. In lineage A, the partial sequences successfully clustered sub-lineage A1 and A3, while the sequences were too conserved to classify the A2 sub-lineage. The HPV45 isolate in our study was classified under sub-lineage A1. HPV33 nucleotide sequences in the GP5+/PGMY09 region could not provide distinct lineages nor sub-lineages, therefore the two samples positive for HPV33 in our study could not be characterised. A total of 10 single nucleotide polymorphism (SNPs) were identified. Three of the HPV16 non-European isolates (2658, 7244 and 9939) shared one non-synonymous mutation at

nucleotide position 1057. This substitution resulted in an amino acid change from threonine to proline. In the European lineage, only isolate 9462 had a mutation which was located at position 1190 of the genome, resulting in an amino acid change of threonine to isoleucine. In addition, HPV33 isolates also showed a non-synonymous mutation at nucleotide position 1155 for both isolates resulting in an amino acid substitution of glutamic acid to aspartic acid.

This research study reported HPV variants that are associated with HNSCC in a South African cohort of head and neck cancer patients, and the SNPs that can occur in the region, though their significance is still unknown. The discriminatory power of the partial L1 sequences obtained from the GP5+/PGMY09 region varied depending on the HPV type, when compared to complete L1 and complete genome sequences.

3.2 Introduction

Human papillomaviruses (HPV) are an alternative risk factor to tobacco and alcohol abuse, radiation exposure and long-term betel nut chewing in the development of head and neck squamous cell carcinoma (HNSCC) [1,2], the sixth most common cancer globally[3]. This group of viruses have a double stranded, circular DNA genome that is approximately 8 kb in length[4]. The genome is organised into eight protein-coding genes, including capsid protein encoding genes (L1 and L2), genes encoding proteins involved in transcription, replication and transformation (E1, E2, E4, E5, E6 and E7), and a noncoding region called the regulatory long control region (LCR)[4]. Analysis of nucleotide sequence data for HPV has shown that the L1 gene is the most conserved within and between types, and therefore, suitable for constructing the genetic relationship between members of the *Papillomaviridae* [5].

The *Papillomaviridae* family is comprised of over 200 HPV types that are differentiated based on percentage similarity of the L1 gene. Different types have less than 90% similarity in nucleotide sequence of the L1 gene[6]. HPV types are grouped into five genera: alpha, beta, gamma, mu and nu papillomavirus[7] with 40% genomic difference between genera. Within each genus, there exist viral species which are HPVs from the same genus with 30-40% genomic difference. HPV types, which consist of HPVs with 10-30% viral genetic variation, are further sub-divided into sub-types. A sub-type (variant lineage) is assigned to isolates within the same type with 1-10% heterogeneity in the L1 gene while sub-lineages have less than 0.5-1% nucleotide difference[7-9].

Among the identified HPV types, HPV16 is phylogenetically classified within the *Alphapapillomavirus* 9 species and is a highly oncogenic HPV type, which is associated with 70-90% of cancers of the oropharynx[9-11]. For HPV16, a report published in 1993 on the phylogenetic analysis of the cloned LCR segment (7478 to 7841) of 301 viral isolates from 25 different ethnic groups and geographic regions in Africa, Asia, America and Europe, identified four major HPV16 lineage branches. These were named based on the population groups in which they were predominantly detected, namely African 1, African 2, Asian-American and European [12]. The latter two intra-typic variant lineages were further divided into sub-lineages, where the European lineage was divided into European (E) and European Asian (E(As)), while the Asian-American lineage was divided into Asian-American 1 and 2 (AA1, AA2) and North American 1 (NA1). This analysis utilized 412 HPV16 positive isolates from GuaCaste, Costa Rica based on complete genome and partial L1, E6 and URR sequences[13]. Sixty-two of these

isolates were further classified into lineages and sub-lineages based on an alphanumeric system, where European, Asian-American and African were represented by A (A1 to A4), D (D1 to D2) and B/C (B1 to B2) respectively [14].

In addition to HPV16, another important HPV type implicated in head and neck cancer is HPV18, which is classified under *Alphapapillomavirus* 7[15]. Initially, HPV18 variants were classified into two main variant lineages based on a cloned segment of the long control region (LCR) from position 7585 to 7805 of the genome. The isolates were of different human ethnic groups from various geographical locations including Germany (Erlangen), Scotland (Glasgow), Greece (Athens), Japan (Sapporo) and Singapore (Chinese patients) which represented the non-African lineage, while samples from patients originating in different parts of Tanzania resulted in the African lineage[16]. Subsequently, Chen and colleagues performed an intra-typic analysis of HPV18 upstream regulatory region (URR), E6 region and complete genome sequences using cloned samples from Costa Rica. Based on the analysis, the previously obtained non-African lineage was further sub-divided into European 1 (E1) and European 2 (E2), while the African lineage was sub-divided into African 1 (Af1) and African 2 (Af2)[17]. Further intra-typic phylogenetic analysis of the HPV18 genome using complete genome sequences of samples from different parts of the world including Costa Rica, Taiwan, Thailand, Rwanda, Burkina Faso and Zambia introduced the use of an alphanumeric system in lineage/sub-lineage naming as an alternative to geographic region naming. The non-African (European) variant lineage was designated as the A lineage while the African variant lineage was designated B and C. The HPV18 A lineage was further sub-divided into five sub-lineages (A1-A5) where A1 and A2 are Asian American and A3 to A5 are European, and the B lineage was sub-divided into three sub-lineages, B1-B3[14].

The HPV16 related alpha-9 types HPV31, 33 and 52 variants were also classified based on samples from different parts of the world including Costa-Rica, Taiwan, Thailand, Rwanda, Burkina Faso and Zambia. Classification was done using partial sequences of the URR and/or E6 ORF for screening, while the complete genome sequences were used to construct phylogenetic trees. The phylogenetic tree constructed using HPV31 variants clustered into three major branches, A, B and C. The HPV31 prototype (HPV31. REF) was grouped under the A lineage, while lineage C differed from the A and B lineages by mean values of 1.2±0.11% and 1.2±0.12% respectively. The HPV33 phylogenetic tree clustered into two main branches, A and B, using URR/E6 nucleotides sequences. The A lineage was further sub-divided into

sub-lineages (A1 and A2) that were 0.6±0.7% different from each other and 0.9±0.09% separated from lineage B. The phylogenetic topology of HPV52 variants clustered into four distinct lineages, A, B, C, and D, with lineages A, B and C sharing a common ancestor and 0.8%-2.0% separated from each other. The monophyletic group was equally distant to the D lineage (1.8%-2.0%). Classification of this HPV type was performed using the URR/E6 region for screening and the complete genome for constructing the phylogenetic tree[18]. For HPV45 variants (alpha-7), phylogenetic analysis was performed based on variants from Costa Rica, Rwanda, Zambia, and Burkina Faso, and using other HPV45 nucleotide sequences including reference isolates from GenBank. The phylogenetic tree topology was separated into two lineages, A and B, which were both further sub-divided into sub-lineages, with three in lineage A (A1-A3) and two in lineage B (B1 and B2)[14,15].

Regardless of phylogenetic relatedness, HPV variants show different oncogenic potential[19,20]. Therefore, large scale screening is required, which includes a large sample size to determine different variants for each HPV type circulating in a setting. Variant analysis is best described using sequence heterogeneity across the whole genome[21]. The viral genome is preferably extracted from fresh frozen tissue samples, however currently there is no standardised procedure for processing and storing of fresh-frozen tissue samples in large numbers, hence their availability is limited[22]. Formalin fixed paraffin embedded tissues (FFPE), which are stable at room temperature and can be stored for long periods of time, are most commonly available in archives worldwide and are therefore important for large scale and retrospective analysis of HPV variants[23]. Nevertheless, whole genome extraction from FFPE samples is challenging because in most cases the nucleic acid is fragmented[24]. Hence use of FFPE samples may be ideal in the analysis of partial HPV genome sequences. Although it has been reported that use of the L1 segment is sufficient in classification of HPV types, there is also loss of clear resolution if L1 is used to define distinct variant lineages and sub-lineages within types, as variants of the same type are closely related.

In a related study, high risk HPV16, 18, 31, 33, 52 and 45 were identified from FFPE tissues from patients with histologically confirmed HNSCC in South Africa. Subsequently, our study investigated HPV variant lineages using sequence data derived from these samples, as there is currently no information regarding which HPV variant lineages are circulating among head and neck carcinoma patients in the country. Due to the nature of the samples, complete genome or complete L1 ORF sequence data could not be amplified. Hence, to determine the variant

lineages/sub-lineages and phylogenetic relatedness, a partial L1 gene nucleotide sequence was used. This data was available from a previous study (Chapter 2). In retrospect, the non-coding region may have given better resolution and less conserved data, however, the L1 gene is still a commonly targeted region and the aim was to determine if we could identify lineages based on limited data. The constructed phylogenetic trees were further compared to the L1 ORF and complete HPV genome sequence phylogenies constructed using published nucleotide sequences obtained from GenBank.

3.3 Aim

The aim of this chapter was to investigate the phylogenetic relationship of the high risk HPV strains from the patients through construction of the phylogenetic trees using the Maximum Likelihood model method

3.4 Objectives

- To investigate phylogenetic relatedness of high risk HPV types obtained using partial L1 nucleotide sequences
- To validate the reliability of the phylogenetic trees of all the HPV variants constructed using the partial sequences, the trees were compared to those generated from the complete genome sequences and complete L1 gene nucleotide sequences

3.5 Materials and Methods

Table 3.1 below, is a summary of patient's demographic information where HPV DNA was successfully extracted and amplified. In total, 20 samples (20/39; 51%) were successfully amplified using the primer pairs provided in Table 2.4, and amplicon sizes ranging from 326-424bp were obtained depending on the HPV type [HPV16, 18 & 31 (410bp), HPV45 (413bp), HPV33 (424bp) and HPV52 (326bp)]. HPV types were confirmed by analysis of nucleotide sequence data using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website and the expected HPV types were confirmed.

Table 3.1: Anatomical sites and demographic data of HNSCC patients with amplifiable HPV DNA

Total number of cases	20	**HPV type**
Mean Age of patients and range	54 years (29-73 years)	
Gender		
Female	7/20 (35%)	
Male	13/20 (65%)	
Anatomical site		
Larynx	5/20 (25%)	33, 18, 16
Oropharynx	4/20 (20%)	52, 16
Oral cavity	7/20 (35%)	31, 16
Nose	4/20 (20%)	45, 18, 16

In summary, sequence data available for phylogenetic analysis of high risk HPV types were as follows: HPV16 (n=12), HPV18 (n=2), HPV31 (n=1), HPV33 (n=2), HPV52 (n=1) and HPV45 (n=2) and the constructed phylogenetic trees are depicted in Figures 3.1 to 3.6. These trees included representatives of lineages and sub-lineages selected from GenBank to determine topology and phylogenetic relationship. A list of accession numbers for HPV sequences used in Figures 3.1 to 3.6 from GenBank is provided in the supplementary data.

3.5.1 Phylogenetic and sequence analysis

To explore the phylogenetic relationship of the high risk HPV types identified in the study, six phylogenetic trees were constructed for the partial L1 sequences (GP5+/PGMY09 region) of the samples and sequence data retrieved from GenBank using the Maximum Likelihood model method and the constructed trees topology is shown in Figure 3.1c to 3.6c. To validate the reliability of the phylogenetic trees of all the HPV variants constructed using the partial sequences, the trees were compared to those generated from the complete genome sequences and complete L1 gene nucleotide sequences [Figure (3.1a and 3.b) to (3.6a and 3.6b)]. To identify mutations, nucleotide sequences of the HPV isolates in the study were aligned with reference sequences to identify mismatches. Table 3.2 and 3.3, demonstrate a total of 10 nucleotide mutations detected in the partial L1 sequences among HPV types analysed. Three non-synonymous mutations were identified in HPV16 and one in HPV33.

HPV16 partial L1 sequences were resolved into two lineages, namely the European (A) and Non-European (B, C and D) lineages. The majority of the SA isolates clustered with the reference strain (NC001526.4) under the European lineage (7/12: 58%), while five isolates grouped with the non-European lineage (5/12: 42%) (Figure 3.1c). Phylogenetic analysis further showed that the 410 bp (GP5+ and PGMY09-R) L1 fragment does not contain sufficient nucleotide variability to enable discrimination between sub-lineages. To confirm this, the tree derived from partial nucleotide sequences was compared to trees constructed using complete HPV16 and complete L1genomes in Figure 3.1a and Figure 3.1b. Similar loss of clear resolution of sub-lineage discrimination was noted in the complete L1 phylogeny where only the A (European), B (African) and C/D (Asian-American) lineages could be identified. The SA isolates in the non-European lineage (1609, 5706, 2658, 7244, 9939) differed from the published reference strain genome by 3-5 point mutations within the 410bp amplicon. Three of these isolates (2658, 7244 and 9939) share one non-synonymous mutation at nucleotide position 1057. This substitution resulted in an amino acid change from threonine to proline. Of the other SA isolates in the European lineage, only isolate 9462 had a mutation which was located at position 1190 of the genome, resulting in an amino acid change of threonine to isoleucine as depicted in Table 3.2.

HPV18 sequences clustered into three groups; A previously known as the non-African lineage, B formerly designated as African and a third branch made up of two isolates (Qv39775 and BF226) designated lineage C, which is part of the African lineage and differs from lineage B variants by 1.0%-1.2%[14,17]. The partial L1 phylogenetic tree also segregated into three major lineages with the two SA HPV18 sequences classified as African (5228) and non-African (5267) according to Figure 3.2c. Within lineages A and B, partial nucleotide sequences were highly conserved with average nucleotide distances of 0.04 and 0.11 respectively and were therefore unable to form distinct sub-lineage branches. As shown in Figure 3.2b, by contrast, the complete L1 ORF was able to resolve all A and B sub-lineages identified by complete genome phylogeny. A number of SNP patterns were detected in samples, with no amino acid changes. A total of four SNPs (G1290A, G1320A, C1413G and G1488A) were detected in isolate 5228, while one was detected in isolate 5267 (C1413G) within the 410bp amplicon when compared to the prototype NC_001357.

As displayed in Figure 3.3a and 3.3b, the phylogenetic trees generated from the complete L1 gene and complete genome nucleotide sequences from GenBank clustered HPV31 variants into

three lineages labelled A, B and C. The phylogenetic tree inferred from the partial L1 region of the gene in Figure 3.3c, resulted in similar topology. HPV31 isolate (2687) clustered under lineage B. A total of four point mutations (G1221A, G1245A, C1266A, C1311T) were detected along the 410bp region compared to the reference strain (J04353.1). None of the nucleotide variations resulted in amino acid changes.

As seen in Figure 3.4a and 3.4b, the topology of the tree constructed using the HPV33 complete genome sequences and L1 ORF revealed two distinct lineages, named A and B. Lineage A was further sub-divided into three sub-lineages, A1, A2 and A3. Use of partial L1 sequencing was unable to properly group both the A and B lineages as most of the sequences in this lineages showed a high level of conservation throughout the amplified L1 fragment, with the nucleotide divergence range of 0.003 to 0.017 in lineage A, while the B lineage had 0.003 nucleotide diversity. Therefore, we could not classify our samples 1673 and 7018 as they both had no nucleotide divergence in the partial L1 and were scattered along with the rest of the A and B lineage sequences. Mutations were determined across the amplified L1 gene (424bp) by aligning unknown HPV33 isolates with the reference strain, which showed that both the sequences possess point mutations at position 1071 and 1155 as depicted in Table 3.3, with an additional mutation at position 1111 for isolate 7018. The mutation at nucleotide position 1155 for both isolates resulted in an amino acid substitution of glutamic acid to aspartic acid.

Amplification and sequencing of the partial L1 region of HPV52 isolate 1704, was performed. Figure 3.5a to 3.5c shows the phylogenetic tree inferred from the complete genome, complete L1 genome and partial L1 nucleotide sequences which clustered HPV52 isolates into four distinct lineages A, B, C and D, with lineages B and C further divided into sub-lineages B1 and B2 and C1 and C2 respectively. There was no nucleotide variation in the 326bp L1 region of the 1704 sequence compared to the reference strain X74481.1, which therefore falls within the A lineage.

Lastly, a total of twenty-four previously reported HPV45 complete genome sequences were selected for inclusion in the phylogenetic analysis. HPV45 isolates clustered into two lineages designated A and B. Lineage A was further sub-divided into three sub-lineages, A1, A2, A3 while lineage B, was sub-divided into two sub-lineages B1 and B2 (Figure 3.6a). The two unknown SA variants clustered with variants in the A1 sub-lineage, with only a G1332A silent

mutation and nucleotide variation of 0.003 detected within the 413bp product of both isolates which therefore shared high similarity with the reference strain X74479.1. Although genetic distances were lower when using partial L1 sequences, differentiation of sub-lineages was possible.

Table 3.2: Genetic variability and amino acid variation of the partial HPV16 L1 gene from isolates of head and neck cancer patients

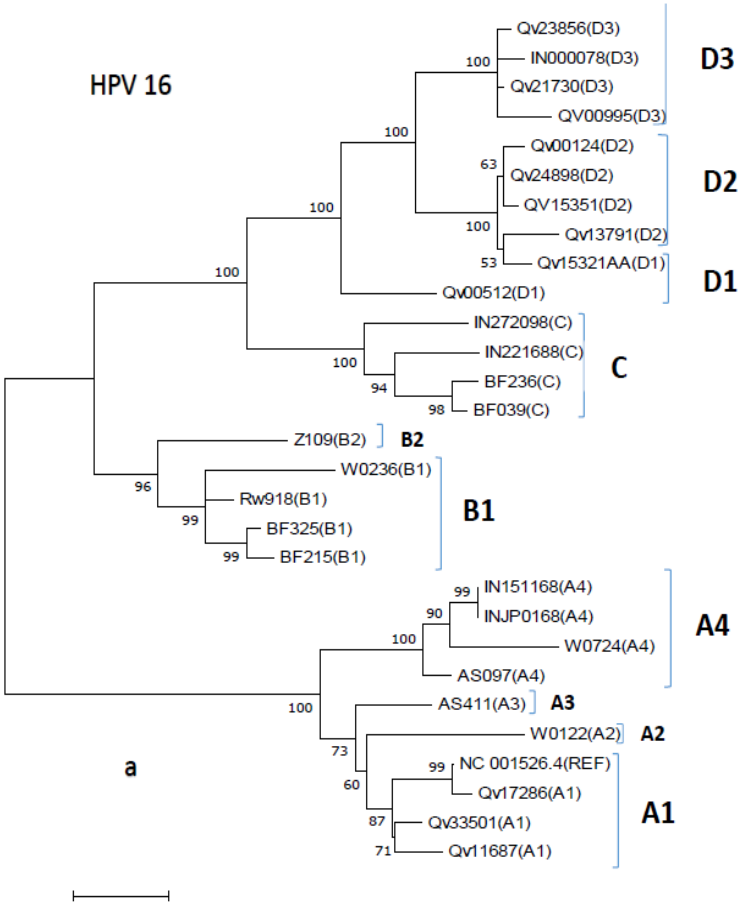
Strain type	L1 Nucleotide position	1057	1083	1190	1216	1227	1230	1332
	Non-synonymous mutation	T353P		T397I	-	-	-	-
	Proto-type (NC001526.4)	A	G	C	C	C	A	C
European	9462	-	-	T	-	-	-	-
Non-European	1609	-	A	-	T	-	G	T
Non-European	5706	-	A	-	T	-	-	T
Non-European	2658	C	A	-	T	T	-	T
Non-European	7244	C	A	-	T	T	-	T
Non-European	9939	C	A	-	T	-	-	T

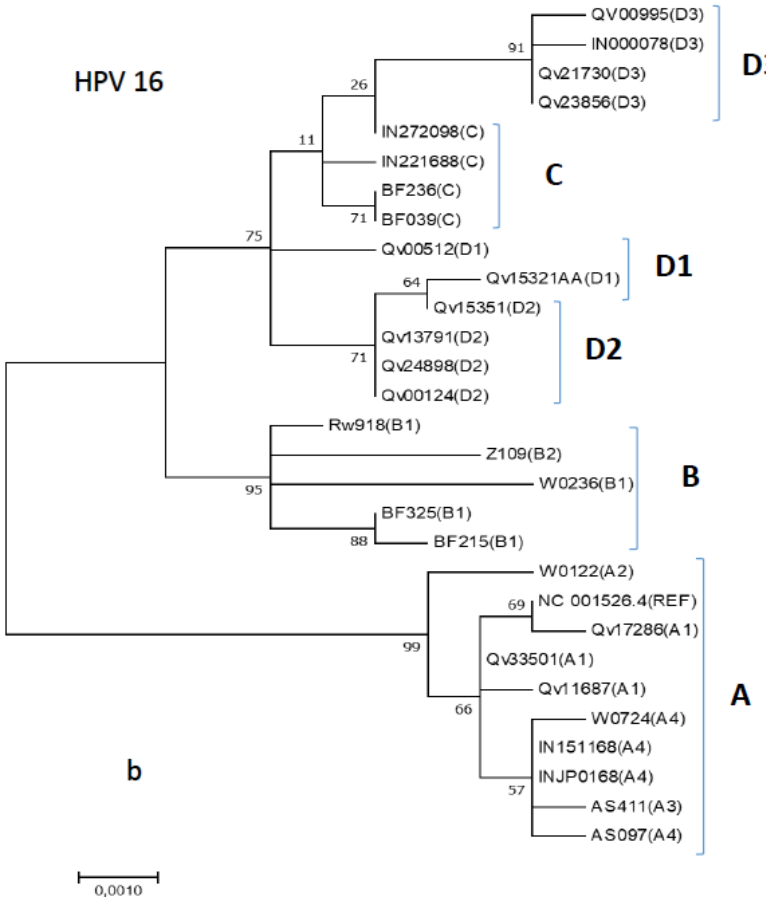
Nucleotides (A-Adenine, C-Cytosine, T-Thymine, G-Guanine)
Amino acids (T-Threonine, P-Proline, I-Isoleucine)
Prototype: Kirnbauer, R. et al. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. J Virol **67**, 6929-6936 (1993)

Table 3.3: Genetic variability and amino acid variation of the partial HPV33 L1 gene from isolates of head and neck cancer patients

Strain types	L1 Nucleotide position	1071	1111	1155
	Non-synonymous mutation	-	-	E385D
	Proto-type (M12732.1)	A	C	A
B	7018	G	T	C
A	1673	G	-	C

Proto-type: Cole, S.T., Streeck, R.E. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. J. Virol **58,** 991-995 (1986)


HPV 16
Qv23856(D3)
IN000078(D3)
Qv21730(D3)
QV00995(D3)
D3
Qv00124(D2)
Qv24898(D2)
QV15351(D2)
Qv13791(D2)
D2
Qv15321AA(D1)
Qv00512(D1)
D1
IN272098(C)
IN221688(C)
BF236(C)
BF039(C)
C
Z109(B2)
B2
W0236(B1)
Rw918(B1)
BF325(B1)
BF215(B1)
B1
IN151168(A4)
INJP0168(A4)
W0724(A4)
AS097(A4)
A4
AS411(A3)
A3
W0122(A2)
A2
NC 001526.4(REF)
Qv17286(A1)
Qv33501(A1)
Qv11687(A1)
A1
a


HPV 16
QV00995(D3)
IN000078(D3)
Qv21730(D3)
Qv23856(D3)
D3
IN272098(C)
IN221688(C)
BF236(C)
BF039(C)
C
Qv00512(D1)
Qv15321AA(D1)
D1
Qv15351(D2)
Qv13791(D2)
Qv24898(D2)
Qv00124(D2)
D2
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NC 001526.4(REF)
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Qv33501(A1)
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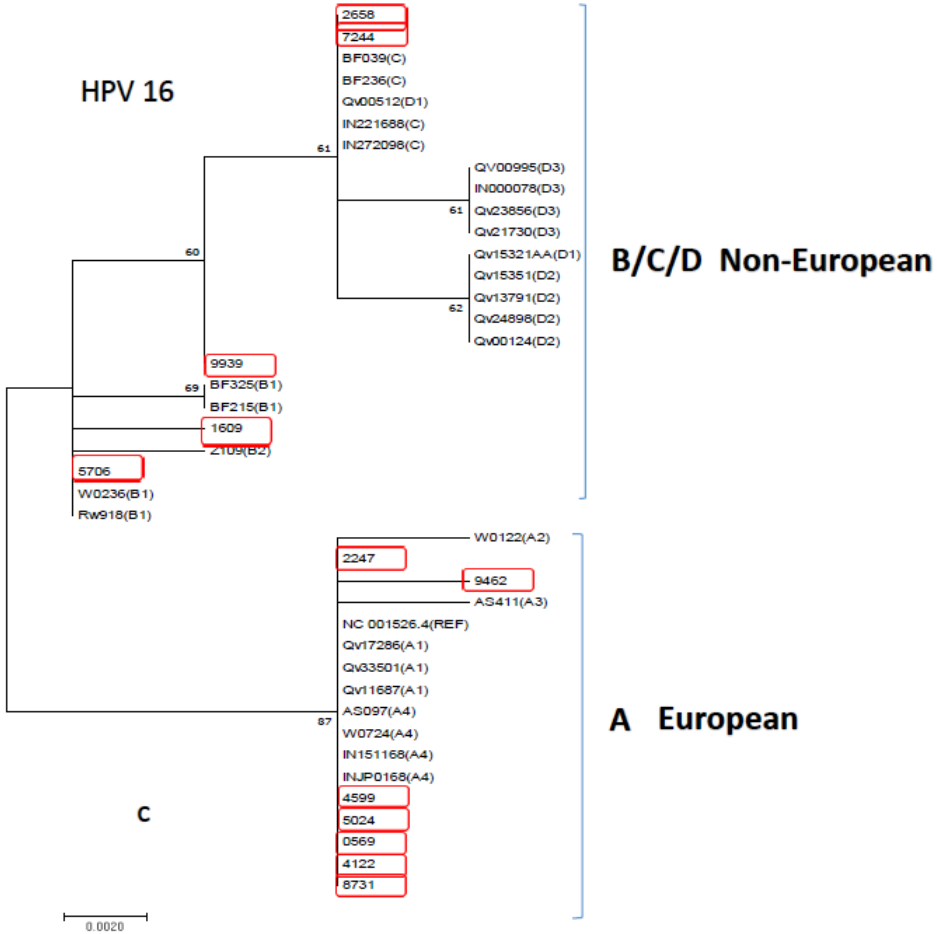
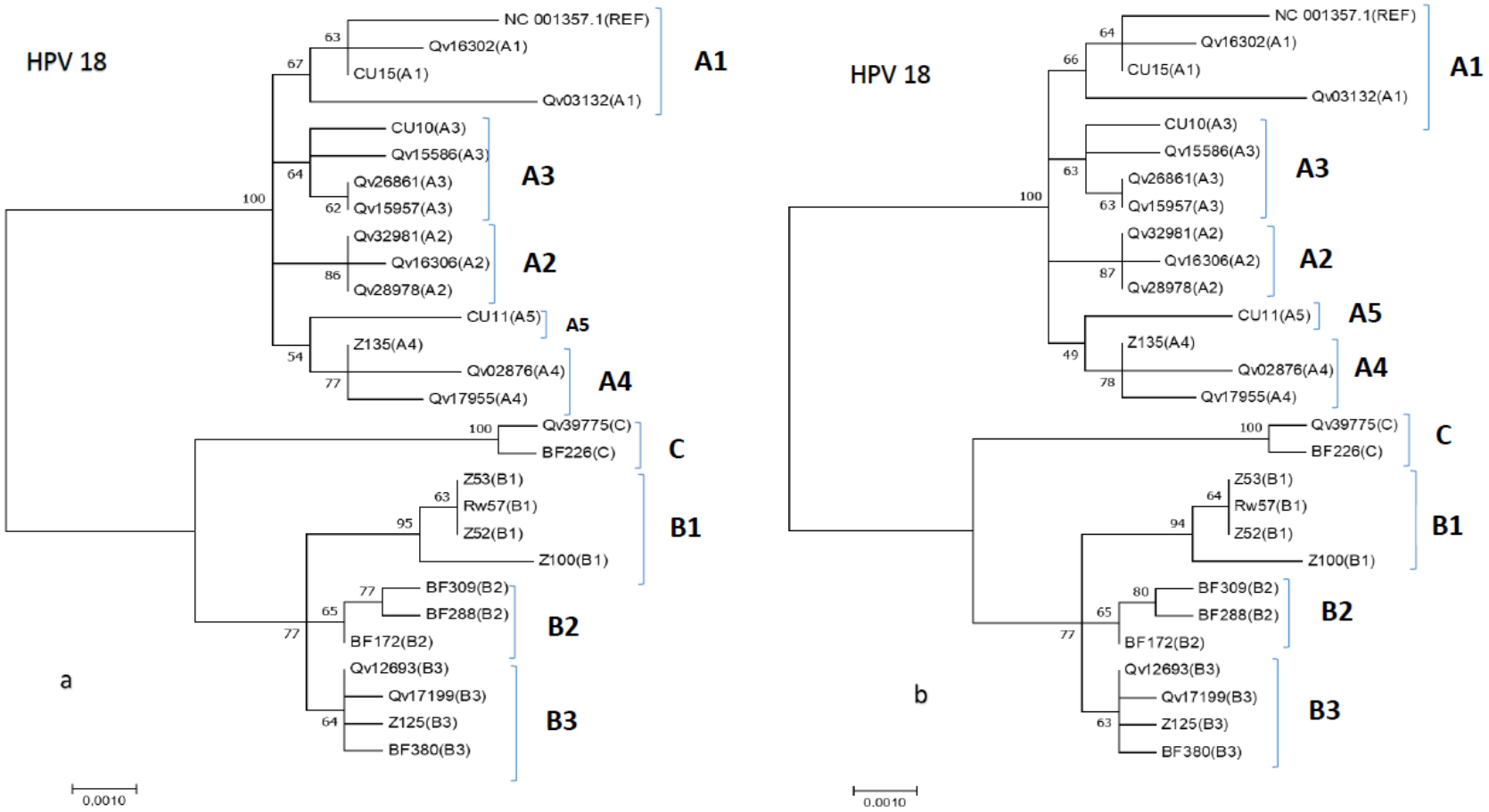


Figure 3.1: Phylogenetic trees showing representatives of variant lineages and sub-lineages of HPV16 cervical cancer specimens from GenBank database constructed by Maximum Likehood method. (a) the complete HPV16 genome, (b) the complete L1 and (c) the partial L1 gene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CU15(A1)
Qv03132(A1)
A1
CU10(A3)
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A3
Qv32981(A2)
Qv16306(A2)
Qv28978(A2)
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A5
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Qv17955(A4)
A4
Qv39775(C)
BF226(C)
C
Z53(B1)
Rw57(B1)
Z52(B1)
Z100(B1)
B1
BF309(B2)
BF288(B2)
BF172(B2)
B2
Qv12693(B3)
Qv17199(B3)
Z125(B3)
BF380(B3)
B3
a
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HPV 18
NC 001357.1(REF)
Qv16302(A1)
CU15(A1)
Qv03132(A1)
A1
CU10(A3)
Qv15586(A3)
Qv26861(A3)
Qv15957(A3)
A3
Qv32981(A2)
Qv16306(A2)
Qv28978(A2)
A2
CU11(A5)
A5
Z135(A4)
Qv02876(A4)
Qv17955(A4)
A4
Qv39775(C)
BF226(C)
C
Z53(B1)
Rw57(B1)
Z52(B1)
Z100(B1)
B1
BF309(B2)
BF288(B2)
BF172(B2)
B2
Qv12693(B3)
Qv17199(B3)
Z125(B3)
BF380(B3)
B3
b
0.0010

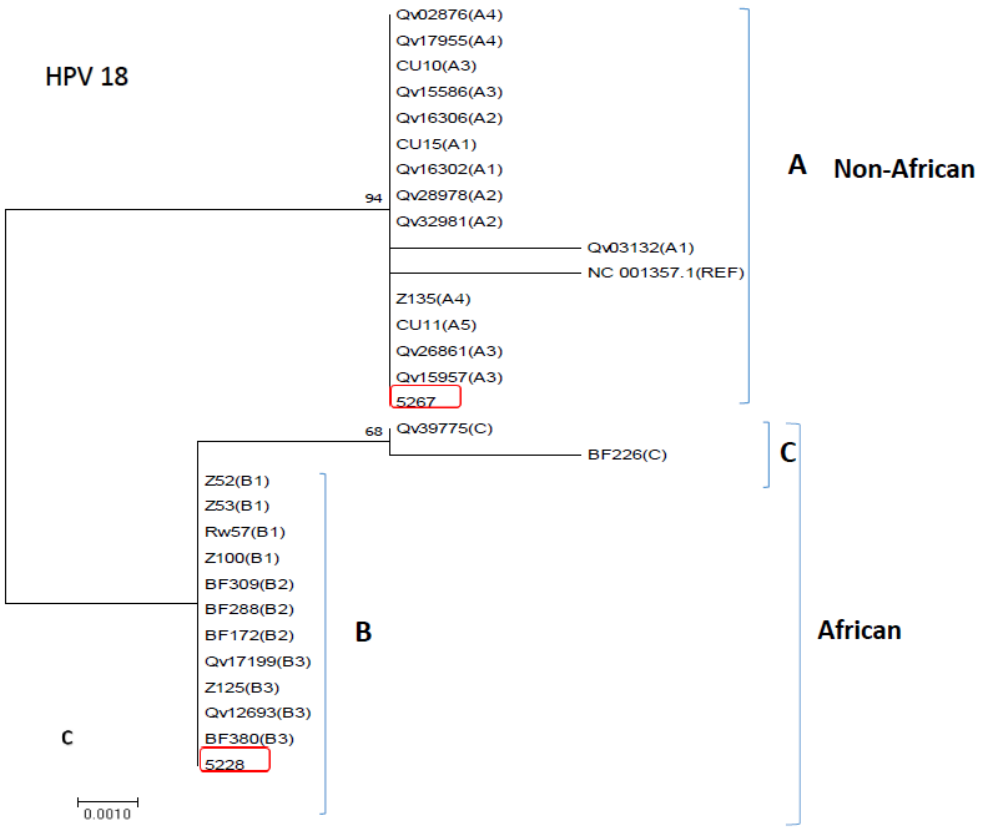
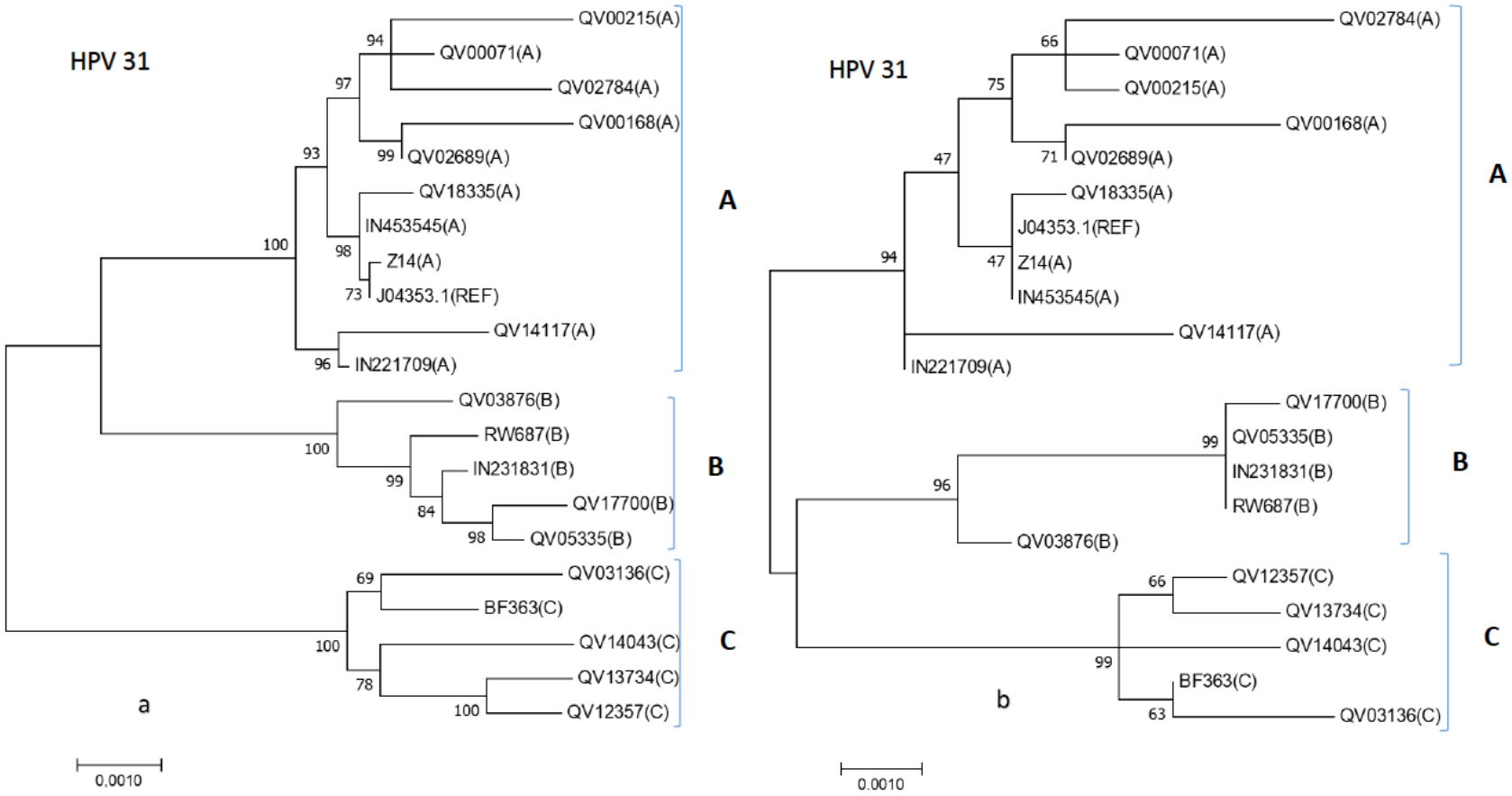


Figure 3.2: Phylogenetic trees showing representatives of variant lineages and sub-lineages of HPV18 cervical cancer specimens from GenBank database constructed by Maximum Likehood method. (a) the complete HPV18 genome, (b) the complete L1 and (c) the partial L1 gene


HPV 31
QV00215(A)
QV00071(A)
QV02784(A)
QV00168(A)
QV02689(A)
QV18335(A)
IN453545(A)
Z14(A)
J04353.1(REF)
QV14117(A)
IN221709(A)
QV03876(B)
RW687(B)
IN231831(B)
QV17700(B)
QV05335(B)
QV03136(C)
BF363(C)
QV14043(C)
QV13734(C)
QV12357(C)
A
B
C
a
0,0010
HPV 31
QV02784(A)
QV00071(A)
QV00215(A)
QV00168(A)
QV02689(A)
QV18335(A)
J04353.1(REF)
Z14(A)
IN453545(A)
QV14117(A)
IN221709(A)
QV17700(B)
QV05335(B)
IN231831(B)
RW687(B)
QV03876(B)
QV12357(C)
QV13734(C)
QV14043(C)
BF363(C)
QV03136(C)
A
B
C
b
0.0010

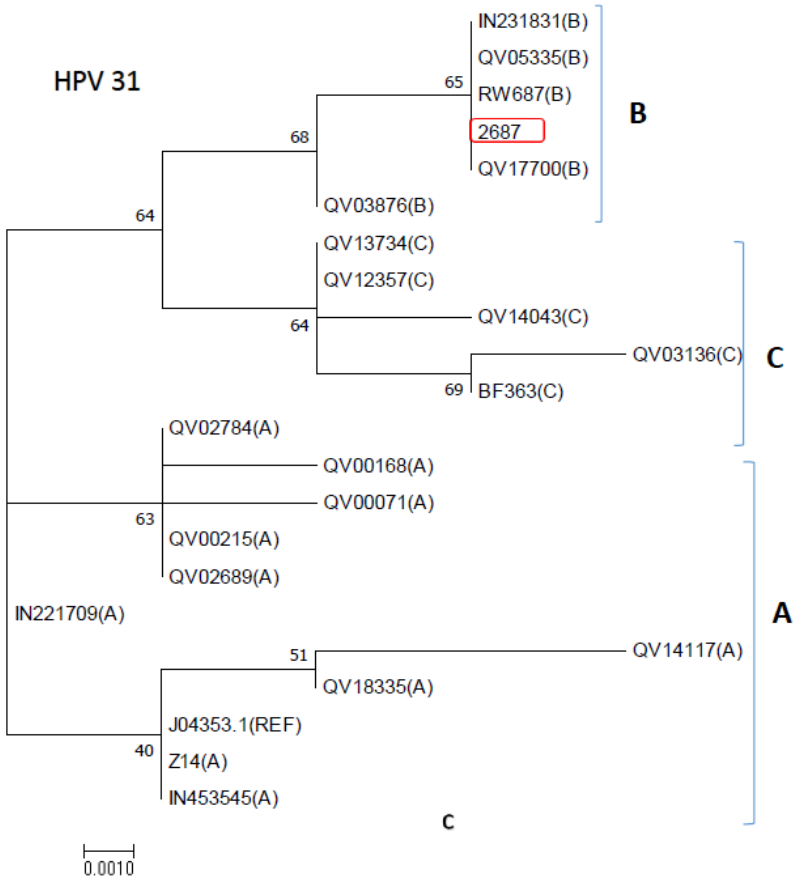
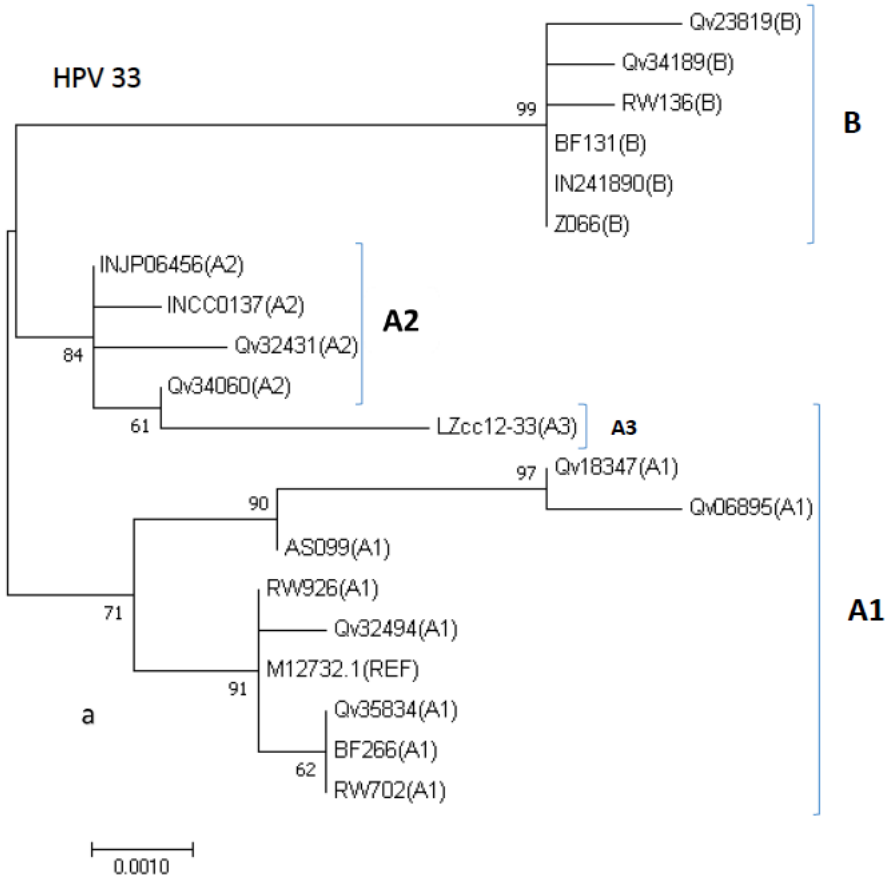
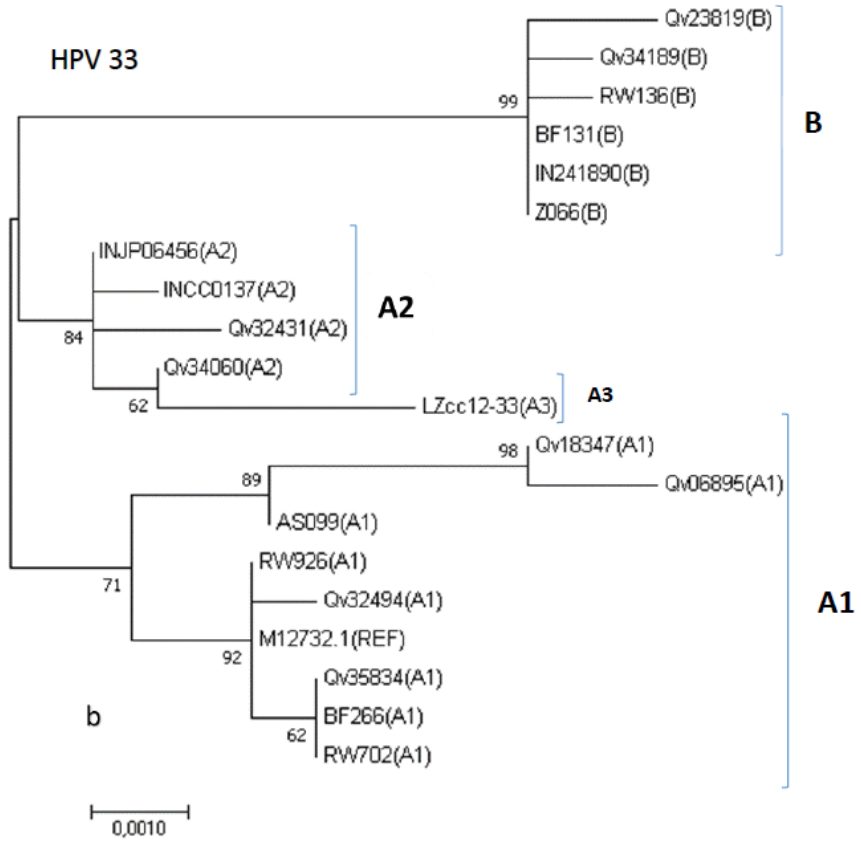


Figure 3.3: Phylogenetic trees showing representatives of variant lineages and sub-lineages of HPV31 cervical cancer specimens from GenBank database constructed by Maximum Likehood method. (a) the complete HPV31 genome, (b) the complete L1 and (c) the partial L1 gene


HPV 33
Qv23819(B)
Qv34189(B)
RW136(B)
BF131(B)
IN241890(B)
Z066(B)
B
99
INJP06456(A2)
INCC0137(A2)
Qv32431(A2)
Qv34060(A2)
A2
84
61
LZcc12-33(A3)
A3
97
Qv18347(A1)
Qv06895(A1)
90
AS099(A1)
RW926(A1)
Qv32494(A1)
71
M12732.1(REF)
91
Qv35834(A1)
BF266(A1)
62
RW702(A1)
A1
a
0.0010


HPV 33
Qv23819(B)
Qv34189(B)
RW136(B)
BF131(B)
IN241890(B)
Z066(B)
B
99
INJP06456(A2)
INCC0137(A2)
Qv32431(A2)
Qv34060(A2)
A2
84
62
LZcc12-33(A3)
A3
98
Qv18347(A1)
Qv06895(A1)
89
AS099(A1)
RW926(A1)
71
Qv32494(A1)
M12732.1(REF)
92
Qv35834(A1)
BF266(A1)
62
RW702(A1)
A1
b
0,0010

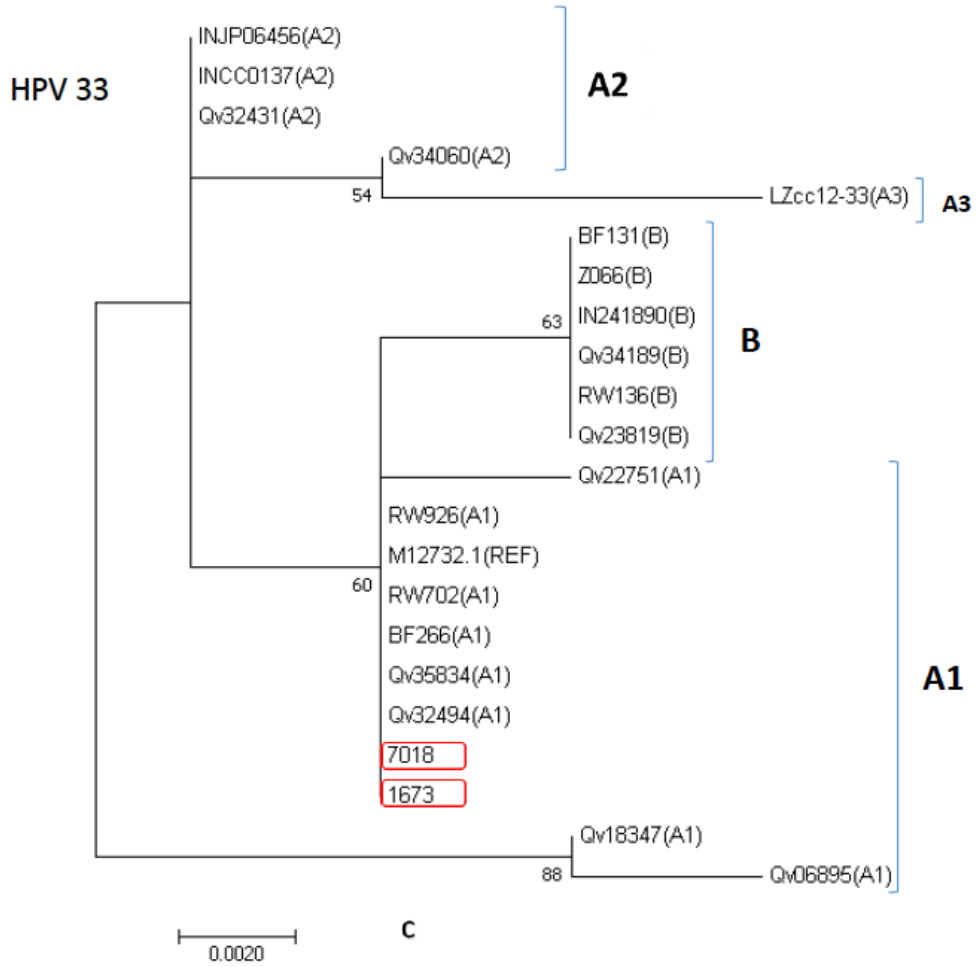
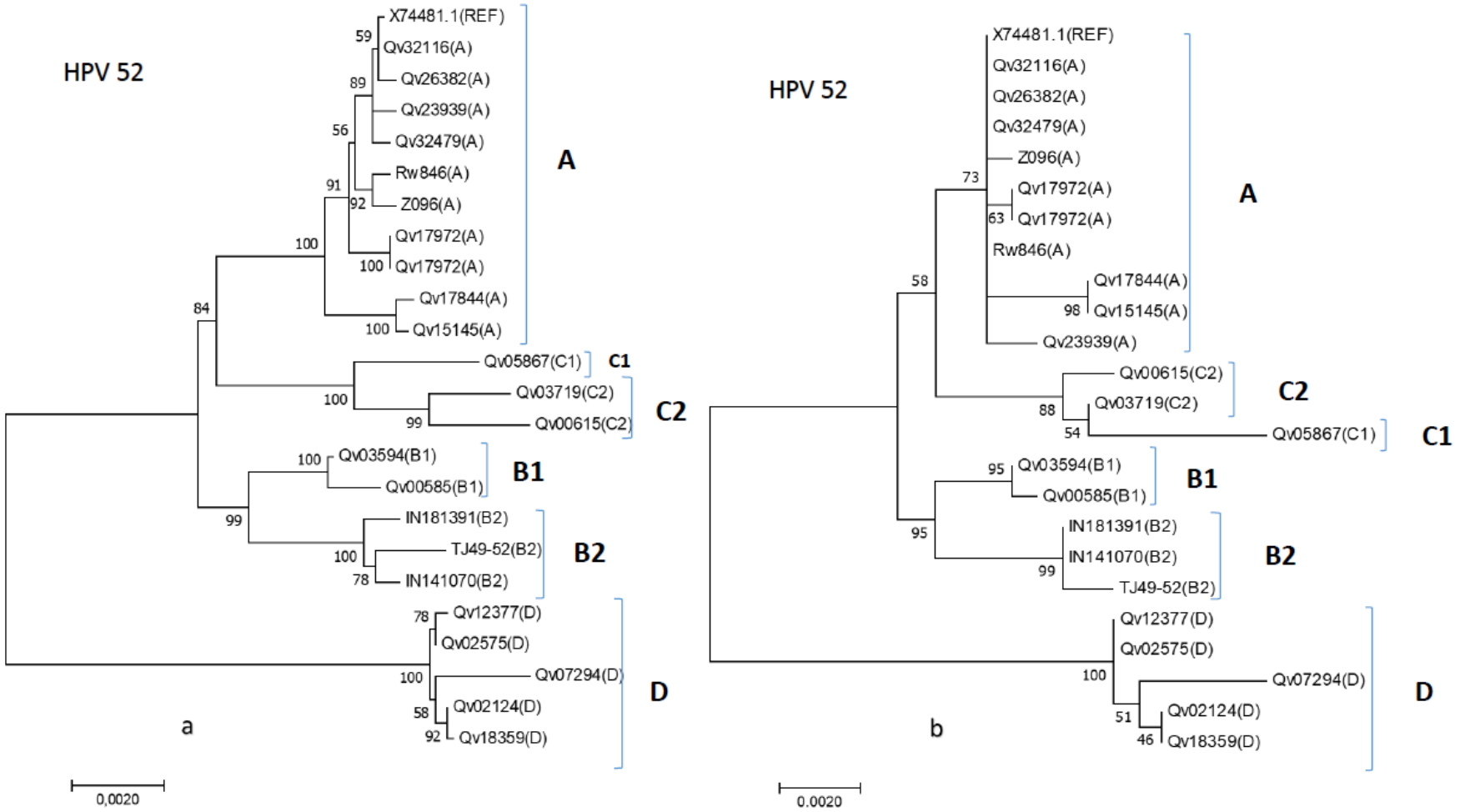


Figure 3.4: Phylogenetic trees showing representatives of variant lineages and sub-lineages of HPV33 cervical cancer specimens from GenBank database constructed by Maximum Likehood method. (a) the complete HPV33 genome, (b) the complete L1 and (c) the partial L1 gene


HPV 52
X74481.1(REF)
Qv32116(A)
Qv26382(A)
Qv23939(A)
Qv32479(A)
Rw846(A)
Z096(A)
Qv17972(A)
Qv17972(A)
Qv17844(A)
Qv15145(A)
A
Qv05867(C1)
C1
Qv03719(C2)
Qv00615(C2)
C2
Qv03594(B1)
Qv00585(B1)
B1
IN181391(B2)
TJ49-52(B2)
IN141070(B2)
B2
Qv12377(D)
Qv02575(D)
Qv07294(D)
Qv02124(D)
Qv18359(D)
D
a
0,0020
HPV 52
X74481.1(REF)
Qv32116(A)
Qv26382(A)
Qv32479(A)
Z096(A)
Qv17972(A)
Qv17972(A)
Rw846(A)
Qv17844(A)
Qv15145(A)
Qv23939(A)
A
Qv00615(C2)
Qv03719(C2)
C2
Qv05867(C1)
C1
Qv03594(B1)
Qv00585(B1)
B1
IN181391(B2)
IN141070(B2)
TJ49-52(B2)
B2
Qv12377(D)
Qv02575(D)
Qv07294(D)
Qv02124(D)
Qv18359(D)
D
b
0.0020

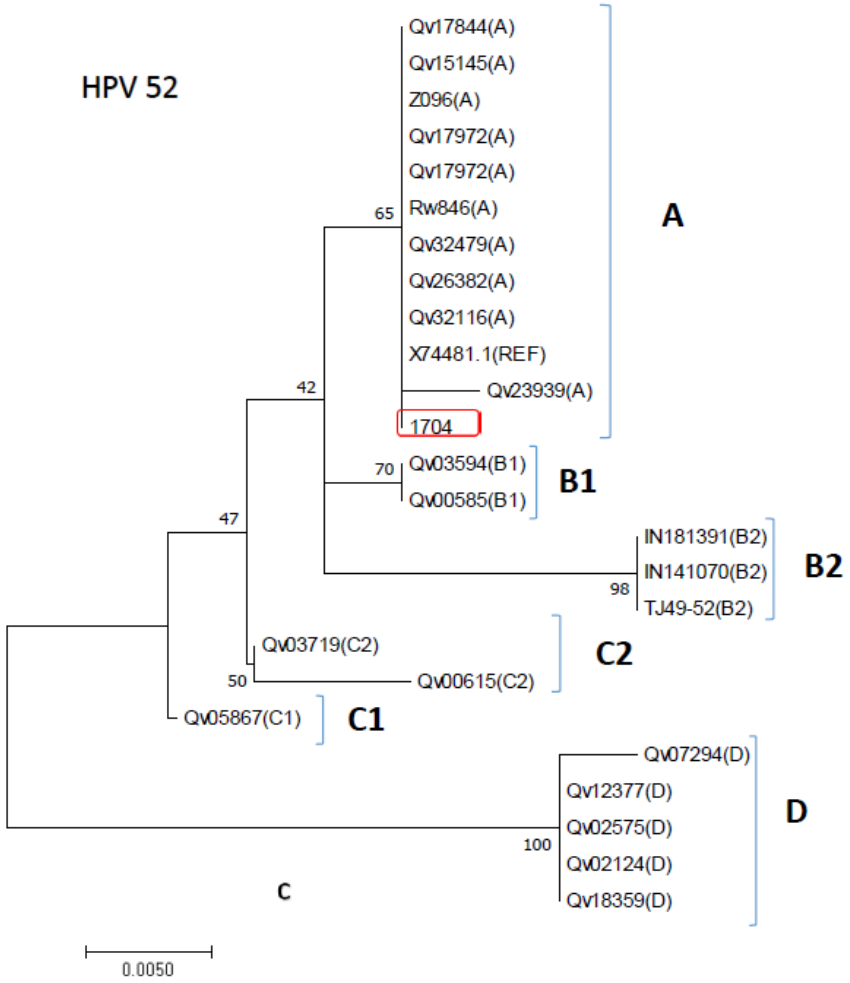
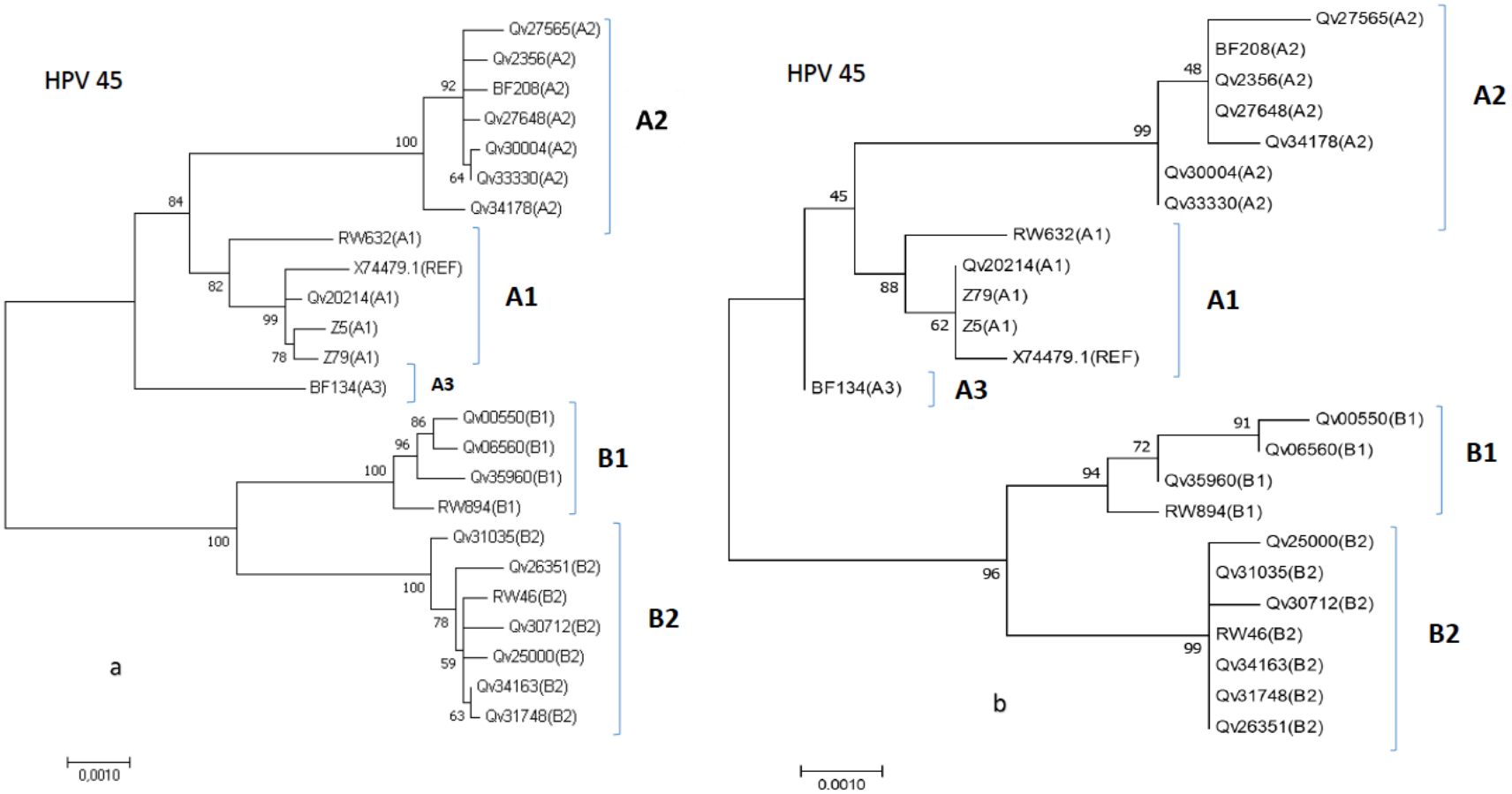


Figure 3.5: Phylogenetic trees showing representatives of variant lineages and sub-lineages of HPV52 cervical cancer specimens from GenBank database constructed by Maximum Likehood method. (a) the complete HPV52 genome, (b) the complete L1 and (c) the partial L1 gene


HPV 45
Qv27565(A2)
Qv2356(A2)
BF208(A2)
Qv27648(A2)
Qv30004(A2)
Qv33330(A2)
Qv34178(A2)
A2
RW632(A1)
X74479.1(REF)
Qv20214(A1)
Z5(A1)
Z79(A1)
A1
BF134(A3)
A3
Qv00550(B1)
Qv06560(B1)
Qv35960(B1)
RW894(B1)
B1
Qv31035(B2)
Qv26351(B2)
RW46(B2)
Qv30712(B2)
Qv25000(B2)
Qv34163(B2)
Qv31748(B2)
B2
a
0,0010
HPV 45
Qv27565(A2)
BF208(A2)
Qv2356(A2)
Qv27648(A2)
Qv34178(A2)
Qv30004(A2)
Qv33330(A2)
A2
RW632(A1)
Qv20214(A1)
Z79(A1)
Z5(A1)
X74479.1(REF)
A1
BF134(A3)
A3
Qv00550(B1)
Qv06560(B1)
Qv35960(B1)
RW894(B1)
B1
Qv25000(B2)
Qv31035(B2)
Qv30712(B2)
RW46(B2)
Qv34163(B2)
Qv31748(B2)
Qv26351(B2)
B2
b
0.0010

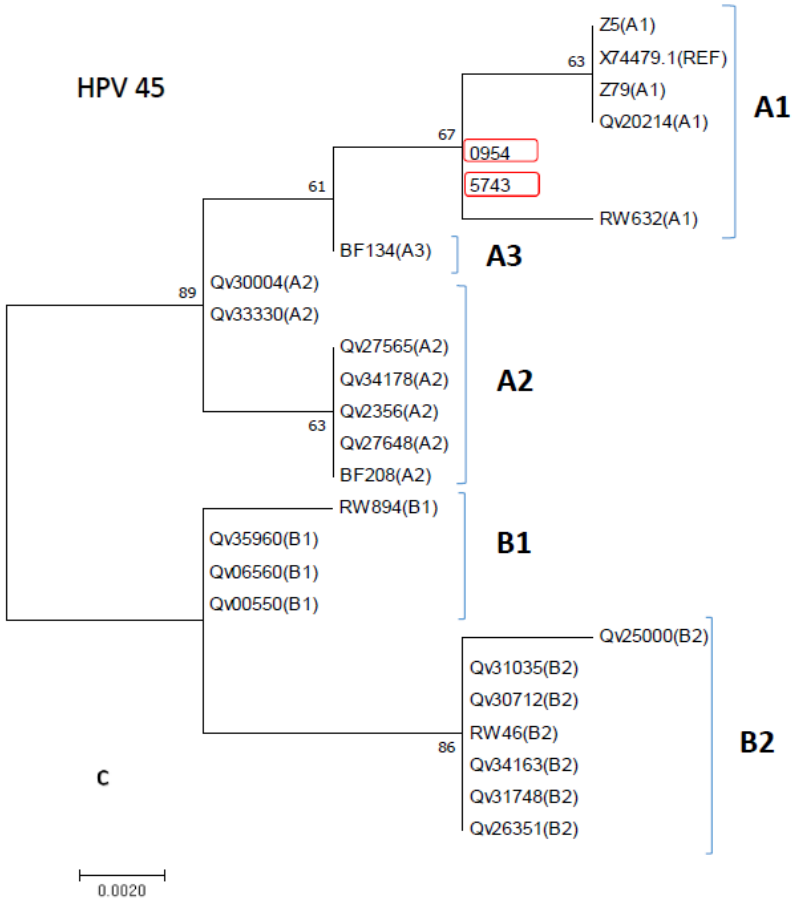


Figure 3.6: Phylogenetic trees showing representatives of variant lineages and sub-lineages of HPV45 cervical cancer specimens from GenBank database constructed by Maximum Likehood method. (a) the complete HPV45 genome, (b) the complete L1 and (c) the partial L1 gene

3.6 Discussion

HPV is a major causative agent for cervical cancer and is also known to be one of the etiologic agents of cancer of the head and neck. In head and neck cancer, the prevalence and distribution of the virus varies in different geographical regions of the world[25]. Several studies have reported HPV prevalence in association with head and neck cancer from different parts of South Africa, however, there is no study to our knowledge that has reported molecular variants within these HPV types. HPV variant investigation has been of great interest to many researchers worldwide, as it has been reported that genome variations may result in differing infectivity and pathogenicity[19,20]. Studies on cervical cancer have reported three fold or more increased risk of progression to cervical cancer if a person is infected with Asian-American (AA) or African (AF) variants of HPV16 compared to European (E) variants. In HPV18, the non-European variant lineage is commonly associated with malignant tissues and high grade cervical lesions[26-30]. In HPV33, C7732G SNP and HPV58 G760A SNPs are linked to a high risk of progression to cervical cancer[31,32]. However, little is known regarding infectivity and pathogenicity of other high risk HPV variants. Hence in the current study we investigated the molecular characterisation of HPV16, 18, 31, 33, 52, and 45 in order to determine variants circulating in our setting using genomic sequencing within the L1 region. The study included 20 high risk HPV isolates from confirmed HNSCC patients.

In the study, most isolates were HPV16 (12/20; 60%). Based on previous studies conducted worldwide, there is a high genetic diversity within HPV16, which resulted in a phylogenetic tree with multiple clusters reflecting the geographic origin of the variants[33,34]. According to literature on cervical cancer, there is a strong association of HPV16 variants of non-European lineage (B, C, D) with increased pathogenicity resulting in increased carcinogenic potential and risk of development of cervical intraepithelial neoplasia (CIN) as compared to the European lineage (A)[35]. In the current study on HNSCC, the European lineage showed a slight predominance (7/12; 58%) as compared to the non-European lineage (5/12; 42%) [Figure 3.1(c)]. All the non-European isolates had SNPs (2658, 7244, 9939, 1609 and 5706), resulting in an amino acid change from threonine to proline in isolates 2658, 7244 and 9939. A SNP was noted at nucleotide position 1190 of European isolate 9462, resulting in a change of amino acid from threonine to isoleucine (Table 3.2).

HPV18, which is the second most common cause of cervical cancer worldwide, is still not well documented as a cause of HNSCC. Two HPV18 positive samples were analysed from HNSCC in our study. The phylogenetic tree constructed with HPV18 sequences from the GenBank database resulted in non-African (A) and African (B/C) lineages which, like HPV16, represent the geographic origin of the variants[14]. One of the two samples analysed belonged to the non-African and the other to the African lineage (Figure 3.2c), although the sub-lineages could not be determined as the amplified region was too conserved. A synonymous mutation was seen in the isolate belonging to the African lineage. Unlike with HPV16, no evidence of an association of different HPV18 variants with risk of cancer has been documented.

The geographic distribution of some HPV types including 31, 33 and 52 variants are not well documented unlike HPV16 and 18[14]. HPV31 variant lineages are phylogenetically divided into three groups designated as A, B and C[18]. Based on studies done on HPV31 variants in association with cervical pathogenesis, lineage C was reported to result in more persistent infections while lineage A/B were more commonly detected in severely abnormal cells (CIN3) on the surface of the cervix, especially lineage B[14]. In our study, the HPV31 isolate was found to cluster with lineage B and the molecular analysis showed four point mutations (G1221A, G1245A, C1266A, C1311T) with no amino acid changes. There is no reported geographic location associated with specific HPV31 variants. In HPV33, distribution of the variant lineages was described to be highly geographically and ethnically specific with variants A and B identified as lineages on the phylogenetic tree[14,18]. The A1 sub-lineage was found to be distributed worldwide, while the A2 sub-lineage was rarely identified in Africa and South-America and A3 sub-lineage was detected in Asia/Oceania[56,57]. The B variant lineage was detected mainly in Africa[18]. The two samples identified as HPV33 were both grouped under lineage A (1673 and 7018), but could not be classified further into sub-lineages due to the conserved nature of the amplicon. There was an amino acid change seen in both the samples (glutamic acid to aspartic acid) (Table 3). With regards to HPV52, four clusters on the phylogenetic tree were obtained which were designated A, B, C and D. The HPV52 isolate detected in the study was grouped under lineage A. In studies done on cervical cancer, it was reported that HPV52 variants are linked to pre-cancerous lesions[14,18], while a study conducted in Canada showed the A1 sub-lineage of HPV52 to be more common in CIN2/3[36].

HPV45, which is among the most common carcinogenic HPV types after HPV16 and 18 in cervical cancer, clustered into two distinct lineages (A and B) with sub-lineages A1-A3 and

B1-B2 when the complete genomic sequences are used[17]. The partial L1 sequences of A2 sub-lineage isolates Qv33330 and Qv30001 showed less nucleotide variation when compared to the reference strain (X74479.1) and other A1 sequences than was seen for the complete L1 or complete genome sequences. The L1 nucleotide variability in the GP5+/PGMY09 region can properly differentiate HPV45 lineages, while at the sub-lineage level it can accurately cluster all but the A2 sub-lineages. In line with other high risk variants, the distribution of variant lineages differs around the world. The HPV45 A1 sub-lineage was largely associated with the Africa region, while B1 and B2 sub-lineages were detected in all regions[15]. In our study, the two samples detected as HPV45 were located within lineage A. Furthermore, in relation to the above mentioned study by Chen et al, HPV45 sub-lineage B2 was implicated to have higher risk of cervical cancer[15].

Phylogenetic analysis conducted in the study was very informative and provided the first data on high risk HPV variants from HNSCC circulating in our setting. Changes in amino acids reported here had no known biological or oncogenic effects, nonetheless this does not exclude the possibility of such associations. In the current research study, it was shown that the discriminatory power of the partial L1 sequences obtained from the GP5+/PGMY09 region vary depending on the HPV type. Tree topology for samples from HPV31 and 52 appeared similar for partial L1, complete L1 and complete genome sequences, therefore showing equivalent discriminatory power, while for HPV18 and 45 samples could be classified into lineages but clear differentiation of sub-lineages was not possible. For HPV16, only the European and non-European lineages could be distinguished. HPV33 showed the highest degree of sequence conservation, with no clear differentiation of lineages using partial L1 sequences. Investigations using larger sample sizes are needed to validate these findings. Knowledge of the circulating variants would also assist in building the global HPV variants database which is used for evolutionary and epidemiological studies. Single nucleotide polymorphisms were identified in the L1 sequences from patients with HNSCC in this setting, however the implications of these polymorphisms need to be evaluated with regards to pathogenesis, severity of infection and carcinogenic potential in this site.

3.7 Methods

3.7.1 Study population

In a previous study on HPV prevalence conducted in our setting, it was shown that the six most prevalent high risk HPV types among HNSCC were HPV16, 18, 31, 33, 52 and 45. The isolates used were from archival FFPE samples from the Department of Anatomical Pathology, Universitas Academic Laboratories, Bloemfontein, South Africa (SA) submitted between January 2004 and December 2014. Samples were amplified using consensus primers, PGMY09/11 as outer primers and GP5+/6+ as inner primers[37-39]. The above mentioned primer pairs amplify a 450bp (PGMY09/11) and 150bp (GP5+/6+) region of the L1 gene of HPV. A total of 39 samples tested positive for the prevailing high risk HPV types and were further analysed in the current work.

3.7.2 Molecular characterisation of HPV samples

The sequenced fragment of the L1 gene of all 39 high risk HPV types from the previous study were found to be too short for intra-typic analysis, hence in the present study, type specific PCR and sequencing reactions were repeated on the samples to obtain a longer L1 region amplicon for phylogenetic analysis. Briefly, integrity of the previously extracted DNA of the samples was confirmed by amplification of a reference gene using the primer pair PCO_4 and GH_{20}, which target a 268 bp region of the human beta globin gene. Samples were subsequently analysed for intra-typic variation by means of a heminested PCR using the primers, PGMY09/11 and GP5+ with type specific PGMY09 as indicated in Table 4. The amplification reaction was performed using GoTaq®Hot Start Polymerase (Promega, Madison, USA) following the manufacturer's instructions. The following PCR conditions were used: initial denaturation, 95°C for two minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for one minute, and a final elongation at 72°C for five minutes. Cycling conditions were the same for the heminested reaction except the annealing temperatures were modified as shown in Table 4 to accommodate the Tm of the type specific primers, and the annealing time was extended to 1 minute.

Table 3.4: Annealing temperatures used for type specific HPV primers in heminested PCR

HPV TYPES	PGMY09 PRIMERS	ANNEALING TEMPERATURE	AMPLICON SIZE
HPV45	PGMY09-N	49°C	413 bp
HPV33	PGMY09-F	49°C	424 bp
HPV31	PGMY09-K	49°C	410 bp
HPV16	PGMY09-R	45°C	410 bp
HPV18	PGMY09-K	49°C	410 bp
HPV52	PGMY09-G	50°C	326 bp

The PCR products were separated by electrophoresis using a 2.5% agarose gel in 1xTAE buffer (pH 8.0) and visualized by staining with GelRed™ (Biotium Inc., CA). Following electrophoresis, PCR products of the predicted size were excised from the gel and purified for nucleotide sequence determination using the Wizard®SV Gel and PCR clean-up system (Promega, Wisconsin, USA) according to manufacturer's instructions. The DNA concentration was determined using a NANODROP 2000 spectrophotometer (Thermo Scientific, lllinois, USA). The purity was determined from the 260nm:280nm absorbance ratio. The nucleotide sequence of each purified amplicon was determined using Sanger sequencing. The reactions were performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystem, Foster City, CA, USA) according to manufacturer's instructions. Bidirectional nucleotide sequence data was edited using Chromas Pro version 1.41 (Technelysium Pty Ltd, Australia). BLAST (Blast.ncbi.nlm.nih.gov, 2015) analysis was performed and the similarity between the nucleotide sequences obtained for each amplicon was compared with the GenBank nucleotide sequence database.

3.7.3 Phylogenetic analysis

To investigate the phylogenetic relationship between isolates from different HPV types, phylogenetic trees using representatives of all HPV variant lineages/sublineages of HPV16, 18, 31, 33, 52 and 45 were constructed using complete L1, partial L1, and complete genome sequence data obtained from GenBank (GenBank Accession numbers provided in supplementary data). Sequences were aligned and edited using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustal/) and Bioedit Sequence Alignment Editor software

respectively. Phylogenetic inference was performed using the Maximum Likelihood method. Maximum Likelihood trees were constructed using the Kimura 2-parameter model correction method of Molecular Evolutionary Genetics Analysis (MEGA) version 7 software. A bootstrap test analysis was performed (1,000 replicates) to assess the reliability of the obtained branching patterns in the phylogenetic trees.

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CHAPTER 4

NON-CODING RNA A POTENTIAL PROGNOSTIC AND/OR DIAGNOSTIC BIOMARKER FOR LARYNGEAL CARCINOMA

The editorial style of the Cancer Research Journal was followed in this chapter

4.1 Abstract

Currently there are no sensitive, specific and non-invasive biomarkers for laryngeal squamous cell carcinoma (LSCC). Long non-coding HOX transcript antisense intergenic RNA (HOTAIR), an example of non-coding RNA, has been suggested as a potential biomarker for cancer of the head and neck. HOTAIR was evaluated as a biomarker for use in diagnosis/prognosis of laryngeal squamous cell carcinoma (LSCC) and the presence of HPV DNA in patients' tissue biopsies was determined.

Total RNA was isolated from blood of five laryngeal cancer patients and five healthy controls. The expression levels of HOTAIR were determined using RT-qPCR. In addition, tumour tissue specimens were collected from the same LSCC patients and DNA was extracted for the detection of HPV by means of a nested PCR. The laryngeal cancer patients were black males between the ages of 50 and 80 years (average, 63.6%) while the controls were healthy male volunteers between the ages of 50 and 63 years (average 56.2%). Most of the cancer patients had advanced disease at diagnosis 80% (4/5), though there was no evidence of metastasis or lymph node invasion. Histopathology revealed moderately differentiated squamous cell carcinoma in one patient, while well differentiated histopathology was seen in the rest of the patients. Among the five tissue biopsies tested, HPV DNA was detected in one. The average HOTAIR expression levels were found to be significantly higher in LSCC patients ($p=0.03$) as compared to control (6.39 ± 2.13 versus 10.19 ± 2.24, respectively) with 13.9- fold overexpression. There was no correlation between HOTAIR expression and patients' demographics including age. Therefore, the study supported the involvement of HOTAIR in LSCC, hence a promising prognostic and diagnostic biomarker.

4.2 Introduction

Early detection of cancers frequently results in patients experiencing significantly better health outcome. Early detection usually refers to detection of a primary tumour before metastasis (1). However, at an early stage, the signs and symptoms of most types of cancer cannot readily be differentiated from many common infections. Use of biomarkers have emerged as a promising tool in understanding tumourigenesis and an ideal potential candidate in an early, accurate characterisation of cancer at a diagnostic/prognostic stage (2). Most of these biomarkers are stable in tissues and/or circulating in blood and other human body fluids (3). The principle of biomarker detection has been applied in differentiating various malignancies in such as the use of progastrin-releasing peptide (ProGRP) for small cell lung cancer (4) and human epididymis protein 4 (HE4) in ovarian cancer (5).

Circulating non-coding RNAs (ncRNAs) which are RNAs that do not code for proteins but rather are involved in the regulation of transcription and post transcription processes, also categorised as short noncoding RNAs (less 200nt) and long noncoding RNAs (more than 200nt) (6, 7), have been suggested as promising effective biomarkers in different cancer types and diseases (8, 9). The HOX transcript antisense intergenic RNA (HOTAIR), which is a member of the long non-coding RNAs has been suggested as a promising biomarker for multiple diseases such as acute myocardial infarction, vascular inflammation and age-related cardio vascular diseases (CVDs) (10, 11). For instance, in acute myocardial infarction, HOTAIR expression was reported to be down-regulated in the early phase of the disease (12), while in CVD patients, HOTAIR overexpression was reported in coronary artery disease, the most common type caused by atherosclerosis. The biomarker was found to be upregulated in plasma and circulating peripheral blood monocytes (13). In addition, HOTAIR biomarker was found to be important in different cancer types including head and neck squamous cell carcinoma (HNSCC) (14,15).

HNSCC, occurs in the upper aero-digestive tract, such as the larynx, oral cavity and oropharynx. Traditionally it was common in people who are tobacco and alcohol abusers (16). Recently, human papillomavirus infections (HPV) were implicated in the genesis of a subset of these cancers (17). Regardless of advances in cancer diagnosis and treatment, laryngeal squamous cell carcinoma (LSCC) is among the most aggressive cancers that occur in the head and neck region. It is the second most common cancer in the head and neck region, responsible for about 30% to 40% of cancers in the region and mostly affects the glottis (18, 19). Hence, a

better understanding of multiple molecular developmental processes of cancers is needed to identify specific predictive biomarkers that can be used as a guide in developing effective cancer treatment strategies.

HOTAIR biomarker is a polyadenylated RNA made up of more than 200 nucleotides (20), transcribed from the HOXC locus based on chromosome12q13.13 (21, 22). It functions as a gene expression regulator through its interaction with RNA, DNA and protein at transcription and post-transcription stages (23). Reduction in HOTAIR expression results in increased transcription of the HOXD locus on chromosome 2 by means of a reduction in H3K27 trimethylation (24). Novel gene expression analysis techniques have made it possible to detect specific genetic patterns and molecular pathways involved in different cancer types including HNSCC (laryngeal cancer), hence facilitating the detection of better screening markers for these cancers (21-29).

Numerous recently published studies that made use of the new techniques have reported detection of several genes and proteins that were significantly overexpressed in tumours of the head and neck (30-33). The majority of these studies focused on comparing potential biomarkers in malignant and adjacent non-malignant tissues in HNSCC patients (21- 33). Though promising, use of tissue samples was proven to be challenging as obtaining tissue involves an invasive procedure and, most frequently, the tumour is too small to be used for all the analysis required. Hence a growing demand for biomarkers that can be detected from non-invasive samples to avoid the need for tissue biopsies in diagnosis and therapy monitoring of the cancer. As a result, blood has been suggested as a promising alternative sample type for biomarker analysis in studies on cancer diagnosis or prognosis, and therapy monitoring, since it can easily be obtained repeatedly in a clinical setting and nucleic acids have proven stable in the presence of the high levels of nucleases in the blood (34).

Regardless of advances in diagnosis and treatment of head and neck cancer, there is still little reduction in mortality and generally the survival rate of patients is still reported to be low. The majority of patients present to the health facility when already at the metastatic stage, hence posing a continuing public health problem (35). Currently, there are no studies conducted on promising biomarkers using blood samples from patients with suspected, or known, laryngeal cancer in South Africa. It is also not clear from the literature whether HOTAIR, the most studied biomarker in different cancer types and diseases, plays a significant role in LSCC. Therefore, the aim of the current study was to determine and compare the expression levels of

the HOTAIR gene in laryngeal cancer patients and healthy individuals as a potential biomarker using relative quantitative real-time PCR (RT-qPCR) analysis on total RNA extracted from blood samples. Furthermore, the tissue biopsies from patients were screened for the presence of HPV DNA using nested PCR.

4.3 Aim

The aim of this chapter was to identify potential biomarkers that can be used as an indicator of HNSCC (laryngeal cancer) in early diagnosis of the cancer and to screen biopsy tissues from patients for HPV DNA

4.4 Objectives

- To extract total RNA from patients and control blood and convert it into cDNA and used it as a template for a relative quantitative real-time PCR (RT-qPCR) analysis
- To Investigate expression of HOX transcript antisense intergenic RNA as potential biomarker of disease using quantitative real-time PCR
- To extract DNA from patients confirmed with laryngeal cancer and perform a nested PCR targeting the L1 region

4.5 Methods

4.5.1 Specimen collection

A total of 10 blood samples and five tissue samples were analysed in the study. Five blood and tumour tissue specimens were collected from patients recruited at the Universitas Academic Hospital, Bloemfontein, South Africa between June 2018 and December 2018 with histologically confirmed SCCs of the larynx. Another five blood samples were obtained from healthy control subjects with no history of any form of cancer. Blood was collected using EDTA BD Vacutainer® tubes (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The patients had not received any form of therapy prior to blood and tissue sample collection. Data including age and gender of the patients were recorded. The clinicopathologic data such as tumour stage, tumour site and histological grade for each patient was obtained from the Department of Otorhinolaryngology, Universitas Academic Hospital. The healthy controls and LSCC patients were “matched” for age and gender where possible. Written informed consent was obtained from all participants in the study. The study was approved by the Health Sciences

Research Ethics Committee of the Faculty of Health Sciences, University of the Free State (137/2013C). The blood specimens were processed immediately after venisection, while the tissues were placed in *RNAlater*® (Thermo Fisher Scientific, SA) after excision and stored at -20ºC until DNA extraction.

4.5.2 Nucleic acid extraction

Total RNA was isolated from blood samples of both the patients and healthy controls taking part in the study, while DNA was isolated from the tissue (biopsy) specimens of the LSCC patients.

4.5.2.1 RNA isolation

Total cellular RNA was isolated from whole blood using Trizol LS reagent (Invitrogen life Technologies, USA). Briefly, 2x specimen volume of cold lysis buffer was added to the blood, mixed by inverting and incubated on ice for 10 minutes to lyse red blood cells. The white blood cells were collected by centrifugation for 2000 rpm for 5 minutes. The procedure was repeated to ensure complete lysis of the red blood cells. A 750µl aliquot of Trizol LS was mixed with 250µl of nuclease free water. A 1ml aliquot of Trizol was added to the cell pellet and the pellet resusupened using a Pasteur pipette until the cells were completely lysed. Then 200µl chloroform (Merck, Darmstadt, Germany) per 1000µl Trizol was added to the homogeneous mixture, shaken vigorously for 15 seconds and incubated on ice for 15 minutes. The mixture was centrifuged for 15 minutes at 4ºC and the aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube. RNA was precipitated from the aqueous phase using 500µl ice-cold isopropanol per 1000µl Trizol, mixed and incubated for 5 hours at -20ºC, then centrifuged at 12000 rpm for 10 minutes at 4ºC. The pellet (precipitated RNA) was washed three times using 75% ice-cold ethanol, and dried for 5 minutes on ice and resuspended in 30ul RNAse free water. RNA quantity and purity were measured on a NanoDrop 250 fluorospectrometer using the A260/A280 ratio, while the integrity was analysed using non-denaturing agarose gel electrophoresis. Isolated RNA was kept at -80 °C until further analysis by RT-qPCR.

4.5.2.2 DNA isolation

DNA was isolated from freshly frozen tissue biopsies using a commercially available DNA extraction kit (QIAmp®DNA Mini kit, Valancia, USA), according to manufacturer's instructions and eluted in a final volume of 100µl AE buffer. The extracted DNA was stored at -80 ºC. Integrity of the DNA was confirmed by amplification of a partial region of a reference

gene using the primer pair GH_{20} (5'GAAGAGCCAAGGACAGGTAC3') forward and PCO_4 (5'CAACTTCATCCACGTTCACC3') reverse, which target a 268 base pair (bp) region of the human beta globin gene. Patient samples were screened for HPV DNA if tested positive for the human beta-globin gene.

4.5.3 Genome amplification

4.5.3.1 cDNA synthesis

RNA isolated from normal and cancer positive samples was reverse transcribed and amplified using a two-step reverse transcription PCR assay (RT-PCR). cDNA was synthesized using forward target specific primers for HOTAIR (5'GGTAGAAAAAGCAACCACGAAGC 3') a nd GAPDH (5'CAGCCTCAAGATCATCAGCA3') and Superscript^{TM} III Reverse transcriptase kit according to the manufacturer's instructions (Invitrogen, Corporation, Carlsbad, CA), from a total RNA starting concentration of 1000ng in a total reaction volume of 20µl.

4.5.3.2 Quantification of HOTAIR gene expression using real-time PCR (RT-qPCR)

For quantification, cDNA was used as a template for a relative quantitative real-time PCR (RT-qPCR) analysis. Amplification and analysis were performed on the Roche LightCycler® 2.0 (Roche, Basel, Switzerland) which is a carousel-based system using LightCycler FastStart DNA Master SYBR Green I according to manufacturer's instructions. Each run was performed using a PCR mix consisting of 2µl cDNA template diluted 1:5, 2µl SYBR Green I MasterMix, 1µl of 20µM HOTAIR forward primer and reverse primer (5'-ACATAAACCTCTGTCTGTTGCC3') (final concentration, 0.4 µM), 0.8 µl of 2 mM $MgCl_2$ and 13.2 µl of ddH_2O in a 20 µl final reaction volume. The PCR conditions were optimised and the amplification conditions consisted of an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation, annealing, and amplification (95°C 10 s, 62°C 5 s, 72°C 8 s). For RT-qPCR specificity check, a melting curve analysis of the PCR products was performed, 95 °C for 10 s, 60 °C for 15 s and 95 °C for 0 s with ramp rate of 0.2 s. The reaction was cooled at 40 °C for 30 s. Results were normalised based on the expression of GAPDH as a reference gene using the primer pair forward 5'-CAGCCTCAAGATCATCAGCA-3' and reverse 5'-TGTGGTCATGAGTCCTTCCA-3'. The analyses of HOTAIR and GAPDH were performed in separate capillary tubes during the RT-qPCR. Each run included nuclease free water as a negative control. Analyses and fold differences of the expression of the target gene

between normal and test samples were determined using the comparative cycle threshold (CT) method. Expression fold change was calculated from the △△CT values with the formula $2^{-\triangle\triangle CT}$ (36). Each reaction was repeated in triplicate and mean values were used for analysis of gene expression. To confirm successful cDNA amplification and reaction specificity, the RT-qPCR products were separated and visualised by electrophoresis on a 2.5% agarose gel.

4.5.3.3 HPV nested PCR

A nested PCR was performed on DNA samples in which the integrity was confirmed by detection of human beta-globin. The nested PCR targeted a 450 bp region of the L1 gene using primers PGMY09/11, while the nested reaction targeted a 150 bp region using primer pair GP5+/GP6+. The nested PCR was performed using GoTaq®Hot Start Polymerase (Promega, Madison, USA) as per the manufacturer's instructions using 5µl of the genomic DNA, and 0.4 µM of each primer. The amplification reaction was performed using the following conditions for the first round, initial denaturation, 95 °C for two minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds and elongation at 72 °C for one minute and a final elongation at 72 °C for five minutes. Cycling conditions were similar for the nested reaction except the annealing temperature was adjusted to 43 °C to accommodate the Tm of the primers.

The PCR products were separated by electrophoresis using a 2.5% agarose gel in 1xTAE buffer (pH 8.0) and visualised by staining with GelRed™ (Biotium Inc., Fremont, CA, USA). The nucleotide sequence of each purified amplicon was determined using Sanger sequencing. The reactions were performed using Big Dye Terminator sequencing ready reaction kit (Applied Biosystem, Foster City, CA, USA). Sample electrophoresis was performed and nucleotide sequence data was edited using Chromas Pro version 1.41 (Technelysium Pty Ltd, Australia). Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov, 2015) analysis was used to compare the similarity between the nucleotide sequencing results obtained from the amplicons with HPV sequence data available on GenBank nucleotide sequence database.

4.5.4 Statistical analysis

For statistical analysis, t-test in Microsoft excel was used to compare HOTAIR expression between patients and healthy controls. Statistical significance was considered for values of $p<0.05$.

4.4 Results

The study group included five patients with histologically confirmed LSCC and five healthy volunteers. For each patient with suspected LSCC blood samples were collected and a tissue biopsy was available. For healthy volunteers, blood samples were collected. As summarised in Table 4.1 below, participants were black males ranging in age from 50 to 80 years (average, 63.6 years) at initial diagnosis of cancer. Healthy individuals ranged in age from 50 to 63 years (average, 56.2 years). There was no significant difference in the age distribution between patients and controls (p=0.721).

Table 4.1: Participant demographic characteristics

Characteristics	**HNSCC-cases N(% total)**	**Control N (% total)**
Total sample	5	5
Gender Male	5 (100%)	5 (100%)
Race Black	5 (100%)	5 (100%)

Clinicopathological status of the patients is summarised in Table 4.2. The larynx was the primary tumour site for all patients. HPV DNA was detected in 1/5 biospy samples. Most of the patients, 80% (4/5) had an advanced tumour (T3 and T4), with advanced stage cancer (stages III and IV). The majority of the patients 80% (4/5) did not show lymph node invasion (N0). No metastasis was evident for any of the patients (M0). Histopathology revealed moderately differentiated squamous cell carcinoma in one patient, while well differentiated histopathology was seen in the rest of the patients

Table 4.2: Clinicopathological characteristics of the HNSCC patients

Spp #	Tumour Site	HPV status	HPV type	Stage	TNM	Histology
1	Larynx	Negative	-	III	T3N0M0	Well-differentiated squamous cell carcinoma
2	Larynx	Positive	35	III	T3N0M0	Moderately differentiated keratinising squamous cell carcinoma
3	Larynx	Negative	-	IV	T4N2cM0	Well-differentiated squamous cell carcinoma
4	Larynx	Negative	-	I	T1N0M0	Well-differentiated squamous cell carcinoma
5	Larynx	Negative	-	III	T3N0M0	Well-differentiated squamous cell carcinoma

To investigate the expression levels of HOTAIR between the two groups, the expression levels of the biomarker in the blood of five HNSCC patients was determined and compared to the level in five blood samples from individuals with no cancer, using RT-qPCR (Table 4.3). The level of gene expression for HOTAIR was normalised to GAPDH and the mean gene expression values were compared between the two groups. The mean expression level of the biomarker was found to be significantly higher ($p=0.03$) in blood collected from patients with laryngeal cancer compared to controls (6.39 ± 2.13 versus 10.19 ± 2.24, respectively). The mRNA expression levels for HOTAIR were further correlated with patient demography such as age range of >59 and ≤ 59 normally used as a cut off age for HNSCC caused by HPV or due to other agents such as alcohol and tobacco.

Table 4.3: HOTAIR expression and correlation with participant characteristics

Characteristic	HNSCC-cases N(% total)	HOTAIR EXP (test) (mean ± SD)	Control N (%)	HOTAIR EXP (C) (mean ± SD)	P-value
Total HOTAIR expression	5 (100%)	6.39±2.13	5 (100%)	10.19±2.24	0.03
>59	2	6.38±3.76	2	12.44±1.62	0.17
≤59	3	6.40±1.44	3	8.70±0.56	0.06

Using a cut-off value of $p<0.05$, the HOTAIR biomarker expression was significantly up regulated ($p=0.03$), with a 13.9-fold overexpression in laryngeal cancer patients' blood as compared to expression in blood from healthy participants (Figure 4.1), with an average Cq value of 28.6 cycles for the test samples, and 31.9 cycles for the controls. The results suggest the presence of malignant tissues in the larynx somehow increases HOTAIR expression significantly in the blood.

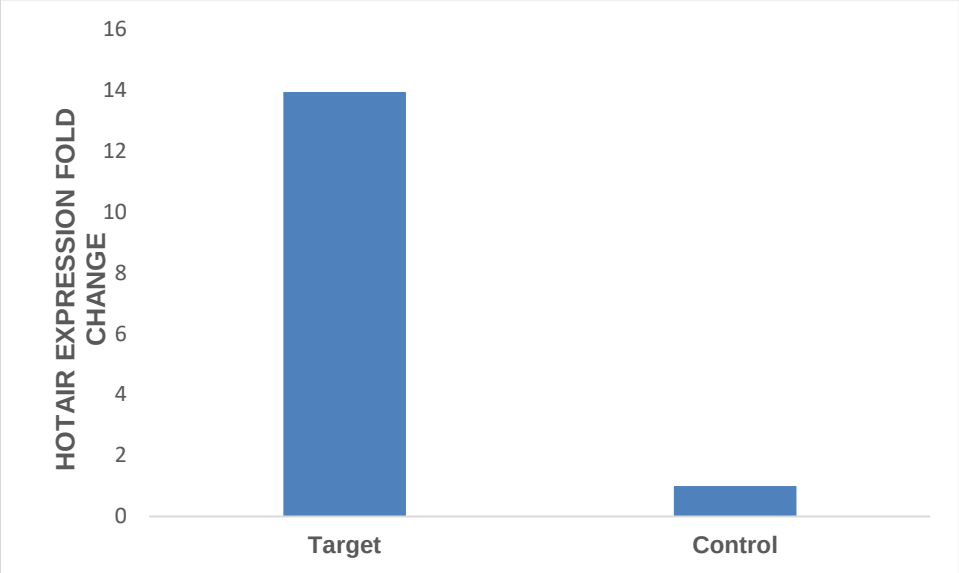

Figure 4.1: Mean HOTAIR gene expression level in whole blood samples of laryngeal cancer patients and healthy controls

4.5 Discussion

HNSCC is rated sixth among the commonly detected cancers in patients worldwide (37, 38). LSCC, which is a subset of the HNSCC, is one of the most aggressive cancers of the head and neck. Regardless of recent advances in HNSCC oncological and surgical treatment, the prognosis for patients with LSCC is still poor. To understand the mechanism of LSCC at the molecular level, it is important to find biomarkers that can be used as prognostic tools as well as for implementation of effective treatment strategies. Hence to achieve successful personalised health care (PHC) (39, 40), the main objective is to find the appropriate treatment at the right dose, specific for the patient at the right time (41).

Discovery of next-generation sequencing and its application in multiple cancer transcriptomes has resulted in detection of numerous non-coding RNAs (ncRNAs) with expression levels linked to carcinogenesis and progression of different cancer types. In literature, ncRNAs are described as taking part in epigenetic regulation of gene translation and as a result, are proposed as potential candidates for drug targets with the aim of interfering with all biological pathways controlled by the targeted ncRNAs (42, 43). HOTAIR is an example of a long non-coding RNA, however its potential as a therapeutic target or a prognostic tool in HNSCC is still in its infancy. Multiple studies have shown that HOTAIR depletion results in tumour cell apoptosis through the mitochondrial pathway of apoptosis in HNSCC, hence suggesting a novel potential therapeutic target in the HNSCC treatment (44). Furthermore, down regulation of HOTAIR and Hu antigen R (HuR), which is a ubiquitous RNA-binding protein that is responsible for the stability and translation of numerous cellular mRNA and also one of the intensely researched regulators of post-transcriptional gene expression in eukaryotes (45), reduce cellular viability, migration and invasion. The significant up regulation of HuR and HOTAIR correlated with metastasis and progression of HNSCC (46).

Therefore, in the current study, the RT-qPCR was performed to determine the expression level of HOTAIR in five LSCC patients and five healthy controls. Instead of using tissue biopsies, blood was tested as an alternative non-invasive sample type for analysis of circulating tumour related nucleic acid. Regardless of the amount of RNase found in blood, total RNA was successfully isolated from samples and used to determine whether HOTAIR was up regulated in patients with LSCC. The results showed that blood of patients with primary LSCC had a significantly increased level of expression of HOTAIR gene compared with that of healthy controls. The number of patients and controls enrolled in the study was relatively small hence no statistical calculations were performed to determine the significance of the correlation between HOTAIR up regulation observed and clinicopathology of the patients. The majority of the LSCC patients in the study had advanced disease, with 4/5 patients presenting with stage III/IV disease, which may account for the marked upregulation of HOTAIR expression. Further studies are required to determine whether similar upregulation is present in the early stages of disease. Despite the limitation, results obtained in the study were generally in line with other studies conducted on laryngeal cancer. In a research study by Li and colleagues, up regulation of HOTAIR in laryngeal tumour was found to be associated with tumour stage, lymph node metastasis, poor differentiation and advanced clinical stages (47). Therefore, HOTAIR was

suspected of playing an oncogenic role in patients with LSCC, thus a potential biomarker, promising prognostic tool and potential target for treatment of LSCC.

Historically, tobacco and alcohol are the common risk factors for HNSCC, with high-risk HPV, especially HPV16, also implicated as an emerging etiological agent in the cancer (48, 49). Subsequently, our study further determined the presence of HPV DNA from all fresh tumour tissues collected concurrently with blood from LSCC patients. Out of five malignant tissues collected, HPV35 was isolated from one patient. Though the study was limited by the small sample size, the low incidence obtained was not unexpected as based on previous studies, HPV is mostly detected in the oropharynx, at 60-70% as compared to other sub-sites of the head and neck such as oral cavity (6-20%), larynx (24%), sinonasal tract (21%) and nasopharynx (31%) (50-52). Alcohol and tobacco abuse are implicated in 75% of cancers of the head and neck region in the literature (35, 38). Because of the low participants number, the significance of these two factors were not analysed in the study, hence further studies will be needed to determine their importance in the LSCC.

4.6 Conclusion

There is a need for more investigations to be conducted, in order to understand the role of biomarkers in tumour genesis, progress, local and distant metastasis of LSCC and HNSCC. The acquired knowledge will be of benefit in the proposed biomarker-driven personalised cancer treatment. Use of several technologies has facilitated the detection of numerous biomarkers suspected of having a potential in diagnosis/prognosis and predicting the response of cancer to specific therapies. Among others, IncRNA, for instance HOTAIR has been intensively studied and proposed as a potential marker to serve in HNSCC prognosis and as a target for the cancer therapy. Our results were in line with this finding as the expression of HOTAIR was significantly higher in LSCC patients' blood compared to controls, therefore providing proof that the presence of necrotic tumour cells might have resulted in an over expression of HOTAIR in the cancer patients. HOTAIR is therefore a potential target for cancer prognosis and therapy, though further studies are needed to verify this. Currently cancer diagnosis is based on tissue biopsy to study biomarkers and genetic nature of the cancer. This sample type is invasive, expensive and time consuming. Hence in our study an alternative sample type, namely blood was utilised in testing the potential of HOTAIR as a biomarker, and the use of blood proved to be effective. The number of samples available for this study were limited by the number of patients admitted during the study period. Although it is difficult to

make any firm conclusions from the study due to the small sample size, the results warrant further investigation of HOTAIR as a prognosic tool and therapeutic target.

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CHAPTER 5

CONCLUSION

5.1 Concluding remarks and future research

Head and neck cancer (HN) refers to cancer diagnosed at different sub-sites of the region excluding brain cancer (Medline, 2014). These heterogeneous group of cancers arise from different anatomical sub-sites such as the nasopharynx, oral cavity, oropharynx, thyroid, hypopharynx and larynx. They are mainly (90%) squamous cell carcinomas (SCC), hence the term head and neck squamous cell carcinoma (HNSCC) and the tumour develops from the epithelial lining of the aero-digestive tract (Suh *et al*., 2014). Globally, it is recorded as the 6th most common cancer (Parkin *et al*., 2005).

Historically, HNSCCs were associated with tobacco and alcohol abuse (Znaor *et al*., 2003). More recently high risk human papillomaviruses (HPV) types are implicated as the cause of a subset of cancers of the oropharynx (Spence *et al*., 2016). More than 200 HPV types have been identified globally. HPV types are classified as high and low risk based on their ability to cause cancer (Muñoz *et al*., 2003; de Villiers *et al*., 2004). HPV is a sexually transmitted virus, and in many cases the immune system clears the infection, however it can transform cells and cause cancer. The virus causes malignancies through the E6 and E7 oncoproteins, which disrupt the normal cell cycle by interfering with tumour suppresser proteins such as P53 and retinoblastoma protein (pRb) (Leemans *et al*., 2011). P53, a tumour suppressor gene, functions by regulating apoptosis at G1-S phase of a cell cycle so that damaged DNA and replication errors are repaired and corrected. E7 interferes with the function of pRB, and hence leaves the G1 cell cycle phase un-regulated. Disruption of these two cell functions results in continuous cell replication with no apoptosis.

Based on recent reports of global incidence, HPV causes an estimated 38,000 to 45,000 cases of HNSCC annually (de Martel *et al*., 2017; Castellsague *et al*., 2017). The incidence varies worldwide and there is variation among the anatomical sub-sites. For instance, according to a recently published pooled data analysis, the prevalence of HPV-related oropharyngeal squamous cell carcinoma (OPSCC) in the United States was 59.3%, in Europe it was 31.1% and in Asia it was 17.5% (Castellsague *et al*., 2016; Anantharaman *et al*., 2017). On the other

hand, information on the epidemiology of HNSCC in Africa, particularly the incidence of the cancer due to HPV at different sub-sites of the upper-aero-digestive tract, is currently limited. Studies conducted in South Africa, have reported HPV infection in some patients presenting with HNSCC, though the prevalence was low. For instance, recently in our setting 112 HNSCC samples were tested for HPV DNA following $p^{16INK4a}$, with 6.3% prevalence reported (Sekee *et al*., 2018). Likewise, in other studies conducted in the country, including oral and oropharyngeal carcinoma in one study (Davidson *et al*., 2014) and another in oropharyngeal carcinoma among men who have sex with another men (MSM) (Müller *et al*., 2016), the authors reported HPV prevalence of 5.6% and 11.5% respectively. In the current study the prevalence of HPV DNA among patients diagnosed with HNSCC at Universitas Academic Hospital in the Free State Province of South Africa was determined.

The study included 994 FFPE samples from histologically confirmed cancers, of which intact DNA was detected in 780 samples. This was the first study of this magnitude to be conducted in South Africa for determining HPV prevalence in HNSCC, in order to gain a better understanding of virus association with HNSCC. To increase sensitivity of detection, a nested PCR using previously published consensus primer pairs was performed for the amplification of HPV DNA in samples. To increase the specificity of the genotyping method, an additional type specific heminested PCR using primer pairs targeting a region of the E6 gene was performed. In total 57/780 (7.3%) samples tested positive for HPV DNA. The majority of samples that were positive for HPV DNA were from tumours located in the sinonasal tract (16.0%) and oropharynx (10.8%). The prevalence obtained here was relatively low as compared to that reported from the developed world such as Europe and Northern America, but corresponded to that obtained in similar studies conducted in South Africa on fewer samples.

The association between high risk HPV types and cancer, especially the oropharynx, have been reported in numerous studies (Castellsague *et al*., 2016; Anantharaman *et al*., 2017). Our study correlated with what has been reported in literature (Dayyani *et al*., 2010) with high risk HPV types detected in 41/57 positive samples (71.9%), though their role in carcinogenesis in the head and neck region is still yet to be proven. High risk HPV DNA was detected in 29 men out of 41 (70.7%) positive samples and in 12 (29.3%) women. In addition to high risk types, low risk HPV types were detected in the study, in malignant tissues of the head and neck. The role played by the low risk types in malignant tissues still needs further investigation. HPV

associated HNSCC, mainly associated with OPSCC, is reported to be more common in younger patients and associated with their sexual practices, which includes high number of sexual partners and multiple oral sex partners (D'Souza *et al*., 2007; D'Souza *et al*., 2010; D'Souza *et al*., 2014). On the contrary, in our study HPV infection was common between the age 50 and 60 years in OPSCC.

HPVs have similar genetic structure with a genome of approximately 8kb in size. The genome is comprised of the early region (E), late region (L) and the non-coding region (NCR) or upstream regulatory region (URR) (de Villiers *et al*., 2004). Taxonomic classification of the viruses is based on the conserved L1 open reading frame (ORF). HPV families have less than 90% similarities in the L1 region, while greater than 40% difference of two HPV L1 sequences describe different genus and species of a genus share 60-70% nucleotides similarities within the ORF. Individual HPV types have more than 10% nucleotide difference, while subtypes and variants have 2-10% and less than 2% difference respectively (Burk *et al*., 2013).

Papillomaviruses are known to evolve slowly resulting in a random mutation rate of about $2\pm0.5x10^{-8}$ per nucleotide annually (Rector *et al*., 2007). They are commonly tissue and host specific (Chen *et al*., 2005). These viruses are known to experience little to no definitive recombination during their evolution, hence nucleotide polymorphism reported within a small number of viral lineages mostly occur due to random mutation over a certain period of time (Chen *et al*., 2005; Chen *et al*., 2009; Schiffman *et al*., 2010). To have an in-depth understanding in the carcinogenesis of HPV related cancers, including cancer of the head and neck, studies should determine not only the HPV types involved but the lineages and sub-lineages prevailing in those cancers and also study the genetic basis of the HPV related tumourigenesis. Hence that challenged us to further characterise high risk HPV types detected in our setting by determining variant lineages and sub-lineages per type through phylogenetic analysis.

Amplifying DNA from FFPE tissues can be limited by fragmentation of the DNA during fixation of the tissues. Hence, to obtain additional sequence data on high risk HPV positive samples, a larger fragment was targeted. A larger fragment was only detectable in 20/39 FFPE tissues that tested positive for high risk HPV DNA. Positive samples included a range of HPV types as follows, HPV16 (n=12), HPV18 (n=2), HPV31 (n=1), HPV33 (n=2), HPV52 (n=1) and HPV45 (n=2). The sequence data for these fragments was used for variant analysis.

Lineage and sub-lineage discrimination is largely based on complete genome, hence use of whole genome sequence is recommended in order to obtain accurate classification. In the absence of complete genome data and in the absence of samples that could be used to obtain complete sequence data, the feasibility of using the L gene and fragments of the L gene were investigated in variant classification. Phylogenetic trees were constructed using representative sequence data for the L1 region and using complete HPV type genome data and the topology of the trees was compared.

In summary, classification of high risk HPV types using the nucleotide sequences within the GP5+ and PGMY09 region, resulted in clear resolution in HPV31 and 52 variants, where partial L1 sequence resulted in phylogenetic trees similar to the complete genome and complete L1 trees. The unknown HPV31 isolate was classified under the B lineage, while in HPV52, the isolate was grouped with the A lineage. In HPV18, the amplified L1 region did not have enough variability to discriminate up to the sub-lineage level, hence the unknown isolates could only be assigned to lineages. Therefore, isolate 5228 was clustered with African (B) lineage while 5267 was grouped under non-African (A). In HPV45, the partial L1 nucleotide sequence could discriminated up to sub-lineage level in the B lineage while for the A lineage, the A2 sub-lineages could not properly be differentiated, and the two HPV45 isolates were classified as A1 sub-lineages. In HPV16, the sequences could only be classified as European (A) or non-European (B/C/D), while in HPV33 the L1 partial sequence totally failed to provide a distinct classification of the unknown isolates due to conserved sequence within the amplified region of the L1. In total, 10 point mutations were identified amongst the 20 samples analysed. There were three non-synonymous mutations, two from HPV16 variants and one from HPV33. The significance of these mutations is still not known.

Head and neck SCC is a heterogeneous cancer whose pathogenic pathways are not fully characterised and can have a poor prognosis. Laryngeal carcinoma is the second most prevailing cancer in the head and neck and is predominantly caused by smoking and alcohol consumption (Muscat and Wynder, 1992). The cancer is mostly seen in patients above the age of 40 years and frequently affects the squamous cells of the region, resulting in laryngeal squamous cell carcinoma (LSCC) (Svero *et al*., 1987; Singh *et al*., 2000). HPV has also been associated with cancer at this region (Koskinen *et al*., 2007; Baumann *et al*., 2009; Coca-pelaz *et al*., 2013; Li *et al*., 2013). This was in agreement with our study where patients presented with laryngeal squamous carcinoma in our setting were mostly in their late fifties with mean

age of 64 years. Generally, HNSCC cancers are challenging to diagnose at an early stage since they are initially asymptomatic hence they are mostly diagnosed and treated at an advanced stage (Suh *et al*., 2014). LSCC was commonly treated by surgery such as total or partial laryngectomy, but later the treatment changed to chemoradiotherapy with the aim to preserve the larynx (American Society of Clinical Oncology *et al*., 2006). The current treatment or management of the HNSCC including the LSCC, still results in long-term side effects.

Therefore, a clinically appropriate gene expression pathway, or biomarker, unique to the cancer synthesis would aid in early detection and prognosis. The biomarker, or the profile, should be specific for the tumour behaviour hence able to distinguish normal and malignant tissues at the molecular level. Biomarkers are the biological molecules that change based on the presence or absence of the disease (Yokota *et al*., 2014). They can be used as diagnostic and prognostic tool in cancers such as HNSCC and also in predicting tumour response to specific treatment (Williams, 2010; Yokota *et al*., 2014). Therefore, they may be useful in determining a personalised treatment, a kind of treatment that could result in patients receiving less intensive therapy, therefore resulting in fewer side effects caused by treatment toxicities. In that case a better prognosis for the patient is likely (Polanska *et al*., 2014).

Use of improved technology in science has helped in deeper understanding of the molecular pathways in HNSCC development, giving rise to identification of numerous biomarkers. The discovery of biomarkers in HNSCC is believed to be a breakthrough in the early detection of primary and relapsed cancers of the head and neck (Lee *et al*., 2011). But currently there are few useful biomarkers for HNSCC in a clinical setting that are routinely used (Suh *et al*., 2014). Numerous potential biomarkers in HNSCC have been investigated such as non-coding RNAs that include long non-coding RNAs growth arrest specific 5 RNA (GAS5RNA), long intergenic non-coding RNA-p21 (LincRNA-p21), X-inactive specific transcript (Xist), Antisense noncoding RNA in the INK4 locus (ANRIL), HOXA transcript at the distal tip (HOTTIP) and HOX transcript antisense intergenic RNA (HOTAIR). There are also small non-coding RNAs including, small interfering RNAs (siRNA), PIWI-interacting RNAs (piRNAs), microRNA (miRNAs) (Kugel and Goodrich, 2012; Ge *et al*., 2013).

Hence, the final aim of our study was to investigate the differential gene expression of HOTAIR biomarker in a non-invasive sample from patients with LSCC and healthy controls. Differential gene expression RT-qPCR was performed and data analysis was done using expression fold

change calculated from the $\triangle\triangle$CT values with the formula $2^{-\triangle\triangle CT}$ (Livak and Schmittgen, 2001). HOTAIR marker was found to be up-regulated in cancer patients with a fold change of 13.9 as compared to the healthy controls. Therefore, the study supported the involvement of HOTAIR in LSCC, hence a promising biomarker. In the current study, none of the patients had tumour metastasis so we could not determine correlation of the expression of HOTAIR in patients with tumour metastases, therefore more research using larger sample sizes are warranted to confirm our findings and also determine the importance of the biomarker in invasion and tumour metastasis.

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APPENDIX A

Table 1: Summary of lineages and sub-lineages used in the phylogenetic trees construction from GenBank database

Species	HPV Types	Lineage	Sub-lineage/variant	Isolate identification	GenBank accession number (NCBI)
Alpha-9	HPV16	A	A1	NC_001526.4 Qv17286 QV33501 QV11687	NC_001526.4 HQ644272.1 HQ644286.1 HQ644268.1
			A2	W0122	AF536179
			A3	AS411	HQ644236
			A4	AS097 W0724 IN151168 INJP0168	HQ644234 AF534061 HQ644248 HQ644251
		B	B1	BF325 BF215 W0236 RW918	HQ644240 HQ644238 AF536180 HQ644293
			B2	Z109	HQ644298
		C		BF039 BF236 IN221688 IN272098	HQ644237 HQ644239 HQ644249 HQ644250
		D	D1	QV00512AA	HQ644257
			D2	QV15351 QV13791 QV24898 QV00124	AY686579 HQ644270 HQ644281 HQ644254
			D3	QV00995 IN000078 QV23856 QV23856	AF402678 HQ644247 HQ644278 HQ644276
	HPV31	A		J04353.1 IN453545 Z14 QV18335 QV14117 IN221709 QV02689 QV00215 QV00071 QV00168 QV02784	J04353.1 HQ537668.1 HQ537666.1 HQ537667.1 HQ537674.1 HQ537675.1 HQ537672.1 HQ537671.1 HQ537669.1 HQ537673.1 HQ537670.1
		B		QV03876 QV17700 RW687 QV05335 IN231831	HQ537676.1 HQ537680.1 HQ537678.1 HQ537681.1 HQ537679.1
		C		BF363 QV03136 QV14043 QV12357	HQ537683.1 HQ537682.1 HQ537684.1 HQ537687.1

				QV13734	HQ537686.1
	HPV33	A	A1	M12732.1 QV18347 QV06895 QV32494 QV35834 BF266 RW702 RW926 QV22751	M12732.1 HQ537695.1 HQ537696.1 HQ537688.1 HQ537690.1 HQ537691.1 HQ537692.1 HQ537694.1 HQ537689.1
			A2	INJP06456 INCC0137 QV32431 QV34060	HQ537700.1 HQ537699.1 HQ537701.1 HQ537698.1
		B		BF131 Z066 IN241890 QV34189 RW136 QV23819	HQ537706.1 HQ537704.1 HQ537703.1 HQ537707.1 HQ537702.1 HQ537705.1
			A3	LZcc12-33	EU918766.1
	HPV52	A		X74481.1 QV17844 QV15145 Z096 QV17972 RW846 QV32479 QV26382 QV32116 QV23939	X74481.1 HQ537738.1 HQ537739.1 HQ537736.1 HQ537737.1 HQ537735.1 HQ537734.1 HQ537732.1 HQ537731.1 HQ537733.1
		B	B1	QV03594 QV00585	HQ537740.1 HQ537741.1
			B2	IN181391 IN141070 TJ49-52	HQ537742.1 HQ537743.1 GQ472848.1
		C	C1	QV05867	HQ537744.1
			C2	QV03719 QV00615	HQ537745.1 HQ537746.1
		D		QV07294 QV12377 QV02575 QV02124 QV18359	HQ537751.1 HQ537747.1 HQ537748.1 HQ537749.1 HQ537750.1
Alpha-7	HPV18	A	A1	NC_001357.1 QV16302 CU15 QV03132	NC_001357.1 EF202144.1 GQ180791.1 EF202143.1
			A2	QV32981 QV16306 QV28978	KC470210.1 EF202146.1 KC470211.1
			A3	CU10 QV15586 QV26861 QV15957	GQ180786.1 EF202147.1 KC470212.1 EF202149.1
			A4	Z135 QV02876 QV17955	KC470213.1 EF202151.1 EF202150.1
			A5	CU11	GQ180787.1

		B	B1	Z53 RW57 Z52 Z100	KC470216.1 KC470219.1 KC470214.1 KC470222.1
			B2	BF309 BF288 BF172	KC470223.1 KC470224.1 KC470225.1
			B3	QV12693 QV17199 Z125 BF380	KC470227.1 EF202152.1 KC470226.1 KC470228.1
		C		QV39775 BF226	KC470229.1 KC470230.1
	HPV45	A	A1	X74479.1 Z5 Z79 QV20214 RW632	X74479.1 KC470251.1 KC470250.1 EF202156.1 KC470252.1
			A2	QV30004 QV33330 QV27565 QV34178 QV2356 QV27648 BF208	EF202160.1 EF202158.1 EF202157.1 EF202159.1 KC470253.1 KC470254.1 KC470255.1
		B	B1	RW894 QV35960 QV06560 QV00550	KC470257.1 EF202162.1 EF202163.1 EF202161.1
			B2	QV25000 QV31035 QV30712 RW46 QV34163 QV31748 QV26351	EF202164.1 EF202166.1 KC470259.1 KC470258.1 KC470260.1 EF202167.1 EF202165.1

APPENDIX B

Ethics approval letter

UNIVERSITY OF THE FREE STATE
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YUNIVESITHI YA FREISTATA

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IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

26 February 2016

MRS A BULANE
DEPARTMENT OF MICROBIOLOGY AND VIROLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Ms Bulane

ECUFS NR 137/2013C (SUB STUDY)
PROJECT TITLE: DETECTION OF HUMAN PAPILLOMAVIRUS TYPES IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

1. You are hereby kindly informed that, at the meeting held on 23 February 2016, the Health Sciences Research Ethics Committee (HSREC) approved the above project after all conditions were met when the signed permission letter from the Free State Department of Health was submitted.

2. The Committee must be informed of any serious adverse event and/or termination of the study.

3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.

4. A progress report should be submitted within one year of approval and annually for long term studies.

5. A final report should be submitted at the completion of the study.

6. Kindly use the ECUFS NR as reference in correspondence to the HSREC Secretariat.

7. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

Yours faithfully

DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
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