Oral cancer with special reference to virus detection and quantitative gene expression

MIRANDA SHOJAЕIAN JALOULI
Abstract

Background. Head and neck cancers (HNC) are among the most common malignancies worldwide, and about 90–92% of oral neoplasias are oral squamous cell carcinomas (OSCC). Alcohol and tobacco consumption have been recognized as the main risk factors for OSCC development. Oncogenic viruses, such as human papillomavirus (HPV) or Epstein-Barr virus (EBV), as well as genetic alterations may also contribute to tumour formation.

Aims. To study the prevalence of HPV, EBV, Herpes simplex type-1 (HSV-1), and HPV-16 and their integration status as well as the molecular mechanisms that can serve as a basis for the development of OSCC.

Results. In Paper I we reported a statistically significant increase in the prevalence of HPV-16 in oral epithelial dysplasia (OED) and OSCC samples compared to controls. A statistically significant increase was also seen in integrated HPV-16 compared to epithelial viral forms when comparing OED and OSCC samples. Paper II reported the detection of HSV-1 in 54% of healthy samples, in 36% of oral leukoplakia samples, and 52% of OSCC samples. However, these differences were not statistically significant. In Paper III we reported a statistically significant increase in the detection of HPV-positive samples when comparing nested polymerase chain reaction (PCR) with single-PCR results in OSCC and fresh oral mucosa. Paper IV reported that the highest prevalence of HPV (65%) was seen in Sudan, while an HSV-1 prevalence of 55% and an EBV prevalence of 80% were seen in the UK. Finally, Paper V reported that the mRNA levels of Bcl-2, keratin 1, keratin 13, and p53 were significantly lower and that the level of survivin was significantly higher in the OSCC samples of the toombak users than in their paired control samples. Significant downregulation in keratin 1 and keratin 13 expression levels was found in the OSCC samples of the non-toombak users relative to their normal control samples.

Conclusion. HPV-16 integration was increased in oral epithelial dysplasia and OSCC compared to normal oral mucosa. Nested PCR is a more accurate method of establishing HPV prevalence in samples containing low copy numbers of HPV DNA. HPV and EBV may be a risk factor in OSCC development. Our findings confirmed the role of survivin in OSCC carcinogenesis and survivin might be interesting as a biomarker to be monitored. The results presented here provide both clinical and biological insights that will bring us closer to the goal of managing this disease and improving treatment and outcomes for future patients.

Keywords: HPV, EBV, HSV-1, Oral Squamous Cell Carcinoma, Leukoplakia, apoptosis, cell cycle regulation, intermediate filament proteins.

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To the memory of my dear father......

To my dear mother and family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Other publications not included in this thesis


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<td>BL</td>
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<td>bp</td>
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<td>DNA</td>
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<td>NNN</td>
<td>N’-nitrosonornicotine</td>
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<td>4-(methylnitrosamine)-1-(3-pyridyl)-1-buta</td>
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<td>OED</td>
<td>Oral epithelial dysplasia</td>
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1. General background

1.1 Head and neck squamous cell carcinoma (HNSCC)

Head and neck cancers are cancers of the lip, tongue, salivary gland, mouth, pharynx, oropharynx, nasopharynx, hypopharynx, nose, sinuses, thyroid, ear, and larynx (1). The tongue is a major location of fatal cancers, which may also occur in the floor of the mouth, cheek lining, gingiva (gums), lips, or palate (roof of the mouth) (2-3) (Figure 1).

Figure 1. Head and neck cancer regions, showing locations of paranasal sinuses, nasal cavity, oral cavity, tongue, salivary glands, larynx, and pharynx (including the nasopharynx, oropharynx, and hypopharynx). Source: National Cancer Institute © 2012 Terese Winslow LLC, U.S. Govt. has certain rights.
There are several types of oral cancers, but oral squamous cell carcinoma (OSCC) is the most frequent tumour of oral cavity, statistically responsible for 90% of oral cancer worldwide diagnosed every year (4). About two thirds of patients with this disease have advanced stage (5).

Epidemiology

Head and neck cancer is the sixth most common cancer worldwide, representing about 6% of all new cancers (6). There is a wide variation in the incidence and mortality rates of head and neck cancer between different regions around the world, and its estimated incidence is approximately 650,000 new cases of cancer and 350,000 cancer deaths worldwide each year (7). Worldwide, most cases occur in South-East Asia, Latin America, and Eastern Europe (8). The incidence of head and neck cancer in Sweden is 1200 new cases per year, being twice as common in men as in women (9). Generally, head and neck cancers have a five-year survival rate of about 60%, though the prognosis differs depending on where the tumour is localized (10).

Historically, most patients with oral cancer are in older age groups with a peak incidence at 64-70 years of age. Recently, however, there have been increasing reports of patients less than 40 years of age presenting with oral cancer (11). The prevalence of oral cancer has increased 5.3-fold in men and two fold in women over the past two decades (12). According to the World Health Organization (2005), cancer might kill 10.3 million people annually by 2020, with an increasing trend in developing and newly industrialized countries (13).

Risk factors

The major risk factors for the development of head and neck cancers, especially cancers of the oral cavity, oropharynx, hypopharynx, and larynx, are tobacco smoking, alcohol abuse, and viral infections such as HPV (14-17). At least 75% of head and neck cancers are caused by tobacco and alcohol use (18). People who use both tobacco and alcohol are at greater risk of developing these cancers than are people who use either tobacco or alcohol alone (19). Alcoholic beverages lead to an increased risk of head and neck cancer, regardless of the type of alcoholic beverage consumed (e.g., beer, wine, or liquor) (20). With regard to the development of OSCC, ethanol has been recognized as the carcinogenic agent within alcoholic beverages (21). Alcohol on its own is not carcinogenic but its conversion to acetaldehyde is carcinogenic, and consumption of at least three alcoholic drinks per day has been shown to increase the risk of developing head and neck cancers (22). It is thought that alcohol acts as a solvent for carcinogens from associated risk factors, and that acetaldehyde also causes cellular DNA damage (22). Based on fair evidence,
cessation of alcohol consumption leads to a decrease in oral cavity cancer, but not until approximately 10 years after cessation. For cancer of the oropharynx, reduction in risk does not occur until approximately 20 years after cessation (23).

Most people with oral cavity and oropharyngeal cancers use tobacco, and the risk of developing these cancers is related to how much and how long these people smoked or chewed tobacco. A meta-analysis showed that oral cavity cancer risk is three times higher in current smokers than in never-smokers (14). About 80% of OSCC patients have a history of smoking tobacco (24). Twenty pack-years seems to be the threshold at which a significantly increased risk of cancer is imparted (22,25), and the risk declines back to baseline 10 years after cessation (15). Smokeless tobacco contains around 28 known carcinogens. These include the non-volatile alkaloid-derived tobacco-specific N-nitrosamine and N-nitrosamine acids as the major group, while volatile tobacco-specific nitrosamines, volatile aldehydes, and some polynuclear agents have also been shown to be present in smokeless tobacco (26). Stopping smoking reduces the risk of oral cancer and premalignant lesions, although it may take 10-20 years for a former smoker’s risk to decline to that of a non-smoker (27).

Virus infections are among the most important causes of cancer (28). The mouth contains a variety of surfaces that are home to a huge diversity of microorganisms, including more than 750 distinct taxa of bacteria; this suggests that the oral squamous epithelium is constantly exposed to a variety of microbial challenges, on both the cellular and molecular levels. It is therefore important to consider how such factors may be related to oral cancer development (29-30). Based on the available evidence, HPV infection is now considered a validated risk factor for OSCC in both men and women, even in the absence of smoking and alcohol consumption (31-32). Additional causes, such as genetic predisposition, diet, or oncogenic viruses, may also help cells override or escape the physiological mechanisms of proliferation control (33).

1.2 Oral leukoplakia

The term leukoplakia was first used in 1877 by Schwimmer to describe an oral white lesion of the oral cavity (34). Oral leukoplakia (OL) is defined by WHO as “a white patch or plaque that cannot be characterized clinically or pathologically as any other disease” (35) and is the most common potentially malignant disorder of the oral mucosa (36).
Clinically, leukoplakia is classified into homogeneous and non-homogeneous lesions. In homogeneous leukoplakia/leukoplakia simplex, lesions are uniformly flat, thin, and predominantly white in colour. The surface of the lesion may be smooth, wrinkled, or corrugated with a consistent texture throughout. These lesions are asymptomatic and have a very low risk of malignant transformation (37). Non-homogeneous leukoplakia/erythroleukoplakia presents as mixed white and red lesions associated with an erythematous component, and patients complain of pain, itching, and discomfort. These lesions have a high risk of malignant transformation (37). The malignant potential of non-homogenous lesions is almost seven times that of homogeneous types (38). Oral leukoplakia often arises in the cheek and alveolar mucosa and is commonly seen on the lips, buccal mucosa, tongue, and gingiva (39). Lesions in the floor of the mouth and the lateral border of the tongue seem to present dysplastic or malignant alterations more frequently (40).

Epidemiology
Various studies have shown the prevalence of leukoplakia to be between 0.2 and 3.6%, with prevalence varying regionally, being 0.2-4.9% in India, 3.6% in Sweden, 1.6% in Germany, and 1.4% in the Netherlands (41-43). Leukoplakia is more common in men than in women, with a male-to-female ratio of 2:1 (44). Most cases of leukoplakia occur at age 50-70 years; less than 1% of patients are under the age of 30 years (45). One study found that men over the age of 60 years with oral leukoplakia on the lateral or ventral aspect of the tongue, who had a non-homogenous lesion with high-grade dysplasia, were most at risk of malignant transformation (46).

Risk factors
Several factors, such as tobacco carcinogens, alcohol, HPV infection, and genetic predisposition, are suspected causative agents for leukoplakia (46). About 70-90% of oral leukoplakias are related to smoking used, and there is a direct relationship between the frequency and duration of cigarette, pipe, or cigar smoking and the prevalence of oral leukoplakia (25). Alcohol is also an important risk factor, having a multiplicative synergistic carcinogenic effect with tobacco (16,47). Extensive molecular biology and virology studies have been carried out to determine the role of human papillomavirus (HPV) in the aetiology and oncogenesis of oral leukoplakia. HPV type 16 has been demonstrated in oral leukoplakias and carcinomas. In a more aggressive variant of leukoplakia, proliferative verrucous leukoplakia (PVL), HPV-16 and 18 were isolated (38).
1.3 Human papilloma virus (HPV)

Viruses were first visualized with an electron microscope in 1949, and their circular double-stranded DNA genome was demonstrated in 1963 (48). In 1983, it was first suggested (49) that HPV might be the agent involved in the development of at least some types of oral SCCs (50). Papillomavirus infection in the oral mucosa was first demonstrated in animals by Demoubren et al. in 1932 (51). Yet not until 1967 did Frithiof et al. present the first ultra-structural evidence of the presence of papillomavirus in human oral papillomas. Jenson et al. were the first to detect HPV antigen in oral verrucae, multiple papillomas, and condylomata (52). Later, human papillomaviruses (HPVs) were associated with benign epithelial lesions and cancer development (53).

The HPV genomic organization

The papillomaviruses that replicate in the nucleus of squamous epithelial cells belong to the papovavirus group, and are small, non-enveloped DNA viruses with a symmetrical icosahedral shape. Papillomavirus particles (52-55 nm in diameter) consist of a single molecule of double-stranded, circular DNA with approximately 8000 bp, contained within a capsid (spherical protein coat) composed of 72 capsomeres (repeating subunits of the capsid) (54-55). In a virus, only the genome is present, with no cellular machinery for replicating the genome or manufacturing the capsid. The host cell must supply the necessary ingredients for the assembly of the viral components. Thus, a HPV virus reproduces by first adhering to and penetrating an epithelial cell. Following insertion into the intercellular medium, the virus loses its capsid. The viral genome entering the nucleus produces the enzyme necessary to cut the host DNA (the viral DNA is protected through a modification in its DNA). The remaining viral genes, having the capacity to code the cell’s structure and produce proteins, promote the cell’s replication of the virus’s own DNA and all of the components that are part of the virus. These then assemble, giving origin to the capsid, the tail, the tail fibres, nucleic acid, and necessary enzymes, which together form, intracellularly, a new duplicate virus. In the last step, one of the viral genes directs the cell’s lysosome to release contents that digest the cell wall. The new viruses are then released, and are free to infect other cells. When cellular death does not occur, a chronic infection can probably succeed (56-57).
The small, double-stranded DNA genome of all HPVs is approximately eight kilobases (kb) in size. On average, HPV encode eight major open reading frames (ORFs), which are expressed from polycistronic mRNAs transcribed from a single DNA strand (59). The papillomavirus genome is divided into three regions: an early region (E) comprising 45% of the genome, encoding six (E1, E2, E4, E5, E6, and E7) ORFs that are expressed immediately after initial infection of a host cell and that are expressed from polycistronic mRNAs transcribed from a single DNA strand; a late region (L) extending for 40% of the genome, encoding a major capsid protein L1 and a minor capsid protein L2; and the long control region (LCR), which is non-coding but contains the origin of replication and enhancer elements for regulating gene expression (Figure 2) (58-62).

Early ORFs encode for E1, E2, E4, E5, E6, and E7 proteins necessary for replication, cellular transformation, and controlling viral transcription. E1 and E2 maintain viral DNA in an episomal form and facilitate the segregation of the viral genome during cell division. During productive infection, E6 and E7 stimulate cell-cycle progression. E1, E2, E4, and E5 are required for and expressed during viral DNA amplification, which occurs in differentiated cells in upper epithelial layers. The late region encodes the structural proteins or capsid proteins that take part in virion assembly. L1 ORF encodes for major
capsid protein and L2 ORF for minor capsid protein. The non-coding upstream regulatory region encompassing the origin of replication, the E6/E7 gene promoter, and the enhancers and silencers is located between the early and late regions. The LCR is necessary for viral DNA replication and transcription (60-62). The early proteins, E1, E2, E6, and E7, are expressed early in infection in undifferentiated cells and have drastically different functions. Sequences within the upstream regulatory region (URR) located in the non-coding region of the genome are responsible for regulating viral transcription and replication. Expression of HPV gene products is directed from two different promoters, the early promoter and the late promoter (59). The early promoter, termed P97 in HPV-31, is located upstream of the E6 ORF and directs expression of early (E) gene product in undifferentiated cells. Early proteins include E1, E2, E6, E7, E1/E4, and E5. Translation of HPV messages occurs by means of a leaky scanning mechanism, resulting in high levels of E6 and E7 but low levels of E1/E4 and E5 protein synthesis. The E1 and E2 proteins function in replication and transcription control, while E1/E4 modulates late viral functions. The promoter P742 in HPV-31 directs the expression of the late (L) gene products and is located within the E7 ORF. Importantly, P742 is activated upon epithelial cell differentiation. Late proteins include L1 and L2 as well as E1/E4 and E5, and these are all expressed from P742 (59).

**HPV infection and life cycle**

Human papillomaviruses (HPV) are highly prevalent, species- and tissue-specific DNA viruses that infect epithelial cells (63-64). Upon infection, the virus establishes its double-stranded DNA genome in the nuclei of infected host cells (59). There are five stages in the HPV life cycle: (a) genome maintenance (occurs in the basal layer); (b) genome maintenance and cell proliferation (occurs in suprabasal layers); (c) genome amplification (occurs in suprabasal layers); (d) virus assembly and release (occurs in granular layers); and (e) virus release (occurs in cornified layers) (65). During genomic amplification, the expression of L1 and L2 is upregulated at both the RNA and protein levels. During the initial phase of infection, the viral genome undergoes episomal replication, and few copies of the viral DNA are present per host cell. The episomal form acts as a reservoir of infected cells and is responsible for the latent state of infection (66). HPV gains entry to cells in the basal layer of the epithelium, which becomes exposed through micro-abrasions. Infections of the basal layer allow the virus to establish a persistent infection, as the basal cells are the only cells of the epithelium undergoing active replication (59). Since the HPV genomes are only 8 kb in size, they do not encode viral polymerases or other enzymes required for viral replication. The virus must therefore rely on host cell replication machinery to facilitate viral DNA synthesis. Following entry, the viral genome becomes established in the nucleus as extra chromosomal plasmids, or episomes. In the infected basal cells, early viral
gene expression is activated, and genome copy numbers are maintained at approximately 20-100 copies per cell (59). In HPV-infected basal cells, E1 and E2 proteins are expressed, and they regulate early viral DNA transcription. When expression of E2 is more pronounced, E2 represses viral DNA replication by blocking cellular transcription factors, thus controlling the number of HPV DNA copies in the basal cell by a process analogous to negative feedback (66-67).

E2 also plays a role in ensuring that the viral DNA moves into new cells during mitosis and acts as a transcription activator or repressor, controlling the expression of other E genes, such as the viral oncogenes E6 and E7. E6 and E7 are expressed early in the infection to promote cell division. The dividing cells carry the HPV-DNA with them (63). E6 and E7 also associate with cellular tumour-suppressor proteins, deregulating their normal function and driving cell proliferation. Interestingly, as levels of E2 increase, E6 and E7 are down-regulated. This results in a loss of the cellular environment necessary for viral DNA replication. This change in gene expression favours the production of HPV virions. Once the infected cells reach terminal differentiation, the late genes L1 and L2 are expressed (63) (Figure 3). Human papillomaviruses are characterized by a special tropism for squamous epithelial cells, keratinocytes. The synthesis of viral DNA and the expression of viral genes are linked to the level of keratinocyte differentiation (68-69). As HPV-infected basal cells divide, one of the infected daughter cells remains in the basal layer. The other daughter cell migrates away from the basal layer and begins to differentiate, resulting in the activation of the late viral promoter. This results in the onset of the productive phase of the life cycle, which includes viral DNA amplification, with the copy number increasing to over 100 copies of HPV-DNA per cell, and the onset of capsid gene expression. Finally, the synthesis of viral capsids and the packaging of the viral genome occur in the uppermost-differentiated layer of the epithelium, ultimately resulting in the release of the progeny virions. Furthermore, studies have indicated that viral proteins E6, E7, E1/E4, and E5 are needed for this activation (59).
Figure 3. Regulation and deregulation of the HPV life cycle. (A) The papilloma-virus life cycle is regulated during epithelial cell differentiation. The upregulation of viral proteins necessary for genome amplification (i.e., E1 and E2) requires activation of the viral late promoter in the upper epithelial layers (cells shown in green with red nuclei), with virus particles subsequently being released from the epithelial surface. (B) Production of infectious virions is restricted to smaller and smaller areas near the epithelial surface. This situation is thought to be accompanied by elevated E6/E7 expression, and represents a non-productive or poorly productive abortive infection. Integration of HPV-DNA into the host cell genome is facilitated by deregulated E6/E7 expression. If integration disrupts the E1/E2 region, this can allow the persistent high-level expression of E6 and E7 and the accumulation of genetic errors in the host genome. Eventually, the productive virus life cycle is no longer supported and viral episomes are lost (63).

The possibility of evolving in the direction of malignancy depends on the type of virus, synergistic action with various physical, chemical, and biological agents, the genetic constitution, and the immune defence mechanisms of the host, all of which can modify the course of HPV infection. In the case of high-risk HPV infection and under favourable conditions, the viral genome is integrated into the host genome, which is necessary for keratinocyte immortality (61). During this process of integration, the circular form of the viral genome breaks at the level of the E1 and E2 regions. The loss of E2 during this process of integration results in the loss of E6 and E7 control. Therefore, the E6 and E7 sequences are directly involved in the cellular cycle by inhibiting the normal functions of p53 and pRb, respectively (60-61). There are 100 different types of HPV; high-risk and low-risk HPV have already been identified because of their medical importance (65). About a dozen high-risk HPV types, which can cause cancer, have been identified. Two of these, HPV types 16 and 18, are responsible for most HPV-caused cancers (70-71).
Epidemiology of HPV

High-risk HPV type 16 is found to be highly prevalent (~90%) in OSCC; other HPV types (e.g., HPV-18, 31, 33, 58, 59, 62, and 72) are less common, and HPV has been demonstrated to be episomal or integrated into the cellular genome (72-73). Some studies have examined the relationship between HPV and SCC in the head and neck region. In most cases, HPV infection seems to be latent or subclinical (74). The incidence rate of HPV infection varies between regions, being high in India, Sri Lanka, Vietnam, the Philippines, Hong Kong, and Taiwan, where about 30% of all cancers occur in the oropharynx. India has approximately 56,000 new cases of OSCC per year, probably among the highest incidence rates in the world (75).

1.4 Epstein-Barr virus (EBV)

The Epstein-Barr virus was the first reported human tumour virus. In the 1950s, Denis Burkitt, a British surgeon working in East Africa, was the first to describe the childhood tumour now known as Burkitt’s lymphoma (BL) (76). EBV was initially seen by electron microscopy in lymphatic tissue culture cells derived from Burkitt’s lymphoma (77). Later in 1965, a research group successfully established BL-derived cell lines, and visualized herpesvirus-like particles from a small percentage of cells by electron microscopy (78). This virus was named EBV because it was subsequently shown to be antigenically and biologically distinct from other known human herpesviruses (79). In 1968, EBV infections were first identified as the cause of infectious mononucleosis (80-81). The transformation potential of EBV was confirmed almost concurrently by demonstration of the ability of the virus to transform infected B-cells, causing them to proliferate uncontrollably, and because it was associated with many malignancies. This led to confirmation that EBV was a new herpesvirus and its characterization as the first human tumour virus (80-82).

The EBV genomic organization

EBV is one of eight viruses in the herpes family; it is one of the most common viruses in humans (83) and was the first herpesvirus to be completely cloned and sequenced from an EBV-DNA BamHI fragment-cloned library (84). The virus is approximately 122 nm to 180 nm in diameter and contains a double helix of DNA of about 172,000 bp with about 80 genes (85) that have been described in detail (86). The DNA is surrounded by a protein nucleocapsid. This nucleocapsid is surrounded by a tegument of protein, which is in turn surrounded by an envelope containing both lipids and surface projections of glycoproteins essential to infecting the host cell (87). The virus genome contains a long unique region interspersed with four major internal repeats (i.e.,
IR1 to IR4) and terminal repeats (TR). Nine latent proteins, including Epstein-Barr nuclear antigen 1 (EBNA1), EBNA2, EBNA3A, -3B, -3C, EBNA-LP, and latent membrane protein 1 (LMP1), LMP2A, and -2B are encoded by genes situated in the unique region of the genome (88). Other open reading frames encode capsid proteins, transcription factors, as well as lytic proteins with various functions (89). In addition to protein-coding genes, the EBV genome also encodes non-coding EBV RNAs, such as Epstein Barr virus encoded small RNA 1 (EBER1) and 2 (EBER2), BART-derived microRNAs (miRNAs-BARTs), and BHRF1 microRNAs (miRNAs-BHRF1) (90-91) (Figure 4).

Figure 4. A. The EBV genome. The origin of plasmid replication (OriP) is shown in orange. The latent proteins include the six nuclear antigens (EBNAs 1, 2, 3A, 3B, and 3C, and EBNA-LP) and the three latent membrane proteins (LMPs 1, 2A, and 2B), shown by purple arrows. Transcription is initiated by promoter C (Cp) or Wp and Qp. The EBNA1 transcript starts from the QP promoter, shown by the blue inner line. B. The EBV genome in linear form showing latent genes. The BamHI fragments are named alphabetically according to size, with A being the largest. At both ends of the genome, terminal repeats (TR) are shown (92-93).
EBV-1 and EBV-2, two subtypes known to infect humans, differ in the organization of the EBV nuclear antigen (EBNA) genes (94). EBV-1 is more frequent in most populations and is dominant in the Western hemisphere and South-East Asia, but EBV-2 is primarily found in parts of Africa and is associated with endemic Burkitt lymphoma (94). EBNA 2 and EBNA-LP are the first genes that start to be transcribed under the control of WP, after in vitro infection of B-cells (95).

EBV infection and life cycle

Figure 5. The life cycle of EBV. The image is taken from the article “Endemic Burkitt’s lymphoma: a polymicrobial disease?” (96)

EBV was the first virus of the gamma herpes family shown to be oncogenic in humans (97). EBV is ubiquitous and infects more than 90% of the population before adulthood (98). EBV establishes latency in B-lymphocytes and infection is life-long. Primary infection is usually asymptomatic during early childhood, although a delay in EBV exposure infection results in infectious mononucleosis (IM) in approximately 25% of cases. EBV infects mainly two cell types: epithelial cells, where it undergoes lytic replication, and B cells, where it initially replicates in a lytic manner before it enters a latent or dormant state (99). EBV can be orally transmitted through saliva, primarily infecting oropharyngeal sites and then spreading farther to healthy B-cells. In order to survive, the EBV virus keeps reproducing and shedding into saliva, which helps it to spread and infect other naïve hosts. In addition, it helps constantly contaminate naïve B-cells in its current host (100-101). After infecting the B-
cell, the viral proteins induce cell proliferation, changing the properties of the cell. In response, the immune system starts to react. The virus finds a way to avoid the immune system and remain latent in memory B-cells (Figure 5) (96,100).

Epidemiology of EBV

EBV infection occurs worldwide. More than 90% of the population is affected, either subclinically during early childhood or as infectious mononucleosis during teenage years. EBV infects individuals in all societies (102), even in the most remote and isolated tribes, such as those in the Melanesian islands (103) and the Amazonian plateau (104). EBV infection takes place at a much lower age in primitive societies than in industrial societies, where there are significant differences between socio-economic classes (105). In Uganda, all children less than three years of age have been infected with EBV, whereas in Singapore, only 20% of children are EBV infected at that age (105). There are, however, geographic and/or racial differences in the type of EBV-associated disease that affects populations. The Chinese in Hong Kong and Singapore have a high incidence of nasopharyngeal carcinoma; Malays and Indo-Pakistanis have a low risk of nasopharyngeal carcinoma and Burkett’s lymphoma; Ugandans have a high prevalence of infectious mononucleosis (105).

1.5 Herpes simplex virus 1 (HSV-1)

The clinical manifestations of oral herpetic infections have been known for 250 years (105). Herpes simplex virus type 1 (HSV-1) is an important pathogen that causes a variety of clinical manifestations in humans. It has the ability to remain latent in host neurons for life and reactivate to cause lesions at or near the site of initial infection. Reactivation from the latent state results in productive infection that ultimately leads to the lytic destruction of distal epithelial cells. During the lytic cycle of the virus in cultured cells, regulation of HSV-1 replication occurs mainly at the transcriptional level and involves the coordination of three phases of gene expression (106).

The HSV-1 genome organization

Herpes simplex virus type 1 (HSV-1) is a member of the Alphaherpesvirinae subfamily of the Herpesviridae family (107). The HSV-1 genome is a linear double-stranded DNA molecule of about 152-261 bp that becomes circularized immediately after being released from the capsid into the nucleus of the cell (108). The herpesvirus genome has two covalently joined segments, i.e., L (long) and S (short), flanked by inverted repetitions of terminal repeat (TR) and internal repeat (IR) regions for both segments at the end of each terminus.
and at the L/S joint, an a-sequence is present as a direct repeat (DR) in an inverted form (109). This a-sequence allows for internal recombination between the L and S regions and can present four isomeric forms (110). A typical herpesvirus genome has a promoter region 50-200 bp upstream and a transcription initiation site 20-25 bp downstream of the TATA box. A 5’ untranslated leader sequence of 30-300 bp is followed by a single major open reading frame (ORF), and 10-30 bp of 3’ untranslated sequence is prototypical of the genome. A polyadenylation signal can be found in the end regions (111). Of the 90 genes present in the genome, HSV-1 encodes at least 84 proteins (112) that have various functions for virus growth in culture, entry of virus into hosts, regulation of gene transcription and translation, and final assembly of virions (113).

Figure 6. HSV-1 virion structure and genome organization.

The HSV-1 has four major features: the viral double stranded DNA, packaged as a tightly wrapped spool, an icosahedral capsid shell; a tegument layer containing numerous viral proteins, and a lipid membrane envelope studded
with viral glycoproteins. The HSV-1 genome is organized into two components, long (L) and short (S). Each component contains a unique region (U) flanked by inverted repeat regions (114) (Figure 6).

HSV-1 infection and life cycle

Herpes simplex belongs to a family of eight related viruses, including herpes simplex virus types 1 (HSV-1) and 2 (HSV-2), varicella-zoster virus, Epstein-Barr virus, and cytomegalovirus. All of these are double-stranded DNA viruses, which affects the skin, mucous membranes and, less frequently, the oesophagus and brain. Skin infections are usually located in the orolabial, genital, or anorectal areas. Of the two serotypes, HSV-1 infection is primarily oropharyngeal and HSV-2 infection is primarily genital. However, HSV-1 has been found in genital lesions and HSV-2 has been found in oral lesions. Orofacial herpes affects the trigeminal ganglion, whereas genital herpes involves the sacral ganglion. (115-116). The life cycle of productive HSV-1 replication in a cell (Figure 7).

![Figure 7](image)

**Figure 7.** The stages of HSV-1 infection: receptor binding and membrane fusion, release of the viral nucleocapsid and tegument into the cell cytoplasm and transport of the nucleocapsid to the nuclear pore, release of viral DNA into the nucleus, transcription and translation of the viral immediate early (IE) and early (E) genes, viral DNA synthesis, transcription and translation of the viral late (L) genes, capsid assembly and DNA packaging, and egress of progeny virions (114).
Herpes simplex infection generally occurs in two phases: the initial, primary infection, followed by secondary, recurrent disease at the same site. In the first phase, the virus spreads by close person-to-person contact with lesions or mucosal secretions (e.g., saliva and cervical discharge) as well as by respiratory droplets. Contrary to previous belief, the virus can be transmitted during asymptomatic periods, though the risk of transmission is higher during symptomatic reactivation. Once the virus is transmitted, incubation takes from 2 to 10 days. The virus then spreads to regional lymph nodes, causing tender lymphadenopathy. Pain and tenderness, paresthesias, and burning at the inoculation site may follow, accompanied by malaise, fever, and headache. Grouped vesicles appear on an erythematous base and then umbilicate, erode, and form crusts. At this point, the lesions are numerous and more scattered than in recurrent disease (116-117).

Epidemiology of HSV-1

Herpes simplex virus type 1 (HSV-1) infections are frequently asymptomatic but can produce a variety of signs and symptoms. These include oral or peroral lesions, ocular infections, congenital skin lesions, genital skin or mucous membrane lesions, and serious systemic illnesses such as encephalitis and neonatal disease. HSV-1 is transmitted from person to person via infected oral secretions during close contact. Infection occurs worldwide, equally between the sexes, and without seasonal variation. In the United States, there are estimated to be approximately 500,000 primary infections per annum (118). While infection is lifelong, it is rarely fatal in the immunocompetent host, producing either asymptomatic or mild clinical disease. HSV-1, one of eight closely related human herpesviruses, is among the most common infectious agents in humans. Infection with HSV-1 usually results from direct contact with the infected oral secretions, skin lesions, or mucous membrane lesions of an infected person, who may or may not be symptomatic. Airborne transmission by aerosolized droplets or desquamated skin cells is also possible. The incubation period for HSV-1 infections is 2-14 days (119). While nearly two thirds of primary infections in adults are symptomatic, only one third of primary infections in children are reported to be symptomatic, with the greatest concentration of virus shed during these symptomatic infections (120-121). Patients with primary HSV-1 infections may shed virus for one week or for as long as several weeks. HSV-1 infections are endemic worldwide, affecting nearly 75% of the general population at some time during life (122). In the United States, the prevalence of HSV-1 infection tends to increase in a roughly linear fashion with increasing age, with the greatest rate of acquisition occurring during childhood and adolescence (123). The prevalence of HSV-1 reaches more than 40% by age 15 years and rises to 60-90% in older adults. Recent data from several European countries show that the prevalence of HSV-1 infection has dropped from 34% to 24% among 10-14 year olds in the
last decade (124). For many adolescents and adults in Europe and the United States, sexual activity is the means of initial exposure, resulting in an increase in genital herpes infections caused by HSV-1 (124). A multicentre study conducted in the USA showed that the number of new genital HSV-1 infections occurred at the same rate as new oral HSV-1 infections among young adults (120).

1.6 Candidate genes

p53

p53 is a tumour-suppressor gene located on the short arm (p) of chromosome 17 (125). It is involved in cell-cycle control, apoptosis, and DNA repair, and participates in the process of carcinogenesis (126). Loss of p53 function can occur through gene mutation, deletion, or inactivation (127). Mutation of the p53 tumour-suppressor gene may represent the most common genetic change in human cancer (128). Prostate (129), ovarian (130), brain (131), breast (132), gastric cancer (133), and SCC of the head and neck (134) have been reported to be associated with p53 mutations. Some studies have reported associations between smoking and alcohol use and the frequency of p53 mutations and p53 overexpression (135-136). Studies have shown a higher frequency of p53 gene mutations in (toombak) snuff-associated oral SCC obtained from Sudan compared to Sudanese non-users (137), and a significantly lower frequency of p53 protein expression in OSCCs from toombak dippers compared to those from non dippers in Sudan and Scandinavia (138). An immunohistochemical study also showed overexpression of the p53 protein and Ki-67 in snuff-induced lesions (139).

p16INK4A and p21WAF1/CIP1

Cell-cycle regulatory genes include p16INK4A and p21WAF1/CIP1, which code for cyclin-dependent kinase inhibitor proteins essential for cellular growth, differentiation, and apoptosis (140), and cyclin regulatory subunits, inducing G-1 arrest (141). p16INK4A is thought to act as a tumour suppressor, as inactivation or deletion is observable in various types of malignancies (142). The cyclin-dependent kinase inhibitor p21WAF1/CIP1, encoded by the WAF1/CIP1 gene, plays an important role in the regulation of the G1-S transition of the cell cycle. The p21WAF1/CIP1 protein functions as a main downstream effector of p53 protein. In response to DNA damage, wild-type p53 accumulates and binds to the promoter region of the WAF1/CIP1 gene, inducing p21WAF1/CIP1 expression. The expression of functional p21WAF1/CIP1 inhibits the activity of the cyclin/cyclin-dependent kinase complex to promote cell-cycle progression (140). p21WAF1/CIP1 expression
can also be induced by p53-independent pathways such as the effects of geno-
toxic drugs and growth factors (141). p21WAF1/CIP1 overexpression has
been reported to be associated with poorer prognosis in bladder (142), ovarian
(143), breast (144), and oesophageal carcinoma (145), as well as oral SCC
(146).

Survivin
Overexpression of some IAP family members has been noted in carcinomas,
suggesting that IAP-mediated inhibition of apoptosis may contribute to tumor-
igenesis (147-148). Among the IAP proteins, there has been recent interest in
survivin, which encodes for a multifunctional protein that suppresses apopto-
sis by association with caspases and second mitochondria-derived activator of
caspases (SMAC)–direct IAP binding protein with low pI (DIABLO) and reg-
ulates mitosis by interacting with other chromosomal passenger proteins, such
as inner centromere protein (INCENP) and aurora-B (149). Survivin does not
directly inhibit caspases. Survivin’s effects on cell death inhibition appear to
be mediated via interactions with other proteins, including another IAP pro-
tein, XIAP (150-151), and the hepatitis B X-interacting protein (HBXIP)
(152). Overexpression of survivin has been noted in virtually all cancers, in-
cluding lung (153-154), breast (155), colon (156), pancreas (157), and hema-
tological malignancies (158-160). The association of some IAP family mem-
bers, especially survivin, with human oral SCC has been extensively reported
(161). Studies have shown a higher survivin expression level in both primary
oral SCC and pre-malignant lesions than in normal oral tissues (162), as well
as increased survivin expression in specimens obtained from toombak-user
OSCC samples compared with toombak-user control samples (163). The
mechanisms of IAP overexpression in cancer are largely unknown. Amplifi-
cation of the survivin locus on chromosome 17 and DNA demethylation of its
promoter region have been suggested as possible mechanisms of survivin up-
regulation in some types of cancer (164).

Bcl-2
The B-cell lymphoma 2 (Bcl-2) gene belongs to a family of oncogenes and is
implicated in cancer development through inhibiting apoptosis (165). In 1984,
it was first discovered that Bcl-2 was activated by a chromosome translocation
in human follicular lymphoma (166). In 1988, Bcl-2 was identified as a regu-
lator of apoptosis (167). Bcl-2 family proteins share one or more of four so-
called Bcl-2 homology (BH) domains named BH1, BH2, BH3, and BH4. It is
the BH domains that are responsible for the functionality of each of the Bcl-
2-related proteins by dictating their ability to bind to neighboring Bcl-2 pro-
teins or other necessary proteins (168). Depending on their function and BH
domains, Bcl-2 family members can structurally be divided into three classes
of proteins: the Bcl-2, Bax, and Bik subfamilies. The Bcl-2 subgroup includes proteins such as Bcl-2 (169), Bcl-xL (167), Bcl-xs, and Bcl-w (170); with the exception of Bcl-Xs anti-apoptotic properties contain all four of the BH domains (BH1–4). The pro-apoptotic Bcl-2 family members include Bax (171), Bak, and Bad, which contain three of the four BH domains, i.e., BH1, BH2, and BH3. The pro-apoptotic Bik subfamily includes proteins such as Bic, Bid, and Bim, which contain BH3 only (172).

Keratins

Cytokeratins (CKs) are intermediate filament genes expressed in specific cell types, such as muscle and glia cells (173), and are essential for cell shape, motility, and structural integrity (174). Changes in some keratins have been documented in several studies. Loss of CK13 has been observed in tongue SCC, which is related to the invasive and metastatic ability of cancer cell lines (175-176). A decrease in the expression of CKs has been observed in cytokeratin-2E, 2P, 6A-F, 7, 13, 14, 15, 17, 18, and 19 in normal tissue, and their expression is almost absent in cancer cell populations, which may reflect the loss of differentiation in tumour cells (176). Other studies have reported down-regulated expression of CK13 in oral SCC (177-178), as well as a decrease in CK13 expression associated with an increase in the grade of malignancy in a transitional cell carcinoma (179). Jalouli et al. showed that CK13 and CK1 were downregulated in oral SCC samples from toombak users and non-toombak users compared with control samples (163). Lack of CK13 expression has been observed in the majority of invasive carcinoma cells (180).
2. Aims of the thesis

I. To evaluate the prevalence of consensus HPV, and HPV-16 and its integration status, in healthy oral mucosa, oral epithelial dysplasia (OED), and oral squamous cell carcinoma (OSCC) samples, and to determine whether HPV in the oral cavity may play a role in the onset of oral dysplasia and in the transition to squamous cell carcinoma.

II. To investigate the presence of HSV-1 DNA by means of nested PCR (NPCR) in clinically healthy oral mucosa, oral leukoplakia, and OSCC samples.

III. To compare the level of detection of HPV DNA, applying two reliable PCR methods, NPCR and SPCR, to paraffin-embedded OSCC samples from Sudan and fresh tissue specimens from healthy Swedish volunteers.

IV. To examine the prevalence of HPV, HSV-1, and EBV in OSCC samples obtained from eight different countries from subjects from different ethnic groups and continents and with different socio-economic backgrounds.

V. To examine the differential expression of nine selected genes related to apoptosis (survivin and Bcl2), cell-cycle regulation (p53, p16INK4a, and p21WAF1/CIP1), and intermediate filament proteins (keratin 1, 13, 14, and 19).
3. Material and methods

3.1 Sample collection

**Paper I: Association of human papillomavirus infection in healthy oral mucosa, oral dysplasia, and oral squamous cell carcinoma**

The study examined 53 OED and 27 OSCC paraffin-embedded cases and 26 fresh tissue samples from clinically healthy Swedish volunteers. The patient age range for OED was 23–93 years (34 male, 19 female, mean age = 65 years, SD = 15), for OSCC was 20–84 years (20 male, 7 female, mean age = 63 years, SD = 16), and for normal oral mucosa was 25–86 years (14 male, 12 female, mean age = 62 years, SD = 15). The formalin-fixed, paraffin-embedded tissue sections were obtained from the Department of Oral Pathology, Gothenburg University and the normal oral mucosa specimens were obtained from the Department of Oral and Maxillofacial Surgery at Uppsala University. The study was approved by the Ethics Committee of Uppsala University.

**Paper II: Nested PCR for detection of HSV-1 in oral mucosa**

The study examined 53 cases of oral leukoplakia and 27 cases of OSCC. The formalin-fixed, paraffin-embedded tissue sections were obtained from the Department of Oral Pathology, Gothenburg University. The patient age range was 23–93 years (34 male, 19 female, mean = 65 years, SD = 15) in patients with oral leukoplakia, and 20–84 years (20 male, 7 female, mean = 63 years, SD = 16) in patients with OSCC.

Twenty-six clinically healthy volunteers, 14 male and 12 female were also included. The patient age range was 25-86 years and the mean age was 62 ± SD 15 years. Local anaesthesia (lidocaine 20 mg mL⁻¹ + 12.5 μg adrenaline; AstraZeneca, Södertälje, Sweden) was used when obtaining biopsy specimens, which were taken from the normal oral mucosa of clinically healthy Swedish volunteers during dentoalveolar surgery. Immediately afterwards, the biopsy specimens were rinsed twice in buffered saline. The specimens were placed in 99% alcohol and kept at room temperature for 24 hours before being stored at −20°C until being analysed. The specimens were obtained from the Department of Oral and Maxillofacial Surgery at Uppsala University. The study was approved by the Ethics Committee of Uppsala University.
Paper III: Comparison between single PCR and nested PCR in detection of human papilloma viruses (HPV) in oral tissues

In this study, 57 paraffin-embedded OSCC samples, from 37 males and 20 females, were obtained from the Department of Oral Pathology, Faculty of Dentistry, University of Khartoum, Sudan. The patient age range was 50–84 years (mean 60 years ±SD). Eight fresh specimens of healthy oral mucosa from healthy volunteers were added, obtained at the Department of Oral and Maxillofacial Surgery, Uppsala University, Sweden. Of these 8 healthy volunteers, 5 were male and 3 female, ranging in age from 58 to 79 years (mean 67 years ±SD). The material was collected after informed consent in accordance with the Declaration of Helsinki.

Paper IV: Human papillomavirus, herpes simplex virus and Epstein Barr virus in oral squamous cell carcinoma from eight different countries

The study population consisted of 155 patients diagnosed with OSCC from eight different countries in Europe, Asia, Africa, and North America. Of the 155 OSCCs, 41 were diagnosed in the tongue (26%) and 23 in the floor of the mouth (15%); the other 91 OSCCs were diagnosed in other locations (59%). Due to socio-economic differences between the eight countries, we also divided the patients into two groups: those from industrialized countries, namely, Sweden, Norway, the UK, and the USA (n = 77), and those from developing countries, namely, Sudan, India, Sri Lanka, and Yemen (n = 78).

Paper V: Differential expression of apoptosis, cell cycle regulation and intermediate filament genes in oral squamous cell carcinomas associated with toombak use in Sudan

Specimens of primary OSCCs from 26 patients (23 male, 3 female, age range 23–85 years, mean age 54.9, ± SD 17 years) were obtained from the Department of Oral and Maxillofacial Surgery at the Khartoum University Dental Teaching Hospital, Khartoum, Sudan. Immediately after surgery, the biopsies were submerged in the tissue storage and RNA stabilization solution, RNAlater (Ambion, Applied Biosystems, Foster City, CA, USA) and stored at −20°C until RNA extraction. Clinicopathological information, including age, gender, toombak use, smoking and alcohol intake, tumour site, and histopathological differentiation, was recorded.
3.2 DNA extraction

Molecular studies of fresh tissues may be quite easy to perform, since DNA or RNA of good quality can usually be extracted easily. Nucleic acid obtained from old material, however, is often impure and of low molecular weight, and may contain many single-strand nicks and breaks that make it difficult to analyse. Furthermore, the available tissue biopsies are often very small. The most frequently used method to preserve human tissue is fixation in formalin, followed by paraffin embedding. Paraffin (wax), which is obtained from certain petroleum oils, consists mainly of carbon hydrates with a molecular formula of C_{20}H_{40}. The carbon hydrates are dissolvable in benzene, xylene, chloroform, and ether but not in water, acetone, or ice-cold ethanol. Paraffin is colorless or white having a density of 0.90 g L\(^{-1}\) and a melting point of 50–57\(^\circ\)C. Since paraffin interferes with subsequent PCR, thorough dewaxing is mandatory.

Paraffin-embedded tissue

All of the tissue specimens were fixed with formalin and embedded in paraffin. Ten 5-μm sections were cut from each paraffin block. Paraffin was dissolved in xylene, and the tissues were digested with proteinase K. DNA was purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA concentrations and DNA quality were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All DNA samples were tested by PCR with a housekeeping gene and were positive for β-actin.

Fresh tissue

Total DNA was extracted from fresh oral biopsies using the QIAamp tissue DNA Mini Kit with the manufacturer’s protocol (QIAGEN, Hilden, Germany). Briefly stated, tissue samples were weighed, cut into small pieces, and incubated at 56\(^\circ\)C with the addition of 180 μL of ATL buffer supplied with 20 μL of proteinase K per 25 mg of sample. When tissues were completely lysed, 200 μL of lysate was transferred into a 2-mL microcentrifuge tube, and DNA extraction with QIAamp Mini spin columns was carried out using a QIAcube. Final elution of DNA extracted from tissue samples was performed with 200 μL of double-distilled water. DNA concentrations and DNA quality were measured using a NanoDrop spectrophotometer (NanoDrop Technologies). All DNA samples were tested by PCR with a housekeeping gene and were positive for β-actin.
3.3 RNA extraction and cDNA synthesis

RNA was isolated with combined TRIzol reagent (Gibco BRL, Carlsbad, CA, USA) and an E.Z.N.A. Tissue RNA kit (Omega Bio-tek, Doraville, GA, USA) as recommended by the manufacturer. DNase I digestion (20 U μL⁻¹ RNase-Free DNase I; E.Z.N.A. Tissue RNA kit) was performed on the extracted RNA to ensure removal of residual genomic DNA. The RNA quantity was measured by obtaining an A260/A280-nm wavelength ratio using a NanoDrop spectrophotometer (NanoDrop Technologies). The quality of the RNA was checked using a denaturing Flash gel system (Cambrex Bio Science, Rockland, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A high-capacity cDNA Archive Kit system supplied by Applied Biosystems (Foster City, CA, USA) was used to generate PCR-ready cDNA from 300 ng of total RNA. Finally, the cDNA was dissolved in a 50-μL reaction volume, and 5 μL separated on a 1.5% agarose gel.

3.4 Polymerase chain reaction (PCR)

The choice of the primer annealing temperature is probably the most critical factor in designing high-specificity PCR.

Table 1. Primer sequences used in the semi-nested and nested PCR techniques.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Target gene</th>
<th>Size (bp)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MY 11</td>
<td>HPV L1</td>
<td>450</td>
<td>5’ GCM CAG GGW CAT AAY AAT GG 3’</td>
</tr>
<tr>
<td>MY 9</td>
<td>HPV L1</td>
<td>500</td>
<td>5’ CGT CC MARR GGA WAC TGA TC 3’</td>
</tr>
<tr>
<td>GP5+</td>
<td>HPV L1</td>
<td>150</td>
<td>5’ TTTGTACTGTGTTAGATACTAC 3’</td>
</tr>
<tr>
<td>GP6+</td>
<td>HPV L1</td>
<td>152</td>
<td>5’ GAAAAATAACTGTTAAATCATTTAC 3’</td>
</tr>
<tr>
<td>HPV-16 F</td>
<td>HPV-16 L1</td>
<td>351</td>
<td>5’ GGGTTTTGACAGTTAATACA 3’</td>
</tr>
<tr>
<td>HPV-16 R</td>
<td>HPV-16 L1</td>
<td>254</td>
<td>5’ TTGGTCACGTGTTGCTAC 3’</td>
</tr>
<tr>
<td>E2 F</td>
<td>E2</td>
<td>209</td>
<td>5’ GTG CTT TGA TGG AAG TGG AGG TA 3’</td>
</tr>
<tr>
<td>E2 R</td>
<td>E2</td>
<td>142</td>
<td>5’ GAG CGG GAA ATC GTG GGT GAC ATT 3’</td>
</tr>
<tr>
<td>β-actin 1</td>
<td>β-actin 2</td>
<td></td>
<td>EBV-1 5’ ATC GTG GTG CAC GAG TTG C 3’</td>
</tr>
<tr>
<td>EBV</td>
<td>EBNA-1</td>
<td></td>
<td>EBV-2 5’ ACT CAA TGG TGT AAG ACG AC 3’</td>
</tr>
<tr>
<td>HSV-1</td>
<td>D</td>
<td></td>
<td>EBV-3 5’ AAG GAG GTG TTC GAA AC 3’</td>
</tr>
<tr>
<td>HSV-1</td>
<td>D</td>
<td></td>
<td>HSV-4 5’ TCT CGG TCC AGT CGT TTA TC 3’</td>
</tr>
</tbody>
</table>
Single and nested PCR

A single-PCR assay was used to detect HPV, EBV, and HSV. DNA extracted from samples was used to amplify HPV, EBV, and HSV by two-step (single and nested) PCR amplifications. Before testing the samples, the specificity of the methods was examined using positive and negative control samples. As positive control, Raji cell DNA for EBV and green monkey kidney (GMK) cell DNA for HSV was used (Virology, Gothenburg University, Sweden). EBV was amplified with EBV-1/EBV-2 in the first round and with HSV was amplified with the primers HSV-1/HSV-2 in the first round and with HSV-3/HSV-4 in the second round. The final products included a 209 bp product of the Epstein-Barr nuclear antigen (ebna 1) gene from EBV and a 142 bp fragment of the Dgene of HSV-1. The PCR mixture contained 0.4 M of the appropriate primer (10 nmol), 1 x PCR buffer (Gene Amp 10× PCR buffer II), 200μM of each dNTP (Gene Amp, dNTP Mix with dTTP), 1.25 units of Taq DNA polymerase (Ampli Taq Gold, 5U/μl), nuclease-free water and 1.5 mM (HSV), 2.5 mM (EBV) of MgCl2 (25 mM MgCl2 solution), all from PE Applied Biosystems, Foster City, CA, USA, in a final volume of 25 μl. Viral DNA, human DNA and reaction controls were included in each run. DNA amplification was performed in an automated thermal cycler (Gene Amp PCR system 2700; PE Applied Biosystems). Reactions were incubated at 95°C for 10 min, followed by 30 cycles consisting of a denaturing step for 30 s at 94°C, an annealing step for 30 s at 60°C (EBV), 50°C (HSV-1 first round) or 60°C (HSV-1 second round), and an extension step for 30 s at 72°C. A final extension step at 72°C was carried out for 5 min. A total of 2 μl of the first round product was used in the second round of amplifications. The amplified PCR products were found to be 209 bp long for positive EBV and 142 bp long for positive HSV-1 samples. The samples were also screened for the presence of HPV. A single and nested PCR assay was used to detect HPV. The samples were screened for the presence of HPV using the standard single PCR approach consisting of the MY09/MY11 primer set. Each PCR mixture was diluted with 2.5 μL ten times PCR buffer (500 Mm KCl, 100 Mm TRIS-PH 8.5), 0.6 μL ten times mix dNTP (25μM), 3.5 μL MgCl 2 (25 mM), 0.3 μL oligonucleotide MY09 primer (100 mM), 0.3 μL oligonucleotide MY11 primer (100 mM), and 14.2 μL H2O to a final volume of 21.4 μL. In addition, 3.5 μL of the sample and 0.15 μL of the Taq polymerase (0.75 U, AmpliTaq DNA polymerase, Applied Biosystems, Foster City, CA, USA) were added to the reaction mixture. Each cycle consisted of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. A final extension step at 72°C was carried out for 5 min, and then at 4°C. Five microlitres of the amplified DNA was used as the template for the second PCR with the GP5+/GP6+ primer pair. For HPV-DNA detection by nested PCR (NPCR), two pairs of primers, GP5+/GP6+, were used. The DNA amplifications were performed in 5×1 of PCR buffer, 2 mM MgCl2, 0.2 mM dNTP, 2 pmol of primer GP5+/GP6+, and 1 U of AmpliTaq.
The thermocycler temperature programme consisted of denaturation at 95°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 1 min for 35 cycles. Each PCR was initiated by a 5 min denaturation step at 95°C and finished by a 10 min extension step at 72°C. The PCR assays were performed using Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA), and the PCR products were analysed on 2% agarose gels. Two controls were used for each PCR assay reaction, described as negative control (containing all reagents except the template DNA) and positive control (DNA HeLa cells). The primer sequences used in the PCR reactions are shown in Table 1.

3.5 Quantitative real-time PCR

Real-time quantitative RT-PCR was performed for nine genes, i.e., survivin (Hs00977611_g1), Bcl2 (Hs00608023_m1), p53 (Hs00153340_g1), p16INK4a (Hs00923894_m1), p21WAF1/CIP1 (Hs00355782), keratin 1 (Hs00196158_m1), keratin 13 (Hs00999762), keratin 14 (Hs00559328), keratin 19 (Hs00372324_m1), and β-actin (Hs99999903_m1), using the ABI 7900HT real-time PCR system and 384-well optical plates (Applied Biosystems, Foster City, CA, USA). Each reaction contained 1 µL of cDNA, 5 µL of 2× TaqMan Universal Master Mix (Applied Biosystems), 0.5 µL of Taqman probe, and H2O to a final volume of 10 µL; each reaction was run in triplicate. The PCR reaction consisted of an initial enzyme activation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative threshold cycle (Ct) method, $2^{-\Delta\Delta \text{Ct}}$, was used to determine the relative gene expression levels of each target gene. Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The β-actin gene was used as a housekeeping gene control. Briefly stated, the mean Ct value of the target gene in each sample was normalized to that sample’s average housekeeping gene ΔΔCt value to give a Ct value. This was then normalized to the control sample’s Ct value, and finally the $2^{-\Delta\Delta \text{Ct}}$ value was obtained. For the unknown samples, evaluation of the $2^{-\Delta\Delta \text{Ct}}$ value indicates the fold-change in gene expression relative to the reference sample.

3.6 Gel electrophoresis

Aliquots of 15 µL of the PCR product were analysed on 2% agarose gel (DNA Agar; Marine Bio Products, Quincy, MA, USA) containing 0.5 g mol⁻¹ of ethidium bromide (Merck, Darmstadt, Germany) and visualized under ultraviolet light. The size of the amplified product was determined by comparison with a base-pair ladder size marker for (GeneRuler, 100bp, 50 bp DNA Ladder Plus; MBI Fermentas, St. Leon-Rot, Germany).
3.7 Sequencing

PCR products from HPV, EBV, and HSV-1 were sequenced with fluorescent dye-labelled dideoxynucleotides and cycle sequencing methods utilizing the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were purified of unincorporated dye-labelled dideoxynucleotides by processing through Centri-Sep spin columns (Applied Biosystems). Sequence analysis was automatically performed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and the Basic Local Alignment Search Tool (BLAST) was used to compare sequencing results from HPV, EBV, and HSV-1 nested PCR products.
4. Statistics

Study I: Statistical analyses were performed using the SPSS software package (SPSS for Windows, version 16.0; SPSS, Chicago, IL, USA). P-values and 95% confidence intervals (CI) were calculated using Anova and Fisher’s exact test. Results were considered significant if the p-value was less than 0.05 (5%).

Study II: Statistical analyses were performed using SPSS for Windows, version 16.0. Age differences were investigated using Student’s t-test and gender analysis was performed using Fisher’s exact test. McNemar’s test was used to compare paired proportions.

Study III: Statistical analyses were performed using SPSS for Windows, version 16.0. Rates of HPV positivity were compared between the two method groups, SPCR OSCC and NPCR OSCC, using Fisher’s exact test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Results were considered significant if the P-value was less than 0.05.

Study IV: Fischer’s exact test was used to evaluate the distribution of viral infections (HPV, HSV, and EBV) among the samples studied. For all statistical analyses, SPSS for Windows version 16.0 was used. Values were considered significantly different if p < 0.05.

Study V: All the data were analysed using SPSS version 15.0. The Wilcoxon signed-rank test was used to compare gene expression between OSCCs and their corresponding control samples. P < 0.05 was considered to indicate statistical significance.
5. Results and discussion

5.1 Paper I

*Association of human papillomavirus infection in healthy oral mucosa, oral dysplasia, and oral squamous cell carcinoma*

Aim

To evaluate the prevalence of consensus HPV, and HPV-16 and its integration status, in healthy oral mucosa, OED, and OSCC samples, and to determine whether HPV in the oral cavity may play a role in the onset of oral dysplasia and in the transition towards squamous cell carcinoma.

Brief description of material and methods

The study material consisted of 26 fresh, normal oral mucosa samples and 53 and 27 paraffin-embedded OED and OSCC samples, respectively. The specimens were DNA extracted and investigated for the presence of HPV, and for HPV-16 and its integration status, by polymerase chain reaction (PCR) and DNA sequencing.

Main results (Figure 8)

- 72% of OED samples were found to be HPV-DNA positive; the majority of these were HPV-16+ (82%) and 84% showed integration of viral DNA into the host genome.
- 59% of OSCC samples were found to be HPV-DNA positive; the majority of these were HPV-16+ (94%) and 87% showed integration of viral DNA into the host genome.
- 46% of the fresh control samples were found to be HPV-DNA positive; none of these were HPV-16+ (0%) and none showed integration into the host genome.
Discussion

Human papillomavirus is a common infectious disease that affects oral health. Under normal circumstances, HPV remains in an episomal state and infection with the virus is transient. However, for reasons that are so far not understood, the viral DNA can break and integrate into the host genome. This is believed to be an important step in the cellular transformation of HPV-infected mucosa. HPV genome integration into the host cell genome is thought to induce deregulation of the cell-cycle control, and therefore uncontrolled cellular proliferation, resulting in disruption of the E1-E2 region. The disruption of E2 leads to the deregulated expression of early viral genes and increased proliferative capacity. However, although the relationship between integration and increased viral oncogene expression has been clearly shown in cell line models, it is still unclear whether it exists in primary carcinomas (181-182). In our study, the presence of HPV in cases of OED and OSCC suggests that the virus may play an etiological role in carcinogenesis in the oral cavity, in accordance with other reports that consider the presence of HPV an independent risk factor (183-184). Substantial molecular evidence indicates that persistent HPV infection, especially with type 16, plays a significant role in the pathogenesis of oropharyngeal SCC. Overall, we found a high prevalence of HPV-16 in both premalignant and malignant lesions of the oral cavity. An association of HPV-16 with a subset of head and neck cancers has previously been established. High-risk HPV-16 has clearly been shown to be the dominant type in head and neck cancers (185-186). According to previous studies, integration of HPV-16 DNA correlates with a selective growth advantage and may allow cancer
cells to outgrow their competitors, which can be an important step in oncogenesis (187). Therefore, in our study we measured the integration of HPV-16 as a complementary tool for the assessment and identification of patients at risk of developing SCC. We showed a high prevalence of integration in OED and OSCC samples.

Conclusion
In conclusion our data have shown a significant rise in the detection of HPV and incorporated HPV-16 in OED and OSCC compared to healthy controls. This finding supports that HPV-16 plays an aetiological role in oral cancer development.

5.2 Paper II

*Nested PCR for detection of HSV-1 in oral mucosa*

Aim
To investigate the presence of HSV-1 DNA in clinically healthy oral mucosa, oral leukoplakia, and oral squamous cell carcinoma (OSCC) samples.

Brief description of material and methods
The study material consisted of 26 fresh, normal oral mucosa samples and 53 and 27 paraffin-embedded OED and OSCC samples, respectively. The specimens were DNA extracted and investigated for the presence of HSV-1 by nested polymerase chain reaction (NPCR) and DNA sequencing.

Main results (Figure 9)
- 36% of the oral leukoplakia samples were found to be HSV-1 positive.
- 52% of the OSCC samples were found to be HSV-1 positive.
- 54% of the healthy samples were found to be HSV-1 positive.
Discussion

HSV-1 is one of the most common causes of infectious disease in humans (181). It has been shown that up to 90% of the general population have antibodies against HSV-1 (114). In this study, we found a high prevalence of HSV-1 in all study groups. However, whether HSV-1 plays an active role in OSCC development or is only a passive bystander in the local immune-deficient tumour area remains unclear. Thus, it is still not clear what role HSV-1 plays in OSCC development. One study showed that HSV-1 had no important role in OSCC (188). However, another study showed that HSV infections are statistically associated with oral carcinogenesis and that HSV-1 antibody levels are increased in OSCC patients (189). Furthermore, in a population-based study from the USA, the authors concluded that HSV-1 may enhance the development of OSCC (190); in another study, HSV-1 antibody was found to be associated with a slightly increased risk of head and neck cancer, although not statistically significantly (191). HSV-1 infection of human cell cultures has been shown to be mutagenic (192-194), to induce DNA synthesis (192-193), and to inhibit apoptosis (195-196), all of which may contribute to carcinogenesis (197-198). Animal experiments also support the hypothesis that HSV-1 contributes to tumour development (193,199). A study found that HSV-1 was particularly associated with OSCC risk when other risk factors, such as cigarette smoking or a history of HPV infection, were present (200). Another study also showed that a combination of HSV-1 seropositivity and a history of cigarette smoking was associated with a higher risk of oral cancer than would be expected from a purely additive effect (201). This finding was compatible with
the study showing that HSV-1 seropositivity did not increase the risk of OSCC in the absence of other risk factors (191). The simultaneous exposure of oral mucosa to HSV-1 and tobacco products was first reproduced in rats by Hirsch et al. (202). These authors developed an ingenious system in which a surgical procedure was used to make a canal inside the lower lip in which a wad of chewing tobacco could be placed. This wad would be retained for several days, after which it could be replaced. Some animals were also inoculated with HSV-1, and it was found that exposure to both the tobacco and the virus produced more tumours than did exposure to either agent alone (191). Interestingly, animals exposed to HSV-1 and tobacco, or to HSV-1 and chemical carcinogens, have an increased incidence of tumours at extra-oral as well as oral sites, although no explanation for this is available (202). Previously, in two studies conducted by our research group on specimens from India and Sudan, low HSV-1 prevalence was seen in OSCC samples (203-204). In the present study, we also found no link between the expression of HSV-1 and OSCC in specimens from Sweden.

Conclusion

We found that HSV-1 infection was not statistically associated with an increased risk of oral leukoplakia or OSCC.

5.3 Paper III

_Comparison between single PCR and nested PCR in detection of human papilloma viruses in paraffin-embedded OSCC and fresh oral mucosa_

Aim

To compare the level of detection of HPV-DNA, applying two reliable PCR methods, NPCR and SPCR, to paraffin-embedded OSCC samples and fresh tissue specimens.

Brief description of material and methods

The study material consisted of 8 fresh samples from healthy volunteers from Sweden and 57 paraffin-embedded oral squamous cell carcinoma (OSCC) samples from Sudan. The specimens were tested by single PCR (SPCR) with primer pair MY9/MY11 and by nested PCR (NPCR) with the GP5+/GP6+ primer sets.
Main results (Figure 10)

- 32% of the paraffin-embedded OSCC samples and 62% of the fresh clinically healthy samples were found to be HPV positive with NPCR.
- 7% of the paraffin-embedded OSCC samples were found to be HPV positive with SPCR. A statistically significant difference between HPV-positive and -negative samples was found when comparing NPCR and SPCR in OSCC and fresh oral mucosa ($p < 0.0001$). The comparative test between SPCR and NPCR showed 100% sensitivity and 69% specificity for OSCC.

![Comparison between SPCR and NPCR](image)

Figure 10. Comparison between single PCR (SPCR) with MY09/MY11 and nested PCR (NPCR) with GP5+/GP6+ for the detection of human papillomavirus. A. Paraffin-embedded tissues, SPCR; B. Paraffin-embedded tissues, NPCR; C. Fresh tissues, SPCR; D. Fresh tissues, NPCR.

Discussion

Some studies have already demonstrated that the use of the primer pairs MY09/11 and GP5/GP6 in a nested-PCR assay increases the sensitivity of HPV detection compared with a single-PCR assay (205-207). Nested PCR techniques have proven to be more sensitive for the detection of HPV than PCR methods based on only one amplification reaction. This study aimed to develop and validate a highly sensitive nested PCR methodology for HPV-DNA detection. In this study, the development of a methodology based on nested-PCR technique, using the MY09/11 primer pair in a first amplification and the G5+/G6+ primer pair in the second reaction, enabled sensitive HPV detection in oral samples. We compared the sensitivity of NPCR with SPCR using paraffin-embedded OSCC specimens and fresh samples of normal oral mucosa from clinically healthy volunteers. Based on our results comparing
NPCR with SPCR, it is evident that NPCR is the superior method for detecting HPV-DNA in paraffin-embedded, as well as in fresh, tissue samples. Our results confirm earlier studies. In one study, DNA from several oropharyngeal carcinomas was subjected to PCR with the MY09/MY11 primer set, followed by a second PCR of the previously amplified products with GP5+/GP6+ primers (NPCR), and three more positive samples were detected (208). Another study found that the NPCR system using GP5+/GP6+ primers was the most sensitive method; the authors showed that determination of HPV-DNA viral loads from the oral cavity was better performed with an NPCR system (209). The high sensitivity of the GP5+/6+ NPCR system allows detection of low-copy HPV and wider HPV-type sensitivity, especially in determining the viral types in multiple-infected samples. Standardization and use of the MY/GP PCR system could help physicians provide more efficient screening and better treatment for patients (210-211). We conclude that the use of GP5+/GP6+ nested PCR significantly increased the positivity rate, efficiency, and sensitivity of HPV detection in oral samples and should be considered the method of choice.

**Conclusion**

The increase in HPV DNA detection suggests that nested PCR is the most sensitive method for detecting HPV DNA in OSCC specimens. By applying nested PCR we minimized the risk of having false negative results.

**5.4 Paper IV**

*Human papillomavirus, herpes simplex virus and Epstein Barr virus in oral squamous cell carcinoma from eight different countries*

**Aim**

To examine the prevalence of HPV, HSV-1, and EBV in OSCC samples obtained from eight different countries from subjects from different ethnic groups and continents and with different socio-economic backgrounds.

**Brief description of material and methods**

The study population consisted of 155 patients diagnosed with OSCC from eight countries in Europe, Asia, Africa, and North America. The specimens were DNA extracted and investigated for the presence of HPV, HSV-1, and EBV by means of polymerase chain reaction (PCR) and DNA sequencing.
Main results

- 55%, 35%, and 15% of the OSCC samples were found to be EBV, HPV, and HSV-1 positive, respectively.
- The highest prevalence of HPV was seen in Sudan (65%), while HSV-1 (55%) and EBV (80%) were most prevalent in the UK.
- 34% of all the samples showed co-infection with two (30%) or three (4%) virus specimens. The most frequent double infection was HPV with EBV in 21% of all OSCCs.
- HPV with EBV co-infection was the only co-infection detected in all eight examined countries, with Sudan (40%) and Norway (30%) having the highest prevalence.
- All three virus types were detected in 4% of the samples.
- There was a statistically significantly higher proportion of samples with HSV and EBV from the industrialized countries than from the developing countries.

![Prevalence of viral infection in oral squamous cell carcinoma in each studied Country.](image1)

![Prevalence of viral infections in studied industrialized and developing countries.](image2)
Table 2. Comparison of co-infections between industrialized and developing countries.

Discussion

In this study, we investigated 155 OSCC samples from eight countries. The countries are located on four continents, and have different socio-economic profiles and religious traditions. We investigated the presence of viral (HPV, HSV-1, and EBV) infections as well as alcohol and tobacco habits. In previous global studies, the single most common localization of intraoral SCC was the tongue (212). This was confirmed in our study, where 26% of the OSCCs were diagnosed in the tongue. Several factors are involved in oral carcinogenesis, such as age, sex, race, lifestyle, genetic background, status of health, and exposure to one or more oncogenic factors. In several epidemiological studies, tobacco smoking and alcohol drinking have been well documented as two major risk factors for oral cancer, with attributable fractions of approximately 90% (213). In six of the countries, most OSCC patients were smokers, which was expected since smoking has been shown to be a major risk factor in OSCC development (214). Alcohol consumption has also previously been shown to be a major risk factor for the development of OSCC (215). In our study, alcohol consumption was not high. Previous studies have indicated associations between viral infection and known causes of oral cancer, such as tobacco, alcohol, and betel quid (216). We also showed that HPV prevalence in OSCC was 35%. The single highest HPV prevalence was seen in Sudan (65%), followed by India (45%) and the UK (45%). There was a statistically significant difference between HPV prevalence in Sudan (65%) and Yemen (6%). No difference in HPV prevalence was seen between industrialized and developing countries. The prevalence of oral carcinomas reported to be associated with HPV has varied widely, due to differences in both the population studied and the sensitivity of the assay used for HPV detection (217). It has previously
been shown that HPV-positive oropharyngeal cancer as a proportion of all oropharyngeal cancer varied according to the geographical region, being 56% in North America, 52% in Japan, 45% in Australia, and 38% in Northern and Western Europe (218). A number of authors emphasize the role of EBV in the development of OSCC (219-221). In our study, the highest overall virus prevalence was of EBV, which was seen in 55% of the OSCCs. There was a huge range in EBV prevalence in OSCC, ranging from 22% in Yemen to 80% in the UK, with a statistically significantly higher EBV prevalence in the industrialized countries than in the developing countries. This finding is comparable to the those of earlier studies performed in Sweden, Sudan, and India regarding EBV prevalence in OSCC (203-204, 222). Furthermore, we have shown that the overall prevalence of HSV-positive OSCC was 15%. When comparing the different countries, there was a huge range in the prevalence of HSV-positive OSCC as a proportion of all OSCC from 0% in the USA and India to 55% in the UK. In our work, there was a statistically significantly higher HSV prevalence in the industrialized countries than in the developing countries. The role of HSV in the development of OSCC has been less investigated. However, animal studies (202,223) and clinical studies (224) have shown a possible interaction between the use of smokeless tobacco and HSV-1 in the development of OSCC. Infection by high-risk human papillomaviruses (HPVs) and Epstein-Barr virus (EBV) are very frequent in the adult human population, and have been associated with several human carcinomas, especially oral cancers. However, only a few studies have examined the association between high-risk HPV and EBV in the progression of human oral cancers. Some authors find an association between EBV infection (especially co-infection with papillomaviruses) and squamous cell carcinoma of the tongue and oropharyngeal sites (225-226). In our work, co-infection with HPV and EBV was seen in all eight countries, with an overall prevalence of 21% of all oropharyngeal cancer. This suggests a possible synergistic effect or the interaction of both viruses in the pathogenesis of such tumours. Further detailed studies at both the molecular and cellular levels will be needed to confirm this relationship in oncogenesis.

Conclusions

- Co-infection with HPV and EBV may be a risk factor in OSCC development.
- No differences in HPV and only minor differences in EBV prevalence was seen between industrialized and developing countries.
- Ethnicity did not seem to be of importance regarding HPV prevalence.
- Co-infection with HPV and EBV may be a risk factor in OSCC development.
• No differences in HPV and only minor differences in EBV prevalence was seen between industrialized and developing countries.
• Ethnicity did not seem to be of importance regarding HPV prevalence.

5.5 Paper V

_Differential expression of apoptosis, cell cycle regulation and intermediate filament genes in oral squamous cell carcinomas associated with toombak use in Sudan._

**Aim**

To examine the differential expression of genes related to apoptosis, cell-cycle regulation, and intermediate filament proteins by means of real-time quantitative RT-PCR in samples from Sudanese patients with OSCCs and their matched normal controls.

**Brief description of material and methods**

The study population consisted of 26 patients diagnosed with OSCC from the Department of Oral and Maxillofacial Surgery at the Khartoum University Dental Teaching Hospital, Khartoum, Sudan. Clinically normal control samples were obtained pair-wise from the site opposite the tumour and were microscopically verified as normal. The specimens were tested by means of real-time quantitative RT-PCR for nine genes: survivin Bcl2, p53, p16INK4a, p21WAF1/CIP1, keratin 1, keratin 13, keratin 14, keratin 19, and β-actin.

**Main results**

• The level of survivin in the OSCC of the _toombak_ users was significantly higher than in their normal control samples.
• The levels of BCL2, P53, keratin 1, and Keratin 13 in the OSCC of the _toombak_ users were significantly lower than in their normal control samples.
• The levels of keratin 1 and keratin 13 in the OSCC of the non-_toombak_ users were significantly lower than in their normal control samples.
• No significant differences were detected in keratin 14, keratin 19, p16INK4a, and p21WAF1/CIP1 levels in the OSCC and control samples in relation to _toombak_ use or non-use.
Discussion

The aim of the present study was to examine the differential expression of nine selected genes related to apoptosis (survivin and Bcl2), cell-cycle regulation (p53, p16INK4a, and p21WAF1/CIP1), and intermediate filament proteins (keratin 1, 13, 14, and 19) by means of real-time quantitative RT-PCR in 26 samples from Sudanese patients with OSCCs and their matched normal controls. The overexpression of survivin, which is a member of the inhibitor of apoptosis (IAP) gene family, has been demonstrated in many types of tumour (227-228). Survivin has been shown to be involved in cell division, antiapoptosis, and cell-cycle control (229-230), and it has been hypothesized that survivin interacts with p21 to regulate cell apoptosis (231). The survivin–p21 axis is important for the proliferation of normal hematopoietic cells and in the regulation of apoptosis through the p21WAF1/Cip1-dependent pathway (232). In our study, we found that survivin expression was significantly increased in the toombak users’ OSCCs samples, which was consistent with previous studies (233-234). Survivin is the one representative of the IAP group of proteins that has been recognized to have a role in the nucleus; hence, it could be presumed that normal epithelium is dedifferentiated while the development and progression of carcinoma result in the reexpression of survivin (235). Therefore, survivin expression can be helpful in evaluating the progress of head and neck carcinomas.

The expression of the anti-apoptotic marker, Bcl-2, showed a statistically significant decrease in the toombak users’ OSCC samples compared with their normal controls, which was in accordance with previous studies (236) in which the dysregulation of Bcl-2 and loss of its expression were noted with increasing grades of carcinoma. The downregulation of Bcl-2 expression, with increasing grades of SCCs, indicated that this oncprotein could play a role in relatively early events seen in the development and progression of OSCC (237). Other study have reported sporadic Bcl-2 expression or lack of expression (238). The mechanism underlying the toombak-dependent downregulation of Bcl-2 is unknown, suggesting that the mechanism(s) by which toombak regulates Bcl-2 expression may involve indirect biological pathway(s).

p53 is a tumour-suppressor gene involved in cell-cycle regulation and apoptosis (239). The importance of the p53 tumour-suppressor gene in the process of carcinogenesis is well established. In particular, a high incidence of p53 mutation has been demonstrated in tobacco-related cancers.

In Western countries, the high incidence of p53 mutations in OSCCs is associated with known risk factors, specifically tobacco use and alcohol consumption (240). However, the incidence of p53 mutations was much lower in betel quid (BQ) and tobacco-associated tumours in studies from India (240) and
Taiwan (241). In the present study, the p53 levels were significantly downregulated in the *toombak* users’ OSCC samples compared with their normal controls. Previously, we showed a higher frequency of p53 gene mutations in *toombak*-associated OSCC from the Sudan versus OSCC in non-users (137), but a significantly lower relative frequency of p53-protein expression in OSCCs from *toombak* dippers compared with those from nondippers in Sudan and Scandinavia (242), indicating that *toombak* use could play an important role in increased p53 mutation and possibly tumour development. Loss of keratin 13 related to the invasive nature and ability to form metastases of the cancer cell line has previously been observed in tongue OSCC (243). In the present study, keratin 1 and keratin 13 expressions were downregulated significantly in the OSCC samples from the *toombak* users and the non-users compared with normal control samples, respectively. Disappearance of keratin 13 is often seen in OSCC lesions, while keratin 13 is expressed in normal non-cornified oral mucosa (244). In addition, keratin 13-negative OSCC is associated with a high potential for local recurrence (245). Although the loss of keratin 13 is correlated with the cellular transformation of oral epithelial cells, the epigenetic mechanisms by which keratin 13 are repressed in OSCCs remain unclear.

**Conclusion**

- Tumour cells are unable to synthesize keratin 1 and 13, a finding that may be valuable for cancer therapy.
- Our findings confirmed the role of survivin in OSCC carcinogenesis. Survivin expression continuously increases with the development of the disease.
- Survivin might be interesting as a biomarker to be monitored.
- *Toombak* use downregulate p53 expression and may be of importance for malignant cell transformation.
6. General conclusions

- The presence of HPV and the high prevalence of the HPV-16 in cases of OED and OSCC support the involvement of HPV-16 and its integration into the host genome. This suggests that the virus may play an etiological role in carcinogenesis in the oral cavity in the development of some oral carcinomas (paper I).

- We observed a high incidence of HSV-1 in healthy oral mucosa, oral leukoplakia, and OSCC tissues, but no association between OSCC development and the presence of HSV-1 was detected. Future study is required to determine whether HSV can increase the risk of OSCC in the presence of other risk factors, such as cigarette smoking and alcohol consumption (paper II).

- Nested PCR could be a valuable screening method for detecting low-copy HPV-DNA (paper III).

- Some tumours are associated with papillomaviruses and some with viruses of the herpes family; however, the exact role of these viruses must still be evaluated carefully. These viruses may provide targets for therapy and for diagnostic tests, and may broaden our understanding of the mechanisms by which the tumours develop (papers I, II, and IV).

- Our data suggest that survivin expression in oral SCC may identify patients at risk of more aggressive and disseminated disease. This may be relevant to the institution of closer follow-up protocols and/or alternative combined therapeutic regimens. These findings reiterate the importance of the deregulation of apoptosis as a critical pathogenetic component of tumour progression, and identify survivin as a potential novel molecular marker of aggressive neoplasia (paper V).
• Survivin is a potential prognostic marker of OSCC. Future studies with larger sample sizes and well-designed inclusion criteria will be needed to dissect the role of survivin expression in determining the clinicopathological features and/or prognosis of OSCC (paper V).

• The mechanism underlying the toombak-dependent downregulation of Bcl-2 is unknown, suggesting that the mechanism(s) by which toombak regulates Bcl-2 expression may involve indirect biological pathway(s) (paper V).

• Differential expressions of genes related to apoptosis, cell-cycle regulation, and types I and II keratin could be useful diagnostic markers providing valuable information to advance our understanding of oral malignancy in relation to toombak use. The expression profiles of the genes could also provide a valuable screening tool for patients at risk of developing OSCCs in Sudan. Such screening could also provide important information concerning the molecular mechanisms underlying the development of OSCC, helping in the elaboration of new forms of treatment (paper V).
Squamous cell carcinoma is the most important type of oral cancer and represents 90–95% of all oral malignancies. Tobacco use and/or alcohol consumption are the main risk factors for this cancer form and account for about 66–90% of squamous cell carcinoma cases. Both epidemiological and molecular data suggest that certain types of human papillomavirus (HPV) and certain members of the herpesvirus family, such as the Epstein-Barr virus (EBV) and human herpes simplex virus type 1 (HSV-1), can also have carcinogenic capacity. Several studies have identified specific genetic alterations in oral carcinomas and in premalignant lesions of the oral cavity. These genetic changes lead to the functional loss of tumour-suppression genes and/or increased function of protooncogenes, which in turn deregulate more biological pathways for cell proliferation, differentiation, and apoptosis. Differences in the expression and/or mutation of the genes coding for molecules involved in cell-cycle progression, growth control, apoptosis, oncogene, tumour suppression, growth factors, and cytokines have been reported in squamous cell carcinoma. The degree to which viral infections may contribute to the increased risk of cancer of the mouth cavity in the presence of alcohol, tobacco, and toombak and the molecular mechanisms involved in the initial stage are currently unclear. The exact molecular changes that are crucial to pathogenesis are also still largely unknown. We studied the pre-existence of HPV, EBV, and HSV-1 in oral premalignant and malignant lesions. Furthermore, we studied the impact of genetic mechanisms on squamous cell carcinoma by examining the expression of nine selected genes related to apoptosis, cell cycle regulation, and filament proteins.

This thesis addressed the following aims:

1. Study the incidence of viral infections with consensus HPV, and HPV-16 and its integration status, in clinically healthy oral mucosa, oral leukoplakia, and squamous cell carcinoma samples.
2. Study the presence of HSV-1 DNA by means of nested PCR (NPCR) in clinically healthy oral mucosa, oral leukoplakia, and oral squamous cell carcinoma samples.
3. Detect and compare the level of HPV-DNA using single and nested PCR in paraffin-embedded squamous cell carcinoma samples from Sudan and oral mucosa samples from healthy Swedish volunteers.
4. Study the prevalence of HPV, HSV-1, and EBV in squamous cell carcinoma samples from eight different countries.
5. Identify genes that are transcriptionally down- and upregulated in squamous cell carcinoma samples and paired normal controls from Sudanese patients among toombak users and non-users.

Tissue samples used in our study were from different countries and different continents. These samples came from patients with oral squamous cell carcinoma and with oral leukoplakia with and without dysplasia, as well as from healthy Swedish volunteers. We used an agile method that our group had previously developed for DNA extraction from old formalin-fixed, paraffin-embedded biopsies. We also used an efficient and sensitive method called nested PCR to detect the level of viruses in both paraffin-embedded squamous cell carcinoma samples and fresh clinical specimens. We observed statistically significant differences in the integrated and episomal forms of the virus in leukoplakia and squamous cell carcinoma samples. A statistically significant difference was also found when we compared the prevalence of HPV-16 in the control group samples and in the leukoplakia and squamous cell carcinoma samples. Furthermore, in the material from Sweden, we found a high prevalence of HSV-1 in healthy oral mucosa, oral leukoplakia, and squamous cell carcinoma tissues. However, we could not show any correlation between the occurrence of HSV-1 virus and the development of squamous cell carcinoma. We found a higher proportion of HSV-1 and EBV in developed countries than in developing countries as well as higher co-infection with HSV-1 and EBV in developed countries. No difference in HPV prevalence was seen between developed and developing countries. Co-infection with two or more viruses therefore may be a risk factor in the development of oral squamous cell carcinoma. Investigation of genes related to apoptosis, cell-cycle regulation, and filament proteins also gave us insight into the understanding of the genetic processes involved in the development of oral squamous cell carcinoma.

Continued research is needed to obtain important information about virus infections and exposure to tobacco, alcohol, and toombak, which are factors that contribute to the development of oral tumours. Further research could elucidate the molecular mechanisms that underlie the development of squamous cell carcinoma. This could help to improve patient prognosis by advancing the development of new forms of treatment.
8. Populärvetenskaplig sammanfattning

Skivepitelcancer är den viktigaste typen av munhålecancer och utgör 90–95% av alla orala maligniteter. Användningen av tobak och/eller alkohol är den viktigaste riskfaktorn för denna cancerform och orsakar ca 66–90% av alla skivepitelcancer fall. Både epidemiologiska och molekylära data tyder på att vissa typer av humant papillomvirus (HPV), samt vissa medlemmar av Herpesvirus familjen, såsom Epstein-Barr-virus (EBV) och humant herpes simplex virus typ 1 (HSV-1), också kan ha cancerogen kapacitet. Multipla genetiska förändringar kan, i en flerstegsprocess, leda till skivepitelcancer. Dessa genetiska förändringar leder till funktionsförlust av tumörsuppressor gener och/eller uppreglering av protonkogener, vilket i sin tur avreglerar flera biologiska vägar för cell-proliferation, differentiering, och apoptos. Skillnaderna i expression, uttryck och/eller mutationer i ett antal gener som kodar för molekyler som deltar i cellcykels progression, tillväxtkontroll, apoptos, onkogenes, tumorsuppression, tillväxtfaktorer och cytokiner har rapporterats i skivepitelcancer.

I vilken grad virus infektioner kan bidra till ökad risk för cancer i munhålan i närvaro av alkohol, tobak och toombak och de molekylära mekanismer som är involverade i ett inledande skede är för närvarande oklart. De exakta molekylära förändringar som är av avgörande betydelse i patogenesen är också fortfarande i stor utsträckning okända. Vi studerade förekomsten av HPV, EBV och HSV-1 i orala premaligna och maligna lesioner. Vidare studerade vi genetiska mekanismer i skivepitelcancer genom att undersöka uttrycket av nio utvalda gener relaterade till apoptos, cellcykel reglering och filamentproteiner.

Fokus i denna avhandling ligger på följande delmål:

1. Studera förekomsten av HPV generellt och HPV-16 specifikt och dess integration i kliniskt frisk munslemhinna, oral leukoplaki, och skivepitelcancer.

2. Studera förekomsten av HSV-1 DNA genom nested PCR (NPCR) i kliniskt frisk munslemhinna, oral leukoplaki och skivepitelcancer.
3. Detektera och jämföra nivån på HPV-DNA, genom användning av singel och nested PCR i paraffininbäddade orala skivepitelcancer prover från Sudan och munslemhinneprover från friska svenska volontärs.

4. Studera förekomsten av HPV, HSV-1 och EBV i skivepitelcancer prover från åtta olika länder.

5. Identifiera gener som är ned- eller uppreglerade i oral skivepitelcancer och parvis normala kontroller från sudanesiska patienter bland toombak-användare och icke toombak -användare.

Vävnadsprover som användes vid våra studier var från olika länder och från olika kontinenter. Dessa bestod av patienter med oral skivepitelcancer, oral leukoplakia med och utan dysplasi samt prover från friska svenska volontärs. Vi använde oss av en smidig metod som vår grupp hade utvecklat tidigare för DNA extraktion från gamla formalin-fixerade paraffininbäddade biopsier. Vi använde oss dessutom av en effektiv och känslig metod så kallad nested PCR för att detektera nivån på virus både i paraffininbäddade skivepitelcancer och klinisk friska prover.

Vi observerade en statistisk signifikant ökning av integrerat HPV-16 i leukopakler och skivepitelcancer prover. En statistiskt signifikant skillnad konstaterades också när vi jämförde förekomsten av HPV-16 i kontrollgruppen jämfört och leukoplakier samt skivepitelcancerar. Vidare kunde vi påvisa i vårt material från Sverige en hög förekomst av HSV-1 i prover från frisk munstehinna, leukoplaki och skivepitelcancer vävnad. Dock kunde vi inte påvisa något samband mellan förekomst av HSV-1 och utvecklingen av skivepitelcancer. Vi hittade en högre andel med HSV-1 och EBV i industriländerna jämfört med utvecklingsländerna och även en högre co-infektion av HSV-1 och EBV i industriländer. Ingen skillnad i HPV prevalens sågs mellan industrieliserade länder och utvecklingsländer. Co-infektion av två eller flera virus kan vara en riskfaktor i utvecklingen av oral skivepitelcancer. Undersökning av gener relaterade till apoptos, cellcykel reglering och filamentproteiner gav oss också en inblick i förståelsen av genetiska processer som är involverade i utvecklingen av skivepitelcancer. Detta skulle kunna bidra till att förbättra prognos och i utarbetandet av nya former av behandlingar.

Fortsatta forskning behövs för att få viktig information om virusinfektion och exponering av tobak, alkohol och toombak som kan vara faktorer som kan bidra till utvecklingen av orala tumörer. Vidare vill vi få svar på de molekylära mekanismer som kan ligga till grund för utvecklingen av skivepitelcancer. Detta skulle kunna bidra till att förbättra prognos och i utarbetandet av nya former av behandlingar.
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