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Human Papillomavirus
in Tonsillar Cancer

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Abstract

Human papillomviruses (HPVs) are known to be causative agents for the development of cervical carcinoma. To what extent HPV is associated with head and neck cancer remains to be clarified, but a connection to tonsillar cancer has been proposed. Importantly, the oncogenes of HPV-16, namely E6 and E7, are generally expressed in tonsillar cancer. It has been suggested that HPV positive and negative tonsillar cancer differ with respect to histopathology, oncogene profile as well as patient group features.

The aim of this thesis was to examine for the presence of HPV in tonsillar cancer, to study the relation of HPV to p53 immunostaining and DNA aberration, and to study the influence of these markers on clinical outcome in tonsillar cancer.

It was shown that HPV, mainly HPV-16, was commonly found in tonsillar cancer (~ 45%), when assayed by PCR. Furthermore, HPV was found to be of significant prognostic value for patients with tonsillar cancer. HPV positive tonsillar cancer patients were to a higher degree tumor free three years after diagnosis and had a better cause-specific survival than the HPV negative group. We therefore investigated if the response to radiotherapy was influenced by HPV or p53 overexpression (determined by immunohistochemistry (IHC)). P53 overexpression by IHC was common in HPV positive as well as in negative cancer. A correlation between HPV and p53 status and response to radiotherapy was not observed in the initial study, including forty patients. When extending the study material, preliminary data showed that HPV positive tonsillar tumors responded better to radiotherapy, although the results were not statistically significant. Complete response (CR) after radiotherapy seemed to be more crucial for clinical outcome than HPV status. Nevertheless, among the group with a CR after radiotherapy, the HPV positive patients appeared to have the highest survival rate.

The physical state of HPV-16 in tonsillar cancer was analyzed by a method based on restriction enzyme cleavage, ligation and PCR. HPV-16 was found to be mainly episomal. When quantifying HPV-16 positive tonsillar cancers by real-time PCR, it was found that the viral load shows a wide range. Moreover, the clinical outcome appeared to be better when the HPV load was higher.

Using image cytometry, the degree of DNA aberration was investigated. Tonsillar cancer displayed a high degree of aneuploidy. HPV positive tumors had a lower degree of aneuploidy than HPV negative tumors. HPV status had a stronger prognostic value in tonsillar cancer than DNA ploidy.

In conclusion, HPV-16 was common in tonsillar cancer and had a prognostic impact. It could not be determined if the presence of HPV lead to an increased sensitivity to radiotherapy. Furthermore, p53 IHC and HPV varied independently. HPV-16 was mainly episomal and the data suggest that a high viral load could be of an advantage for clinical outcome in patients with HPV positive tonsillar tumors. Independently of HPV status, there was a high degree of aneuploidy in tonsillar cancer, although to a lower degree in HPV positive tumors. Future studies may reveal further clinically relevant differences in HPV positive and negative tonsillar cancer.

Contents

List of Original Papers

I   **Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse and survival.** H. Mellin, S. Friesland, R. Lewensohn, T. Dalianis and E. Munck-Wikland, *International Journal of Cancer;* 2000; **89**: 300-4

II   **Human papillomavirus (HPV) and p53 immunostaining in advanced tonsillar carcinoma - relation to radiotherapy response and survival.** S. Friesland, H. Mellin, E. Munck-Wikland, A. Nilsson, J. Lindholm, T. Dalianis and R. Lewensohn. *Anticancer Res* 2001; **21**: 529-34

III   **Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer.** Mellin H, Dahlgren L, Munck-Wikland E, Lindholm J, Rabbani H, Kalantari M, Dalianis T. *International Journal of Cancer;* 2002, **102**: 152-58

IV   **Human papillomavirus and DNA ploidy in tonsillar cancer-correlation to prognosis.** Mellin H, Friesland S, Auer G, Dalianis T, Munck-Wikland E. *Manuscript*

Abbreviations

bp	base pairs
BPV	bovine papillomavirus
CIN	cervical intraepithelial neoplasia
CR	complete remission
CRPV	cottontail rabbit papillomavirus
CTL	cytotoxic T cell
E	early
EGFR	epidermal growth factor receptor
EV	epidermodysplasia verruciformis
HLA	human leukocyte antigen
HPV	human papillomavirus
IARC	International Agency for Research on Cancer
ICM	Image cytometry
IHC	immunohistochemistry
ISH	*In situ* hybridization
L	late
LCR	long control region
MAP	mitogen-activity protein
NCR	non-coding region
ORF	open reading frame
PCR	polymerase chain reaction
pRb	retinoblastoma protein
SV40	vacuolating simian virus
Taq	Thermus aquaticus
TIL	tumor infiltrating lymphocyte
UICC	International Union Against Cancer
URR	upstream regulatory region
VLP	virus-like particles

Introduction

Human Papillomavirus

Human papillomviruses (HPVs) have the potential to cause both benign and malignant tumors in humans, and HPV is e.g. the causative agent of verruca vulgaris, (common warts), condylomata acuminata (genital warts) as well as cervical carcinoma. To what extent HPV is associated with head and neck cancer remains to be elucidated. Among all head and neck cancer subtypes HPV is to this date most closely connected to tonsillar cancer and this is the subject of the thesis below.

Historical background
Common skin warts and genital warts have been known since many centuries and the latter were named condyloma by the antique Greeks. The hypothesis that the etiology of the common wart was an infectious disease was not proven until 1907, by Ciuffo, who showed the development of common skin warts after the inoculation of ultra-filtered wart lysates (zur Hausen, 1999).

In the 1930s, after the finding of cancer development in rabbits that had been infected with cottontail rabbit papilloma extracts, the first reports appeared on papillomavirus as a carcinogen (Rous and Beard, 1934; zur Hausen, 1999). However, already in 1842 a sexually transmitted infectious etiology for cervical cancer was proposed, when a high prevalence of cervical cancer in prostitutes and its absence in nuns was noted in an epidemiological investigation (Rigoni-Stern, 1842; zur Hausen, 1999). Nonetheless, it was not until the early1970s that HPV was singled out to be the most likely candidate for cervical cancer and in 1983 several specific HPV types were isolated from cancer of the cervix as well as from its precancerous precursors (zur Hausen, 1996). In 1995 the International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence to categorize HPV type 16 and 18 as human carcinogens ((IARC), 1995).

Taxonomy
HPVs belong to the Papovaviridae family, which includes the subfamilies papillomaviruses and polyomaviruses (Shah and Howley, 1990). The family name of papovavirus is a combination of papillomaviruses, polyomaviruses and vacuolating simian virus, the latter is an early name of SV40, a virus now included in the polyoma subfamily (Shah and Howley, 1990). Papillomaviruses infect a broad spectrum of

vertebrates, including man. They are highly species specific and do not infect over the species barrier. Therefore the different papillomaviruses are named after the species they infect, and they are numbered according to the order of their discovery. The papillomaviruses that have been studied most extensively are cottontail rabbit papillomaviruses (CRPVs), bovine papillomaviruses (BPVs) from cattle and human papillomaviruses (HPVs). So far 85 different HPV genotypes have been fully characterized and approximately 30 additional putative genotypes have been partially described (zur Hausen, 1999). Initially, the definition of an HPV type was based on viral DNA re-association according to the liquid hybridization technique (Coggin and zur Hausen, 1979). Later, in the early 1990's a novel HPV type was defined according to differences observed in the nucleotide sequences of the E6, E7 and L1 open reading frames (ORFs). Since 1995 a new HPV type is defined by a sequence difference exceeding 10% in the L1 ORF, when compared to that of established genotypes.

HPVs have a strict tropism for epithelial cells and are often divided into two major groups depending on which epithelial surface they most commonly infect. There are mucosal HPV types and cutaneous HPV types, of which selected examples are listed in Table I (zur Hausen, 1996).

Table I. HPV type and associated lesions

HPV type	Preferentially found in:	Subgroup
1	Plantar warts	Cutaneous
2, 4	Common warts	Cutaneous
5	Benign/ malignant EV[1] lesions	Cutaneous
6,11	Condyloma, laryngeal papilloma	Mucosal, low-risk
7	"Butcher's warts", oral papilloma (HIV pat.)	Mucocutaneous
16	Anogenital cancer and precursors	Mucosal, high-risk
18	Anogenital cancer and precursors	Mucosal, high-risk
19-25	EV lesions	Cutaneous
30	Laryngeal carcinoma	Mucosal
31, 33	Anogenital cancer and precursors	Mucosal, interm.-risk
42, 43, 44	Anogenital benign lesions	Mucosal, low-risk
45, 56	Anogenital cancer and precursors	Mucosal, high-risk
51-52, 58	Anogenital cancer and precursors	Mucosal, interm.-risk
53-55	Anogenital benign lesions	Mucosal
70	Vulvar papilloma	Mucosal
72, 73	Oral papilloma (HIV patient)	Mucosal
80	Normal skin	Cutaneous

The list is comprised from zur Hausen, 1996 and the HPV database (http://hpv-web.lanl.gov/stdgen/virus/hpv/) EV[1]= Epidermodysplasia verruciformis.

Some of the cutaneous types (Table I) are preferably found in epidermodysplasia verruciformis (EV), a hereditary rare disease which is linked to a high risk of development of skin cancer at UV-exposed sites. Furthermore, HPV can be divided into so called low-risk, intermediate risk and high-risk HPV types, depending on if they are commonly isolated from benign or malignant cervical lesions (Table I) (Lorincz et al., 1992).

Viral particle

Human papillomaviruses are non-enveloped, small circular double-stranded DNA viruses with an icosahedrical capsid (Syrjanen and Syrjanen, 1999). The capsid of 72 capsomers is approximately 55 nm in diameter (Figure 1.). The DNA of HPV is chromatin-like and covered by histones (zur Hausen, 1996).
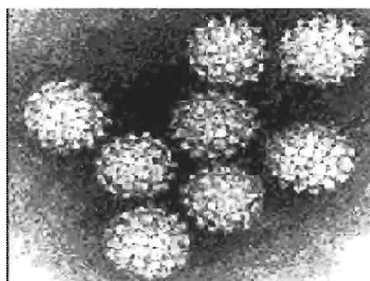
Figure 1- Electron microscope picture of HPV type 2, an HPV type commonly found in common skin warts.

Genomic organization

The viral genome consists of 7200-8000 base pairs (bp). Only one strand encodes for viral proteins, leading to that transcription occurs in one direction (Engel et al., 1983; zur Hausen, 1996). The genome is dived into three regions; the long control region (LCR) and the two open reading frames: the early (E) and the late (L) region (Figure 2.). The LCR is a non-coding region where the regulation of viral gene expression is controlled (see below). The E region codes for regulatory proteins E1, 2, 4-7 and the L region codes for structural proteins L1 and L2.

HPV protein functions

The E1 protein is essential for episomal papillomavirus DNA replication and consequently plays a critical role for episomal maintenance (Ustav et al., 1991). The protein has site–specific DNA binding functions and binds to the origin of replication in the LCR (zur Hausen, 1996). E1 interacts with E2, and it is reported that this interaction is needed for maximum

13

efficiency of replication although E1 can initiate replication alone (Del Vecchio et al., 1992; Gopalakrishnan and Khan, 1994; Sakai et al., 1996; Ustav and Stenlund, 1991). The E1 ORF is, after L2, the most conserved region of the papillomavirus genome (zur Hausen, 1996).

The E2 protein acts as a transcription factor and regulates viral transcription and is therefore important for the viral life-cycle. As mentioned above E2 also assists E1 in viral replication (Syrjanen and Syrjanen, 1999). The protein represses the expression of E6 and E7, the oncogenes of HPV high risk types (Thierry and Howley, 1991). Deletion in the E2 ORF is frequently seen in cervical cancer, which may result in a growth advantage for the malignant cell, since E6/E7 transcription is then dysregulated (Cullen et al., 1991; Jeon et al., 1995; Schwarz et al., 1985). However, it seems as a deletion of E2 is a late event in cancer development since most premalignant lesions do not contain disrupted E2 (zur Hausen, 1996). In addition, in 15-30% of the HPV-16 positive cases of cervical cancer contains episomal forms of HPV-16, and thus disruption of E2 may not be necessary for HPV induced carcinogenesis (Cullen et al., 1991; Das et al., 1992; Kalantari et al., 2001).
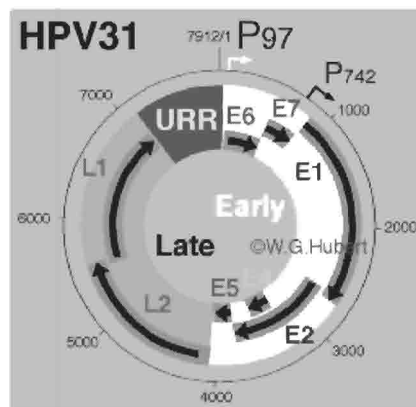
Figure 2- Circular map of the HPV-31 genome indicating the location of the open reading frames and the upstream regulatory region (URR or long control region, LCR).

The E4 protein originates from an mRNA formed by a splice from E1, and is one of the major transcripts in warts. The role of this protein has yet to be eluded (zur Hausen, 1996). E4 associates with the keratin cyto-skeleton of cultured epithelial cells, causing a collapse of the intermediate filament network (zur Hausen, 1996). The protein is exclusively found in the differentiated layer of the epithelium and it has been speculated that the protein may disrupt normal differentiation in order to establish favorable conditions for viral particle formation. E4 seems thus to be important for productive infection (zur Hausen, 1996).

14

The E5 protein is highly hydrophobic and is found in the Golgi apparatus and in the plasma membrane. E5 seems to have a weak transforming capacity in some HPV types such as e.g. HPV-16 (Leptak et al., 1991; Pim et al., 1992). The ORF of E5 is often disrupted in cervical cancer, and thus E5 does not seem to be essential for maintaining the malignant phenotype, but could possibly be important for the initiation of transformation (Schwarz et al., 1985; zur Hausen, 1996). Recently, it has been shown that the epidermal growth factor receptor (EGFR) is required for the E5 protein of HPV-16 to transform murine cells (3T3) (Syrjanen and Syrjanen, 1999). Furthermore, it has been suggested that the E5 protein of HPV-16 is able to reduce degradation of internalized EGFRs (Syrjanen and Syrjanen, 1999; zur Hausen, 1996). E5 has also other transforming capacities, e.g. has it been reported that E5 increases the response to growth stimulating factors by enhancing the mitogen-activity protein (MAP) pathway (Gu and Matlashewski, 1995).

The E6 protein is together with the E7 protein one of the two main oncoproteins of the high-risk HPV types and the constant expression of E6 and E7 in cervical cancer is necessary to maintain the malignant phenotype (zur Hausen, 1996). The E6 protein cooperates with E7 in immortalization and transformation, but E6 can alone immortalize human mammary epithelial cells (zur Hausen, 1996). The most significant ability of E6 of the high-risk types is to be able to bind and degrade p53 (Scheffner et al., 1990; Werness et al., 1990). P53 is a tumor suppressor protein required for growth arrest and/or apoptosis following DNA damage, and cells without a functioning p53 display genetic instability (zur Hausen, 1996). E6 degrades p53 by binding to the cellular protein E6 associate protein (E6-AP), leading to ubiquitin proteolyses of p53 (zur Hausen, 1996). It has been shown that E6 enhances telomerase activity, a necessity in immortalized cells (Klingelhutz et al., 1996). E6 has also p53 independent transforming functions, which will not be discussed here, however for details see reviews by (Rapp and Chen, 1998; Syrjanen and Syrjanen, 1999).

The E7 protein is the other major oncoprotein of high-risk HPV types and can alone immortalize human keratinocytes (Halbert et al., 1991). E7 drives the cell into S-phase by binding to the retinoblastoma tumor suppressor protein (pRb), which in turn leads to the release of the transcription factor E2F from the pRb complex, and thereby transcriptional activation of genes regulating cell proliferation (Bandara et al., 1991; Munger et al., 1989). E7 has been found to up regulate the G1-S cyclin; cyclin A and cyclin E, and activates cdk2 activity (Alani and Munger, 1998; Syrjanen and Syrjanen, 1999) E7 of high risk types also associates with other proteins that are important for cell cycle regulation such as p107 and p130. (Dyson et al., 1992; Tommasino et al., 1993). The binding affinity of the E7 protein of high-risk HPV types to pRb is

15

approximately 10 times higher than that of the E7 protein of low-risk HPV types (zur Hausen, 1996).

The L1 and L2 proteins are the two capsid proteins forming the 72 capsomers that enclose the viral DNA (Doorbar and Gallimore, 1987). The L1 protein is the major capsid protein, and contributes to 80-90% of the capsid and is highly conserved among different human papillomavirus (Favre et al., 1977) Furthermore, L1 and L2 can self-assembly into in virus-like particles (zur Hausen, 1996).

Viral transcription

Viral gene expression is controlled by the LCR, which contains viral promoter and enhancer sequences and the origin of replication (Figure 2 and 3). The LCR is also commonly called the upstream regulatory region (URR) or the non-coding region (NCR). The complex regulation of viral gene expression is controlled by both cellular and viral transcription factors (Figure 3). Examples of cellular transcription factors that bind to the LCR are NF-1, AP-1, Oct-1, TEF-1, TEF-2, SP-1, YY-1 and the glucocorticoid receptor (zur Hausen, 1996). Dysregulation of these transcription factors seems to be of importance for carcinogenesis in HPV induced lesions (zur Hausen, 1994). The viral protein E2 acts as a viral transcription factor and has four binding sites in the LCR of HPV-16. E2 represses E6/E7 expression by inhibiting the promoter p97.
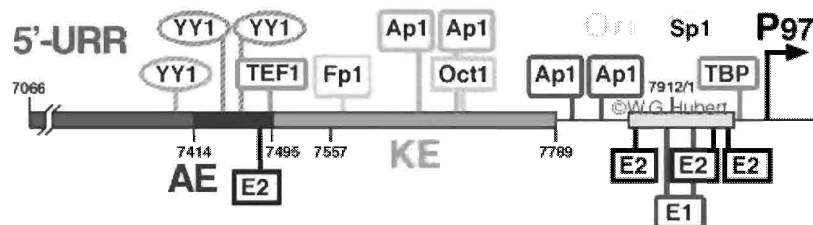
Figure 3 - The LCR (long control region or URR) in HPV-31 with cellular and viral transcription factors (E2). Ori= origin of replication where E1 binds in assistance of E2. P97 is the promoter of E6 and E7 open reading frames.

Route of HPV infection

Knowledge of the virus replication cycle is limited since HPV needs an advanced tissue raft system, imitating a multi-layer epithelium, in order to infect and replicate *in vitro*. HPV infects the basal layers of epithelial surfaces and the transmission is facilitated by minor epithelial damage (Figure 4) (zur Hausen, 1996). Which cell surface receptor for entry into epithelial cells is under debate, but alpha-6 integrin as well as heparin and

cell-surface glycosaminoglycans resembling heparin have been suggested as possible candidates (Evander et al., 1997; Joyce et al., 1999). Papillomaviruses replicate exclusively in differentiating epithelial cells, hence a high level of viral proteins and viral assembly occurs in the upper layer of the epithelium (stratum spinomsum and granulosum) (Figure 4) (Stanley, 2001). The action of E6 and E7 bring the differentiated cells into S-phase which facilitates the viral replication. Viral expression is tightly regulated in the infection cycle, initially permitting early gene expression in the lower epithelial layers, and later when the infected cells start to differentiate permitting expression of all genes (zur Hausen, 1999).

The most thoroughly studied infections HPV are in the anogenital region in women, and therefore a normal route of infection will be described below in that context if not indicated otherwise. HPV infection is either clinical (manifest symptoms or visible lesion), subclinical (symptom free and visualized only by acetic acid or colposcopy) or latent (presence of HPV DNA without yielding any symptoms and no morphologic abnormality). Anogenital infections are mainly transmitted by sexual contact and HPV infections and their associated lesions are extremely common among young, sexually active women (Oriel, 1971; Schiffman and Brinton, 1995).
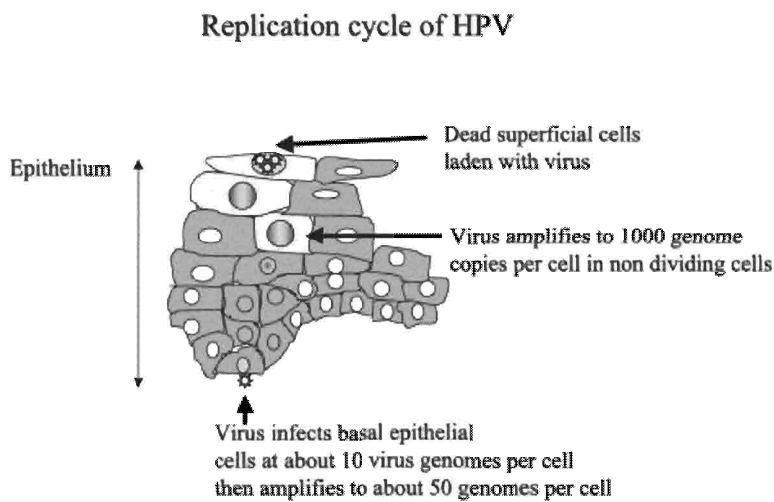
Replication cycle of HPV

Figure 4- The replication cycle of HPV in the epithelium.

17

When estimating the life-time risk, up to 79% of Finnish females will have at least one HPV infection within 20 to 79 years of age (Syrjanen and Syrjanen, 1990). There is a correlation between the number of sexual partners and low age at sexual debut and prevalence of HPV infection. Oral–genital contact my lead to HPV infections at oral sites as well, see below (Maden et al., 1992; Schwartz et al., 1998; Smith et al., 1998).

Most often the host immune system clears infection spontaneously within months to a few years (Schiffman and Brinton, 1995). Rarely, HPV infections and/or low grade lesions persist and progress to high grade lesions. It has been estimated that 15% of all HPV infection will progress into a low or high grade lesions and in total 1% into cervical carcinoma (Beskow and Gyllensten, 2002; Rome et al., 1987; Syrjanen and Syrjanen, 1990). There is a long latency period of 10-30 years between infection and development of dysplasia and subsequent progress into invasive carcinoma (Schiffman and Brinton, 1995). Accordingly, low grade dysplasia in the cervix has a peak in incidence at 25-35 years of age, whereas the incidence of cervical cancer peaks at 55-65 years of age (de Villiers et al., 1992)

The risk factors for progression are mainly unknown, but include HPV type, impaired cell-mediated immunity, genetic susceptibility and co-factors as smoking (Beskow and Gyllensten, 2002; Schiffman and Brinton, 1995). E6 and E7 transcription of high-risk types of HPV seems crucial for malignant transformation, since they are constantly expressed in cervical cancer, and blocking these genes leads to the reversion of the malignant phenotype, as well as cessation of cell growth of cervical carcinoma cell lines (zur Hausen, 1996). Cancer cells have a defect regulation of normal cell proliferation and homeostasis (Hanahan and Weinberg, 2000). E6 and E7 disturb cell cycle regulation directly as well as by recruiting/blocking cell host factors (see function of viral proteins). However, HPV alone is not sufficient for cancer development and carcinogenesis of the cervix is a multistep pathway, which includes modification of cellular genes prior to malignant conversion (zur Hausen, 1999a)

An impaired cellular mediated immune system results in an increase of the number of HPV induced lesions and in persistence of HPV infections. Accordingly HPV associated cancers (including tonsillar cancer, although a limited number of cases have been studied) increase in immunosuppressed persons such as in HIV infected and transplanted patients (Berkhout et al., 1995; de Villiers et al., 1997; Frisch et al., 2000a). Regression of anogenital warts is accompanied by a CD4 T cell dominated Th1 response (Stanley, 2001). Evasion of the cell-mediated immune system is thus critical for HPV transformed tumor cells and in most cervical carcinomas HLA class I antigen and accessory molecules expression are down regulated (Cromme et al., 1994; Stern, 1996).

The role of humoral immunity in HPV infection is not well understood. The majority of HPV infected women develop an IgG response to HPV-16 and HPV-18, and this usually occurs 4-12 months after HPV DNA detection, whereas antibodies to HPV-6 and HPV-11 are detectable already at the time of HPV DNA detection (Carter et al., 1996; Lehtinen and Paavonen, 2001) While capsid antibodies are markers of past or present infection, antibodies against E6 and E7 are markers of malignant disease (Lehtinen and Paavonen, 2001).

Cytological screening in women

Cytological screening for cervical cell dysplasia was introduced in many countries in the 1960's. There is statistical evidence that the screening program may have played an important role in the prevention of cervical cancer as well as reducing the mortality of the disease. In a survey from 12 European countries (including the Nordic countries) the incidence of cervical cancer in women, between 45-49 years of age. was 75 per 100,000 in the mid 1960s and by 1980 it had declined to 35 per 100,000 (Ponten et al., 1995). In Sweden within the screened cohorts and age groups, the incidence was reduced by two-thirds between 1958 and 1980 and there was a decline of mortality in cervical cancer by 34% between 1965-1982 (Laara et al., 1987; Pettersson et al., 1985).

Screening for cervical intraepithelial lesions is performed with Papanicolau (Pap) smears (first described in 1948). Exfoliated cells from the cervix are examined by light microscopy and the morphologic changes are graded as cervical intraepithelial neoplasia (CIN) 1-3 depending on the grade of dysplasia. Typical morphological changes associated to HPV infection are so called koilocytosis and dyskeratosis (Meisels, 1983). The sensitivity of Pap smear test has been estimated to 75% (Ponten et al., 1995).

Common HPV DNA detection methods

Southern blot hybridization has long been used to identify HPV DNA. Purified DNA is cleaved by restriction enzymes and then separated by agars gel electrophoreses. After denaturation the DNA is transferred to a membrane and HPV specific sequences are identified through hybridization (specific complementary nucleotide binding) to labeled cloned HPV DNA. The sensitivity is about 0.1 HPV genome copy per cell (Syrjanen, 1990). Furthermore, the method can give information regarding if the virus DNA is integrated or episomal. The main advantage of this technique is the high specificity in HPV typing, and the disadvantage is that the technique is time consuming and a relatively large amount of DNA is needed (~10 µg) (Syrjanen, 1990).

Dot blot hybridization is a method where extracted DNA, without restriction enzyme digestion, is transferred and bound to a membrane in

Its single-stranded form. Similar to Southern blotting, HPV specific sequences are identified by hybridization with labeled cloned HPV DNA. The sensitivity is about 1 genome copy per cell, using 300-500 ng sample DNA (Syrjanen, 1990). One advantage of this method is that it is relatively fast, however, its specificity is lower than that of the Southern blot and false positive signals should be controlled for. (Syrjanen, 1990). A variant of the assay is to bind HPV PCR products to the membrane, instead of extracted DNA. Reverse dot blot is another variant for HPV typing, where different types of known HPV DNA of are fixed on the membrane and labeled extracted DNA or PCR products are used as probes (de Villiers, 1992).

DNA *in situ* hybridization (ISH) does not only detect the viral DNA, but can also localize the virus within in the natural morphology of the tissue. Tissue sections are put on slides and the tissue sections are hybridized with labeled DNA or RNA probes after denaturation. The sensitivity differs between different ISH methods, but can identify approximately down to 20-25 HPV copies/ cell. ISH can also be used to see if the viral DNA is integrated into the chromosome or is episomal in the nuclei.

Hybrid Capture is a commercially available method designed for detection of selected low-risk and high-risk HPV types. After the polymerase chain reaction (PCR) assay, the hybrid capture assay is the 2nd sensitive detection method, with a lower detection limit of approximately 0.05 HPV DNA copies/ cell (Brown et al., 1993). It is based on non-radioactive hybridization with HPV RNA as probes, which gives the advantage of that the binding is stronger than that of DNA-DNA. The RNA-DNA hybrids are captured in tubes coated with monoclonal antibodies against the hybrid molecules.

Polymerase chain reaction (PCR) is the most sensitive HPV detection method, where even a single gene copy of HPV is possible to detect (Saiki et al., 1988). The principle of the assay is to amplify a specifically selected sequence of DNA. The specificity is based on complementary binding of oligonucleotides, so called primers, to the DNA of interest (in this case HPV). With temperature dependent and heat stable DNA polymerase *Thermus aquaticus (Taq)* polymerase, the DNA part selected by the two primers is amplified up to as much as a million fold, which explains the sensitivity of the assay.

In essence, first the DNA is denaturated (separated) (at~95°C), then the primers anneal to the two DNA strands (at ~50°C), then *Taq* polymerase amplifies the DNA between the two primers situated in the opposite direction (at ~72°C). The PCR cycle is repeated 30-40 times. The amplified DNA, the PCR product, is then usually visualized on an ethidium bromide stained electrophoresis gel or detected by probe hybridization in a Southern blot or dot blot.

The primers can be either so called consensus/general primers, binding to a wide rage of HPV types, which is useful in screening approaches, or type specific primers that are HPV type specific (paper I-IV). General primers are complementary to sequences (often in L1) in HPV that are highly conserved among many HPV types, while HPV type specific primers bind to a sequence found in a single HPV type (often in E6 or E7) and do not cross-bind to other HPV types. Instead of an HPV type specific PCR, HPV typing can also be performed by sequencing the PCR product obtained by a PCR run with general primers (paper III).

Since PCR is so sensitive, laboratory rules to avoid contamination are crucial. DNA handled pre-PCR must strictly be separated from the manifold amplified post-PCR DNA. Negative controls at all steps are thus of great importance to check for false positive results.

Future possible prevention and treatment of HPV infections

HPV DNA is found in nearly all cervical cancers and in more than 50% of all vulvar, penile an anal cancer (zur Hausen, 1996). In addition, oncogenic HPV is associated with non-anogenital cancers such as epidermodysplasia verruciformis, oropharyngeal cancer and non-melanoma skin cancer. (Andl, 1998; de Villiers et al., 1997; Gillison et al., 2000; Gillison and Shah, 2001; Harwood et al., 2000; Klussmann et al., 2001; Mellin et al., 2000; Munger, 2002; Paz et al., 1997; Snijders et al., 1992; Strome et al., 2002; Wilczynski et al., 1998). Cervical cancer represents the second most frequent malignant tumor of women in the world and is the leading cause of cancer mortality in developing counties (Parkin et al., 1999; Pisani et al., 1999). In more developed countries the frequency is lower, much due to the above described screening, but the morbidity of precancerous lesions is still extensive also in these countries and prevention of HPV induced infection is thus desirable globally.

Today research and clinical trials are performed on preventive vaccinations as well as therapeutic vaccinations to cure HPV infection. Thus, the goals of vaccination are two fold; firstly to prevent HPV infection and secondly to eliminate already established infections. Preventive vaccination approaches are often based on the capsid proteins, while therapeutic vaccination tests are generally based on the two early genes E6 and E7. Furthermore, a combination of the two, so called chimeric virus-like proteins, consisting of both late and early proteins has been evaluated for both prophylactic and therapeutic vaccination (Da Silva et al., 2001).

Prophylactic vaccines are as mentioned above often derived from the capsid proteins, which can be expressed in high amounts (e.g. in insects and yeast cells) and spontaneously aggregate into virus-like proteins (VLPs). By using VLPs in canine, rabbit and in bovine systems,

21

successful prevention of papilloma infection and tumor development have been shown (zur Hausen, 1999). *In vitro* VLPs of HPV have been shown to raise neutralizing type specific antibodies (Alani and Munger, 1998). In Phase I trials, immunization with HPV VLPs have induced good serum IgG antibody responses (Stanley, 2001). The key issues are whether the antibodies will be protective, how long the protection will last and to what extent they will be cross protective against infection with other HPV types (Stanley, 2001). Future vaccines may need VLPs derived from several HPV types in order to induce immunity against various HPV types.

A reasonable approach to treatment of already established tumors involves either targeting the crucial E6/E7 gene expression directly or immunization therapy (vaccines) based on the antigenic epitopes of E6/E7. Direct targeting of the oncogenes by antisense E6/E7 oligonucleotides inhibits of growth in cell lines, however clinically efficient methods of introducing and maintaining oligonucleotides is lacking today. Another not yet evaluated therapeutic approach to repress E6/E7 is introducing the E2 protein. Induction of E2 expression in HeLa cells, an HPV-18 positive cervical carcinoma cell line, resulted in growth arrest (Dowhanick et al., 1995). The approach of vaccination therapy can be done by introducing peptides of E6/E7 or vectors producing these proteins. This is ongoing in cervical carcinoma patients and induction of cytotoxic T cell (CTL) responses have been shown. Furthermore, to enhance the immune system, especially the CTL response, against E6/E7 is another future possibility. Hohn *et al.* characterized tumor infiltrating lymphocytes (TILs) from a patient with cervical cancer and the TILs recognized both the patients own HPV-33 positive tumors cells and synthetic E7 peptides of HPV-33 (Hohn et al., 2000). Dendritic cells play a key role in mediating anti-tumor immune responses, being a effective antigen presenting cells and thus activating T-cells. Using dendritic cells pulsed with HPV-16 E7 proteins to stimulate CTLs *in vitro* is an approach under evaluation (Luxton and Shepherd, 2001; Santin et al., 2000).

Today there is only preliminary data from clinical trials on the efficiency of therapeutic vaccination, however, partial response in patients with advanced tumors encourage continuation of ongoing trials (Gissmann et al., 2001).

Head and Neck Cancer

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 gland, as well as the soft tissues of the neck and the ear (Figure 5). However, investigations of cancers of the upper aerodigestive tract, i.e. the oral cavity, and pharyngeal and laryngeal cancer are often handled separately. Tonsillar cancer is the most common tumor in the oropharynx (Nathanson et al., 1999). Essential functions as eating, speaking and breathing may be highly affected in patients with advanced tumors in the aerodigestive tract. Further distress may be added due to cancer growth in the face and the neck. In addition, surgery and radiotherapy treatment can be disabling and disfiguring. Therefore to optimize individual therapy, i.e. to give the most efficient treatment with the lowest possible impact on function and form is of utmost importance.
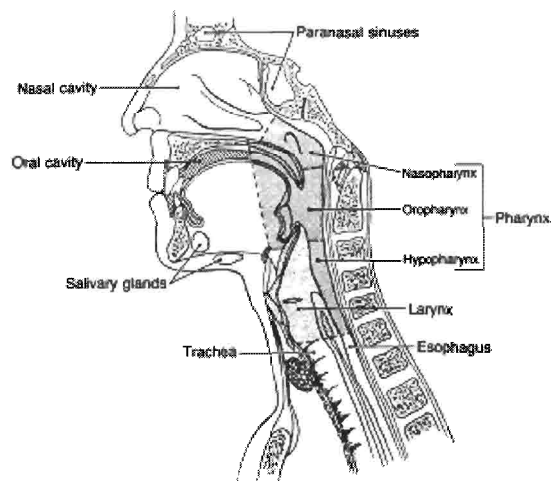

Figure 5- Anatomic overview of the head and neck.

Incidence and prevalence
Cancer in the head and neck region comprises 3-4% of all cancer cases in Sweden, which is slightly lower than what is observed in other developed countries (Nathanson et al., 1999). Approximately 1000 new

23

cases of head and neck cancer are diagnosed per year in Sweden, excluding all cases of esophagus cancer (Nathanson et al., 1999). The incidence is almost twice as high in men than in women (Nathanson et al., 1999). In Europe, the Latin countries have a higher prevalence compared to Northern Europe. In other areas of the world head and neck cancer is much more common, and in Bombay, India, they account for nearly 50% of all cancer cases (Decker and Goldstein, 1982).

Prognosis

The five-year survival is approximately 30-40% for all head and neck cancers. Eighty percent of the relapses occur within 1.5 years (Nathanson et al., 1999). No significant changes in survival for patients with head and neck cancer have been noted the last decades.

Etiology

The large differences in the incidence of head and neck cancer imply that exogenous factors are of importance for its development. As many as 80-90% of all head neck cancer may be contributed to the known risk factors smoking and alcohol abuse (Decker and Goldstein, 1982; Licitra et al., 2002) There is a dose-response relationship between tobacco exposure and risk of head and neck cancer (Gillison et al., 1999). Whether alcohol has an independent carcinogenic effect is debatable and difficult to assess because of its close connection to tobacco. However, it has been established that for a given level of tobacco exposure, increasing levels of alcohol exposure is correlated to an increasing risk for oral cancer and laryngeal cancer (Decker and Goldstein, 1982). A small proportion of head and neck cancer occurs in individuals without a history of tobacco or alcohol use (Gillison et al., 1999). Some work related risk factors are known, e.g. exposure to wood dust as a risk factor for nose and sinus cancer (Nathanson, 1999). In high-risk areas local customs such as bethel chewing are important risk factors (Decker and Goldstein, 1982; Licitra et al., 2002). A reduction in the risk of oropharyngeal cancer by higher intake of fruit and vegetables has been demonstrated in the USA and China (Licitra et al., 2002). If there is a specific genetic susceptibility for developing head and neck cancer is unknown (Licitra et al., 2002). Viruses have been proposed to be connected to head and neck cancer and an association between mainly human papilloma virus (HPV) and Epstein-Barr virus and certain types of head and neck cancer has been found (de Villiers, 1991; Snijders et al., 1992a; Alani and Munger, 1998; Niedobitek, 2000; Gillison et al., 2000; Gillison and Shah, 2001; Mork et al., 2001).

Patients that have had a tumor in the head and neck region are at increased risk of developing new primary tumors in the aerodigestive

tract. To what extent this occurs varies in different studies, but around 17-30% of the patients develop a new, i.e. a second primary tumor (Braakhuis et al., 2002). Moreover, there may be difficulties in separating a second primary from local recurrence (Braakhuis et al., 2002). The cause of this relatively high risk of developing a second primary tumor is unknown, a plausible cause is the continuation of a given risk factor such as smoking, but radiotherapy may also be a co-factor (Nathanson, 1999).

Tumor classification

Each tumor is classified according to the size of the tumor (T), to whether the cancer has spread to the local lymph nodes on the neck (N) and if the cancer has a distant spreading metastasis (M), as viewed in Table IIa. Tumor stage is classified by adding the TNM data together (Table IIb). In all studies included in this thesis the TNM stage was classified according to UICC (International Union Against Cancer), 1997 (Table IIb). The histological grading is based on the structure, degree of differentiation, nuclear polymorphism and number of mitoses, and is divided into well, moderately, poorly and undifferentiated tumors.

Table IIa. Tumor classification for oropharyngeal and oral cavity

T, N, M	Definition
TX	1°[1] tumor size can not be defined
T0	No 1° tumor is found
Tis	Carcinoma in situ[2]
T1	< 2 cm
T2	>2 <4 cm
T3	>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 sign of regional lymph node metastases
N1	One ipsilateral[3] lymph node metastasis <3 cm
N2a	One ipsilateral lymph node metastasis >3 <6 cm
N2b	Multiple ipsilateral lymph node metastases <6 cm
N2c	Bilateral[4] or contralateral[5] lymph node metastases <6 cm
N3	Regional lymph node metastases >6 cm
MX	Distant metastasis status can not be defined
M0	No sign of distant metastases
M1	Presence of distant metastases

[1]1°= primary. [2]Carcinoma in situ= no invasive cancer, the basal membrane is intact. [3]Ipsilateral= on the same side of the neck as the primary tumor. [4]Bilateral= on both sides of the neck. [5]Contralateral= on the other side of the neck as the primary tumor.

Table IIb. Tumor stage classification

TNM Stage	T classif.	N classif.	M classific.
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T1-T2	N1	M0
III	T3	N0-N1	M0
IVA	T4	N0-N1	M0
IVA	Any T	N2	M0
IVB	Any T	N3	M0
IVC	Any T	Any N	M1

Treatment

Head and neck cancer treatment with a curative intent aim implies surgery and/or radiotherapy. Radiotherapy can be given alone, before surgery, i.e. preoperatively, or after surgery, i.e. postoperatively. The radiotherapy dose ranges from 50-68 Gray (Gy), which at the Karolinska Hospital is delivered as fractionated radiotherapy with a 2 Gy daily target dose 5 days a week (Nathanson et al., 1999). The primary goal for surgery is to be radical, i.e. that with an adequate safety margin extirpate all tumor tissue, with the smallest possible damage. If cure cannot be reached, the patients receive palliative therapy, in order to treat pain and discomfort. Palliative therapy includes radiotherapy, chemotherapy, optimal pain relief, nutrition aid and psychological support.

Oropharyngeal cancer

Approximately 150 patients acquire oropharyngeal cancer per year in Sweden (Nathanson et al., 1999). Two-thirds of the patients are men. The oropharynx comprises the region of the soft palate down to the level of the tongue bone and can be divided in to 4 areas: the base of the tongue, the tonsillar fossa, the walls of the oropharynx and the soft palate.

Tonsillar cancer is the most common oropharyngeal cancer. Small tumors in the tonsils do not often give rise to any discomfort. Thus, unfortunately, the patients do not seek counseling until the tumor is fairly large and presents symptoms like pain related to swallowing or difficulties to swallow (Nathanson et al., 1999). Another relatively common first symptom is pain in the ear. Most patients with oropharyngeal cancer already have spreading of the tumor to the lymph nodes of the neck at the time of diagnosis and sometimes the patients have no discomfort from the primary tumor, but notice a nodal swelling on the neck (Nathanson et al., 1999).

More than 80% of all oropharyngeal cancer is squamous cell carcinoma, and the remaining cancers are lymphomas and more rarely sarcomas. Tonsillar cancer is primarily treated with full-dose radiotherapy (64 Gy) against the primary tumor and ipsilateral radiotherapy to the neck. The extent of the surgical treatment depends on the size of the primary tumor, the presence of metastases to the neck lymph nodes and the response to the given radiotherapy.

Patients with stage I-II oropharyngeal cancer have a 5-year survival of 60-70%, while patients with stage III-IV oropharyngeal cancer have a 5-year survival of 10-25%. Overall survival for patients with oropharyngeal cancer is only slightly more than 25% (Nathanson et al., 1999).

HPV in head and neck cancer

In the beginning of the 1980s the first reports appeared on HPV in head and neck cancer (de Villiers et al., 1985; Gissmann et al., 1982; Naghashfar et al., 1985; Syrjanen et al., 1983). Accumulating molecular and epidemiological data indicate that high-risk types of HPV are associated with a subset of head and neck cancer (Alani and Munger, 1998; Gillison et al., 2000; Gillison and Shah, 2001; Mork et al., 2001; Snijders et al., 1992a). The strongest association so far has been found for oropharyngeal cancer, especially tonsillar cancer (Andl, 1998; Gillison et al., 2000; Gillison and Shah, 2001; Paz et al., 1997; Snijders et al., 1992a). Another virus, namely the Epstein-Barr virus, is strongly associated with nasopharyngeal cancer (de Villiers, 1991; Niedobitek, 2000).

The prevalence of asymptomatic HPV infection of the oral cavity in adults is estimated to be between 5-11%, depending on assay and sampling methods. Furthermore high risk and low-risk HPV types have been detected in approximately equal frequency (Gillison et al., 1999). HPV has been suggested to be more common in macroscopically normal-appearing laryngeal mucosal and is found by PCR in approximately 20% of all cases (Aaltonen et al., 2002). So far no HPV has been detected in normal tonsillar tissue, see below (Brandsma and Abramson, 1989; Mellin et al., 2000; Niedobitek et al., 1990; Snijders et al., 1992a; Steinberg and DiLorenzo, 1996). HPV infection has also been associated with benign lesions of the upper airway such as focal epithelial hyperplasia and laryngeal papillomatosis (Aaltonen et al., 2002; Syrjanen et al., 1987). In the latter HPV-6 and -11 are found in almost all cases (Aaltonen et al., 2002). There is an apparent need of a detailed prospective study of the prevalence HPV in normal tissue as well as in mild, moderate and severe dysplasia in persons who later develop invasive carcinoma in the head and neck. However, several reports on a small number of dysplastic lesions have shown the presence of HPV in 10-20% of the cases and in a meta-analyses summarizing 94 reports, it was found that HPV was 2-3

times more common in precancerous oral lesions than in normal oral mucosa (Gillison et al., 1999; Miller and Johnstone, 2001).

The prevalence of HPV in head and neck cancer has varied significantly when comparing different reports and this discrepancy could plausibly be explained by differences in tumor site, the material and methods applied for analysis, as well as number of cases included. However, the overall prevalence is 14-35% when detected by PCR technique, ~25% by Southern blot hybridization, ~18% by *in situ* hybridization, and ~6% by dot blot hybridization (Haraf et al., 1996; Klussmann et al., 2001; McKaig et al., 1997; van Houten et al., 2001). The HPV types observed in head and neck cancer are the same high-risk types found in cervical carcinoma, with HPV-16, -18, -33 and -31 being predominant, which is in contrast to asymptomatic HPV infection of the oral cavity, where low-risk types are detected equally often (Gillison et al., 1999; Smith et al., 1998). Antibodies against the early genes of oncogenic HPV are signs of past or present malignancy, whereas antibodies against the later genes reflect past or present infection (Lehtinen and Paavonen, 2001). In one study the prevalence of antibodies against high-risk proteins E6/E7 was analyzed in sera from 144 patients with head and neck cancer at the time of diagnosis and in parallel in 288 matched controls (Zumbach et al., 2000). Overall, 12% of the cancer patients had antibodies against E6 or E7 of HPV-16 or –18, which was significantly higher than the 3.5% incidence observed in the (Zumbach et al., 2000). Most seropositive sera contained antibodies against HPV-16. HPV DNA was found only in patients with antibodies against HPV (Zumbach et al., 2000). Moreover, a significant association of seropositivity with tumor location in Waldeyer´s tonsillar ring was noted (Zumbach et al., 2000).

HPV infection as a risk factor for head and neck cancer needs to be investigated further. To day there is one extensive seroepidemiological study that reports the analysis of sera from persons who later developed head and neck cancer, as well as from matched controls (Mork et al., 2001). In total 292 patients with head and neck cancer and 1568 controls were tested for HPV capsid antibodies on average 9.4 years prior to diagnosis (Mork et al., 2001). The prevalence of seropositivity for HPV-16 was twice as high in the patient group compared to that of the control group, and was found to be a significant risk factor for head and neck cancer (Mork et al., 2001). For HPV-18, -33 and -73 the seroprevalence was similar in both groups. When investigating the risk to develop cancer according to tumor location, the highest risk was found for oropharyngeal cancer (odds ratio 10.2) and cancer of the base of the tongue (odds ratio 20.7)(Mork et al., 2001). (Please note that cancer of the base of the tongue is usually included in the oropharyngeal cancer group). The prevalence of HPV-16 DNA in the tumors corresponded with prediagnostic seropositivity of HPV-16 (Mork et al., 2001).

28

The route of transmission of HPV to the upper respiratory tract is not established. A possible mode of transmission is oral-genital sexual contact or auto-inoculation from infected genital mucosa (Gillison et al., 1999). Another mechanism that cannot be excluded is transmission from mother to child at birth (Syrjanen and Puranen, 2000). Nonetheless, non-sexual oral HPV transmission is rare according to a study of oral swabs from 392 children, new-born up to 17 years old, where 0.25% of the oral mucosa was HPV positive (Koch et al., 1997). In line with risk factors for cervical cancer, three case-control studies found that individuals with a high number of sexual partners had an increased risk of head and neck cancers (Maden et al., 1992; Schwartz et al., 1998; Smith et al., 1998). One study also found that a young age at first intercourse also was a risk factor for oral cancer (including oropharyngeal cancer) in men (Schwartz et al., 1998).

HPV in tonsillar cancer

HPV DNA is detected in 45-70% of all cases of tonsillar carcinoma, thus more frequently than in other head and neck cancers (Andl et al., 1998; Gillison et al., 2000; Mellin et al., 2000; Mork et al., 2001; Paz et al., 1997; Snijders et al., 1992a; Steinberg and DiLorenzo, 1996). No HPV has been detected in normal mucosa of the tonsils in adults, which is an argument against a non-specific colonization of HPV in the tonsils (Brandsma and Abramson, 1989; Mellin et al., 2000; Niedobitek et al., 1990; Snijders et al., 1992a; Steinberg and DiLorenzo, 1996). Nonetheless, tonsillar cancer is a relatively rare cancer, and even if approximately 1% of all cases with an HPV infection (as in the cervix) would lead to cancer, a great number of controls is needed for detection HPV in normal tissue of the tonsils. There is a clear dominance of HPV-16 in the tonsils, and other types are rarely detected (Andl, 1998; Gillison et al., 2000; Mellin et al., 2000; Niedobitek et al., 1990; Paz et al., 1997; Snijders et al., 1992a; Steinberg and DiLorenzo, 1996).

Using DNA as well as RNA *in situ* hybridization, the viral genome of HPV-16 has been located to the cancer cells and not to the surrounding stroma in the primary tonsillar tumor and, importantly, also to its nodal metastases (Demetrick et al., 1990; Gillison et al., 2000; Niedobitek et al., 1990; Snijders et al., 1992a; Wilczynski et al., 1998). In a study by Gillison *et al.* the PCR HPV positive cancer cases were only confirmed by Southern blot and *in situ* hybridization in cancers of the oropharynx and rarely in tumors located outside the oropharynx.

Nonetheless, the detection of virus DNA does not show that the virus has a causal role in tumor development. Noteworthy, is the fact that the oncogenes of HPV-16, namely E6 and E7, are generally expressed in HPV positive tonsillar cancer (Mork et al., 2001; Snijders et al., 1992a; Snijders et al., 1992b; van Houten et al., 2001; Wilczynski et al., 1998). In

a report from a study by van Houten, 20 out of 84 head and neck cancers were found HPV positive by PCR, all typed HPV-16, but only nine showed mRNA E6 expression. However, seven of nine were located in the oropharynx and the other two in the oral cavity (van Houten et al., 2001). None of these nine oropharyngeal and oral cancers showed p53 mutation (van Houten et al., 2001).

It has been indicated that HPV positive and negative tonsillar tumors have distinctively different histopathological as well as different tumor molecular features. The histopathologic pattern of HPV positive tonsillar cancer cells seems to consist of poorly keratinized cells, with a basaloid morphology with a high nuclear-to-cytoplastic value (Gillison et al., 2000; Wilczynski et al., 1998). An association between HPV and basaloid features has also been reported for cancers of the vulva, the penis and the perianal area ((IARC), 1995). HPV positive tonsillar cancers seem to have a lower frequency of p53 mutations compared to what is found in HPV negative tonsillar cancer (Andl et al., 1998; Brachman et al., 1992; Gillison et al., 2000; van Houten et al., 2001). Moreover, in tonsillar carcinomas the HPV positive tumors have been shown to have decreased levels of pRb according to IHC, although when sequenced they contain wild-type pRb and normal mRNA pRb levels (Andl et al., 1998). The HPV positive, Rb defective tumors had low levels of cyclin D and over-expression of p16 (Andl et al., 1998) In contrast, HPV negative tonsillar cancer showed normal levels of pRb, over-expression of cyclin D1 and low expression of p16 (Andl et al., 1998; Wilczynski et al., 1998). Patients with HPV positive tonsillar cancer appear less likely to be heavy smokers and drinkers, furthermore, HPV-16 has been reported to have a synergistic risk effect in smokers (Gillison et al., 2000; Haraf et al., 1996; Schwartz et al., 1998). Patients with HPV positive tonsillar cancer seem to have a better clinical outcome as compared to patients with HPV negative tumors (Gillison et al., 2000; Mellin et al., 2000).

According to an epidemiological analysis, summarizing the number cases of tonsillar cancer in USA from 1973-1995, the incidence of tonsillar cancer has increased ~40% (from 1.9% to 2.7%), while the incidences for cancers at other oral sites have not (Frisch et al., 2000b). No decline in tonsillectomy could explain the incidence of tonsillar cancer (Frisch et al., 2000b). Furthermore, individuals with a history of an HPV associated anogenital malignancy have an increased risk for a second primary cancer in the tonsils as well as in the oral cavity, and in one study this risk was estimated to be 4.3 fold (Boice et al., 1985; Frisch and Biggar, 1999; Rabkin et al., 1992). In contrast, patients with an HPV unrelated cancer, e.g. colon cancer or breast cancer, had no increased risk of developing tonsillar cancer (Frisch and Biggar, 1999). Another study, in Sweden, also demonstrated an increase in tonsillar cancer in

women > 50 years with a history of *in situ* cervical cancer, and the incidence of tonsillar and tongue cancer were increased in the husbands of cervical cancer patients (Hemminki et al., 2000). Immunosuppressed patients have a higher incidence of HPV related cancers in general, and this was reported e.g. in HIV positive men, who were shown to have an increase of penile, anal as well as tonsillar cancer (Berkhout et al., 1995; de Villiers et al., 1997; Frisch et al., 2000a). Two case reports from transplanted patients who developed oropharyngeal cancer have also been reported (Demetrick et al., 1990; Swoboda and Fabrizii, 1993).

Why the high-risk types of HPV are more commonly found in tonsillar cancer compared to other tumor sites of the head and neck is not yet known. In the cervix it is the so-called transformation zone, where the squamous epithelium of ectocervix meets the columnar epithelium of endocervix, that is especially susceptible to HPV transformation. However, possible explanations could be that the tonsillar epithelium may be more sensitive to the transforming capacity of HPV or that HPV may have a facilitated access to basal mucosal cell in the tonsillar crypts.

Aim of the Thesis

Although the overall survival for tonsillar cancer patients correlates with tumor stage, there is a large discrepancy in clinical outcome among individuals having tumors within the same stage and receiving the same treatment. The biological aggressiveness and the radiotherapy sensitivity of tumors, within the same stage, varies considerably and cannot be predicted by conventional histopathological evaluation. Thus, in order to improve therapy results for these patients there is a strong need for additional predictive and prognostic factors. The aim of this thesis was therefore not only to examine for the presence of HPV in tonsillar cancer, but also to study the relation of HPV to clinical stage, p53 immunostaining and DNA aberration, and the influence of these tumors markers on clinical outcome in tonsillar cancer.

More specifically the aims of this thesis were:

- To estimate the prevalence and type of HPV in tonsillar cancer

- To investigate the prognostic value of HPV in tonsillar cancer and if it is related to response to radiotherapy

- To study how HPV correlates to p53 immunostaining and DNA aberration in tonsillar cancer

- To study the physical state and viral load of HPV in tonsillar cancer

Material and Methods

Patients (paper I-IV)

All patients included in the studies of paper I-IV, had obtained the diagnosis of primary tonsillar squamous cell carcinoma. Furthermore, all of the patients were treated for their disease between 1984-1999 at the Dept. of Otorhinolaryngology, Head and Neck Surgery or Radiumhemmet, Karolinska Hospital. However, in a few cases the diagnostic biopsies that were analyzed in these studies, were also obtained from the Departments of Otorhinolaryngology at Danderyd or Huddinge Hospital. Relevant clinical data, response to therapy and cause of death were collected from the patients charts at the above departments, but only after all the laboratory tests had been completed. Occasionally, the cause of death had to be searched for at other health care institutions.

The study material of paper I, II, and IV shared partly some of the same patients. Including all four papers, in total 104 patients were included, of which 26 were female. The mean age of all patients was 64 years (range 41-89 years) at diagnosis. All patients were treated with radiotherapy, delivered as fractionated radiotherapy with up to a total dose of 64 Gy. Fifty-four of the patients received radiotherapy pre-operatively, 7 patients had radiotherapy post-operatively and 32 had radiotherapy as a single treatment with the exception of the diagnostic biopsy of tonsillectomy (the remaining seven patients either died before therapy, refused therapy or treatment data was lacking). The median follow up time was 59.5 months for the surviving patients and 33 months when deceased patients were also included in the analysis. Only disease specific death was included in the survival analysis.

Tumor specimens (paper I-IV)

All analyzed biopsies were pre-treatment tissues of primary tonsillar cancer. For the HPV analyses three or five 5 μm thin sections from the paraffin-embedded biopsies were collected in tubes (paper I, II, IV). For the HPV analysis of fresh-frozen biopsies in paper III three 20 μm sections were cut from each biopsy. Before the first and after the last of the above sections a section was put on glass and hematoxoylin-eosin stained in order to confirm that all sections contained at leased 80% cancer cells. Control sections between every biopsy were collected to check for HPV contamination from the cryotom.

33

Methodological assays

DNA extraction (paper I-IV)

  DNA was obtained after standard proteinase-K digestion and phenol-chloroform extraction (Skyldberg et al., 1991). The paraffin-embedded samples were initially deparaffinized by melting of the paraffin in a water bath at 65°C for 10 min, and then 1 ml xylen was added to the samples, which were then incubated for 30 min. Thereafter the sample was centrifuged at 13000 rpm for 5 minutes and the pellet was saved. The xylen incubation and centrifugation was repeated. The pellet was washed with 95% ethanol and centrifuged as before. After removal of the supernatant the pellet was vacuum-died carefully, resuspended in 500 µl lysis-buffer and 20 µl proteinase-K (10 mg/ml) was added. The samples were then digested at 37°C for 2-5 days. After lysis, 0.5 ml phenol-chloroform (1:1) was added followed by 10 min centrifugation at 13000 rpm. The water-phase was transferred to new tubes and the DNA was precipitated with 1ml 95% ethanol, stored in -20°C for 15 min and then centrifuged for 30 min at 4°C. After saving the pellet, the DNA was washed with 70% ethanol and the centrifugation was repeated. The pellet was carefully vacuum-dried and suspended in 30 µl of distilled water.

  The DNA extraction from the fresh-frozen material was performed in a similar manner with minor differences such as that the lysis was done in 50° C over-night and that the final DNA pellet was suspended in 150 µl of distilled water.

HPV detection by PCR (paper I-IV)

  HPV screening was performed using broad-spectrum general primers Gp5+/6+ located in a conserved region of L1 (Table III) in all four studies (papers I-IV) (de Roda Husman et al., 1995). These Gp5+/6+ primers have been used extensively in the literature and are known to detect at least the 27 most common mucosal HPV types (de Roda Husman et al., 1995). The primer sequences for all primers used in all HPV detection and typing assays are listed in Table III. For the PCR program of Gp5+/6+ please see paper I, II and with small adjustments for an updated PCR machine in paper III (Friesland et al., 2001; Mellin et al., 2002; Mellin et al., 2000).

  In paper III an additional primer pair that binds to E1 was used. The CpI/IIG, primer pair were tested on the samples negative for Gp5+/6+ (Table III). These primers were used in order to detect HPV that may have lost the L1 region (Tieben et al., 1993). Furthermore, these primers have a different HPV profile, they are known not only to detect the common mucosal HPV types, but also several cutaneous types (Tieben et al., 1993). However, in paper III no additional HPV positive samples

34

were found with CpI/IIG that were not already detected with GP5+/6. For the PCR conditions used for the CpI/IIG primer pair, please see paper III (Mellin et al., 2002).

All primers used yield a fairly short PCR product (Table III), which is known to be an advantage when using paraffin embedded tissues, where the DNA is not always of optimal quality and where the material may contain PCR inhibitors (Shibata, 1994). In all runs negative PCR controls, the section negative controls (see above) as well as positive controls of cloned plasmids of HPV-16, -33 or –6 were included (kindly provided by Dr Kalantari, with permission from Prof. zur Hausen and Prof. de Villiers).

HPV typing by PCR (paper I-IV)

In paper I, II, IV the samples found HPV positive with the consensus primers were typed using type specific primers for HPV-16 and HPV-33 (Table III) (Hagmar et al., 1992). In paper III, all samples were tested with type specific primers for HPV-16, HPV-18 as well as HPV-33 (Hagmar et al., 1992). Controls were the same as for the consensus PCRs.

Table III. HPV primers used for screening and typing

Primer[1]	Sequence	Location[2]	Length[3]
GP5+	5´-TTTGTTACTGTGGTAGATACTAC-3´	6625-6647 (L1)	142 bp
GP6+	5´-GAAAAATAAACTGTAAATCATATTC-3´	6742-6766 (L1)	
CpIIG.[4]	5´-ATGTTAATWSAGCCWCCAAAATT-3´	1776-1798 (E1)	188 bp
CpI[4]	5´-TTATCAWATGCCCAYTGTACCAT-3´	1941-1963 (E1)	
16-1	5´-TCAAAAGCCACTGTGTCCTGA-3´	421-441 (E6)	120 bp
16-2	5´-CGTGTTCTTGATGATCTGCAA-3´	520-540 (E6)	
18-1	5´-CCGAGCACGACAGGAACGACT-3´	533-553 (E7)	173 bp
18-2	5´-TCGTTTTCTTCCTCTGAGTCGCTT-3´	682-705 (E7)	
33-1	5´-AACGCCATGAGAGGACACAAG-3´	567-587 (E7)	212 bp
33-2	5´-ACACATAAACGAACTGTGGTG-3´	758-778 (E7)	

[1]For further details see original papers (de Roda Husman et al., 1995; Hagmar et al., 1992; Tieben et al., 1993). [2] Location in the HPV genome, using HPV-16 as reference for the general primers. [3] Length of the PCR product obtained by the use of the indicated primer pairs. [4]The CpI/IIG primers are so called degenerated primers; W=A,T;S=G,C;Y=A,G.

DNA amplification control by PCR (paper I-IV)

To rule out false negative results, the HPV negative samples were assayed with primers GH26/27 of the HLA DQ locus, in order to check that they contained amplifiable DNA (Ehrlich and Bugawan, 1989). If the HLA PCR turned out to be negative the sample was excluded. The primer GH26 has the following sequence 5´-GTGCTGCAGGTGTAAACTTG-TACCAG-3´ and the sequence of GH27 is 5´-CACGGATCCGGTAG-CAGCGGTAGAGTT-3´. The final volume of 25 µl was a solution of, 1 X PCR Buffer II (Applied Biosystems), 200 µM of each deoxynucleotide, 2 mM magnesium chloride and contained 10 pmol of each primer and 0.5 U of Taq polymerase. Five µl of extracted DNA was included. As positive control extracted DNA from a tonsillar biopsy of non-malignant tissue was used. A negative control with no DNA was always included. Amplification of 40 cycles was run in an automated thermocycler (PE Applied Biosystems). The program consisted of one initial denaturation for 5 min at 95°C, a primer annealing cycle for 30 sec at 55°C and a primer extension cycle for 1 min at 72°C, followed by 38 identical cycles except for the denaturation time of 30 sec. The last cycle differed only with regard to the primer extension time, which was 5 min.

Sequencing of purified PCR products (paper III)

HPV typing results of the HPV type specific PCR were double checked in paper III by direct cycle sequencing of purified PCR products of consensus primers GP5+/6+. The GP5+/6+ PCR was repeated for the GP5+/6+ positive samples in order to have a yield of 50 µl, which was then purified by gel extraction (QIAquick, Qiagen). The PCR products were then sequenced, using the Big Dye Terminator Cycle Sequencing Kit, performed in 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/).

E2 and E1 gene detection (paper III)

In paper III, the physical state of HPV-16 in tonsillar cancer was studied (Mellin et al., 2002). In order investigate the integrity of E2 and E1 found in episomal HPV, both entire genes were amplified in two separate PCR assays as described previously (Das et al., 1992; Kalantari et al., 2001). For E2 amplification the following primers were used; ´5-AGGACGAGGACAAGGAAAA-3´ and 5´-TGTTTAGAACTATGACGTAG-G-3´ (Das et al., 1992). For E1 amplification the following primers were used; 5´-TGTGCCCCATCTGTTCTCA-3´ and 5´ GGCGCATGTGTTTCC-AATAG-3´ (Kalantari et al., 2001).

RIiPCR (paper III)

In order to investigate if tonsillar cancers harbor integrated and/or episomal HPV-16 a method, described earlier and developed by Kalantari *et al.*, based on restriction enzyme digestion, ligation and inverse PCR (*rIi*PCR) was used (overviewed in Figure 6) (Kalantari et al., 2001; Mellin et al., 2002). Running a long template PCR, using inverse primers (the Long A-S/AS primer pair) which are specific for HPV-16, the entireepisomal HPV-16 genome (7904 bp) is amplifiable in one single PCR, since episomal DNA is circular (Figure 6A). In contrast, integrated, linear, HPV-16 will not be amplified by inverse primers (Figure 6B). However, if the samples are digested with a restriction enzyme, which cuts human DNA fairly regularly and then are circularized by self-ligation, both episomal and integrated HPV-16 can be detected at the same time (Figure 6C). Integrated HPV detected this way will also yield PCR fragments containing virus-human junction sequences
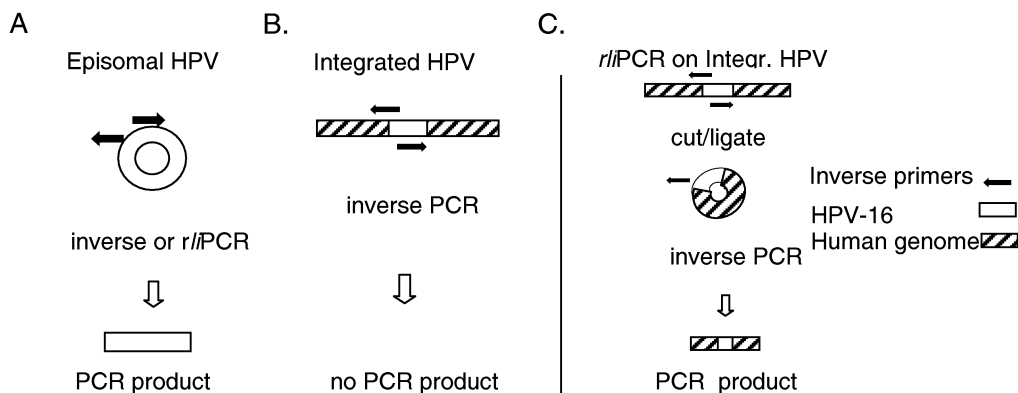

Figure 6- Methodical overview of restriction enzyme digestion, ligation and inverse PCR (*rIi*PCR). (*a*) Circular DNA as episomes yield PCR products directly with inverse PCR, and also after cutting and re-circulating as in *rIi*PCR. (*b*) Integrated HPV gives no PCR product on inverse PCR. (*c*) *RIi*PCR on integrated HPV gives PCR products with human-viral junctions.

Inverse PCR was run on undigested DNA (shows only episomal), as well as cleaved DNA and on cleaved and ligated samples (*rIi*PCR). As controls SiHa (1-2 copies of integrated HPV-16 per cell), two cervical cancer samples (F826, F3155) with known integration sites, and the HPV negative cell line (MCF-7) were used (Kalantari et al., 2001). For further PCR conditions please see paper III (Mellin et al., 2002).

DNA sequencing by primer walking (paper III)

In paper III, PCR products from an inverse PCR on undigested and digested and ligated DNA different from the episomal length of 7904 bp were gel-extracted (Qiagen Kit) and cycle sequenced as described above. The sequencing was initiated using the Long A-S primer, and was continued by constructing a new primer from the sequence obtained from the previous run, i.e. "primer walking". Sequencing was performed for episomal HPV from the Long A-S primer until the sequence of the Long A-AS primer was reached or for integrated HPV-16 where human DNA also was sequenced, until the other end of HPV-16 was reached (reference sequence of HPV-16 complete genome in BLAST GenBank was considered NC_001526).

HPV quantification by real-time PCR (paper III)

In Paper III the amount of HPV-16 copies per human genome equivalent was estimated by a quantitative real-time PCR method (TaqMan), based on the 5'-3' exonuclease activity of Taq DNA polymerase. Each time Taq polymerase amplifies a DNA strand, a fluorescent reporter molecule is released. The sequence detector monitors the emission and compares the accumulated amount to a specific standard. In order to create a standard with a known amount of virus copies, a plasmid (pGEM-T) containing HPV-16 E6 was created, see paper III. A dilution series of the pGEM-T plasmid with an HPV-16 E6 insert was made and used in each real time assay as a standard to calculate the number of viral copies. The quantitative real-time PCR was carried out with the HPV-16 specific primers, with the addition of a fluorogenic probe (16E6TQP) located in HPV-16 E6 as designed by Dr. Kalantari with the following sequence 6-FAM-CCGGTCCACCGACCC-CTTATATTATGGAATC-TT-TAMRA-3'-phosphate. For more details on the real-time conditions see paper III (Mellin et al., 2002). The 11 tonsillar tumors that were found HPV-16 positive in paper III, two HPV-16 negative tonsillar tumors, two cervical cancers biopsies (F3155, F725), CaSki and negative water blanks were run in duplicates and in parallel with the standard dilutions of HPV-16 plasmid in each analysis. As an internal positive control and as an estimation of the human genome content in the tonsillar samples, a quantitative real-time PCR was run on ß-actin as instructed by the kit suppliers (Applied Biosystems, Part Nr 401970).

P53 immunohistochemistry (paper II)

In paper II overexpression of p53 was assessed. The murine monoclonal antibody DO-1 was used for the immunohistochemical staining of p53. All tissue sections were 4 μm thick and prepared from formaline fixed, paraffin embedded specimens. For further details of the

procedure, see paper II (Friesland et al., 2001). The p53 immunostaining was considered positive when at least 5% of tumor cell nuclei were stained. Normal oral epithelial cells were used as an external control. Counting was carried out using an objective magnification of 40x (field diameter 400 µm) and evaluated by two independent observes.

Statistical analysis (paper I-IV)

In all papers the Pearsons χ^2 test was used to correlate the frequency of HPV or other findings to clinical data. Logistic regression was used in paper I and IV to test if correlation between the clinical data and the presence of HPV (and in paper IV also the degree of aneuploidy) influenced clinical outcome, e.g. if a confounding factor like small tumor stage influenced the results. Survival analysis was done in all papers by using the Kaplan-Meier method (Kaplan and Meier, 1958). The significance of the survival rate was analyzed by the log-rank test. Cox regression (uni- and multivariate) was used in paper I and IV to evaluate factors influencing the mortality risk.

Results and Discussion

HPV frequency and HPV type in tonsillar cancer (papers I-IV)

A central aim of this thesis was to study the frequency of HPV in tonsillar squamous cell cancer and to investigate which HPV types that dominate.

In paper I, II and IV, the presence of HPV was investigated by PCR in pre-treatment paraffin-embedded biopsies of primary tonsillar cancer. In total, 27 out of the 62 patients tested for HPV were found to be positive, hence the frequency of HPV was 43.5% in the paraffin-embedded biopsies (in paper I 26/60 patients were HPV positive, in paper II 14/34 patients were HPV positive and in paper IV 27/60 patients were HPV positive). In paper III the presence of HPV was investigated in 22 fresh-frozen biopsies and 12 were HPV positive, consequently the observed HPV frequency was 55% and somewhat higher than that observed in the paraffin-embedded biopsies. In summary, the overall frequency of HPV in paper I-IV was found to be 46% (39/84). Thirty-seven out of the 39 HPV positive samples were typed as HPV-16, one was typed as HPV-33 and one contained both HPV-16 as well as HPV-33. Both findings, i.e. the frequency of HPV in tonsillar cancer as well as the clear dominance of HPV type 16 were in line with other reports (Andl et al., 1998; Gillison et al., 2000; Klussmann et al., 2001; Mork et al., 2001; Paz et al., 1997; Snijders et al., 1992a; van Houten et al., 2001; Wilczynski et al., 1998). The typical finding is that 90-100% of the HPV positive biopsies in tonsillar cancer are typed HPV-16 and 0-7% are typed HPV-33, while HPV-31, HPV-59 or non-typeable HPV (HPV-X) are found more rarely. In contrast to cervical cancer where HPV-18 is found in approximately 15% of the cases, this type has not yet been reported to be present in tonsillar cancer (Munoz, 2000).

HPV and prognosis in tonsillar cancer (papers I-IV)

Another central aim of the thesis was to investigate if the presence of HPV is relevant with regard to the clinical outcome of patients with tonsillar cancer. A question that follows naturally is that if this is the case, then through what mechanism can HPV be a prognostic factor for these patients. Another related issue was if the prognostic relevance of HPV could be correlated to specific tumor markers.

40

In all papers the presence of HPV was correlated to prognosis. However, in paper I this was the main focus and thus this particular question is most thoroughly answered in that paper (Mellin et al., 2000). In paper I, we sought not only to examine the frequency of HPV in tonsillar cancer, but also to correlate the presence of HPV with tumor stage, nodal status, grade of differentiation, risk of tumor relapse and progression, and with survival. We could show that 52% of the patients with HPV positive tonsillar cancers were tumor free three years after diagnosis, compared to 21% of the patients with HPV negative tumors (OR=4.18, p=0.025, χ-test). The number of tumor free patients after three years in each TNM stage according to HPV status is shown in Figure 7. There were only 10 patients in the lower tumor stages (stage I-II) included in the study, and 3/4 in stage I and 4/8 in stage II were found to be HPV positive (Mellin et al., 2000). However, using logistic regression and taking into account the somewhat uneven distribution of HPV in stage I, the odds ratio was still significantly in favor of the HPV positive patients (OR=19,6; p=0.014). The influence of nodal status, age, gender and tumor differentiation grade was also adjusted for, and patients with HPV positive tonsillar cancer still remained disease free to a higher degree compared to patients with HPV negative tonsillar cancer.
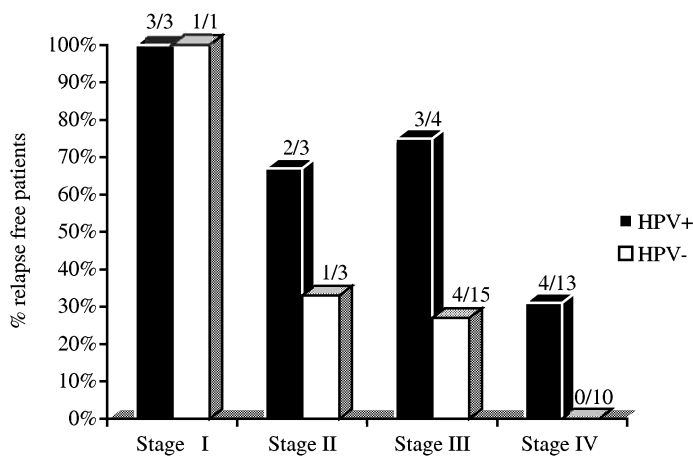

Figure 7- Number and percent of disease free patients at three years after diagnosis for each TNM stage according to HPV status. Graph from paper I.

41

Furthermore, as shown in Figure 8 and in paper I, the cause-specific survival was found to be significantly better for patients with HPV positive tonsillar cancer than for the group of patients with HPV negative tonsillar cancer (p=0.047, log rank test). Three years after diagnosis the HPV positive group had a survival rate of 65.3% compared to 31.5% in the HPV negative group. At five years the survival rate was 53.5% in the HPV positive group and 31.5 % in the HPV negative group. When estimating the cause-specific mortality risk over time, the HPV positive group had half the risk of dying of tonsillar cancer as compared to the HPV negative group (p=0.049, Cox regression analysis). The better survival of patients with HPV positive tumors was independent of stage, nodal status, age and gender (p=0.023, Cox multivariate analysis). The result of a better outcome for patients harboring HPV in their tonsillar cancer included both staying disease free as well survival were also confirmed in study IV. Please note that to a high degree the same patients were included in both paper I and in paper IV.
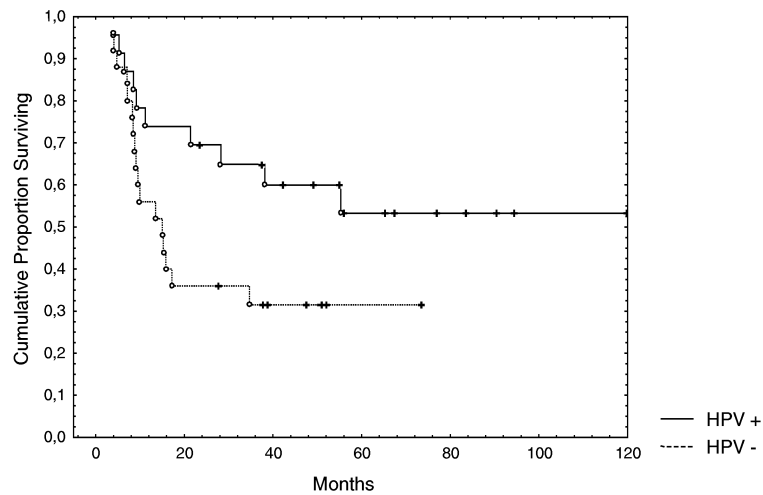

Figure 8 - Kaplan-Meier graph from paper I showing that patients harboring HPV positive tonsillar cancer had significantly better survival as compared to patients with HPV negative cancers (p=0.047, log rank test).

Most patients that were analyzed in paper II had advanced tumors and all 34 patients that were tested for HPV, except for two of the patients, had stage III and IV tumors. This HPV distribution in study II probably influenced the results of the prognosis analysis and only patients with stage IV HPV positive tumors had a significantly better survival (p=0.0431, log rank test) than patients with stage IV HPV negative tumors. This was plausibly due to that 10 out of 14 HPV positive tonsillar cancers, were observed in patients with stage IV tumors (while the remaining 4 HPV positive tonsillar cancers were all stage III). The same was found in a study by Haraf *et al.*, where also most HPV positive tonsillar cancers had advanced stage (Haraf et al., 1996). Consequently, only a significant better survival for patients with HPV positive tonsillar cancer, compared to patients with HPV negative tonsillar cancer was noted in patients with stage IV tumors (Haraf et al., 1996). In paper III, 20 patients were included in the prognostic evaluation, and once again the HPV positive group had a better outcome both regarding staying disease free as well as survival. However, the results in paper III demonstrated a statistically non-significant tendency (p=0.09,χ^2 test; p=0.08, log rank test respectively), possibly due to the few patients included.

A favorable prognostic value of HPV in tonsillar cancer has also been reported by Gillison *et al.* (Gillison et al., 2000). In that study 253 head and neck cancer patients were analyzed of which 60 were patients with oropharyngeal cancers (mostly tonsillar cancers) (Gillison et al., 2000). Disease-specific survival was significantly improved for the HPV positive oropharyngeal cancer group as compared to the HPV negative group (Gillison et al., 2000). In contrast, among patients with cancer at sites other than the oropharynx the disease-specific survival was similar regardless of HPV status (Gillison et al., 2000). Accordingly, the prognostic value of HPV does not seem to hold for head and neck cancer in general, but for tonsillar cancer more specifically (Gillison et al., 2000; Paz et al., 1997; Riethdorf et al., 1997; Snijders et al., 1996). Another study presented a survival analysis on 31 patients with tonsillar cancer according to the pRb expression of the cancers and demonstrated a significantly better survival for patients with tumors lacking pRb expression (Andl et al., 1998). HPV presence and survival were not analyzed separately, but only indicated since there was a significant correlation between lack of pRb expression and presence of HPV (Andl et al., 1998). A third study, of 52 patients with tonsillar cancer, reported a survival advantage in patients with HPV positive tonsillar cancer (Strome et al., 2002). However, in this study, the HPV positive patients were substantially younger than the HPV negative patients and the survival advantage was thus not statistically significant when adjusting for age in a multivariate analysis (Strome et al., 2002). A study by Portugal *et al.*demonstrated that patients with HPV positive tonsillar cancer had a

significantly better five year survival rate than HPV negative patients, however, neither survival analysis over time (as Kaplan-Meier analysis) nor Cox multivariate analysis was shown (Portugal et al., 1997).

The mechanisms responsible for a better outcome for patients with HPV positive tonsillar tumors remains to be revealed. In general, the explanation may be found either in the patient profile (e.g. genetic factors, age or life-style related) or in tumor biological differences between HPV positive and negative tumors, which in turn results in a different clinical behavior. These factors can be connected e.g. smoking may yield p53 mutations and hence an adverse clinical outcome. However, heavy smoking may also be correlated to decreased health status and thus yield a negative impact on clinical outcome. Concerning the patient category, it has been reported that alcohol consumption and smoking occurs less frequently among patients with HPV positive tonsillar cancer (Gillison et al., 2000; Haraf et al., 1996; Klussmann et al., 2001; Koch et al., 1999; Lindel et al., 2001; Schwartz et al., 1998; Strome et al., 2002). However, it is clear that some of the patients with HPV positive tumors are smokers and a study by Schwartz et al. found that smoking and HPV-16 together increased the risk of oral cancer more than each of these factors alone (Schwartz et al., 1998). Furthermore, there is a tendency but no clear evidence that HPV positive tonsillar cancer patients are significantly younger than patients with HPV negative tumors (Gillison et al., 2000; Haraf et al., 1996; Klussmann et al., 2001; Koch et al., 1999; Lindel et al., 2001; Mellin et al., 2000; Schwartz et al., 1998; Strome et al., 2002).

HPV, response to radiotherapy and prognosis (paper II)

In a study by Friesland *et al.* it was noted that there was a significantly better survival for patients with tonsillar cancer that had a complete remission (CR) after radiotherapy than those who had non-CR, i.e. no response, a partial response or progressive disease (Friesland et al., 1999). The evaluation of radiotherapy outcome was performed one month after completion of radiotherapy by clinical examination and, in non-surgical cases when required, also in combination with a biopsy at the primary tumor site. When no evidence of the tumor could be observed the outcome was classified as CR and when there was a viable tumor left the outcome was classified as non-CR. After the finding of a favorable outcome for patients with HPV positive tonsillar cancer, one aim of paper II was to analyze if HPV positive tumors were more sensitive to radiotherapy than HPV negative tonsillar tumors.

**TABLE IV – HPV and p53 status according to complete response
(CR) to radiotherapy (paper II)**

Response[1]	HPV+	HPV-	p53+	p53-
CR	8 (57%)[2]	10 (50%)	11 (58%)	10 (50%)
Non-CR	6 (43%)	10 (50%)	8 (42%)	10 (50%)
Total no[3]	14 (100%)	20 (100%)	19 (100%)	20 (100%)

[1]Response evaluated one month after
completed radiotherapy. [2]Percent of the column,
i.e. percent of responders per HPV or p53
status. [3]34 patients were HPV tested and 39
patients were analyzed for p53 status.

For that purpose 21 complete responders and 19 non-complete
responders were selected. Among these 40 patients, 34 had tumors with
amplifiable DNA and these tumors could be evaluated for HPV status.
There were no significant differences between the response to
radiotherapy between patients with tumors with different HPV status, and
57% of the patients with HPV positive tumors and 50% of the patients
with HPV negative ones had a CR (Table IV).

However, in a recent analysis, when including data from 65 patients
with tonsillar cancer, i.e. when including almost the double amount of
patients evaluated in paper II, we note a possible although not significant
difference with regard to HPV status and response to radiotherapy. As
viewed in Table V, 71% of the patients with HPV positive tumors were
CRs compared to 53% of the patients with HPV negative tumors
(p=0,134, χ^2 test). Patients with post-operative radiotherapy were
excluded.

**TABLE V – HPV status according to complete response (CR)
to radiotherapy (unpublished data)**

Response[1]	HPV+	HPV-
CR	22 (71%)[2]	18 (53%)
Non-CR	9 (29%)	16 (47%)
Total no	31 (100%)	34 (100%)

[1]Response evaluated one month after
completed radiotherapy. [2]Percent of the
column, i.e. percent of responders per HPV
status.

Moreover, when including these patients in a Kaplan-Meier survival analysis, one finds that a complete response to radiotherapy could be more crucial than HPV status (Figure 9; p=0,00002; unpublished data). In the HPV positive group, the patients that have CR have a significantly better survival compared to the HPV positive patients that have a non-CR (Figure 9, p=0.0011, log rank). Accordingly, in the HPV negative group, the patients that have CR have a significantly better survival as compared to the HPV negative patients that have a non-CR (Figure 9, p=0.00006, log rank). However, in the CR group, the HPV positive patients have a 82% 5-year survival rate compared to the HPV negative patients that have a 61.5% 5-year survival rate (Figure 9, p=0.26, log rank test).
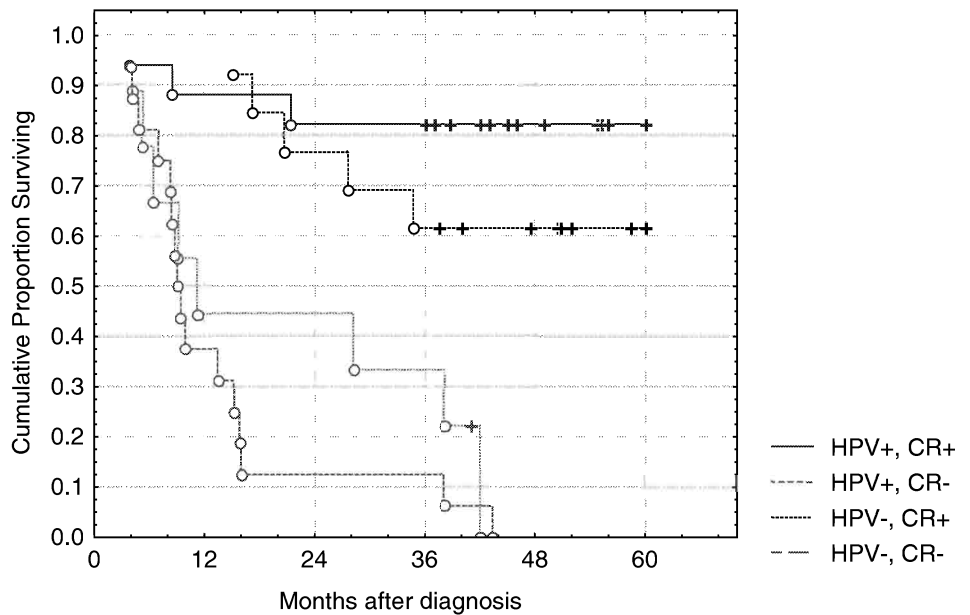
Figure 9 - Kaplan-Meier survival curve showing the cause-specific survival in HPV positive and negative tonsillar cancer according to complete response (CR+) to radiotherapy or non-CR (CR-) (p=0.00002). There is a significant difference according to CR or non-CR regardless of HPV status. The highest survival rate is seen in patients with HPV positive tumors that respond with complete remission to radiotherapy. Unpublished data.

Please note that these unpublished survival data, have neither been tested with uni- nor multivariate analysis. In conclusion, slightly more HPV positive patients have CR than the HPV negative patients, the response to radiotherapy is crucial for survival, and among the CR group the HPV positive patients seem to have the highest survival rate.

In line with this recent finding, a study on HPV and local control after radiotherapy in oropharyngeal cancer, reported that HPV positive tumors seemed to be more radiotherapy sensitive (Lindel et al., 2001). However, only 14 percent of the patients were found HPV positive in this study and the results were not significant (Lindel et al., 2001).

Thus, there is an indication although still weak that HPV positive tonsillar cancer responds better to radiotherapy. Tumor biological differences, such as p53 status, between tonsillar cancers harboring oncogenic HPV and tumors lacking HPV may implicate differences in radiotherapy sensitivity. Obata *et al.* found that oropharyngeal tumors with wild type p53 respond better to radiotherapy than tumors with mutated p53 (Obata et al., 2000).

HPV, p53 and prognosis (paper II)

In addition to the aim of investigating the correlation of HPV to radiotherapy response, we also wished in paper II to analyze the relation of HPV to p53 overexpression, and the correlation of both these factors to the response to radiotherapy and survival in tonsillar cancer. HPV was analyzed in 34 primary tonsillar tumors, and p53 immunohistochemistry (IHC) in 39 tumors (Friesland et al., 2001). In normal cells with wild-type p53, the tumor suppressor protein has a short half-life and is thought to be undetectable with immunohistochemistry (Soong et al., 1996). In cells with mutated p53, the level of the protein is stabilized, "overexpressed", at least partly due to the inability of mutated p53 to induce transcription of MDM2, a protein which both degrades p53 and inhibits p53 transcription (Haupt et al., 1997; Thut et al., 1995). In paper II the distribution of p53 positive tumors, i.e. tumors showing p53 overexpression, and p53 negative tumors, i.e. tumors with undetectable p53, were even in the CR and the non-CR groups (Table IV). There was no difference in survival according to p53 immunohistochemistry. Out of the 14 HPV positive cases, 6 (43%) were p53 positive and 8 (57%) were p53 negative. Negativity of p53 IHC was thus not demonstrated in all HPV positive cases. Out of the remaining 20 HPV negative tumors, 11 (55%) were found to be p53 positive and 9 (45%) were p53 negative. No correlation was found between the four groups of HPV+/p53+, HPV+/p53-, HPV-/p53+, and HPV-/p53- tumors and response to radiotherapy (Friesland et al., 2001). The only difference in survival was that patients with HPV+/p53- stage IV tumors (the majority of the tumors in paper II were in stage IV) had a better survival compared to patients with HPV-/p53-

47

tumors, according to Kaplan-Meier analysis (p=0.02, log rank test) (Friesland et al., 2001).

Our finding that p53 overexpression was rather common in HPV positive cases is in line with a study by Snijders *et al.,* who found that 4/8 (50%) of the HPV positive tonsillar tumors showed elevated p53 expression when tested with IHC (Snijders et al., 1994). In that study, p53 mutation was in contrast to p53 IHC not as common in HPV positive tonsillar cancers, and only 1/10 (10%) showed a p53 mutation (in addition to one silent mutation, yielding no change in amino acids and p53 function) (Snijders et al., 1994). The fact that in our study tonsillar tumors with HPV were not found to be p53 negative may depend on that p53 overexpression does not exclusively reflect p53 mutation status (Mineta et al., 1998; Riethdorf et al., 1997). It has been suggested that wild-type p53 may be stabilized in tumors by disturbances in the degradation of p53 or be normally elevated due to the presence of DNA damage (Mineta et al., 1998, and ref. therein). Furthermore, there may be p53 mutations that do not stabilize p53 sufficiently enough to be detected by IHC. Alternatively, p53 mutations may alter the p53 structure in such a way that the antibody does not react with the epitope of the protein (Saunders et al., 1999). Riethdorf *et al.* reported that in 80% of the p53 mutated head and neck tumors, p53 was IHC also positive, while only 54% of the IHC positive cases had mutated p53 when examined by sequencing (Riethdorf et al., 1997). It has been shown by others that tonsillar cancers harboring HPV most often contain wild-type p53 when compared to HPV negative tumors (And et al., 1998; Brachman et al., 1992; Gillison et al., 2000; van Houten et al., 2001). In a study by Gillison *et al.* e.g., 2/17 (10.5%) of the HPV positive oropharyngeal tumors (mostly tonsillar cancer) had mutated p53, while 5/15 (67%) of the HPV negative oropharyngeal tumors had mutated p53 (Gillison et al., 2000). Furthermore, in oropharyngeal cancer, it has been reported that p53 mutation is more common in smokers than in former and non-smokers and that HPV is more common in the latter group (Koch et al., 1999).

As mention above, it has been reported that oropharyngeal tumors with wild type p53 respond better to radiotherapy than tumors with mutated p53 and accordingly, patients with wild-type 53 had significantly better survival than patients with p53 mutations (Obata et al., 2000) Furthermore, it has been found that especially p53 DNA contact mutations have a strong negative impact on clinical outcome in head and neck cancer in general (Erber et al., 1998)

HPVs physical state and prognosis (paper III)
In cervical cancer the HPV genome is mainly integrated in the host genome (Cullen et al., 1991; Klaes et al., 1999). Integration leads to

48

disruption and deletion of the viral genes E1 or E2 open reading frames (ORFs), which encode for proteins of importance for viral replication and viral transcription (zur Hausen, 1999). Disruption of E2 may allow dysregulation of the E6/E7 genes, which encode proteins which are required in HPV associated cervical cancer and essential for maintenance of the malignant phenotype (zur Hausen, 1999). However, in 15-30% of the HPV-16 positive cases of cervical cancer only the episomal form of HPV-16 is found (Cullen et al., 1991; Das et al., 1992; Kalantari et al., 2001). The E6 and E7 transcription level is regulated by a promoter in the long control region (LCR) and is influenced by viral as well as cellular transcription factors (zur Hausen, 1999). In cervical cancer as well as in oral cancer biopsies with episomal HPV-16, genetic changes in the LCR and subsequent elevated activity of the E6/E7 promoter have been reported (Dong et al., 1994; May et al., 1994; Chen et al, 1997; Watts et al., 2001).

The aim of paper III was therefore to investigate the physical state and the viral load of HPV-16 in tonsillar cancer and to correlate these findings with clinical outcome. To distinguish between integrated and episomal forms of HPV, 22 fresh-frozen tonsillar cancer samples were analyzed by a method based on restriction enzyme cleavage, ligation and PCR (rliPCR, see Methodological assays). Only extra-chromosomal forms of HPV-16 were observed. Full-length episomal HPV was detected exclusively in 7/11 of the cancers, while both full-length and deleted forms of episomal HPV-16 were found in parallel in two other tumors. In one tumor only a deleted episomal form of HPV-16 was present. In the remaining HPV-16 positive tumor both full-length episomal as well as an 11 kbp PCR product were detected and if the 11 kbp product contained integrated HPV, or was off-size linearized episomal could not be determined. In two cervical cancer controls, HPV-16 was integrated and could be chromosome located. The physical state of HPV-16 in tonsillar cancer could not be correlated to prognosis, since all HPV positive cancers contained episomal HPV.

Our results that HPV-16 is mainly episomal in tonsillar cancer are in line with the findings by Snijders et al. where the physical state of HPV-16 in two tonsillar cancer biopsies was investigated, by Southern blot and two-dimensional gel electrophoresis analysis, and where both tumors contained only episomal forms of HPV (Snijders et al., 1992b). Both tumors were also found to express E6 and E7 (Snijders et al., 1992b). The fact that HPV-16 in cervical cancer has been found to harbor HPV in its episomal form in up to one third of the cases, suggests that HPV-16 can transform even without integration into the host genome (Cullen et al., 1991; Das et al., 1992; Kalantari et al., 2001; Watts et al., 2002). As mentioned above, in oral cancer as well as in cervical cancer it has been shown that extra-chromosomal HPV-16 can exhibit genetic modifications

in the LCR, leading to an enhanced activity of the LCR, which in turn may influence the promoter activity of E6/E7 transcription (Dong et al., 1994; May et al., 1994; Chen et al, 1997; Watts et al., 2001). If the LCR is mutated in a similar way in HPV-16 positive tonsillar cancer is unknown but could in fact be possible.

In paper III, HPV-16 was quantified by real-time PCR and most tonsillar cancers contained between 10 to a few hundred copies of HPV per ß-actin. The HPV-16 positive tumors could be divided into two groups according to their viral load, either they harbored over 190 viral copies per human genome or they harbored below 60 viral copies per human genome. Unexpectedly, there was a significant difference in prognosis in the two viral load groups (Figure 10). The six patients with tumor sections with ≥190 HPV-16 copies/ß-actin remained tumor free (p=0.026) as well as had a better survival rate (p=0.039) when compared to the five patients with tumors sections with ≤ 60 HPV-16 copies/ß-actin (Figure 10).
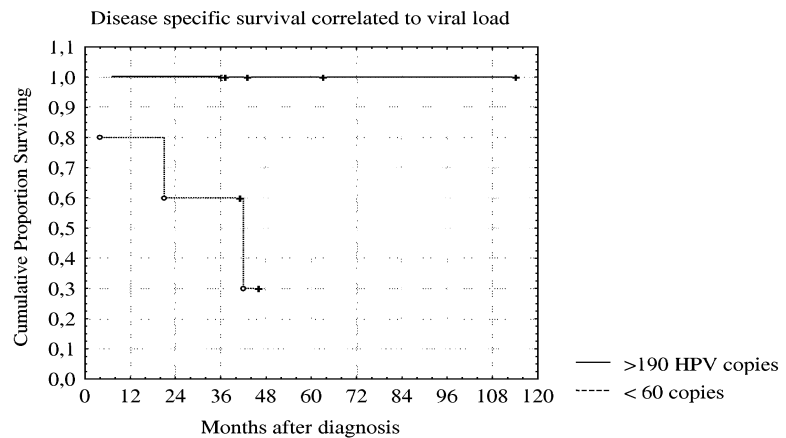

Figure 10 - Kaplan-Meier graph from paper III showing significantly better disease specific survival in patients with tonsillar tumors with ≥190 HPV-16 copies/β-actin when compared to patients with tumors with ≤ 60 HPV-16 copies/β-actin.

Our estimation of viral load in tonsillar cancer is similar to the study of Klussman *et al.*, in which six tonsillar cancers and corresponding metastasis were analyzed, and the viral copy number per ß-goblin varied

between 5.8-152.6 (Klussmann et al., 2001). Our finding that a high viral load is correlated with a better prognosis for patients with in tonsillar cancer should be confirmed in extended studies since few patients were included (n=11).

HPV and DNA aberrations (paper IV)

Using image cytometry (ICM) the degree of DNA aneuploidy can be investigated, which has been found to correlate to prognosis in some head and neck cancers. Oncogenic HPV may cause aneuploidy through two mechanisms. One is through the ability of the viral protein E7 to uncouple the duplication of the centrosome from the cell division cycle, probably by targeting the pRb pathway. The second mechanism would be by the disturbing effect of E6 on the checkpoint function of the cell cycle, by degrading p53 (Duensing and Munger, 2001). In paper IV we wished to investigate if HPV positive and negative tonsillar cancer differ in DNA content and if the degree of DNA aberration also influences clinical outcome. The DNA content was estimated in 58 primary tonsillar tumors. The normal diploid cell nuclear DNA content was indicated as the 2c value (c is the haploid genome equivalent). The fraction (percent) of cancer cells exceeding 2.5c was referred to as the 2.5c exceeding rate (2.5c ER), while the percent of cancer cells exceeding 5c was referred to as the 5c exceeding rate (5cER). Cancer cells with a DNA content value above 2.5c are considered as either proliferating diploid cells or aneuploid cells, whereas cells with a DNA content value above 5c ER are considered to be aneuploid (hyperploid). A lesion was classified as diploid if none of the cells exceeded 5c ER and less than 35% of the cells were between 2.5-5c ER.

Most of the analyzed tumors exhibited a high degree of aneuploidy, harboring a mean of 17.5% in 5cER and only 7 (12%) of the tumors were found diploid (Figure 11). Patients with a cancer with a 5c ER below the mean value were to a higher degree disease free after 3 years and displayed a better survival as compared to patients harboring tumors with a 5c ER above the mean value. These differences were however not statistically significant. HPV positive tumors had a tendency to have a lower mean 5c ER, 13%, as compared to 22% for the HPV negative tumors (p=0.066, χ^2 test). Furthermore, significantly fewer HPV positive tumors had a 5c ER above the mean value compared to the HPV negative tumors (p=0.026, χ^2 test).
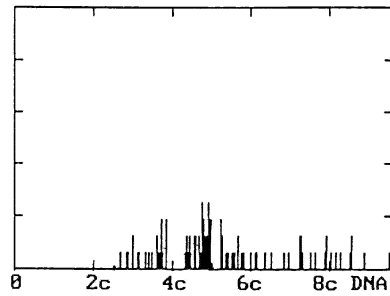
Figure 11 - A representative DNA histogram from paper IV of a tonsillar cancer displaying a high degree of aneuploidy. The 100 selected cancer cell nuclei have 100% >2.5 c ER and >39% 5c ER.

Conclusively, tonsillar cancer has a high degree of aneuploidy, although HPV positive tumors have a lower degree of aneuploidy than HPV negative tumors. The latter is now being studied further, and an ongoing investigation of specific chromosome amplifications and deletions (using comparative genomic hybridization) may reveal information on differences in genetic patterns between HPV positive and HPV negative tonsillar cancers (Dahlgren et al., Manuscript). It may even be possible that specific chromosomal changes in the two groups correlate to clinical outcome. HPV status seems to have a stronger prognostic value in tonsillar cancer than DNA content.

The results that HPV positive as well as HPV negative tumors contained a high degree of DNA aberrations and few tumors displayed diploidy are in accordance with what has been observed in cervical cancer (Lorenzato et al., 2001; Rihet et al., 1996; Skyldberg et al., 2001). In fact, in one report most cervical cancers had 20% in 5c ER, and only a very limited number were shown to be diploid and in another study all cervical cancers included were reported to be aneuploid (Skyldberg et al., 2001; Steinbeck and Auer, 2000).

Conclusions

The studies presented in this thesis have shown the following:

- HPV is found more frequently (~ 45%) in tonsillar cancer compared to what has been reported for other head and neck cancers. HPV-16 is mainly detected, although HPV-33 may be found occasionally.

- Presence of HPV is of prognostic value, i.e. patients with HPV positive tonsillar cancer are to a higher degree tumor free three years after diagnosis and have better cause-specific survival than patients with HPV negative tonsillar cancer.

- HPV positive tonsillar cancers tended to respond better to radiotherapy compared to HPV negative cancers, however not statistically significant. Complete response after radiotherapy seems to be prognostically more important than HPV status for patients with tonsillar cancer. Nonetheless, among the group with a complete response after radiotherapy, the HPV positive patients appear have the highest survival rate.

- P53 overexpression evaluated by immunohistochemistry is common in both HPV positive and negative tonsillar cancer and does not seem to have any impact on either response to radiotherapy or survival.

- HPV-16 is mainly episomal in tonsillar cancer. The viral load shows a wide distribution and our results suggest that clinical outcome may be better for patients with tumors containing a high HPV load.

- Tonsillar cancer displays a high degree of aneuploidy. However, DNA content is not an optimal prognostic factor. HPV positive tumors generally have a lower degree of aneuploidy than HPV negative tumors. Independently of DNA ploidy, HPV status appears to be of better prognostic value in tonsillar cancer than DNA content.

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54

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