HUMAN PAPILLOMAVIRUS TYPE 16 IN HEAD AND NECK CARCINOGENESIS

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Human Papillomavirus Type 16 in Head and Neck Carcinogenesis

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1. ABSTRACT

The etiology of squamous cell carcinomas of the head and neck (HNSCC) is considered to be multifactorial. Besides the main risk factors of tobacco and alcohol, infection by human papillomavirus (HPV) and genetic alterations are likely to play a role. Oncogenic HPVs are a causative agent in uterine cervical cancer, and molecular biology approaches have provided evidence for specific mechanisms. The role of HPV infection in head and neck carcinogenesis is less well established. HPV DNA has been repeatedly detected in HNSCC, especially in squamous cell carcinomas of tonsils. Little is known about HPV infection in tumor-free tonsils.

To investigate the role of tumor suppressor genes, *PTEN* and *LKB1*, in laryngeal tumorigenesis, we analyzed somatic mutations of these genes from laryngeal carcinoma and laryngeal papillomatosis by genomic sequencing and single strand confirmation polymorphism assay, respectively. We observed no mutation in these two genes in our studied samples.

In an attempt to elucidate HPV status in head and neck carcinomas, we studied the frequency of HPV DNA and viral genotypes in HNSCC by means of SPF10 PCR and INNO-LiPA hybridization. We found that more than half the patients harbored HPV DNA in their HNSCC samples, and oncogenic HPV-16 was predominant. Copy numbers of viral E2 and E6 genes in the HPV-16 positive HNSCC samples were quantitated by real-time PCR. We observed a considerable variation in the viral copy numbers among different samples, and tonsillar carcinomas had a remarkably higher viral load than did carcinomas in the other sites of the head and neck region. HPV-16 existed in episomal, integrated, or mixed forms and tumors harboring only episomal virus were significantly larger in size at diagnosis than those with only integrated or mixed forms. The presence of HPV DNA did not correlate with a better prognosis in HNSCC patients.

Furthermore, we also observed that 6% of patients with tonsillitis or tonsillar hypertrophy harbored oncogenic HPV-16 but no other types in their tonsillar tissue, as determined by a sensitive nested PCR assay. The presence of HPV-16 DNA in tonsils did not correlate with serum antibodies against viral proteins. Our findings imply that tonsillar epithelial cells serve as a reservoir for HPV-16 virus.

To establish an *in vitro* model to study mechanisms involved in HPV-associated tonsillar carcinogenesis, normal human tonsillar epithelial (HTE) cells were transfected with full-length HPV-16 DNA. All the transfected HTE cell lines were cytogenetically abnormal. They exhibited altered morphology and impaired expression of cytokeratins in organotypic culture. They failed to form colonies in soft agarose and formed no tumors in nude mice within 6 months. They have been cultured over 100 passages and considered as immortalized cell lines and thus provide a useful model for studying further the multistep molecular events of HPV-16 associated tonsillar carcinogenesis.

2. ORIGINAL PUBLICATIONS

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

- I. **Chen RW**, Avizienyte E, Roth S, Leivo I, Mäkitie AA, Aaltonen L-M, Aaltonen LA. *PTEN* and *LKB1* genes in laryngeal tumors. *J Med Genet* 1999; 36; 943-4.
- II. Koskinen W*, **Chen RW***, Leivo I, Mäkitie A, Bäck L, Kontio R, Suuronen R, Lindqvist C, Auvinen E, Molijn A, Quint WG, Vaheri A, Aaltonen L-M. Prevalence and physical status of human papillomavirus in squamous cell carcinomas of the head and neck. *Int J Cancer* 2003; 107; 401-6. *These two authors contributed equally to this article.
- III. **Chen RW**, Sehr P, Waterboer T, Pawlita M, Leivo I, Vaheri A, Aaltonen L-M. Presence of DNA of human papillomavirus 16 but no other types in tumor-free tonsillar tissue. Resubmitted (*J Clin Microbiol*)
- IV. **Chen RW**, Aalto Y, Teesalu T, Dürst M, Knuutila S, Aaltonen L-M, Vaheri A. Establishment and characterization of human papillomavirus type 16 DNA immortalized human tonsillar epithelial cell lines. *Eur J Cancer* 2003; 39; 698-707.

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3. ABBREVIATIONS AND DEFINITION OF SOME ESSENTIAL TERMS

AJC	American Joint Cancer Committee
ATCC	American Type Culture Collection
DEIA	DNA enzyme immunoassay
E	early (gene)
EGF	epidermal growth factor
FISH	fluorescence <i>in situ</i> hybridization
HES	human embryonic skin
HNSCC	squamous cell carcinoma of the head and neck
HPV	human papillomavirus
HTE	human tonsillar epithelium
IARC	International Agency for Research on Cancer
IHC	immunohistochemistry
ISH	<i>in situ</i> hybridization
kb	kilobase(s)
L	late (gene)
LiPA	line probe assay
MMAC	mutated in multiple advanced cancers
NCR	non-coding region
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
<i>PTEN</i>	phosphatase and tensin homolog gene
SCC	squamous cell carcinoma
SPF	short PCR fragment
SSCP	single strand confirmation polymorphism
<i>LKB1/STK11</i>	serine/threonine kinase 11
UICC	International Union Against Cancer
VLP	virus-like particle

EPISOMAL VIRUS – viral DNA exits extrachromosomally in a cell.

INTEGRATED VIRAL GENOME – a viral genome which is incorporated into the cellular DNA and is replicated with it.

INTEGRATION – the process of insertion of viral DNA into the host genome. It usually involves a virus-coded enzyme, the integrase. The viral DNA is then replicated by the host nucleic acid replication mechanism.

TRANSFECTION – Two different approaches serve to transfer DNA into eukaryotic cells: transient transfection and stable transfection. In transient transfection, recombinant DNA is introduced into a recipient cell line in order to obtain a temporarily but high level of expression of the target gene. The transfected DNA does not necessarily become integrated into the host chromosome. Stable transfection is used to establish clonal cell lines in which the transfected target gene is integrated into chromosomal DNA.

FISH – This procedure describes fluorescence in situ hybridization of biotin- or digoxigenin-labeled probes to denatured metaphase chromosomes and interphase nuclei. The hybridized probes are detected and visualized with fluorochrome-conjugated reagents.

4. INTRODUCTION

The etiology of squamous cell carcinomas of the head and neck (HNSCC) is considered to be multifactorial. Besides the main risk factors, tobacco use and alcohol intake (Decker and Goldstein 1982; Franceschi *et al* 1990), infection by oncogenic human papillomavirus (HPV) as well as genetic alterations are likely to play a role in it. Several tumor suppressor genes play a role in human tumorigenesis. Somatic mutations of the *PTEN* gene, a major new tumor suppressor gene located at human chromosome 10q23, have been found in several types of cancers, including gliomas and glioblastoma, as well as carcinomas of prostate, kidney, and breast (Daniel *et al* 1995; Fallani *et al* 1997; Steck *et al* 1997). Germline mutations in *LKB1*, a tumor suppressor gene mapped to 19p13.3, have been associated with Peutz-Jeghers syndrome (PJS) (Hemminki *et al* 1998; Jenne *et al* 1998) whereas somatic mutations in this gene are a rare event in most sporadic tumors (Avizienyte *et al* 1998; Avizienyte *et al* 1999; Bignell *et al* 1998; Park *et al* 1998; Resta *et al* 1998). Little is known about the role of *PTEN* and *LKB1* genes in the development of laryngeal tumors, among which approximately 25% (range 0 to 100%) of laryngeal carcinomas are associated with infection of oncogenic HPVs and 83 to 100% of the laryngeal papillomatosis are associated with infection by HPV types 6 or 11 (Syrjänen and Syrjänen 2000; Aaltonen *et al* 2002).

HPVs are small double-stranded DNA viruses infecting epithelial cells of the skin, as well as anogenital and oropharyngeal mucosa. To date, 96 HPV genotypes have been identified and fully sequenced (de Villiers *et al* 2004). An increasing number of studies have reported on the presence of HPV in HNSCC; its frequency varies greatly, depending on the number of patients included and tumor site, as well as detection methods applied. By a sensitive PCR, the overall prevalence of HPV DNA in HNSCC has been reported from 9 to 60% and the majority of HPV-positive tumors contained the high-risk HPV-16 (Snijders *et al* 1996; Paz *et al* 1997; McKaig *et al* 1998; Gillison *et al* 2000; Mork *et al* 2001). Sero-epidemiological data showed that a significantly increased risk of HNSCC is associated with HPV-16 seropositivity (Schwartz *et al* 1998; Mork *et al* 2001; Herrero *et al* 2003). The prevalence of HPV, particularly the high-risk types, suggests a causal association

between HPV and a subset of HNSCC (Gillison *et al* 2000; Herrero *et al* 2003; Dai *et al* 2004; Smith *et al* 2004). HPV is more commonly found in squamous cell carcinoma (SCC) of the tonsil than at other sites of HNSCC (Snijders *et al* 1992; Paz *et al* 1997; Klussmann *et al* 2001; Mellin *et al* 2000). The reason for the close association of HPV with tonsillar SCC remains unclear. Investigation of HPV infection in the tumor-free tonsils would additionally elucidate the role of HPV in tonsillar carcinogenesis.

Oncogenic HPVs play an etiological role in cervical cancer, and molecular biological approaches have provided evidence for specific carcinogenic mechanisms. Besides the most-studied tumor suppressor proteins p53 and pRb, which are degraded by the viral oncoproteins E6 and E7, respectively, several other cellular proteins have also been found to interact with these two viral oncoproteins (Mantovani and Banks 2001; Munger *et al* 2001; Scheffner and Whitaker 2003). Study of HPV in cell culture has been hindered because a complete viral life cycle cannot be mimicked *in vitro*. Although terminal differentiation of squamous epithelial cells has been achieved by use of a raft culture system, infectious virion production is very limited (Meyers *et al* 1992; Meyers *et al* 2002). Transfection of primary human epithelial cells with high-risk types of HPV DNA often results in cell transformation and immortalization (McDougall 1994). The HPV-immortalized cell lines such as human cervical epithelial cells and foreskin keratinocytes, as well as the naturally infected cell lines UT-Dec-1 and W-12 have served as an *in vitro* model for studying the role of HPV in human carcinogenesis (Dürst *et al* 1987a; Stanley *et al* 1989; Zheng *et al* 1994; Hietanen *et al* 1998). To establish an HPV-immortalized tonsillar epithelial cell line may provide a useful *in vitro* model to study HPV in tonsillar carcinogenesis.

5. REVIEW OF THE LITERATURE

5.1. HUMAN PAPILLOMAVIRUS

Human papillomaviruses (HPVs) belong to the *Papillomaviridae* family and are small, nonenveloped, icosahedral DNA viruses. The viral particles are 55 to 60 nm in diameter and consist of a single molecule of a double-stranded covalently closed circular DNA genome of approximately 7900 bp. The genomic organization of all papillomaviruses is remarkably similar (Fig 1). Viral DNA is associated with cellular histones to form a chromatin-like complex. All coding sequences are located on one DNA strand only. Most papillomaviruses contain six early ORFs and two late ORFs. There is a region with no ORFs which is designated the long control region (LCR), the upstream regulatory region (URR), or the noncoding region (NCR).

Figure 1. Genomic organization of HPV-16. ORFs deduced from the DNA sequence are designated E1 to E7, L1, and L2, indicated in grey boxes. A non-coding region (NCR) is indicated by a black box. Main functions of genes are listed.

5.1.1. Classification of human papillomaviruses

Genotypes

Determination of HPV genotypes is based on their degree of homology within the L1 ORF (de Villiers 1997). If the DNA sequence of the L1 ORF of a new isolate differs by more than 10% from the closest related known type, it is regarded as a novel type; a difference between 2 and 10% is referred to as a subtype; and at less than 2%, a variant, as agreed at the International Papillomavirus Workshop held in Quebec in 1995 (zur Hausen 1996). Thus far, 96 HPV genotypes have been fully sequenced and identified (de Villiers *et al* 2004).

Mucosal and cutaneous types

Depending on the location of the lesion from which a specific HPV type was first isolated, HPV can be classified as mucosal or cutaneous types. HPV types associated with skin warts are for example HPV-1, -2, and -4. A wide range of HPV types including HPV-5, -8, -9, -23, and -47 cause epidermodysplasia verruciformis lesions, which can be transformed to malignancy upon exposure to ultraviolet light (Androphy 1994; Pfister 2003). The largest subgroup is represented by HPV types infecting mainly mucosal surfaces of the genital and respiratory tracts. More than 40 of the identified HPV types belong into this group (de Villiers 1997).

High- and low-risk types

Classification of HPVs can be extended to cover the carcinogenic potential of different virus types. According to a recent publication by Munoz and colleagues, 15 are classified as high-risk types including HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82; 3 are classified as probable high-risk types which are HPV-26, -53, and -66; low-risk types consist of HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81, and CP6108 (Munoz *et al* 2003).

5.1.2. HPV life cycle

HPV has a specific tropism for squamous epithelial cells. Several modes of viral DNA replication during the infection were suggested in a review (Chow and Broker 1994) and are elucidated in Fig 2. Thus, HPV proteins stimulate the proliferation of cells and interfere with normal epithelial cell differentiation, leading to an increased thickness of the infected epithelial cells which produces neoplasia.

Figure 2. Schematic representation of HPV life cycle in stratified epithelium. (1) HPV infects basal cells of the squamous epithelium through micro-abrasions, and wound-healing promotes the proliferation of basal cells and activates the expression the viral early genes, resulting in a temporary amplification of the viral DNA; (2) after wound healing, most basal cells return to a quiescent state and divide infrequently, and viral transcription and replication are autoregulated at a low level, resulting in a steady-state, low-level maintenance mode; (3) after a cell leaves the cell cycle and undergoes progressive differentiation, a vegetative amplification mode for the production of progeny virus occurs.

5.1.3. Function of viral proteins

E1 and E2 proteins

The E1 protein has site-specific DNA-binding functions and it binds to the origin of replication in the NCR (Ustav *et al* 1991; Holt *et al* 1994). The E2 open reading frame (ORF) encodes at least two or three different proteins which all act as transcription factors and regulate viral transcription (Cripe *et al* 1987; Baker *et al* 1987; Bouvard *et al* 1994). The E2 proteins bind to the E1 and stimulate viral DNA

replication (Chow and Broker 1994). During early stages of viral infection, the E2 protein represses transcription of the oncogenes E6 and E7 (Thierry and Howley 1991). Integration of the viral genome, which often takes place during carcinogenic progressing, disrupts the E2 and E1 ORFs and results in elevated expression of E6 and E7 (Jeon and Lambert 1995).

E4 protein

The E4 ORF is located in an early gene region, yet it appears to be expressed as a late gene with a role in productive infection. It is exclusively localized within the differentiating layer of the infected epithelium and induces the collapse of the cytokeratin network (Doorbar *et al* 1991; zur Hausen 1996).

E5 protein

The E5 of HPV-16 is a small hydrophobic polypeptide and with weak transforming activity (Leptak *et al* 1991; Pim *et al* 1992). The E5 ORF is often disrupted in cervical cancer and does not seem to be essential for maintaining the malignant phenotype but is possibly important for initiation of transformation (Schwarz *et al* 1985; zur Hausen 1996). The E5 stimulates the transforming activity of the epidermal growth factor receptor resulting in increased cell proliferation (Pim *et al* 1992). In a raft culture model, the E5 was shown to support cellular DNA synthesis in suprabasal cells and neither viral late gene expression nor epithelial differentiation was affected by the E5 (Genther *et al* 2003). The E5 protects human foreskin keratinocytes from ultraviolet B-irradiation-induced apoptosis (Zhang *et al* 2002). On the other hand, it sensitizes human keratinocytes to apoptosis induced by osmotic stress (Kabsch and Alonso 2002).

E6 protein

The E6 protein of HPV-16 is a small polypeptide of approximately 150 amino acids. It contains two zinc-binding domains, each of which contains two copies of a Cys-X-X-Cys motif (Barbosa *et al* 1989). The E6 protein first binds to a cellular ubiquitin-ligase, the E6-associated protein (E6-AP) forming a complex which is

then able to bind the p53 protein, resulting in p53 degradation mediated by the cellular ubiquitin proteolysis system (Werness *et al* 1990; Scheffner *et al* 1990; Scheffner *et al* 1993). Because p53 is required for the growth arrest following cellular DNA damage, cells without functional p53 are not arrested appropriately in G1, but display genomic instability (Werness *et al* 1990). The HPV-16 E6 protein activates telomerase (Klingelhutz *et al* 1996), an enzyme that maintains the telomeric DNA at the ends of linear chromosomes (Greider and Blackburn 1985). Without telomerase, telomers shorten upon each cell division, until they reach a critically short length. Beyond this point further division would induce damage in the coding regions of the chromosome and cause cell senescence. Telomerase activity is undetectable in most normal human somatic cells, but nearly all human cancers and immortalized cell lines have highly active telomerase (Kim *et al* 1994; Shay and Bacchetti 1997). Besides the p53 and telomerase, the E6 proteins also interact with various cellular proteins (Table 1).

E7 protein

The E7 protein of HPV-16 is a small, predominantly nuclear polypeptide of approximately 100 amino acids. Interestingly, the carboxy terminus of E7 contains a similar zinc-binding domain as the E6. The E7 binds to retinoblastoma protein (pRB) and the related pocket proteins p107 and p130, results in releasing the transcription factor E2F from pRB complexes, and consequently activating genes regulating cell proliferation (Dyson *et al* 1989; Bagchi *et al* 1990; Bandara *et al* 1991). Besides the pRb, the E7 also interacts with various proteins (Table 1), most of which are important regulators of cell growth, especially the transition of a cell from the G1 to the S-phase of mitosis. The E7 protein has recently been shown to induce abnormal centrosome duplication, resulting in multipolar, abnormal mitoses, aneuploidy, and genomic instability, one of the hallmarks of a cancer cell (Duensing *et al* 2000; Duensing *et al* 2001). This function of E7 as a mitotic mutator may directly contribute to generating the host chromosomal changes that are necessary for carcinogenic progression of a high-risk HPV positive precursor lesion.

Table 1. List of HPV-16 oncoproteins associated cellular proteins

Viral oncoproteins	Cellular proteins	Reference
E6	E6-associated protein, p53	Werness <i>et al</i> 1990; Scheffner and Whitaker 2003
	telomerase	Klingelhutz <i>et al</i> 1996
	hTERT	Kiyono <i>et al</i> 1998; Gewin and Galloway 2001
	homolog of disc large (hDlg)	Lee <i>et al</i> 1997; Kiyono <i>et al</i> 1997; Gardiol <i>et al</i> 1999
	human Scribble (Vartul)	Nakagawa and Huibregtse 2000
	MUPP1	Lee <i>et al</i> 2000
	MAGI-1, -2, -3	Glaunsinger <i>et al</i> 2000; Thomas <i>et al</i> 2002
	Myc	Veldman <i>et al</i> 2003
E7	pRb, p107, p130	Dyson <i>et al</i> 1989; Bagchi <i>et al</i> 1990; Bandara <i>et al</i> 1991; Scheffner and Whitaker 2003
	AP-1 transcription factors (c-Jun, JunB, JunD, c-Fos)	Antinore <i>et al</i> 1996
	cyclin-dependent kinase inhibitors (p21, p27)	Funk <i>et al</i> 1997; Zerfass-Thome <i>et al</i> 1996; Jones <i>et al</i> 1997
	histone deacetylases complexes (HDAC)	Brehm <i>et al</i> 1999
	cyclins and Cdks	Chakrabarti and Krishna 2003
	pCAF	Avvakumov <i>et al</i> 2003

Both E6 and E7 proteins play a role in cell transformation and immortalization. The E6 protein alone cannot immortalize human foreskin keratinocytes (Halbert *et al* 1991), but it is capable of immortalizing human mammary epithelial cells (Band *et al* 1991; Band *et al* 1993; Liu *et al* 1999) and inducing carcinomas in transgenic mice (Song *et al* 1999). The E7 alone is capable of immortalizing human foreskin keratinocytes (Halbert *et al* 1991). However, the E6 and E7 together can immortalize human epithelial cells cooperatively (Hawley-Nelson *et al* 1989; Munger *et al* 1989). In E6- and E7-transgenic mice experiments, the E7 primarily causes tumor promotion, whereas the E6 contributes weakly to the early stages, acts more strongly during tumor progression, and accelerates the malignant conversion of benign tumors (Song *et al* 2000).

Late proteins

The L1 and L2 proteins are 72 capsomers encapsidating viral DNA. The L1 is the major capsid protein, with an average molecular mass of 57 KDa, and it contains reactive epitopes for type-specific neutralization. The L2 protein is a minor component of the viral capsid with an about 78 kDa polypeptide (Doorbar and Gallimore 1987).

5.2. HISTORICAL BACKGROUND OF HPV AND CANCER

The hypothesis of infectious etiology of the common wart was shown by Ciuffo, who demonstrated in 1907 that cell-free extracts from common warts were transmissible from person to person upon intradermal inoculation, as reviewed in (zur Hausen 1999a). Papillomavirus as a carcinogen was first reported by Drs. Rous and Beard in 1934 (zur Hausen 1999a). It was not until the mid 1970s that HPV was singled out to be the most likely candidate for the cause of cervical cancer (zur Hausen *et al* 1974; zur Hausen 1976), and from 1983 to 1986, HPV-16, -18, -31, and -33 were isolated from cervical cancer and its precursors (Dürst *et al* 1983; Boshart *et al* 1984; Lorincz *et al* 1986; Beaudenon *et al* 1986). In 1995, the International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence to categorize HPV types 16 and 18 as human carcinogens (IARC 1995). An association between HPV and a subset of head and neck cancer was noticed already in the 1980s (Syrjänen *et al* 1982; Syrjänen *et al* 1983a; Löning *et al* 1985; de Villiers *et al* 1985).

5.3. *PTEN* AND *LKB1* TUMOR SUPPRESSOR GENES

PTEN (also known as *MMAC1*) is a major new tumor suppressor gene located at human chromosome 10q23 (Steck *et al* 1997). It comprises 9 exons with a coding sequence of 1212 bp and encodes a 403 amino acid phosphatase protein that shares sequence homology with the dual-specificity phosphatase family (Li and Sun 1997). *PTEN* is a tumor suppressor gene based on the presence of inactivating mutations in human brain, breast, and prostate cancers (Li *et al* 1997; Steck *et al* 1997). Further studies extended initial observations in a variety of different tumor

entities. Somatic mutations of this gene occur in several types of cancers, including gliomas and glioblastoma, as well as prostate, kidney and breast carcinomas (Fallani *et al* 1997; Daniel *et al* 1995; Steck *et al* 1997). Germline mutations of *PTEN* gene underlie Cowden disease, an autosomal dominant disorder associated with an increased risk for breast and thyroid cancer, and possibly also for endometrial malignancy (Liaw *et al* 1997). In addition, multiple oral papillomas are associated with Cowden's disease (Greer *et al* 1976).

LKB1 (also called *STK11*) is a tumor suppressor gene mapped to 19p13.3, and it encodes a serine/threonine kinase which is highly homologous (84%) to *Xenopus* serine/threonine kinase XEEK1 (Su *et al* 1996). Germline mutations in the *LKB1* gene have been associated with Peutz-Jeghers syndrome (PJS) (Hemminki *et al* 1998; Jenne *et al* 1998), which is characterized by hamartomatous intestinal polyposis, mucocutaneous pigmentation, and increased risk for cancer of multiple organ systems (Phillips *et al* 1994). Somatic mutation in *LKB1* is a rare event in most sporadic tumor types: melanoma, pancreatic, gastric, ovarian granulosa, cervical, lung, soft tissue, renal tumors, colorectal, testicular, breast, gastric carcinoma, and colon cancer (Avizienyte *et al* 1998; Avizienyte *et al* 1999; Bignell *et al* 1998; Park *et al* 1998; Resta *et al* 1998). Thus far, reports on the mutation analysis of this gene in HNSCC are few.

5.4. SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Squamous cell carcinoma of the head and neck (HNSCC) is a group of malignant epithelial diseases arising from the mucosa of the upper aerodigestive tract including the lips, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, maxillary sinus, nasal cavity and ethmoid sinus, and salivary glands (Fig 3). This disease is characterized by local tumor aggressiveness, early recurrence, and high frequency of second primary tumors. HNSCC represents the eighth most prevalent malignancy worldwide, with nearly half a million new cases per year (Parkin *et al* 1999). According to the report by Finnish Cancer Registry in 2002, each year approximately 600 new cases of head and neck cancer are diagnosed in Finland, and the age-adjusted incidence rates is 10.7 per 100,000 in male and 3.8

per 100,000 in female (Finnish Cancer Registry 2002). Treatment of head and neck cancers is based on surgery or radiotherapy, or their combination, or on chemo-irradiation (Luukkaa *et al* 2003; Forastiere *et al* 2003).

Figure 3. Anatomic overview of head and neck region, modified from (Netter 2001) in Plate 57.

5.4.1. Etiology and risk factors for the head and neck cancer

As many as 80 to 90% of all head and neck cancers except cancer of the salivary glands may be attributed to the known risk factors tobacco smoking and alcohol consumption (Decker and Goldstein 1982; Franceschi *et al* 1990; Spitz 1994; Licitra *et al* 2002), although a minority of head and neck cancer patients have no history of tobacco or alcohol use, suggesting other possible contributing exposures (Gillison *et al* 1999). Several oncogene activations (*Ras*, *Myc*, epidermal growth factor receptor, and cyclin D1) and tumor suppressor gene inactivations (*p53*,

p16^{INK4A}, *Rb*) have been identified in primary HNSCC, as reviewed in (Forastiere *et al* 2001; Mao *et al* 2004). The *p53* gene is probably the most widely studied gene in HNSCC. The reported frequency of *p53* mutations in HNSCC varies widely, from 30 to 70%, and this may be a reflection of the techniques used to detect mutations, the regions of *p53* subjected to analysis, and differences in mutation rates between anatomical sites. Many studies analyzing gene sequence have focused on exon 5-8 of *p53*, since the majority of mutations occur here (Gasco and Crook 2003; Blons and Laurent-Puig 2003). The frequency of *p53* mutations in HNSCC seems to be correlated with the dose-dependant effect of tobacco and inversely associated with HPV infection, as reviewed in (Blons and Laurent-Puig 2003). The spectrum of chromosomal loss increased progressively at each histopathological step from benign hyperplasia to invasive cancer in HNSCC (Califano *et al* 1996). Viruses, mainly HPV and Epstein-Barr virus (EBV), have been noticed to be connected with head and neck cancer development. The association of infection with HPV and cancers of the oral cavity, larynx, and nasal and paranasal regions was originally reported in (Syrjänen *et al* 1982; Syrjänen *et al* 1983b; Syrjänen *et al* 1983a). The association of infection by EBV and nasopharyngeal carcinomas was first suggested by Old *et al* in 1960s, as reviewed in (zur Hausen 1999b).

5.4.2. Tumor classification

The International Union Against Cancer (UICC) TNM classification is identical to that of the American Joint Cancer Committee (AJC). The T categories are defined by tumor dimension, the N categories are according to whether the cancer has spread to the regional lymph nodes, and the M categories indicate whether the cancer has any distant spreading metastasis (Table 2). Tumor stage is classified by adding the TNM data together (Table 3).

Table 2. Tumor classification for oropharyngeal cancer (UICC 2002)

TNM	Definition
TX	primary tumor cannot be assessed
T0	no evidence of primary tumor
Tis	carcinoma <i>in situ</i>
T1	tumor # 2 cm
T2	tumor > 2 cm but # 4 cm
T3	tumor > 4 cm
T4a	tumor invades any of the following: larynx, deep/extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible
T4b	tumor invades any of the following: lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, skull base; or it encases the carotid artery
NX	regional lymph nodes cannot be assessed
N0	no regional lymph node metastasis
N1	metastasis in a single ipsilateral lymph node # 3 cm
N2a	metastasis in a single ipsilateral lymph node > 3cm but # 6cm
N2b	metastasis in multiple ipsilateral lymph nodes # 6cm
N2c	metastasis in bilateral or contralateral lymph nodes, # 6cm
N3	metastasis in a lymph node > 6 cm
MX	distant metastasis cannot be assessed
M0	no distant metastasis
M1	presence of distant metastases

Table 3. Tumor stage classification for oropharyngeal cancer (UICC 2002)

TNM stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T1, T2	N1	M0
III	T3	N0, N1	M0
IVA	T1-T3	N2	M0
IVA	T4a	N0-N2	M0
IVB	T4b	any N	M0
IVB	any T	N3	M0
IVC	any T	any N	M1

5.4.3. HPV in HNSCC

To date, more than 1900 studies on the presence of HPV DNA in HNSCC have emerged. The frequency of HPV DNA in HNSCC has a large variation depending on tumor site, material and methods applied, as well as number of cases included, in average range from 9 to 60% (Snijders *et al* 1996; Paz *et al* 1997; McKaig *et al* 1998; Gillison *et al* 2000; Mork *et al* 2001). Several case-control studies as well as a meta-analysis suggested that high-risk HPVs are associated with HNSCC (Miller and Johnstone 2001) (Table 4). *In situ* hybridization (ISH) reveals that HPV DNA is located in tumor cells but not in the surrounding stroma or the nondysplastic surface epithelium in HNSCC (Niedobitek *et al* 1990; Gillison *et al* 2000). Klussmann *et al* documented for the first time that viral loads as determined by quantitative real-time PCR in microdissected HPV-16 positive tonsillar tumors were higher than in whole-sample DNA preparations, indicating that HPV DNA is present in the tumor and not in the stroma or other cells (Klussmann *et al* 2001). By the more sensitive PCR assay, however, HPV DNA has also been detected in tumor-free resection margins of HPV-positive HNSCC (Snijders *et al* 1996;

Badaracco *et al* 2000; Klusmann *et al* 2001). Approximately half the HPV DNA-positive HNSCC expresses E6/E7 mRNA in the tumors (Wiest *et al* 2002; Balz *et al* 2003). HPV DNA-positive tumors with no E6/E7 mRNA expression possibly underwent a deletion within the E6 gene (Wiest *et al* 2002). Interestingly, E6/E7 mRNA expression occurs mostly in tumors with wild-type *p53*, reduced pRb, and overexpressed p16, whereas tumors without E6/E7 expression are associated with mutant *p53*, normal pRb, and low levels of p16 (Wiest *et al* 2002). HPV-16 E6 transcript expression is correlated with basaloid morphology (van Houten *et al* 2001). A rather frequent coexistence of HPV DNA and *p53* mutations was found in HNSCC (Barten *et al* 1995; Chiba *et al* 1996; Scholes *et al* 1997), which is in contrast to the situation in cervical and other anogenital cancers, which show an almost complete inverse correlation between HPV and *p53* mutations and share a conspicuous basaloid morphology (zur Hausen 1996).

Table 4. High-risk HPVs in HNSCC (case-control studies)

Reference	Tumor stie	Material	No of case / control	HPV%	Odds ratio
Schwartz <i>et al</i> 1998	Oral cavity	Oral exfoliated cells	237 / 435	5.9% / 4.1%	1.3
Nishioka <i>et al</i> 1999	head and neck	Fresh-frozen tissue	74 / 70	16.2% / 4.2%	4.3
Strome <i>et al</i> 2002	Tonsil	Paraffin-embedded tissue	52 / 48	40.1% / 6.3%	NA*
Chen <i>et al</i> 2002	Oral cavity	Paraffin-embedded tissue	29 / 29	85.7% / 32% (HPV-16)	12.8
				71.4% / 11.5% (HPV-18)	19.2
Herrero <i>et al</i> 2003	Oral cavity and oropharynx	Oral exfoliated cells	601 / 613	5.3% / 6.9%	NA*
Smith <i>et al</i> 2004	Oral cavity and oropharynx	Oral exfoliated cells	201 / 333	28.4% / 10.8%	2.6

*not available

5.4.4. HPV in SCC of tonsils

HPV DNA is detected in 42 to 100% of all cases of tonsillar carcinoma, thus appearing more frequently than in other head and neck carcinomas (Snijders *et al* 1992; Steinberg and DiLorenzo 1996; Paz *et al* 1997; Andl *et al* 1998; Gillison *et al* 2000; Mellin *et al* 2000; Mork *et al* 2001; Mellin *et al* 2002; Syrjänen 2004; Li *et al* 2004). Tonsillar SCC often contains episomal HPV with or without the deletion of E2 sequence (Mellin *et al* 2002). HPV-positive tonsillar SCC has been suggested as a distinct biological group by several studies. For instance, HPV-16 is predominant; it has rather frequent oncogene E6/E7 expression with wild-type *p53* (Wiest *et al* 2002; Balz *et al* 2003), reduced pRb and cyclin D1 expression and increased p16 expression (Andl *et al* 1998; Wilczynski *et al* 1998; Klussmann *et al* 2003; Li *et al* 2004). Patients with HPV-positive tonsillar SCC are younger in age, use less tobacco and alcohol, and have better prognosis than do patients with the HPV-negative cancers (Mellin *et al* 2000; Strome *et al* 2002; Li *et al* 2003). Epidemiological data showed that patients with HPV-associated anogenital cancers had a 4.3-fold increased risk for tonsillar SCC, whereas patients with HPV-unrelated cancer (cancers of colon, stomach and breast) had no increased risk for developing tonsillar SCC, suggesting that HPV may be a common etiological factor for the link between tonsillar and anogenital SCC (Frisch and Biggar 1999).

5.4.5. HPV DNA in tumor-free tonsils

The detection of HPV DNA in tumor-free tonsils has been reported in only a few studies (Smith *et al* 1993; Watanabe *et al* 1993; Fukushima *et al* 1994; Tominaga *et al* 1996; Strome *et al* 2002) and others failed to detect the viral DNA in tonsillitis samples (Brandsma and Abramson 1989; Niedobitek *et al* 1990; Snijders *et al* 1992; Klussmann *et al* 2001). The overall frequency of HPV DNA in normal tonsillar mucosa or benign lesions is 8.5% (17 of 200), consisting of 70% with HPV-16 and 30% with HPV-6/11, as summarized in a recent review (Syrjänen 2004). The reason for the association of HPV with the tonsil remains unclear. HPV seems to

be important, especially in tonsillar carcinogenesis, and data on HPV infection in tumor-free tonsils is based on small study populations.

Presence of the IgG antibody to the HPV-16 L1 capsid protein is an indication of persistent infection, and presence of an antibody against HPV-16 E6 or E7 may suggest the HPV-associated malignancy (Herrero *et al* 2003; Zumbach *et al* 2000). Seroepidemiological data showed a significantly increased risk for HNSCC is associated with HPV-16 seropositivity (Mork *et al* 2001). Serum antibodies against HPV-16 proteins have been detected among healthy individuals (Hamsikova *et al* 1998). The concomitant of presence of HPV DNA in tumor-free tonsillar tissue and seropositivity, however, has not been investigated as yet.

6. AIMS OF THE STUDY

The etiology of HNSCC is considered to be multifactorial. Besides the well-known main risk factors, tobacco and alcohol, the presence of some DNA tumor viruses as well as the genetic alteration also play a role. An association between infection by HPV and HNSCC has been based mainly on the detection of HPV DNA in tumor samples and on the case-control studies. Little is known about the physical status of HPV in HNSCC, with no systematic study in infection by HPV in tumor-free tonsillar tissue. Thus far no *in vitro* model exists to study mechanisms of HPV in tonsillar carcinogenesis.

More specifically, the aims of this thesis were to study:

1. the role of somatic mutations of *PTEN* and *LKB1* genes in laryngeal tumors
2. the prevalence, viral load, and physical status of HPV DNA in HNSCC
3. the frequency of HPV DNA and seroprevalence in patients with tumor-free tonsils, and
4. to establish and characterize HPV-16 DNA immortalized HTE cell lines

7. PATIENTS, MATERIALS, AND METHODS

7.1. PATIENTS AND MATERIALS (I, II, III)

All patients in Studies I, II, and III were from Helsinki University Central Hospital, Department of Otorhinolaryngology – Head and Neck Surgery (Table 5). Fresh tissue obtained from surgical operations and tonsillar exfoliated cells collected with Cytobrush Plus Cell Collector (Medscand Medical AB, Sweden) into PBS were immediately stored at -70°C till analysis.

Table 5. Patients and clinical samples

	Patients' diagnosis (number of cases)		Specimens for HPV-PCR	Luminex	IHC/ISH*
Study I	Laryngeal carcinoma (n=13) Laryngeal papilloma (n=3), one with malignant transformation		Fresh-frozen tissue containing at least 40% neoplastic cells		
Study II	Carcinomas of	hypopharynx (n = 10) larynx (n=18) mobile tongue (n = 6) base of tongue (n = 9) oral cavity (n=13) tonsil (n=5)			
Study III	Tonsillitis (n = 135) Tonsillar hypertrophy (n = 77)		Fresh-frozen tissue containing at least 30% epithelial cells	Serum	Formalin-fixed and paraffin-embedded tissue
	Normal tonsil (n=189)		Cytobrushed exfoliated cells		

* immunohistochemistry / *in situ* hybridization

7.2. CELL LINES (IV)

Primary human tonsillar epithelial (HTE) cells were derived from children's bilateral tonsillectomies. In these tonsils, HPV-PCR analysis showed no pre-existing infection with HPV. HTE-114/K1, HTE-114/K2, HTE-114/K3, and HTE-114/B have derived from HPV-16 immortalized primary HTE cells. The HPV-16 positive cervical carcinoma cell line SiHa and spontaneously transformed HaCaT human keratinocyte cell line were obtained from the American Type Culture Collection (ATCC).

7.3. PLASMIDS (IV)

The plasmids, pHPV16 114/K and pHPV16 114/B, containing full-length HPV-16 DNA, were the kind gift of Dr. Matthias Dürst, University of Jena, Germany.

7.4. METHODS

<u>Method</u>	<u>Described in</u>
DNA extraction from patients' samples	I, II, III
Mutation analysis of <i>PTEN</i> and <i>LKB1</i> genes	I
Detection and genotype of HPV DNA by PCR	I, III
Determination of HPV-16 viral load and physical status	III
Conventional cell culture	IV
Transfection of cells	IV
Raft culture	IV
Anchorage-independent growth in soft agarose	IV
Tumorigenicity assay	IV
Electron microscopy examination	IV
Cytogenetic analysis	IV
Fluorescence in situ hybridization (FISH)	IV
Southern blot hybridization	IV
Northern blot hybridization	IV
Immunohistochemistry (IHC)	II, IV
Statistical analysis	II, III

In situ hybridization (ISH)

Five- μ m sections of formalin-fixed and paraffin-embedded tonsillar SCC tissue were deparaffinized and rehydrated. In situ hybridization was performed using the Ventana Discovery automated ISH-IHC Slide Stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). The sections were digested with protease 2 (Ventana) for 4 min before hybridization at 57°C for 14 hours with a Digoxigenin-labeled full-length HPV-16 DNA probe in ribohybe™ (Ventana) and followed with low- and high-stringent wash. The hybrids were detected with the BlueMap Detection Kit (Ventana) and counterstained with nuclear Fast Red (Ventana). After dehydration, the slides were mounted with Eukitt mounting medium (O. Kindler GmbH & Co., Freiburg, Germany). The positive signals appeared in blue.

8. RESULTS AND DISCUSSION

8.1. SOMATIC MUTATION IN *PTEN* AND *LKB1* GENES IS A RARE EVENT IN LARYNGEAL TUMORS (I)

To determine whether *PTEN* and *LKB1* tumor suppressor genes are mutated in laryngeal tumors, we screened 16 laryngeal tumor samples by genomic sequencing and SSCP assay. When we performed direct sequencing of the amplicon for *PTEN* mutations analysis, no mutations were evident in the coding sequences and exon/intron boundaries; to screen for *LKB1* mutations, the SSCP assay was applied after PCR amplification of the nine exons of *LKB1*. Differences in mobility pattern in the SSCP assay are usually suggestive of a variant, the nature of which should be examined by sequencing. In our hands, SSCP analysis of *LKB1* always revealed known mutations, and it is reasonable to assume the 70 to 80% sensitivity that is commonly reported for the method (Jordanova *et al* 1997). Our SSCP protocol was specifically optimized for the *LKB1* gene analysis. To validate the SSCP results, exon 1 was directly sequenced from each sample with no mutations observed in exon 1 and the respective exon/intron boundaries. The methods we used to detect mutations would not have detected some mutation types, such as large genomic deletions or alterations in the promoter region.

Several tumor suppressor genes have been demonstrated to play an important role in human tumors, including head and neck cancers. Our results are in agreement with reports showing that in HNSCC, somatic mutations in *PTEN* gene are infrequent (Henderson *et al* 1998; Gasparotto *et al* 1999; Hu *et al* 1999; Chen *et al* 2000; Mavros *et al* 2002). Other studies, however, imply that mutation of *PTEN* might play a role in the genesis of some HNSCC, ie., carcinomas of the oropharynx, larynx, and hypopharynx (Okami *et al* 1998; Shao *et al* 1998; Poetsch *et al* 2002). Analysis of *PTEN* expression at protein level is probably more relevant for the elucidation of its role in head and neck tumors. In laryngeal papillomas, *PTEN* protein is consistently overexpressed (Zhang and Steinberg 2000). Levels of

PTEN protein vary from negative to highly expressed in SCC of the tongue and oral cavity, and loss of PTEN expression is significantly associated with poorer prognosis (Lee *et al* 2001; Squarize *et al* 2002). The rate of PTEN inactivation at protein level may be more frequent than that identified at the genomic level in HNSCC, and the inactivation of PTEN by either genetic alteration or by the loss of a PTEN transcript may play a role in the pathogenesis of some oral cancer (Shin *et al* 2002).

8.2. PREVALENCE AND PHYSICAL STATUS OF HPV IN HNSCC (II)

8.2.1. Prevalence, genotype, viral load, and physical status of HPV DNA in HNSCC

By SPF10 PCR screening and INNO-LiPA HPV genotyping assay, we found that 61% (37 of 61) of the tumors of HNSCC patients harbored HPV DNA. The most frequently detected type was HPV-16, followed by HPV-33 (Table 6). None of our samples were positive for the cutaneous HPVs. Our findings support the role of high-risk types of HPV, especially HPV-16, in head and neck carcinogenesis. In line with other reports (Snijders *et al* 1992; Paz *et al* 1997; Mellin *et al* 2000; Klussmann *et al* 2001), we also observed a high prevalence of high-risk HPV-DNA in tonsillar carcinoma (n = 5, 100%). We were able to demonstrate by ISH that HPV-16 DNA was localized only in tumor cells of a tonsillar SCC (Fig 4).

Figure 4. Photomicrograph shows that HPV-16 DNA (in blue) located in poorly differentiated dysplastic epithelial cells in tonsillar SCC. Magnification $\times 400$.

Table 6. Frequency and genotypes of HPV DNA in 61 HNSCC patients

Site of tumor (n)	HPV-16	HPV-33	HPV-6/16	HPV-16/33	HPV-6/11/16/51/52	Unknown type	Total HPV-DNA(%)
Hypopharynx (10)	2	1	1	1	0	0	5 (50)
Larynx (18)	5	0	1	2	1	0	9 (50)
Base of tongue (9)	2	4	0	1	0	0	7 (8)
Mobile tongue (6)	3	0	0	1	0	0	4 (7)
Oral cavity (13)	6	0	0	0	0	1	7 (54)
Tonsil (5)	5	0	0	0	0	0	5 (100)
Total (61)	23	5	2	5	1	1	37 (61)

Viral load of HPV-16 DNA in HNSCC was analyzed by real-time PCR. Even considering that some differences existed in the proportion of carcinoma cells (40 to 100%) and in the amount of material used for DNA extraction, large variation appeared in the viral load between tonsillar SCC and non-tonsillar HNSCC. The median viral load in tonsillar specimens was thus approximately 80,000 times as high as in non-tonsillar HNSCC specimens (Fig 2, Study II). Furthermore, the median viral load in tonsillar SCC that harbored episomal viral DNA was 11.6 times that in the integrated group. This high viral load of HPV-16 DNA in tonsillar carcinomas has also been observed by others (Table 7). High viral load in tonsillar SCC favors detection of HPV, and this might explain the high prevalence of HPV-DNA in tonsillar SCC samples. It is unclear why the viral load in tonsillar carcinomas is markedly higher than at other sites of HNSCC.

Table 7. Viral load in HNSCC

Site of tumor	HPV-16 DNA (copies/genome)
Tonsillar SCC	2.3 – 245 (Table II, study II)
	10 – 15,400 (Mellin <i>et al</i> 2002)
	6.6×10^4 – 172 (Klussmann <i>et al</i> 2001)
HNSCC (mainly oropharyngeal SCC)	1.8×10^2 – 322 (Ha <i>et al</i> 2002)
Non-tonsillar HNSCC	5×10^5 – 3.4×10^2 (II)

Viral DNA integration often leads to the disruption of E1/E2 genes in HPV-associated cervical lesions (Kalantari *et al* 1998). In our study, determination of viral physical status was based on the assumption that the E2 gene is disrupted in integrated viral DNA. Thus, episomal viral DNA has equivalent copy numbers of the E2 and the E6 genes, and mixed forms have smaller copy numbers of the E2 than of the E6 (Peitsaro *et al* 2002). We observed that HPV-16 DNA existed in all three forms: only episomal (n = 8), only integrated (n = 11), and mixed (n = 4). The disrupted E2 gene is quite common in our study: 15 of 25 (60%). The minor disadvantages of our assay for determination of viral physical status stem from the assumptions that viral integration occurs within the selected site of the E2 gene and no mutations exist at sites of PCR primers of E2 region. Although disruption of the E2 gene has been considered as consequence of viral integration, episomal viral DNA with no detectable E2 DNA also occurs in tonsillar SCC (Mellin *et al* 2002).

8.2.2. Absence of viral capsid protein expression in HNSCC

Despite the high copy numbers of episomal HPV-16 DNA in tonsillar carcinomas, HPV L1 capsid protein was surprisingly undetectable by immunohistochemistry in any of the 36 HPV-positive tissue sections, whereas it was expressed strongly in control samples from a cervical intra-epithelial neoplasia type III and in a juvenile-onset laryngeal papilloma. Thus, undetectable HPV L1 protein in our tonsillar SCC samples was not due to technical reasons. It would, however, be of interest to look at L1 mRNA expression in the HPV-containing tonsillar SCC samples. In tonsillar carcinoma, HPV was reported to be correlated with a poor differentiation, no terminal differentiation, and basaloid histological appearance (Klussmann *et al* 2001; Wilczynski *et al* 1998). Our observation supports the view that the expression of viral capsid proteins requires terminal differentiation of host cells.

8.2.3. Correlation of HPV DNA status and clinical outcome

Figure 5. Kaplan-Meier survival graph showing that patients harbouring HPV-positive HNSCC had a similar survival profile as the HPV-negative patients. Fifty-nine HNSCC patients were included in the analysis.

By Kaplan-Meier survival analysis, we observed that HPV-positive patients had similar survival profile as the HPV-negative ones (Fig 5). Viral load had no statistically significant effect on the survival among those patients with HPV-16 positive HNSCC specimens (data not shown). Interestingly, we found that large tumors (T3 and T4) were significantly over-represented among the samples harboring episomal HPV-16 DNA, as compared to those which contained integrated or mixed forms of the viral DNA (Fig 3, Study II). However, the number of patients was rather small, and follow-up time quite short. Reports on the impact of HPV-positivity on prognosis in HNSCC have been controversial. Some studies reported that the prognosis of patients is not correlated with the detection of HPV DNA or with the viral physical status (Pintos *et al* 1999; Badaracco *et al* 2000). Others, however, observed that patients with HPV-positive HNSCC had a better overall and disease-specific survival than did the HPV-negative group (Ringstrom *et al* 2002). This correlation has also been observed

among tonsillar carcinoma patients, plus the fact that higher viral load correlated with better clinical outcome in tonsillar SCC harboring episomal HPV-16 (Mellin *et al* 2002). The number of cases in those studies was relatively small (11 to 101). Thus, large study populations and a long follow-up time are required for clarification of this issue.

8.3. PRESENCE OF HPV-16 DNA BUT NO OTHER TYPES IN TUMOR-FREE TONSILS (III)

8.3.1. Presence of HPV DNA and HPV genotypes in tumor-free tonsils

We analyzed HPV DNA in tumor-free tonsillar tissue by nested PCR using the consensus primers My09/11 and GP5+/6+; the overall prevalence of HPV-16 DNA in tonsillar tissue was 6.1% (13 of 212) in patients operated on for tonsillitis or tonsillar hypertrophy, and no other type of HPV was detectable (Table 1a, Study III). No significant difference was observed between the positivity of HPV DNA in patients with tonsillitis (7%, 10 of 135) and tonsillar hypertrophy (3.9%, 3 of 77). In contrast, only one tonsillar exfoliated cell sample from 189 control subjects with normal tonsils had detectable HPV DNA, and this one was typed as HPV-58. Thus, the presence of HPV-DNA in tonsillar tissue was significantly higher ($p < 0.05$, Fisher's exact test, two-tailed) than that in tonsillar exfoliated cells. The frequency of HPV DNA in normal tonsils or tonsillitis was summarized in a recent review that out of 200 samples from 10 original studies, 6% (12 of 200) were positive for HPV-16 and 2.5% (5 of 200) were positive for HPV-6/11, thus, the overall frequency of HPV DNA was 8.5% (17 of 200) (Syrjänen 2004).

Since in our study the samples from patients and control subjects were not obtained in the same way, we were not able to determine the concordance of HPV-DNA positivity in tonsillar tissue and that in tonsillar exfoliated cells. Data on this are controversial. One study showed a lower prevalence of HPV DNA in oral exfoliated cells, scraped superficially with soft toothbrushes, than that in oral biopsies among HNSCC patients (Herrero *et al* 2003). These authors suggested that the HPV status in exfoliated cells did not accurately reflect that in the tumor; however, other study observed that 57.9% of the patients ($n = 190$) with cancer of

oral cavity or oropharynx were positive for HPV in both tumor tissue and oral exfoliated cells, collected by oral rinse, indicating good correlation of HPV-DNA positivity from tumor tissue and that from oral exfoliated cells (Smith *et al* 2004). Reliability of the exfoliated cells for analysis of HPV DNA by PCR needs to be evaluated further regarding the sampling methods, tumor or tumor-free samples, and specific anatomic sites of the head and neck.

Interestingly, in our study viral DNA prevalence was highest in preschool children aged between 1.5 and 6 years (11.5%, 3 of 26) among all age groups. It is unknown how these children originally acquired the HPV-16 DNA in their tonsils. Vertical transmission of HPV from mothers to their children, or additional sources such as breast milk, siblings *via* kissing, or exposure to contaminated fomites is feasible, as suggested in one review (Syrjänen and Puranen 2000). In our study population, no patients older than 33 years was positive for HPV DNA in their tonsils, allowing speculation that, if these patients did not develop pre-cancer or cancer lesions, HPV-16 DNA had been cleared by their immune system. Infection by HPV is common, but it rarely develops into cancer (Bauer *et al* 1991).

8.3.2. Seroprevalence of antibodies against HPV-16 L1, E6, and E7

The overall seroprevalence of HPV-16 L1 was 11.8% (25 of 212) in patients group and 8.5% (16 of 189) in control subjects (Table 1, Study III). The highest seroprevalence appeared in the group aged between 26 and 40 years in both patients and control subjects, but not significantly. It is unknown whether these patients and control subjects had had HPV infection at sites other than their tonsils, since the HPV-16 L1 antibody can persist for many years and is a marker for persistent infection by HPV-16 (Shah *et al* 1997; af Geijerstam *et al* 1998), and in females is associated with lifetime number of sexual partners (Dillner *et al* 1996). Although serum antibodies against HPV-16 E6 and E7 proteins were detectable in our patients and control subjects, prevalences were low (0 to 2.8%) (Table 1, Study III). Detection of serum antibodies against HPV-16 E6 and E7 proteins among all age healthy individuals has been observed by several studies (Muller *et al* 1995; Mund *et al* 1997; Hamsikova *et al* 1998). Antibody responses to

HPV-16 E6 and E7 fusion proteins are intriguing, because these E6 and E7 genes are involved in HPV-mediated cellular transformation, and such antibodies are markers for HPV-16 associated oropharyngeal cancer (Zumbach *et al* 2000; Herrero *et al* 2003).

8.3.3. Non-concordance between presence of HPV DNA in tonsillar tissue and seropositivity to HPV proteins

Double positivity of HPV-16 DNA in tonsillar tissue and serum antibodies against any HPV-16 proteins was apparent in 2 of 212 patients; both were male young adults and had elevated serum antibodies against HPV-16 L1 proteins (all coefficients < 0.10 , all $p > 0.4$, Spearman correlation coefficients). No concordance thus exists between the presence of HPV-16 DNA in tumor-free tonsillar tissue and seropositivity against HPV-proteins. Our patients' HPV-16 DNA was detected at only one visit, and we lack knowledge of how long the HPV DNA had been in their tonsillar tissue and whether it was a transient infection. A single detection of viral DNA is less likely to lead to seroconversion than is the case for patients in whom HPV-16 DNA is detectable at several visits (Carter *et al* 2000; Carter *et al* 1995; Carter *et al* 1996; de Gruijl *et al* 1997). Most HPV-16 seroconversions were detected 6 to 12 months after detection of HPV-16 DNA, and transient HPV-DNA is associated with a failure to seroconvert following incident HPV infection (Carter *et al* 2000). Conversely, those seropositive patients with no detectable HPV DNA in their tonsils may harbor the viral DNA at other anatomic sites than one of their tonsils.

8.4. ESTABLISHMENT AND CHARACTERIZATION OF HPV-16 DNA IMMORTALIZED HUMAN TONSILLAR EPITHELIAL CELL LINES (IV)

8.4.1. Growth and differentiation of HPV-16 DNA transfected human tonsillar epithelial cells

The introduction of full-length HPV-16 plasmid with its own promoters, 114/K or 114/B, into primary human tonsillar epithelial (HTE) cells from two donors, respectively, resulted in cell infinite growth. The immortalization properties of

HPV-16 have been mapped to oncogenes E6 and E7 (Hawley-Nelson *et al* 1989; Liu *et al* 1999). Subculturing HPV-transfected HTE cells on plastic dishes revealed no significant changes in cellular morphology. *In vitro*, the differentiated phenotype of epithelial cells can be induced by growing cells at the air-liquid interface on collagenous substrata. If HPV-immortalized cells represent the tissue culture counterpart to naturally occurring premalignant cells, they should share many characteristics of dysplastic cells (Blanton *et al* 1991). We therefore used a raft culture system, which reproduces *in vitro* the differentiation patterns of epithelial cells *in vivo*, to search for the differences between normal and HPV-transfected HTE cells. We observed that the appearance of the HPV-16 transfected HTE cells in raft culture varied considerably in size and shape from the primary HTE cells. They showed disturbed cellular polarity, vacuolated cytoplasm, and an increased nucleus-to-cytoplasm ratio which resembled epithelial dysplasia *in vivo*. A weak-to intermediate expression level of cytokeratins (determined with pan cytokeratins 1, 5, 10, and 14) was detected in the HPV-transfected cells in rafts (Fig 2, Study IV). The poor differentiation of HPV-transformed cells was possibly a result of the expression of viral oncogenes E6 and E7 which stimulate cell proliferation in raft culture and leads to the formation of disorganized epithelial layers (Halbert *et al* 1992). By electron microscopy, we observed that HPV-16 transfected HTE cell lines presented numerous desmosomes and microvilli, characteristic components of epithelial cells.

8.4.2. Tumorigenicity of HPV-16 transformed cell lines

All HPV-transformed HTE cells failed to form colonies in soft agarose and tumors in nude mice, suggesting that HPV-transformed cells are non-malignant. The presence of HPV alone does not necessarily result in malignancy. Other co-factors may be necessary for full oncogenic expression, of which fibroblasts are perhaps one of the most important (Zheng and Vaheri 1995; Atula *et al* 1997; Olumi *et al* 1999). We also observed that a co-culture of HES fibroblasts with HPV-transformed HTE cells in soft agarose resulted in colony formation (Fig 6). Several growth factors are known to modulate cascades of signaling pathways in

epithelial cell lines. Dysregulation of such cascades may lead to cell transformation and tumorigenesis (Ridley *et al* 1995; Dhawan *et al* 1999; LaRochelle *et al* 1999). For example, upregulation of keratinocyte growth factor (KGF) receptor in HPV-immortalized cells may subsequently result in KGF-induced anchorage-independent growth of the cells (Zheng *et al* 1995). KGF is a member of the fibroblast growth factor family and has unique target-cell specificity restricted to the epithelial cell type (Finch *et al* 1989; Aaronson *et al* 1991).

Figure 6. HES fibroblasts induced anchorage-independent growth of HPV-transformed HTE cells in soft agarose. a. Co-culture of HPV-transformed HTE cells and HES fibroblasts formed large colonies (*arrow*) in soft agarose. b. Culture of only HPV-transformed cells in soft agarose formed no colony.

8.4.3. Cytogenetic aberration

Unbanded metaphase spreads from all HPV-16 transfected HTE cell lines showed chromosome abnormalities such as chromosomal breakage, chromatid elongation (premature chromosome condensation), dicentric and acentric chromosomes, and numerical chromosomal changes ($n = 80 - 100$) (Fig 4, Study IV). Our observations are in line with that by others (Dürst *et al* 1987a; Hukku *et al* 2000). Mechanisms underlying HPV integration and chromosomal instability need to be further studied.

8.4.4. Physical status and chromosomal localization of viral DNA in HPV-16 transformed HTE cell lines

Southern blot analysis indicated that all four HPV-transformed HTE cell lines harbored only integrated HPV-16 DNA (Fig 5, Study IV). We used real-time PCR with primers (Table 1, Study II) specific for the HPV-16 E2 and E6 regions to quantitate the copy numbers of the E2 and E6 DNA and found that each cell line contained equal amounts of E2 and E6 DNA copies, suggesting that integration did not occur within the selected E2 region. Although the E2 gene is often disrupted upon viral DNA integration *in vivo* (Kalantari *et al* 1998), integration sites do not necessary occur within the E2 gene *in vitro* transfection. The viral DNA integrated exclusively at chromosomal 7q31 in three cell lines originating from the same transfectant (HTE-114/K); and in a polyclonal cell line from another transfectant of a different donor (HTE-114/B), the viral DNA integrated at single locus near the telomere of the long arm of chromosome 9 (Fig 6, study IV). According to previous reports viral DNA integration sites seem to be random: Kahn and colleagues reported a tonsillar carcinoma case with HPV-6a integration at chromosomal 10q24 in tumor cells (Kahn *et al* 1994). In cervical carcinomas, integration of HPV has been mapped in many chromosomes, i.e., chromosomes 1, 2, 3, 6, 8, 10, 13, 14, 16, 17, 18, 20, and 22, with the integration event occurring at a single locus in each case (Dürst *et al* 1987b; Wilke *et al* 1996; Reuter *et al* 1998; Koopman *et al* 1999; Thorland *et al* 2000; Luft *et al* 2001). However, multi-site integration has also been evident in a cell line derived from oral carcinoma containing six viral integrated loci within chromosomal region 15q14-15 and five unidentifiable chromosomes (Steenbergen *et al* 1995). Several studies have reported no specific preferred chromosomal sites for viral DNA integration in HPV-immortalized cell lines (Weijerman *et al* 1997; Dürst *et al* 1995; Smith *et al* 1992; Popescu and DiPaolo 1990). In those studies, the authors also observed that viral DNA integrated in either a single locus or multi-site in chromosomes. It is unclear whether host-cell origin, HPV variants, transfection condition, or any combination of these may favor specific loci for viral integration.

8.4.5. Viral gene expression in HPV-16 transformed HTE cell lines

The viral DNA was transcriptionally active in all transfected cells. Two major transcripts of 1.7 and 3.7 kb and one minor transcript of 2.4 kb existed in all four immortalized cell lines by northern blot hybridization with a full-length HPV-16 DNA probe as well as with an E7 specific probe (Fig 7, Study IV). In contrast, no viral transcript was detectable when the filter was hybridized with a fragment corresponding to the L1 region. We failed to demonstrate viral particles in the raft culture, which can be explained by the fact that these cells contained no episomal viral DNA. Viral particles have been identified in raft cultured cells derived from a cervical intraepithelial neoplasia type I lesion and HPV-immortalized keratinocytes containing episomal viral DNA (Meyers *et al* 1992; Frattini *et al* 1996). When a constructed chimeric HPV-18/16 plasmid DNA was introduced into primary keratinocytes and maintained episomally, the viral late functions (capsid gene expression and synthesis of virion) were active in raft cultures (Meyers *et al* 2002).

Our HPV-immortalized HTE cells are non-malignant and thus provide a useful *in vitro* model for studying further the multistep molecular events of HPV-16 associated tonsillar carcinogenesis, as well as the effect of anti-virus and anti-tumor compounds.

9. CONCLUDING REMARKS

The studies presented in this thesis have shown the following:

- Although mutations in many tumor suppressor genes play a role in head and neck carcinogenesis, our study suggests that *PTEN* and *LKB1* are not among them in laryngeal tumors.
- Infection by high-risk types of HPV plays a role in the development of head and neck cancers, particularly in tonsillar carcinogenesis. HPV-16 DNA existed in episomal, integrated, and mixed forms in HNSCC. Tonsillar SCC harbored a remarkably higher viral load than did the others in HNSCC. Patients with HPV-positive tumors had a prognosis similar to those with HPV-negative ones.
- Only HPV-16 DNA, but no other types, presented in tumor-free tonsillar tissue. Detection of HPV-16 DNA in tonsillar tissue did not correlate with seropositivity against HPV proteins. HPV DNA is seldom detected in the exfoliated tonsillar cells in normal tonsils, suggesting that tonsillar crypt epithelial cells may be a reservoir for HPV-16 or normal tonsils seldom harbor HPV.
- Primary human tonsillar epithelial cells can be immortalized by introduction of full-length HPV-16 DNA which provides an *in vitro* model to study further multistep molecular events of HPV-16 associated tonsillar carcinogenesis, as well as treatment for the HPV-associated tonsillar carcinomas.

10. PERSPECTIVES

Detection of HPV DNA in tumor or precancer samples does not on its own indicate that the virus plays a causal role in tumor development. Additional molecular data argue, however, that the virus may play such a role in a subset of HNSCC. Understanding the fundamental molecular changes of HNSCC may lead to improvements in therapy for patients with this disease.

Studies on the molecular mechanisms underlying HPV-associated head and neck carcinogenesis is hindered, because the HPV is not easy to propagate *in vitro*. We have established and characterized HPV-immortalized human tonsillar epithelial cell lines which allow us to further study multistep in molecular events (co-factors) in HPV-16 associated tonsillar carcinogenesis. In addition, these cell lines may provide an *in vitro* model to test effects of anti-viral and anti-tumor agents, as well as to study the response to radiotherapy.

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