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STUDIES ON THE OCCURRENCE AND EFFECTS OF HUMAN PAPILLOMAVIRUS IN TUMORS OF THE HEAD AND NECK

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ABSTRACT

The presence of human papillomavirus (HPV) in squamous cell carcinoma of the head and neck (HNSCC) was first reported in 1985. Since then, this association has been studied intensively and today there is substantial evidence for HPV as a causative agent and positive prognostic factor for clinical outcome in tonsillar cancer, but the association to other HNSCC is still unclear.

The aim of this thesis was first to examine the presence of HPV in tongue cancer and to study its possible influence on disease outcome. Thereafter, the association between HPV and cdk inhibitor $p16^{INK4a}$ expression and a possible correlation to response to radiotherapy (RT) and survival was studied. A third aim of this thesis was to investigate if HPV is a potential risk factor for the increase in incidence of tonsillar cancer that has been observed in Sweden. Furthermore, presence of HPV, viral load and expression of the viral oncogenes E6 and E7 in tonsillar cancer was investigated and correlated to clinical outcome.

In tongue cancer, HPV DNA detected by PCR was more commonly found in base of tongue cancer (40%) as compared to mobile tongue cancer (2.4%), and was a positive prognostic factor for survival in patients with base of tongue cancer. This finding indicates that HPV might not only be involved in tonsillar cancer, but also in base of tongue cancer, which has a similar histology and is also a part of oropharynx.

In tonsillar cancer there was a strong correlation between a high expression of $p16^{INK4a}$ detected by immunohistochemistry (IHC) and presence of HPV detected by PCR. However, only high expression of $p16^{INK4a}$, and not the presence of HPV, was shown to be a predictive factor for complete response to RT in tonsillar cancer. Nevertheless, both $p16^{INK4a}$ and HPV were positive predictive factors for clinical outcome.

The incidence of tonsillar cancer and presence of HPV was studied in the Stockholm area during 1970-2002. HPV was detected by PCR in 49% of the patient samples and 87% of these were positive for HPV-16. The frequency of HPV positive tonsillar cancer increased 2.9-fold from 1970 to 2002 and during the same time period a parallel 2.8-fold increase in the incidence of tonsillar cancer was observed. These results strongly support HPV as a risk factor for the increase in incidence of tonsillar cancer.

In the tonsillar cancer patients above, the finding of HPV as a positive prognostic factor in tonsillar cancer for clinical outcome was confirmed. In addition, HPV viral load and expression of the viral oncogenes E6 and E7 was analyzed with real time quantitative PCR and reverse transcriptase PCR in the HPV-16 positive tonsillar cancer samples. In most HPV-16 positive tumors, expression of E6 and E7 was ascertained. However, in contrast to earlier studies a high viral load was not correlated to survival.

The findings of an increase in incidence of tonsillar cancer and a parallel increase in frequency of HPV positive tumors, a better disease specific survival, and the expression of viral oncogenes strongly support previous findings that HPV positive tonsillar cancer should be considered a different disease entity. If the now available prophylactic vaccines are included in the childhood vaccination program for girls, the possible effects on HPV positive tonsillar cancer should be discussed, since most patients with HPV positive tonsillar cancer are men.

LIST OF PUBLICATIONS

I. Dahlgren, L., Dahlstrand, H. M., **Lindquist, D.,** Hogmo, A., Bjornestal, L., Lindholm, J., Lundberg, B., Dalianis, T. & Munck-Wikland, E. (2004). 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. *Int J Cancer* **112**, 1015-9.

II. Mellin Dahlstrand, H., **Lindquist, D.,** Bjornestal, L., Ohlsson, A., Dalianis, T., Munck-Wikland, E. & Elmberger, G. (2005). P16(INK4a) correlates to human papillomavirus presence, response to radiotherapy and clinical outcome in tonsillar carcinoma. *Anticancer Res* **25**, 4375-83.

III. Hammarstedt, L*., **Lindquist, D*.,** Dahlstrand, H., Romanitan, M., Dahlgren, L. O., Joneberg, J., Creson, N., Lindholm, J., Ye, W., Dalianis, T. & Munck-Wikland, E. (2006). Human papillomavirus as a risk factor for the increase in incidence of tonsillar cancer. *Int J Cancer* **119**, 2620-3.

IV. **Lindquist, D*.,** Romanitan, M*., Hammarstedt, L., Näsman, A., Dahlstrand, H., Lindholm, J., Onelöv, L., Ramqvist, T., Ye, W., Munck-Wikland, E. & Dalianis, T. (2007). Human papillomavirus is a favourable prognostic factor in tonsillar cancer and its oncogenic role is supported by the expression of E6 and E7. *Molecular Oncology* **1(3)**, 350-355.

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CONTENTS

LIST OF ABBREVIATIONS

aa	amino acids
bp	base pairs
BSA	bovine serum albumin
Cdk	cyclin dependent kinase
CR	complete response
E	early
E6-AP	E6-associated protein
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMEA	European Medicines Agency
FDA	food and drug association
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
ISH	in situ hybridization
L	late
LCR	long control region
$MgCl_2$	magnesium chloride
NCR	non-coding region
ORF	open reading frame
PCR	polymerase chain reaction
Rb	retinoblastoma
RT	radiotherapy
SCC	squamous cell carcinoma
UICC	International Union Against Cancer
URR	upstream regulatory region
VLP	virus-like particle

1 INTRODUCTION

In the beginning of the 20th century, there were studies suggesting that a virus could be the cause of human hand and foot warts. Since then, several viruses have been found to have the capacity of inducing tumors and some examples are demonstrated in Table I. Approximately 15% of all human cancer is caused by viruses (zur Hausen 1999) and around one third of these cancers are due to infection with human papillomaviruses (HPV) (Parkin, Bray et al. 2005).

Table I Examples of viruses causing human cancers

Virus	Associated human cancers
Human papillomavirus	Cervical cancer, other anogenital cancers, oropharyngeal cancer
Hepatitis B virus	Liver cancer
Epstein-Barr virus	Nasopharyngeal carcinoma, Burkitt's lymphoma
Human Herpes virus 8	Kaposi's sarcoma
Human T-cell lymphotropic virus-1	Adult T-cell leukemia

1.1 HUMAN PAPILLOMAVIRUS

1.1.1 Taxonomy

Human papillomavirus belongs to the family Papillomaviridae, which includes viruses infecting many different vertebrates. They are strictly species specific and are therefore named based on the species they infect. The taxonomic classification is based on sequence variation in the L1 open reading frame (ORF) and the taxonomic levels within the family are "genus", "species", "types", "sub-types" and "variants". The criteria for different genera are less than 60% nucleotide sequence identity in the L1 ORF and a full length sequence identity of 23-43% (reviewed by (de Villiers, Fauquet et al. 2004)). Species within a genus share 60-70% nucleotide identity and have common biological and pathological properties. For the lower levels, the L1 ORF sequence should differ more than 10% between types, 2-10% between sub-types and less than 2% between variants.

1.1.2 Genomic organization

The relatively small viral genome of 8000 base pairs (bp) is organized in three different regions: the long control region (LCR) (also commonly called the upstream regulatory region (URR) or the non-coding region (NCR)) and the two coding regions called the late (L) and the early (E) coding regions (Syrjanen and Syrjanen 1999) (fig 1). The late region encodes the major (L1) and minor (L2) capsid proteins, that are only

expressed in terminally differentiated squamous epithelial cells and found in all HPV types (McKaig, Baric et al. 1998). The ORFs in the early coding region found in most HPV types are E1, E2, E4, E5, E6 and E7 and these genes encode for the proteins described below (Syrjanen and Syrjanen 1999).

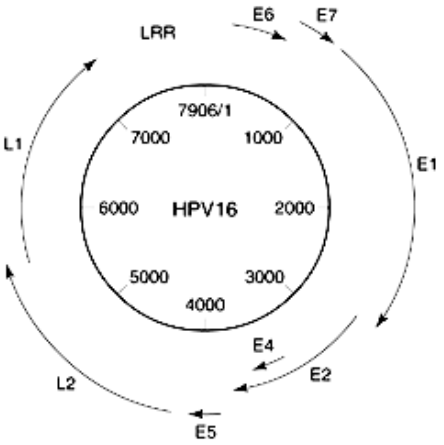

Figure 1 The HPV-16 genome

1.1.3 Viral capsid

The viral capsid of HPV is icosahedral in shape and is approximately 55 nm in diameter. It is built up by 72 pentameres of the major capsid protein and only a few molecules of the minor capsid protein (Modis, Trus et al. 2002). The virus lacks an envelope and is therefore very stable and remains infectious outside its host.

Figure 2 The viral capsid

1.1.4 The viral life cycle and the viral proteins

1.1.4.1 The normal viral life cycle

The goal for the viral life cycle is to infect the target cell, ensure that the target cell proliferates, amplify the viral genome and ultimately produce and release new viral particles.

An HPV infection begins when virus particles gain access to the basal cells of the epithelium. This has been suggested to require micro wounds or more obvious damage to the epithelium, but in some anatomical sites access to the basal cell layer is naturally occurring, for example the base of the hair follicle and in the transformation zones of the cervix and the anus where columnar and stratified epithelia meet each other. The tonsillar crypts, with a monolayer epithelium, are of particular interest since they also may be a site where the virus can gain access to the basal epithelial cells. The entry to the cell has been suggested to be mediated by association between the virus particles and proteoglycans on the cell surface (reviewed by (Doorbar 2007)), but other receptors such as the alpha-6-integrin have also been suggested to facilitate viral uptake (McMillan, Payne et al. 1999).

After infection, the papillomavirus genome is replicated, and this is partly controlled by E1 and E2 as described more in detail below. Stimulation of cell cycle progression is initially driven by growth factors near the basal membrane, but is enhanced by the E6 and E7 oncoproteins. As the cells migrate upwards in the epithelium and start to differentiate, expression of the oncogenes influence the control of the cell cycle (reviewed by (Doorbar 2007)). Upon further differentiation, there is a switch to the late promoter and the expression of E1, E2, E4 and E5 then increases which leads to viral replication and accumulation of viral DNA.

The major (L1) and minor (L2) capsid proteins are expressed from the late promoter when there are high levels of E4 in the cytoplasm. Then the viral capsid forms in the nucleus by assembly of L1 capsomeres facilitated by L2 and E2 localized to the promyelocytic leukemia bodies (reviewed by (Doorbar 2007)). Finally, the viral particles are released, facilitated by an association of the E4 protein with cytokeratin networks as described below.

1.1.4.2 The viral genes and the function of their proteins

The proteins from the early ORFs described below codes for proteins with different regulatory functions, and the proteins from the late ORFs build up the viral capsid. The early genes E3 and E8 are expressed by papillomaviruses of some species, but not in humans.

1.1.4.3 E1

The E1 ORF encodes a 68 kilodalton (kDa) protein and is the largest and most highly conserved region, making this region a good target for general HPV polymerase chain reaction (PCR), in order to detect many different HPV types (see material and methods). The E1 protein controls episomal DNA replication by binding weakly to AT-rich sequences near the origin of replication (Frattini and Laimins 1994). Interaction

with the E2 protein facilitates this binding leading to enhanced effects (Frattini and Laimins 1994; Frattini and Laimins 1994). Other functions of E1 in the DNA replication are binding to DNA polymerase, recruiting cellular replication complexes and interaction with cyclins A and E (reviewed by (Longworth and Laimins 2004)). Furthermore, the E1 ATPase and helicase activities are important functions when the HPV genome is replicated (Hughes and Romanos 1993).

1.1.4.4 E2

The E2 protein is a 50 kDa protein that facilitates DNA replication (Frattini and Laimins 1994) and acts as a transcription factor. The levels of E2 expression are low when the cell is first infected, and at that point, the transcription of early genes is controlled by cellular transcription factors, enhanced by the low levels of E2 (Steger and Corbach 1997). However, when the cell starts to differentiate, there is a switch to the late promoter which is not controlled by E2 and this leads to increased levels of the E2 protein (Klumpp and Laimins 1999). Higher levels of E2 have the reverse effect on the early promoter and the expression of early genes will therefore decrease. This effect is achieved when the E2 product binds to and inhibits cellular transcription factors (reviewed by (Longworth and Laimins 2004)). There is also an increase in DNA replication by recruiting the E1 protein to its binding site (as described above), to start producing viral DNA to new viral particles.

1.1.4.5 E4

The 17 kDa E4 protein synthesis originates mainly from the late promoter which is activated when the cell has started to differentiate. The E4 protein is then produced in higher amount than all other HPV proteins (Howley 1996). The E4 ORF is translated as a fusion with the first 5 amino acids of E1 to generate an E1^E4 fusion protein, which is necessary since the E4 ORF lacks an initiator codon (Howley 1996). The E4 proteins from high-risk types have been described to associate with and destroy keratin networks which facilitates the release of viral particles (Doorbar, Ely et al. 1991; Roberts, Ashmole et al. 1997; Wang, Griffin et al. 2004). Other suggested functions are regulation of gene expression and induction of G2 arrest (discussed by (Longworth and Laimins 2004)). The consequences of the latter function are still under debate, but it has been proposed that when expression of E7 facilitates progression from the G1 to the S phase, expression of E4 inhibits progression towards mitosis, rendering an S-phase environment in the cell which allows accumulation of the viral genomes.

1.1.4.6 E5

The E5 protein is a strongly hydrophobic 83 amino acid (aa) protein localized to the Golgi apparatus, endoplasmic reticulum, nuclear membrane and occasionally in cellular membranes of the host cell (Conrad, Bubb et al. 1993). The most well studied function of E5 is the impaired down regulation of the epidermal growth factor receptor (EGFR) leading to an increased activation of the downstream signaling cascade (reviewed by (Tsai and Chen 2003)). Other suggested effects are interactions with other receptors in the growth factor receptor family and suppression of expression of the cyclin dependent

kinase (cdk) inhibitor p21. Inhibiting expression of p21 leads to activation of cdk4-cyclin D complexes known to inactivate the Rb-check point control (reviewed by (Tsai and Chen 2003)). Like the E4 ORF, E5 is mainly expressed from the late promoter (Howley 1996) and the E5 gene is frequently deleted when the HPV genome is integrated into the host genome (Pater and Pater 1985). If integration in the human genome is necessary for development of HPV-induced cancer (discussed below), and the viral genome is integrated before there has been a switch to the late promoter, the E5 ORF will not be expressed to a large extent. It is therefore unclear in what phase of the viral life cycle E5 has effects on cancer development in humans and needs to be investigated more in detail.

1.1.4.7 E6

The E6 ORF encodes for one of the two oncoproteins and is in HPV-16 a 151 aa protein containing two zinc finger domains. Together with E7, the E6 ORF is expressed early in the viral life cycle. The E6 protein alone can immortalize primary human mammary epithelial cells (Liu, Chen et al. 1999), but to immortalize human foreskin keratinocytes additional activity from the E7 protein is required (Kiyono, Foster et al. 1998). The most well studied effect of E6 is the ability to induce ubiquitin dependent proteolysis of p53 by interacting with the E6-associated protein (E6-AP) (Scheffner, Werness et al. 1990), resulting in blocking of apoptosis and then accumulation of genomic abnormalities. E6 can also suppress the effects of p53 by inhibiting its translocation to the nucleus (Mantovani and Banks 1999) and blocking apoptosis in p53 independent ways (reviewed by (McMurray, Nguyen et al. 2001)). Another important E6 function is to increase telomerase activity (Kiyono, Foster et al. 1998) which is a feature observed in normal proliferating cells and in most tumors. This increased activity results in longer telomeres, which are suggested to control the number of cell divisions a cell can undergo. The E6 protein has also been suggested to alter gene transcription and interacts with other proteins (reviewed by (McMurray, Nguyen et al. 2001)).

The effects of E6 to prevent p53 mediated arrest and apoptosis could be very important during infection since both expression of E2 and E7 has been shown to increase the amount of p53 (reviewed by (McMurray, Nguyen et al. 2001)).

1.1.4.8 E7

The 98 aa protein E7 is a nuclear protein and it is the protein considered to have the major transforming effects. As mentioned above, E7 is expressed early in the viral life cycle and E7 alone is sufficient to immortalize human foreskin keratinocytes (Halbert, Demers et al. 1991). The most important effect of E7 known so far is the binding to the retinoblastoma (Rb) family members. When bound, the transcription factor E2F is released and this induces transcription of cyclins and cdks necessary for progression of the cell cycle from G1 to S phase (reviewed by (McMurray, Nguyen et al. 2001)). Several other effects of E7 have been described, such as association with histone deacetylases, AP-1 transcription factors, cyclins and cdk inhibitors (reviewed by (McMurray, Nguyen et al. 2001)), but the consequences of these associations are not fully understood (reviewed by (McMurray, Nguyen et al. 2001)).

1.1.4.9 L1

The L1 ORF is like E1 a well conserved region between different HPV types and codes for the major capsid protein (Howley 1996), which is only expressed in terminally differentiated cells. The L1 protein self-assembles into pentameres, and 72 pentameres then self-assemble to form one virus-like particle (VLP). This feature has been used for development of prophylactic vaccines, discussed further in chapter 1.5.

1.1.4.10 L2

The L2 ORF encodes for the minor capsid protein and is usually present in far lower amounts than L1 in the viral particle and as L1, it is expressed only in terminally differentiated cells (Howley 1996). Papillomavirus can assemble to viral particles in the absence of L2, however when L2 is present packing occurs more efficiently (Stauffer, Raj et al. 1998) and the viral infectivity is much higher (Roden, Day et al. 2001).

1.1.5 Integration of the viral genome

In cervical cancer, the HPV genome is mainly integrated in the host genome (reviewed in (Pett and Coleman 2007)), which frequently leads to disruption of the E2 gene regulating the expression of E6 and E7. Integration of the viral genome as one step in malignant progression would provide evidence for a clonal relationship and therefore support HPV as an etiologic agent also in tonsillar cancer. However, the studies of the physical status of HPV in tonsillar cancer has not been conclusive, some studies report that HPV is mainly episomal (Snijders, Cromme et al. 1992; Mellin, Dahlgren et al. 2002) while others report the opposite (Begum, Cao et al. 2005; Kim, Koo et al. 2007). All these studies used different methods to address this issue, and could explain the discrepancy, but the physical status of HPV in tonsillar cancer has to be clarified in further studies.

1.2 HPV IN HUMAN DISEASES

HPVs are divided into low risk types and high risk types depending on their potential to cause benign lesions or malignant disease.

1.2.1 Benign lesions

Common warts on hands and feet are caused by low risk HPVs (reviewed by (zur Hausen 1996)) and are spread through contact. A common way of spreading is through sharing public changing rooms and showers.

Genital warts (condyloma accuminata) are caused by the HPV types 6 and 11 and condyloma accuminata is one of the most common sexually transmitted infections which occur in the genital tract in both men and women. Recurrent respiratory papillomatosis is a rare disease occurring both in infants (early onset) and in adults (adult onset) and is also caused by HPV types 6 and 11 (reviewed by (Aaltonen, Rihkanen et al. 2002)).

1.2.2 Malignant diseases

High risk HPVs were in 2002 estimated to cause 5.2% of all cancer worldwide (Parkin 2006). HPVs are associated with cancer in the genital tract, in skin and in the head and neck region. The distribution of HPV positive cases is shown in table II.

Table II The distribution of HPV positive tumors (Parkin 2006)

Anatomical site	Percentage of high risk HPV
Cervix	100%
Anus	90%
Penis	40%
Vulva, vagina	40%
Oropharynx	12%
Oral cavity	3%

1.2.2.1 Cervical cancer

Cervical cancer is the second most common cancer in women worldwide with approximately 493,000 new cases and 274,000 deaths each year (Parkin 2006). The highest incidence is observed in developing countries, while the incidence in industrialized countries has decreased during the past decades, due to a highly effective screening program, for example the one in England (Quinn, Babb et al. 1999). The early detection and treatment of pre-malignant lesions has decreased the cases of cervical cancer substantially. High risk HPV is considered a necessary cause of cervical cancer (Bosch, Manos et al. 1995; Walboomers, Jacobs et al. 1999).

1.2.2.2 Other anogenital cancers

Cancer of the penis, vagina and vulva has been reported to harbor high risk HPV in 40% of all cases. All these cancers are rare and the etiological link between them and HPV is not as strong as for cervical cancer (Parkin 2006).

1.2.2.3 Head and neck cancer

HPVs have been suggested to contribute to 12% of oropharyngeal cancers and 3% of oral cavity cancers when the global health burden of infection-associated cancers was estimated for the year 2002 (Parkin 2006). However, the presence of HPV in squamous cell carcinoma of the head and neck (HNSCC) has been reported by others to be around 25% (Gillison, Koch et al. 2000). Tonsillar cancer is the head and neck tumor where HPV most frequently is found and several recent reports detect HPV DNA in 40-75% of all cases (reviewed by (Dahlstrand and Dalianis 2005)). The data

suggesting 12% presented by (Parkin 2006) is based on a study including patients from nine countries known to have a higher prevalence of tobacco smoking or chewing (Herrero, Castellsague et al. 2003), compared to for example the Scandinavian countries and some parts of the US. Geographic variations of HPV frequency in oropharyngeal cancer has also been reported recently (Li, Tran et al. 2007) and could explain this discrepancy.

1.3 HPV DNA DETECTION METHODS

The most commonly used method for detection of HPV DNA is the PCR method. A general HPV PCR is often performed to screen for presence of several different HPV types (Tieben, ter Schegget et al. 1993; de Roda Husman, Walboomers et al. 1995). If positive, the HPV type can thereafter be determined either by type specific PCR with primers targeting a region not well conserved between different HPV types (Hagmar, Johansson et al. 1992; Karlsen, Kalantari et al. 1996), by direct sequencing of the general HPV PCR product or by two different reverse hybridization techniques. The latter methods are reverse line blot (van den Brule, Pol et al. 2002) and line probe assay (Kleter, van Doorn et al. 1999) and the choice of method depends on the primers used for initial HPV detection.

There is also an hybridization method commonly used for HPV screening called the Hybrid Capture 2, which is the only FDA approved HPV detection test. This assay has synthetic RNA probes complementary to DNA sequences in five different low risk types and 13 different high risk types (Clavel, Masure et al. 1999) and has been reported to have a lower sensitivity than the PCR method (Hesselink, van den Brule et al. 2004).

There are other methods for detection of HPV DNA, for example southern blot hybridization, dot blot hybridization and DNA in situ hybridization (ISH). All these methods are less sensitive than the PCR method and usually require larger amounts of material, and are therefore not frequently used for HPV detection today. One advantage of the DNA ISH method is that it can localize the viral DNA within the tissue analyzed and also whether the DNA is found in the cytoplasm or in the nucleus.

1.4 HEAD AND NECK CANCER

There are approximately 644,000 new cases of head and neck cancer diagnosed in the world each year excluding esophageal cancer (462,000 new cases) (Parkin, Bray et al. 2005). In Sweden, head and neck cancer comprises 3-4% of all cancer cases diagnosed each year, rendering approximately 1000 new cases (Hammarstedt, Dahlstrand et al. 2007). The number of new cases and the age standardized incidence in Sweden for tonsillar cancer, base of tongue cancer and mobile tongue cancer in 2006 is presented in table III (including only squamous cell carcinomas (SCC)) (Socialstyrelsen 2006).

Table III Number of new cases and age standardized incidence per 100,000 for tonsillar and tongue cancer in Sweden 2006

Tumor type	Cases	Incidence women	Incidence men
Tonsillar cancer	150	0,93	2,12
Base of tongue cancer[1]	46	0,26	0,48
Mobile tongue cancer[2]	129	0,93	1,57

[1]The part of the tongue that belongs to the oropharynx or
[2]the oral cavity, separated by the circumvallate papillae

1.4.1 Anatomy and symptoms

Head and neck cancer includes cancer of the lip, the oral cavity, the nose and sinuses, the nasopharynx, the oropharynx, the hypopharynx, the larynx, esophagus, the salivary glands and the soft tissues of the neck and ear (fig 3). Patients with cancer in these anatomical sites may have problems with swallowing, persistent hoarseness, changes of the voice, persistent pain, thickening of the soft tissues, a persistent mucosal wound or bleeding from the head and neck region. A common first symptom of oropharyngeal cancer is a lump on the neck, and hence sometimes the primary tumor gives no symptoms until it is fairly large.

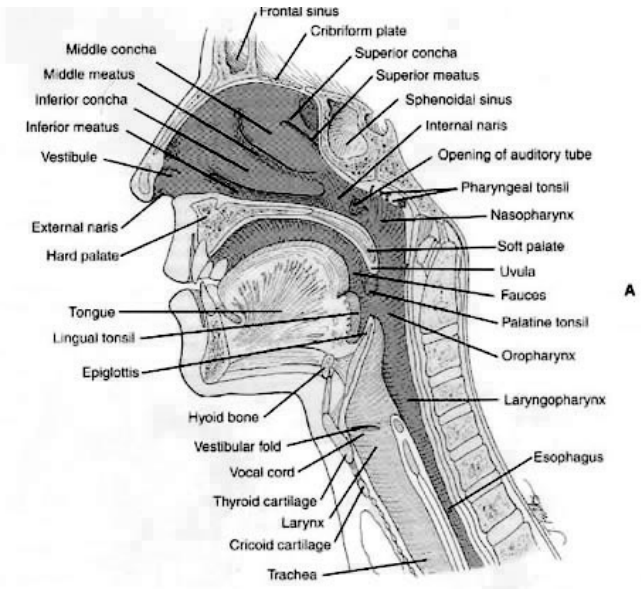

Figure 3 Anatomy of the head and neck

1.4.2 Risk factors

Smoking and alcohol are well established classical risk factors for development of head and neck cancer (IARC 1986). Other well known risk factors are betel nut chewing and tobacco chewing. Viral agents also contribute to the development of head and neck cancer, for example, Epstein-Barr virus is associated with nasopharyngeal cancer and there is evidence that high risk HPVs, mainly HPV-16, cause oropharyngeal cancer (Gillison and Lowy 2004). In one recent case-control study including 100 cases and 200 matched controls (D'Souza, Kreimer et al. 2007), several other risk factors for development of oropharyngeal carcinoma were observed: a high lifetime number of vaginal-sex partners, a high lifetime number of oral-sex partners, high risk HPV infection and seropositivity for the HPV-16 L1 capsid protein. It has also been shown that there is an increased risk of tonsillar cancer for patients with a previous anogenital tumor (Frisch and Biggar 1999; Hemminki, Dong et al. 2000).

1.4.3 Histopathology

Most tumors in the head and neck region are SCC and are graded depending on differentiation as well-, moderately-, poorly- and undifferentiated. In HPV positive tonsillar cancer, the grade is more frequently poorly differentiated as compared to HPV negative tonsillar cancer and the tumor has a “basaloid” morphology (Gillison, Koch et al. 2000; Klussmann, Weissenborn et al. 2001). Another feature commonly seen in HPV infected cells is the appearance of halos around the nucleus. This phenomenon is called koilocytosis and can also sometimes be observed when the cells are infected by other viruses.

1.4.4 Classification

The most widely used classification system for oral cavity and oropharyngeal tumors is the TNM system developed by the International Union Against Cancer (UICC) (Sobin 1997). The classification is performed prior to treatment and is based on the size of the primary tumor (T), presence, size and localization of regional lymph node metastasis (N) and presence of distant metastasis (M). The TNM characteristics are then combined in stages (fig 4). There is an updated version from 2002 of the TNM classification system with minor changes, but none of the patients in our study are classified according to this version.

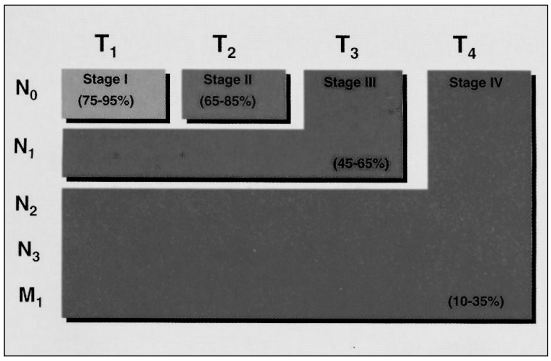

Figure 4 The TNM classification system (survival rates depending on stage)

1.4.5 Genetic alterations in HNSCC

Activation of proto-oncogenes and inactivation of tumor suppressor genes are the two most common genetic alterations which contribute to malignant progression. Common tumor suppressor genes lost in HNSCC are $p16^{INK4a}$ and p53, and common oncogenes activated in HNSCC are EGFR and Her2 (Perez-Ordonez, Beauchemin et al. 2006). When comparing HPV positive and negative tonsillar cancer, it has been shown that HPV positive tonsillar cancer has a higher degree of aneuploidy (Mellin, Friesland et al. 2003) and a different pattern of DNA aberrations (Dahlgren, Mellin et al. 2003) as compared to HPV negative tonsillar cancer. Interestingly, in one recent report, the gene expression profile in HPV positive and negative oropharyngeal (tonsillar and base of tongue only) cancer and in normal epithelium was studied, and several differentially expressed genes were reported (Martinez, Wang et al. 2007), which supports the idea that HPV positive tonsillar cancer has a different molecular biology. The gene expression pattern and proteomic profiling are important approaches to further characterize the biology of HPV positive tonsillar cancer.

1.4.6 Treatment

Treatment with curative intention is based on radiotherapy (RT), surgery or in most cases a combination of both. When combining the two treatment modalities, RT can be given pre-operatively or post-operatively and this differs between different hospitals and countries. At the Karolinska University Hospital, RT is frequently given pre-operatively, which has the advantage of better blood flow and hence better oxygen supply. The advantage of post-operative RT is that the surgeon can operate in non-radiated tissue. There are no reported differences in survival between the two methods. Treatment of all head and neck tumors can result in extensive surgery and reconstruction of different anatomical structures. In this thesis, tonsillar cancer and tongue cancer are the head and neck tumors studied, therefore only treatment of these tumors will be described more in detail.

The treatment for tongue cancer differs depending on if the primary tumor is from the part of the tongue localized to the oral cavity (mobile tongue) or to the base of the

tongue which is localized to the oropharynx (see below). Small mobile tongue tumors (T1) without metastasis are treated with local surgery only. For larger tumors (T2-T4) RT is added and directed both to the primary tumor and to the neck, since relapse with regional lymph node metastasis is common. When there is regional metastasis, a neck dissection is also performed.

Tonsillar cancer and base of tongue cancer with localized disease is usually only treated with RT towards the primary tumor and the neck. When there is presence of regional lymph node metastasis (N1-N3), RT is given towards the neck region and then a neck dissection is performed. If there are signs of viable tumor at the site of the primary tumor, surgery is also performed here. In the past years, chemotherapy has been added in some cases to the treatment but the benefit of this therapy has not been evaluated to a large extent yet.

1.4.7 Prognosis

The five-year survival is 30-40 % for patients with head and neck cancer, but it varies substantially depending on the site and sub-site of the primary tumor (Sant, Aareleid et al. 2003) (table IV) and also depending on the stage at diagnosis (fig 4). A higher stage correlates to a worse prognosis.

Table IV 5-year survival in head and neck cancer

Site of primary tumor	5-year relative survival
Lip	94%
Larynx	62%
Salivary glands	61%
Tongue	39%
Oral cavity	45%
Oropharynx	32%
Nasopharynx	43%
Hypopharynx	25%
Oesophagus	10%

1.4.8 The incidence of tonsillar cancer is increasing

In Sweden 414 new cases of tonsillar cancer were diagnosed between 2000-2003, hence approximately 100 new cases are presented each year (Hammarstedt, Dahlstrand et al. 2007). In one study, performed by our group, we showed an increase in incidence by 2.6% per year in men and 1.1% per year in women between 1960-2003 in Sweden (Hammarstedt, Dahlstrand et al. 2007). This finding is in line with what has previously been reported in Finland and the US (Frisch, Hjalgrim et al. 2000; Syrjanen 2004). This is interesting since the incidence of all other head and neck cancers as well as lung

cancer, all known to be related to smoking, have decreased substantially during the same time period in Sweden, most likely as a consequence of decreased prevalence of smoking (Hammarstedt, Dahlstrand et al. 2007). At the same time, there is now accumulating evidence for HPV as a causative agent in tonsillar cancer (see below) and it is therefore reasonable to suspect HPV as a risk factor for the increase in incidence.

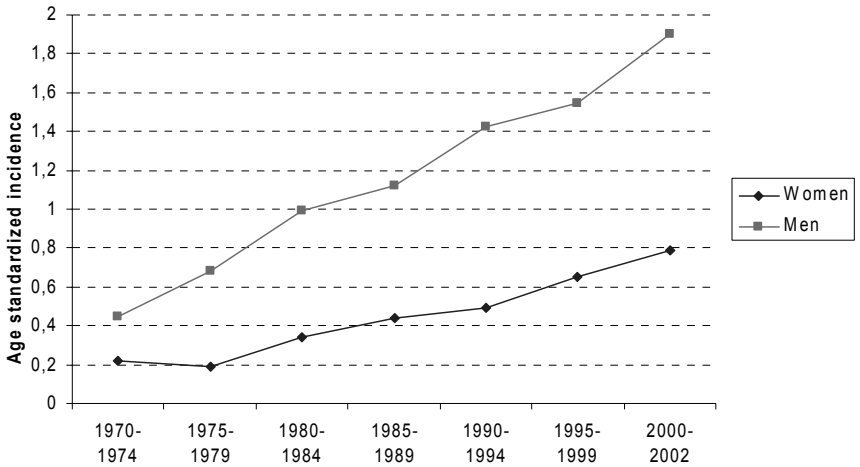

Figure 5 The incidence of oropharyngeal cancer per 100,000 inhabitants (excluding base of tongue cancer) in Sweden 1970-2002 (Socialstyrelsen 2006)

1.4.9 HPV in tonsillar cancer

From the end of the 20th century it has become clearer that there is an association between HPV and oropharyngeal cancer, but to what extent HPV is involved in other HNSCC is still uncertain. When studying HNSCC as a group, HPV is commonly found in approximately 25-28% of the tumors (Gillison, Koch et al. 2000; Kreimer, Clifford et al. 2005; Ragin and Taioli 2007), but in oropharyngeal cancer it is almost always found in a higher frequency (Snijders, Cromme et al. 1992; Paz, Cook et al. 1997; Gillison, Koch et al. 2000; Mellin, Friesland et al. 2000; Dahlstrand and Dalianis 2005). When evaluating presence of HPV as a risk factor for SCC, the occurrence of HPV in normal tissue must also be considered. The prevalence of HPV in non-cancerous tissue in the oral cavity has been reported to be around 10% (reviewed by (Franceschi, Munoz et al. 1996; Miller and Johnstone 2001)). However, less is known about presence of HPV in non-cancerous oropharyngeal tissue, possibly due to difficulties in collecting material. In most studies performed, material is collected from patients who undertake surgery for other reasons than malignancy, most commonly tonsillectomy due to chronic tonsillitis or tonsillar hypertrophy. This usually raises the problem that these patients are younger and thus could not be accurately compared to patients with tonsillar cancer. In three studies performed so far, including patients at older ages, the prevalence of HPV in non-cancerous oropharyngeal tissue was estimated to 6.3-14% (Fukushima, Ogura et al. 1994; Chen, Sehr et al. 2005; do Sacramento, Babeto et al.

2006). In conclusion, when comparing these findings with the prevalence of HPV reported for tonsillar cancer patients, HPV is found at a higher frequency in tonsillar cancer, supporting HPV as a causative agent in tonsillar cancer.

One large seroepidemiological cohort study has been performed so far (Mork, Lie et al. 2001) including 292 patients with head and neck cancer and 1568 controls. They were tested for HPV capsid antibodies and seropositivity increased the risk for developing head and neck cancer. Interestingly, the risk was high for tonsillar cancer with an odds-ratio (OR) of 10.2, and an even higher OR (20.7) was observed for base of tongue cancer. The higher risk of developing base of tongue cancer, as compared to oropharyngeal cancer as a group, was not unexpected since previous studies have shown that HPV is more common in tonsillar and base of tongue cancer compared to tumors at other sites in the oropharynx (Gillison, Koch et al. 2000; Klussmann, Weissenborn et al. 2001).

HPV has in many studies shown to be a predictive factor for better prognosis in tonsillar cancer (Alani and Munger 1998; Andl, Kahn et al. 1998; Gillison, Koch et al. 2000; Mellin, Friesland et al. 2000; Mellin, Dahlgren et al. 2002; Li, Thompson et al. 2003) and in one recent meta analysis, HPV positive patients had a better survival only when the tumor originated from oropharynx as compared to other head and neck sites (Hobbs, Sterne et al. 2006). A number of recent epidemiological studies also supports HPV as a risk factor for development of oropharyngeal cancer (Herrero, Castellsague et al. 2003; Hansson, Rosenquist et al. 2005; D'Souza, Kreimer et al. 2007).

The route of transmission is still under debate. However, there is now one recent case-control study suggesting that oral HPV infection is sexually acquired (D'Souza, Kreimer et al. 2007). In this study both a high number of vaginal- and oral-sex partners, and high risk HPV infection, were risk factors for developing oropharyngeal cancer. There are also other epidemiological studies supporting a sexual route of transmission for HPV infection in the head and neck region (Maden, Beckmann et al. 1992; Schwartz, Daling et al. 1998; Smith, Hoffman et al. 1998), but other means of transmission, such as mother to child at birth (Syrjanen and Puranen 2000) or mouth-to-mouth contact can not be excluded.

1.5 HPV VACCINES

There are today two approved prophylactic vaccines for prevention of HPV infection with completed phase III trials: one quadrivalent vaccine targeting HPV types 6, 11, 16 and 18 (2007; Garland, Hernandez-Avila et al. 2007) and one bivalent vaccine targeting HPV types 16 and 18 (Paavonen, Jenkins et al. 2007). The vaccines consist of the major capsid protein L1 which self-assembles to form VLPs. The VLPs are purified after expression in yeast cells or in insect cells using baculoviruses, and are administered with an adjuvant. They contain no genome and are thus not infectious. The vaccines are administered intramuscularly in three doses given during six months. The phase III trials of both the vaccines have shown a 95-100% efficacy in preventing HPV 16- and 18-related precancerous lesions, given that the vaccine has been administered before exposure to the virus (2007; Garland, Hernandez-Avila et al. 2007; Paavonen, Jenkins et al. 2007).

In Sweden, the quadrivalent HPV vaccine was approved for use in females 20th September 2006 by the European Medicines Agency (EMEA) (Läkemedelsverket 2006) and a Swedish consensus meeting in May 2006 was held by invited experts. The conclusion was the recommendation of a general vaccination program for schoolgirls between 11 and 15 years old, but no decision has been made yet.

2 AIM OF THE THESIS

The aim of this thesis was to study the presence and influence of HPV in tonsillar and tongue cancer and in particular:

- To confirm HPV as a positive prognostic factor for disease specific survival in tonsillar cancer
- To clarify if the frequency of HPV varies in different sub-locations of cancer in the tongue and to investigate if HPV is a positive prognostic factor for disease specific survival in these patients
- To evaluate $p16^{INK4a}$ as a molecular marker for presence of HPV in tonsillar cancer and as a prognostic marker for response to radiotherapy and survival
- To evaluate if HPV is a potential risk factor for the increase in incidence of tonsillar cancer in the Stockholm area
- To study the presence of HPV, HPV-16 viral load and expression of HPV-16 E6 and E7 mRNA in tonsillar cancer and to study the possible influence of these factors on prognosis

3 MATERIAL AND METHODS

3.1 PATIENTS AND SAMPLES

All patients included in papers I-IV were diagnosed with primary squamous cell carcinoma of the tonsils or the tongue in the Stockholm County 1970-2002. Patients with tonsillar cancer were treated at one of the hospitals in the Stockholm area and all patients with tongue cancer were treated at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery at Karolinska University Hospital. The clinical data and tumor characteristics from all patients were collected from patient files and are summarized in Table I in papers I and IV. Archival paraffin blocks were collected for all patients except for 11 patients, where fresh frozen biopsies were collected.

A total of 25-30 µm from the paraffin blocks or from the fresh frozen pre-treatment biopsies, cut in either 5 or 10 µm sections, were collected for DNA and RNA extraction. Before and after the sections a 4-µm section was taken for staining with H&E to verify presence of tumor tissue in the sample. Each sample was studied by a pathologist for confirmation of diagnosis and to verify that the biopsies contained at least 70% tumor material. All samples with less tumor material were microdissected to avoid the presence of normal tissue that might affect the result of the PCR. To check for HPV contamination, sections from an empty paraffin block were collected between each tumor sample and treated in the same way as the tumor samples.

3.2 DNA AND RNA EXTRACTION

DNA extraction from paraffin embedded material was performed using either a standard phenol-chloroform protocol, the QIAamp DNA Mini kit (Merck Eurolab AB) according to the manufacturer's instructions, or as in most cases, using the High Pure RNA kit (Roche Diagnostics, Stockholm, Sweden) according to the manufacturer's instructions, excluding DNase treatment. RNA extraction of the samples in paper IV was performed using the High Pure RNA kit (Roche Diagnostics, Stockholm, Sweden) according to the manufacturer's instructions. DNA and RNA content was measured with a spectrophotometer. All samples analyzed by real time PCR (see below) were extracted with the High Pure RNA kit.

In paper II, DNA extraction and PCR analysis of the samples from the included patients had already been performed in earlier studies (Mellin, Friesland et al. 2000; Mellin, Dahlgren et al. 2002). Eleven of the 51 biopsies were extracted from fresh frozen material according to a standard phenol-chlorophorm protocol with proteinase K treatment and washing with ethanol.

3.3 GENERAL HPV PCR

For detection of at least 27 HPV types, consensus primers GP5+/6+, located within the conserved L1 region, were used (de Roda Husman, Walboomers et al. 1995). The final volume of 50 µl was a solution consisting of 5 µl 10×PCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 3.5 mM magnesium chloride ($MgCl_2$), 25 pmol of each primer, 0.5 µg/µl bovine serum albumine (BSA) and 1 U Taq

polymerase (AmpliTaq DNA polymerase, Applied Biosystems). Cloned plasmids of HPV-16 were used as positive controls and a negative control with no DNA (water) was always included. The program started with 4 min denaturation at 94°C followed by 40 cycles of 94°C for 1 min, 44°C for 1 min, 71°C for 2 min, and finally 71°C for 10 min.

The samples negative for GP5+/6+ were further tested using CpI/IIG primers, located within the E1 region (Smits, Tieben et al. 1992). The 50 µl used for this amplification was a solution of 5 µl 10×PCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 3 mM $MgCl_2$, 17 pmol of CpI, 26 pmol of CpIIG, 0.5 µg/µl BSA and 2.5 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). Positive and negative controls were used as described above. This program started with 5 min denaturation at 94°C followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, and finally 72°C for 10 min.

3.4 TYPE SPECIFIC HPV PCR

To determine the HPV type, type specific primers for HPV-16, -18 and -33 were used (Karlsen, Kalantari et al. 1996) and in some cases the samples from the general HPV PCR were also sequenced for type verification (see below). The 50 µl PCR mix used for these amplifications consisted of 5 µl 10×PCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 3.5 mM $MgCl_2$, 20 pmol of each primer and 1 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). Cloned plasmids of HPV-16, -18 and 33 were used as positive controls and a negative control with no DNA (water) was included. The PCR program started with 4.5 min denaturation at 95°C followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, and finally 72°C for 4 min.

3.5 VERIFICATION OF AMPLIFIABLE DNA BY PCR

To check if amplifiable DNA could be obtained from the samples, 127 bp of the ribosomal S14 gene was amplified with S14 sense/anti-sense primers. The 50 µl PCR mix consisted of 5 µl 10×PCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide, 1.5 mM $MgCl_2$, 15 pmol of each primer, 0.5 µg/µl BSA and 1 U Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems). A positive control (human DNA) and a negative control (water) were included in each run. A 40-cycle amplification was run in an automated thermocycler (Perklin-Elmer, Norwalk, CT). The program started with 1 min denaturation at 94°C followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, and finally 72°C for 10 min.

3.6 SEQUENCING OF HPV POSITIVE PCR PRODUCTS

The results from the type specific PCR were in some cases confirmed by direct sequencing of the PCR products from the general HPV PCR. The general HPV PCR was re-run to increase the yield. The products were purified by gel extraction (QIAquick PCR Purification Kit, Merck Eurolab AB) and were then sequenced using the Big Dye Terminator Cycle Sequencing Kit and an ABI PRISM 377 DNA Sequencer (Applied Biosystems). Both DNA strands were sequenced and aligned to

those available at NCBI BLAST GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

3.7 REAL TIME QUANTITATIVE PCR

In paper IV, the number of HPV copies per genome equivalent was estimated using a real time quantitative TaqMan PCR. A dilution series of a cloned plasmid of HPV-16 was used in order to create a standard curve with known numbers of viral copies. The primers and the probe used are described in (Mellin, Dahlgren et al. 2002). Ten µl of sample DNA were run in three different concentrations in triplicates in 25 µl reactions containing 2.5 µl 10x PCR buffer II (Applied biosystems), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer, 5 pmol probe and 0.5 U of Taq DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems). A 40-cycle amplification was run in an iCycler iQ (iCycler iQ real-time PCR detection system, BioRad) and the program consisted of an initial step at 50°C for 2 min, and 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 sec, and annealing and elongation at 60°C for 1 min. As an internal reference gene, a commercial kit was used according to the manufacturer's instructions to estimate the gene content of the human RNase P gene (TaqMan RNase P Detection Reagents Kit, Applied Biosystems, Stockholm, Sweden). Calculation of viral load/genome-equivalent was performed as described by (Si, Tsao et al. 2003).

The detection of E6 and E7 mRNA was also performed as a real time PCR with the primers presented in paper IV and the same standard dilution series as for the estimation of the viral load was used. After RNA extraction as described above, cDNA was synthesized from the extracted RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Copenhagen, Denmark). An HPV-16 type specific PCR was run as described above before cDNA synthesis to verify that no contaminating DNA from the RNA extraction was present. Ten µl of cDNA were run in a SybrGreen protocol in an iCycler iQ with the 25 µl reaction mix containing 12.5 µl iQMN SYBR Green Supermix (BioRad Laboratories) and 10 pmol of each of the HPV-16 primers. The program started with 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, then a melting curve was applied to verify specificity, starting at 40°C and increasing by 0.5°C every 10^{th} second until 120°C was reached. The commercial kit used for quantification of DNA (see above) was used as a control to verify presence of cDNA.

3.8 IMMUNOHISTOCHEMICAL ANALYSIS OF $p16^{INK4a}$ AND OF L1

In paper II, $p16^{INK4a}$ was evaluated as a biomarker for the presence of high risk HPV. Immunohistochemistry (IHC) was performed with the primary monoclonal mouse anti-human $p16^{INK4a}$ antibody (clone E6H4; DakoCytomation A/S) as described in detail in paper II. The staining was evaluated by light microscopy and was scored on a semi-quantitative scale either as negative (<10% of tumor cells positive), 1+ (10-50% of positive tumor cells), 2+ (50-90% of positive cells) or 3+ (>90% positive cells).

HPV L1 capsid protein was also detected by IHC, performed with a monoclonal mouse anti-HPV antibody (clone K1H8; DakoCytomation A/S), using the same protocol as with $p16^{INK4a}$ described in detail in paper II. This antibody binds to a non-conformational internal epitope of the major capsid protein of HPV-1 and has been found to stain nuclei of cells infected by HPV-6, -11, -16, -18, -31, -33, -42, -52, -56 and -58. A strong nuclear staining, evaluated by light microscopy, of the L1 capsid was considered a positive staining.

A positive tissue control section from a cervical carcinoma in situ and a negative antibody control, a monoclonal mouse IgG2a, was included with all samples.

3.9 METHODOLOGICAL CONSIDERATIONS

3.9.1 DNA and RNA extraction

In total, three different DNA extraction methods have been used. The High Pure RNA Kit is the method most extensively used in all papers taken together, since it yields very pure products and was possible to use both for DNA and RNA. The QIAamp DNA Mini Kit also gives a pure product and shows no major differences compared to the High Pure RNA kit, but it could not be used for RNA extraction. Both kits have the advantage of being clean to work with and not containing organic material that can inhibit the following PCR, compared to the traditional phenol-chlorophorm protocol.

3.9.2 HPV detection by PCR

In this thesis, screening for HPV has been performed by using the two consensus primer pairs Gp5+/6+ and CpI/IIG targeting the conserved regions L1 and E1. The main reason for including the second general HPV PCR protocol was due to the risk of not having the L1 gene retained in tumor cells. However, the E1 region is also sometimes lost, for example when the viral DNA is integrated in the host genome (zur Hausen 1999). Therefore we also performed an HPV-16 type specific PCR in all samples investigated in paper III and IV. With this method we discovered 19 more HPV positive patients in a material of 203 patients. One explanation for this might be that it has been shown by others in the group that the HPV-16 type specific PCR is more sensitive than the general HPV PCR methods.

However, theoretically, this is a methodological bias since we know very little about the types of HPV present in tonsillar cancer. One can speculate that these cases where E1 and L1 not could be detected by PCR but the E6 region could be found, the virus had integrated in the host genome and lost most genes but not the oncogenes E6 and E7. If such cases of integration do exist in our material for HPV types other than HPV-16, for example HPV-33 integrated with only E6 and E7 intact, the HPV DNA would not be found in our current assay, since we only detect E6 from HPV-16.

In the future, more emphasis should be made in determining the HPV type, since prophylactic vaccines are now available (see below), in order to define which groups of patients could benefit from vaccination and for the development of new vaccines.

3.9.3 Real time PCR

Two different protocols were used for real time PCR: the TaqMan technique and the SybrGreen technique. The SybrGreen system is not specific for the target gene, but instead it binds to all double stranded DNA and emits a florescent light at 490 nm when bound, hence the technique is highly dependent on the specificity of the primers. To verify the specificity a melting curve is performed when the 40 cycles are run as described above, and with high specificity of the amplification, a peak melting temperature depending on the length of the amplicon is found.

Using the TaqMan technique on the other hand, no melt curve is needed since a flourogenic probe specific for the amplicon is used and this probe only emits light when it is "pushed off" by the DNA polymerase. Another approach not used in this thesis is to connect the flourophore to the primers directly.

The aim when choosing method for detection of RNA was to have a method as comparable as possible to the method for viral load estimation, in order to compare these results. However, little is known about the expression of the RNase P gene and to have reliable results to estimate the amount of expression, not one, but three different well characterized house keeping genes with assays designed for that purpose should be used. Therefore, we chose only to determine the presence of expression of E6 and E7 mRNA.

3.10 STATISTICAL ANALYSIS

All significance testing was performed at the 0.05 level and two sided p-values were reported. A non-parametric method was always used where applicable.

3.10.1 Paper I

The methods used for statistical analysis in paper I were exact logistic regression and Fisher's exact 2-tailed test, when correlating the HPV frequency to clinical parameters. In the survival analysis, a multivariate Cox proportional hazard regression model was used, and the factors analyzed were grade of differentiation, presence of HPV, gender, TNM stage and age at diagnosis. The survival data was presented graphically in a Kaplan-Meier graph, and differences in survival rate were analyzed with the Log-rank test.

3.10.2 Paper II

To compare the different methods in paper two and to analyze the predictive value of the markers analyzed on the response to radiotherapy, being tumor-free after three years or dead from tonsillar carcinoma, exact logistic regression or Fisher's exact 2-tailed test was used. When analyzing the influence of the methods on response to radiotherapy and survival, the results were adjusted for age at diagnosis, gender, differentiation grade of the tumor and TNM stage.

3.10.3 Paper III

When analyzing the frequency of HPV positive cases of tonsillar carcinoma during the different decades, a Chi square test was used and the 1970 population was used for direct standardization of incidence rates. When comparing the age in patients with HPV positive tumors versus patients with HPV negative, a Mann-Whitney test was used.

3.10.4 Paper IV

When correlating the HPV status to clinical parameters, a Chi square test was used. The survival analysis was performed with a multivariate Cox proportional hazard regression model controlling for age at diagnosis, gender and TNM stage, and graphically presented in a Kaplan-Meier graph where differences in survival rate were analyzed by the Log-rank test.

4 RESULTS

4.1 PAPER I

4.1.1 Presence and type specificity of HPV in tongue cancer

One hundred and ten tongue cancer samples had amplifiable DNA and the over all frequency of HPV was 12/110 (10.9%) when analyzed using consensus primers Gp5+/6+ (10 samples) and CpI/IIG (2 samples). HPV was significantly more common in base of tongue cancer (10/25, 40%) than in mobile tongue cancer (2/85, 2.4%) irrespective of gender or age ($p<0.001$, Fischer's exact 2-tailed test). When analyzing different stages, grouped as stage I+II and stage III+IV respectively, HPV was significantly more common in base of tongue cancer for the two lower stages ($p<0.0068$, Fischer's exact 2-tailed test), but this correlation was not found for stage III+IV. However, the number of available cases was limited (2/13 for mobile tongue cancer and 7/16 for base of tongue cancer).

When analyzing all tumors in a multivariate analysis, the only predictive factor for having an HPV positive tumor was tumor localization with an odds ratio of 26.4 for base of tongue cancer. However, when analyzing the different tumor characteristics separately, HPV was found at a higher frequency in poorly differentiated tumors compared to moderately differentiated ($p<0.0077$, exact logistic regression) and well differentiated tumors ($p<0.0009$, exact logistic regression) and in large tumors (Stage III+IV) compared to small tumors (Stage I+II) ($p<0.001$, Fischer's exact 2-tailed test).

When determining HPV type, HPV-16 was found in 9 cases, 2 from mobile tongue and 7 from base of tongue cancer, and HPV-33 and 35 in one case respectively. One sample could not be typed due to limited amount of material.

4.1.2 HPV and tumor characteristics in relation to prognosis

The clinical data for the patients and tumor characteristics for HPV positive and negative mobile tongue cancer and base of tongue cancer is presented in table I, paper I. One month after their primary treatment, 93 patients were free of disease, and nine had residual cancer, while eight had no or palliative treatment. The distribution of the different tumor locations is presented in table V.

Table V Response after primary treatment

	Disease free 1 month	**Residual cancer**	**No or palliative treatment**
Mobile tongue cancer	76	3	6
Base of tongue cancer	17	6	2

The recurrence rate of base of tongue cancer was compared with regard to presence or absence of HPV in the tumors. For the 10 patients with HPV positive tumors the mean time to recurrence was 17 months, and for patients with HPV negative tumors the mean time to recurrence was only 8 months. The recurrence rate could not be analyzed for patients with mobile tongue cancer due to too few HPV-positive cases.

A 3-year survival analysis was performed for patients with base of tongue cancer including all patients with a minimum of 36 months follow up time or if they died of the disease within the same time period. Nineteen patients fulfilled the criteria and could be evaluated. However, when performing survival analysis regarding tumor stage, only 17 patients were included, since tumor stage was unknown/undetermined for two patients. In a Cox multivariate regression model, HPV status and tumor stage were predictive factors for prognosis. Patients with HPV positive base of tongue cancer had significantly longer survival compared to patients with HPV negative tumors (6/8 patients with HPV positive tumors were alive after 3 years as compared to 2/11 patients with HPV negative tumors, $p<0.0159$). This survival analysis is illustrated in a Kaplan-Meier graph in figure I, paper I. When analyzing the 17 patients regarding tumor stage, four patients had tumor stages I or II and 13 patients had tumor stages III or IV. The patient group with stage I or II base of tongue cancer had a significantly better disease specific survival compared to the higher stages III and IV ($p<0.0376$, Cox regression, multivariate). When performing a 5-year survival analysis, HPV was still a positive factor for disease specific survival, but tumor stage was not (for p-values, see paper I).

4.2 PAPER II

4.2.1 Presence and type specificity of HPV in tonsillar cancer

HPV was detected by PCR in 25/51 (49%) with the consensus primers Gp5+/6+ and no additional HPV positive tumors were found with the CpI/IIG primers. Twenty four of the tumors were HPV-16 positive and one tumor was positive for HPV-33.

4.2.2 $P16^{INK4a}$ as a biomarker for HPV detected by PCR

The relation between $p16^{INK4a}$-staining and presence of HPV is summarized in table VI and in table II, paper II.

Table VI Staining of $p16^{INK4a}$ in relation to HPV

$p16^{INK4a}$ -staining	**1+**	**2+**	**3+**	**Neg**	**Total**
HPV positive	5	7	8	5	25
HPV negative	6	1	0	19	26
Total	11	8	8	24	51

Twenty-seven of the 51 (53%) patients had tumors positive for $p16^{INK4a}$ by IHC. Of these, 20/25 (80%) were HPV positive and 7/26 (27%) were HPV negative, resulting in an 80% sensitivity and a significantly more common $p16^{INK4a}$-positivity among the HPV positive tumors ($p<0.001$, logistic regression). Thus, 20/27 $p16^{INK4a}$-positive cases were HPV PCR positive, resulting in a specificity of 73%. As indicated in table VIII, the specificity increases with higher $p16^{INK4a}$ grading, resulting in a specificity of 76% for 1+staining; 95% specificity for 2+staining; and 100% specificity for 3+staining. The expression of $p16^{INK4a}$ was significantly more common in HPV positive tumors compared to HPV negative tumors for grades 2+ and 3+ ($p<0.003$ and $p<0.001$ respectively, logistic regression). The odds for HPV positive cases to stain 2+ or 3+ was 49 times more likely than staining negative and 16 times more likely than to stain 1+ ($p<0.001$ and $p=0.017$, respectively, logistic regression).

4.2.3 Evaluation of other biomarkers for HPV detected by PCR

With ISH, 10/51 (20%) of the tumors were positive for high risk HPV and none were positive for low risk HPV. All cases positive for HPV with ISH were also positive for HPV by PCR, resulting in a sensitivity of 40% and a specificity of 100%.

The L1 capsid protein could only be detected in 4/51 (8%) tumors, three of these cases were also positive for HPV by PCR. Thus, the sensitivity was 12% and the specificity 95% for L1 capsid detection compared to HPV PCR.

4.2.4 Predictive markers for response to radiotherapy

The 49 patients who received full dose RT were included and 33/49 (67%) had a complete response (CR) to RT. A tendency towards a better response to RT was observed when comparing patients with HPV positive and negative tumors, and when comparing $p16^{INK4a}$-positive and negative patients (table II, paper II). However, when studying high-grade (2+ and 3+) $p16^{INK4a}$-staining and combining these two groups, 15/16 patients had a CR to RT and this was significantly better than that observed in the 1+ group, and among the $p16^{INK4a}$-negative cases ($p=0.017$ and $p=0.018$ respectively, logistic regression). This significant observation remained irrespective of gender, tumor stage, differentiation grade and age. Neither in situ DNA hybridization, nor L1 capsid protein detection correlated to response to radiotherapy.

4.2.5 Predictive markers for survival

The parameters staying disease free and survival were correlated with the different detection methods. When analyzing whether the patient was free of tonsillar cancer three years after diagnosis 43 patients were included. As illustrated in table VII, patients with HPV PCR positive tonsillar cancer were significantly more often free of disease after 3 years and had a better disease specific survival.

Table VII Survival is better for patients with HPV positive tumors

	HPV+	HPV-	p-value[1]
Disease free 3 years	17/22 (77%)	6/21 (29%)	0.004
Disease specific survival	17/22 (77%)	5/19 (26%)	0.004

[1]Patients with HPV positive tumors as compared to HPV negative tumors, logistic regression, irrespective of gender, tumor stage, differentiation grade or age

When comparing survival in patients with $p16^{INK4a}$ positive and negative tumors, no correlation was found when comparing positive with negative staining (for p-values see paper II), However, when combining 2+ and 3+ staining into one group, there was a significantly better survival in patients with high $p16^{INK4a}$ staining (2+/3+) as compared to patients with low $p16^{INK4a}$ staining as well as no $p16^{INK4a}$ staining (table VIII). Hence, a high staining of $p16^{INK4a}$ in the tumors correlated to both being disease free after 3 years and disease specific survival.

Table VIII Survival is better in patients with tumors with a high $p16^{INK4a}$ staining

	$p16^{INK4a}$ 2-3+	$p16^{INK4a}$ 1+ [1,2]	$p16^{INK4a}$ neg [1,3]
Disease free 3 years	12/14 (86%)	7/18 (39%)	3/9 (33%)
Disease specific survival	12/14 (86%)	7/18 (39%)	3/9 (33%)

[1]A high $p16^{INK4a}$ staining as compared to low, or no $p16^{INK4a}$ staining, logistic regression, irrespective of gender, tumor stage, differentiation grade or age, [2]p=0.025 and [3]p=0.025

No correlation between disease free status after 3 years and survival could be found for either in situ DNA hybridization or L1 capsid detection (for details see paper II).

4.3 PAPER III

4.3.1 Incidence of tonsillar cancer

The age-standardized incidence of tonsillar cancer increased 2.8-fold between 1970 and 2002 in the Stockholm region. This increase was 2.6-fold in men and 3.6-fold in women (fig 1, paper III).

4.3.2 Presence and typing of HPV in tonsillar cancer

Ninety-nine patients had HPV DNA in their tumors, detected by either the general primers targeting the L1 or the E1 region (80 cases), or the HPV-16 type specific primers targeting the E6 region (19 cases). The type distribution, determined by either type specific primers or direct sequencing, is shown in table IX.

Table IX HPV type distribution in tonsillar cancer

Type	HPV-16	HPV-33	HPV-35	HPV-45	Undetermined
Cases	86 (87%)	3 (3%)	1 (1%)	1 (1%)	7 (7%)

The distribution over the decades is shown in table X. As illustrated, there was a significant increase in the proportion of HPV-positive tonsillar cancer 1990–1999 and 2000–2002 compared to 1970–1979, 2.5-fold (p=0.003, Chi-square test) and 2.9-fold (p<0.001, Chi-square test), respectively. When comparing the 1990–1999 and 2000–2002 with the calendar years 1980-1989, the increase is still significant, 2.0-fold (p=0.0045, Chi-square test) and 2.4-fold (p<0.001, Chi-square test), respectively.

Table X Presence of HPV in tonsillar cancer over time

Calendar years	Presence of HPV	p-value[1]
1970-1979	7/30 (23%)	
1980-1989	12/42 (28%)	0.79
1990-1999	48/84 (57%)	0.0025
2000-2002	32/47 (68%)	<0.001
Total	99/203 (49%)	

[1] Compared to the frequency of HPV in the 1970-1979 calendar period

The number of cases with amplifiable human DNA possible to evaluate for presence of HPV DNA (see also table I, paper III) was for the different time periods; 30/39 (77%) from the 1970s; 42/46 (91%) from the 1980s; 84/86 (98%) from the 1990s and 47/47 (100%) from 2000–2002.

4.3.3 Patient features

The distribution of gender and age for the 99 patients with HPV positive tumors and the 104 patients with HPV negative tumors was compared. No difference in distribution of gender was observed, but the patients with HPV positive tumors had a significantly lower median age (55 years) compared to the patients with HPV negative tumors (65 years) (p<0.001, Mann–Whitney test). The age distribution is shown in fig 6.

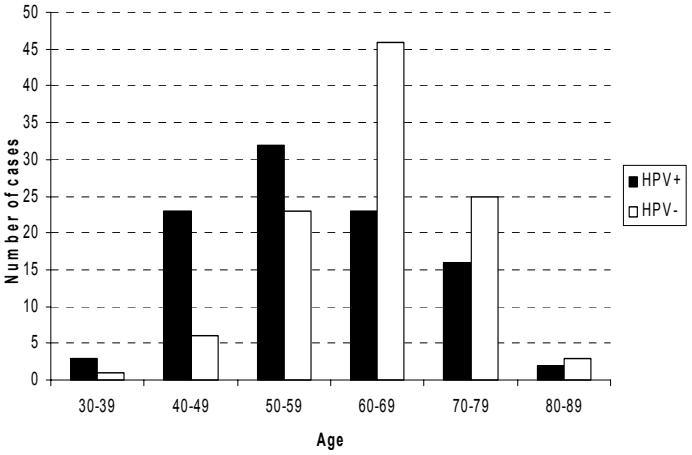

Figure 6 Age distribution among patients with HPV positive and negative tumors

4.4 PAPER IV

4.4.1 Patient and tumor features

The same 203 patients as in paper III were included in this study and their clinical data and tumor characteristics are presented in table I, paper IV. HPV status and distribution over time is discussed above. Complete case reports were available for 192 patients. HPV-positive tumors were more often poorly differentiated (p=0.02, Chi-square test) and patients with HPV-positive tumors had more often regional lymph nodes at the time of diagnosis (p=0.03, Chi-square test), but no correlation between stage and HPV distribution could be found (TNM stages I and II, compared to TNM stages III and IV) (p= 0.63, Chi-square test).

4.4.2 HPV and survival in tonsillar cancer

Only patients receiving curative treatment and with reliable survival data were included in the survival analysis, and 150 patients could be included. As shown in fig I, paper IV, the HPV positive patients had a significantly better 5-year survival ($p<0.001$, Log-rank test) independent of age, gender and tumor stage, as determined with a multivariate Cox proportional hazard regression model (table II, paper IV). The improved survival was consistent over time during the 1980s, the 1990s and 2000-2002, but not during the 1970s, where the number of patients was limited (for p-values, see paper IV).

Eighty-two patients had a local or regional relapse and patients with HPV positive tumors (24 patients, 29%) had significantly less often relapses compared to patients with HPV negative tumors (58 patients, 71%) ($p<0.01$, Chi square test).

4.4.3 HPV, smoking habits and survival in tonsillar cancer

Smoking data could be obtained for 119 patients, including only the cases where the patients smoking habits clearly were documented in the patient files. Smokers (including ex-smokers) and non-smokers were compared with regard to presence of HPV (table XI).

Table XI Smoking habits in patients with HPV positive and negative tumors

	Non-smokers	Smokers
HPV+	12	42
HPV-	62	3

The 12 non-smoking patients with HPV positive tumors were all alive and free of disease with a minimum of two years follow up time, compared to 71% of the smokers with HPV positive tumors and 34% of the smokers with HPV negative tumors. The remaining three patients were either heavy drinkers or had multiple primary tumors at several different locations. When performing a 5-year survival analysis, the survival was significantly better for the non-smokers with HPV positive tumors, both when compared to smokers with HPV positive tumors and to smokers with HPV negative tumors ($p<0.05$ and $p<0.05$, respectively, Log-rank test). Also, smokers with HPV positive tumors had significantly better survival compared to smokers with HPV negative tumors ($p<0.001$, Log-rank test) (fig 7).

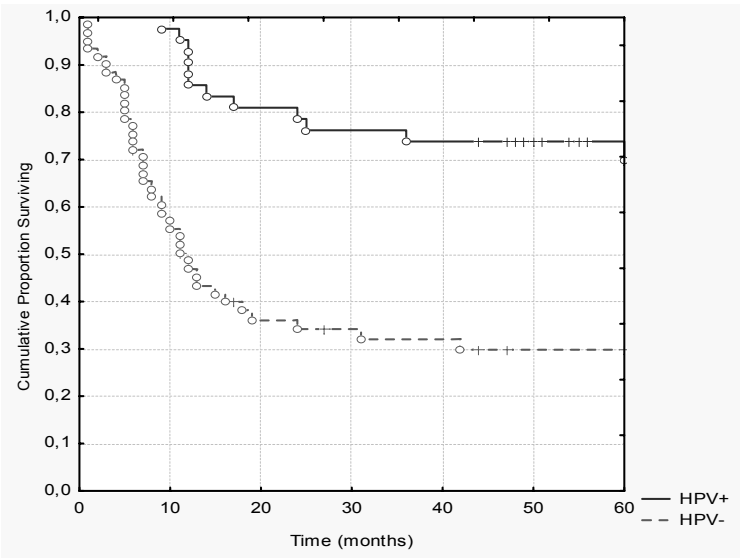

Figure 7 Smokers with HPV positive tumors had significantly better survival compared to smokers with HPV negative tumors

4.4.4 HPV-16 viral load and survival in tonsillar cancer

The viral load was estimated for the 86 HPV-16 positive tumors analyzed in paper III and it varied between 0.08-130 copies/genome equivalent. When correlating with survival, using quartiles as cut-offs for dividing the patients in comparable groups, no clear correlation between survival and viral load could be observed. The cut-offs measured in copies/genome equivalent and the Kaplan-Meier graph are illustrated in fig 8, where the patients with intermediate viral load had the best survival, whereas the patients with the highest and the lowest viral load had a lower survival rate. However, all the groups had significantly better survival compared to patients with HPV negative tumors.

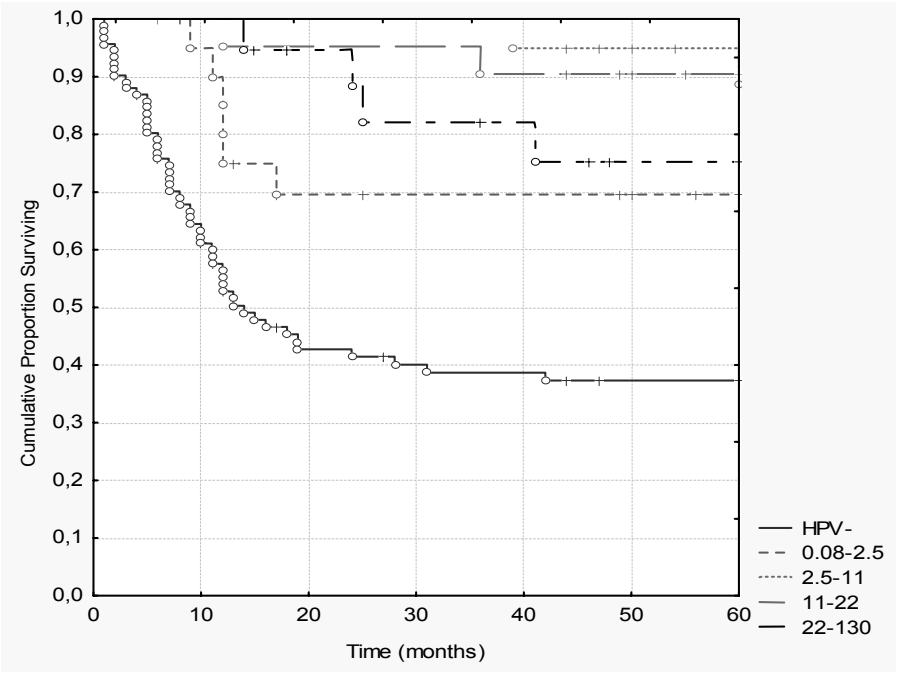

Figure 8 Viral load in correlation to survival

4.4.5 Expression of HPV-16 mRNA in tonsillar cancer

Fifty-four of the 86 HPV-16 positive tumors analyzed in paper III were available for further analysis of expression of mRNA. The tumors not available for analysis were either not available from the archive for unknown reasons (20 cases) or contained too little material in the paraffin block (12 cases). Presence of mRNA and successful cDNA synthesis could be ascertained in 53/54 cases, determined by the presence of the housekeeping gene RNase P. Fifty of the 53 tumors (94%) contained E7 mRNA and 42/50 of the E7 expressing tumors also expressed E6 mRNA (table 3, paper IV). In summary, three samples with no expression of the viral oncogenes could be found despite high levels of the housekeeping gene and despite presence of HPV-16 DNA. To

make sure no DNA was present after RNA extraction, but prior to cDNA synthesis, an HPV-16 type specific PCR was performed. When DNA could be detected in this PCR, the RNA was purified again, using the same kit and performing the steps including DNase treatment and the subsequent washing procedure.

The levels of the housekeeping gene where very variable, and to calculate an accurate level of expression of viral oncogenes related to the levels of the housekeeping gene was not possible. Hence, the exact number of oncogenes expressed per equivalent housekeeping gene were not calculated and the tumors where considered only either positive or negative for expressing HPV oncogenes (if they also were positive for the housekeeping gene).

4.5 HPV TYPES IN OROPHARYNGEAL CANCER

In all papers the HPV type has been determined, either by type specific PCR or direct sequencing, or both for verification. When combining the HPV typing results from all patients with oropharyngeal cancer in papers I-IV, a total of 108 HPV DNA positive tumor samples were detected. Among these HPV positive tumors 93/108 (86%) were HPV-16, 4/108 (3.7%) were HPV-33, 2/108 (1.9%) were HPV-35, 1/108 (0.9%) was HPV-45 and in 8/108 (7.4%) the HPV type could not be determined due to limited amount of material (fig 9). Interestingly, all types, except one, belongs to the HPV-16 species, while the remaining type, HPV-45, belong to the HPV-18 species.

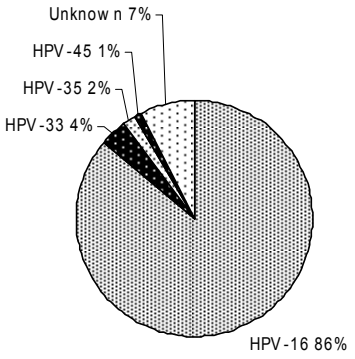

Figure 9 HPV types in oropharyngeal cancer in paper I-IV

5 DISCUSSION

In this thesis, the occurrence and effects of HPV in tonsillar and tongue cancer were studied. The patients included were diagnosed with primary squamous cell carcinoma of the tonsil and the tongue in the Stockholm area during 1970-2002. All patient material analyzed was taken before treatment and either archival paraffin embedded or fresh frozen. HPV DNA and mRNA were analyzed by general, type specific and real time quantitative PCR and in paper II also by ISH. The tumor suppressor gene product $p16^{INK4a}$ and the L1 capsid protein were detected by IHC and the patient files were reviewed for clinical data.

In paper I, HPV was detected in 12/110 (10.9%) of the investigated tongue cancer patient samples and was found significantly more often in base of tongue cancer, where 10/25 (40%) were HPV positive, as compared to 2/85 (2.4%) in mobile tongue cancer. These findings are in line with what previously has been reported in studies where the tumor location has been clearly specified (Gillison, Koch et al. 2000; Kreimer, Clifford et al. 2005). The OR for patients with a base of tongue cancer to be HPV positive was 26.4 (CI: 4.95-160.1) and for mobile tongue cancer only 2.8. A similar correlation has been presented when HPV was detected by serology, and compared with risk of having an HPV positive tumor depending on tumor location (Mork, Lie et al. 2001). The results that HPV is more commonly found in tumors originating from the oropharynx is hence in line with what has been published previously (Frisch and Biggar 1999; Gillison, Koch et al. 2000; Mellin, Friesland et al. 2000). Furthermore, in a recent meta-analysis (Ragin and Taioli 2007) it has been shown that HPV is a positive factor for survival only in patients with oropharyngeal cancer, and that this is not observed for patients with HPV positive cancer originating from other sites of the head and neck. It has also been concluded in epidemiological studies that HPV may contribute only to a small proportion of SCC of the oral cavity (Schwartz, Daling et al. 1998; Herrero, Castellsague et al. 2003). The frequency of HPV positive oral cavity SCC has been reported to be around 26% (Kreimer, Clifford et al. 2005), which is lower than what usually is observed in oropharyngeal SCC (Kreimer, Clifford et al. 2005). Hence, only a small proportion of oral cavity SCC is likely to be caused by HPV, and a possible explanation for the higher frequency in these tumors compared to what has been reported for normal tissue (around 10%, as discussed in the introduction) could also be due to the often ulcerating cancer in the oral cavity that might serve as en entry site for the virus when there is exposure.

One explanation to the fact that HPV seems to be more important in the development of oropharyngeal cancer might be the inflammatory activity that is present in the cervix as well as in the tonsils. Theoretically, these immunologically competent cells may produce growth factors when responding to foreign antigens, facilitating tumor development. Another theory is that HPV may play a part in tumor development to some extent in different locations of the head and neck, but the better prognosis is explained by the presence of HPV-specific immune cells in the tonsils. These cells may be stimulated by an HPV antigen in the infected tumor, enhancing the immunological response against the tumor. A third theory is that the tonsillar crypts make it easier for the virus to infect the epithelium and there is therefore a higher frequency of HPV in the tonsils.

In paper II, the presence of HPV DNA analyzed by ISH, the presence of HPV L1 capsid protein and $p16^{INK4a}$ overexpression detected by IHC, were evaluated as biomarkers for HPV in comparison to HPV detected by PCR. As illustrated in table VI, there is a significant association between having a tumor positive for HPV by PCR and overexpression of $p16^{INK4a}$, but the association was not 100%. This association as well as discrepancy has been observed by others (Andl, Kahn et al. 1998; El-Mofty and Lu 2003; Hafkamp, Speel et al. 2003; Klussmann, Gultekin et al. 2003; Li, Thompson et al. 2003; Begum, Cao et al. 2005; Weinberger, Yu et al. 2006) and in one study it was hypothesized and confirmed that patients with tumors overexpressing $p16^{INK4a}$ and positive for HPV by PCR represent the patients with a clinically relevant HPV infection and thus a better prognosis (Weinberger, Yu et al. 2006). This is further supported by findings where loss of $p16^{INK4a}$ was reported as an early event in tobacco related HNSCC (Cairns, Mao et al. 1994; Reed, Califano et al. 1996). Hence, patients with HPV positive tumors without $p16^{INK4a}$ overexpression would then represent a group of patients with tumors mainly caused by tobacco and alcohol. In another study, overexpression of $p16^{INK4a}$ was observed in the non-cancerous crypt epithelium of the tonsil with neoplasia, in the crypt epithelium of the contralateral tonsil and in tonsils in control patients without cancer (Begum, Cao et al. 2005). Therefore, $p16^{INK4a}$ expression, at least low expression as seen in paper II, might not be an excellent marker for having an HPV positive tonsillar cancer, but the absence of $p16^{INK4a}$ might indicate that even if HPV is present, it has no beneficial effects on prognosis. $P16^{INK4a}$ has also been suggested as a marker to determine if a lymph node metastasis is of oropharyngeal origin (Begum, Gillison et al. 2003). However, a better understanding of the natural history of HPV infection in the head and neck region is required for these conclusions.

The biological markers in paper II were also evaluated as predictors for response to RT and prognosis. A high $p16^{INK4a}$ staining was correlated to a better response to RT. When studying the prognostic value of the different detection methods, being positive for HPV by PCR compared to being HPV negative and a high staining of $p16^{INK4a}$ compared to a low $p16^{INK4a}$ staining or no $p16^{INK4a}$ staining, were significantly better predictors for staying disease free 3 years and for survival. These results suggest that $p16^{INK4a}$ may be a useful molecular marker when predicting prognosis and tailoring treatment for patients with tonsillar carcinoma, but before introduction in clinical praxis, the cut-off level for high $p16^{INK4a}$ staining should be evaluated more systematically. The reason for $p16^{INK4a}$ being a positive predictive factor is not entirely clear. It is known that $p16^{INK4a}$ is often inactivated in HNSCC (Perez-Ordonez, Beauchemin et al. 2006) and when this inactivation (e.g. by a deletion) has not occurred, the tumors expressing $p16^{INK4a}$ (mainly HPV positive tumors) have at least one important tumor suppressor gene intact. This might indicate different tumor biology with a genetic profile providing better conditions for adequate responses to the cellular stress induced by RT and the survival for the patients would then be higher. Interestingly, when studying HPV positive cell lines and the response to genotoxic treatment, such as for example cisplatin, it has been shown that there is an intact cellular response to this damage, which can induce apoptosis (Butz, Geisen et al. 1996; Ferris, Martinez et al. 2005). Therefore, the impact of the inclusion of chemotherapy in the treatment of patients with HNSCC on survival will be interesting to follow.

In paper III, we attempted to include all patients in the Stockholm area diagnosed with primary squamous cell carcinoma of the tonsils during 1970-2002. From these

patients 203 pre-treatment biopsies were available to study the presence of HPV, in order to investigate if HPV was a potential risk factor for the increase in incidence of tonsillar cancer that we had observed previously (Hammarstedt, Dahlstrand et al. 2007). The incidence of tonsillar cancer increased 2.8-fold and during the same period the proportion of HPV positive tonsillar cancer increased 2.9-fold. The median age was significantly lower for patients with the HPV positive tumors, as compared to patients with the HPV negative tumors. Interestingly, in one recent study from Colorado, US, a parallel increase in incidence of oropharyngeal cancer and an increase in the frequency of HPV positive tumors was reported (Ernster, Sciotto et al. 2007). In that study, it was also demonstrated that patients with HPV positive tumors had a better disease specific survival and a significantly lower median age (53.6 vs. 65.1 years) as compared to patients with HPV negative tumors. This is in line with what has been shown in this thesis.

In paper IV, the presence of HPV, estimation of HPV-16 viral load and presence of HPV-16 E6 and E7 mRNA were examined. In the five year survival analysis, 150 patients were included and a better disease specific survival was observed for patients with HPV positive tumors compared to patients with HPV negative tumors, independent of age, gender and tumor stage. This finding was expected and is in line with what previously has been reported in several studies and in one meta analysis (Gillison, Koch et al. 2000; Mellin, Friesland et al. 2000; Mellin, Dahlgren et al. 2002; Ragin and Taioli 2007).

Smoking data were gathered from patient files in the cases where smoking habits were clearly defined, and could be found for 119 patients. Patients were classified as smokers (including ex-smokers) or non-smokers. Interestingly, all non-smokers with HPV positive tumors were alive and disease free with a follow-up time of at least two years, compared to 71% of smokers with HPV positive tumors and 34% of the smokers with HPV negative tumors. The disease specific survival was significantly better for patients with HPV positive tumors both among non-smokers and smokers. This has also been observed in one study where also EGFR expression was investigated (Kumar, Cordell et al. 2007). In this study, smokers were less likely to have HPV positive tumors and their tumors more likely to express EGFR, smokers had a worse prognosis, as compared to never-smokers with HPV positive, EGFR negative tumors (Kumar, Cordell et al. 2007). These findings might also reflect that the HNSCC mainly induced by smoking may have more genetic alterations, which in turn facilitates tumor development and contributes to a worse response to RT as discussed above. However, to evaluate the effects of smoking, the data must be gathered more systematically than what has been done in our study.

A previous finding in our group (Mellin, Dahlgren et al. 2002), where viral load was estimated in fresh frozen tonsillar cancer biopsies and where a high viral load was correlated to a better survival, could not be confirmed in paper IV. The copy numbers we detected were in line with what previously has been reported (Klussmann, Weissenborn et al. 2001). In one recent study, HPV viral load was reported to be associated with better response to therapy and survival, however, in this study a new treatment modality with an organ-sparing protocol was also evaluated (Kumar, Cordell et al. 2007). Therefore, there are many differences in the study of Kumar et al compared to our study and the patient material used in this study is not clearly

specified. Interestingly, it was also found that patients who were smokers had a lower HPV viral load in their tumors (Kumar, Cordell et al. 2007). However, the prognostic value of HPV viral load should be further investigated, and the analysis of viral load should be conducted from fresh frozen material for obtaining the best results.

Extraction of mRNA was successfully performed in 53/54 samples monitored by synthesis and detection of cDNA of the housekeeping gene RNase P. Expression of E6 and E7 mRNA was detected from the same cDNA as the housekeeping gene, both E6 and E7 were detected in 42 samples and 8 samples expressed only E7. In three samples none of the viral genes could be detected despite high levels of the housekeeping gene. Thus, 94% of the investigated patient samples expressed mRNA, which confirms previous pilot studies including only a small number of patients (Snijders, Cromme et al. 1992; van Houten, Snijders et al. 2001; Wiest, Schwarz et al. 2002; Venuti, Badaracco et al. 2004). These results in paper IV strongly support an oncogenic role of HPV in tonsillar cancer.

There are today many retrospective studies showing that patients with HPV positive oropharyngeal cancer have a better survival (Mellin, Friesland et al. 2000; Gillison and Shah 2001; Mellin, Dahlgren et al. 2002; Dahlgren, Mellin et al. 2003; Li, Thompson et al. 2003), as well as a meta-analysis addressing this issue (Ragin and Taioli 2007), and one serological study showing that HPV-16 seropositivity increases the risk of oropharyngeal cancer (Mork, Lie et al. 2001). In a recent case-control study (D'Souza, Kreimer et al. 2007) it was also shown that oral HPV-16 infection increased the risk for oropharyngeal cancer. Other factors also associated with oropharyngeal cancer were a high number of vaginal-sex partners (a known risk factor for cervical cancer) and a high number of oral-sex partners. These data combined with the known molecular events by the viral oncogenes (discussed in the introduction), the presence of HPV in precancerous lesions (Bouda, Gorgoulis et al. 2000), the localization of the HPV DNA to the nucleus (Gillison, Koch et al. 2000; Klussmann, Weissenborn et al. 2001) and the presence of HPV in nodal metastasis (Gillison, Koch et al. 2000; Klussmann, Weissenborn et al. 2001; Hoffmann, Gottschlich et al. 2005) provide strong evidence for HPV as a causative agent in tonsillar cancer. This conclusion is strongly supported by the increase in incidence of tonsillar cancer and the parallel increase in the frequency of patients with HPV positive tumors, in combination with the detection of mRNA expression of viral oncogenes, which has been presented in this thesis.

The incidence and frequency of HPV positive tonsillar cancer should be carefully followed when preventive vaccines are introduced on the market. Determining the HPV type in the tonsils will be more important when following the incidence of tonsillar cancer and the frequency of HPV positive tonsillar cancer in the future, since the two HPV vaccines currently available only prevent infection with high-risk HPV types 16 and 18. Determining the type is also important for future development of new vaccines. The impact of vaccination on tonsillar cancer incidence will be interesting to follow, a decrease in incidence would further support the oncogenic role of HPV, but the effects of the vaccine will not show in many years to come. Also, currently the vaccine is only recommended for young females, something that might be necessary to reconsider since two thirds of tonsillar cancer patients are men.

Finally, HPV positive tonsillar cancer should now be considered a different disease with a better prognosis and a different risk factor profile than HPV negative tonsillar

cancer and it should be further studied how this type of tonsillar cancer could be diagnosed in clinical practice ($p16^{INK4a}$ or HPV PCR) and if a different treatment strategy is needed.

6 CONCLUSIONS

The main findings in this thesis were the following:

- HPV was confirmed to be a strong positive prognostic factor for disease specific survival, irrespective of age, gender or TNM stage in tonsillar cancer
- HPV was found at a higher frequency in base of tongue cancer as compared to mobile tongue cancer, and was a positive prognostic factor for disease specific survival in base of tongue cancer
- A high staining of $p16^{INK4a}$ correlated with presence of HPV detected by PCR and was a predictor for complete response to radiotherapy and a better survival in tonsillar cancer
- A parallel increase in the incidence of tonsillar cancer and the proportion of patients with HPV positive tonsillar cancer in the Stockholm area was observed in the Stockholm area
- In most HPV-16 positive tonsillar cancer, expression of the oncogenes E6 and E7 could be ascertained

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