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Studies on the presence and influence of human papillomavirus (HPV) in head and neck tumors

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To my family

Above all powers, above all kings
Above all nature and all created things
Above all wisdom and all the ways of men
You were there before the world began

(L. LeBlanc, P. Baloché)

Abstract

The aim of this thesis was to confirm the presence of human papillomavirus (HPV) in tonsillar cancer and the previous finding that presence of HPV in tonsillar cancer is prognostic favorable for patient survival. Furthermore, the physical state and viral load of HPV in tonsillar cancer, and the influence of HPV on chromosomal patterns and on the clinical outcome for tonsillar cancer patients were examined. In addition, the presence of HPV in tongue cancer and influence of HPV on clinical outcome for tongue cancer patients were studied. Finally, the presence of HPV, viral load and proliferation rate were determined in recurrent respiratory papillomatosis in response to Interferon- α treatment.

It was shown that HPV was present in 62.2% of examined tonsillar cancer and HPV was shown to be a favorable prognostic factor. Furthermore, the viral genome was shown to be mainly episomal, hence integration of the viral genome was not a requisite for malignant transformation. Quantification of HPV-16 was performed by real-time quantitative PCR. The viral load was shown to vary greatly and patients with a higher viral load in their tumors had a significantly better disease specific survival than patients with a lower viral load.

The pattern of chromosomal aberrations in tonsillar cancer was examined by comparative genomic hybridization. Generally, more aberrations were seen in HPV negative tumors compared to HPV positive tumors. In addition, there were specific differences between HPV positive and HPV negative tonsillar cancers. Gain of chromosome 3q was statistically more common in the HPV positive versus negative tonsillar tumors, while gain of chromosome 7 was more common in HPV negative tumors. Survival could not be correlated to gain or loss of any specific chromosome since there were too few cases in each group, however, HPV was a prognostic favorable factor.

HPV was found in 40% of examined base of tongue cancer patients and patients with HPV positive tumors had a significantly better disease specific survival compared to patients with HPV negative tumors. In contrast, only 2.4% of examined mobile tongue cancer patients were HPV positive and hence presence of HPV could not be correlated to prognosis.

Finally, the presence of HPV, viral load and proliferation rate were determined in recurrent respiratory papillomatosis (RRP) in response to Interferon- α (IFN- α) treatment. The majority of the patients that were subjected to IFN- α therapy responded to the treatment. The rate of proliferation was generally high, and remained unchanged during IFN- α treatment. The viral load varied between the biopsies and both patients with a low, as well as a high, viral load responded to IFN- α treatment, indicating that the viral load is not a determinant for responsiveness.

List of publications

This thesis is based on the following original papers, which will be referred to in the text by their roman numeral.

- I. Mellin, H., **Dahlgren, L.**, Munck-Wikland, E., Lindholm, J., Rabbani, H., Kalantari, M. and Dalianis, T.: *Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer.*
International Journal of cancer (2002) 102:152-58
- II. **Dahlgren, L.**, Mellin, H., Wangsa, D., Heselmeyer-Haddad, K., Björnestål, L., Lindholm, J., Munck-Wikland, E., Auer, G., Ried, T. and Dalianis, T.: *Comparative genomic hybridization analysis of tonsillar cancer reveals a different pattern of genomic imbalances in human papillomavirus-positive and -negative tumors.*
International Journal of cancer (2003) 107:244-249
- III. **Dahlgren, L.**, Dahlstrand (Mellin), H., Lindquist, D., Högmo, A., Björnestål, L., Lindholm, J., Lundberg, B., Dalianis, T. and Munck-Wikland, E.: *Human papillomavirus is more common in base of tongue than in mobile tongue cancer and is a favorable prognostic factor in base of tongue cancer patients.*
International Journal of Cancer (2004) 112:1015-1019
- IV. **Dahlgren, L*.**, Szeps, M*, Aaltonen, M-L., Öhd, J., Kanter-Lewenshon, L., Dahlstrand, H., Munck-Wikland, E., Grandér, D. and Dalianis, T.: *Human papillomavirus (HPV), viral load and proliferation rate in recurrent respiratory papillomatosis in response to interferon alpha (IFN- α) treatment.*
Accepted for publication in Journal of General Virology
*shared first authorship

Abbreviations

aa	amino acids
ANCA	average number of chromosomal aberrations
bp	base pair(s)
BPV	bovine papillomavirus
BSA	bovine serum albumin
CDK	cyclin dependent kinases
CIN	cervical intraepithelial neoplasia
CR	complete remission
CGH	comparative genomic hybridization
DNA	deoxy nucleic acid
E	early
EBV	Epstein-Barr virus
EGF	epidermal growth factor
FISH	fluorescent in situ hybridization
GAG	glycosamino-glycan
HN	head and neck
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
IFN- α	interferon alpha
ISH	<i>in situ</i> hybridization
kb	kilobase
kDa	kilodalton
L	late
LCR	long control region
NES	nuclear export sequence
NR	no response
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PD	progressive disease
POD	promelytic oncogenic domains
PR	partial remission
pRb	retinoblastoma protein
<i>rl</i> iPCR	restriction enzyme, ligation, inverse PCR
RRP	recurrent respiratory papillomatosis
RT	radio therapy
SCC	squamous cell carcinoma
Taq	<i>Thermus aquaticus</i>
UICC	union internationale contre le cancer
VLP	virus like particle
Q-PCR	quantitative PCR

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1. Introduction

History

When reading the literature from early studies on warts it becomes clear that accidents, and experiments, which would not be performed today, based on their risky nature, are the foundation on which the knowledge of the infectious nature of warts is based. The first report confirming the infectious etiology of warts was published in 1845 by Chandler. He was removing a condyloma with a knife when he accidentally injured his assistant who later developed a wart on the injured site (cited in (Aaltonen et al., 2002, Ullmann, 1923)). Another similar story is reported by Ullman who to confirm an accidental transmission of laryngeal papillomas, inoculated himself with extracts from laryngeal papillomas. After a period of nine months, warts appeared on his forearm where he had applied the extract (Aaltonen et al., 2002, Ullmann, 1923). Other experiments, such as serial inoculations in humans (Kingerey, 1921) and observations based on hand and foot warts (cited in (Munger et al., 2004)) are also reported. In addition, in 1917 a paper was published regarding a study on the infectious nature of the agent causing genital warts, contradicting the notion that genital warts and cervical cancer were results of venereal diseases such as syphilis and gonorrhea ((Jördens, 1800, Waelsch, 1917) cited in (Munger et al., 2004)). Instead this study showed that genital warts are distinct diseases caused by a transmissible agent not necessarily related to other venereal diseases.

Tumor viruses

Approximately 15% of all human cancer is caused by tumor viruses (Wyke, 1999, zur Hausen, 1999b). Examples of viruses that cause cancer are presented in Table I. Viruses can cause tumors indirectly or directly when they adopt the host cell machinery in order to proliferate. Indirect ways involve e.g. suppression of the immune system in order to avoid the elimination of virally infected cells, host cell cycle activation by the excretion of growth factors, the mimicking of growth receptor ligands by virions, or the stimulation of immune cells resulting in e.g. lymphomas. Direct ways include the so-called “hit and run” mechanism and insertion of the viral genome into the host cell genome (mutagenic effect on the host genome). “Hit and run” comprise events where the virus initiates something that eventually leads to the development of a tumor growth, although the virus itself is not longer present in the affected cell. In addition, the viral genome may carry oncogenes which may, or may not be a part of the viral life cycle (Wyke, 1999). The studies of viral oncogenes have provided great knowledge, not only related to the virus, but also concerning what controls the normal cell cycle.

The first reports that viruses may cause tumors were published in 1908 and 1911 and described that filterable agents induced erythroblastosis in chickens (Ellermann, 1908) and sarcomas in fowls (Rous, 1911). In 1923 Ullman inoculated himself with laryngeal papilloma tissue (as mentioned above), and after a few months flat warts appeared on the inoculated site, demonstrating the viral etiology of papillomas

(Aaltonen et al., 2002, Ullmann, 1923). In the 1930s the first reports showing that virus induced carcinomas and papillomas in rabbits were published (Rous, 1935, Shope, 1933). Since then, it has been shown that papillomaviruses can cause warts and carcinomas in various hosts e.g. humans, rabbits and cattle.

Table I. Viruses associated with human tumors.

Virus	Associated human tumors
Human papillomavirus (HPV)	
HPV type 16, 18, 33 ¹	Cervical cancer, head & neck tumors
HPV type 6, 11	Condyloma acuminata
Polyomavirus	
Simian Virus 40 (SV40)	Mesothelioma
Hepatitis B	Liver cancer
Herpes viruses	
Epstein-Barr virus (EBV)	Nasopharyngeal carcinomas, Burkitt's lymphoma
Human Herpes virus 8 (HHV8)	Kaposi's sarcoma
Human T-cell leukemia virus (HTLV)	Adult T-cell leukemia
Hepatitis C	Liver cancer (indirect)

¹Other HPVs found in malignant lesions include types 31, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, and possibly a few others

Normal cell proliferation and apoptosis

In the normal cell, there is a highly regulated balance between cell proliferation and cell death (reviewed in (Ivanchuk & Rutka, 2004, Sandal, 2002, Sherr, 1996)). The cell cycle consists of two main steps; the doubling of the genome (S-phase) and the division into two daughter cells, a process called mitosis (M-phase) (Figure 1). Between these two steps are time gaps (the G₁ and G₂-phases) with check points to make sure that, before the cell continues to the next phase, the genome is replicated in a satisfactory way and that there is no damage to the DNA. The main check point in the transition between the G₁-S-phases is called the restriction point or “point of no return” and is controlled by cyclin dependent kinases (CDKs) that are regulated by cyclins. In the G₁-phase, growth factors, oncogenes, cyclins and CDKs stimulate the cell to grow in size, while tumor suppressor genes and CDK inhibitors are counterbalancing the pro-growth actions. The main biochemical basis for regulation of the components involved are phosphorylation, dephosphorylation and proteolytic degradation (Sandal, 2002).

The cyclin/CDK complexes are inhibited by two protein families; the Cip/Kip family (p21, p27 and p57) and the INK4a family (p15, p16 and p18) (Figure 1). To exit the G₁-phase, cyclin D-dependent kinases must phosphorylate their substrates and one of their targets is the Retinoblastoma protein (pRb). When pRb becomes phosphorylated it releases the transcription factor E2F, which is bound to promoters of genes involved in S-phase progression and transcription of these genes becomes activated (Longworth & Laimins, 2004).

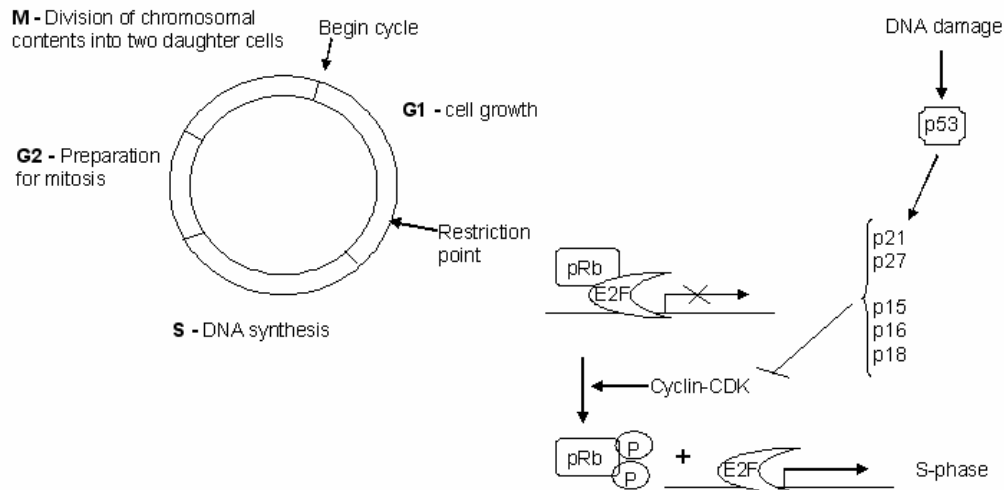


Figure 1. Phases in the normal cell cycle; G₁, S, G₂ and M. In the G₁-phase the cell grows and prepares the chromosomes for replication. In the S-phase DNA is synthesized, resulting in a duplication of the genome. The G₂-phase is for preparation for mitosis which occurs in the M-phase.

The pRb is a so-called tumor suppressor and together with the p53 tumor suppressor it is one of the most important cell cycle regulators. P53 is a DNA binding protein that determines cell fate through regulation of expression of genes involved in cell cycle arrest and apoptosis. P53 is present in low amount in the cell due to rapid degradation through a ubiquitin dependent mechanism. When the DNA becomes damaged, for instance through UV irradiation, p53 becomes stabilized and the result is either cell growth arrest (to repair the damaged DNA) or cell death (apoptosis). The p53 gene is mutated in more than 50% of human cancers leading to accumulation of genomic instability (Vogelstein et al., 2000).

Another control of cell proliferation is at the level of chromosome shortening. For each cell division the telomeres (i.e. chromosomal ends) become shorter until they reach a critical length beyond which the cell can no longer divide. To maintain the chromosomal ends over the critical threshold, the cellular telomerase complex adds hexanucleotide repeats onto the ends of telomeric DNA (Bryan & Cech, 1999). However, every cell has only the potential to divide a finite number of times and finally the chromosomes are too short in spite of the telomerase activity. The majority of malignant cells have an increased telomerase activity (Shay & Bacchetti, 1997) and it has been shown that this can lead to an infinite life span (Hanahan & Weinberg, 2000).

Hallmarks of cancer

In 2000 it was proposed (Hanahan & Weinberg, 2000) that in order for a normal cell to become a malignant cell, several criteria have to be fulfilled. The cell has to be self-sufficient in growth signals, insensitive to growth inhibitory signals, able to evade apoptosis, obtain limitless replicative potential, have sustained angiogenesis and be able to invade the surrounding tissue and metastasize. In addition to these hallmarks the ability to avoid the immune system has also been proposed as a critical feature for tumor development.

1.1 Basic virology – *Papillomaviridae*

Taxonomy and classification

The first evidence that it was not one, but different agents that caused the various clinical pictures that were observed when cell-free extracts from common warts were inoculated into humans, was presented by Gissmann and zur Hausen in 1976. They showed that different cleavage patterns could be obtained when HPV genomes are cleaved with restriction enzymes (Gissmann & Hausen, 1976).

HPV was first identified in 1949 (Strauss et al., 1949) and today, nearly 100 different HPV types have been characterized and several sub-genomic sequences are being considered as novel HPV types (de Villiers et al., 2004). The papillomavirus family is a separate family of viruses called the *Papillomaviridae*. The taxonomic classification of papillomaviruses is based on the traditional criteria of L1 open reading frame (ORF) sequence variation, which is the most well conserved gene within the genome. The taxonomic levels are; “family”, “genus”, “species”, “types”, “sub-types” and “variants”. The L1 ORF sequence should differ by at least 10% between “types”, with 2-10% between “sub-types” and with less than 2% between “variants”. The term “genus” is used for grouping of papillomaviruses with less than 60% nucleotide (nt) sequence identity, and often biologically diverse but phylogenetically related species are united in the same genus. Human papillomaviruses are divided into five different genera, (Alpha, Beta, Gamma, Mu and Nu) with some biological properties shared within the genera (de Villiers et al., 2004). For instance, in the Alpha-papillomavirus genus, 15 different species are found, which infect cutaneous or mucosal epithelia, causing benign or malignant lesions (Table II).

Table II. Papillomaviruses divided into genus, species and types.

Genus	Species	Types in species	Comment
Alpha ¹	7	18, 39, 45, 59, 68, 70	high risk ² , mucosal
	9	16, 31, 33, 35, 52, 58, 67	high risk, mucosal
	10	6, 11, 13, 44, 74	low risk ² , mucosal
Beta	1	5, 8, 12, 14, 19, 20, 21, 25, 36, 47	cutaneous/mucosal benign/malign

¹three of 15 species in the Alpha genus ²High risk HPV can induce malignant lesions while low risk HPVs are not associated with malignant lesions

Genomic organization

Papillomaviruses have relatively small genomes which has made them easy to study and their genomic structure was unraveled already in 1963 (Crawford & Crawford, 1963) (Figure 2). The genome consists of circular, double stranded DNA of approximately 8 kilo bases (kb) that associates with cellular histones to form chromatin like complexes (Howley, 1996). All papillomaviruses have the same basic genomic organization, with a long control region (LCR, 10% of the genome), an early transcription region (E, 50%) and a late transcription region (L, 40%). The genomes

have up to 10 ORF, which are found exclusively on one of the strands, and hence transcription occurs in one direction (zur Hausen, 1996).

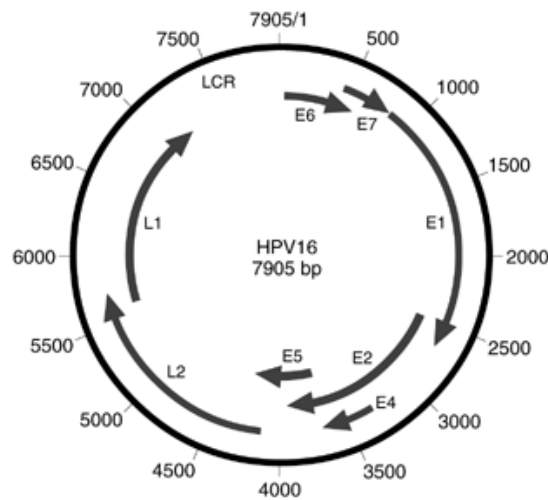


Figure 2. Genomic organization of HPV-16, with the three functionally determined regions; the long control region, the early region and the late region.

Viral capsid

Human papillomaviruses are small with a diameter of approximately 55 nm. The capsid is icosahedric in shape and lack an envelope, rendering the virus very stable and infectious for years, as well as resistant to heat, organic solvents and to many therapeutic agents (Bonnez et al., 1994, Nebesio et al., 2001). The major capsid protein L1 forms the viral capsid, which is built up by 72 capsomers while approximately 12 molecules of the minor capsid protein L2 bind to the inside of some of the capsomers (zur Hausen, 1996).

1.2 Epidemiology

Tissue tropism and transmission

Papillomaviruses infect a broad spectrum of vertebrates e.g. humans, rabbits and cattle. They are highly species restricted and infect the basal cells in either skin or mucosal epithelia where they may be latent and persist during long time without clinical manifestations. In humans, the single layer of proliferating basal cells can be accessed by HPV through micro wounds e.g. at squamo-columnar junctions such as the transformation zone in the cervix (Lowy, 2001). HPVs are suggested to be transmitted by direct contact, since they are shed by the infected host cell (Munger, 2002) (Figure 3), but the mechanism of viral transmission from person to person is not well understood. In normal epithelia, the cells become flattened and elongated, the nucleus is dissolved and a cornified cell envelope develops as the cells enter the stratum corneum (Bryan & Brown, 2001) (Figure 3). These cells are continuously shed from the epithelial surface. However, in the HPV infected epithelia, the nucleus is

retained in stratum corneum and the cornified cell envelope, which normally is very stable, is thin and fragile (Brown & Bryan, 2000). In order for HPV to infect a new host the envelope needs to be broken, which possible occurs through mechanical trauma. Anogenital infections are mainly transmitted through sexual contact, and there is a correlation between the number of sexual partners and HPV infection (zur Hausen, 1996).

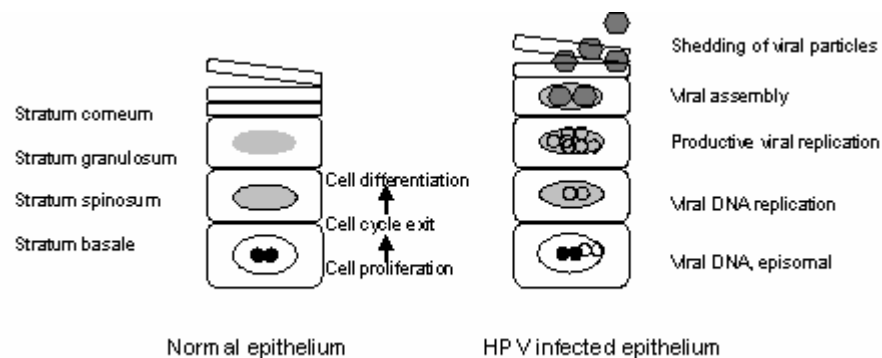


Figure 3. Schematic presentation of the normal and HPV infected epithelia. Cell proliferation, exit from the cell cycle and differentiation are indicated for the normal epithelia to the left and viral replication, productive viral replication, viral assembly and the shedding of viral particles are indicated for the HPV infected epithelium to the right.

1.3 Papilloma viral life cycle

Viral cell entry

Papillomavirus infection is highly restricted to basal cells in mucosal or epithelial cell layers. Little is known about the initial steps of papillomavirus uptake, due to lack of a good infectivity assay, which has led to dissonance on the specifics regarding viral cell entry. However, it has been shown that HPV interacts with molecules on the cell surface that are highly conserved within the animal kingdom, and that a protein component is involved in the binding (Muller et al., 1995, Qi et al., 1996, Volpers et al., 1995). In 1997 it was suggested that alpha-6-integrin constituted part of the receptor for HPV-6 (Evander et al., 1997), but this has since been questioned by others (Giroglou et al., 2001, Shafit-Keramat et al., 2003). Cell-surface glycosamino-glycans (GAGs) have been found to be of importance for initial attachment, and in several HPV types the L1 protein has been found to have a heparin binding region in its C-terminal (Joyce et al., 1999) supporting the theory. Others have shown that heparin, a ubiquitous polysaccharide, inhibits pseudo-infection of HPV-33 VLPs while heparan sulphate (similar to heparin but has a lower proportion of N-sulphation) is essential (Giroglou et al., 2001). Heparan sulphates are a group of membrane bound proteoglycans which can be divided into two main subgroups with cell-, tissue- and development specific expression; syndecans and glypicans. Syndecan-1 is the major heparan sulphate protein found on human epithelial cells and the expression is increased during epithelial differentiation and

wound healing. This heparan sulphate has been proposed to be a putative primary receptor protein for infection of keratinocytes (Shafti-Keramat et al., 2003).

Viral genome replication and regulation of gene expression

HPV replication occurs within the nucleus of the infected cell and is dependent on S-phase entry since it requires the cellular DNA machinery including DNA polymerase α /primase, DNA polymerase δ , replication protein A, PCNA and topoisomerases (Deng et al., 2004). The virus is present in a latent form in proliferating cells, while the productive large scale viral DNA replication, translation, functional activities of the late proteins and viral assembly are restricted to the differentiating layers of skin and mucosa (zur Hausen, 1996).

Regulation of viral gene expression is complex and involves both viral and host factors and is controlled by elements present in the LCR, and in some cases by elements within the gene itself. In the basal cells of the squamous epithelium low levels of early transcripts (E6, E7, E5, E1 and E2) are detected. These transcripts are initiated at the early promoter, located upstream of the E6 ORF, in the LCR. When the cell has divided, one daughter cell migrates towards the suprabasal regions (stratum spinosum and higher) and starts to differentiate. The uninfected cell leaves the cell cycle when it starts to differentiate, while the HPV infected cell re-enters the S-phase after reaching the suprabasal layer (Doorbar et al., 1997) (Figure 3), giving that both cell cycling and differentiation occur at the same time (McMurray, 2000). The entry into the S-phase initiates productive viral replication and results in a viral copy number of several thousands (Lambert, 1991). At this stage the late promoter is activated and the expression results in synthesis of the E1, E4, L1 and L2 proteins and assembly of viral particles (Fehrman & Laimins, 2003).

Viral genes and functions of viral proteins

The early ORFs (E1, E2, E4, E5, E6 and E7) encode for proteins involved in regulatory functions, DNA replication and activation of the lytic cycle, while the late ORFs (L1 and L2) encode for proteins that build up the viral capsid (Table III). The early genes E3 and E8 are found in some papillomaviruses, but not in HPVs.

E1

The E1 ORF is the largest and most highly conserved of all HPV ORFs and codes for a polycistronic RNA, which is translated into 68-85 kDa proteins with both ATPase and helicase activities (Hughes & Romanos, 1993). The E1 protein is expressed at low levels in HPV positive cells and has site-specific DNA binding sequences that bind, weakly, to the origin of replication and initiate DNA replication (reviewed in (Longworth & Laimins, 2004, zur Hausen, 1996)). DNA binding is stabilized by complex formation with the E2 protein (Longworth & Laimins, 2004) and once bound, E1 forms hexamers with high binding affinity for DNA (Sedman & Stenlund, 1998). Without E1, there would be no viral DNA replication, since E1 is

essential for its initiation (Wilson et al., 2002). The helicase activity of E1 allows for separation of viral DNA strands ahead of the replication complex (Hughes & Romanos, 1993). E1 is suggested to be regulated through interactions with cyclin A and cyclin E by CDK mediated phosphorylation (Ma et al., 1999), resulting in inactivation of the E1 nuclear export sequence (NES), keeping E1 in the nucleus (Deng et al., 2004), where it can initiate viral DNA replication.

E2

The E2 ORF codes for 2-3 proteins all acting as transcription factors (reviewed in (zur Hausen, 1996)). These proteins have a DNA binding region in their C-terminal and regulate viral transcription and replication (Syrjänen, 1999) by forming dimers at specific binding sites. The E2 protein is essential for viral replication, since it directs E1 to its DNA binding sites and enhances the binding affinity of E1 to DNA.

It is suggested that E1 and E2 control the copy number of episomal HPV copies/cell since the number of HPV copies increases with increased E1 or E2 expression (McMurray, 2000). In addition, the E2 protein is involved in the assembly of DNA containing virions since it provides high affinity binding to specific regions in the viral genome. The E2 ORF is frequently disrupted when the HPV genome is integrated. This leads to a more malignant phenotype as seen in cervical biopsies and cell lines derived from cervical cancer (zur Hausen, 1996) and has been suggested to be due to the loss of the E2 repression of the E6 and E7 transforming proteins (Syrjänen, 1999).

E4

The E4 ORF is expressed in low amounts early in the viral life cycle, however upon epithelial cell differentiation the expression of E4 becomes high and E4 is the most highly expressed HPV protein (Howley, 1996). The E4 ORF is translated from spliced transcripts together with the five first amino acids of E1, resulting in an E1^{E4} fusion protein, where the E1 sequence is used for initiation of translation (Howley, 1996). E4 proteins are exclusively found in the differentiating layer of the infected epithelium (zur Hausen, 1996). High risk HPV E4 is suggested to be involved in facilitating release of viral particles (Longworth & Laimins, 2004), since E4 interacts with the keratin networks and causes their collapse (Doorbar et al., 1991, Sterling et al., 1993). Furthermore, E4 may play a role in regulating gene expression and has been shown to induce G₂ arrest in a variety of cell types (Longworth & Laimins, 2004).

E5

The E5 ORF codes for a small highly hydrophobic membrane bound protein which is primarily expressed late in the viral life cycle, in differentiated epithelial cells (Longworth & Laimins, 2004). The E5 protein of high risk HPVs has weak transforming activities (Fehrman & Laimins, 2003, Longworth & Laimins, 2004), while the corresponding E5 protein in bovine papillomavirus (BPV) is the major transforming protein (Burkhardt et al., 1987). BPV E5 and HPV E5 do not share

sequence homology and while BPV E5 acts through interactions with the platelet-derived growth factor (PDGF) receptor the HPV E5 is proposed to interact with the epidermal growth factor (EGF) receptor. In HPV E5 over expressing cells, increased numbers as well as increased phosphorylation of EGF receptors are seen. This is due to impaired degradation of receptors and to recycling of the receptors to the cell membrane (Straight et al., 1993).

Table III. HPV proteins

Protein	Size	Interactions and functions	Cellular location	Expressed in
Early				
E1	68-85 kDa	DNA binding ATPase and helicase activities Initiation of viral replication	Nucleus	Basal and differentiating epithelial cells
E2	43-48 kDa	DNA binding Regulation of viral transcription and replication	Nucleus	Basal and differentiating epithelial cells
E4	17-16 kDa	Interacts with keratin networks Maturation and release of viral particles Regulating gene expression, G ₂ arrest	Cytoplasm	Differentiating epithelial cells
E5	44-91 aa	Interacts with EGF receptor	Golgi apparatus, ER ¹ and plasma membrane	Differentiated epithelial cells
E6	16-18 kDa	Interacts with E6-AP Gene transcription Prolonging of cellular lifespan Up-regulation of telomerase activity	Nuclear matrix	Basal and differentiating epithelial cells
E7	14-21kDa	Interacts with pRb proteins, cyclin A and E, p21, p27 and HDACs ² Induces cellular proliferation, Immortalization and transformation	Nucleus	Basal and differentiating epithelial cells
Late				
L1	55 kDa	Self-assembles into viral particles Large capsid protein, 360 copies/viral particle	Nucleus	Terminally differentiated epithelial cells
L2	76-78 kDa	Interacts with L1 and E2 Small capsid protein, 12 copies/viral particle Relocalizes L1 and E2 to PODs	Nucleus	Terminally differentiated epithelial cells

¹ER, endoplasmic reticulum, ²HDACs, histone deacetylases

E6

The E6 ORF codes for a basic protein with two zinc-binding motifs in the C-terminal (Barbosa et al., 1989, Grossman & Laimins, 1989, Kanda et al., 1991). The E6 protein of high risk HPVs is classified as an oncoprotein and can transform human mammary cells (Kiyono, 1998, Liu et al., 1999) and cooperate with E7 in transforming primary human foreskin keratinocytes. E6 is expressed early after viral infection and

facilitates viral production by conferring several cellular changes, and by prolonging of cellular lifespan through blocking of apoptosis and by increasing telomerase activity. HPV E6 interacts with a ubiquitin ligase, the E6 associated protein (E6-AP), and forms complexes with p53, which is then degraded through ubiquitin dependent mechanisms (Huibregtse et al., 1995, Scheffner et al., 1990, Werness et al., 1990). Moreover, E6 can block translocation of p53 into the nucleus (Mantovani & Banks, 1999) and thereby inhibit the gene regulatory functions of p53. The consequence of p53 degradation and blocking of p53 transport into the nucleus is disruption of p53 mediated cell cycle control and the cell can continue to divide in spite of DNA damage.

E7

The E7 ORF codes for an acidic phosphoprotein with two zinc-binding motifs in the C-terminal half of the protein (Syrjänen, 1999), which are essential for proper protein folding and stability. The E7 protein is primarily found in the nucleus and has been shown to induce cellular proliferation, immortalization and transformation (McMurray, 2000). The high risk HPV E7 confers the major transforming activities and can immortalize human keratinocytes, by interactions with factors involved in the regulation of cell growth (Halbert et al., 1991). One of these interactions is with the proteins of the Rb family (Dyson et al., 1989). Binding of E7 to pRb results in release of the E2F transcription factor, leading to activation of gene transcription (Longworth & Laimins, 2004). In addition, high risk HPV E7 mediates degradation of pRb (Wang et al., 2001). The ability of E7 to bind pRb is shared between the high and low risk HPVs, although the interaction between low risk HPV E7 and pRb is much weaker (Ciccolini et al., 1994, Heck et al., 1992). Furthermore, high risk HPV E7 interacts with cyclin A and cyclin E and increases the levels of these proteins. In addition, E7 associates with inhibitors of CDKs; p21 and p27, blocking their activity (Longworth & Laimins, 2004). E7 proteins also interact with the histone deacetylases (HDACs) which are normally recruited to the E2F inducible promoters by pRb (Longworth & Laimins, 2004) and represses the E2F function.

L1

The L1 ORF is highly conserved between different HPV types and is only expressed in terminally differentiated epithelial cells (Howley, 1996). It codes for the major capsid protein, which is present in 360 copies per virion. The L1 protein self-assembles into pentamers, which are the building blocks of the viral capsid and they can also self-assemble into virus-like particles (VLP), but L1 does not bind DNA and therefore L1 VLPs are generally devoid of DNA (Zhou et al., 1994).

L2

The L2 ORF codes for the minor capsid protein, present in around 12 copies per virion. Expression of L2 protein is restricted to terminally differentiated cells of the epithelium (Howley, 1996). The L2 protein is highly phosphorylated and suggested to be required for encapsidation of viral DNA into the capsid (Zhao et al., 1999, Zhou et al., 1993), and proposed to act through relocation of the L1 protein to

subnuclear domains called promyelocytic oncogenic domains (PODs) (Day et al., 1998). In addition, the L2 protein also directs the E2 protein to the PODs and thus facilitates viral assembly and viral genome packaging (Day et al., 1998). Together L2 and L1 proteins can self-assemble into VLPs.

Escape from cell cycle control

When the epithelial cell starts to differentiate the cell proliferation machinery is turned off, a process regulated mainly through the Rb family proteins (Longworth & Laimins, 2004). Since some of the viral ORF is not expressed until the host cell starts to differentiate, it is essential for viral production that the cellular proliferation machinery is running in parallel with the differentiation program. To achieve this, some viral gene products interfere with the cell cycle to ensure viral DNA replication. For instance, high risk HPV E7 directly competes with the E2F for pRb binding, which leads to more unbound E2F and transcription of its target genes (Figure 4). In addition, E7 is proposed to interact and inhibit cyclin A and cyclin E, which are the main cyclins in the late G₁-phase and in the S-phase. HPV E6 interacts with a ubiquitin ligase, the E6-AP, and forms complexes with p53, which is then degraded more rapidly (Huibregtse et al., 1995, Scheffner et al., 1990, Werness et al., 1990). Moreover, the gene regulatory functions of p53 can be blocked by inhibition of translocation of p53 into the nucleus (Mantovani & Banks, 1999). In these various ways p53 mediated cell cycle control is disturbed and the cell continues to divide in spite of DNA damage (Figure 4). Finally, E6 also confers prolonged life span by up-regulation of the telomerase activity.

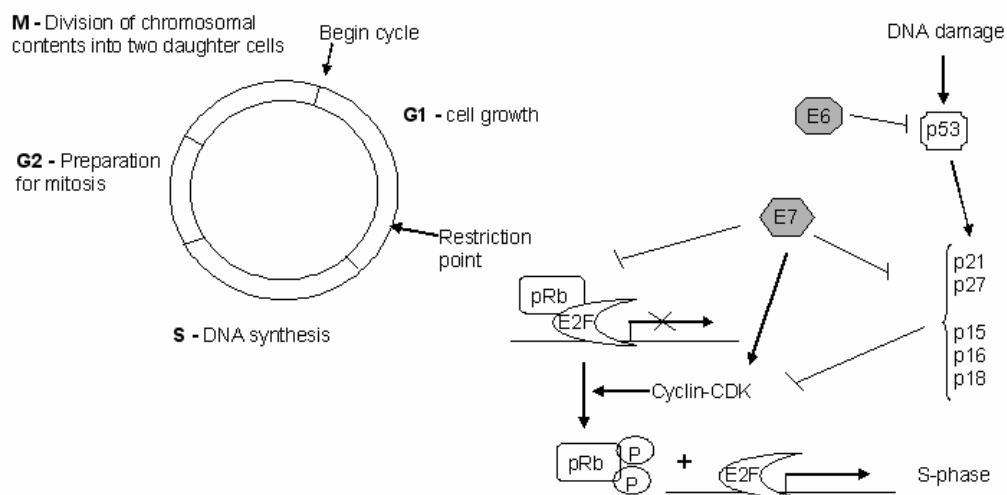


Figure 4. High risk HPV proteins E6 and E7 disrupt the tightly controlled cell cycle check points in order to keep the cellular proliferation machinery running in parallel to the differentiation program.

1.4 Pathogenesis

Association of HPV with human diseases

Human papillomaviruses are divided into two groups based on their potential to cause malignancies. The so-called *high risk* HPV types have been found to be associated with human tumors, of which cervical carcinoma is the most well studied. The so-called *low risk* HPV types are not associated with malignancies but might cause benign hyperplastic lesions e.g. warts (Table II).

Non-malignant

Hand and foot warts and Condyloma Acuminata

Low risk HPVs can potentially cause warts on hands and feet, as well as in the genital tract. Hand warts are often caused by HPV-2 and -4, while warts on the feet (plantar warts) are caused by HPV-1 and -63 (zur Hausen, 1996). Warts are spread through contact and it is not uncommon that children and teenagers have plantar warts contracted through sharing of public changing rooms and showers. These benign hyperplastic lesions do not undergo malignant transformation and the HPV infection causing the warts is often cleared within a few years.

Genital warts (i.e. condyloma acuminata- one of the most common sexually transmitted diseases) are caused by HPV-6 and -11 and can occur in the genital areas of both men and women including the vagina, cervix, vulva, penis, and rectum. They spread through skin-to-skin contact in a very contagious manner and the number of infected persons is high, although many do not have any symptoms.

Respiratory papillomatosis

Human papillomaviruses can also cause benign warts in the upper airways, i.e. recurrent respiratory papillomatosis (RRP), most commonly located in the larynx (Aaltonen et al., 2002). On-set of RRP has two age distributions; the disease affects children (juvenile papillomatosis) or adults (adult on-set papillomatosis). The low risk HPV-6 and -11 are the main cause of this rare disease (Mounts et al., 1982) but the route of transmission is not fully understood.

Malignant

High risk HPVs can cause lesions that may undergo malignant transformation a long time after the initial infection. These lesions are generally found in the genital tract (cervix, vulva and anus), in skin, (non-melanoma skin cancer) and in the head and neck (oropharynx). Several HPV types have been found in malignant lesions, and the high risk HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, and possibly a few others.

Integration of the viral genome into the host DNA causes loss of some viral genes leading to non-productive infection, i.e. no viral particles are produced (Munger, 2002), however viral RNA is often detected in the tumors. The E2 ORF is often deleted at integration leading to a deregulated expression of E6 and E7 genes and to

a more malignant phenotype (Munger, 2002). Integration does not occur at a specific site in the human genome, but from the viral point of view, there is a specific pattern of gene deletions and retentions. The minimal region that is retained after viral integration includes the E6, E7 and the LCR.

Cervical carcinomas

The most common lesion that is at risk of malignant transformation is the cervical intraepithelial neoplasia (CIN), a pre-malignant lesion that may convert into cervical carcinoma. In 1983, HPV-16 was isolated from cervical cancer (Durst et al., 1983) and since then high risk HPV types have been found in almost all cases of cervical carcinoma. The etiologic link between HPV and cervical cancer is strong, and HPV seems to be the cause of almost all cases of cervical cancer around the world (Gillison & Shah, 2003). According to the WHO, the prevalence of HPV infections in the world is around 630 million of which 190 million are clinical infections leading to 510,000 new diagnoses of cervical carcinoma each year. Cervical carcinoma is the second largest cause of death in cancer for women, with 288,000 reported deaths annually (2005). In the 1960's cytological screening programs for cervical dysplasia were introduced in many countries. Cytological specimens (exfoliated cells) from the cervix are examined by light microscopy and possible morphologic changes are recorded according to the degree of dysplasia. CIN lesions are divided into three groups, CIN I-III, where a higher number indicates a higher degree of dysplasia. These programs have led to early detection and decreased mortality of cervical neoplasia.

Upper aerodigestive tract

Human papillomavirus has been suggested to be involved in the development of some cancer of the upper aerodigestive tract, i.e. the oral cavity, nasopharynx, pharynx, esophagus and the larynx. In the year 2000, the worldwide 5 year prevalence for these cancers was predicted to be 2,001,400 (Parkin et al., 2001), and the mortality was around 194,000. The main etiologic agents for tumor development in the upper aerodigestive tract are smoking and alcohol consumption (Sturgis et al., 2004). However, HPV is suggested to be involved in some of the tumors especially in the oropharynx and the oral cavity.

Diagnostics

Two main approaches are used for diagnosis of HPV infection; direct detection of HPV DNA or detection of specific antibodies against HPV i.e. serologic testing. HPV DNA can be detected using several methods varying in their specificity and in requirements of DNA amount. Three commonly used techniques are hybrid capture, *in situ* hybridization (ISH) and polymerase chain reaction (PCR), where PCR is the most sensitive method.

The hybrid capture method (Brown et al., 1993, Lörincz, 1992) is commercially available and targets high risk versus low risk HPV types, with hybridization of non-radioactive RNA probes to the HPV DNA present in the sample. After hybridization

the RNA-DNA hybrids are captured in monoclonal antibody coated tubes and detected by chemiluminescent.

In situ hybridization (ISH) is commonly used since it in addition to detection of HPV also localizes the viral DNA within the analyzed tissues as well as within the analyzed cells (cytoplasm vs. nucleus). Archival paraffin embedded material can be analyzed using ISH (Unger, 2000, Unger et al., 1998). The tissue slides are denatured and hybridized with probes against either high risk or low risk HPV types and detected by microscopy.

PCR is the most sensitive technique for detection of viral DNA (Dabic et al., 2004). The primers used in the PCR determine the spectrum of detected HPV types. General primers can be designed against sequences in the viral genome that are conserved between the species (e.g. L1), and hence the PCR reaction detects a wide range of HPV types. These types of primers are often used for screening purposes. On the other hand, if the primers are designed towards highly specific sequences in the respective HPV type genomes, the PCR only detects one single HPV type and can be used for typing of HPV positive samples.

The advantages of PCR techniques are multiple. The design of primers allows for highly adaptable protocols depending on the aim of the study. PCR can be performed in a variety of materials, fresh samples, frozen samples, cell cultures, and even in formalin fixed and paraffin embedded samples. Furthermore, the needed amount of DNA is quite low since the amplification provides high sensitivity. The high sensitivity is also one of the major problems with PCR protocols; since the risk for contamination is high one needs to be extra careful in avoiding contact between pre- and post PCR DNA.

Serological detection of HPV can be indicative of an on-going HPV infection but can also give information regarding a previous, cleared infection. However, detection of HPV specific antibodies does not give any information regarding the site of the HPV lesion. In addition, not all HPV infected individuals seroconvert, and this leads to false negative results.

Pathology and histopathology

It appears that individual virus types induce a somewhat specific histopathology and differ in their growth-stimulating potential (Zur Hausen 1996). Benign HPV types induce lesions characterized by hyperplasia, parakeratosis and papillomatosis. The differences in these features vary between HPV types. High risk HPV types can potentially induce lesions with intraepithelial neoplasia characterized by disorganized architecture of the epithelia, abnormal mitotic figures and nuclear atypia. These lesions are graded depending on how much of the epithelia that are affected. In addition, in HPV infected cells halos appear around the nucleus, a phenomenon that is called koilocytosis.

Immune response

Papillomavirus infection does not usually induce a strong immune response. However, HPV infections are to a high degree cleared within a couple of years, and 18 months after infection, 80% have cleared the infection (Konya & Dillner, 2001).

How infected individuals clear the infection is not fully understood, but the host immune systems and in particular the cell-based response is suggested to be involved. Antibodies against HPV are type specific and might be effective in prevention of infection, while T-cell responses are involved in the clearance of established infections (Wang & Hildesheim, 2003). The serum antibody levels in humans are stable over time and are used as markers of HPV infection, even though not all HPV infected individuals have been shown to seroconvert (Konya & Dillner, 2001).

Prevention and vaccination

The incidence and mortality of cervical cancer have decreased in countries where a program exists for screening of HPV infections in women, i.e. the Papanicolaou test (Ponten et al., 1995). However, these tests are expensive and the world wide frequency of HPV induced diseases is still high. Since HPV is mainly transmitted through sexual contacts, with a 4% increased risk for each change of sexual partner (Dillner et al., 1996), information campaigns might lead to a decrease in the number of infections. However, HPV can possibly also be transmitted through other routes e.g. vertically from mother to child (Quick et al., 1980, Silverberg et al., 2003). In order to decrease the world-wide incidence and mortality of HPV induced diseases two kinds of vaccines are currently being developed; prophylactic vaccines for prevention of HPV infection and therapeutic vaccines for clearance of HPV infection and regression of HPV induced lesions.

Prophylactic vaccines are based on the type specific activity of neutralizing antibody responses (Konya & Dillner, 2001), and vaccines based on noninfectious VLPs built up by the L1 protein have provided prevention of HPV infection in animal models (Breitburd & Coursaget, 1999). In humans, VLPs have been used to vaccinate against HPV infection and hopefully vaccination will prevent development of HPV induced neoplasia (Harper et al., 2004, Koutsky et al., 2002). These VLP-vaccines have been shown to induce a strong immune response with type-specific antibodies against epitopes in the L1 protein (Harro et al., 2001). In addition, an L1 specific T-cell response has also been observed after vaccination with L1 based VLPs (Pinto et al., 2003).

In addition to the prophylactic vaccines, there is a need for therapeutic vaccines for treatment of already established HPV induced lesions. Therapeutic vaccines are based on the induction of a cellular immune response against HPV proteins expressed in tumor cells, and the aim is to promote regression of HPV induced lesions. Several protocols are under investigation for their effectiveness in therapeutic vaccination, however, several technical difficulties have to be resolved (Breitburd & Coursaget, 1999). Consistently expressed HPV proteins e.g. the products of the early genes E6 and E7 are potential target antigens for therapeutic vaccines. Expression of these genes are required for malignant maintenance and expressed already in pre-malignant lesions (zur Hausen, 1999a).

1.5 Head and neck tumors

Squamous cell carcinoma

Anatomy

The head and neck (HN) includes several regions; the nasopharynx, the lip and oral cavity (including floor of the mouth, tongue, buccal mucosa, gingival and the hard palate) the salivary glands, the oropharynx (including base of tongue, tonsillar region, soft palate and pharyngeal walls), the hypopharynx, the larynx, the nasal cavity and paranasal sinuses (Figure 5). The esophagus is often regarded as a separate entity. Tumors can arise at any of these sites.

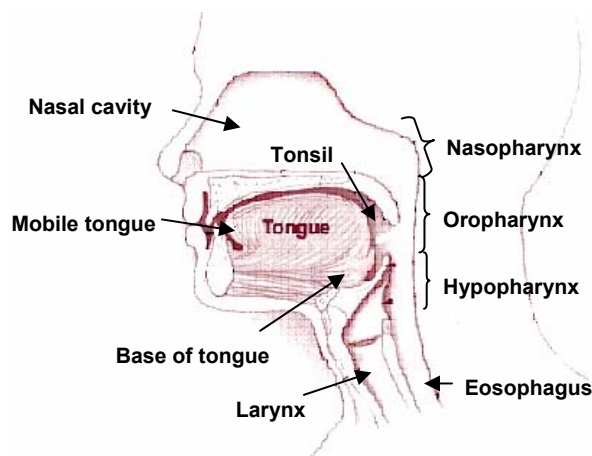


Figure 5. The head and neck region includes the nasopharynx, the oral cavity, the salivary glands, the oropharynx, the hypopharynx, the larynx, the nasal cavity and paranasal sinuses.

Tumors with different histological origin can arise in the HN region but the most common type of cancer is the squamous cell carcinoma (SCC) (Braakhuis et al., 2002). Other types of HN cancer are adenocarcinomas, lymphoepitheliomas, spindle cell carcinomas, verrucous cancers, and lymphomas. In addition, a variety of benign tumors may be found in the HN region out of which respiratory papillomatosis is a special entity.

Epidemiology

In 2000 it was estimated that the global incidence rate of HN cancer (including the esophagus) would in 2005 be 2,001,400, with 542,700 cases in females and 1,458,700 cases in males (Parkin et al., 2001). However, the frequency of HN cancer varies considerably over the world. Countries such as India and the Latin countries in Europe have a high incidence rate while the Scandinavian countries have a low incidence rate. In Sweden, HN cancer accounts for approximately 3-4% of all cancer cases with approximately 1,000 new cases each year.

The incidence of HNSCC increases with age, with most cases diagnosed in patients over 40 years of age (Burzynski et al., 1992).

Etiology/Risk factors

Known risk factors for HN cancer are tobacco (smoking or chewing) and alcohol use, separately or in combination. The association between smoking and HNSCC has been demonstrated in several case-control studies and odds ratios in the 3-12 fold range have been shown (Sturgis et al., 2004). Furthermore, long time exposure to environmental tobacco smoke has been shown to give a 2 fold increased risk of developing HNSCC (Tan et al., 1997, Zhang et al., 2000). Leukoplakias, white patches that can be observed in the oral mucosa are also considered to be a risk factor for cancer development, since such lesions may progress to cancer. Furthermore, dietary factors have been shown to be of importance for the development of oral and pharyngeal cancers (Levi et al., 1998). There are no indications of any hereditary causes of cancer in the HN region, however, since not all smokers develop HNSCC, there could be a genetic susceptibility involved in the etiology of HNSCC. This variability might depend on dysfunctions in DNA repair systems, cell cycle control or apoptotic pathways that influence their sensitivity to tobacco (Sturgis et al., 2004).

Viral agents have also been proposed to be involved in the development of some HNSCC. Epstein-Barr virus (EBV) is associated with nasopharyngeal cancer and during the last decades HPV has been found to play a role of in the development of HNSCC (Sturgis et al., 2004). The data supporting high risk HPV-16 as a causative agent of oropharyngeal carcinomas are mounting (Dahlstrom et al., 2003, Frisch & Biggar, 1999, Gillison et al., 2000, McKaig et al., 1998, Mellin et al., 2000, Mork et al., 2001, Schwartz et al., 1998, Sturgis et al., 2004).

The risk of a second primary tumor is high (15-30%) for patients who have suffered from a HNSCC tumor and the prognosis is worse the second time (Braakhuis et al., 2002, Nathanson, 1999).

Symptoms

Various symptoms can be signs of a tumor in the HN region; a persistent pain in the throat, pain or difficulty in swallowing, persistent hoarseness or a change in voice, pain in the ear or otosalginitis. Other possible symptoms include; a lump or thickening of soft tissue, a mucosal lesion which does not heal, bleeding from the mouth or throat or poorly fitting dentures. In addition, a lump on the throat can be a sign of a neck node metastasis from a HN cancer and the first symptom.

Tumor histopathology

Except for salivary gland tumors, the majority of the malignant HN tumors are SCC. The histopathological grade of differentiation for SCC depends on nuclear polymorphism, mitoses, degree of keratinization and tissue architecture. The grades are; well-, moderately-, poorly- and undifferentiated. In well-differentiated tumors the cells resemble that of normal tissue, while the resemblance is lower in moderately differentiated tumors, and even lower in poorly differentiated, and undifferentiated tumors.

Tumor classification and staging

There are different tumor classification systems but the Union Internationale Contre le Cancer (UICC) system is most widely used in Europe. Clinical findings prior to treatment constitute the basis for the so-called TNM classification system. The tumor is described and classified in regard to size and extension of the primary tumor (T), degree of regional lymph node involvement (N) and the presence or absence of distant metastasis (M) (Table IV).

Table IV. UICC TNM classification system for oral and oropharyngeal tumors.

T/N/M	Definition
TX	primary tumor size can not be defined
T0	no primary tumor is found
Tis	carcinoma in situ
T1	primary tumor less than 2 cm
T2	primary tumor between 2 and 4 cm
T3	primary tumor larger than 4 cm
T4	invasion of bone or deep infiltration in e.g. skin or muscle
NX	lymph nodal status can not be defined
N0	no signs of regional lymph node metastasis
N1	one ipsilateral lymph node metastasis less than 3 cm
N2a	one ipsilateral lymph node metastasis larger than 3 cm
N2b	multiple ipsilateral lymph node metastases all less than 6 cm
N2c	bilateral or contralateral lymph node metastases less than 6 cm
N3	regional lymph node metastases larger than 6 cm
MX	distant metastasis status can not be defined
M0	no sign of distant metastasis
M1	presence of distant metastasis

The T, N and M are combined in stages (se Table V) and together with the morphological characteristics they lay the foundations for decisions regarding therapy.

Table V. Tumor stage classification

TNM stage	T	N	M
stage 0	Tis	N0	M0
stage I	T1	N0	M0
stage II	T2	N0	M0
stage III	T1-T2	N1	M0
	T3	N0-N1	M0
stage IVa	T4	N0-N1	M0
	any T	N2	
stage IVb	any T	N3	M0
Stage IVc	any T	any N	M1

Molecular genetic findings

Accumulation of genetic alterations is one of the main features of tumor cells. This has been shown with e.g. the comparative genomic hybridization technique (Gebhart

& Liehr, 2000, Struski et al., 2002). Multiple genetic changes are involved in the development of HNSCC and the karyotypes observed in HNSCC are among the most complex described so far in solid tumors (Gebhart & Liehr, 2000, Kim & Califano, 2004, Struski et al., 2002) (Table VI). Genetic alterations may lead to two common features of tumor cells; inactivation of tumor suppressors and activation of proto-oncogenes. Examples of tumor suppressor genes that are frequently inactivated in HN cancer are p16/p14^{ARF} and p53 (Scully et al., 2000). In addition, regions on chromosome 3p which harbors genes responsible for tumor suppressor activities have been suggested to be inactivated in more than 50% of all HN cancer (Gebhart & Liehr, 2000) and it is seen early in the precancerous lesions (Califano et al., 1996). The epidermal growth factor (EGF) influences several cellular processes for instance cell division, differentiation and apoptosis and it has been shown that the level of EGF expression influences prognosis (Kim & Califano, 2004). Furthermore, the over-expression of EGF seems to be correlated with increased severity of the pre-malignant lesion. The most commonly gained chromosomal region in HN cancer is found on 3q. More than 50% of all HN tumors have been shown to have gained copy numbers in parts of 3q (Gebhart & Liehr, 2000). Amplification of 11q13 is seen in 40% of HNSCC cancer. Several putative proto-oncogenes are found in this region, for instance the cyclin D1, which is involved in the regulation of pRb (Figure 1) and has been shown to be inversely proportional to HPV infection (Wilczynski et al., 1998).

Table VI. Genetic alterations commonly seen in HN cancer

Location/gene	Frequency (%) and type of alteration
Tumor suppressor genes	
9p21-22	70% loss of heterozygosity
p16/p14 ^{ARF}	66% mutated and 21% hypermethylated
3p	60% loss of heterozygosity
17p13	
p53	50-79% mutated
Oncogenes	
4q25	
EGF	90% alteration of pathway via over-expression and other mechanisms
17q11	
Her2/neu	40% over-expression
11q13	
cyclin D1	40% amplification

Treatment

Curative treatment for a HN cancer aims at elimination of the disease and the treatment options are radiotherapy (RT) and surgery. These modalities can be used alone, in combination, or with chemotherapy. For palliative treatment, the options are the same, but often used less extensively and the treatment is aiming at reducing pain, discomfort, anxiety and stress.

RT can be given in combination with surgery, either pre- or post-operatively. There are no clear survival benefits with the pre- vs. post-operative RT treatment,

and there are advantages with both methods. Radiotherapy depends on good oxygen supply, and while pre-operative RT treatment has the advantage of a better blood flow, the circulation after surgery is hampered and higher doses are required for post-operative RT treatment. On the other hand, if RT is given post-operatively, the surgeon has the advantage of working in a non-radiated tissue. In cases where surgery is not possible or refused by the patient, RT can be given as sole treatment. Radiotherapy can result in either no sign of the tumor, i.e. complete response (CR), >50% reduction of the tumor, i.e. partial response (PR), <50% reduction of the tumor, i.e. no response (NR), or tumor progression during treatment, i.e. progressive disease (PD).

Various side effects are seen as a result of HN cancer treatment. Radiotherapy may cause mucositis, dry mouth, loss of taste, difficulties in swallowing and esophageal strictures. In addition, surgery may cause disfiguring cosmetics, impaired swallowing and neurological sequelae.

If cure is not possible to achieve, palliative treatment is given to ease the discomfort for the patient. Surgery, RT and chemotherapy can all be given in palliative modalities to decrease the tumor burden aiming at decreased discomfort since tumors in the HN region may affect eating, speaking and are disfiguring. Pain and anxiety can be medically treated.

Prognosis

There have been improvements in the treatment of HNSCC cancer during the last years, leading to a decreased morbidity and increased quality-of-life. However, the 5-year survival rate has remained unchanged at approximately 30-40%. For oropharyngeal cancer the overall 5-year survival is around 25%. Most recurrences (80%) occur within 1.5 years (Mellin et al., 2000, Nathanson, 1999).

The prognosis for patients with HNSCC cancer depends mainly on the tumor stage at the time of diagnosis. For oral cavity cancer patients with stage I-II tumors the survival is 60-80% after five years, while for patients with stage III-IV tumors the 5-year survival is 20-40%. Tumor spread to regional lymph nodes gives a 50% reduction in survival (Nathanson, 1999). For patients with oropharyngeal cancer survival rate is 60-70% after five years for tumors in stage I-II, and 10-20% for patients with stage III-IV tumors. If the tumor spreads to the local lymph nodes, survival decreases with approximately 20% (Nathanson, 1999).

Prevention

The majority of HNSCC is related to tobacco and alcohol use and hence the best way to prevent HNSCC would be to educate the general population about the risks and to help them stop smoking. Furthermore, identifying individuals with inherited dysfunction in their DNA repair systems, cell cycle control and apoptotic pathways rendering them sensitive to tobacco induced carcinogenesis would have a great impact on prevention as well as early detection (Sturgis et al., 2004). In addition, prevention of HPV induced HNSCC would include changes in sexual behaviour since each alteration of sexual partner increases the risk for HPV infection (Dillner et al., 1996, Schwartz et al., 1998, Smith et al., 2004).

Vaccines against HPV induced tumors have been developed by pharmaceutical companies and different vaccines are currently being evaluated. If these vaccines succeed in prevention of HPV induced warts and carcinoma of the genital tract, it would also be of interest to evaluate their effect on HPV induced HNSCC.

HPV in HNSCC

HPV is detected in approximately 20% of all HNSCC and in 50% of oropharyngeal SCC (Gillison, 2004). This can be compared to a 5-11% detection rate in normal HN tissue (Franceschi et al., 1996). The most commonly detected HPV type in HNSCC is HPV-16 followed by HPV-31, -33 and -18. Different molecular characteristics are found in HPV negative versus HPV positive HNSCC (reviewed in (Gillison, 2004)). For instance, in HPV positive HNSCC, detection of HPV DNA, expression of E6 and E7, wild type p53, decreased expression of Cyclin D and pRb and up-regulation of p16 are found. On the other hand, in HPV negative HNSCC, mutated p53, increased cyclin D expression, and normal, increased or decreased levels of pRb are seen (Gillison, 2004).

HPV induced SCC is predominately found in the oropharyngeal region (tonsil and base of tongue) although tongue- and laryngeal cancers also have been associated with HPV (Herrero et al., 2003, Mork et al., 2001). In comparison to HPV negative tonsillar cancer, HPV positive tonsillar cancer is suggested to be more common in non-smokers, younger patients, non alcohol users, and in individuals who have had oral-genital sex, but the results are not conclusive between studies (Gillison, 2004, Smith et al., 2004).

In an epidemiological study, HPV-16 seropositive individuals had a 14-fold increased risk of developing oropharyngeal cancer compared to seronegative individuals (Mork et al., 2001). Another factor that may increase the risk of developing tonsillar cancer is history of a HPV induced malignant disease, e.g. women with a history of cervical cancer have a higher risk of developing a cancer in the tonsils. Their husbands were also at increased risk of tonsillar cancer compared to the general population (Frisch & Biggar, 1999). Furthermore, HIV-seropositive individuals have a high risk of any HPV induced malignancy (Frisch et al., 2000).

In HPV induced anogenital cancer, there is clear evidence that HPV infection precedes development of dysplasia (Schiffman & Brinton, 1995), while in HNSCC this is not as clear. Oropharyngeal tumors are often not detected before they have reached advanced stages, and therefore the assessment of HPV with pre-malignant lesions is difficult to perform. However, the frequency of HPV infection in normal tonsils or in tonsillitis appear to be low (Syrjanen, 2004). Furthermore, screening for HPV in potentially pre-malignant lesions in the oral cavity have resulted in some indications that high risk HPV is detected in dysplastic lesions, but not in normal mucosa (Bouda et al., 2000, Gillison, 2004).

Presence of HPV has been suggested to influence the disease specific survival rate for patients with oropharyngeal cancer (Dahlstrand et al., 2004), while the HPV status is of no importance for tumors at other sites than the oropharynx (Gillison et al., 2000).

Respiratory Papillomatosis

Recurrent respiratory papillomatosis (RRP) is a rare, epithelial, exophytic, benign neoplastic growth that is most frequently (>90%) located on the vocal cords i.e. in the larynx (Figure 5) (Aaltonen et al., 2002, Corbitt et al., 1988, Go et al., 2003, Kimberlin & Malis, 2000, Pou et al., 1995, Rabah et al., 2001). The growth may spread in the respiratory tract and affect the trachea, bronchi and rarely the lungs.

Epidemiology

The disease has two age distributions; one with onset in childhood, defined as juvenile respiratory papillomatosis and the other with onset in adults, usually with a wide age distribution defined as adult recurrent respiratory papillomatosis. Juvenile RRP has an even distribution between the sexes, while adult RRP affects men more often than women (Aaltonen et al., 2002).

Etiology/Risk factors

RRP is regarded to be induced by HPV-6 and -11 (Mounts et al., 1982), although the route of infection is not fully understood. HPV can be detected in clinically normal laryngeal and tracheal tissue, where it exists in a latent form (Abramson et al., 2004, Abramson et al., 1987). It is not fully understood what activates HPV in these lesions, however a failure of generating an effective T-cell immune reaction has been suggested (Bonagura et al., 1999, DeVoti et al., 2004). Juvenile papillomatosis has been suggested to result from vertical transmission of HPV from mother to child, linking the genital condylomas, also caused by HPV-6 and -11, to RRP (Aaltonen et al., 2002, Quick et al., 1980, Silverberg et al., 2003). For adult onset papillomatosis there are speculations that the disease is sexually transmitted, although clear evidence is lacking (Aaltonen et al., 2002). Nonetheless, it has been shown that adult onset RRP patients have more life time sex partners and a higher frequency of oral sex (Kashima et al., 1992). The high risk HPV-16, -18, -31, -33 and -51 have also been detected in some cases of RRP (Dickens et al., 1991, Go et al., 2003).

Symptoms

The main symptoms are related to the voice; hoarseness, weakness, breathy, strained and low in pitch. In addition, airway obstruction may occur. Juvenile RRP is often more aggressive than adult RRP and the papillomas might be found in lower parts of the respiratory tract (Aaltonen et al., 2002).

Treatment

RRP shows a variable clinical course (Abramson et al., 1987, Bonagura et al., 2004, Pou et al., 1995, Rabah et al., 2001) and approximately one tenth of the patients are cured after the first surgical intervention and the primary therapeutic method is endoscopic surgery. However, the disease has a tendency to recur, (hence the name recurrent respiratory papillomatosis) and some patients have to endure repeated

surgery often in combination with other treatment modalities, to keep the disease under control. Since RRP has a viral etiology, antiviral agents e.g. Interferon- α are sometimes given in addition to surgical treatment (Deunas et al., 1997).

Prognostic factors

Although the HPV-6 and -11 are so-called low risk HPVs, there have been cases of malignant transformation of RRP (Corbitt et al., 1988, Go et al., 2003). This is more common in HPV-11 induced RRP than in HPV-6 induced RRP and smoking is a potential co-factor for malignant transformation. So far, no clear prognostic markers exist for RRP (Aaltonen et al., 2002, Abramson et al., 1987) although some studies have seen a worse prognosis for RRP patients positive for HPV-11 compared to patients positive for HPV-6 (Corbitt et al., 1988, Pou et al., 2004, Rabah et al., 2001).

2. Aim of the thesis

- To confirm the presence of HPV in tonsillar cancer and its prognostic favorable impact on patient survival, and to examine how HPV has a positive influence on patient survival
- To study the presence of HPV in tongue cancer and to examine if HPV is a prognostic favorable factor in these lesions
- To study the response to Interferon- α treatment of recurrent respiratory papillomatosis (RRP) in regard to presence of HPV, viral load and proliferation rate

3. Materials and methods

Patients

Patients with squamous cell carcinoma of the tonsils (Paper I-II)

Patients included in papers I and II were diagnosed with primary tonsillar carcinoma and treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery or Radiumhemmet at the Karolinska Hospital between 1990-2001.

The clinical data for the patients included in the study was obtained from the files of Radiumhemmet and the Department of Oto-Rhino-Laryngology, Head and Neck Surgery at Karolinska Hospital.

TNM stage classification was done according to the International Union against Cancer (UICC 5th ed. 1997) and the differentiation grade according to the WHO International Histological Classification of tumors.

In total, 37 patients with tonsillar cancer (11 females and 26 males) were analyzed in paper I and II. Ten patients were included in both paper I and II. The age at diagnosis ranged from 29-87 with a mean at 65 years (median 64 years). The stage distribution was 1 in stage I, 8 in stage III and 28 in stage IV and the differentiation grade distribution was 10 moderately differentiated, 20 poorly differentiated, 6 undifferentiated and 1 unknown. The primary treatment was pre-operative radiotherapy for 25 patients, radiotherapy only for 8 patients, surgery only for 1 patient and 3 patients received no or palliative treatment only. The response to primary treatment was complete for 20 patients, partial for 9 patients and 4 patients did not respond to treatment.

For a summary of the patient data and the tumors see Table I in papers I and II respectively.

Patients with squamous cell carcinoma of the tongue (Paper III)

Patients included in paper III were diagnosed with primary carcinoma of the tongue and treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery or Radiumhemmet at the Karolinska Hospital between 1970-2002.

More specifically the tumors originated either from the dorsal surface and lateral borders, anterior to vallate papillae (anterior two-thirds, ICD-O code C02.0,1) and the inferior (ventral) surface (ICD-O code C02.2) or the base of the tongue *i.e.* posterior to the vallate papillae (posterior third, ICD-O code C01). For simplicity, sites encoded by C02.0,1 and C02.2 are thereafter called the “mobile tongue”, since it is the movable part of the tongue.

The clinical data of the patients were retrieved from the files of Radiumhemmet and of the Department of Oto-Rhino-Laryngology, Head and Neck Surgery at the Karolinska University Hospital.

TNM stage classification was done according to the International Union against Cancer (UICC 5th ed., 1997) and the differentiation grade according to the WHO International Histological Classification of tumors.

In total 110 patients were included in the study; 85 patients were diagnosed with mobile tongue cancer (34 females and 52 males) and 25 patients with base of tongue

cancer (7 females and 18 males). The age at diagnosis ranged between 30-92 years for patients with mobile tongue cancer with a mean at 62 years (median 64), and between 35-81 years for patients with base of tongue cancer with mean at 62 years (median 62). Of the examined tumors, 70 were in stage I, 9 in stage II, 8 in stage III, 21 in stage IV and for 1 tumor the stage was undefined. Twenty-one tumors were poorly differentiated, 50 moderately differentiated, 36 well differentiated and for 3 tumors the grade of differentiation was not determined. The majority of the patients with a stage I tumor of the mobile tongue were treated with local resection, while most patients with more advanced tumors and tumors at the base of the tongue received pre-operative radiotherapy followed by surgery. Only a limited number of patients received no- or palliative treatment. The response to primary treatment was complete for 93 patients, partial for 9 patients and unknown for 8 patients.

For details on tumor stage, differentiation grade, treatment and clinical response see Table I in paper III.

Patients with respiratory papillomatosis (Paper IV)

Patients in paper IV were diagnosed with recurrent respiratory papillomatosis and treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery at the Karolinska Hospital between 1977-1994 or at the Helsinki University Hospital, Helsinki, Finland between 1989-2002. The clinical data of the patients were retrieved from the files of the Karolinska University Hospital and from the Helsinki University Hospital.

Twenty-five patients (5 females and 20 males) were included in the study and the age at on-set ranged from 0.5-67 years with mean at 21, and median at 13 years.

Three patients were cured after the first surgical intervention while the remaining 22 patients suffered from recurrent papillomas and 18 of these were in addition to surgery, treated with Interferon- α (IFN- α). Twelve of the IFN- α treated patients showed a clinical response as defined by absence of recurrence after primary IFN- α treatment, or if no or only one relapse occurred after one year of IFN- α treatment.

Detailed information on the patients' gender and age as well as available biopsies from before, during and after treatment is shown in Table I, paper IV.

Tumor tissue

Fresh frozen material (paper I and II)

Fresh frozen pre-treatment tumor material was obtained at the time of diagnosis from patients with primary squamous cell carcinomas of the tonsil as described in papers I and II. A pathologist confirmed the diagnosis on hematoxylin-eosin sections and calculated the percentage of cancer cells in the material. Only samples with at least 70% tumor cells were used in the studies.

DNA was extracted from three 20 μ m sections from each biopsy in paper I and from 2x50 μ m sections in paper II. Before, in-between and after the sections a section was taken for staining with hematoxylin-eosin for tumor tissue verification. To check for HPV contamination an empty block was cut and the sections collected between

every tumor block. In paper I three fresh-frozen cervical cancer biopsies, previously characterized, (F725, F826, F3155) were used as controls.

Paraffin embedded material (paper II, III and IV)

Paraffin embedded archival material of pre-treatment biopsy samples obtained at the time of diagnosis from patients with primary squamous cell carcinoma of the tonsils were collected as described in Paper II. A pathologist confirmed the diagnosis on hematoxylin-eosin sections and calculated the percentage of cancer cells in the material. Only samples with at least 70% tumor cells were used in the studies. If the percentage of tumor cells was less than 70%, micro dissection was used to remove the surrounding tissue.

DNA was extracted from five 5 μ m sections. Before, in-between and after the sections a section was taken for staining with hematoxylin-eosin for tumor tissue verification. To check for HPV contamination an empty block was cut and the sections collected between every tumor block.

Paraffin embedded archival material of pre-treatment biopsy samples obtained at the time of diagnosis from patients with primary squamous cell carcinoma of the tongue were collected as described in Paper III. A pathologist confirmed the diagnosis on hematoxylin-eosin sections and calculated the percentage of cancer cells in the material. Only samples with at least 70% tumor cells were used in the studies. If the percentage of tumor cells was less than 70%, micro dissection was used to remove the surrounding tissue. For three patients with stage I cancer, the available tissue block contained only cancer in situ lesions, however, the patients had invasive cancer and were treated accordingly and were hence included in the HPV analysis, and subsequent analysis.

DNA was extracted from five 5 μ m sections from each biopsy in paper III. Before, in-between and after the sections a section was taken for staining with hematoxylin-eosin for tumor tissue verification. To check for HPV contamination an empty block was cut and the sections collected between every tumor block.

In paper IV, paraffin embedded archival material of pre-, ongoing- and post-treatment samples were obtained from 25 patients diagnosed with respiratory papillomatosis. A total of 23 pre-treatment, 18 on-going treatment and 13 post-treatment samples were obtained and the diagnosis of respiratory papillomatosis was confirmed by histopathology on hematoxylin-eosin sections. For details on available samples from each patient see Table I in paper IV.

DNA was extracted from five 5 μ m sections from each biopsy in paper IV. Before, in-between and after the sections a section was taken for staining with hematoxylin-eosin for tumor tissue verification. To check for HPV contamination an empty block was cut and the sections collected between every tumor block.

Methodology

DNA extraction

DNA extraction from fresh frozen material

In paper I the DNA was extracted according to a standard phenol-chloroform protocol with proteinase K treatment, and subsequent ethanol washing. In paper II the DNA was extracted either according to a high salt protocol, with proteinase treatment or according to a standard phenol-chloroform protocol.

DNA extraction from paraffin embedded material

In paper II the DNA was extracted according to a standard phenol-chloroform protocol.

In paper III three different methods were used for DNA extraction from paraffin embedded material; either a standard phenol-chloroform protocol, or according to the manufacturers protocol for use of the QIAamp® DNA Mini Kit (Merck Eurolab AB) or the High Pure RNA Paraffin Kit, with exclusion of DNase treatment (Roche, Diagnostics GmbH).

In paper IV the paraffin was removed by xylene treatment, followed by subsequent ethanol washing. The pellet was air dried and incubated with proteinase K in 1x PCR buffer II (Applied Biosystems) at 60°C, followed by inactivation of proteinase K at 98°C for 8 minutes.

For 21 of the samples the DNA was instead extracted using the High Pure RNA paraffin kit (Roche Diagnostics GmbH) according to the manufacturers' instructions, but with exclusion of DNase treatment.

Before running real-time quantitative PCR, all samples that had not been extracted with the High Pure method were cleaned from cell debris using DNA binding columns from the High Pure RNA Paraffin Kit (Roche Diagnostics GmbH).

Methodological considerations – DNA extraction

In this thesis DNA extraction has been performed using 4 protocols, with different advantages and disadvantages. The standard phenol-chloroform protocol for DNA extraction has the disadvantage of using phenol and chloroform, which may present a health hazard. In addition, any trace amounts of organic agents still in the DNA after extraction could impair on the subsequent analysis.

The advantage of using a high salt DNA extraction protocol is mainly that it avoids the use of phenol-chloroform. The disadvantages, when extracting DNA from paraffin embedded material where the amount of material is limited, is that several washing steps are required to wash away the salt and with each step there is a risk of losing material.

Two different DNA extraction kits, one from QiaGen and one from Roche Diagnostics have also been used. The advantages with these kits are that they are "clean" to work with, i.e. no harmful chemical agents and the end product is clean,

the disadvantage is the cost. If one compares the kits there is one major difference; the High Pure RNA paraffin kit from Roche includes one extra Proteinase K treatment step to remove any trace amounts of proteins still bound to the DNA after the first treatment step. This gives an end product that is clean enough to use in highly sensitive analysis such as real-time quantitative PCR, and hence this protocol has been used for all samples extracted where quantification of HPV viral load was of interest.

HPV detection by PCR

General HPV PCR

For detection of HPV DNA in tumor samples two different broad-spectrum general PCR protocols were used.

The GP5+/6+ protocol (de Roda Husman et al., 1995) uses primers that bind to the L1 gene of several HPV types (Table VIII) and gives an amplicon 130-150 base pairs (bp) in length depending on the HPV type.

Between 100-200 ng of sample DNA was run in 50 µl reactions containing 5 µl 10x PCR buffer II (Applied Biosystems), 200 µM of each dNTP, 3.5 mM MgCl₂, 25 pmol of each primer and 1 U of Taq DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems). The amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The program consisted of an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 44°C for 60 sec and elongation at 72°C for 90 sec. The program was ended with an extended elongation at 72° for 10 min. Cloned plasmids with HPV-6 or HPV-16 were used as positive controls. PCR products were run on a 2.5-3% agarose gel stained with ethidium bromide and visualized under UV light.

The CPI/IIG protocol (Tieben et al., 1993) uses primers that bind to the E1 gene of several HPV types (Table VIII) and gives an amplicon of approximately 190 bp.

Between 100-200 ng of sample DNA was run in 50 µl reactions containing 5 µl 10x PCR buffer II (Applied Biosystems), 200 µM of each dNTP, 3 mM MgCl₂, 0.05% BSA, 17 pmol CPI, 26 pmol CPIIG and 2.5 U of Taq DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems). The amplification was run in an automated thermocycler (GeneAmp PCR system 9700). The program consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 60 sec, annealing at 55°C for 60 sec and elongation at 72°C for 120 sec. The program was ended with an extended elongation at 72° for 10 min. Cloned plasmids with HPV-6 or HPV-16 were used as positive controls. PCR products were run on a 2.5-3% agarose gel stained with ethidium bromide and visualized under UV light.

Type-specific HPV PCR

Type-specific HPV PCR was used to determine the type of HPV present in samples positive in the general HPV PCRs. Type specific primers for HPV-6, -11, -16, -18 and -33 were used in separate runs to detect respective HPV type. Not all primer

pairs were used for all samples, the priority, depending on available tumor material, was; HPV-16, -18 and -33 for squamous cell carcinomas and HPV-6, -11 and -16 for respiratory papillomatosis.

Table VIII. List of primers used to detect and type HPV, for verification of amplifiable DNA and the HPV-16 probe

Primer name	Oligo sequence	Position	Length of amplimers (bp)
general HPV primers			
GP5+	5'-TTT GTT ACT GTG GT AGAT ACT AC-3'	6765-6784 in HPV-16 ¹ (L1)	138
Gp6+	5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'	6903-6882 in HPV-16 ¹ (L1)	
CPI ²	5'-TTA TCW TAT GCC CAY TGT ACC AT-3'	1942-1964 ³ in HPV-16 ¹ (E1)	187
CPIIG ²	5'-ATG TTA ATW SAG CCW CCA AAA TT-3'	1777-1799 ³ in HPV-16 ¹ (E1)	
type specific HPV primers			
HPV-6.1	5'-GTA TTA GAC CTG CAA CCT CCA-3'	564-584 in HPV-6 ⁴ (E7)	154
HPV-6.2	5'-CTG TCA CAT CCA CAG CAA CAG-3'	718-698 in HPV-6 ⁴ (E7)	
HPV-11.1	5'-CGC AGA GAT ATA TGC ATA TGC-3'	221-241 in HPV-11 ⁵ (E6)	89
HPV-11.2	5'-AGT TCT AAG CAA CAG GCA CAC-3'	310-290 in HPV-11 ⁵ (E6)	
HPV-11 F	5'-AAT GAA AAC CCTA GGG CAC GCA GA-3'	4420-4437 in HPV-11 ⁵ (L1)	179
HPV-11 R	5'-GGT TAG GTA TTG GTA CAG GG-3'	4580-4599 in HPV-11 ⁵ (L1)	
HPV-16.1	5'-TCA AAA GCC ACT GTG TCC TGA-3'	421-441 in HPV-16 ¹ (E6)	119
HPV-16.2	5'-CGT GTT CTT GAT GAT CTG CAA-3'	520-540 in HPV-16 ¹ (E6)	
Long A-S	5'-CAG TAG TGG AAG TGG GGG AGA GGG TGT TAG TG-3'	1376-1407 (E1)	440
Long A-AS	5'-CCT GTA TCA CTG TCA TTT TCG TTC TCG TCA TC-3'	967-998 (E1)	
E1 gene 1	5'-TGT GCC CCA TCT GTT CTC A-3'	830-848	2036
E1 gene 2	5'-GGC GCA TGT GTT TCC AAT AG-3'	2847-2866	
E2 gene 1	5'-AGG ACG AGG ACA AGG AAA A-3'	2734-2752	1139
E2 gene 2	5'-GGA TGC AGT ATC AAG ATT TG-3'	3873-3853	
HPV-18.1	5'-CCG AGC ACG ACA GGA ACG ACT-3'	533-553 in HPV-18 ⁶ (E7)	172
HPV-18.2	5'-TCG TTT TCT TCC TCT GAG TCG CTT-3'	705-682 in HPV-18 ⁶ (E7)	
HPV-33.1	5'-AAC GCC ATG AGA GGA CAC AAG-3'	567-587 in HPV-33 ⁷ (E7)	211
HPV-33.2	5'-ACA CAT AAA CGA ACT GTG GTG-3'	778-758 in HPV-33 ⁷ (E7)	
probes			
HPV-16	FAM-5'-CCG GTC CAC CGA CCC CTT ATA TTA TGG AAT CTT T-3'-TAMRA	504-471 in HPV-16 ¹ (E6)	
control primers			
S14 sense	5'-TCG AAA GGG GAA GGA AAA GA-3'	2275-2256 ⁸	127
S14 antisense	5'-CAG TGA CAT GGA CAA AAG TG-3'	2148-2167 ⁸	
β-microglobulin F	5'-GTG GAG CAT TCA GAC TTG TC-3'	24753-24772 ⁹	98
β-microglobulin R	5'-ACG GCA GGC ATA CTC ATC TT-3'	24832-24851 ⁹	
GH26	5'-GTG CTG CAG GTG TAA ACT TGT ACC AG-3'	1-26 ¹⁰	242
GH27	5'-CAC GGA TCC GGT AGC AGCGGT AGA GTT-3'	216-242 ¹⁰	

¹HPV-16 NC-001526, ²degenerate primers; W=A, T, S=G, C, Y=A, G, ³position alignment of CPI/IIG primers according to (Tieben et al., 1993), ⁴HPV-6 NC_000904, ⁵HPV-11 NC_001525, ⁶HPV-18 AY2 62282, ⁷HPV-33 NC_001528, 6 nt of primer 1 lies up-stream of the E7 ORF, ⁸M13934, ⁹AC087828, ¹⁰AF126253

One hundred ng of sample DNA was run in 50 µl reactions containing 5 µl 10x PCR buffer II (Applied Biosystems), 200 µM of each dNTP, 3 mM MgCl₂, 20 pmol of

each primer and 1 U of Taq DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems). The amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The program consisted of an initial denaturation step at 95°C for 4.5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 60 sec. The program was ended with an extended elongation at 72° for 4 min. Cloned plasmids with respective HPV type were used as positive controls. PCR products were run on a 2.5-3% agarose gel stained with ethidium bromide and visualized under UV light. The lengths of the amplimers from the different HPV types are stated in Table VIII.

Verification of amplifiable DNA by PCR

HLA PCR

In papers I and IV a HLA DQ locus based PCR was used for verification of amplifiable DNA after extraction. Five µl sample DNA was run in 25 µl reactions containing 2.5 µl 10x PCR buffer II (Applied Biosystems), 200 µM of each dNTP, 2 mM MgCl₂, 4 µg/µl BSA, 10 pmol of each primer GH26/GH27 (Ehrlich, 1989) and 0.5 U of Taq DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems). The amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The program consisted of an initial denaturation step at 95°C for 4.5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 60 sec. The program was ended with an extended elongation at 72° for 4 min. DNA extracted from normal tissue was used as positive control. PCR products were run on a 3% agarose gel stained with ethidium bromide and visualized under UV light and samples with a band at 250 bp was considered to have amplifiable DNA.

S14 PCR

In paper II, III and IV a S14 PCR protocol was used for verification of amplifiable DNA after extraction. Between 100-200 ng sample DNA was run in 50 µl reactions containing 5 µl 10x PCR buffer II (Applied Biosystems), 200 µM of each dNTP, 1.5 mM MgCl₂, 4 µg/µl BSA, 15 pmol of each primer (Table VIII) and 10 U of Taq DNA polymerase (AmpliTaq DNA polymerase, Applied Biosystems). The amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The program consisted of an initial denaturation step at 94°C for 60 sec, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and elongation at 72°C for 45 sec. The program was ended with an extended elongation at 72° for 5 min. DNA extracted from normal tissue, fibroblasts or SiHa cells (Baker et al., 1987) were used as positive control. PCR products were run on a 2.5-3% agarose gel stained with ethidium bromide and visualized under UV light and samples with a band at 127 bp was considered to have amplifiable DNA.

Methodological considerations – PCR protocols

Several different PCR protocols have been used in this thesis; general HPV PCR protocols (GP5+/6+ and CPI/IIG), HPV type specific PCRs and PCR protocols targeting house keeping genes in normal cells (HLA and S14). These last protocols have been used in order to avoid false negative HPV results due to unclean DNA (trace amounts of proteins and chemical agents that are still present after DNA extraction might inhibit the PCR reaction), and damaged DNA (due to formalin fixation and storage).

One other consideration that needs to be done regarding which PCR protocol/primer pair to use, is that formalin fixation results in DNA bridges that might lead to DNA breaks and this has implications on the length of the DNA obtained after DNA extraction. For this reason we chose to, in paraffin embedded material, work with the S14 protocol that gives amplicons of 127 bp. The HLA DQ locus primers gives PCR products that are 250 bp in lengths which is borderline to what, in our hands, can be detected in formalin fixated materials. However, the latter primers can be useful, to analyze the quality of DNA and estimate if it also can be used for PCR reactions where primers are used that result in larger bands.

In this thesis, two different general HPV protocols were used in order to avoid false negative results due to disruption of viral genes. Both protocols give PCR products of less than 200 bp, and are hence possible to use even in formalin fixed materials. The GP5+/6+ protocol targets the L1 gene that is highly conserved between HPV types and 22 HPV types can be detected using these primers (Table IX). This gene however is not always retained in tumor cells (Munger, 2002) and hence false negative results might be obtained. The CPI/IIG protocol on the other hand targets the E1 gene, which is also highly conserved and 24 different HPV types can be detected using this primer pair. Using both the GP5+/6+ protocol as well as the CPI/IIG protocol the number of false negative results should be very limited and the number of HPV types that can be detected is higher than when only one primer set is used (Table X).

Table IX. HPV types detected with the general primers used in the thesis as determined in (de Roda Husman et al., 1995, Tieben et al., 1993)

Primer pair	HPV-1	-2	-3	-4	-5	-6	-7	-8	-10	-11	-12	-13	-14	-16
GP5+/6+	- ¹	-	-	-	-	+	-	-	-	+	-	+	-	+
CPI/IIG	+	+	+	+	+	+	+	+	+	+	+	-	+	+
	HPV-18	-19	-20	-21	-22	-24	-25	-30	-31	-32	-33	-35	-36	-39
GP5+/6+	+	-	-	-	-	-	-	+	+	+	+	+	-	+
CPI/IIG	+	+	+	+	+	+	+	-	+	-	+	-	+	-
	HPV-40	-43	-45	-46	-51	-52	-54	-55	-56	-58	-59	-66		
GP5+/6+	+	+	+	-	+	+	+	+	+	+	+	+		
CPI/IIG	-	-	-	+	-	-	-	-	-	-	-	-		

¹ + detected, - not detected

General primers are good for screening of tumors samples for presence of HPV since they are able to detect a broad range of different HPV types. However, to determine the HPV type, one needs to use HPV type specific primers or run a Southern blot against the amplimers or sequence them. In this thesis we chose to run type specific PCR and in some cases we sequenced the amplimers from the general PCRs.

In addition, the general primers are not as sensitive as type specific primers since they need around 10x more DNA to be able to detect HPV in a tumor sample. When we compared the detection limit for the GP5+/6+ and HPV-16 type specific protocols using dilutions of DNA extracted from either fresh frozen or formalin fixated paraffin embedded SiHa cells the HPV-16 protocol was the most sensitive for both DNA preparations (Table X).

Table X. Detection limit for HPV PCR protocols in fresh frozen vs. paraffin embedded SiHa cells

PCR protocol	Fresh frozen SiHa cells	Paraffin embedded SiHa cells
GP5+/6+	1.5 ng	65 ng
HPV-16	0.2 ng	1 ng

Sequencing of HPV positive PCR-products

In addition to type specific PCR, direct sequencing of the GP5+/6+ or CPI/IIG PCR products was used for sub-typing of some HPV positive samples. Samples positive in on or the other of the general HPV PCR protocols were re-run in order to increase the yield. The products were purified by gel extraction (QIAquick PCR Purification Kit, (Merck Eurolab AB) and sequenced using the Big Dye Terminator Cycle Sequencing Kit and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). One or 2 strands were sequenced and aligned to the sequences available at NCBI BLAST GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>).

Restriction enzyme, ligation inverse (rli-) PCR

In paper I the physical status of HPV-16 was investigated using a technique based on restriction enzyme digestion, ligation and inverse PCR (*rli*PCR) (Figure 6) (Kalantari et al., 2001). Extracted DNA (0.3-1 µg) was digested over night with either Hind III (does not cleave HPV-16) or Taq I (cleaves at one site in HPV-16 E6), in 37°C or 65°C respectively. Ten µl of the cleaved DNA was saved for PCR and 40 µl was ligated by T4 ligase (Rapid DNA ligation Kit, Roche).

A long template inverse PCR specific for HPV-16 (primers Long A-S and Long A-AS in Table VIII) was run on undigested, digested and digested & ligated DNA. The amplification was run using the Expand TM long Template PCR System (Roche) in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). Five µl sample DNA was run in 50 µl reactions containing 5 µl 10x PCR buffer II (supplied in the kit), 350 µM of each dNTP, 50 pmol of each primer and 2.265 U of

Taq DNA polymerase mix (supplied in the kit). The program consisted of an initial denaturation step at 94°C for 2 min, followed by 16 cycles of denaturation at 94°C for 15 sec, annealing/elongation at 68°C for 15 min, followed by 14 cycles with the same conditions except for 15 sec increments per cycle of each annealing/elongation step. The program was ended with an extended elongation at 72° for 10 min. PCR products were run on a 0.8% agarose gel stained with ethidium bromide and visualized under UV light. As controls SiHa (1-2 copies of integrated HPV-16 per cell (Baker et al., 1987)), two cervical cancer samples (F826, F3155) with known integration sites, and HPV negative cell line (MCF-7) were used (Kalantari et al., 2001).

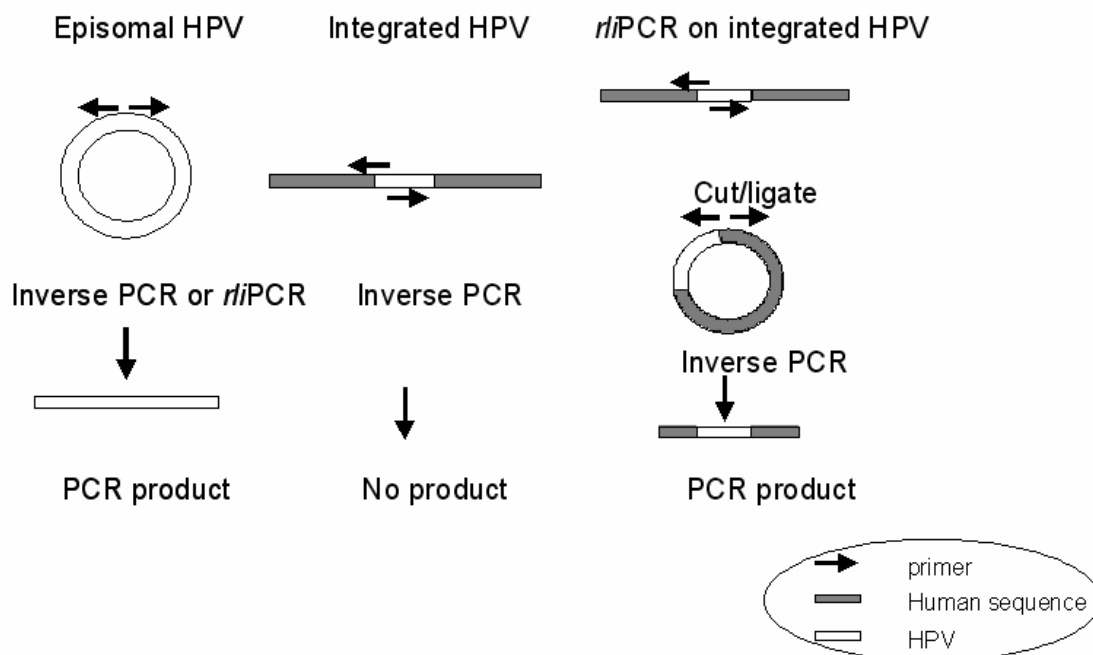


Figure 6. Schematic over-view for the *rliPCR* method.

Methodological considerations *rliPCR*

It is possible to amplify the entire HPV-16 genome (7904 bp) in one single PCR reaction by running a long template PCR using inverse primers, if the HPV genome is episomal (circular), since inverse primers will amplify circular DNA (Figure 6). If, on the other hand, the HPV-16 genome is integrated (linear), it will not be amplified by inverse primers (Figure 6) unless it is first cleaved with a restriction enzyme, which cuts human DNA fairly regularly (but leaves the HPV genome unaffected) and then circularized by self-ligation (Figure 6). Integrated HPV detected this way will also yield PCR fragments containing virus-human junction sequences. The inverse PCR protocol was run on undigested DNA in order to detect episomal forms of HPV-16 in the samples, on digested DNA, which should be unaffected in episomal forms and on digested and ligated DNA in order to detect integrated forms of HPV-16.

DNA sequencing by primer walking

The PCR products from the inverse PCR of undigested and digested & ligated DNA that had a different band length than the expected (7,904 bp) were gel extracted and sequenced as described above. The Long A-S primer was used for the initial sequencing and new primers were constructed based on the obtained sequence, i.e. “primer walking” until the sequence of Long A-AS was reached (for episomal HPV) or until the other end of HPV-16 was reached (for integrated HPV). The standard HPV-16 complete genome NC_001526 (BLAST Gen Bank) was used for reference.

E2 and E1 gene detection

In paper I the HPV-16 genes E1 and E2 were sequenced in order to investigate the integrity of the genes in episomal forms of HPV-16. The genes were amplified in 2 separate PCR reactions using primers specific for the E1 and E2 genes respectively (Table VIII) (Das et al., 1992, Kalantari et al., 1998).

HPV quantification by real-time quantitative PCR

HPV-16 quantification by real-time TaqMan PCR

In paper I the amount of HPV-16 was estimated using real-time quantitative (Q-PCR) TaqMan PCR, based on the 5'-3' exonuclease activity of Taq DNA polymerase. Cloned HPV-16 was used to create a standard curve with known amount of HPV-16. First a HPV-16 type specific PCR was run as described above and the product was inserted into a pGEM-T easy vector (Promega). Cloned HPV-16 was diluted and used to correlate the number of viral copies in each sample with the known number in the standard.

The real-time PCR was run with the HPV-16 specific primers described in Table X and a fluorogenic probe located in between the HPV-16.1 and HPV-16.2 primers in the E6 region of HPV-16 (Table VIII). Ten µl sample DNA was run in duplicates in 25 µl reactions containing 2.5 µl 10x PCR buffer II (Applied Biosystems, Foster City, CA), 200 µM of each dNTP, 1.5 mM MgCl₂, 10 pmol of each primer, 5 pmol probe (Mellin et al., 2002) and 0.5 U of Taq DNA polymerase (AmpliTaQ Gold DNA polymerase, Applied Biosystems). The amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The program consisted of an initial step at 50°C for 120 sec and at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing and elongation at 60°C for 60 sec. As an internal control of the human genome content in the samples, quantitative real-time PCR was run on β-actin as instructed by the manufacturer (Applied Biosystems). Only sample values that were concordant in triplicates or duplicates were accepted, all other samples were re-run until acceptable concordant triplicate or duplicate values were obtained.

HPV-6 quantification by real-time quantitative PCR using SybrGreen

In paper IV, the HPV-6 positive samples were run in triplicates in a type-specific quantitative PCR with a SybrGreen protocol in an iCycler iQ (iCycler iQ real-time

PCR detection system, BioRad) with the HPV-6 type specific primers described in Table VIII.

Three ng sample DNA was run in 25 µl reactions containing 12.5 µl iQ™ SYBR Green Supermix (BioRad Laboratories) and 10 pmol/µl of each of the HPV-6 primers. The program started with the initial steps of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and elongation at 74°C for 30 sec. Finally a melting curve, starting at 40°C and increasing by 0.5°C every 10th second until 120°C was reached, was run in order to verify specificity of the obtained amplicons. Cloned HPV-6 was diluted to contain 10²-5x10⁵ copies of HPV-6 per test tube and used in triplicates as standard series. For estimation of viral copies per cell, 1 ng of DNA was considered be equivalent to 2.000 cells. Only sample values that were concordant in triplicates or duplicates were accepted, all other samples were re-run until acceptable concordant triplicate or duplicate values were obtained.

When following individual patient samples over time for variation in viral load, a 0.5 log difference was considered as a significant quantitative increase or decrease (Yun et al., 2003).

HPV-11 quantification by real-time quantitative PCR using SybrGreen

In paper IV the HPV-11 positive samples were run in triplicates in a quantitative PCR based on the GP5+/6+ protocol with adjustments to the SybrGreen system. The reaction was run in an iCycler iQ (iCycler iQ real-time PCR detection system, BioRad) with newly designed HPV-11 type specific primers (described in Table VIII).

Three ng was run in 25 µl reactions containing 12.5 µl iQ™ SYBRGreen Supermix (BioRad Laboratories) and 10 pmol/µl each of the GP5+ and GP6+ primers. The program started with the initial steps of 50°C for 2 minutes and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 30 sec and elongation at 71°C for 30 sec. Finally a melting curve, starting at 40°C and increasing by 0.5°C every 10th sec until 120°C was reached, was run in order to verify specificity of the obtained amplicons. Cloned HPV-11 were diluted to contain 10-10⁶ copies of HPV-11 per test tube and used in triplicates as standard series. For estimation of viral copies per cell, 1 ng of DNA was considered be equivalent to 2.000 cells. Only sample values that were concordant in triplicates or duplicates were accepted, all other samples were re-run until acceptable concordant triplicate or duplicate values were obtained.

When following individual patient samples over time for variation in viral load, a 0.5 log difference was considered as a significant quantitative increase or decrease (Yun et al., 2003).

Methodological considerations viral quantification

In papers I and IV three different protocols were used for viral load estimation. In paper I, HPV-16 was quantified with a TaqMan technique based on the 5'-3' exonuclease activity of the Taq DNA polymerase, and in paper IV a SyberGreen technique was used to quantify HPV-6 and -11. The use of a flourogenic probe in

addition to the specific primers increases the specificity of the reaction as the probe only emits light as it is “pushed off” the DNA by the polymerase.

The SybrGreen system on the other hand is not specific for viral DNA as the dye binds to any double stranded DNA and emits a fluorescent light at 490 nm that is proportional to the amount of bound DNA. In this case it is entirely dependent on the primers how specific the reaction will become.

For all three protocols only sample values that were concordant in triplicates or duplicates were accepted, all other samples were re-run until acceptable concordant triplicate or duplicate values were obtained.

Comparative genomic hybridization

In paper II the comparative genomic hybridization (CGH) technique was used to determine the pattern of chromosomal gains and losses in tonsillar carcinomas. Tumor and control DNA (obtained from lymphocytes of healthy donors), were labeled by nick translation with biotin-dUTP and digoxigenin-11-dUTP (DIG), respectively and 500 ng-1 µg nick translated test and control DNA were co-precipitated in 3 M NaAc and ethanol using an excess of human Cot-1 DNA (Invitrogen) and salmon sperm DNA (Sigma). The precipitate was re-suspended in 5 µl de-ionized formamide, pH 7.5 at 37°C for 1 hour and then an equal volume of master mix (20% dextran sulfate and 2xSSC, pH 7.0) was added to the probes followed by incubation at 37°C for 30 minutes. The probes were denatured at 85°C for 5 minutes followed by a pre-annealing at 37°C for 1-2 hours. Normal lymphocytes from healthy donors were stimulated with phytohemagglutinin and used for metaphase spreads. The probes were hybridized to the metaphase slides for 72 hours at 37°C and detected with avidin conjugated fluorescein isothiocyanate (FITC) antibodies for test DNA and anti-DIG antibodies conjugated to tetramethylrhodamine isothiocyanate (TRITC) for control DNA. Chromosomes were stained with 4,6-diamidino-2-phenylindole. At least 10 metaphases per case were imaged using a Leica DM RXA microscope with a cooled CCD camera (Sensys, Roper Scientific). The ratio between the FITC:TRITC intensities was analyzed with CW 4000 software from Leica (Leica Microsystems). FITC:TRITC ratios greater than 1.2 defined a gain, and greater than 1.4 amplifications. Ratios below 0.8 defined loss of genomic material.

Immunohistochemical detection of Ki-67

Immune staining was performed according to the standard ABC-technique (Elite Standard Kit Vector). Paraffin sections were deparaffinized, re-hydrated and pre-treated with citrate buffer at pH 6 in a microwave oven for 20 minutes (700W). After rinsing, the endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide, 0.5% for 30 minutes. The sections were rinsed and incubated with blocking serum (1% bovine serum albumin) for 20 minutes, and subsequently incubated with the primary antibody, Ki-67 (Mib-1) (1:160) (DAKOPATTS AB) overnight in a moist chamber at 8°C. As a secondary antibody a biotinylated anti-mouse IgG antibody (DAKOPATTS AB) was added to the slides and after 30 minutes incubation,

followed by rinsing, the ABC complex was added for 30 minutes. The peroxidase reaction was developed using 3,3-diaminobenzidine for 6 minutes. Nuclear counterstaining was performed with Mayers haematoxylin. Tris buffered saline pH 7.4 was used for rinsing between the steps. The staining was checked with positive controls (intestinal mucosa and a lymph node). The Ki-67 labelling index was determined by light microscopy with a 40-power objective magnification randomly counting cells with positive nuclear staining and expressing the results as the number of immunoreactive cells among the 200 nuclei of respiratory papilloma cells.

Statistical analysis

Paper I

Pearson's χ^2 test was used to analyze the results when correlated to number of disease free patients. Survival analysis was done using the Kaplan-Meier method. The significance between the differences in survival rate was analyzed by the log rank test.

Paper II

Fisher's exact 2-tailed test was used to correlate the frequency of HPV and chromosomal imbalances to clinical data. Logistic regression was used to estimate if the correlation of HPV and genetic imbalances with clinical data influenced the outcomes of the patients. Survival analysis was done using the Kaplan-Meier method. Significance between differences in survival rate was analyzed by the log rank test. Cox regression (uni- and multivariate) was used to evaluate factors influencing mortality risk.

Paper III

Exact logistic regression and Fischer's exact 2-tailed test were used to correlate the frequency of HPV in tongue cancer patients to clinical data. The association between diagnosis and different endpoints were assessed using a Cox proportional hazard regression model controlling for confounders. The assumption of time independent hazard ratio was investigated by including the covariates as a function of time. Kaplan-Meier curves were used for graphical presentation of survival and log rank test was used to analyze significant differences in survival rate. Cox regression model was used for 3- and 5-year survival analysis. For the multivariate Cox proportional hazard regression model a backward selection was used, and the factors analyzed were grade of differentiation, presence of HPV, gender, stage and age at diagnosis.

Paper IV

A chi-square exact test was used for all statistical analysis. A 95% confidence interval (C.I.) and $p=0.05$ was considered significant. Non-categorical variables were tested with two-tailed t-test after performing Levene's test for normality, $p=0.05$ was considered significant.

4. Results

Paper I-II

Frequency and type specificity of HPV in tonsillar cancer

In papers I and II the frequency and type specificity of HPV was analyzed in 37 cases of tonsillar cancer. HPV was detected in 23/37 (62.2%) analyzed samples. Twenty-one of these, harbored HPV-16, 1 harbored HPV-33 and for 1 sample the HPV type was not possible to determine due to inadequate amount of material.

Physical state and viral load of HPV-16 in tonsillar cancer

In paper I, the physical state of HPV-16 in tonsillar cancer was examined for 11 tumors by *rli*PCR. All HPV-16 genomes could be amplified and for 7 cases only full length (7,904 bp), episomal bands were detected. For 1 tumor (F7) two different sized bands (2,071 bp and 4,140 bp) were obtained by both inverse PCR on undigested DNA and by *rli*PCR. Both bands were shorter than the full length HPV-16 genome and when sequenced it was shown that the bands consisted of a disrupted form of HPV-16 with a 5,455 bp deletion in the E1 ORF at nt 2,388, and the sequence continued in the LCR at nt 7,843. The longer band was present in a dimeric form and hence the double length. The final 3 tumors (F14, F25 and F12) all showed both full length episomal bands and bands with “off size” lengths. The “off size” bands (2,712 bp for F14, 1,969 bp for F25 and 11kb for F12) were sequenced and for F14 and F25 and were shown to be deleted episomal forms of HPV-16. The 4,814 bp deletion in F14 was found in the E1 ORF at nt 2,786 and the sequence continued in the LCR at nt 7,600. In F25 a 5,557 bp deletion started at nt 2,640 in the E1 ORF and the sequence continued in the E6 ORF at nt 294. In addition to the full length episomal band found in F12 by *rli*PCR an 11 kb band was also found. This band was not detected after *rli*PCR of undigested DNA and unfortunately it was not possible to sequence so it could not be verified that F12 contained an integrated form of HPV-16 in addition to the episomal form.

The copy number of HPV-16 in tonsillar cancer was examined for 11 patients by quantitative PCR and correlated to the copy number of β -actin to give a value of HPV-16/cell. The viral load ranged between 10-15,500 HPV-16 copies/ β -actin, with a median at 190 copies/ β -actin. The median value divided the cases in two groups; one group consisted of cases with an equal or higher viral load than the median value and the other group consisted of cases with a viral load lower than the median value of 190 copies/ β -actin (see Table I in paper I).

Chromosomal aberrations in tonsillar cancer

In paper II, the chromosomal aberrations in tonsillar cancer were examined. Both HPV negative and positive cases analyzed by the CGH technique were found to exhibit aberrations on all chromosomes except chromosome 6. The mean number of gains was 3.0 and losses 2.1, giving an average number of chromosomal aberrations (ANCA value) of 5.2. The most commonly gained region were the long arms (q) of

chromosome 3 (60%), 8 (32%), 20 (24%) 11 (20%), 17 (20%) and the short arm (p) of chromosome 12 (20%). The most commonly lost regions were found on chromosome 11q14-qter (44%) and chromosome 13 (20%). The specific gains and losses for each case are described in Table II and shown in Figure 1 in paper II.

There were some differences in chromosomal aberrations between the HPV positive and negative cases (Table XI). In the HPV positive group the mean number of gains and losses were 2.5 and 2.1 respectively, which resulted in an ANCA value of 4.6 while in the HPV negative group the mean number of gains and losses were 4.1 and 2.1 respectively, giving an ANCA value of 6.2. Chromosome 3q was the most commonly gained chromosomal arm in both groups,

Table XI. Average number of aberrations in HPV positive and negative tonsillar cancer.

	All cases	HPV positive	HPV negative	HPV+ vs. HPV-
Average number of aberrations (ANCA)	5.2	4.6	6.2	no diff
Mean number of gains	3.1	2.5	4.1	no diff
Most common gain	3q (60%)	3q (73%)	3q, 7q, 8q (40%)	3q: p=0.049
Mean number of losses	2.1	2.1	2.1	no diff
Most common loss	11q (44%)	11q (47%)	11q (40%)	

however it was significantly more frequently gained in the HPV positive cases (73%) (p=0.049, Fisher's exact 2-tailed test, Table XI) compared to the HPV negative group (40%). In addition, in the HPV negative group gains of 7q and 8q were as frequent as 3q gains (Table XI). The chromosome that was most commonly lost was 11q, which was lost in 47% of the HPV positive cases and in 40% of the HPV negative cases. A more detailed description of the specific gains and losses are shown in Table II in paper II.

HPV, physical state, viral load and genomic aberrations in relation to clinical outcome

In both papers I and II the impact of HPV on the clinical outcome was investigated for patients with tonsillar cancer. In paper I, a 3 year follow up period showed that the HPV positive patients were to a higher degree disease free compared to the HPV negative patients, and the disease specific survival was also better for HPV positive patients compared to HPV negative patients. However these differences were not statistically significant (p=0.09, χ^2 test and p=0.08, log rank).

In paper II, a disease specific survival benefit was observed for patients with HPV positive tonsillar cancer compared to patients with HPV negative tonsillar cancer (p=0.002, log rank). Only patients who received treatment and were followed for at least 2 years were included in the survival analysis.

The importance of HPV-16 physical state (episomal vs. integrated) could not be evaluated since all cases analyzed exhibited episomal forms of HPV-16.

The impact of viral load on survival was also examined, and patients with a high viral load (equal to or higher than the median value) were statistically more often tumor free after three years compared to the patients with a viral load lower than the median value ($p=0.026$, χ^2 test). In addition, the disease specific survival was also statistically better for patients with a high viral load compared to patients with a low viral load ($p=0.039$, log rank).

Paper III

Frequency and type specificity of HPV in tongue cancer

In paper III the frequency and type specificity of HPV in tongue cancer was examined by PCR. Of 85 mobile tongue cancer cases and 25 base of tongue cancer cases only 12 (10.9%) were found to be HPV positive. More specifically, 10/25 base of tongue cancer cases and 2/85 mobile tongue cancer cases were HPV positive ($p<0.001$, Fischer's exact 2-tailed test). The difference in presence of HPV between the mobile tongue cancer cases and the base of tongue cancer cases was irrespective of gender or age group and seen also when only stage I and II tumors were analyzed. However, when only more advanced stages, (stages III and IV), were analyzed, this difference was not seen which could be due to that the number of cases in the more advanced stages of mobile tongue cancer was limited (13 cases).

The most common HPV type was HPV-16, which was found in 9 samples (2 from mobile tongue cancer cases and 7 from base of tongue cancer cases), while HPV-33 and HPV-35 were found in one base of tongue cancer case each. One sample was not typed due to limited amount of material.

HPV was more common in undifferentiated tumors than in moderately differentiated ($p=0.0077$, exact logistic regression) and in well differentiated tumors ($p=0.0009$, exact logistic regression). HPV was also more frequently found in more advanced stages tumors (stage III and IV) compared to that observed in smaller tumors (stage I and II) ($p<0.001$, Fischer's exact 2-tailed test). Nevertheless, the only factor in a multivariate analysis that could explain the presence of HPV was the base of tongue cancer diagnosis with an Odds Ratio for base of tongue cancer to be HPV positive at 26.4.

Tumor characteristics and HPV in correlation to clinical outcome

Ninety-three out of 102 patients, who received treatment with intent to cure, were disease free 1 month after primary treatment. Nine patients, 6 with advanced stage mobile tongue cancer and 3 patients with base of tongue cancer had residual disease.

The 3-year disease specific survival was influenced by the tumor location, tumor differentiation, and tumor stage at the time of diagnosis (data not shown).

A separate survival analysis was performed for 19 base of tongue cancer patients that fulfilled the criteria (at least 36 months follow-up or death of cancer disease within 3 years from diagnosis). HPV as well as tumor stage (stage I + II vs. III + IV) were of importance for the 3-year disease specific survival ($p=0.0159$, Cox regression, multivariate and $p=0.0376$, Cox regression, multivariate respectively). However,

when analyzing the 5-year disease specific survival only the presence of HPV was of importance ($p=0.0362$, Cox regression, multivariate) (Figure 1 in paper III).

Papers I-III

Overall 2-year survival in HPV positive and negative tonsillar and base of tongue cancer patients

Together tonsillar cancer and base of tongue cancer make up for a majority of all oropharyngeal cancer. To estimate if HPV could be a prognostic favourable factor for patients with oropharyngeal cancer we combined all patients with oropharyngeal cancer from papers I, II and III in a survival analysis. The overall survival was analyzed for 52 patients with at least 2 years of follow-up data. Patients with HPV positive tumors were found to have significantly better survival compared to patients with HPV negative tumors ($p=0.002$, log rank, Figure 8), and when correlated for stage, an even stronger impact of HPV was seen ($p=0.001$, Cox regression).

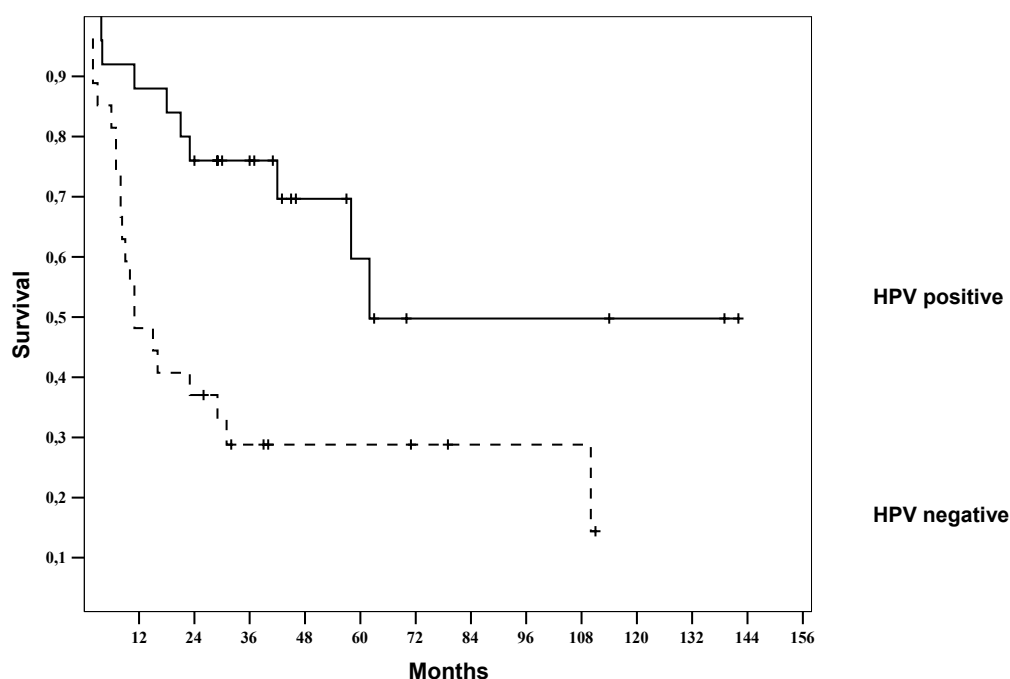


Figure 8. Kaplan-Meier graph showing significantly better overall survival two years after primary diagnosis for patients with an HPV positive tumor compared to patients with HPV negative tumors ($p=0.002$, log rank).

In addition, the overall survival for patients with a stage IV oropharyngeal cancer was 30% (10/33) after two years (Table XII). For patients with HPV positive tumors the survival rate was 50% (9/18) while for patients with HPV negative tumors only 6.7% (1/15) ($p=0.009$, Fisher's exact 2-sided test).

Table. XII. Overall 2-year survival for patients with stage IV oropharyngeal cancer. HPV positive patients have a significantly better overall survival compared to HPV negative patients ($p=0.009$, Fisher's Exact test, 2-sided)

	Alive	Dead	Total
HPV negative	1 (6.7%)	14 (93.3%)	15 (45.5%)
HPV positive	9 (50%)	9 (50%)	18 (54.5%)
Total	10 (30.3%)	23 (69.7%)	33 (100%)

Paper IV

Frequency and type specificity of HPV in recurrent respiratory papillomatosis

In paper IV the presence and type specificity of HPV in 25 patients with RRP was determined by PCR. Twenty out of 25 patients (80%) were found to be HPV positive, 12 for HPV-6 (12/20, 60%), 6 for HPV-11 (6/20, 30%) and the final two 2 (2/20, 10%) were HPV positive although HPV-type could not be determined due to a limited amount of material.

Response to IFN- α treatment of recurrent respiratory papillomatosis

The effect of IFN- α treatment on RRP, was evaluated for 18 patients. Response was defined as absence of recurrence in one patient treated primarily with IFN- α . All other patients were treated after recurrence, and were considered as responders if no or only one relapse occurred during a one-year-period throughout or after IFN- α treatment period. Twelve of the treated patients, (aged 0.5-40 years) showed a clinical response, and the remaining 6 (aged 5-67 years) were judged as non-responders (Table I in paper IV).

Viral load in HPV-6 and -11 positive recurrent respiratory papillomatosis and in response to IFN- α treatment

Viral load of HPV-6 and HPV-11 were estimated in 3 ng sample DNA by Q-PCR and correlated to the number of cells using the approximation that 1 ng DNA equals 200 cells. The HPV-6 load ranged between 0.25-533 copies/cell and the HPV-11 load between 0.30-87 copies/cell. No general conclusions could be drawn regarding the importance of viral load on the response to IFN- α treatment since patients with different pre-treatment viral loads (between 6.2-126 HPV-6 copies/cell and 0.07-0.1 HPV-11 copies/cell) responded, and since the viral load for the 3 patients that did not respond to IFN- α treatment did not differ from the viral load of those that responded.

HPV-6 viral load could be compared before and during treatment for 4 IFN- α responding patients. The viral load was somewhat lower in all patients during treatment, but this decrease was significant (>0.5 log) only for one patient and not for the remaining 3 patients. HPV-6 viral load was available for only 2 patients post-

treatment and in both cases a clear increase in viral load was observed. For details see Table II in paper IV.

HPV-11 viral load could be compared before and during treatment for 2 IFN- α responding and one non-responding patients. The viral load was somewhat higher in all patients during treatment, but this increase was not significant (<0.5 log) for 2 IFN- α responsive patients. For the IFN- α non-responsive patient, a significant increase in viral load was seen initially during treatment after which it dropped (increasing from 0.55 to 6.04 and then down to 0.08 HPV-11 copies/cell) and finally, the viral load increased to 87.6 HPV-11 copies/cell after termination of IFN- α treatment. For details see Table II in paper IV.

Proliferation before- during and after IFN- α treatment of recurrent respiratory papillomatosis

The proliferation rate was determined for 22 patients with RRP by immunohistochemical detection of Ki-76. A high proliferation rate was generally seen in all samples, with Ki-67 indexes ranging from 8-54% (median 35%). There were however, no major differences in proliferation rate between pre-treatment samples from IFN- α responders (8-50%, median 35%), and non-responders (22-35%, median 35%), or during (10-54%, median 28%), and after IFN- α treatment (19-47%, median 37.5%). For details see Table II in paper IV.

5. Discussion

In this thesis the presence, physical state and viral load of HPV in tonsillar cancer, and the influence of HPV on chromosomal patterns and on the clinical outcome for tonsillar cancer patients were examined (papers I and II). Further, the presence of HPV in tongue cancer and influence of HPV on clinical outcome for tongue cancer patients were examined (paper III). Finally, the presence of HPV, viral load and proliferation rate were examined in recurrent respiratory papillomatosis (RRP) in response to Interferon- α treatment (paper IV).

HPV was present in 62.2% of examined tonsillar carcinomas (papers I and II). The majority were HPV-16 positive, while one patient was HPV-33 positive and for one patient HPV type could not be determined due to inadequate amount of material. These results are in line with previous reports on HPV frequency and type specificity in tonsillar carcinoma (Gillison, 2004).

Furthermore, the viral genome was shown to be mainly episomal, hence integration of the viral genome was not a requisite for malignant transformation. This is in line with previous findings where HPV-16 was found to be episomal in two out of two ISH analyzed cases of tonsillar cancer (Snijders et al., 1992). Moreover, in seven of the samples examined in paper I, the full-length genome was detected exclusively, indicating that the E2 ORF, which is commonly lost during integration, is still present in the malignant cell exhibiting its repressor functions on E6 and E7 ORFs. However, in the study by Snijders et al, E6 and E7 transcripts were detected even from tumors with episomal HPV-16 (Snijders et al., 1992).

In two samples a deleted episomal form was detected in addition to the full-length episomal form and in one sample only a deleted episomal form was observed. The deletions in these three samples were not exactly the same, but in all three cases the E1 and E2 ORFs were deleted. Furthermore, the loss of E1 and E2 ORFs could be verified in E1 and E2 specific PCRs for the sample where only deleted episomal forms of HPV-16 could be detected.

The physical status of HPV-16 in HNSCC has since been investigated by other methods. Koskinen et al used a method based on the assumptions that in integrated viral DNA, the E2 ORF is disrupted and that episomal viral DNA has an equal copy number of E2 ORF and E6 ORF, while mixed forms have smaller copy number of E2 ORF than of E6 ORF (Koskinen et al., 2003). They found that HPV-16 was integrated in 48%, episomal in 35% and present in mixed forms in 17% of 61 HNSCC cases. In their material only five tonsillar carcinomas were included and the result showed that HPV-16 was episomal in two and integrated in three of the five cases. However, the method did not allow for detection of deleted episomal forms of HPV-16, and hence they cannot exclude that the three tumors where E2 was not detected could in fact contain deleted episomal forms of HPV-16.

Quantification of HPV-16 was performed in paper I, and the viral load was shown to vary between 10-15,500 copies/ β -actin with a median at 190 copies/ β -actin. Patients with a viral load in their tumors that were equal to or higher than the

median load of 190 HPV-16 copies/ β -actin copies had a significantly better disease specific survival than patients with a lower viral load ($p=0.026$). However, only a few patients were included in this study, so the results should only be regarded as preliminary and hence interpreted with caution. In addition, the viral load has been shown to vary within the tumors, and in our study we did not perform micro dissection before determining the viral load (Klussmann et al., 2003, Klussmann et al., 2001).

The pattern of chromosomal aberrations in tonsillar cancer was examined by comparative genomic hybridization (CGH) (paper II). Generally, more aberrations were seen in HPV negative tumors (ANCA 6.1) compared to HPV positive tumors (ANCA 4.5) although the difference was not significant. This results were in accordance with a study by Mellin et al (Mellin et al., 2003), where HPV positive tumors showed less aneuploidy than HPV negative tumors. Moreover, the findings of paper I were supported by a recent publication by Braakhuis et al, (Braakhuis et al., 2004) who showed that HNSCC with transcriptionally active HPV-16 had fewer chromosomal losses than HNSCC with no HPV DNA. However, in the study by Braakhuis et al, 28 microsatellite markers on 7 chromosomal arms, were analyzed for allelic loss, and hence no data on chromosomal gain was presented. In our material we saw that gain of chromosomal regions were more abundant than loss of chromosomal regions. In addition, there were specific differences between HPV positive and HPV negative tonsillar carcinomas regarding gain of copy number on two chromosomal arms. Gain of chromosome 3q was statistically more common in the HPV positive versus negative tonsillar cancers ($p=0.049$), even though this is borderline and based on few patients. Furthermore, none of the HPV positive tumors had gain on chromosome 7q, while 40% of the HPV negative tumors had such gain ($p=0.017$). The effect of these differences on patient survival was not possible to evaluate due to the very few number of patients included in each group. However, as mentioned above, HPV was a prognostic favorable factor in this study ($p=0.002$).

The presence of HPV in tongue cancer and the influence of HPV on clinical outcome for patients with tongue cancer were examined (paper III). Earlier publications on presence of HPV in tongue carcinoma have presented a wide range in HPV frequency, partly based on the use of different HPV detection techniques and partly due to material composition. Therefore, we chose to focus on a highly selected material of mobile tongue carcinoma detected in stage I. When the study was initiated our hypothesis was that this disease which has a relatively good prognosis would frequently be HPV positive. We also assumed that a subgroup therein, with a DNA aberration comparable to that of advanced carcinomas and prone to local and/or regional recurrence would be HPV negative (Hogmo et al., 1998). In parallel we collected a number base of tongue carcinomas to investigate presence and possible influence of HPV on the prognosis of these tumors as well. However, none of the stage I and only 2/17 stage II-IV mobile tongue cancer samples were HPV positive, and hence the result could not be correlated to prognosis. In contrast, 40% of the base of tongue cancer patients were HPV positive and these patients showed a significantly better disease specific survival compared to the HPV negative patients

($p=0.0362$). Since this study was initiated, others have also reported that HPV is more often present in oropharyngeal tumors (including base of tongue) than in oral tumors (including mobile tongue) (Dahlstrom et al., 2003, Gillison et al., 2000, Schwartz et al., 2001).

The influence of HPV in tonsillar and base of tongue cancer on overall 2-year survival was also estimated in this thesis. Combining the results for tonsillar cancer patients included in papers I-II, it was found that patients with HPV positive tumors had a better overall 2-year survival compared to patients with HPV negative tumors ($p=0.03$ log rank). Furthermore, when combining the 29 tonsillar cancer and 24 base of tongue patients included in papers I-III, the overall 2-year survival was better for patients with HPV positive tumors compared to patients with HPV negative tumors ($p=0.002$ log rank). These results indicate that HPV indeed is a prognostic favorable factor for patients with oropharyngeal cancer.

Finally, the presence of HPV, viral load and proliferation was examined in RRP in response to IFN- α treatment (paper IV). RRP is a disease with a clinical course that not only varies between patients but may also differ from time to time for one patient, making it a challenge to plan treatment and to evaluate response. IFN- α is one of few substances that have been successfully used in the treatment of RRP, although the underlying mechanisms have not fully been elucidated. Furthermore to achieve remission, long term treatment is necessary with severe side effects and great cost as a consequence. In order to find possible mechanisms of IFN- α action, we investigated the presence and type specificity of HPV in 25 patients diagnosed with RRP. The majority of the patients that were subjected to IFN- α therapy responded to the treatment, with a tendency for HPV-6 positive patients to respond better than HPV-11 positive patients ($p=0.067$). HPV-11 associated papillomas have been suggested to be more aggressive than HPV-6 associated disease and this may be reflected in the responsiveness to IFN- α treatment (Rabah et al., 2001). This would be in line with that a correlation between virus genotype and response to IFN- α has been noted for other viruses such as hepatitis C (Tan & Katze, 2001). The rate of proliferation was generally high, and remained unchanged during IFN- α treatment. The viral load varied between the biopsies and both patients with a low, as well as a high, viral load responded to IFN- α treatment, indicating that the viral load is not a determinant for responsiveness. The viral load was generally not affected during IFN- α treatment, however, an increased viral load was observed in four patients when IFN- α was withdrawn. Clinically withdrawal of IFN- α can result in a rebound phenomenon observed as an increase in the number of papillomas (Kimberlin & Malis, 2000). The absence of influence of IFN- α on viral load during treatment was not anticipated, and could possibly be due to the limited number of patients included in this study. However, Steinberg et al have reported similar results but not by quantitative PCR (Steinberg et al., 1988). It is possible that IFN- α exerts its mechanisms by other means. One possibility is enhancement of an immunological response against the HPV infected cells, for example by increasing MHC expression (Grander & Einhorn, 1998). Another possibility could be that IFN increases the rate of apoptosis in virus-infected cells. IFN- α has been shown to act pro-apoptotic in

neoplastic cells, and it was recently shown that expression of the HPV-16 E7 oncogene may sensitize tumour cells to IFN-induced apoptosis (Thyrell et al., 2005). If apoptosis induction explains the clinical effects of IFN- α in respiratory papillomatosis however, still remains to be investigated.

6. Concluding remarks

The studies presented in this thesis have shown the following:

- HPV-16 is commonly found in tonsillar cancer and is a prognostic favorable factor for patient survival
 - HPV-16 load varies in tonsillar cancer, and a higher load may correlate to a better prognosis
 - HPV-16 is mainly episomal in tonsillar cancer, and hence integration of the genome is not a requisite for malignant transformation, or correlated to prognosis
 - HPV positive tonsillar cancer exhibit a tendency of displaying fewer chromosomal aberrations compared to HPV negative cancer and gain of 3q is statistically more frequently observed in HPV positive compared to HPV negative tonsillar cancer
- HPV-16 is commonly detected in base of tongue cancer, but not in mobile tongue cancer, and is a prognostic favorable factor for patient survival for patients with base of tongue cancer
- RRP is frequently responsive to IFN- α treatment, but presence of HPV, viral load and proliferation are generally not affected by IFN- α therapy

7. Future perspectives

In this thesis we have demonstrated that HPV is not only commonly found in tonsillar and base of tongue cancer but that HPV also is a favorable prognostic factor in these malignancies. However, all patients with HPV positive tumors do not survive. Nevertheless, in a pilot study a high viral load was shown to be beneficial for prognosis.

There is a need for further investigations regarding the molecular differences between HPV positive and negative HNSCC and how these differences affect prognosis. The optimal outcome of these studies would be ways to better understand which treatment modalities that are the best for each patient group.

Based on the present findings it would be interesting to expand the investigations to examine if a high viral load is indeed a favorable prognostic factor in tonsillar cancer and possibly also in base of tongue cancer. Moreover, the viral and molecular differences seen between HPV positive and negative tumors, as well as between different HPV positive tumors, and their possible correlation to clinical outcome needs further attention. Additional investigation of the expression levels of E6 and E7 could possibly give insights regarding the divergence in survival that is seen among the patients with HPV positive tonsillar or base of tongue cancer.

In order to further unravel the mechanisms behind IFN- α responsiveness in RRP prospective studies are crucial. These would include collection of fresh frozen biopsies for expanded analysis on host cell and viral markers, as well as collection of blood samples for immunological analysis, e.g. serological responses and cellular responses to HPV.

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Psalm 145

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